MICROBIAL MERCURY RESISTANCE AND POTENTIAL METHYLATION RATES IN THE UPPER WISCONSIN RIVER

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ABSTRACT

Microbial mercury resistance and potential mercury methylation rates were examined in water, sediment-floc, and sediment cores from the Upper Wisconsin River. Mercury (II) resistance was quantified using aerobic and anaerobic heterotrophic media containing 0-75 µg/ml Hg^{+2}. Methylation activity was determined by measuring $CH_3{}^{203}Hg^{+}$ formation from $^{203}Hg(NO_3)_2$. Sediment incubations were carried out under strict anaerobic conditions. Aerobic and anaerobic heterotrophic bacteria were highly resistant to 14 µg/ml Hg^{+2}. Anaerobic heterotrophic bacteria were more resistant to higher concentrations of Hg^{+2} than aerobic heterotrophic bacteria. Mercury methylation activity was near background in the water, highest in surface sediments, and decreased with increasing sediment depth. More than 98% of the added ^{203}Hg was bound to sediments within 4 hrs of inoculation, while more than 3% was methylated during a 10-day incubation. As much as 7% of the added ^{203}Hg was methylated in other experiments. This suggests that bound Hg^{+2} was available for methylation. Organically enriched sediments exhibited higher methylation activity than less eutrophic sediments. The addition of peptone to sediments caused highly significant ($p < 0.01$) increases in methylation activity, while vitamin B_{12} and sewage sludge caused significant ($p < 0.05$) increases. The presence of oxygen in sediments inhibited methylation activity. This indicates that mercury methylation in the Upper Wisconsin River is primarily an anaerobic process. The optimum temperature for methylation was 35°C, although the maximum *in situ* temperature was 24°C. A seasonal summer peak in methylation activity was observed in water, floc, and

sediments. These data suggest that the Upper Wisconsin River sediments have the potential to release large amounts of toxic methylmercury to the overlying water.

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INTRODUCTION

This study examined the effects of mercury on microbial populations in the Wisconsin River, the degree of mercury methylation in water and sediments, and the effects of varying environmental parameters on methylation. This research was initiated due to the general lack of knowledge concerning mercury methylation as it occurs *in situ* and the effects of various environmental parameters on this process.

The Wisconsin River received heavy inputs of mercury from numerous pulp and paper mills until the late 1950s. The presence of several impoundments in the Wausau area (Lake Wausau, Mosinee Flowage, and Lake DuBay) served as sinks for mercury (25). During 1970-71, elevated levels of mercury were reported in fish and sediments in the Wisconsin River (53, 56). In 1978, Sheffy (88) reported that mercury was present in Wisconsin River crayfish, indicating that mercury was still biologically available many years after the major reductions in industrial releases.

Most of the mercury discharged into this waterway by pulp and paper mills was the less toxic phenylmercuric acetate. This organic form of mercury is rapidly degraded to inorganic mercury, which can be converted by bacteria to the highly toxic methylmercury (4, 36, 60, 97, 105, 110).

Characteristics of the impoundments on the Wisconsin River are favorable for the methylation of mercury (78) and high rates of methylation have been reported in its sediments (43). The majority of mercury in aquatic environments is sorbed onto particulate and organic matter which readily settles out of solution. Up to 97% of the mercury formed

in aquatic systems is associated with bed sediments (48), and it can remain biologically available for a long period of time (65, 83).

Methylmercury is not readily retained by sediments, but it is commonly released into overlying waters where it can be taken up by aquatic organisms. It accumulates in body fat tissues and biomagnification occurs as larger animals consume mercury-laden prey. Consequently, concentrations of methylmercury in fish tissues are often thousands of times greater than in the water (20).

Sediments are largely anaerobic, except for a narrow, oxidized microzone at the sediment-water interface (80, 81). Because most of the mercury in an aquatic system is present in the sediments, the highest concentrations of mercury occur in anaerobic environments. However, few studies of anaerobic mercury methylation have been attempted.

Most mercury research has been performed in the laboratory with pure cultures of specific mercury-resistant (15, 28, 38, 39, 64, 74, 75, 95, 106, 108) or mercury methylating bacteria (36, 54, 69, 72, 105, 116). Few studies have attempted to determine mercury resistance and methylation activity of natural bacterial populations in aerobic and anaerobic aquatic ecosystems. Information gained from studies of native populations is needed, because results obtained from pure culture studies may not reflect processes occurring under natural conditions.

The specific objectives of this study were to:

1. determine the resistance of aerobic and anaerobic heterotrophic bacteria to different concentrations of mercury in water and sediment cores at each sampling site;

2. determine seasonal variations of bacterial resistance to mercuric ion and to compare these variations to physical and chemical changes occurring in the river;

3. measure seasonal variations in methylation activity at one site on the river;

4. determine the effects of temperature, oxygen, and different organic compounds on methylation activity; and

5. examine the influence of mercury binding to sediments on methylation activity.

Accomplishment of the above objectives will provide needed information on the effects of mercury contamination and the potential availability of methylmercury in the Wisconsin River. Bacteria play a vital role in aquatic ecosystems by degrading allochthonous and autochthonous organic matter in water bodies. This is especially important in anaerobic sediments where anaerobic decomposition of organic matter involves several trophic levels of microorganisms, which consist largely of bacteria (109). Therefore, an examination of the effects of mercury on these microorganisms is an important, yet often overlooked, aspect of mercury pollution. Finally, a knowledge of the effects of varying environmental conditions on methylation activity may aid in controlling the production of methylmercury in mercury contaminated systems.

LITERATURE REVIEW

Historical Background

Mercury compounds have long been known to be toxic, although the hazards of mercury in aquatic environments have only been recently recognized. Several episodes focused worldwide attention on the hazards of pollution with mercury compounds. Between 1953 and 1960, 116 people were poisoned and 43 died from consuming methylmercury contaminated fish in Japan (20). The source of contamination was traced to a vinyl chloride factory located in Minamata Bay. A similar episode took place in Niigata (20). One hundred twenty people were poisoned, and five deaths occurred during that incident. In Sweden, a drastic decrease in bird populations was caused by the use of methylmercury dicyandiamide, a fungicide (20).

Perhaps the largest epidemic of mercury poisoning occurred in Iraq in 1971 and early 1972 (20). This incident was caused by human consumption of alkylmercury-treated seed grain. The Iraqi Ministry of Health officially recognized 50 cases of poisoning in January 1972, and hospital admissions reached 400 per day. Unofficial estimates indicated that up to 60,000 peasants could have eaten enough alkylmercury-treated grain to cause adverse effects (18). The government officially acknowledged that 6530 victims were hospitalized and 459 died (20).

The Canadians also reported problems of mercury contamination in waterways. Chlorine and caustic soda plants discharged high concentrations of mercury into the Wabigoon River, and fish in the Wabigoon-English River system contained the highest levels of mercury reported in the Western Hemisphere. Healthy-looking northern pike, walleyed

pike, and burbot contained up to 28, 20, and 10 μg/ml of mercury, respectively (24). Fish containing 24 μg/ml of methylmercury have been taken from Lake Wabigoon in the Saskatchewan River System. Consumption of only a few fish polluted at this level could be fatal (98). Huckabee et al. (40) reported that almost all freshwater fish tested had detectable levels of mercury in their tissues, and the majority (usually greater than 80%) of this mercury is in the methylated form.

Worldwide mercury contamination of waterways is a major environmental problem that has resulted in numerous isolated deaths and cases of acute toxicity (20). In 1970, elevated levels of mercury were found in waterways in 33 states, and many of these have been closed indefinitely to fishing (111).

Toxicity of Mercury

Inorganic Mercury Compounds

The toxicity of heavy metals is a result of their binding to active sites of important enzyme systems in the cells and their binding to ligands in the cell membrane, thereby resulting in a variety of toxic effects (76). Early symptoms of inorganic mercury poisoning are so general that they are difficult to detect. Inorganic mercury compounds concentrate in the kidney, liver, and spleen. They are readily excreted, however, and do no damage unless the threshold tolerance level of the organ is exceeded (20). Prolonged exposure to inorganic mercury compounds is required for toxic symptoms to develop.

The symptoms of inorganic mercury poisoning develop gradually. The first clear physical symptoms are numbness of the fingers and toes and then of the lips and tongue (20). Weakness, fatigue, anorexia, loss of weight, and disturbances of gastrointestinal functions are

associated with fully developed clinical forms of chronic poisoning (26). Late phases are characterized by mercurial tremor, psychic disturbances, and changes in personality (26). Prolonged exposure to high concentrations of inorganic mercury can result in death.

Organic Mercury Compounds

Mercury is covalently linked to a carbon atom in organic mercury compounds (71). Most organic mercury compounds are rapidly excreted and therefore, pose no serious health problems (45). However, the short-chain alkylmercury compounds, such as methylmercury, are formed in aquatic environments via methylation of inorganic mercury. Methylmercury is among the most toxic of all mercury compounds (12, 20).

The mercury-carbon bond in methylmercury is extremely stable, and the attachment of the alkyl radical increases lipid solubility. This facilitates penetration of the blood-brain barrier and cell membranes (23). Nervous tissue tends to accumulate the greatest concentrations of methylmercury (13). Methylmercury rapidly diffuses through the cell membrane and enters the cell, where it is rapidly bound by sulfhydryl groups. Inside the cell, methylmercury inhibits protein and RNA syntheses (45).

Methylmercury concentrates in the body during a latent period during which no symptoms are observed. After threshold levels are exceeded, severe effects on the central nervous system may occur (20). Symptoms of methylmercury poisoning include fatigue, headache, numbness of the extremities, blurred vision that can progress to blindness, and poor muscular coordination (45). Severe methylmercury poisoning usually results in death due to the toxic effects on the brain and body. Three to eleven cases of death from mercury poisoning have occurred in the United States annually from 1957 to 1970 (111).

The effects of prolonged exposure to low levels of mercury before permanent damage occurs are not known. The United States Food and Drug Administration, however, considers fish unacceptable for human consumption if they contain concentrations of mercury greater than 1.0 µg/ml wet weight. Canadian health agencies consider fish with $\geq$ 0.5 µg/ml of mercury to be unacceptable for human consumption.

Sources of Mercury

Natural Sources

Natural inputs of mercury into the environment are not well quantified because of the difficulties and uncertainties with analytical measurements. On a worldwide scale, natural emissions are estimated to be more than double the man-related discharges (Table 1). However, anthropogenic discharges exceed natural discharges in the United States (Table 1).

Land. In localized areas, cinnabar (HgS) and metacinnabar ($HgS\text{-}(HgS)_{80}(HgSe)_{20}$) can contribute significant quantities of mercury to the atmosphere and water. Most of this is discharged into the air from the combustion of fossil fuels, or the element leaches out of the ground into the waterways. This has become problematic in areas of Canada, where high levels of mercury have been leached into new reservoirs from recently covered soils containing naturally-occurring concentrations of cinnabar (J.W.M. Rudd, personal communication).

On a worldwide scale, natural sources of mercury from land are low. Normal mercury content in igneous rock and minerals has been estimated at 50 ng/g and in many cases less than 10 ng/g (3). Natural source discharges from land are included under air and water data in Table 1.

Table 1. Mercury discharges (metric tons) in the USA and the world by environmental media[a] (107).

	1975	1985	2000	2025
World				
Anthropogenic				
air	2374.2(31.6)	2822.1(30.5)	2970.4(29.8)	4008.0(29.6)
water	293.3(3.9)	299.5(3.2)	390.6(3.9)	372.2(2.8)
land	4850.5(64.5)	6138.8(66.3)	6608.6(66.3)	9149.0(67.6)
total	7518.0(32.3)	9260.4(37.0)	9969.6(38.7)	13529.2(46.2)
Natural				
air	14460.2(91.8)	14460.2(91.8)	14460.2(91.8)	14460.2(91.8)
water	1330.0(8.2)	1300.0(8.2)	1300.0(8.2)	1300.0(8.2)
total	15760.2(67.7)	15760.2(63.0)	15760.2(61.3)	15760.2(53.8)
USA				
Anthropogenic				
air	473.4(29.1)	506.2(27.7)	461.3(28.4)	614.7(30.7)
water	59.8(3.7)	52.7(2.9)	50.7(3.1)	49.1(2.5)
land	1091.5(67.2)	1270.8(69.5)	1111.1(68.5)	1335.9(66.8)
total	1624.7(57.1)	1829.8(60.0)	1623.2(57.1)	1999.6(62.1)
Natural				
air	1018.7(83.6)	1018.7(83.6)	1018.7(83.6)	1018.7(83.6)
water	200.0(16.4)	200.0(16.4)	200.0(16.4)	200.0(16.4)
total	1218.7(42.9)	1218.7(40.0)	1218.7(42.9)	1218.7(37.9)

[a]Numbers in parentheses are percentages. For anthropogenic and natural estimates, the percentages are of total discharges. For air, water, and land estimates, they are percentages of the indicated higher level category.

Atmosphere. The total mercury content of the air over nonmineralized land appears to be in the range of 2-9 ng/m^3 (61). Volcanic gases and emissions from geothermal areas contain mercury vapor. Worldwide emissions to the atmosphere from volcanoes has been estimated at 2×10^7 g/hr (103). Mercury emissions to the atmosphere from the United States have been estimated at 1.78×10^{10} g/yr (103). Table 1 estimates that worldwide, greater than 90% of the total natural discharge of mercury occurs from the atmosphere. The atmosphere contributes more than 80% of the total natural discharge of mercury in the United States.

Water. On a global basis, approximately 8% of the total natural input of mercury accumulates in water (Table 1). This figure is greater than 16% in the United States. Thirteen hundred metric tons are discharged annually into the world's waterways from natural sources (107). About 200 metric tons enter United States waters (102). Most of the mercury entering water bodies comes from land and air sources.

Anthropogenic Sources

Due to the natural inputs of mercury, release by human activities are responsible for only a small part of the environmental mercury problem. In localized areas, however, pollution by man is the major source of mercury contamination. This pollution has been responsible for all of the mercury-contamination disasters.

Land. Land receives the largest percentage of anthropogenic discharges of mercury to the environment. Discharges to land in the United States are expected to increase from 1092 metric tons in 1975 to 1336 metric tons in 2025 (Table 1). Most mercury discharges to land occur when materials containing mercury are placed in landfills. This includes instruments such as thermometers, electrical tubes, switches, relays, and primary batteries.

One of the largest industrial sources of mercury discharges to land originate from mercury-cell chlorine plants. Most of the mercury discharged from these plants is present in sludge generated by the brine and caustic purifiers. The sludge is concentrated in settling ponds and deposited in local industrial landfills.

Anthropogenic sources of mercury in cultivated soils include the fertilizers, lime, and fungicides used in modern agriculture. The 13 types of lime for agricultural purposes showed mercury contents ranging from less than 5 to 20 ng/g (3). The quantity of mercury annually introduced into cultivated soils from normal fertilization may reach 1.0 g/ha, but is more commonly in the range of 0.05 to 0.3 g/ha (3).

Organic fertilizers of municipal origin, such as sewage sludge and composts from solid wastes, may contain more mercury than agriculturally fertilized soils. This results in a considerably greater mercury concentration. A single application of sewage sludge may double or triple the normal mercury content in the soil (3).

Since 1900, mercury compounds have been utilized in agriculture as fungicides and seed disinfectants. Because of this, an average of 1-2 g/ha annually has been distributed in cereal-producing soils (3).

Atmosphere. The majority (86%) of mercury introduced into the atmosphere is from anthropogenic sources. Approximately 30% of the total anthropogenic discharges in the United States are emitted into the air (Table 1). A summary of anthropogenic atmospheric emissions in the United States is given in Table 2. Approximately 60% of the total atmospheric release are due to the final consumption of manufactured goods containing mercury. These include paints, batteries, pesticides, and dental materials.

Table 2. Anthropogenic emissions of mercury to the atmosphere in the USA (61).

Source	Emissions ($\times 10^{-3}$ kg/yr)	Percent of total
Mercury mining and processing	7.8	1.7
Other mining	50.7	10.8
Fuel burning (except utilities)	53.8	22.2
oil 19.2		
coal 17.1		
gas 15.5		
Utilities (oil, coal and gas)	52.7	--
Manufacturing	26.0	--
Final consumption of manufactured goods	282.2	59.8
Total	471.3	100.0

The second largest anthropogenic source is fossil fuel burning. Fossil fuels contain varying amounts of mercury that are emitted to the atmosphere upon burning. Sewage treatment plants are also a major point-source of atmospheric mercury. Raw sewage contains an average of 2 ng/ml of mercury (102). Much of this is emitted in an alkyl-mercury form and in some cases, can be detected several kilometers from the plant (61).

Water. A large part of airborne mercury eventually reaches surface waters. Similarily, initial discharges to land can reach water unless landfills are carefully designed and managed. Only about 3% of the total anthropogenic emissions are directly deposited in water (Table 1). A large percentage of mercury discharges to water are from pharmaceuticals contained in human wastes and from mercury lost in the preparation of dental amalgams (107). Anthropogenic point sources can be of local significance. Chlor-alkali plants are sources of mercury contamination. A portion of the total mercury lost from these plants enters receiving waters.

Additional point sources include plants that manufacture vinyl chlorides and urethane plastics. Until 1970, pulp and paper mills used mercury compounds on pulp to prevent the growth of bacteria and fungi. This process introduced these mercury compounds into the adjacent waters.

Mercury Contamination of the Wisconsin River

Until the late 1950s, pulp and paper mills on the Wisconsin River used phenylmercuric acetate as a slimicide on manufactured pulp, resulting in discharges of mercury compounds to the river. During 1970-71,

elevated levels of mercury were reported in both fish and sediments (53, 56). At that time, average mercury concentrations in the axial musculature of fish in the Wisconsin River below Rhinelander, Wisconsin, were above the 0.5 μg/ml (wet weight) guideline established for human consumption by the United States Food and Drug Administration (53). Konrad (56) reported the highest concentrations of mercury in sediments downstream of pulp and paper mills and a chlorine-caustic soda plant. In 1978, Sheffy (88) demonstrated that mercury was present in Wisconsin River crayfish and biologically available many years after the reduction of the industrial releases of mercury.

Impoundments on the Wisconsin River have several characteristics favorable for the methylation of mercury (78). These include: (1) high rates of primary production, (2) periods of high flow that mobilize methylmercury from sediments, and (3) sufficient mixing to prevent thermal stratification and maintain mildly oxidized bottom waters. Jacobs and Keeney (43) found that in situ formation of methylmercury was rapid in Wisconsin River sediments experimentally treated with phenylmercuric acetate. They hypothesized that the rapid methylation rate was due to the organic nature of phenylmercuric acetate and high organic content of Wisconsin River sediments.

Findley et al. (25) recently reported large amounts (10-750X greater than background) of mercury in bed sediments at certain sites in the Upper Wisconsin River. According to the United States Environmental Protection Agency's criteria for harbor sediments, surficial sediments from the Upper Wisconsin River are moderately to heavily polluted with mercury (25). Metal content of surface sediments was strongly related to texture and organic content. Generally, the greatest concentrations

of mercury occurred in the fine-grained fraction of sediments. The largest concentration of Hg was 7.6 µg/g (dry weight) and occurred in the Brokaw area.

Effects of Mercury Contamination on Microorganisms

Effects on Microbial Activities

Few studies have been attempted to determine the effect of mercury contamination on other microbial activities. Pedersen and Sayler (77) found that $HgCl_2$ had no significant effects on methanogenesis. Research by Winfrey (unpublished) confirmed these results. The sediment environment may protect the methanogenic population from the toxic effects of mercury (77). Effects of mercury on other microbial activities have apparently not been investigated.

Ecology of Mercury-Resistant Bacteria

Many bacteria possess a variety of resistance mechanisms to the toxic effects of mercury. Resistance depends on the strain, species, and genus of bacteria. Nelson and Colwell (70) reported that the majority of mercury-resistant bacteria in Chesapeake Bay water and sediments belonged to the genus Pseudomonas. They also showed that H_2S production is not an exclusive property of mercury-resistant bacteria. Phenylmercuric acetate was found to be considerably more toxic to bacteria than $HgCl_2$ in Cheasapeake Bay samples (70). Chesapeake Bay bacteria also exhibited a peak in mercury-resistant populations during spring. The highest percentages of resistant bacteria were observed from the study site with the highest mercury contamination. Percentages of mercury-resistant bacteria at this site were 22.8 and 28.5 in sediment and water, respectively.

Mercury Resistance Mechanisms

Bacterial resistance to heavy metals can occur by a variety of mechanisms, due to multiple interactions that can occur among microbial cells, heavy metal ions, and other environmental constituents. Resistance mechanisms to many metals have been implicated, but only mercury and cadmium have been extensively studied.

Hydrogen sulfide production. Hydrogen sulfide production by microorganisms can significantly decrease mercury toxicity because insoluble mercuric sulfide is formed in the presence of mercuric ion and hydrogen sulfide. Consequently, H_2S-producing organisms can exhibit tolerance to mercury. This mechanism is plasmic linked in Clostridium cochlearium because mercury tolerance and the ability to produce H_2S are lost when C. cochlearium is cured of its plasmid (37).

In some cases, sulfide producing organisms can protect sensitive organisms from the toxic effects of mercury. Staphylococcus aureus was more tolerant of mercury in the presence of Escherichia coli. The positive effect of E. coli was partly due to H_2S production (101).

Production of organic compounds. Yeast produce organic compounds that reduce the toxicity of mercury to the organism. For example, Saccharomyces cerevisiae requires methionine to produce a substance that acts as a detoxifying agent (90).

Uptake and accumulation of mercury by microorganisms. Some microorganisms accumulate mercury from their environment. This may reduce the toxic effects of mercury to other microbes within the community. Saccharomyces cerevisiae binds mercury to cell walls (68). Zoogloeal bacteria, which are common in aquatic habitats, adsorb and precipitate heavy metals within their cellular matrix (27). Removal of mercury

from the environment in this manner may allow more sensitive organisms to survive, but may also present a hazard if they are consumed by other organisms.

Mercury reduction. The most common mechanism of mercury resistance is reduction of mercuric ion. Mercury resistance by this method has been observed in Escherichia coli (54, 55, 95, 97), Staphylococcus aureus (97), and several species of Pseudomonas (29, 30, 31, 97). Mercury resistance depends on the ability of the organisms to detoxify mercury by reducing it from the divalent inorganic (Hg^{+2}) or organomercurial form to metallic mercury (Hg°) (54, 86, 96). This results in volatilization of mercury from the medium. This reaction is carried out by the enzyme mercuric reductase which is coded for by the mer gene. This gene is located on plasmids in Escherichia coli (95), Pseudomonas sp. (15), and Staphylococcus aureus (108).

Most studies on plasmid-mediated resistance have been done with clinical isolates of microorganisms. Thus, the importance of the survival mechanism under environmental conditions is unclear. Nevertheless, plasmid-mediated mercury reduction may be ecologically important because resistance could be rapidly transferred from resistant to sensitive bacteria. This would enable microorganisms in natural habitats to adapt to mercury toxicity faster than by mutation or natural selection (33).

Transformations of Mercury

Chemistry of Mercury in the Aquatic Environment

Inorganic mercury forms. Mercury can exist as a wide variety of chemical species, complicating detailed studies of the physicochemical states of mercury in aquatic systems. Mercury can be present with

valences of 0 (Hg°, elemental), 1^+ (Hg_2^{+1}, mercurous), and 2^+ (Hg^{+2}, mercuric).

Most of our knowledge concerning soluble forms of mercury in natural waters was achieved by equilibrium calculations based on chemical equilibrium constants and standard redox potentials.

Benes and Havlik (5) concluded from predominance diagrams that $Hg(OH)_2$ or $HgCl_2$ molecules will predominate in most fresh waters. Under reducing conditions in the sediment, the presence of sulfide ion will immobilize mercury by forming insoluble mercuric sulfide. Morel et al. (67) concluded that mercury should be present almost exclusively as $HgCl_2$ in well-oxygenated water, while soluble Hg°, $Hg(SH)_2$, and HgS_2^{-2} should predominate under reduced conditions.

Mercury complexes with organic ligands. Ionic mercury can form stable complexes with organic ligands. These associations depend on the concentration and nature of the organic species present and on the pH and composition of the water. Divalent mercury has a great affinity for a large number of organic substances, especially proteinaceous materials containing sulfhydryl (R-SH) groups (84). When organic particles and divalent mercury ions are present, mercury is adsorbed on particulates and may eventually settle to sediments (84). Determination of the actual nature of organic forms of mercury in natural waters is difficult. Consequently, much work remains to be done before the nature and abundance of organic complexes of mercury will be understood.

Organomercurial compounds. Organomercurial compounds are the most toxic forms of mercury and can be divided into two categories: those in which the mercury atom is bonded to one organic radical and those in which it is bonded to two organic radicals. The first type

dissociates in water to yield the $R\text{-}Hg^{+}$ cation and the X^{-} anion, making it soluble in water. Methylmercury is an example of this type of organomercurial. Dimethylmercury is an example of the second type of organomercurial. The presence of organomercurial compounds in water may result from natural processes or human activities. Natural processes of methylmercury formation are discussed in detail in the following sections.

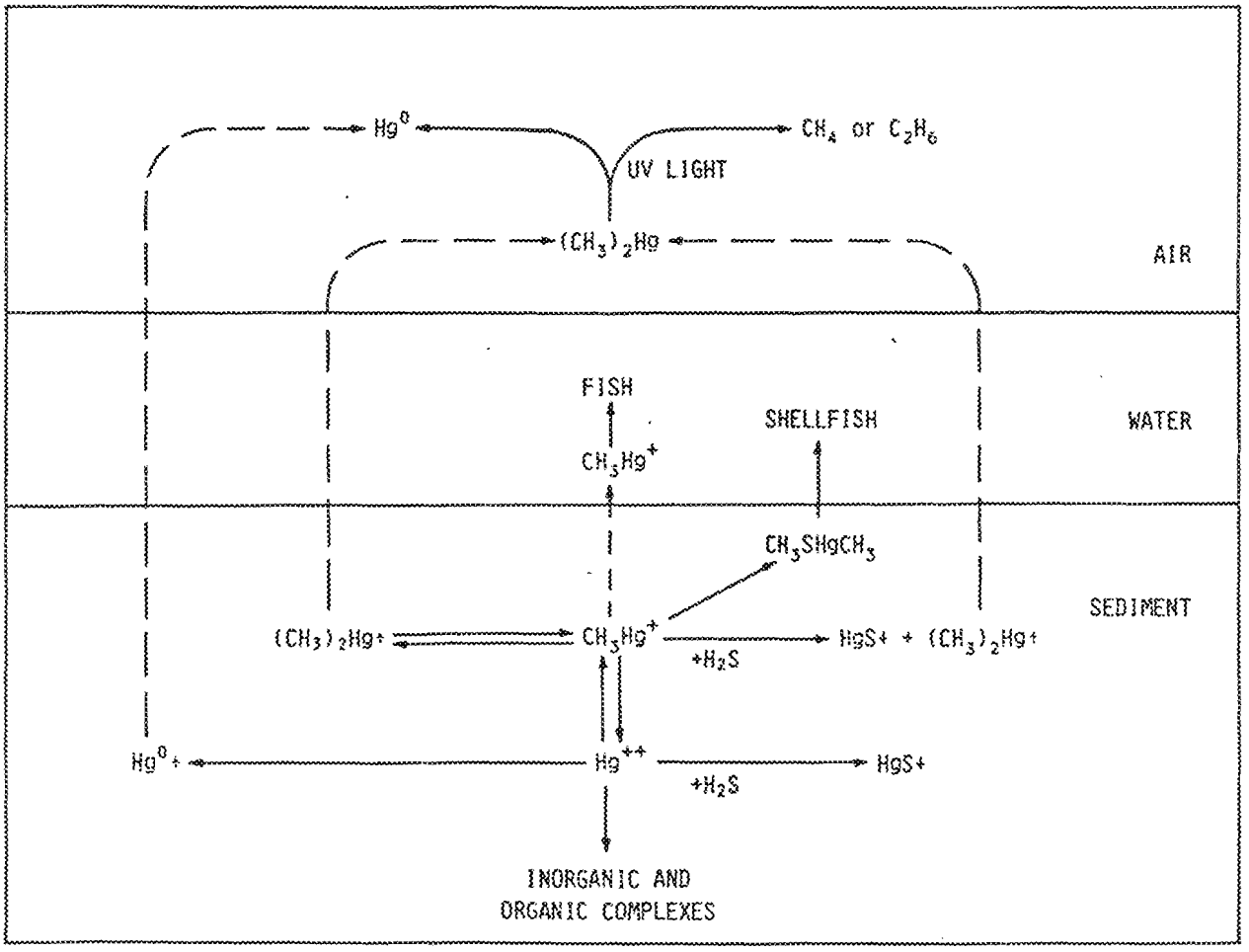
The Mercury Cycle

Several mercury cycles depicting mercury transformations in aquatic environments have been published (19, 34, 58). Mercury is rapidly adsorbed onto sediments, which can serve as a source of mercury for years (58, 62, 65, 83). For this reason, most of the mercury available for cycling in freshwaters is present in bed sediments. Figure 1 illustrates the fate of ionic mercury (Hg^{+2}) after it enters an aquatic system.

In the presence of hydrogen sulfide (H_2S), mercuric ions (Hg^{+2}) spontaneously precipitate as mercuric sulfide (HgS). Under anaerobic conditions, the formation of mercuric sulfide effectively reduced availability of mercuric ion for biological conversions. In the presence of oxygen, mercuric sulfide (HgS) may be converted to methylmercury by bacteria; however, this occurs at a rate 100-1000 times slower than mercuric ion methylation (21). Therefore, the presence of sulfide, commonly formed by sulfate-reducing bacteria, prevents methylmercury formation. Methylmercury (CH_3Hg^{+}) will chemically form dimethylmercury ($(CH_3)_2Hg$) and mercuric sulfide in the presence of hydrogen sulfide.

Mercuric ion may also be reduced to the volatile elemental mercury by resistant bacteria. This reaction results in the release of mercury

Figure 1. Transformations of mercury in aquatic ecosystems.


Hg^0
CH_4 or C_2H_6
UV LIGHT
$(CH_3)_2Hg$
AIR
FISH
SHELLFISH
WATER
CH_3Hg^+
CH_3SHgCH_3
SEDIMENT
$(CH_3)_2Hg\uparrow$
CH_3Hg^+
$+H_2S$
$HgS\downarrow + (CH_3)_2Hg\uparrow$
$Hg^0\downarrow$
Hg^{++}
$+H_2S$
$HgS\downarrow$
INORGANIC AND
ORGANIC COMPLEXES

from aquatic systems (17). Mercury volatilization might be expected to occur more readily than methylation due to the large numbers of bacteria capable of carrying out this reaction in aquatic sediments (17) and the kinetics of volatilization in bacterial cultures compared to methylation (Anne Summers, personal communication).

Methylmercury can be formed from mercuric ion by a variety of microorganisms, including anaerobes, facultative anaerobes, and aerobes. Thus, the potential for microbial methylation exists under both aerobic and anaerobic conditions. The product is primarily monomethylmercury (CH_3Hg^+) under neutral and acidic conditions, while formation of dimethylmercury ($(CH_3)_2Hg$) dominates under basic conditions (4). Monomethylmercury is water-soluble and is released into the water column. Dimethylmercury is volatile and is released into the atmosphere, where it is degraded into organic compounds (CH_4 or C_2H_6) and elemental mercury (Hg°) in the presence of UV light.

Fish are unable to methylate mercury; however, microorganisms (Pseudomonas sp.) living on their external slime and in their intestines can synthesize methylmercury from inorganic mercury (85). This could account for the accumulation of methylmercury by fish, because methylmercury should pass through the gill and gut membranes due to its high lipid solubility.

Methylmercuric methylsulfide ($CH_3S\text{-}HgCH_3$) may also be formed from methylmercury. Shellfish can metabolize methylmercury and store it as methylmercuric methylsulfide crystals (20). This was the major methylmercuric compound isolated from toxic Minimata Bay shellfish (20).

Methylmercury is not readily decomposed and is persistant to all but specific biochemical processes (4). Microbial degradation of methylmercury, reported by several investigators (8, 86, 91), results from hydrolysis and reduction of methylmercury to metallic mercury (Hg°) and methane (CH_4). Biological decomposition of methylmercury occurs in mercury-polluted sediments from lakes and rivers (8, 91), however, little is known about the rates of this process. Therefore, methylation is reported as a net rate, because individual rates of methylation and demethylation are not quantified.

Chemical methylation also occurs in aquatic environments in the presence of suitable methyl donors. Jewett and Brinkman (49) demonstrated transalkylation reactions among several metals in aqueous solutions. Wood (113) suggested that methylation could occur via transmethylation between mercury and lead and tin gasoline additives.

Methylation of Mercury

Rates of Methylation

Culture studies. Many culture studies have been performed in the laboratory with mercury-methylating microorganisms. Anaerobic cultures of Clostridium cochlearium produced 0.12, 0.06, 0.045, and 0.008 µg/ml of methylmercury from 10, 20, 30, and 40 µg/ml of added inorganic mercury in a five-day period (116). Vonk and Sijpesteijn (105) demonstrated that several pure cultures of bacteria could aerobically methylate $HgCl_2$. Aerobic, mercury-methylating bacteria included Bacillus megaterium, Escherichia coli, and Pseudomonas fluorescens. Wood et al. (110) reported that extracts of a methanogenic bacterium could enzymatically and non-enzymatically methylate mercury via methylcobalamine. Results from pure culture laboratory experiments cannot be extrapolated

to include actual environmental reactions; consequently, it is therefore not possible to compare the conditions in the laboratory to in situ conditions.

Natural studies. Several attempts have been made to determine the kinetics of mercury methylation and decomposition under natural conditions (6, 9, 21, 32, 43, 44, 57, 92, 115). However, results to date cannot be generalized to a wide range of environmental conditions.

In 1969, Jensen and Jernelöv (44) published the first study on methylation rates. These studies involved batch studies on bottom sediment and showed that methylmercury formation increased in proportion to the amount of inorganic mercury added to the sediment. Jernelöv (46) demonstrated that surface sediments exhibit greater methylation rates than deeper sediment layers.

Factors Affecting Methylation

The effects of various environmental factors on methylation have been extensively studied. Accumulation of mercury by fish placed in the microcosms above mercury-contaminated sediments indicated that methylation rates in river sediment were dependent on total mercury concentration, microbial activity, organic matter concentration, and pH in the sediment (60).

Organic loading. Supplementation of sediments with organic nutrients can increase the net methylmercury production. Bishop and Kirsch (9) noted an increase in anaerobic methylmercury production after supplementation with nutrients. Olson and Cooper (73) found that sediments with high organic content produced the highest methylation rates in both aerobic and anaerobic environments. Jacobs and Keeney (43) observed greater methylation in the more organic Wisconsin River sediments than

the less organic Fox River sediments. Others have observed increased methylation rates in sediment after the addition of tryptic soy broth (32, 115).

Temperature. Bishop and Kirsch (9) reported that anaerobic mercury methylation increased with increasing temperature. Wright and Hamilton (115) reported that methylmercury production was greater at 20°C than at 4°C.

Oxygen. Studies on the effects of oxygen on mercury methylation rates have yielded conflicting results. Methylation rates in aerobic and anaerobic San Francisco Bay sediments showed that methylation was much greater in anaerobic sediments than in aerobic sediments (73). Other investigators, however, have reported that when sediments containing mercury were exposed to air, the rate of biological methylation of mercury was greater (10^3-10^4 times "normal" methylation rates in the aquatic environment) (21). Vonk and Sijpesteijn (105) found that Escherichia coli methylated mercury slower anaerobically than aerobically.

Anaerobic methylation may not be ecologically significant in high sulfate sediment due to the simultaneous presence of hydrogen sulfide. Under these conditions, mercuric sulfide forms, which effectively prevents mercury from being methylated. This may account for the lack of methylmercury formation that has been found in some anaerobic sediments (48). Blum and Bartha (10) reported an inverse relationship between salinity and methylation in saltwater. The large sulfate concentrations in saltwater would lead to increased hydrogen sulfide production in the presence of mercuric ion. This would effectively make mercuric ion unavailable for methylation. For this reason, methylmercury may not be as great of a potential problem in saltwater environments.

pH. The pH of a system can affect the form of methylmercury released from sediments. Fagerström and Jernelöv (22) reported that overall methylation was minimally affected by pH variations between 5 and 9. At higher pH values, however, dimethylmercury is the dominant species formed, while monomethylmercury is the primary product at low pH values.

Methylation Mechanisms

The mechanisms of methylation have been the subject of many studies and are still not fully understood. Methylmercury formation requires a molecule capable of donating a methyl group. In an aquatic system, most of these molecules are biologically synthesized.

Mercury methylation studies have been performed in two areas: nonenzymatic methylation and enzymatic methylation. Nonenzymatic methylation requires the products of active metabolism as methyl donors, whereas enzymatic methylation requires actively metabolizing organisms.

Nonenzymatic methylation. Nonenzymatic methylation via methylcobalamine (Vitamin B_{12}) is well documented (7, 42), although questions concerning the reaction mechanisms remain (59, 112). Methylation via vitamin B_{12} is probably a major route for methylation and mobilization of mercury from bottom sediments in polluted waters. Bacteria living in bottom sediment and sludge can carry out mercury methylation by excreting vitamin B_{12}, which serves as an *in vitro* methyl donor.

Photochemically induced methylation occurs in aqueous solutions in the presence of acetate ion or acetic acid (1). However, this reaction is not likely to be significant in natural environments.

Enzymatic methylation. A number of mechanisms of enzymatic methylation have been proposed (113). In biological systems, three major coenzymes are known to be involved in methyl transfer: S-adenosyl

methionine, N^5-methyltetrahydrofolate derivatives, and methylcobalamine (vitamin B_{12}) derivatives.

A transfer of carbanion methyl groups is required for the methylation of the mercuric ion. The vitamin B_{12} derivatives are the only known agents capable of doing this directly. Therefore, they are believed to be the active agents in most microbial methylation of mercury (82). Enzymatic methylation via vitamin B_{12} occurs inside the cell, whereas nonenzymatic methylation via vitamin B_{12} occurs by a chemical reaction outside of the cell.

Another potential mechanism involves N^5-methyltetrahydrofolate-homocysteine transmethylase (methionine synthetase). Microorganisms that use this enzyme to synthesize methionine from homocysteine can also produce methylmercury in the presence of mercuric ion.

Landner (59) proposed a third enzymatic mechanism of methylation involving S-adenosyl methionine by an isolated fungal species of Neurospora crassa, which appears to lack vitamin B_{12} enzymes. Landner suggested that the methylation might result from "incorrect" synthesis of methionine, which is normally formed through methylation of homocysteine.

Mercury methylation by methanogenic bacteria may also be an important environmental process. Methanogens have a methylating mechanism and they are abundant in the sediments where mercury accumulates. Research needs to be performed in this area.

In summary, mercury undergoes a variety of complex physical, biological, and chemical transformations in the environment. Our understanding of the fate and effects of mercury in the environment has increased substantially since the numerous disasters involving mercury contamination in recent decades. Much remains to be learned, however, about the

specific rates of various mercury transformations, the organisms responsible, and the effects of environmental conditions on these processes.

DESCRIPTION OF STUDY AREA

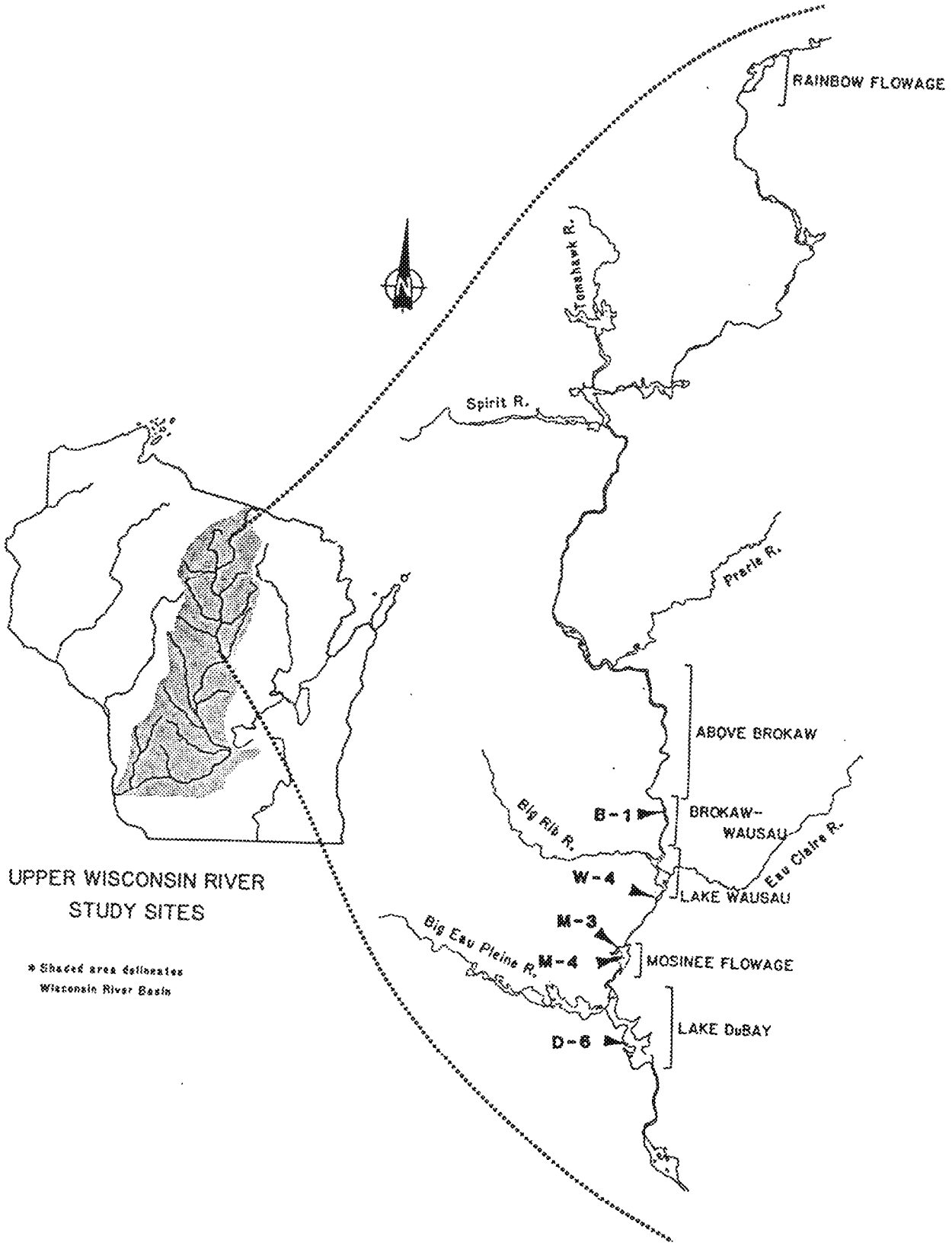
The Upper Wisconsin River study area is located in the Northern Highlands Geographical Province of Wisconsin (63) and extends from Lake DuBay to above Brokaw (Fig. 2). This stretch of the river contains numerous tributaries that form a dendritic drainage system. The drainage system is overlain by Pleistocene glacial drift that mantles an eroded Precambrian surface of granitic rock (100).

During 1977, land in the entire Wisconsin River basin included 42% in forest, 29% in cropland, 12% in grassland, 4% in urban development, 6% in other uses, and 6% in water and Federal land (99). Well-drained sandy upland soils are predominant in the northern portion of the Upper Wisconsin River Basin. Heavier loamy upland soils predominate in the central part of the basin (99).

The Wisconsin River is soft and weakly buffered water. The mean annual alkalinity near Wausau is about 28 mg/l (as $CaCO_3$). Hardness and pH are about 40 mg/l (as $CaCO_3$) and 6.8, respectively. The Wisconsin River has had intermittent violations of water quality standards, most notably low dissolved oxygen (14, 16). Water quality problems generally occurred within one or two miles of major point sources, frequently pulp or paper mills (100).

The Upper Wisconsin River contains large concentrations of nitrogen and phosphorus (66, 94). These support algal blooms, especially in the slower moving waters of the reservoirs. Melosira, Aphanizomenon, and Cyclotella are the dominant taxa of phytoplankton within impoundments on the river (94).

Figure 2. The Upper Wisconsin River study area.



UPPER WISCONSIN RIVER
STUDY SITES

* Shaded area delineates
Wisconsin River Basin

Four dams (DuBay, Mosinee, Rothschild, and Wausau) form a series of reservoirs in the study area (Fig. 2). Lake DuBay (Fig. 3) is the largest reservoir in the study area. It drains a watershed with an area of about 12,700 km^2 (99). The surface area is 28.5 km^2 and the volume is 1.04 x $10^8 m^3$. The mean depth is 3.7 m. Turnover time is 9.8 days, and the average annual trapping efficiency is approximately 28%. A sewage treatment plant (RM 248.8) and a pulp and paper mill (RM 248.9) discharge waters into Lake DuBay. Highly eutrophic waters from the Big Eau Pleine Reservoir also affects its water quality (11, 50, 51, 87, 101). Massive blooms of Aphanizomenon are common during the summer (66, 94). Silty sands and sandy silts are the primary sediment types in Lake DuBay (25).

Lake DuBay samples were collected from a site (D-6) located in a large bay on the west side of the lake (Fig. 3). Samples were taken from a stump field approximately 50 yards from shore with a water depth of approximately 2 m. Sediments were mostly sand and contained wood chips and fibers.

The Mosinee flowage (Fig. 4) is formed by the Mosinee dam (RM 248.9). It has a surface area of 5.57 km^2, a volume of 1.04 x $10^8 m^3$, and a mean depth of 2.1 m. The turnover time is 1.4 days. Two pulp and paper mills (RM 258.2 and RM 256.4) and one sewage treatment plant (RM 257.8) are located on this segment of the river. Sediments from Mosinee flowage are composed of silty sands, clayey silts, and silty clays (25).

Lake Mosinee samples were collected from a site (M-3) located at the confluence of Half Moon Lake and the river channel (Fig. 4). The water depth was approximately 1.5 m. Another sampling site (M-4)

Figure 3. Lake DuBay study area.

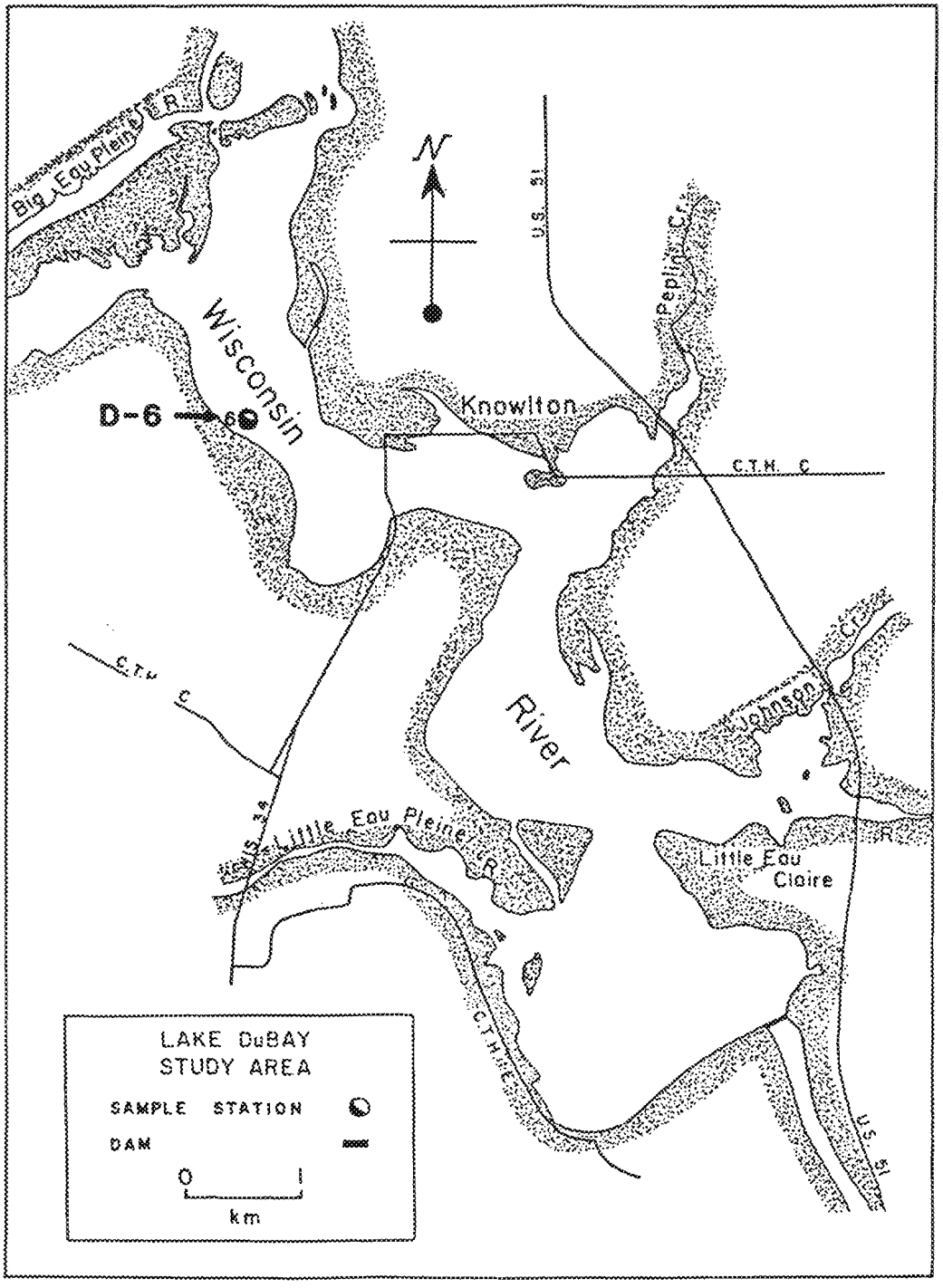
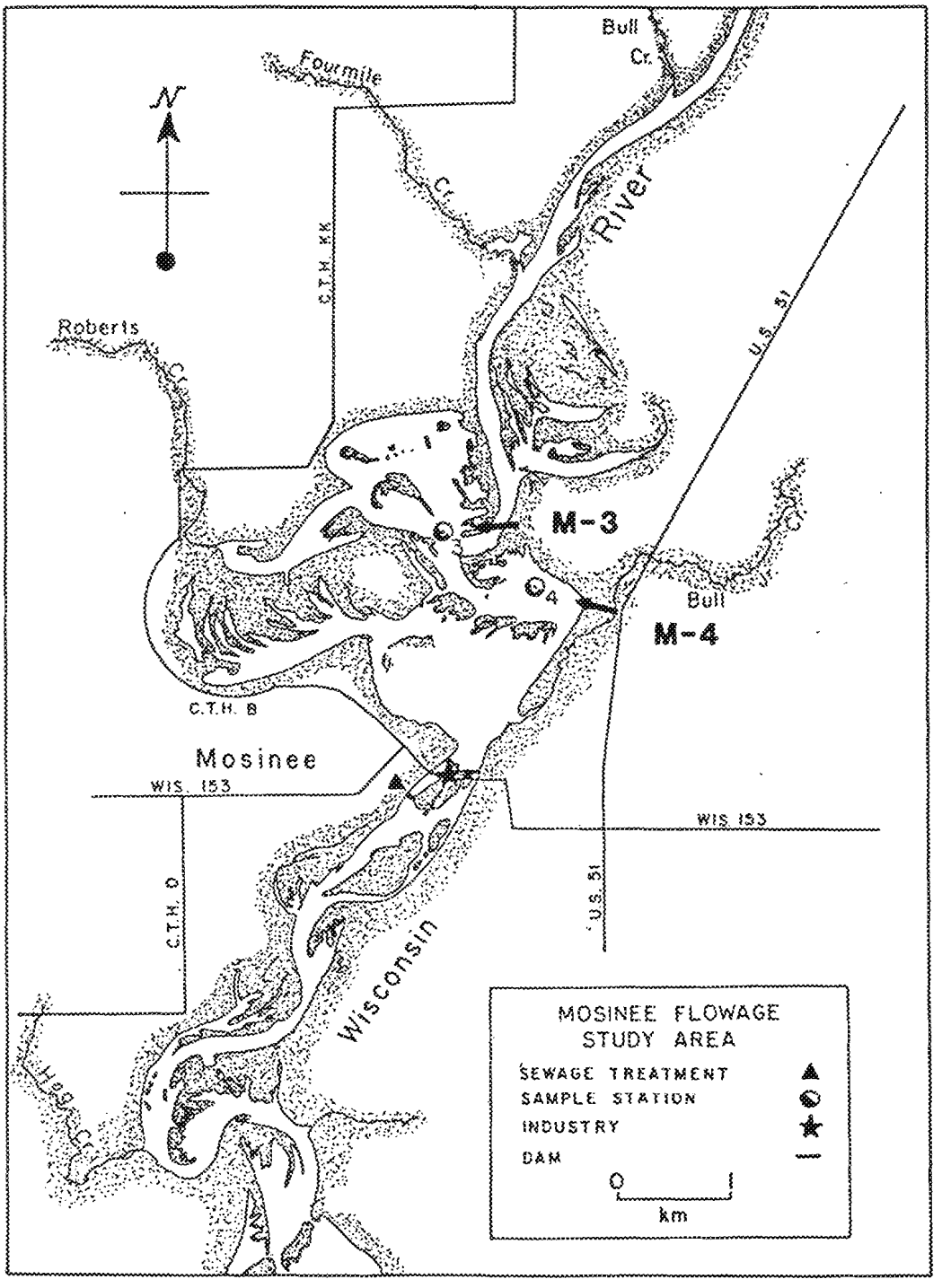

Big Eau Plein R.
N
U.S. 51
Pepling Cr.
Wisconsin
D-6
Knowlton
C.T.H. C
C.T.H. C
River
Johnson Cr.
Little Eau Pleine R.
Little Eau Claire
C.T.H. E
U.S. 51
LAKE DuBAY
STUDY AREA
SAMPLE STATION
DAM
0
1
km

Figure 4. Mosinee Flowage study area.


Bull
Cr.
Fourmile
Cr.
N
C.T.H. KK
River
Roberts
Cr.
U.S. 51
M-3
O4
Bull
M-4
Cr.
C.T.H. B
Mosinee
WIS. 153
WIS 153
C.T.H. O
U.S. 51
Wisconsin
MOSINEE FLOWAGE
STUDY AREA
SEWAGE TREATMENT
SAMPLE STATION
INDUSTRY
DAM
0
km

was located in a stump field on the extreme north shore of Lake Mosinee (Fig. 4). The water depth was approximately 1 m. Wood chips and fibers were present in sediments in both study areas.

Lake Wausau (Fig. 5) is formed by the Rothschild dam. It has a surface area of 7.76 km^2, a volume of 1.72 x $10^7 m^3$, and a mean depth of 2.2 m. The turnover time is 2 days and trapping efficiency is 18%. The drainage basin for this reservoir covers 10,360 km^2. One sewage treatment plant (RM 263.8) is located along this stretch of river. Lake Wausau sediments vary from silty sands to silty clays. Clay concentrations generally increase with distance downstream in the reservoir (25).

Lake Wausau samples were collected from a site (W-4) located approximately 300 yards north of the Rothschild dam in a dike area (Fig. 5). The water depth was approximately 4 m. Processed wood chips and fibers were present in the sediments.

Between Wausau and Brokaw (Fig. 6), the Wisconsin River is free-flowing and is not impeded by dams. Sediments are mainly clay-filled sands and silty clays (25). A municipal sewage treatment plant (RM 270.4) and a pulp and paper mill (RM 271.0) are located on this stretch of the river.

Brokaw samples were collected from a site (B-1) located in a small, backwater area approximately 1/2 mile south of the sewage treatment plant (Fig. 6). The water depth was approximately 0.5 m and the surface of the water was oily from upstream discharges.

Findley et al. (25) provided data on sediment analyses and mercury contents present in the sampling areas of the Upper Wisconsin River. Table 3 shows the sediment composition of the mercury content of the

Figure 5. Lake Wausau study area.

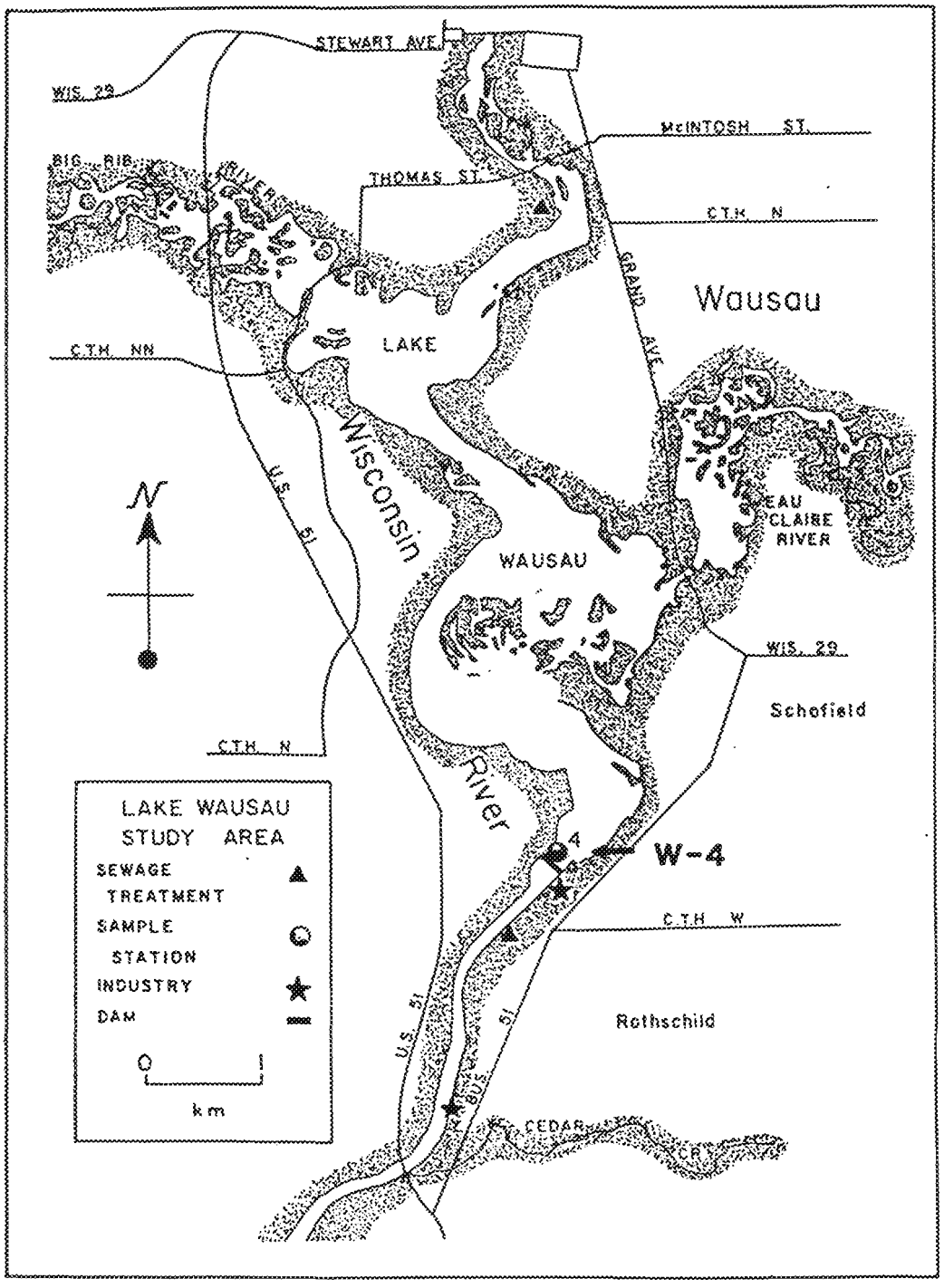

STEWART AVE
WIS 29
McINTOSH ST
BIG RIB
RIVER
THOMAS ST
C.T.H. N
GRAND AVE
Wausau
LAKE
C.T.H. NN
Wisconsin
U.S. 51
EAU CLAIRE RIVER
WAUSAU
WIS 29
Schofield
C.T.H. N
River
LAKE WAUSAU STUDY AREA
SEWAGE TREATMENT
SAMPLE STATION
INDUSTRY
DAM
km
W-4
C.T.H. W
U.S. 51
Rothschild
CEDAR CR

Figure 6. Brokaw to Wausau study area.

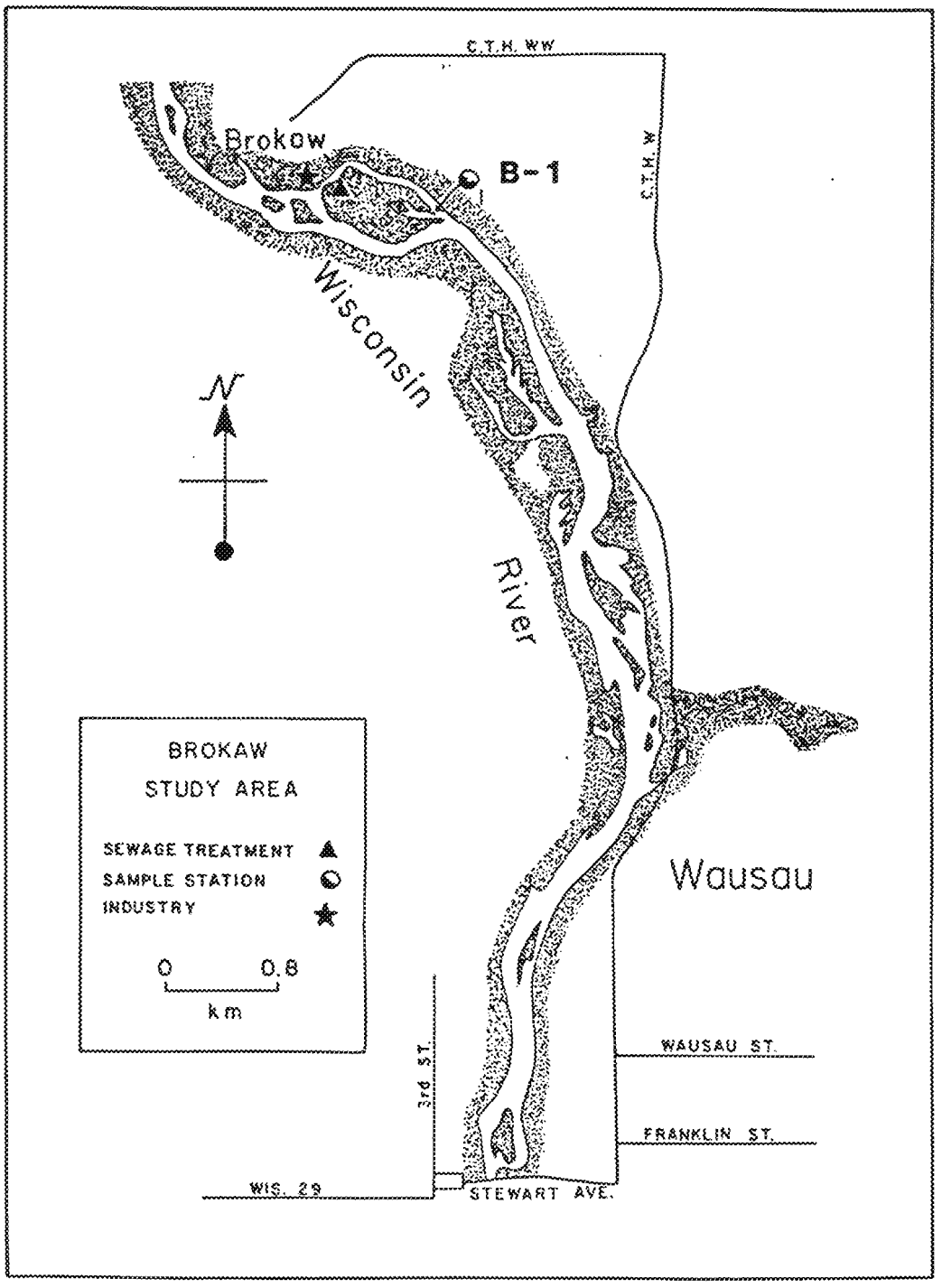

C.T.H. WW
Brokow
B-1
C.T.H. W
Wisconsin
River
N
BROKAW
STUDY AREA
SEWAGE TREATMENT
SAMPLE STATION
INDUSTRY
0
0.8
km
Wausau
WAUSAU ST.
FRANKLIN ST.
3rd ST.
WIS. 29
STEWART AVE.

Table 3. Sediment texture, organic content, and mercury concentrations of bottom sediments collected from the Upper Wisconsin River during August, 1981 (25).

Location	Site	Depth(cm)	Textured comp(%) Clay	Silt	Sand	Organic content(%)	Mercury content(µg/g)
Lake DuBay	4*	0-7	5.7	15.9	78.4	1.9	0.02
		7-14	7.0	21.9	71.2	1.9	0.02
Lake Mosinee	3	0-7	49.3	43.3	7.3	17.1	0.53
		7-14	45.8	49.5	4.7	17.0	0.57
	4	0-7	13.8	32.5	53.8	8.2	0.36
		7-14	14.5	32.0	53.5	8.3	0.32
Lake Wausau	4	0-7	40.4	51.2	8.4	16.6	0.39
		7-14	37.0	54.6	8.4	20.4	0.40
Brokaw	1	0-6	38.3	49.8	11.9	20.4	1.76
		6-11	45.4	46.5	8.2	19.7	2.09

*Sampling site 4 was located near site D-6 but is not data from D-6. Site D-6 was not examined by Findley et al. (25).

sediment at the Upper Wisconsin River study sites. In general, mercury content of the sediments increased with increasing organic content. Sampling site B-1 had the highest concentration of mercury in its sediments. Site D-4 had the lowest concentration. Sampling site D-4 was relatively low in organic material and consisted mainly of sand. In this study, site D-6 was used for all comparison studies. Site D-6 was located near site D-4 and the sediment was similar. All other sampling sites had high percentages of clay and silt in the sediments.

METHODS AND MATERIALS

Collection and Processing of Samples

Sediment Sampling

Sediment and sediment-floc samples were collected with an Ekman dredge. The Ekman dredge collected approximately a 4-liter sample from the top 10-12 centimeters of sediment. To collect sediment-floc samples, the Ekman grab was rocked until the floc was suspended in the overlying water. This suspension was then collected in a 50-ml screw cap test tube.

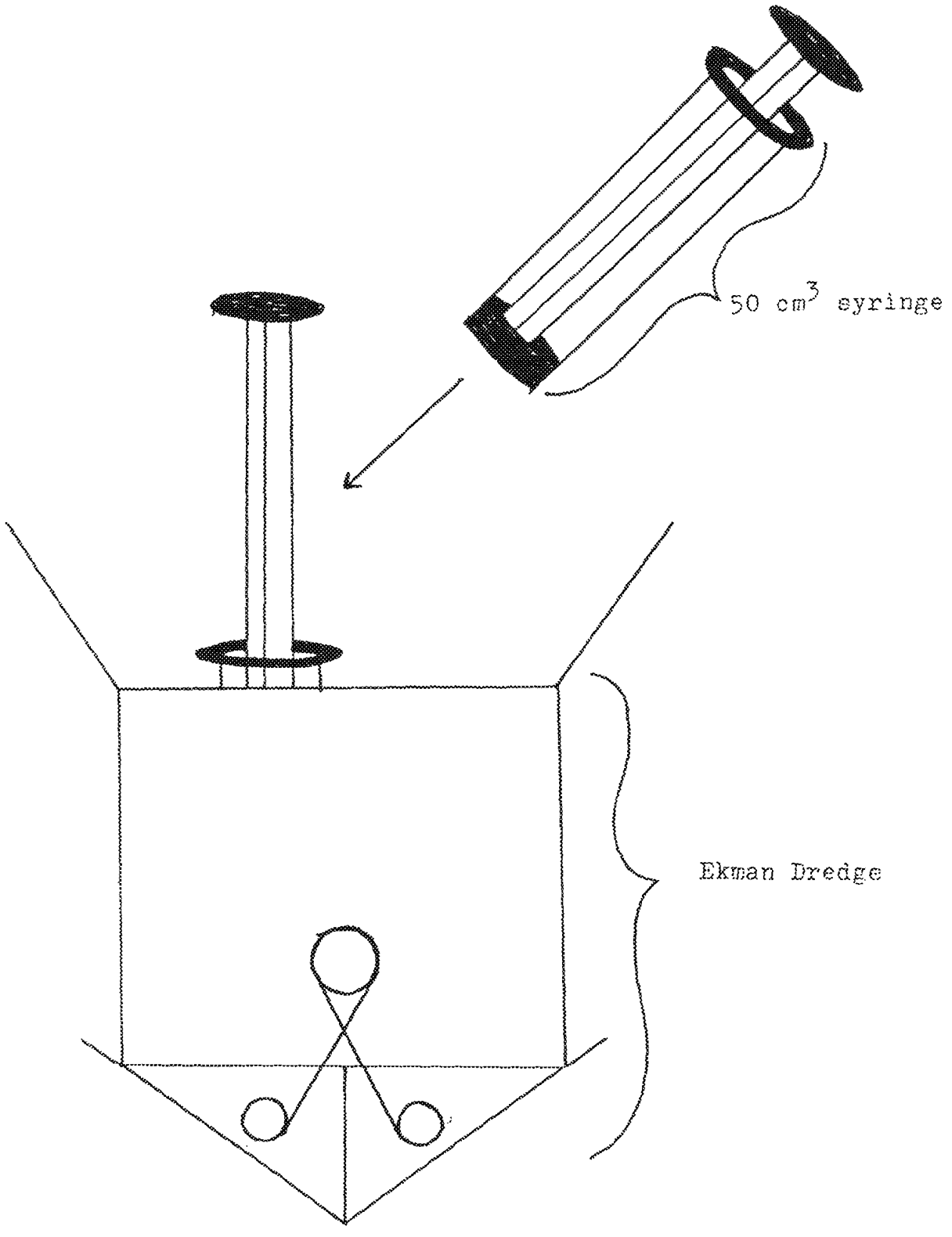
Sediment cores were collected from the center of an Ekman grab with a 50-ml plastic syringe with the top removed (Fig. 7). The plunger of the syringe was held fixed at the surface of the sediment while the 50 cm^3 tube was pushed down into the sediments. This resulted in the collection of an intact core in the syringe. After collection, syringes were sealed with rubber stoppers to prevent loss of sediment.

To collect bulk sediments, an Ekman grab of sediment was placed into a plastic dish pan, homogenized, and transferred into an amber glass jar. The jars were completely filled and sealed with butyl rubber stoppers to minimize further oxygen contamination.

Water Sampling

Water samples were obtained from approximately 0.5 m above the sediment-water interface. Three different methods were used for collecting water. Where possible, a 1-L plastic bottle was lowered and filled by hand from the desired depth. In deep water, a horizontal Van Dorn water sampler was used, but close intervals could not be sampled. After

Figure 7. Method for collecting sediment cores.


50 cm3 syringe
Ekman Dredge

October 1982, water samples were routinely collected using a peristaltic pump (Horizon Ecology Company). The pump was connected to a thick walled 3/16 inch tygon® tubing that was weighted on one end. The weighted end was lowered to a depth of 0.5 m above the sediments and water was pumped directly into a 1-L plastic bottle. All samples were stored on ice and transported as quickly as possible to the laboratory for processing. Transportation time was 2-4 h.

Physical Measurements

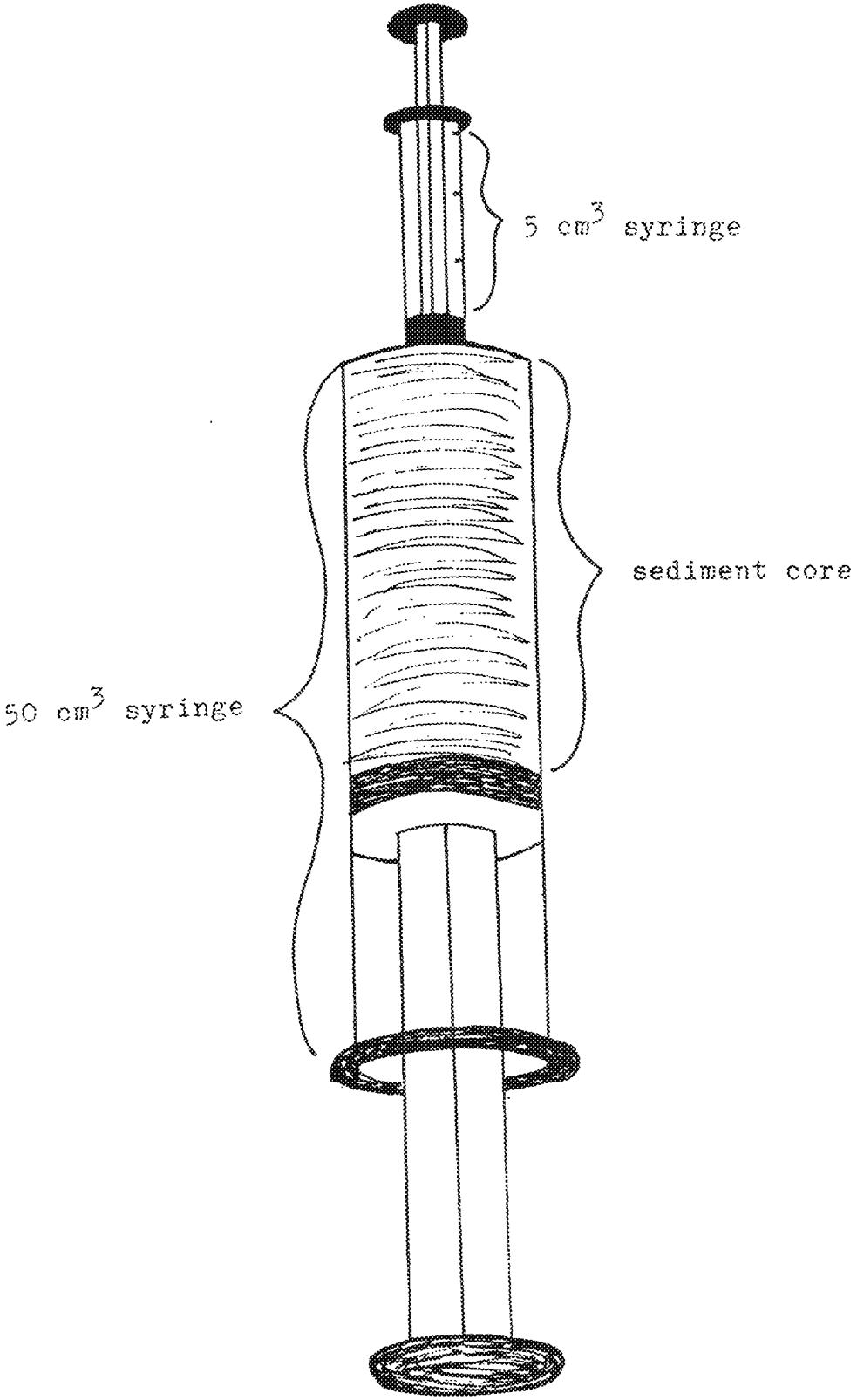
Temperature and dissolved oxygen measurements were recorded at all sampling sites using a dissolved oxygen meter (Yellow Springs Instrument--Model 54). Readings were taken at 0.5 m intervals from the water surface to the bed sediments. At sampling sites used for seasonal studies (B-1 and D-6), secchi disc readings were taken.

In the laboratory, water samples from B-1 (Brokaw) and D-6 (DuBay) were collected monthly from April - October to determine seasonal variations in turbidity. Turbidity readings were obtained using a Ratio® Turbidimeter (Hach Chemical Co.).

Sample Processing

All samples were processed immediately after returning from sampling. Sediment cores were sectioned by removing subcores from various intervals. The plunger of the 50-cm^3 coring syringe was used to push the desired sediment interval to the end so that a subcore could be extracted. A 5-ml plastic syringe with the tip removed was used to remove 1-ml subcores from the center of the sediment cores at various depth intervals (Fig. 8). This technique insured collection of completely anaerobic sediment subsamples. Oxygen readily diffuses through plastic and it was assumed that the outer sediments became exposed to oxygen during

Figure 8. Method for collecting sediment subcores.


5 cm3 syringe
sediment core
50 cm3 syringe

transport. Subcores were transferred to dilution blanks for enumeration experiments or to incubation bottles for methylation assays. One ml subcores were used for enumerations, and 1 ml or 3 ml subcores were used for methylation experiments. Methylation experiments were performed in duplicate on subcores from the same sampling site.

Anaerobic sediment slurries of B-1 and D-6 sediments were prepared by mixing sediments (vol/vol) with sterile, anoxic river water (2:1). Slurries were placed in amber glass bottles, gassed with N_2, sealed with rubber stoppers, and refrigerated. Slurries were then used as needed for methylation experiments. Water samples (15 ml) were taken as needed from 1-L plastic bottles and placed into test tubes or BOD bottles for methylation experiments.

Laboratory Analyses

Enumerations of Mercury-Resistant Heterotrophic Bacteria

Aerobic heterotrophic bacteria. Enumerations of mercury-resistant aerobic heterotrophic bacteria were determined using standard pour plates (2). Varying concentrations (7, 15, 37, 75 µg/ml) of mercuric ion (as $HgCl_2$) were added to test tubes containing 12 ml of Standard Plate Count Agar (2). Mercuric ion was added to the agar from a 5000 µg/ml stock ($HgCl_2$). Agar was kept molten in a 45-50°C water bath until used.

Sterile petri dishes were inoculated with 0.1 ml of serial tenfold dilutions, and agar was poured onto plates, mixed, and allowed to solidify. Samples from three dilutions of each sample were inoculated to insure countable plates. Plates were incubated at 20°C for 48 hours. After incubation, plates were counted to determine microbial resistance to

mercuric ion. The pour plate method was used due to initial problems encountered with spread plates. Inoculated spread plates were quickly overpopulated with colonies of Bacillus cereus var. mycoides and accurate colony counting was impossible. Overgrowth by Bacillus was not encountered with standard pour plate technique.

Anaerobic heterotrophic bacteria. All anaerobic culture media was prepared on the day before sampling using a variation of the anaerobic roll tube technique described by Hungate (41). Anaerobic growth media was Standard Plate Count Agar with added agar to give a final concentration of 2%. This prevented agar from slipping down the side of the roll tube. Varying concentrations of mercuric ion (7, 15, 37, 75 µg/ml) were added to the agar. All anaerobic tubes and vessels were continuously gassed with N_2 by inserting a bent gas cannulae made from a 2-in 12 gauge steel needle that was connected to a gas source. The gas was passed over heat copper filings to remove any trace of oxygen.

Resazurin (1 µg/ml from a 100 µg/ml stock solution) was added to all anaerobic media as a redox indicator. The media was boiled and cooled under a stream of O_2-free N_2 to remove all oxygen. After cooling, 0.01% (final concentration) cystein-HCl was added as a reducing agent. The pH of the media was then adjusted to 7 with 1N NaOH.

Molten anaerobic growth media (6 ml) was transferred to 18 x 142 mm anaerobic roll tubes (Bellco Glass, Inc.), gassed with N_2, and sealed with a no. 2 butyl rubber stopper. The stopper was moistened in distilled water before inserting it in the tube to form a better seal. This aided in maintaining anaerobic conditions over long incubation times.

Anaerobic roll tubes were kept molten until inoculation by storing in a water bath at 45-50°C.

Nine ml anaerobic dilution blanks (H_2O) were prepared using strict anaerobic techniques as described above. Initial dilutions were prepared by anaerobically transferring a 1 ml subcore to an anaerobically prepared 9 ml dilution blank. Subsequent ten-fold dilution series were prepared by anaerobically transferring 1.0 ml of inoculated dilution blanks to successive blanks.

Sterile N_2-purged plastic syringes (1.0 cm^3) were used to inoculate roll tubes with 0.1 ml of the appropriate dilutions. Three dilutions were used to obtain countable tubes (30-300 colony forming units). Inoculated roll tubes were gently mixed and spun on a roll tube spinner (Bellco Glass, Inc.) under ice water to harden the agar. Roll tubes were incubated at 20°C for 56 h. Colony forming units were then counted to determine mercuric ion resistance.

Binding of mercuric ion to aerobic and anaerobic culture media. Percentages of added mercuric ion being bound to aerobic and anaerobic culture media were determined to insure that differences in mercuric ion resistance were not due to differential binding of mercury in one of the culture media. Anaerobic and aerobic culture media samples (10 ml) were prepared. Broth was used instead of agar for the culture media due to difficulties encountered with centrifuging the agar. Samples with and without 75 µg/ml mercuric ion were inoculated with 0.1 ml of 10^{-2} dilution of sediments to obtain bacterial growth. Radiolabelled mercuric ion ($^{203}Hg(NO_3)_2$, 1 x 10^6 DPM) was added to all samples and samples were incubated at 20°C for 48 h. After incubation, samples were transferred to centrifuge tubes and centrifuged in a high speed

centrifuge (Beckman, Model J-21) at 17,600 x g for 10 min. Subsamples were then pipetted from the supernatant, transferred into scintillation vials containing PSC cocktail (Amersham Chemical Co.), and counted to determine the percentage of labelled mercury remaining unbound in the culture broth.

Mercury Methylation Activity Experiments

Most incubations were started immediately upon return from sampling. Some preliminary methylation experiments were performed on older sediment, however, unless otherwise stated, reported results were obtained from fresh sediments.

Radiolabelled mercuric ion was introduced into samples via a microliter syringe or Eppendorf micropipet. For all samples, 1-4.5 μCi of $^{203}Hg(NO_3)_2$ was added to samples (Table 4). Each batch of $^{203}Hg(NO_3)_2$ was diluted to a stock of 400 μCi/ml. To avoid adding varying amounts of mercury to each experiment, the same molar amount was added for each batch and the amount of radioactivity varied with decay.

Sample preparation. Seasonal methylation activity experiments were performed on sediments, sediment-floc, and water. Samples were transferred into 30-ml screw cap test tubes, $^{203}Hg^{+2}$ was added, mixed, and samples were incubated at 20°C.

Homogenized sediments (5 ml-wet weight) for seasonal sediment methylation activity were transferred via a 5 cm^3 plastic syringe into test tubes. Sterile river water (10 ml) was then added to test tubes. Anaerobic techniques were not employed with seasonal studies; however, control experiments showed that methylation activity in these sediments was the same as that observed in identical samples incubated in BOD bottles using strict anaerobic techniques (Table 5). These results

Table 4. Amount of $^{203}Hg(NO_3)_2$ added to mercury methylation experiments.

Assay date of batch	Specific Activity (μCi/μg)	μCi added	μg added/ 10μl
9/28/81	0.96	1.3 - 4.0	8.34
7/16/81	0.96	1.2 - 4.5	4.65
10/16/81	1.46	1.1 - 4.0	2.74

Table 5. Mercury methylation activity in aerobically and anaerobically prepared sediment samples.

Sample Preparation	Percent Methylation[a]
Aerobic (test tube)	2.58 ± 0.02
Anaerobic (BOD bottle)	2.60 ± 0.06

[a] ± 2SD, n = 2

$p \leq 0.05$

suggest that sediments for seasonal studies rapidly became anaerobic. Floc and water samples were prepared in test tubes using 10 and 15 ml, respectively.

All other methylation activity experiments were performed using strict anaerobic techniques. Previously prepared sediment slurry (10 or 20 ml) and $^{203}Hg^{+2}$ were added to 60-ml BOD bottles (Wheaton Company) under a steady stream of O_2-free N_2 gas. All additions were made from sterile, anoxic stock solutions stored in gassed, sealed serum bottles. After inoculation, BOD bottles were gassed for 30 s and sealed with glass stoppers. The glass stoppers were moistened before sealing to make a better seal and help insure anaerobic conditions. BOD bottles were shaken with a vortex apparatus to insure complete mixing. The rims of sealed BOD bottles were covered with water to maintain an air tight seal and then sealed with parafilm to prevent evaporation.

In experiments where aerobic conditions in sediments were desired, 300-ml BOD bottles were inoculated, sealed with cotton plugs, and incubated on a rotary shaker. Aerobic water samples were incubated in 60-ml BOD bottles and stoppered with loosely fitting glass stoppers. Whenever possible, samples were prepared in duplicate or triplicate and incubated with "killed" control cultures. Control cultures were killed and incubation terminated by the addition of 6N HCl (1 ml). After acidification, samples could be stored for up to five days without loss of activity (32). All samples were incubated for four days at 20°C unless otherwise stated.

Methylmercury assay procedure. All mercury methylation activity assays were performed using techniques developed by Uthe et al. (104)

as modified to water and sediment samples by Furutani and Rudd (32). This procedure involved extracting the methylmercury into toluene as methylmercuric bromide, partitioning the bromide salt into 20% ethanol as a thiosulfate complex, and re-extracting into benzene as methylmercuric iodide. To accomplish this, the following steps were used (Fig. 9):

1) After "killing", 2.0 ml of 0.5 M $CuSO_4$ and 5.0 ml of 3 M NaBr in 11% H_2SO_4 were added to samples to convert methylmercury into methylmercuric bromide. Samples were shaken by hand for 1 min to ensure complete conversion to methylmercuric bromide.

2) After shaking, sediment samples were quantitatively transferred into 30-ml glass centrifuge tubes and centrifuged for 5 min.

3) After centrifuging, all of the supernatant, which contained soluble methylmercury as methylmercuric bromide, was transferred into a 125- or 250-ml glass separatory funnel containing 25 ml of glass-distilled toluene (Fisher Scientific Co.) or scintanalized toluene (Fisher Scientific Co.). Water samples were added directly to separatory funnels.

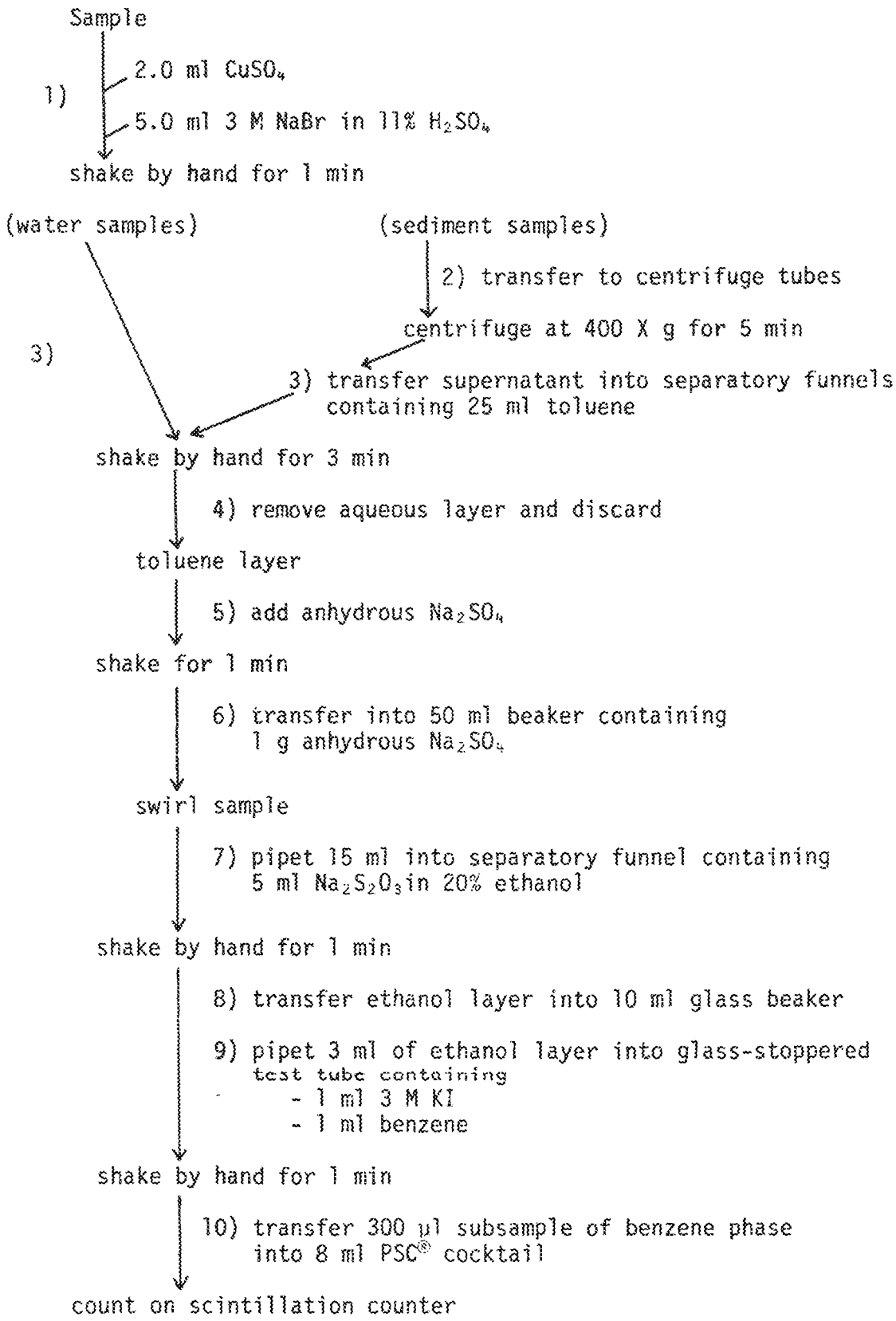
4) All separatory funnels were shaken by hand for 3 min and the resulting layers were allowed to separate. After complete separation of the layers, the methylmercuric bromide was present in the toluene layer. The aqueous layer (bottom) was removed and discarded.

5) Approximately 1 g of anhydrous sodium sulfate was added to the toluene layer as a drying agent, and the samples were shaken for 1 min to remove water.

6) The toluene phase was then transferred into an acid-washed 50-ml glass beaker containing approximately 1 g of anhydrous sodium sulfate. Samples were swirled in beakers to insure complete removal of water.

7) The dried toluene (15 ml) containing the methylmercuric bromide was pipetted into a second acid-washed 125-ml glass separatory funnel containing 5.0 ml of 0.0025 N sodium thiosulfate in 20% ethanol. Samples were shaken by hand for 1 min to partition the methylmercuric bromide into the aqueous ethanol as a thiosulfate complex.

Figure 9. Methylmercury assay procedure.


Sample
1)
2.0 ml $CuSO_4$
5.0 ml 3 M NaBr in 11% H_2SO_4
shake by hand for 1 min
(water samples)
(sediment samples)
2) transfer to centrifuge tubes
centrifuge at 400 X g for 5 min
3)
3) transfer supernatant into separatory funnels containing 25 ml toluene
shake by hand for 3 min
4) remove aqueous layer and discard
toluene layer
5) add anhydrous Na_2SO_4
shake for 1 min
6) transfer into 50 ml beaker containing 1 g anhydrous Na_2SO_4
swirl sample
7) pipet 15 ml into separatory funnel containing 5 ml $Na_2S_2O_3$ in 20% ethanol
shake by hand for 1 min
8) transfer ethanol layer into 10 ml glass beaker
9) pipet 3 ml of ethanol layer into glass-stoppered test tube containing
- 1 ml 3 M KI
- 1 ml benzene
shake by hand for 1 min
10) transfer 300 µl subsample of benzene phase into 8 ml PSC® cocktail
count on scintillation counter

8) After the separation of layers, the lower ethanol layer was transferred into an acid-washed 10-ml glass beaker.

9) The aqueous ethanol phase (3.0 ml) was then pipetted into a 15-ml screw-cap or glass-stoppered test tube that contained 1.0 ml of 3 M potassium iodide and 1.0 ml of glass-distilled benzene (Fisher Scientific Co.). The test tubes were shaken by hand for 1 min to transfer the methylmercuric iodide into the benzene layer.

10) Subsamples (300 µl) of the benzene layer (containing the extracted monomethyl mercuric iodide) was transferred via a 100-µl pipet (Eppendorf) into a plastic scintillation vial containing 8.0 ml PSC cocktail (Amersham). Samples were mixed and counted on a scintillation counter (Beckman, Model LS 7500).

This procedure did not account for methylmercury remaining in the interstitial water. To correct for this, sediment pellets remaining in centrifuge tubes were weighed to the nearest 0.01 g and then dried at 105°C to a constant weight. The difference yielded the amount of water remaining in the sediments. Results were corrected to account for the percentage of methylmercury present in the water remaining in the sediment pellet.

Isotope counting procedures. ^{203}Hg is a beta-gamma-emitting isotope. For this reason, samples could be counted on a beta or gamma scintillation counter. All radiolabelled methylmercury formed during methylation experiments was counted using a programmable beta scintillation counter (Beckman, Model LS 7500). Samples were counted for 20 min or until counts were within a 2 sigma percent error.

Counting efficiency was monitored using a previously prepared quench curve. Prepared standards of ^{203}Hg were not available for this research. Instead, quench curves of isotope standards having energies slightly above and below ^{203}Hg were used. Commercially

prepared standards of carbon-14 (β^-=0.159 MeV) were used to construct the lower energy curve, while technetium-99 (β^-=0.29 MeV) standards were prepared in the laboratory to construct the higher energy curve. Technetium-99 quenched standards were prepared by adding varying concentrations of acetone (0.1 - 1.0 ml) to scintillation vials containing 15,000 DPM ^{99}Tc.

The counting efficiency of quenched samples was compared using the channels ratio method and the automatic external standardization (H-number). Discriminations for channel 1 were set at 400 and 800 to monitor the upper end of the energy spectrum. Discriminations for channel 2 were set at 0 and 800 to include most of the pulse height distribution of ^{203}Hg. A ratio of the counts in the two channels was then obtained. The window in channel 1 was set too wide. Therefore, channel ratios varied little with decreased efficiency. For this reason, the automatic external standardization (H-number) was used to construct the quench curve (Fig. 10). The quench curve for ^{203}Hg (β^-= 0.21 MeV) was extrapolated to fit between ^{99}Tc (β^-=0.29 MeV) and ^{14}C (β^-=0.159 MeV).

Calculations. The percentage of added radiolabelled mercuric ion methylated was determined by the following equation:

$$\text{percent methylation} = \left(\frac{[\text{DPM counted} \times \text{df}] + X[\text{DPM counted} \times \text{df}]}{Y} \right) 100$$

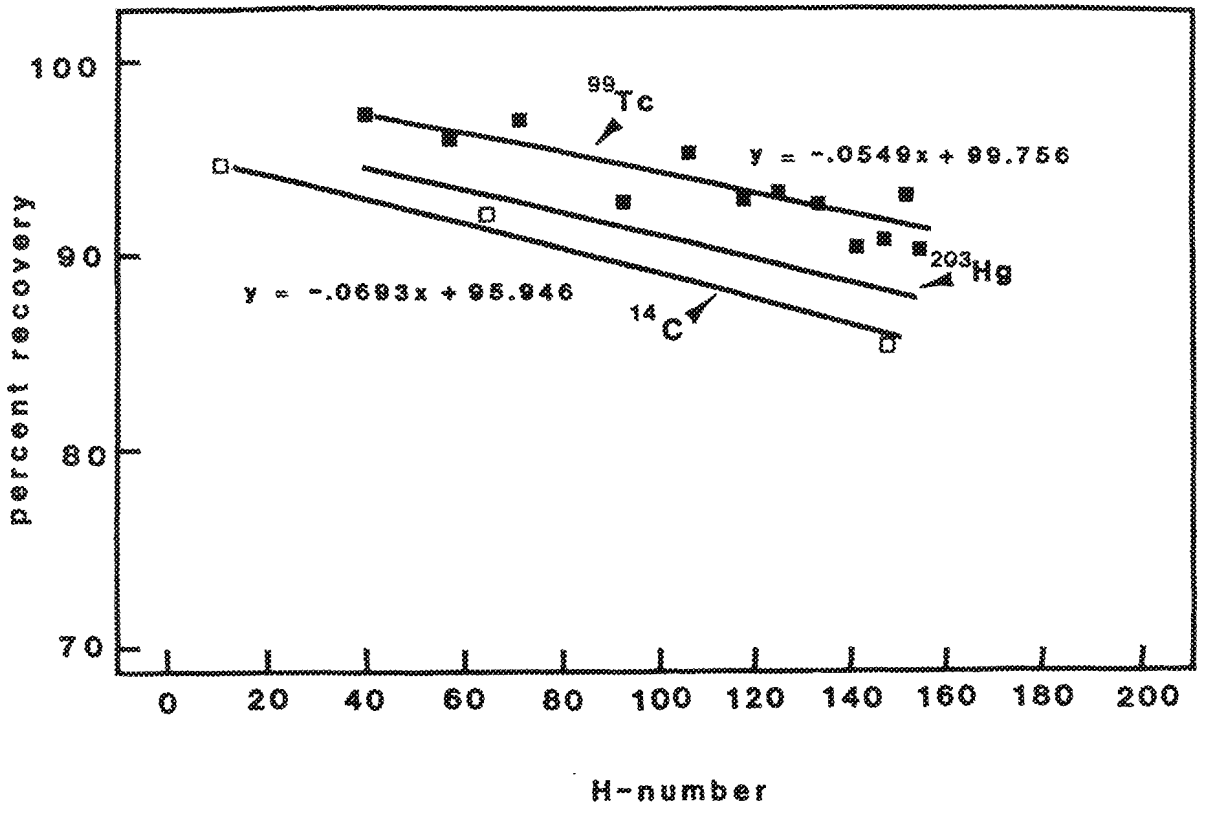
where DPM counted = DPM from scintillation counting

$$\text{df} = \text{dilution factor} = \left(\frac{25\text{ml}}{15\text{ml}}\right)\left(\frac{5.0\text{ml}}{3.0\text{ml}}\right)\left(\frac{1.0\text{ml}}{0.3\text{ml}}\right) = 9.29$$

Y = DPM's ^{203}Hg added to sample

X = percentage of H_2O remaining in sediment pellet

Figure 10. Mercury-203 quench curve.


100
90
80
70
percent recovery
^{99}Tc
y = -.0549x + 99.756
^{203}Hg
y = -.0693x + 95.946
^{14}C
0
20
40
60
80
100
120
140
160
180
200
H-number

The half-life of ^{203}Hg is 46 days. To monitor this, the decay rate of the ^{203}Hg was determined as follows:

$$\% \text{ radioisotope left} = 100e^{\frac{-0.693t}{x}}$$

where e = natural log base

t = elapsed time expressed in the same units as the half-life

x = half-life of ^{203}Hg = 46 days

The amount (μg) of methylmercury formed was determined by the following equation:

μg methylmercury = (x)(y)

where x = μg Hg^{+2} added to the sample*

y = percentage of added ^{203}Hg methylated

*the amount (μg) of Hg^{+2} added was calculated as follows:

$$\mu g/\mu l = \left(\frac{\mu Ci/\mu l}{(SA)\ (\mu Ci/\mu g)}\right)$$

where SA = specific activity of the Hg^{+2}

Binding of $^{203}Hg^{+2}$ to Sediment

The percentage of added ^{203}Hg bound to sediment was determined by adding ^{203}Hg to 10 ml of sediment slurry in a test tube, mixing with a vortex apparatus, and incubating samples for varying lengths of time. After incubation, sediments were quantitatively transferred with 10 ml of H_2O into plastic 30-ml centrifuge tubes. Samples were centrifuged at 17,600Xg for 10 min. An aliquot of supernatant (0.5 ml) was then transferred into a plastic gamma-counting tube and counted as described above.

Separate samples were dried at 105°C to constant weight, and the amount of H_2O present was calculated. To determine the amount of ^{203}Hg remaining unbound in the water, the following equation was used:

$$\text{percentage unbound } ^{203}Hg = \left(\frac{(x)(y)}{\text{standard}}\right)100$$

where x = DPM in 0.5 ml H_2O

$$y = \text{dilution factor} = \left(\frac{\text{ml } H_2O \text{ in sample*}}{0.5 \text{ ml}}\right)$$

standard = DPM ^{203}Hg added to sample

* = supernatant + water in sediment

Quality Control

Methylmercury Extraction Efficiency

To determine the extraction efficiency, each extraction step in the assay procedure was extracted a second time and the rest of the analysis was carried to completion. This provided a determination of the amount of methylmercury not extracted at each extraction. Figure 11 shows where second extractions were performed in the assay procedure. Radioactive methylmercury in the original and second extractions were counted after completion to determine the percentage of recovered methylmercury. The DPM of $CH_3{}^{203}Hg^+$ recovered with the first extraction is compared to the DPM of $CH_3{}^{203}Hg^+$ recovered with the second extractions in Table 6. Approximately 99% of the radiolabelled methylmercury was recovered with the first extraction.

Methylation Kinetics

Mercury-203 is only commercially available at fairly low specific activities. This resulted in the addition of large molar amounts of

Figure 11. Double extraction procedure for methylmercury assay.

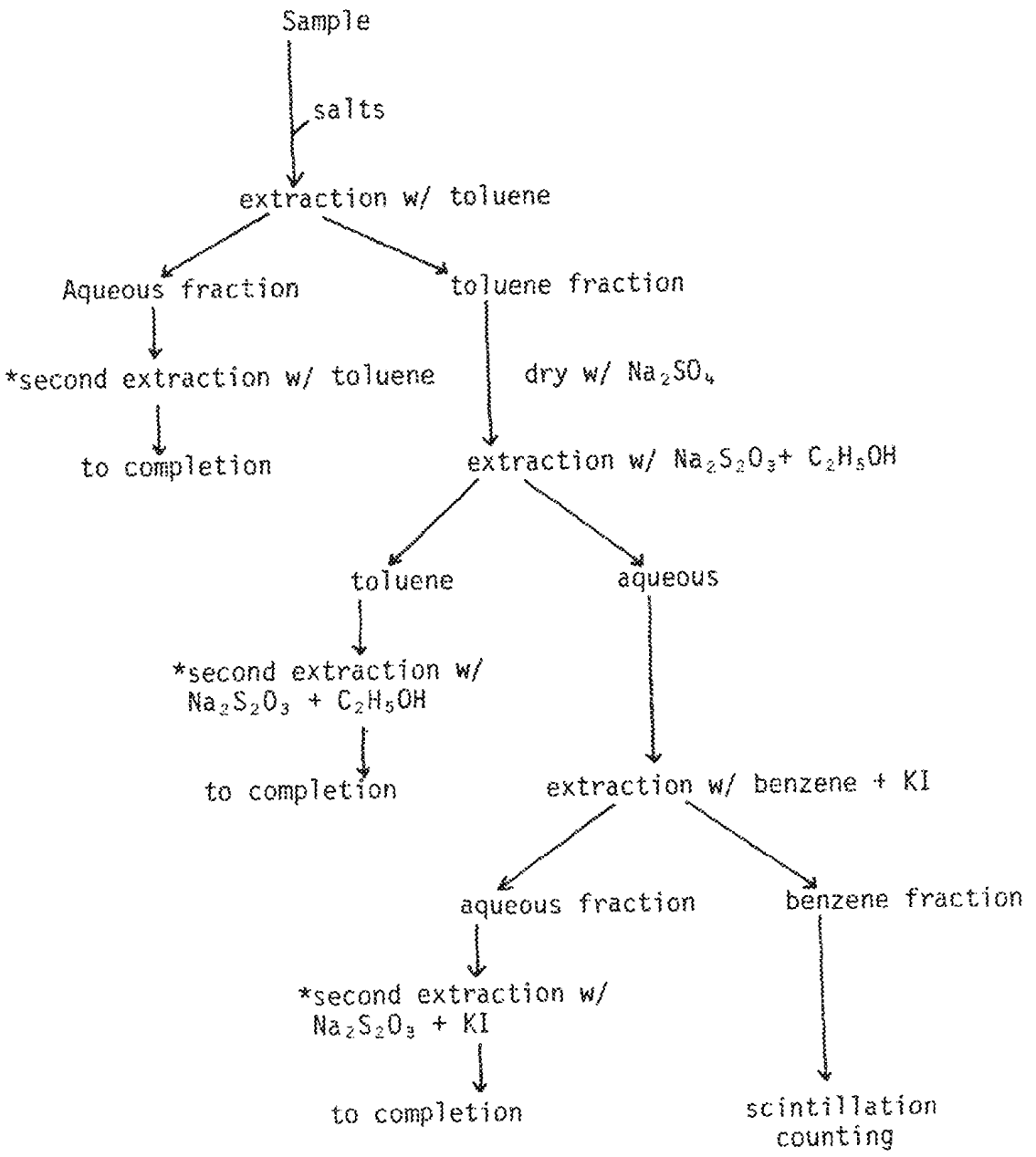

Sample
salts
extraction w/ toluene
Aqueous fraction
toluene fraction
*second extraction w/ toluene
dry w/ Na_2SO_4
to completion
extraction w/ $Na_2S_2O_3$+ C_2H_5OH
toluene
aqueous
*second extraction w/ $Na_2S_2O_3$ + C_2H_5OH
to completion
extraction w/ benzene + KI
aqueous fraction
benzene fraction
*second extraction w/ $Na_2S_2O_3$ + KI
to completion
scintillation counting

Table 6. Efficiency of methylmercury extraction procedure.

Extraction counted	DPM $^{203}CH_3Hg^+$/sample
complete extraction of sample	49939
2nd toluene extraction	Not detectable
2nd sodium thiosulfate extraction	Not detectable
2nd benzene extraction	600
Total DPM recovered	50539

Percent efficiency of first extraction = $\frac{49939}{50539} \times 100 = 99$

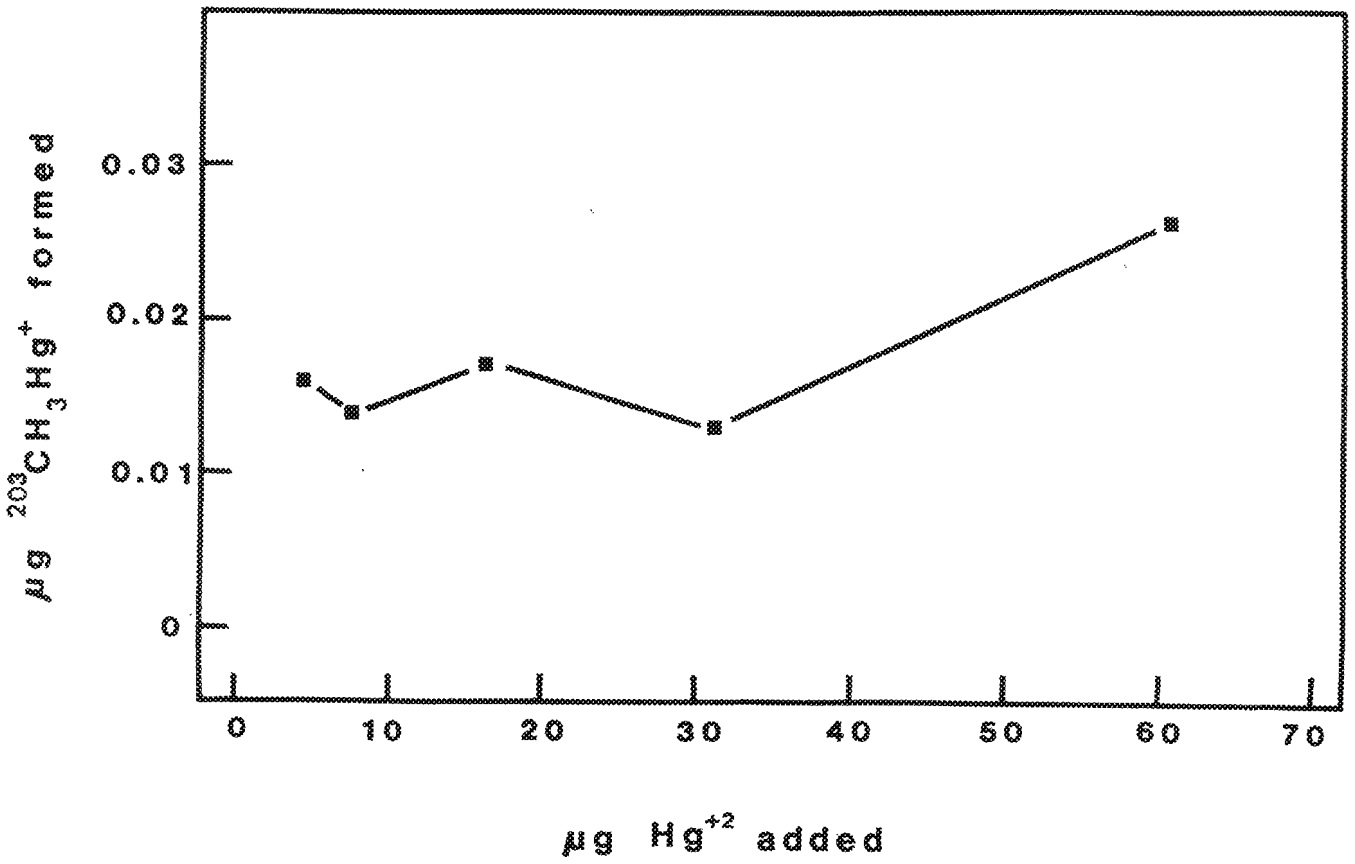
Hg^{+2} in isotope experiments. For this reason, an experiment was performed to evaluate the kinetics of the methylmercury formation when varying amounts of unlabelled mercuric ion were present in the sediments.

At all concentrations of unlabelled Hg^{+2} in the sediments, essentially the same amount (μg) of the added Hg^{+2} was methylated (Fig. 12). This linear response indicated that the amount of Hg added in isotope experiments saturated the reaction. This means that we did not affect the net methylation rate by adding varying amounts (μg) of Hg^{+2}. Therefore, the experimental methylation percentage is an accurate representation of methylation activity taking place even though varying amounts of mercuric ion was added with different batches.

Statistical Analysis of Data

All experiments were performed using a minimum of duplicate samples, although triplicate or quadriplicate samples were run when possible. Variations between sets were evaluated using standard deviations and pooled t-tests and results were reported as significant ($p \leq 0.05$) or highly significant ($p \leq 0.01$).

Figure 12. Kinetics of methylmercury formation in B-1 sediments.


μg $^{203}CH_3Hg^+$ formed
0.03
0.02
0.01
0
0
10
20
30
40
50
60
70
μg Hg^{+2} added

RESULTS

Enumerations of Mercury-Resistant Heterotrophic Bacteria

Aerobic Heterotrophic Resistance

Total aerobic heterotrophs and mercury-resistant heterotrophs were enumerated in water, sediment-floc, and surface sediments (0-1 cm). Table 7 shows an estimate of the numbers of aerobic heterotrophs present in samples collected on June 16. Resistance of bacteria to 7 μg/ml Hg^{+2} ranged from 0.3-100%, while resistance to 74 μg/ml ranged from 0-0.5% from the June 16 sampling trip (Table 8). In general, the highest aerobic resistances were exhibited in the water samples. All samples had bacteria resistant to 7 μg/ml Hg^{+2}. Minimal resistance was observed at concentrations greater than 7 μg/ml Hg^{+2}. Similar results were obtained when this experiment was repeated.

The seasonal variation in anaerobic heterotrophs and mercuric ion resistant heterotrophic bacteria at site B-1 was determined (Table 9). The seasonal variation in mercury resistance of aerobic heterotrophic bacteria at site B-1 was also determined (Table 10). Large fluctuations in resistance to 7 μg/ml of Hg^{+2} were observed throughout the sampling season; however, resistance to higher concentrations (>15 μg/ml) remained low. The samples collected during July 25 and October 1 were 100% resistant to 7 μg/ml Hg^{+2} and resistance to higher concentrations of Hg^{+2} were generally greatest in these samples. Lower resistance was observed in all samples from the June 16 sampling trip. Throughout the sampling season, the highest percentage of mercury-resistant aerobic heterotrophic bacteria were observed in water samples.

Table 7. Density of aerobic heterotrophic bacteria in the Upper Wisconsin River (16 June 1982).

Sampling site	Sample	Density (CFU[a]/ml x 10^{-3})
B-1	H_2O	1.3
	Floc	15.0
	0-1 cm	88.0
W-4	H_2O	0.3
	Floc	14.0
	0-1 cm	300.0
M-3	H_2O	0.5
	Floc	28.0
	0-1 cm	290.0
M-4	H_2O	2.3
	Floc	1.9
	0-1 cm	120.0
D-6	H_2O	3.0
	Floc	9.2
	0-1 cm	150.0

[a]Colony forming units

Table 8. Percentage of aerobic heterotrophic bacteria displaying resistance to mercuric ion in the Upper Wisconsin River (16 June 1982).

Sampling Site	Sample Type	Hg^{+2} concentration (µg/ml)			
		7	15	37	75
B-1	H_2O	24.6	13.1	0.0	0.0
	Floc	6.0	0.1	0.1	0.1
	0-1 cm	0.3	0.1	0.1	0.1
W-4	H_2O	52.9	1.2	0.0	0.0
	Floc	30.7	3.7	0.4	0.5
	0-1 cm	30.7	0.8	0.1	0.1
M-3	H_2O	80.4	7.8	5.0	0.1
	Floc	8.2	0.5	0.4	0.1
	0-1 cm	23.1	0.1	0.1	0.1
M-4	H_2O	100.0	4.1	0.1	0.0
	Floc	14.7	3.2	0.5	0.5
	0-1 cm	25.0	0.1	0.1	0.1
D-6	H_2O	10.0	0.5	0.0	0.0
	Floc	15.2	0.3	0.1	0.1
	0-1 cm	6.6	2.7	0.1	0.1

Table 9. Seasonal variation in densities of standing crops of aerobic heterotrophic bacteria at site B-1, Upper Wisconsin River.

Sampling date (1982)	Sample	Density (CFU[a]/ml x 10^{-3})
April 30	H_2O	0.36
	Floc	16.0
	0-1 cm	170.0
June 5	H_2O	0.51
	Floc	4.2
	0-1 cm	130.0
June 16	H_2O	1.3
	Floc	15.0
	0-1 cm	88.0
July 25	H_2O	0.28
	Floc	8.0
	0-1 cm	80.0
September 2	H_2O	0.67
	Floc	13.0
	0-1 cm	25.0
October 1	H_2O	0.31
	Floc	3.9
	0-1 cm	87.0

[a]Colony forming units

Table 10. Seasonal variation in the percentage of aerobic heterotrophs that are resistant to mercuric ion at site B-1, Upper Wisconsin River.

Sampling date (1982)	Sample type	Hg^{+2} concentration (µg/ml)			
		7	15	37	75
April 30	H_2O	69.4	11.1	8.3	2.8
	Floc	28.8	5.3	2.3	0.3
	0-1 cm	44.7	0.6	0.1	0.1
June 5	H_2O	49.0	21.6	0.0	0.0
	Floc	28.6	4.5	2.6	1.5
	0-1 cm	5.5	3.2	0.1	0.2
June 16	H_2O	24.6	13.1	0.0	0.0
	Floc	6.0	0.1	0.1	0.1
	0-1 cm	0.3	0.1	0.1	0.1
July 25	H_2O	100.0	96.4	0.0	0.0
	Floc	100.0	65.0	5.6	0.1
	0-1 cm	100.0	100.0	3.9	0.4
September 2	H_2O	40.3	0.6	0.0	0.0
	Floc	10.8	0.1	0.1	0.1
	0-1 cm	0.4	0.4	0.4	0.4
October 1	H_2O	100.0	71.0	19.7	28.4
	Floc	100.0	4.1	0.3	0.3
	0-1 cm	100.0	1.1	1.1	1.1

Anaerobic Heterotrophic Resistance

Many of the bacteria in the Wisconsin River exist under anaerobic conditions. Therefore, it was necessary to determine the percentages of mercury-resistant anaerobic heterotrophic bacteria. The numbers of anaerobic heterotrophic bacteria persent at different depths in samples collected on July 11 (Table 11) were used to determine the percentages of these bacteria exhibiting resistance to varying concentrations (7, 15, 37, 75 µg/ml) of mercuric ion (Table 12). Resistance to 7 µg/ml of Hg^{+2} ranged from 7.6 to 100%, while resistance to 75 µg/ml ranged from 4.1 to 100%. Greater than 25% resistance to 75 µg/ml of Hg^{+2} was obtained in most samples. Anaerobic heterotrophic bacteria were more resistant to higher concentrations of Hg^{+2} than aerobic heterotrophic bacteria at all sites. Similar results were observed for both replicates.

Table 13 shows anaerobic heterotrophic bacteria obtained from site 8-1 sediments during the sampling season. High concentration of mercury-resistant anaerobic heterotrophic bacteria were recovered throughout the sampling season (Table 14). No seasonal variation in anaerobic heterotrophic resistance were observed. High resistance was observed during all sampling trips.

Anaerobic heterotrophic bacteria were more resistant to 75 µg/ml of Hg^{+2} than aerobic heterotrophic bacteria were to 37 µg/ml of Hg^{+2} in the water column and surface sediments (Fig. 13). It should be noted that aerobic and anaerobic heterotrophic resistance in water and sediment-floc were determined from the same samples. Therefore, these differences in resistance cannot be attributed to site variation.

Table 11. Densities of anaerobic heterotrophic bacteria in the Upper Wisconsin River (11 July 1982).

Sampling site	Sample type	Density (CFU/ml x 10^{-3})
B-1	Floc	1.7
	0-1	28.0
	1-3	22.0
	3-5	42.0
	8-10	18.0
W-4	Floc	1.8
	0-1	21.0
	1-3	8.3
	3-5	18.0
	8-10	17.0
M-3	Floc	0.91
	0-1	28.0
	1-3	31.0
	3-5	29.0
	8-10	17.0
M-4	Floc	0.71
	0-1	16.0
	1-3	14.0
	3-5	27.0
	8-10	33.0
D-6	Floc	0.96
	0-1	6.8
	1-3	11.0
	3-5	22.0
	8-10	18.0

Table 12. Percentages of anaerobic heterotrophic bacteria displaying resistance to mercuric ion in the Upper Wisconsin River (11 July 1982).

Sampling site	Sample type	Hg^{+2} concentration (μg/ml)			
		7	15	37	75
B-1	Floc	56.5	100.0	100.0	55.3
	0-1	60.7	100.0	64.3	42.9
	1-3	54.5	100.0	100.0	100.0
	3-5	42.9	100.0	100.0	61.9
	8-10	7.6	32.9	31.9	17.1
W-4	Floc	94.4	83.3	43.9	33.9
	0-1	66.7	100.0	52.4	100.0
	1-3	97.6	100.0	100.0	48.2
	3-5	94.4	100.0	100.0	33.3
	8-10	100.0	100.0	100.0	35.9
M-3	Floc	73.6	100.0	100.0	100.0
	0-1	60.7	53.6	82.1	32.9
	1-3	25.2	45.2	13.5	6.5
	3-5	75.9	30.3	27.9	4.1
	8-10	94.1	100.0	24.1	10.6
M-4	Floc	100.0	100.0	100.0	100.0
	0-1	75.0	100.0	100.0	36.3
	1-3	78.6	100.0	100.0	48.6
	3-5	70.4	100.0	100.0	51.9
	8-10	42.4	100.0	100.0	78.8
D-6	Floc	100.0	100.0	71.9	60.4
	0-1	100.0	100.0	100.0	86.8
	1-3	85.5	100.0	69.1	55.5
	3-5	40.9	90.9	63.6	21.4
	8-10	33.9	100.0	40.6	8.3

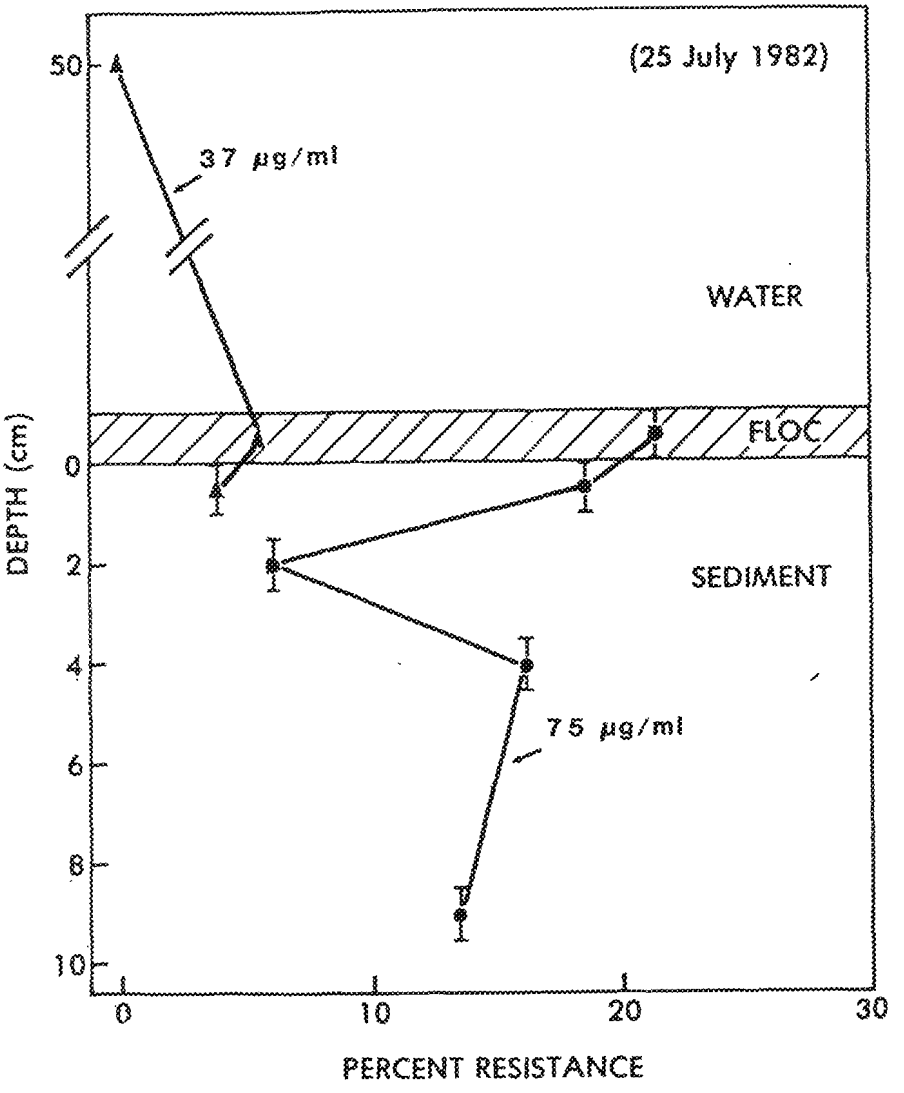
Table 13. Seasonal variation in anaerobic heterotrophic bacteria at site B-1, Upper Wisconsin River.

Sampling date (1982)	Sample type	Density (CFU/ml x 10^{-3})
May 2	Floc	1.1
	0-1	72.0
	1-3	21.0
	3-5	45.0
	8-10	44.0
July 11	Floc	1.7
	0-1	28.0
	1-3	22.0
	3-5	42.0
	8-10	210.0
July 25	Floc	13.0
	0-1	39.0
	1-3	28.0
	3-5	40.0
	8-10	54.0
September 2	Floc	3.2
	0-1	31.0
	1-3	41.0
	3-5	54.0
	8-10	49.0
October 1	Floc	4.0
	0-1	61.0
	1-3	90.0
	3-5	97.0
	8-10	120.0

Table 14. Percentages of anaerobic heterotrophic bacteria displaying resistance to mercuric ion at site B-1, Upper Wisconsin River, during each sampling date.

Sampling date (1982)	Sample type	Hg^{+2} concentration (µg/ml)			
		7	15	37	75
May 2	Floc	18.2	72.7	27.3	36.4
	0-1	9.3	84.7	43.1	100.0
	1-3	44.8	100.0	100.0	100.0
	3-5	37.8	100.0	97.8	100.0
	8-10	86.4	100.0	100.0	100.0
July 11	Floc	56.5	100.0	100.0	55.3
	0-1	60.7	100.0	64.3	42.9
	1-3	54.5	100.0	100.0	100.0
	3-5	42.9	100.0	100.0	61.9
	8-10	7.6	32.9	31.9	17.1
July 26	Floc	26.9	2.5	32.3	20.8
	0-1	59.0	56.4	14.6	18.7
	1-3	22.1	67.9	12.9	6.1
	3-5	95.0	72.5	17.5	16.3
	8-10	66.7	94.4	79.6	13.5
September 3	Floc	62.5	90.6	100.0	56.3
	0-1	90.3	100.0	100.0	67.7
	1-3	75.6	48.8	58.5	51.2
	3-5	72.2	57.4	83.3	20.4
	8-10	91.8	95.9	100.0	46.9
October 1	Floc	55.0	72.5	62.5	57.5
	0-1	45.9	85.2	47.5	29.5
	1-3	38.9	70.0	97.8	32.2
	3-5	70.1	89.7	33.0	52.6
	8-10	70.0	100.0	34.2	40.8

Figure 13. Depth distribution of mercury resistance at site B-1, Upper Wisconsin River (bars represent depth intervals of sediment core).


(25 July 1982)
37 µg/ml
WATER
FLOC
SEDIMENT
75 µg/ml
DEPTH (cm)
50
0
2
4
6
8
10
0
10
20
30
PERCENT RESISTANCE

This means that anaerobic heterotrophs were more resistant to Hg^{+2} than aerobic heterotrophs. Similar results were obtained at all sampling sites.

Binding of Mercury to Aerobic and Anaerobic Culture Media

The greater mercury resistance exhibited by anaerobic heterotrophs was not due to differential availability of mercury between the aerobic and anaerobic culture medias. This conclusion was based on an experiment performed to determine and compare the amount of added mercuric ion available in aerobic and anaerobic media. Binding of ^{203}Hg in sediment-inoculated aerobic and anaerobic broth media, media containing 75 µg/ml Hg^{+2}, and distilled H_2O controls was determined.

The percentage of soluble ^{203}Hg in the aerobic water controls was low (Table 15). This may have been due to greater binding of ^{203}Hg to the plastic petri dishes than to the glass roll-tubes. For this reason, percentages of soluble ^{203}Hg in all samples were calculated using the initial amount (DPM) of ^{203}Hg added to each sample as the standard.

It should be noted that this experiment compared the amount of soluble mercuric ion in the media. The soluble mercuric ion may have also been bound to organic substances in the media. I did not attempt to determine the type of mercury binding.

In all instances, recovery of ^{203}Hg was lower from samples containing 75 µg/ml of Hg^{+2} than from samples not containing mercuric ion. This was contrary to expectations, because it was anticipated that the previous addition of 75 µg/ml of Hg^{+2} would decrease the number of available binding sites. This should then lead to a greater recovery of soluble ^{203}Hg.

Table 15. Percentages of soluble ^{203}Hg recovered from aerobic and anaerobic culture media.

Environmental conditions	Soluble ^{203}Hg recovered (%)[a]	
	Aerobic	Anaerobic
H_2O	45.0 ± 4.4[b]	81.6 ± 27.4*
0 µg/ml Hg^{+2} (not inoculated)	88.0 ± 30.6	106.2 ± 29.8
0 µg/ml Hg^{+2} (inoculated)[c]	115.9 ± 24.0	31.4 ± 3.6**
75 µg/ml Hg^{+2} (not inoculated)	22.8 ± 8.6	65.6 ± 24.8*
75 µg/ml Hg^{+2} (inoculated)	30.4 ± 7.2	36.0 ± 15.6

[a] ± 2SD, n = 3, experimental means compared to each other using a t-test.

[b] n = 2.

[c] samples inoculated with B-1 sediments to get bacterial growth.

* $p \leq 0.05$

** $p \leq 0.01$

Each media condition yielded significant concentrations of soluble ^{203}Hg, indicating that bacterial cultures were exposed to mercuric ion. It would not be expected that 100% of the soluble mercuric ion would be bound to organic substances in the media. The recovery percentages in matching aerobic and anaerobic samples suggests that the heterotrophic bacteria were exposed to significant concentrations of unbound mercury.

Measurements of Mercury Methylation Activity

Mercury methylation activity was examined under a variety of conditions to evaluate potentials for mercury methylation in the Upper Wisconsin River. The majority of methylation experiments were performed on B-1 sediments because of the high methylation activity and the high mercury concentrations (25) observed in these sediments. These experiments were designed to determine where methylation activity was greatest in the core profile and the effect of changing environmental parameters on these activities. The mercury methylation percentages measured in these experiments indicates potential methylation activity, not actual rates occurring in the Upper Wisconsin River. This is because significant concentrations of Hg^{+2} were added to all samples because of the low specific activity of the mercury isotope. Methylation activity was not observed in any of the killed controls, which suggests that measured methylation potentials were due to biological activity and not abiotic processes.

Depth Distribution of Mercury Methylation

Throughout the sampling season, high methylation activity was observed in the sediments, whereas minimal to undetectable activities

were measured in the water column. Methylation assays were done on H_2O, sediment-floc, and various sections in sediment cores from site B-1 to determine the location of methylation in the sediments. In all sediment cores, methylation was highest in the surface sediments (0-1 cm); activity consistently decreased with depth (Table 16). Deeper sediment layers (9-10 cm) exhibited little methylation activity.

Figure 14 illustrates methylation activity with depth in core #1 from September 16, showing the peak of methylation activity in the surficial sediments. No methylation was detected in the water column. In the floc, 0.22% of the added ^{203}Hg was methylated. As previously stated, methylation activity was greatest in the surficial sediments and decreased with sediment depth.

Effect of Environmental Variables on Methylation Activity

Temperature. Methylation activity in Upper Wisconsin River sediments was lowest at 4°C, increased to maximum at 35°C, and decreased at temperatures above 35°C (Fig. 15). This optimum was considerably higher than the maximum *in situ* temperature of 24°C. Significant mercury methylation activity occurred at temperatures as high as 50°C.

Dissolved oxygen concentration. Significant mercury methylation activity occurred in all anaerobically-incubated sediment samples. Radiolabelled methylmercury was essentially undetectable in the same sediments incubated aerobically. This indicates that anaerobic conditions favor the formation of methylmercury in the Upper Wisconsin River.

Organic loading. Higher methylation potential was observed with B-1 sediments than D-6 sediments on most sampling dates (Table 17). Sediments at site B-1 were more organic enriched than D-6 sediments (Table 3). This led to the hypothesis that increased organic content

Table 16. Vertical distribution of mercury methylation at site B-1, Upper Wisconsin River.[a]

Sample depth	PERCENT METHYLATION			
	2 June 1982[b]		16 Sept. 1982[c]	
	core 1	core 2	core 1	core 2
H_2O	0.00	0.00	0.00	0.00
Floc	0.00	0.00	0.22	0.22
0-1 cm	1.18	1.13	2.53	1.62
1-3 cm	0.63	0.36	2.20	--[d]
3-5 cm	0.17	0.12	2.09	1.14
6-7 cm	--	--	0.64	0.20
9-10 cm	0.23	0.21	0.13	0.10

[a]All samples incubated anaerobically in BOD bottles.

[b]Sediment slurry (1g sediment + 1 ml anoxic river water).

[c]Sediment slurry (3g sediment + 3 ml anoxic river water).

[d]Not determined.

Figure 14. Depth distribution of mercury methylation at site B-1, Upper Wisconsin River (bars represent depth intervals in sediment core).

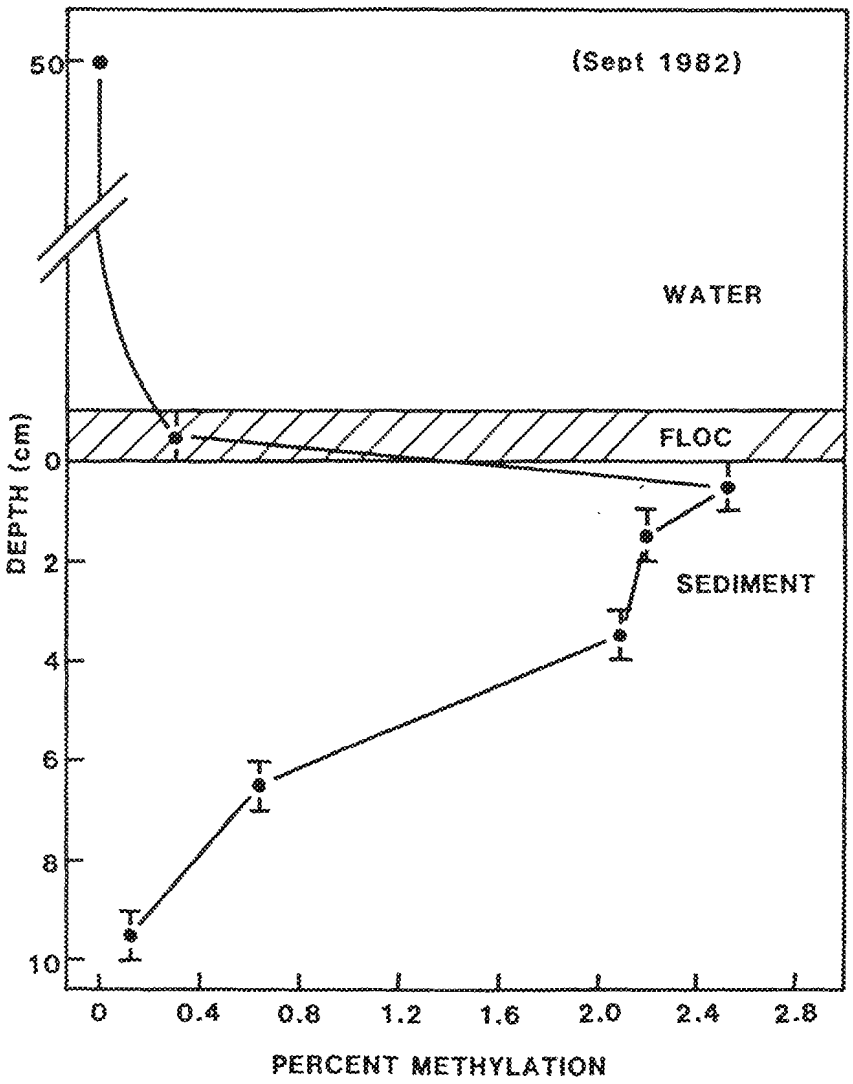

(Sept 1982)
WATER
FLOC
SEDIMENT
DEPTH (cm)
50
0
2
4
6
8
10
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
PERCENT METHYLATION

Figure 15. Effect of temperature on mercury methylation activity (error bars represent values obtained from duplicate samples).

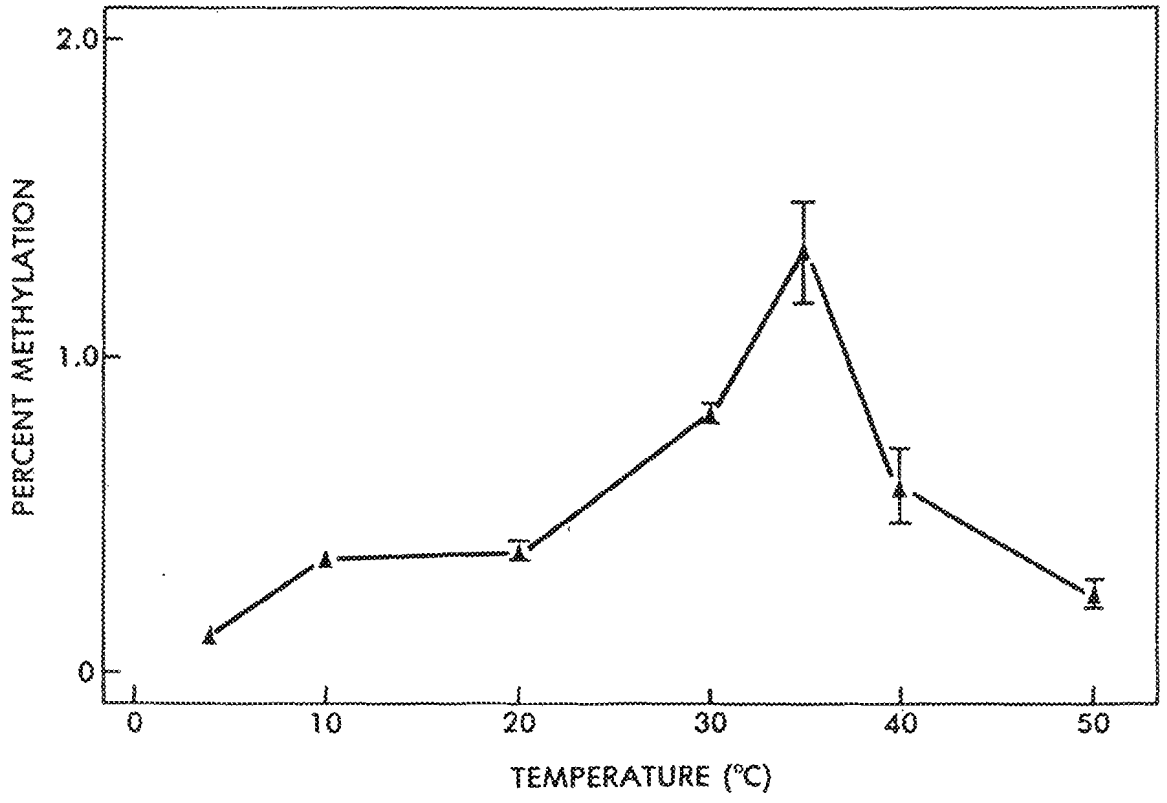

2.0
1.0
0
PERCENT METHYLATION
0
10
20
30
40
50
TEMPERATURE (°C)

Table 17. Mercury methylation at sites B-1 and D-6, Upper Wisconsin River.

Sampling date (1982)	Percent methylation[a]	
	B-1	D-6
May 5[b]	0.58 ± 0.20	0.48 ± 0.00
June 6	0.30 ± 0.00	0.64 ± 0.08**
July 31	2.78 ± 0.50**	0.86 ± 0.56
September 7	3.75 ± 2.06*	0.50 ± 0.00
October 5	0.47 ± 0.16*	0.18 ± 0.20

[a] ± 2SD, n = 2, experimental means compared to each other using a t-test.

[b] 2 days @ 20°C

* $p \leq 0.10$.

** $p \leq 0.05$.

in sediments could stimulate methylation activity. To test this hypothesis, varying concentrations of various organic compounds (starch, sludge, glucose, and vitamin B_{12}) were added to B-1 sediments. The additions of starch, sludge, and glucose did not significantly ($p > 0.05$) increase methylation activity (Table 18). The addition of 0.1% sludge did increase methylation activity; however, this was not repeatable in subsequent experiments. In a separate experiment, the addition of vitamin B_{12} to B-1 sediments did not stimulate methylation activity (Table 19).

Separate organic addition experiments were performed on D-6 sediments because of the lack of stimulation of methylation activity in B-1 sediments. It was anticipated that organic loading would increase methylation activity in D-6 sediments, which contained a lower organic content than those at site B-1. The addition of peptone, 2.0% sludge, and 2 and 10 µg/ml vitamin B_{12} significantly increased methylation activity in D-6 sediments (Table 20). The addition of peptone to D-6 sediments increased methylation activity by as much as three-fold and the amount of stimulation was proportional to the amount of peptone added.

Seasonal Variation in Mercury Methylation

Mercury methylation activities in water, sediment-floc, and sediment from sites B-1 and D-6 from April-October 1982 were analyzed as a function of various physical and chemical parameters (bacterial numbers, turbidity, secchi depth, dissolved oxygen, and water temperature).

The highest methylation activity was observed in B-1 sediments throughout the study (Fig. 16). During May and June, methylation activity was low. The maximal methylation activity in B-1 sediments occurred during September. Methylation activity decreased sharply

Table 18. Effects of organic additions on mercury methylation in site B-1 sediments, Upper Wisconsin River (21 October 1982).

Addition type	Percent methylated[a]	Methylation as a percent of control
none (control)	0.13 ± 0.12	
0.5 mM starch	0.07 ± 0.04	54
2.0 mM starch	0.09 ± 0.12	69
10.0 mM starch	0.15 ± 0.14	115
0.1% sludge	0.36 ± 0.20*	277
0.4% sludge	0.18 ± 0.26	139
2.0% sludge	0.06 ± 0.06	46
0.5 mM glucose	0.12 ± 0.10	92
2.0 mM glucose	0.11 ± 0.06	85
10.0 mM glucose	0.14 ± 0.20	108

[a] ± 2SD, n = 3, experimental means compared to control using a t-test.
* $p \leq 0.05$.

Table 19. Effect of vitamin B_{12} on mercury methylation in B-1 sediment, Upper Wisconsin River (16 November 1982).

Addition type	Percent methylated[a]	Methylation as a percent of control
none (control)	1.03 ± 0.02[b]	
0.05 µg/ml B_{12}	0.97 ± 0.12	94
0.50 µg/ml B_{12}	1.01 ± 0.22	98
2.00 µg/ml B_{12}	0.78 ± 0.34	76
5.00 µg/ml B_{12}	0.99 ± 0.52	96

[a] ± 2SD, n = 3.

[b] n = 2.

Table 20. Effects of organic additions on mercury methylation in D-6 sediment, Upper Wisconsin River (10 December 1982).

Addition type	Percent methylated[a]	Methylation as a percent of control
none (control)	1.24 ± 0.26	
0.10% sludge	1.37 ± 0.16[b]	111
0.50% sludge	1.48 ± 0.32	119
2.00% sludge	1.77 ± 0.16*	143
0.5 µg/ml Vitamin B_{12}	1.79 ± 1.10	144
2.0 µg/ml Vitamin B_{12}	2.12 ± 0.08**	172
10.0 µg/ml Vitamin B_{12}	1.88 ± 0.52*	152
10 µg/ml Peptone	2.13 ± 0.44**	172
50 µg/ml Peptone	2.35 ± 0.34**	190
200 µg/ml Peptone	3.60 ± 1.08*[b]	290

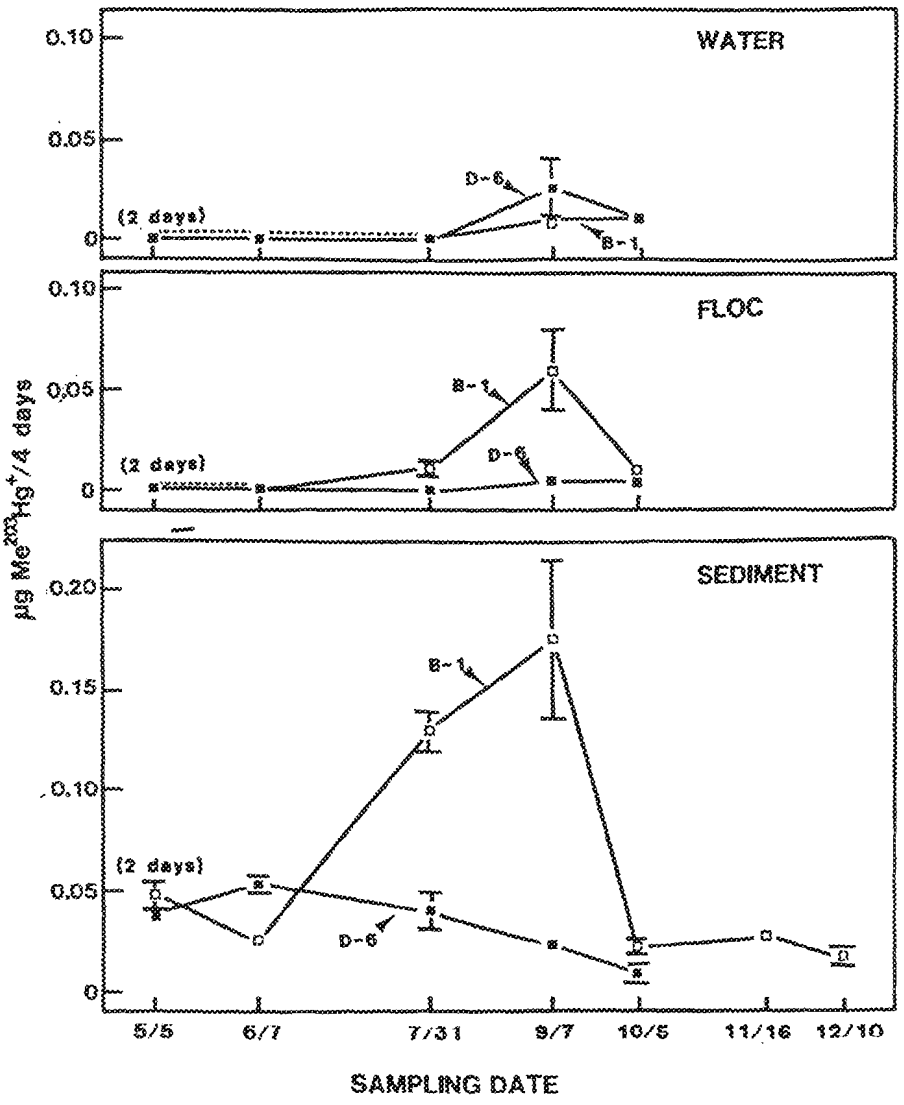
[a] ± 2SD, n = 3, experimental means compared to control using a t-test.

[b] $n = 2$.

* $p \leq 0.05$.

** $p \leq 0.01$.

Figure 16. Seasonal variation in mercury methylation activity in water, floc, and sediments of sites B-1 and D-6, Upper Wisconsin River (error bars represent the high and low values from duplicate samples).


WATER
FLOC
SEDIMENT
0.10
0.05
0
0.20
0.15
(2 days)
D-6
B-1
μg Me^{203}Hg^{+}/4 days
5/5
6/7
7/31
9/7
10/5
11/16
12/10
SAMPLING DATE

during October, November, and December. Methylation activities in sediment-floc and water samples exhibited the same temporal trends but were much lower than that in B-1 sediment. An exception to this trend was noted with D-6 sediments. Peaks in methylation activity were not due to seasonal fluctuations in temperature, because all samples were incubated at 20°C. By estimating methylation activity increases and decreases from seasonal in situ temperatures (from Fig. 15), the seasonal methylation activity peak in B-1 sediments shows little change (Fig. 17).

Bacterial numbers fluctuated throughout the season, but generally showed less than an order of magnitude change (Fig. 18). The greatest increase in densities occurred with anaerobic bacteria in the sediment-floc. This would be expected because sediments became more anaerobic during the summer months. Similar trends were observed in all other samples (B-1 water samples and site D-6 samples). Consequently, it is difficult to associate changes in methylation activity with changes in bacterial numbers.

Seasonal variations in temperature, dissolved oxygen, secchi disc depth, and turbidity at sites B-1 and D-6 are shown in Figure 19. Rises in water temperature coincided closely with decreases in dissolved oxygen concentration. Peaks in methylation activity were observed during the periods with lowest dissolved oxygen periods. The sharp increase in turbidity at site B-1 during July coincided with the maximal methylation activity observed in B-1 sediment, floc, and water samples. The cause of the rise in turbidity was not known.

Figure 17. Seasonal variation of mercury methylation activity in sediments corrected for *in situ* temperature at site B-1, Upper Wisconsin River (error bars represent the high and low values from duplicate samples).

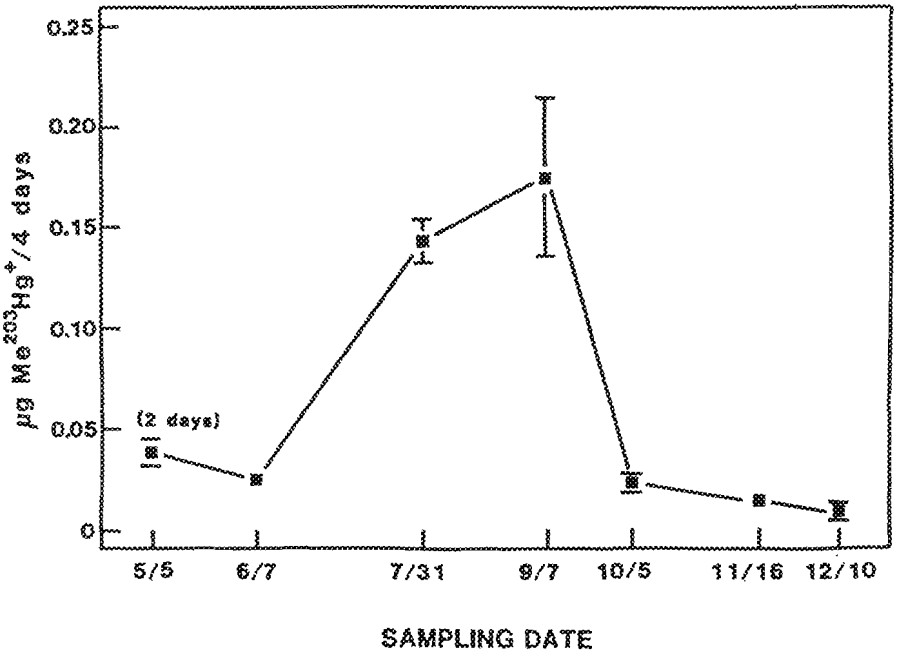

0.25
0.20
0.15
0.10
0.05
0
μg $Me^{203}Hg^{+}$/4 days
(2 days)
5/5
6/7
7/31
9/7
10/5
11/16
12/10
SAMPLING DATE

Figure 18. Seasonal variation in aerobic and anaerobic heterotrophic bacteria in floc and surface sediments at site B-1, Upper Wisconsin River.

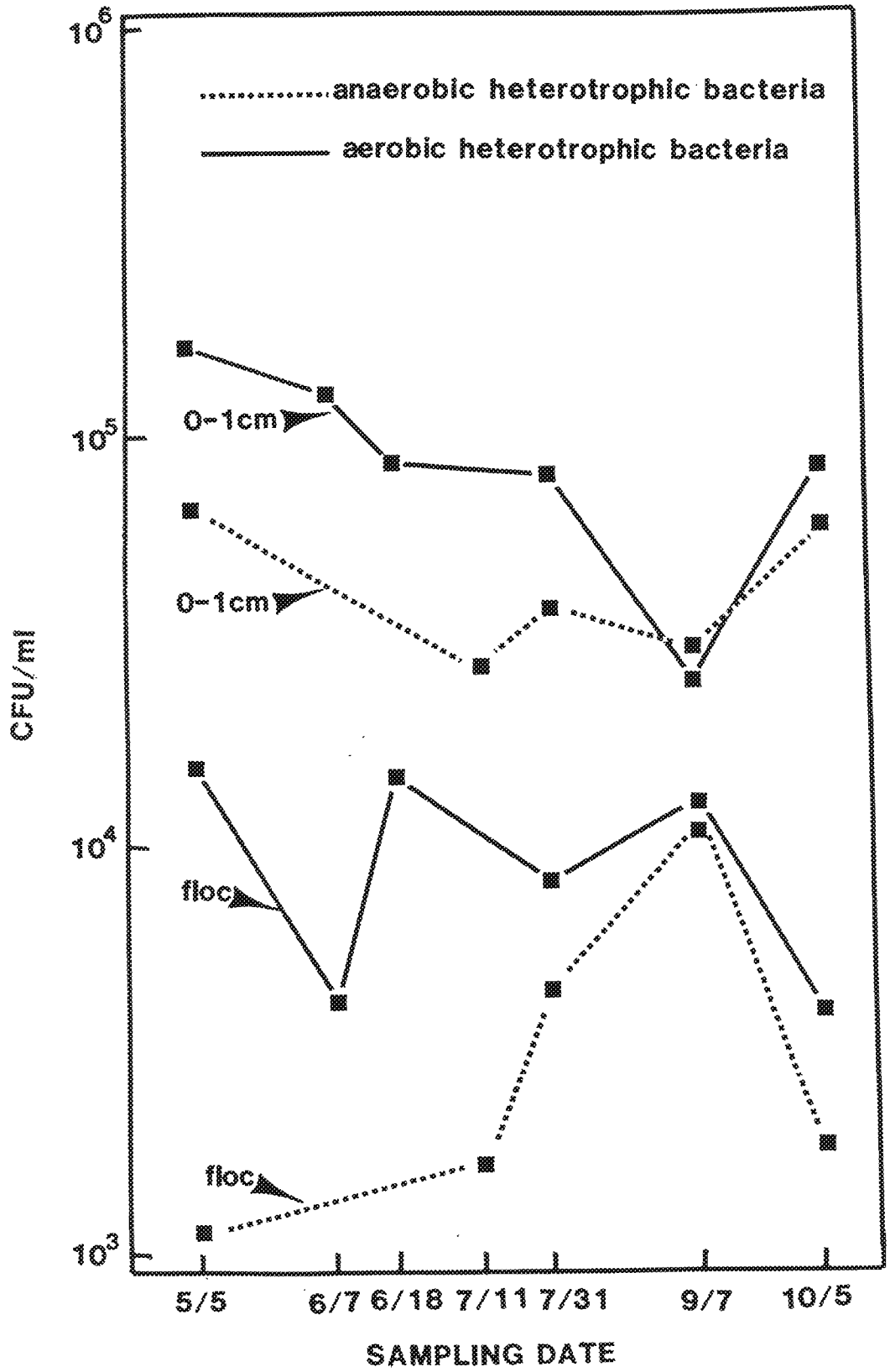
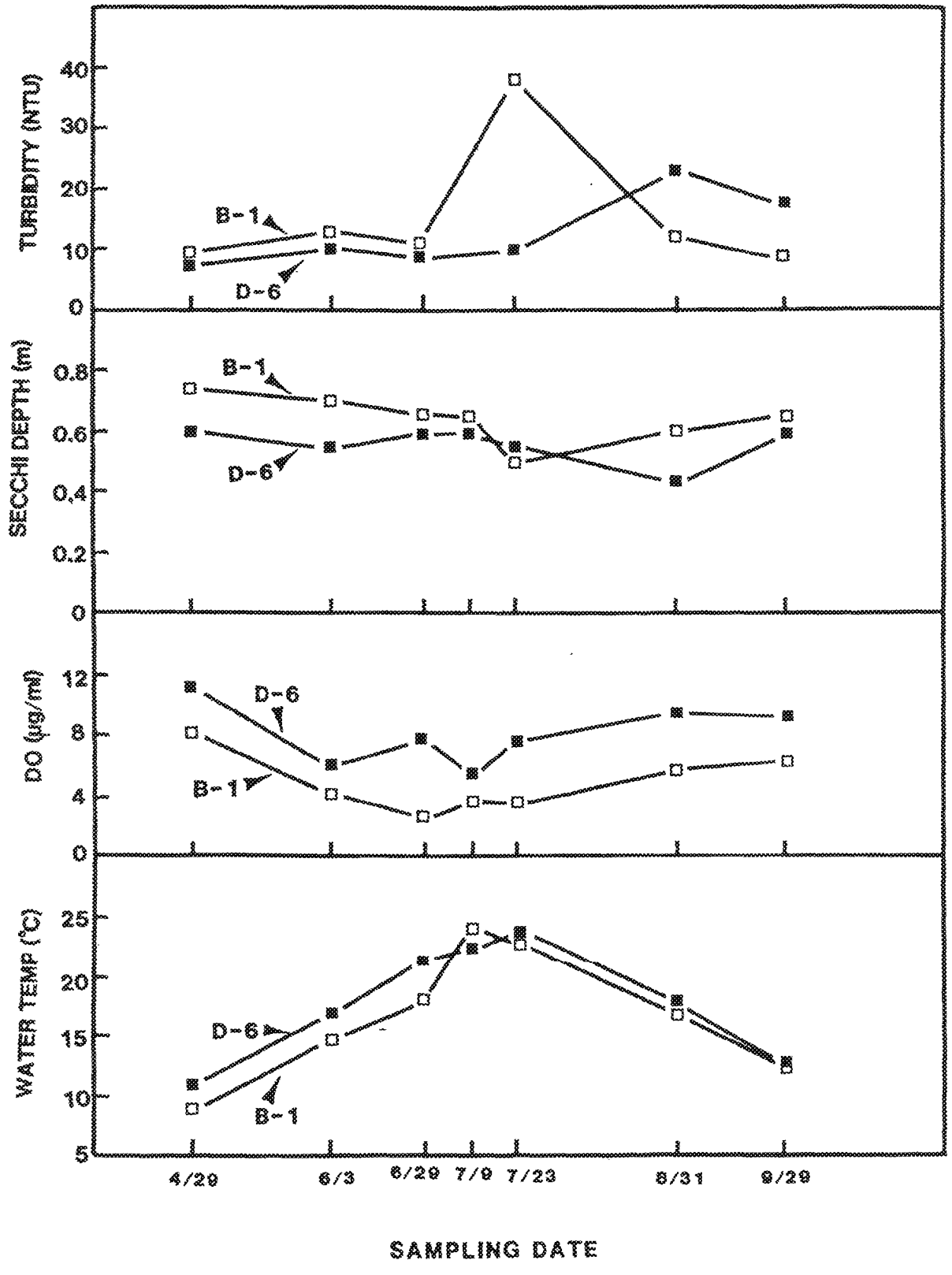

anaerobic heterotrophic bacteria
aerobic heterotrophic bacteria
0-1cm
0-1cm
floc
floc
CFU/ml
10^6
10^5
10^4
10^3
5/5
6/7
6/18
7/11
7/31
9/7
10/5
SAMPLING DATE

Figure 19. Seasonal variations in water temperature, dissolved oxygen, secchi depth, and turbidity at sites B-1 and D-6, Upper Wisconsin River.


TURBIDITY (NTU)
40
30
20
10
0
B-1
D-6
SECCHI DEPTH (m)
0.8
0.6
0.4
0.2
0
B-1
D-6
DO (μg/ml)
12
8
4
0
D-6
B-1
WATER TEMP (°C)
25
20
15
10
5
D-6
B-1
4/29
6/3
6/20
7/9
7/23
8/31
9/29
SAMPLING DATE

Effect of Binding of Mercury to Sediments on Methylation Activity

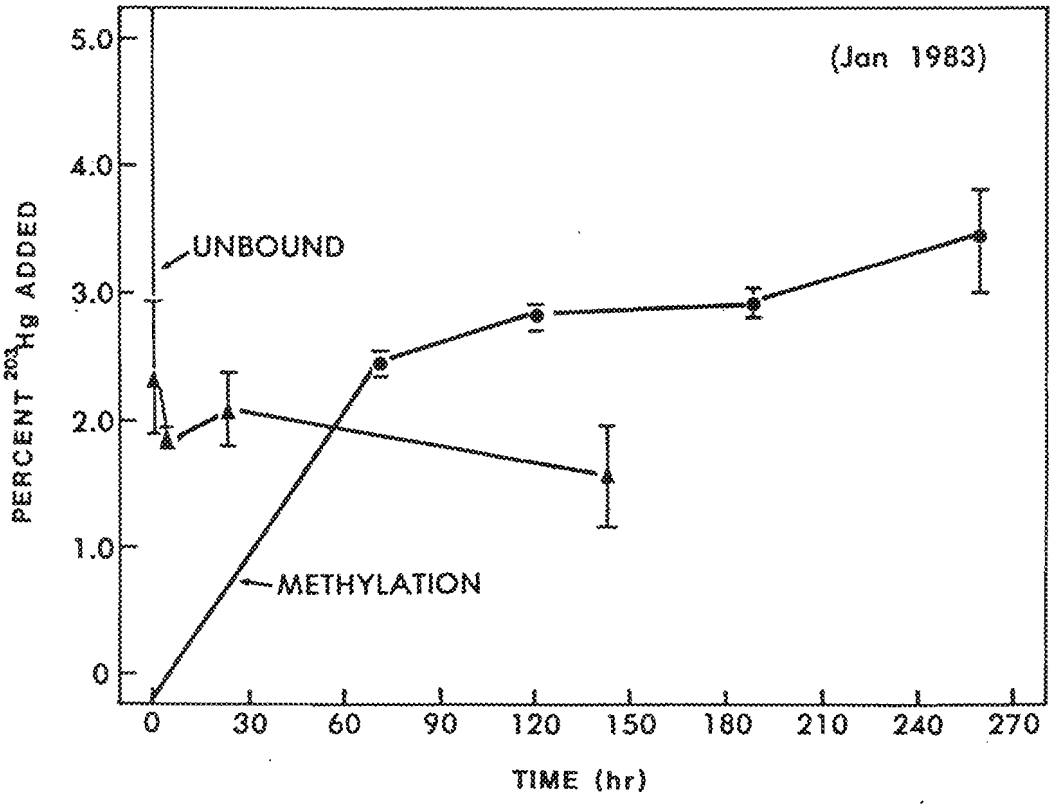
As much as 7% of the added ^{203}Hg was methylated in some preliminary experiments (Table 21). This indicated that mercuric ion bound to sediments remained biologically available for methylation by bacteria. To examine this phenomenon, sediment slurry samples prepared anaerobically in BOD bottles were assayed for methylmercury formation at various incubation times and compared to the amount of ^{203}Hg in the sediment porewater. Sediment samples for this experiment were collected during December 1982 and showed little methylation activity due to the cold temperature of the time of year (see Fig. 16). To overcome this low activity, 1000 μg/ml of peptone was added to all sediment samples to stimulate methylation (see Table 21).

After a 1 hour incubation, less than 2.5% of the added radiolabelled mercuric ion was available in the porewater (Fig. 20). However, the percentage of mercuric ion methylated exceeded the percentage of unbound radioactive mercury as incubation time increased. For example, after 120 hours of incubation, less than 2% of the added radioactivity was unbound, but almost 3% of the added mercuric ion had been methylated. It should be noted that radioactive mercury in the porewater would consist of ^{203}Hg as well as soluble radiolabelled methylmercury formed during the incubation. Therefore, the amount of unbound ^{203}Hg was less than the amount of unbound radiolabelled mercury indicated in Figure 20. These data suggest that anaerobic sediment bacteria are capable of methylating bound mercury.

Table 21. Time course of mercury methylation at site B-1 sediments, Upper Wisconsin River (21 July 1982).

Incubation time (hr)	Percent methylation
20	0.05
24	0.44
53.5	1.43
101.5	3.57
173.5	7.33

Figure 20. Relationship between mercury binding and methylation activity at site B-1, Upper Wisconsin River (error bars represent the high and low values from triplicate samples).


(Jan 1983)
UNBOUND
METHYLATION
PERCENT ^{203}Hg ADDED
TIME (hr)
5.0
4.0
3.0
2.0
1.0
0
0
30
60
90
120
150
180
210
240
270

DISCUSSION

This research quantified bacterial mercury resistance and methylation activity under near *in situ* conditions. To accomplish this, radioactive mercury was used in short incubation experiments to evaluate short-term fluctuations in methylation activity. In addition, the study focused on methylation activities in anaerobic sediments by use of strict anaerobic techniques. Anaerobic sediments are the primary site of microbial activities, and contain high concentrations of mercury in the Wisconsin River. It is important to note that the results presented in this thesis reflect *potential* (not actual) methylmercury activity in the Upper Wisconsin River because radiolabelled mercury was added in all experiments in quantities sufficient to alter the *in situ* pool of mercury.

Aerobic heterotrophic bacteria exhibited significant resistance to mercuric ion. The highest percentages of aerobic, mercury-resistant heterotrophic bacteria were recovered from water. This was expected, because most sediment bacteria exist under anaerobic conditions. As many as 100% of the aerobic heterotrophic bacteria exhibited resistance to 7 μg/ml of Hg^{+2}. It would, however, be unlikely that natural communities would be exposed to concentrations that high. However, these data demonstrate the potential ability of these bacteria to survive sudden increases in mercury concentrations. The most common mechanism of mercury resistance is reduction of mercuric ion to elemental mercury (29, 30, 31, 54, 55, 95, 97), which is volatile and subsequently lost from the medium (54, 86, 96). Therefore, these results

indicate a potential for volatilization of mercuric ion entering the Upper Wisconsin River.

The use of phenylmercuric acetate by pulp and paper mills on the Upper Wisconsin River was discontinued in the late 1950s; however, a significant mercury-resistant community of aerobic heterotrophic bacteria exists. There are other potential sources of mercury contamination to the environment, such as domestic sewage, which probably contributes mercury to the Upper Wisconsin River. A mercury-resistant population of bacteria would evolve in the presence of high concentrations of mercury. It should be noted that aerobic heterotrophs showed essentially no resistance to concentrations of Hg^{+2} greater than 7 µg/ml.

High anaerobic heterotrophic resistance occurred in all sediment samples including sediments with low concentrations of mercury. Lake DuBay (D-6) sediments contained less than 0.1 µg/g of mercury, whereas Brokaw (B-1) sediments contained greater than 2 µg/g of mercury (25).

The presence of mercury-resistant aerobic heterotrophic bacteria appears to be widespread throughout the aquatic environment and not only in areas containing high mercury concentrations. Nelson et al. (69) isolated approximately 900 strains of mercury-resistant bacteria from the Chesapeake Bay; the dominant genus was Pseudomonas. No data is available on densities of mercury-resistant anaerobic heterotrophic bacteria in aquatic environments; however, my results suggest that anaerobic heterotrophs are more resistant than aerobic heterotrophic bacteria to mercury. This implies even greater potential for volatilization of Hg^{+2} from sediments.

Binding experiments indicated that the amount of soluble mercuric ion was essentially the same in both aerobic and anaerobic culture media. However, it is not known if the soluble mercuric ion was being bound to soluble organic compounds. The presence of cystein in the anaerobic media had no effect on mercury resistance (M. R. Winfrey, pers. comm.). Ramamoorthy and Kushner (79) have reported differential binding of mercury in culture media; however, it is likely that the observed increased resistance of anaerobic heterotrophs was valid and not due to differential binding.

Seasonal variations in the densities of mercury-resistant aerobic and anaerobic heterotrophs were not observed. This suggests that bacterial communities in the Upper Wisconsin River have reached a steady state with respect to mercury resistance. These results differ from those of Nelson and Colwell (70), who reported a spring peak in the densities of mercury-resistant aerobic heterotrophic bacteria in the Chesapeake Bay. They also reported that mercury resistance correlated positively with water transparency (Secchi disc), dissolved oxygen, and total mercury concentration in the sediment. My results showed no correlations with these environmental factors. More work needs to be done in this area due to the differing results obtained between separate aquatic ecosystems.

Not surprisingly, the greatest methylation activity was observed in the sediments, because the highest bacterial densities occurred there. This was probably due to greater availability of organic materials in the sediment. Mercury methylation was maximal in the surface layer (0-1 cm) of sediment. Surface sediments exhibit greater

overall microbial activity due to the constant introduction of fresh organic compounds (109). Deeper sediments contain little labile matter, and general microbial activity decreases with sediment depth (109). Therefore, mercury methylation activity in Upper Wisconsin River sediment correlated with typical profiles of microbial activity in sediments.

Surface sediments probably have the greatest potential to release methylmercury into the water column. Jernelöv (46) reported that when macroorganisms are absent, the release of methylmercury occurs almost entirely from the uppermost centimeter of the sediment. Methylmercury produced deeper in the sediments may undergo demethylation before release into the water column. The presence of demethylating microorganisms in methylmercury-producing sediments has been documented (8, 91, 92). Spangler et al. (92) showed that the level of methylmercury in batch-flask sediment cultures dropped drastically after an initial increase in methylmercury levels over a 1.5-month period. They concluded that this was due to demethylating organisms. Billen et al. (8) hypothesized that an equilibrium between methylation and mineralization might eventually be reached. This may have occurred in Upper Wisconsin River sediments; however, the introduction of mercury from natural or anthropogenic sources would alter this balance and significant methylmercury production could be the result.

The effects of various physical and chemical factors clarify the causes of the observed summer peak in methylation. This peak was not due to increases in temperature because all samples were incubated at the same temperature. However, temperature effect experiments showed that increases in incubation temperature increased methylation

activity. The optimum temperature for methylation activity was 35°C, which was substantially higher than the maximum *in situ* temperature of 24°C measured during this study. However, an *in situ* temperature of 33°C was measured at site B-1 in July 1983 (M. R. Winfrey, pers. comm.). This high temperature indicates that significant increases in mercury methylation could occur with increased environmental temperatures. This may be significant in areas receiving heated thermal effluents from power plants.

Significant methylation activity occurred at temperatures as low as 4°C and as high as 50°C. Methylation activity at 4°C indicates that psychrophilic bacteria present in sediments can methylate mercury, whereas methylation at 50°C suggests that thermophilic bacteria can methylate mercury.

Methylation of mercury has been shown to occur under both aerobic and anaerobic conditions (73, 105, 116), although the effect of dissolved oxygen is not completely understood. Olson and Cooper (73) reported greater anaerobic methylation rates than aerobic rates in San Francisco Bay sediments, whereas Vonk and Sijpsteijn (105) reported greater aerobic rates than anaerobic rates in several pure cultures of bacteria. The results of the present study clearly indicate that anaerobic conditions increase the potential for methylmercury production in the Upper Wisconsin River. However, these experiments did not account for demethylation or dimethylmercury production. The formation of volatile dimethylmercury or increased demethylation could account for the observed decrease in methylmercury formation.

Olson and Cooper (73) hypothesized that the presence of oxygen could significantly increase methylmercury decay rate, which would

cause the net methylmercury production to be greater under anaerobic than aerobic conditions. However, in essentially all aerobic samples assayed during the present study, the net amount of methylmercury produced was near zero. Even with increased decay rates, 100% methylmercury decay would not be expected during the short incubation periods. Therefore, the potential for anaerobic formation of methylmercury in the Upper Wisconsin River is greater than the potential for aerobic formation.

Increases in temperature and decreases in dissolved oxygen during the summer months in the Upper Wisconsin River would lead to anaerobic conditions in surface sediments, which would increase methylmercury production. Therefore, the summer peak in methylation activity was probably influenced by an increase in anaerobic conditions.

Greater methylation was observed in B-1 sediments than in D-6 sediments. This was probably due to the greater amount of organic materials at site B-1. Several investigators have shown that methylmercury production is enhanced by increasing nutrient levels (9, 22, 73). The organic effects experiments demonstrated that enrichment with sewage sludge, vitamin B_{12}, and peptone lead to increased methylation activity in D-6 sediments. Increases in methylation activity were more apparent in D-6 sediments than B-1 sediments. This was probably because B-1 sediments were already highly organic and subsequent additions did not affect overall microbial activity.

Stimulation of methylation activity with sewage was variable. This is difficult to interpret because the types and quantities of the organic matter present were not known. Some of the organic matter

present was wood fibers and chips. Therefore, it was not possible to evaluate which compounds were stimulatory to methylmercury production.

The addition of vitamin B_{12} increased methylation activity in D-6 sediments; however, increases were less than would have been expected due to the importance of vitamin B_{12} as a precursor for a methylation mechanism. Ridley et al. (82) reported a mechanism for the vitamin B_{12} dependent methyl transfer to mercuric ion. This evidence suggests that methylation via vitamin B_{12} may be utilized by sediment bacteria in the Upper Wisconsin River, but, the major in situ mechanism is unknown.

The greatest stimulation of methylation activity in both B-1 and D-6 sediments occurred with the addition of peptone. Peptone contains amino acids and peptides that are easily utilized by a wide variety of bacteria. Other investigators (32, 115) have reported increases in methylation activity after enrichment with tryptic soy broth, which contains peptone. Another potential mechanism for methylmercury formation could involve the utilization of amino acids as methyl donors.

Surprisingly, additions of glucose and starch did not stimulate methylation activity. These compounds stimulated other microbial activities such as methane production (M. R. Winfrey, pers. comm.). Therefore, sugar and carbohydrate compounds do not stimulate methylation activity in Upper Wisconsin River sediments.

The actual methylation mechanisms involved under in situ conditions remain unknown. Results of this study suggest that more than one mechanism is utilized. Further work should determine the role of

amino acids on mercury methylation, because peptone caused such a significant increase in methylation activity.

The densities of aerobic and anaerobic heterotrophic bacteria showed random fluctuations during the period of this study. Seasonal methylation experiments, however, showed a peak in methylation activity in water, floc, and sediments from site B-1 during sampling trips in July and August. Similar peaks occurred in water and floc samples from site D-6, but a peak was not observed in D-6 sediments. Furutani and Rudd (32) observed similar seasonal methylation peaks in water and sediment-floc samples from the Wabigoon-English River System. Therefore, increases and decreases in methylation activity were not due to changes in bacterial densities.

The physical and chemical effect experiments partly explained the observed seasonal peaks in methylation activity. Higher temperatures increased the potential for significant methylation activity; however, the potential for methylation existed even to temperature extremes (4° and 50°C). An increase in anaerobic conditions, which occurred during the summer months, favored methylmercury production. Oxygen inhibited net methylation activity. In general, increases in microbial activity appear to have lead to an increase in methylation activity. Factors that increase nutrient availability, such as increased primary production during the summer and input from sewage treatment plants, would increase microbial activity and also increase methylation activity. Increased microbial activity would also decrease oxygen concentration.

Kudo et al. (58) showed that up to 97% of the mercury in aquatic environments is sorbed onto particulate matter and becomes associated with the bed sediments. Mercury binding experiments in the present

study showed that greater than 3% of the added mercury was methylated, while less than 2% remained soluble in the porewater, indicating that bacteria can remove and methylate sediment-bound mercury. This is of considerable ecological significance because of the high mercury concentrations in the Upper Wisconsin River sediments (25). Thus, methylation rates in these mercury contaminated sediments may be greater than was previously considered possible. Increases in turbidity may also lead to increases in methylation activity due to increased bacterial exposure to high concentrations of bound mercury.

The potential for significant methylmercury formation in the Wisconsin River has been demonstrated. This is especially important due to the possibility of further additions of mercury to the system, the continuing availability of mercury already present in the sediments, the potential for redistribution of mercury in Upper Wisconsin River sediments, and the ability of bacteria to methylate bound mercury. The potential for methylation is greatest during the summer months due to increased temperature, increased microbial activity, enhancement of anaerobic conditions, and increased availability of organic material.

Further work is necessary to measure actual in situ methylation rates in the Upper Wisconsin River; however, the experiments in this study were designed to simulate in situ conditions as closely as possible. Questions also exist concerning the amount of mercury available for methylation. High concentrations of the added mercury were not methylated. The form and fate of this unmethylated mercury is unknown. Adsorption, precipitation, volatilization as dimethylmercury or elemental mercury, and demethylation mechanisms are probably

important transformations occurring in the Upper Wisconsin River. The relative importance of these phenomena are not known. The results of this thesis and further experiments designed to answer questions that have arisen from this research should clarify the role of microbial mercury methylation in aquatic ecosystems.

CONCLUSIONS

The following conclusions can be derived from the results of this study:

1) A significant community of mercury-resistant aerobic and anaerobic heterotrophic bacteria exists in the Upper Wisconsin River.

2) Anaerobic heterotrophic bacteria are more resistant to mercury than aerobic heterotrophic bacteria in the Upper Wisconsin River.

3) The densities of mercury-resistant heterotrophic bacteria in the Upper Wisconsin River do not fluctuate seasonally.

4) Mercury methylation in the Upper Wisconsin River is primarily an anaerobic process.

5) Surface sediments have the greatest potential for producing and releasing methylmercury into the water column.

6) Factors that raise water temperatures or increase nutrient availability increase mercury methylating potential.

7) The greatest stimulation of methylation activity occurs after the addition of amino acids.

8) Bacteria can methylate bound mercury in Upper Wisconsin River sediments.

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