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ENVIRONMENTAL METHYL MERCURY PRODUCTION

V. Susan Springthorpe

This thesis is submitted in partial fulfillment of the requirements for a Master of Science Degree at the University of Ottawa.

May 1984

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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ABSTRACT

Despite a wealth of literature on mercury methylation, there is still a lack of concensus on the environmental generation of methyl mercury, and on the role that bacteria or other microorganisms may play in this process.

This study was intended to look at the alternative methods by which methyl mercury can be generated in the environment, and to investigate further the role of microorganisms in methyl mercury production. Any discussion of the environmental production of methyl mercury must also be concerned with the availability and flux of mercury within the environment. Hence, mercury interconversions become an important ancilliary consideration. The research reported here was carried out as a part of the Ottawa River Project.

Bacterial cultures from Ottawa River Sediment, which were tolerant of 10 ppm mercury added to the growth medium, failed to generate detectable methyl mercury levels when grown in Foote & Taylor medium, containing 10 ppm added mercuric chloride or mercuric nitrate, for up to 11 days. Mixed bacterial cultures from the slime layer on the exterior surfaces of fish produced similar results. Even isolates with the reported ability to generate methyl mercury, showed no such production when incubated with mercuric chloride. Cultures which were incubated with mercuric acetate were shown to contain some methyl mercury. However, it was later shown that this methyl mercury was not present as a result of microbial activity. Attempts to show mercury methylation by fungal cultures also gave negative results.

During the course of the methylation experiments, both mixed

and pure cultures of bacteria from the Ottawa River and its resident fish showed some ability to volatilise added inorganic mercury, and hence the potential to affect or effect mercury flux within the environment.

The ability of microorganisms to compete successfully with sediment for mercury in the aqueous environment was also demonstrated. Bacteria could accumulate from water mercury that had previously been bound to the sediment. The addition of a chelating mixture to the water significantly increased the binding of the mercury to the bacteria.

Methyl mercury production proved much easier to demonstrate in defined chemical, rather than biological systems. Ultraviolet or sunlight irradiation of mercuric acetate, mercuric oxide or mercuric sulphide, the last two in the presence of acetic acid, in either distilled or river water, gave substantial quantities of methyl mercury after a 6-day period. However, experiments where simulated water columns, containing realistically low levels of added mercury, were irradiated showed no detectable levels of methyl mercury with or without fish as bioaccumulators.

A hypothesis is proposed which examines alternate mechanisms for methyl mercury generation in the environment.

RÉSUMÉ

Bien qu'il y ait une abondance d'information scientifique sur la methylation du mercure, il est difficile d'établir un consensus sur la production du mercure méthylique dans l'environnement, ainsi que de definer le rôle que peuvent jouer les bactéries ou autres microorganismes dans ce processus.

Cette recherche a pour but d'étudier les methodes diverses par lesquelles le mercure méthylique peut-être produit dans l'environnement et éxaminer davantage la part que jouent les microorganismes dans la production du mercure méthylique. Toute hypothèse relative à la production du mercure méthylique dans l'environnement doit aussi prendre en considération la disponsibilité et le flux du mercure. Les interconversions du mercure sont devenu un apport d'examen imporant et le rôle des microorganismes dans la mobilisation du mercure a aussi été consideré. Cette recherche a été conduite comme faisant partie du projet de la Riviere Ottawa.

Les cultures bacteriennes du sédiment de la Rivière Ottawa, qui peuvent croître dans les cultures contenant 10 ppm de mercure, n'ont pas réussi à produire des nivaux de mercure méthylique détectables lorsque cultivé dans le medium Foote & Taylor contenant 10 ppm de nitrate de mercure ou de chlorure de mercure, jusqu'a 11 jours. Les cultures bacteriennes mixtes, provenant des couches vaseuses qui entourent les surfaces exterieures des poissons, ont produit les resultat sembables. Mêmes les isolés qui ont été reporté d'être capable de produire du mercure méthylique n'ont pas produit de tels effets lorsque

ils ont été incubé avec le chlorure de mercure. Les cultures qui ont été incubé avec l'acetate de mercure contenaient du mercure méthylique, mais il a été prouvé plus tard que ceci n'était pas produit par les cultures bacteriennes. Des essais pour démontrer la méthylation du mercure par des moyens de culture de champignons ont aussi donné des resultats negatifs.

Au cours de l'éxperimentation du procédé de méthylation à la fois des cultures mixtes et pures de bactéries provenant de la Riviere Ottawa et de ses poissons ont indiqué la capacité de volatiliser le mercure inorganique supplié et ainsi le potentiel de modifier ou d'affecter le flux de mercure dans l'environnement.

La capacité des microrganismes de bien rivaliser avec le sédiment pour l'obtention de mercure dans l'environnement aquatique a aussi été démontré. Les bactéries peuvent tirer de l'eau le mercure qui a été auparavant lié au sédiment. Quand l'eau était supplementé avec un melange de chelateurs, les bactéries peuvent liér une plus grande quantité du mercure.

La production du mercure méthylique est plus facile à démontrer dans les systèmes chimiques limites plustôt que dans un système biologique. Les irradiations ultraviolettes ou de lumiere solaire de l'acetate de mercure, de l'oxide de mercure ou de sulfide de mercure, ces deux derniers en présence d'acide acétique soit dans l'eau distillé soit dans l'eau de la rivière ont donné des quantitiés substantielles de mercure méthylique pendant une periode de 6 jours. Cependant, l'expérimentation de colonnes d'eau simulés contenant des niveaux moindres de mercure ajouté, après irradiation, n'ont pas indiqué des nivaux

détectables de mercure méthylique avec ou sans poissons comme bioaccumulateurs.

Une hypothèse est proposé qui examine des autres mechanismes par lesquelles le mercure méthylique peut-être produit dans l'environnement.

THE ENVIRONMENTAL MERCURY PROBLEM

Introduction

Mercury (Hg) has always been present as a minor constituent of the environment, but only in the last two decades has it been considered as a major pollutant. World attention was first alerted by poisoning epidemics in Japan (Fujiki, 1963; Tajima, 1970; Fujiki & Tajima, 1973), Iraq (Bakir et al., 1973), Pakistan (Haq, 1963) and Guatemala (Ordonez et al., 1966; Eyl, 1971) as well as by widespread environmental contamination in Sweden (Borg et al., 1969; Lofroth, 1969; Rissanen & Miettinen, 1971) and North America (d'Itri,1972b; Saha & McKinlay, 1973; Galster, 1975; Hanlon, 1976).

Short-chain alkyl Hg compounds have long half-lives in association with biological material (Lofroth, 1970; Magos, 1978): continuous exposure often allows them to accumulate faster than they can be excreted. This not only leads to toxic damage to individuals at any particular trophic level, but also to amplification throughout the food chains with the most concentrated effects on the terminal predators (Stickel, 1971; Larsson, 1970). Despite this cumulative effect, Hg undergoes a cyclic movement through the environment (Goldwater, 1971).

In the past, there has been a widespread belief that methyl mercury (MeHg), the most toxic form of Hg, is produced by bacteria in aquatic sediment and released to the overlying water from where it is accumulated by fish. It seems likely, as will be discussed later, that this may be an oversimplification of a complex environmental problem. The work described in the experimental section is concerned with the possible role that micro-

organisms play in the environmental generation of MeHg.

The following literature review is divided into three sections. The first discusses the sources, distribution and cycling of Hg and MeHg in the environment. The second is concerned with the aquatic chemistry of Hg, including its interconversions, and the availability of Hg species to microorganisms and other potential methylating agents. The third deals with the present state of knowledge on environmental Hg methylation.

SOURCES, DISTRIBUTION AND CYCLING OF MERCURY AND METHYL MERCURY IN THE ENVIRONMENT

(i) The Natural Occurence and Distribution of Mercury and Methyl Mercury

Mercury occurs naturally in about 25 different minerals, but its principal ore is cinnabar, a mercuric sulphide (HgS) with low solubility and relatively low volatility. It is estimated that the earth's crust contains approximately 10^9 tons of Hg (Bishop, 1972) which is not uniformly distributed (Jonasson & Boyle 1971).

Apart from ore deposits, Hg is found almost ubiquitously in trace amounts. In non-polluted areas rocks and soils usually contain <1 ppm of Hg. Organic-rich shales may contain up to 10 ppm and soils rich in humus up to 2 ppm. Water, fresh or salt, contains only ppb levels of Hg and air contains < 1 ppb. In the vicinity of Hg deposits or other precious metal ores, Hg levels in soil, water and air are elevated; this is used currently as an exploration tool (McCarthy et al., 1970; Williston, 1968).

Rain acts like a 'scrubber' system, returning atmospheric

pollutants to earth. Even near ore deposits, it has been shown that Hg in the atmosphere is almost zero after a rainstorm (McCarthy et al., 1970). Stock and Cucuel (1934) found an average content of 0.2 ppb in rainwater, nearly an order of magnitude greater than in seawater (0.03 ppb). In Sweden, soil receives about 1.2 g of Hg per hectare in the annual rainfall (Rissanen & Miettinen, 1971).

Mercury has a strong tendency to bioaccumulate, and, while there is considerable variation, biota usually contain Hg levels at least an order of magnitude greater than their surroundings (Stock & Cucuel, 1934; Johnels et al., 1967; Hirota et al., 1979).

Detailed information on the abundance and distribution of Hg in the U.S.A. is presented in a compilation of papers by the U.S. Geological Survey (Mercury in the Environment, 1970). The available data for other parts of the world, while rarely so comprehensive, are in general agreement (Warren & Delavault, 1969; Stock & Cucuel, 1934; Andersson, 1967).

Where Hg concentrations are high and geochemical conditions are suitable for effective leaching from the rock, localised areas may be subject to high levels of 'natural' Hg pollution. In general, however, the areas of high Hg pollution which have been the subject of scientific investigation are due, at least in part, to anthropogenic sources.

Sediment levels of MeHg are either undetectable (Spangler et al., 1973a) or very low, and represent only a small percentage of the total Hg present; 0.01-0.075% (Andren & Harriss, 1973); 0.4% (Longbottom & Dressman, 1973); 0.01-0.06% (Batti et al., 1978);

0.2-0.4% (Bartlett & Craig, 1981). Even in the polluted sediments of Minamata Bay, Japan, the highest measured concentration of MeHg was 11 ppb (Fujiki & Tajima, 1973), or about 0.02%.

A positive correlation has been observed between MeHg levels and organic carbon, silt and sulphide up to a limiting concentration (Bartlett & Craig, 1981). However in the presence of higher inorganic sulphide the MeHg level declines sharply (Craig & Moreton, 1983). Levels of MeHg in sediments have been observed to fluctuate (Bartlett et al., 1977; Olsen, 1978; Bartlett & Craig, 1981) but this observation may be partly due to an artefact of the sampling procedure (Bartlett & Craig, 1981). These authors speculate that the introduction of oxygen to anaerobic sediments on sampling decreases the anaerobic demethylation processes and allows methylation to predominate for a short period. This gives a rise and subsequent fall in the levels of MeHg detected. Bartlett and Craig (1981) also predict that MeHg is only likely to be found between +150mV and -100mV, since above 150 aerobic demethylation processes are likely to predominate, and below -100 the sulphide levels would cause MeHg to be converted to dimethyl mercury (diMeHg), which is volatile, and HgS. This may be an artefact of the extraction procedure because sulphide has been reported to convert MeHg to bis-sulphur dimethyl mercury (MeHg-S-S-HgMe) which does not extract with glutathione or cysteine unless copper ions are added (Fujiki, 1970). Also the authors do not seem to have considered the fairly large fraction of organic sulphur found in most soils and sediments. Brinckman & Iverson (1975) have found no simple correlation

between sulphur levels in sediments and immobilisation of Hg as HgS; they detected substantial organic extractable Hg even in the presence of high sulphur concentrations in the surface sediment.

In a study on the equilibrium concentrations of MeHg in sediments Kudo et al. (1977) found the final equilibrium concentration to vary with the type of sediment, being greatest for wood-chip sediment where the methylation process was assumed to occur more rapidly and the demethylation process more slowly. It might, however, be pertinent to consider that lignin is known to have large numbers of bioactive methyl groups (Fruton & Simmonds, 1953), and so its degradation could make available a continual supply of methyl groups to the methylating agent/s.

Although considerable information is available on the levels of total and organic Hg in surface waters, very little is known about levels of MeHg mostly because of the difficulties of measurement. The few published reports (Chau & Saitoh, 1973; Kudo et al., 1982) indicate levels from <1-10 ng/mL (ppt), though this may represent a considerable proportion (25-50%) of the total Hg (Kudo et al., 1982). The low levels found in water are, however, rather misleading, because microorganisms and other biota may be surrounded by microenvironments with much higher concentrations of both Hg and MeHg: >80% of total Hg in water has been shown to be associated with particulate matter (Airey & Jones, 1982).

Less than 1% of airborne Hg exists as the dimethyl form (Summers & Silver, 1978). However, it is of interest that sewage facilities constitute a widespread source of airborne organic Hg emissions (Soldano et al., 1975) which can be correlated with population size. Atmospheric concentrations of organic Hg were

shown to increase for several miles from the site of the sewage treatment plant whereas volatilised elemental Hg declined.

Biota may contain 20-90% of their Hg in the methyl form (Hirota et al., 1979; Miller et al., 1979; Kudo et al., 1982) though this may range from about 10 ppb to several ppm. The best studied example is fish, which tend to carry 70-90% of the Hg body burden as MeHg; an extensive review is available for fish from Swedish lakes (Report of an Expert Committee, 1971). Marine fish at least, presumably due to the relatively constant levels of Hg in the ocean, seem always to have contained Hg (Miller et al., 1972). Mercury and MeHg can be said to be ubiquitous in biota, though certain organisms have developed specialised means of tolerating or detoxifying the Hg species with which they are in contact (Anelli et al., 1973; Bouquegneau, 1975; Ishihara & Suzuki, 1976; Summers & Silver, 1978; Pan Hou & Imura, 1983).

(ii) Mercury and Methyl Mercury Pollution as a result of Human Activity

The anthropogenic sources of Hg pollution, which are superimposed on the natural cycles, fall into two categories - the result of Hg-based technology, and the use of other mineral deposits containing low levels of Hg.

a) Pollution from mercury-based technology

North America and Eurasia contribute the largest proportion of Hg from industrial and agricultural sources: there are about 3,000 distinct uses for Hg (Bailey and Smith, 1964). World consumption of Hg was more than 9×10^6 Kg in 1968 (West, 1969), and Wershaw (1970) estimates that 25% of this may have been

leaked to the environment.

The most immediate impact of Hg loss on the aquatic environment is when wasting occurs directly into a body of water: sediment Hg concentrations of several thousand ppm have been recorded in areas of industrial pollution (d'Itri, 1972b). Mercury lost to the air becomes widely distributed and Hg lost to the soil is usually leached into the water system fairly slowly. The largest direct loss of Hg to water has been in the chlor-alkali industry where losses are estimated by the Hg aquisition needed to replace the loss; this amounted to about 100 tons annually in Canada (Fimreite, 1970), and much larger quantities in Sweden (Van den Berg, 1971) and the U.S. (D'Itri, 1972b). In spite of the reduction in Hg consumption which has occurred since the 1960's in the chlor-alkali and other industries (Flewelling, 1974; De Jong & Rekers, 1974; Van den Berg, 1971), about 50% of the annual Hg purchase is lost to the environment (Flewelling, 1974). A recent report (Marshall, 1983) details about 2.4 million pounds of Hg unaccounted for from the site of the Oak Ridge National Laboratories in Tennessee, and the highest levels of environmental Hg contamination ever recorded in the U.S..

A major source of MeHg and other organic Hg compounds in the environment has been the agricultural use of mercurials as fungicides. The types and properties of such mercurial fungicides have been extensively reviewed by Risannen and Miettinen (1971) and estimates are available for the quantities used in Europe (Granhall, 1965), North America and Japan (Byerly, 1971) and Canada (Fimreite, 1970). It is now well established that alkyl mercurials are among the most toxic and stable Hg compounds

known, and many countries have now outlawed or severely curtailed their use. However, some mercurial fungicides are still in use, and for the most part their potential for conversion to MeHg is unknown.

Direct industrial MeHg contamination of the environment is not known to occur now, though it has been previously reported (Fujiki & Tajima, 1973). However the vast range of chemical wastes which are discharged into waterways may produce this compound more often than is realised without the intervention of microorganisms.

b) Pollution from fossil fuels and ores

A considerable amount of Hg is released by the consumption of fossil fuels and metal ores. There are comparatively few data available on the Hg content of fossil fuels, but coals have been reported with between 1 ppb and 300 ppm Hg (Fleischer, 1970; D'Itri, 1972b; Joensuu, 1971) and oils contain from 1ppm to 500 ppm Hg (D'Itri, 1972a; White et al., 1970). Klein (1971) has estimated worldwide losses of Hg from fossil fuels to be approximately 3,000 tons annually.

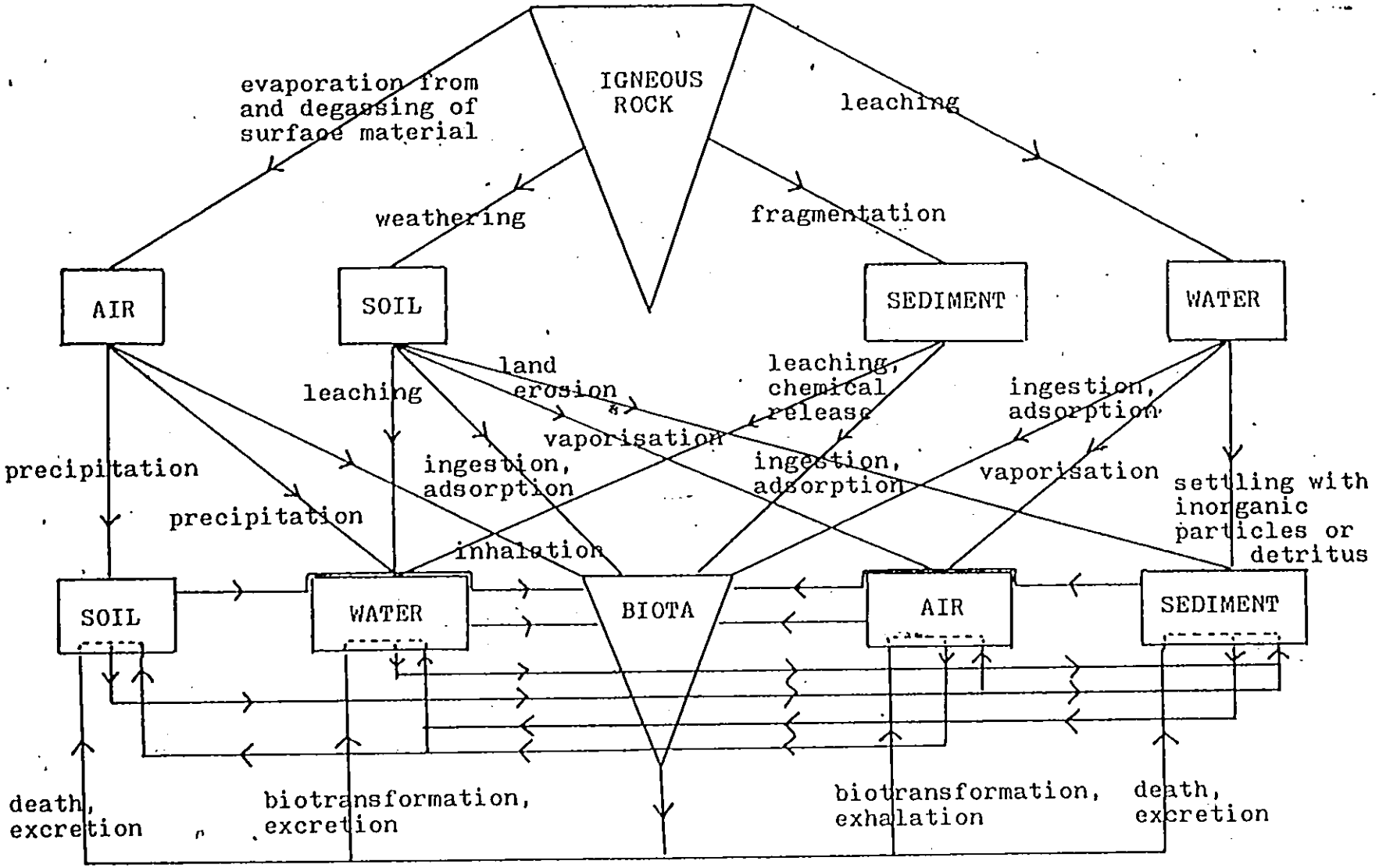
Mercury, in small amounts, occurs commonly in other metal sulphide ores (copper, lead, gold and zinc) and is vapourised when the ores are roasted. Klein (1971) estimates Hg released to the atmosphere from smelting as between 3 and 30 thousand tons annually.

Although Hg pollution from industrial and agricultural sources has declined, the total consumption of fossil fuels and the smelting of metal ores has not decreased.

iii) Mercury and Methyl Mercury Cycling in the Environment

The ultimate source and sink for Hg is the earth's molten core, but apart from active volcanoes, the immediate reservoir of Hg released to the environment is igneous rock. Fig. 1 shows a deliberately complex schematic of natural Hg flux in the environment. Soil, and sediment form the interfaces with the fluid media air and water respectively; Hg from all four of these compartments can be concentrated in biological material, translocated from one compartment to another or recycled within one compartment. Both of the fluid media are of vital importance in Hg dissemination from point sources, but their relative significance may lie in the mode of Hg introduction into the environment. Air is the more important vehicle for widespread dispersion of Hg from smelters and fossil fuels, and for movement of Hg evaporated from the earths surface. Mercury and MeHg have often been found in lakes and fish far from any known pollution sources or areas of Hg mineralisation (Forstner, 1976; Thompson, 1971; Philips & Medvick, 1981). Water is, however, the more important medium for introducing relatively high levels of Hg to biological material by wasting through underwater pipes, leaching of surrounding rock and land run-off. The residence time for Hg in any compartment is governed by a number of factors, but the chemical nature of the Hg and its affinity for the material in that compartment are paramount. Bowen (1975) estimates residence times of heavy metals in the atmosphere and fresh water on the order of days or weeks, while he is unable to estimate their residence times in soils, lake sediments or estuarine deposits. In biota, residence times could be as high as several years.

Fig.I: Pathways and Processes Involved in Environmental Mercury Cycling



Whatever the source of Hg in the environment, the comparative scarcity of the metal leads to the concept that Hg is a localised problem, and does not pose a worldwide threat (Goldwater, 1971). Peakall and Lovett (1972) have also suggested that man's use of Hg is unlikely to affect Hg concentrations in the oceans. Neither of these papers consider the increase in Hg pollution of the atmosphere from fossil fuels and smelting, or the increased leaching due to both land erosion and the increased acidity of rainfall. It has been estimated that the earth receives 100,000 tons of Hg annually in rainfall (Saha & McKinley, 1973). The total atmospheric Hg load and the evaporation rate from the earth's surface are unknown. However, since it has been shown that Hg content of the atmosphere decreases with altitude and is close to zero after a rainstorm (McCarthy et al., 1970), it could be argued that 100,000 tons may represent the bulk of the atmospheric Hg load. If this assumption is made, then by using estimates for the input of Hg from anthropogenic sources, it can be calculated that man's annual input to Hg that is cycled and redistributed is about 20%. In spite of the very high affinity of sulphur compounds for Hg, natural sulphur cycles, and the anthropogenic contribution to them, are frequently ignored in considering Hg transformation and redistribution.

CHEMISTRY, DISTRIBUTION AND MOBILISATION OF MERCURY AND METHYL MERCURY IN AQUATIC SYSTEMS

i) The Aqueous Chemistry of Mercury and Methyl Mercury

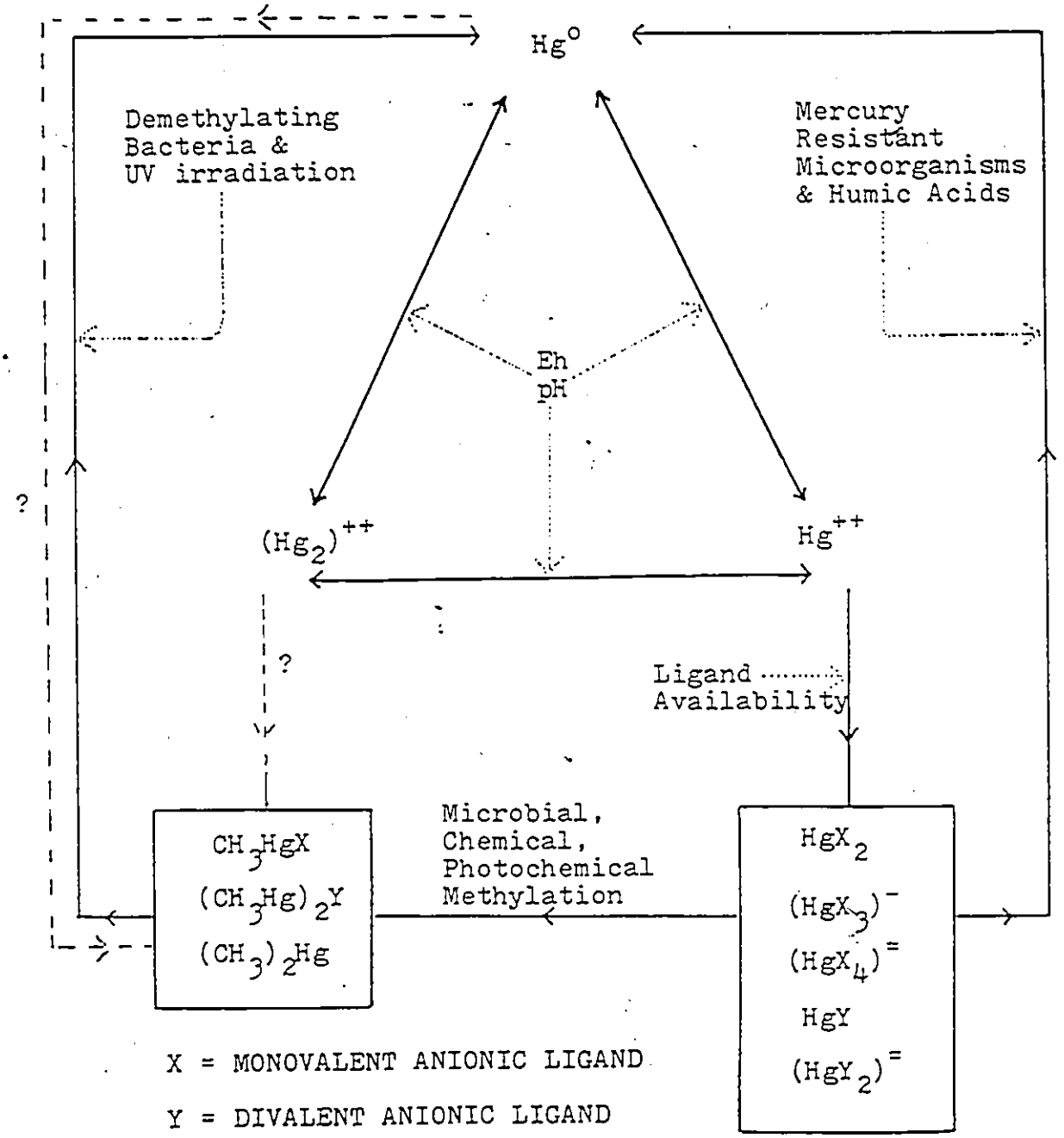
Mercury exists in three oxidation states; one uncharged Hg^{o}, and two ionic forms, Hg_2^{2+} and Hg^{2+}. Mercurous ions (Hg_2^{2+}) are usually relatively unstable, form few compounds, and readily

Undergo dismutation reactions to Hg^{o} and Hg^{2+}. Mercuric ions (Hg^{2+}) are the most oxidised and stable under oxidising conditions; they form many compounds. The interconversion of Hg between these three oxidation states depends on the oxidation reduction potential (Eh), the hydrogen ion concentration (pH) and the chemical composition of the medium with respect to complexing ions or molecules. Figure 2 shows what is known about Hg interconversions in the aquatic environment and the factors affecting them.

Under the normal conditions of Eh (0 - 500mV) and pH (5 - 9) prevailing in the aquatic environment, Hg will most likely be distributed between Hg^{o} and Hg^{2+} forms (Hem, 1970) with the sulphides predominating in anaerobic sediments and thiols or hydroxide or chloride predominating in the more oxidising waters. The concentration and variety of available ligands will determine what proportion of the metal is complexed and what remains as Hg^{o}. The composition of dissolved MeHg species in solution is also dependent on the type and concentration of complexing agents present and on the pH. Complexation of the MeHg group by reduced sulphur species results in mono- and di- methyl mercuric sulphide which are in equilibrium. Baughman et al. (1973) have calculated that these 2 species should account for >95% of the MeHg found in natural waters that contain reduced sulphur species: at high concentrations of reduced sulphur, and in more oxidising conditions in basic waters, $MeHgS^{-}$ would predominate, but $(MeHg)_2S$ becomes the predominant complex for more oxidising conditions in acid waters. In the absence of reduced sulphur, nearly all the

Figure 2

SOME ASPECTS OF MERCURY INTERCONVERSIONS IN AQUATIC SYSTEMS



MeHg would be organic thiols, and, when no suitable sulphur compounds are available, the hydroxide would dominate in basic, and the chloride in acidic, waters.

(ii) Mercury distribution in the aquatic environment.

When a pollutant such as Hg enters the aquatic environment, it is distributed among the various components of that environment to an extent dependent on the following:

a) the chemical and physical nature of the pollutant

b) the affinities of the components for the pollutant

c) the relative masses of the components

and d) the degree of mixing of the system.

Mercury binds avidly to both sediment and biological material, due mainly to its high affinity for sulphur and thiols, and is found in its highest concentrations in association with these components. The concentration of Hg in the water column is usually very low, but the huge mass of water in many aquatic systems can carry a significant proportion of the total Hg load. In the Ottawa River the water contains only 3% of the Hg load at any particular time, but is responsible for the transport of about 90% of the mobilised Hg: 1,300kg/year compared with 290 kg/year for suspended solids, and only 3.4 kg/year for the bed sediment (Ottawa River Project Group, 1979). The biomass in aquatic systems is however very variable, and in a fast flowing river system like the Ottawa River it is small, accounting for only a small fraction of the total Hg load (Ottawa River Project Group, 1979).

The main sink for Hg is the sediment which can be considered to be composed of finely divided complex silicates with varying

degrees of organic content. Sediment Hg concentrations have been studied extensively in the Ottawa River (Rust, 1977) and elsewhere (Andren & Harriss, 1973; Bartlett & Craig, 1981; Craig & Moreton, 1983) and have been found to be highest in sediments with high organic content. The total holding capacity of sediments with respect to Hg is enormous; in situ sediment levels as high as 9,300 ppm have been reported (D'Itri,, 1972b), and desorption is extremely slow, even in the presence of chelators (Ramamoorthy & Rust, 1977). If additional sinks, such as biota, are present then sediment desorption may be accelerated (Ramamoorthy et al., 1977).

The rate of adsorption to, and the form of Hg in, sediments is dependent to a large extent on the nature of the sediment, as well as the Eh, pH and ligand availability in the surrounding waters. Mercury binds very strongly to sulphur containing sediments (Reimers & Krenkel, 1972). At one time it was widely held that in such sediments, under anaerobic conditions, Hg would be immobilised as HgS. It has been shown that a fraction of sediment Hg can be regarded as organic extractable even in sediments with a high sulphur content: up to 45% in the top 4 cm. (Brinckman & Iverson, 1975). Presumably, the organic extractable Hg is bound to live organisms, decaying organic matter and complex soluble sulphides, but the chemistry of complex sulphur compounds in sediments is poorly understood.

Sediment interstitial waters have been reported to be enriched in Hg 10-30 times with respect to the surrounding waters (Lindberg & Harriss, 1974). As this is far in excess of what would be predicted from solubility data on HgS, the authors

suggest that this is due to the formation of organic and polysulphide complexes of Hg. Although very small quantities of MeHg have been found in association with sediments (20-190 ppb) (Batti et al., 1975; Andren & Harriss, 1973), it is obvious that this can only account for a very small fraction of the organic extractable Hg reported by Brinckman and Iverson (1975).

iii) Mercury and Methyl Mercury Mobilisation

Physical mobilisation

Physical processes can play an important role in the mobilisation of Hg and MeHg from aquatic systems. Fluvial systems may show a higher rate of clearance than stationary bodies of water (Van den Berg, 1971). Clearance of Hg from sediment in the Ottawa River has been estimated at more than 50% per year (Ottawa River Project Group, 1979). Flow through studies on the desorption rate from sediments from the same part of the river indicated a much slower removal (Kudo et al., 1975), but physical disruption of the sediment by the annual spring flood and the action of macrofauna during the whole year are believed to contribute to the accelerated clearance. The presence of Tubificid worms was suggested by Jernelov (1970) to be a means of making Hg in the sub-surface layers of the sediment available for methylation. Boddington et al. (1979) have postulated that the action of Tubificid worms could be sufficient to account for Hg clearance from the bed sediments of fluvial waters. The uncharged species Hg^{o} and $(CH_3)_2Hg$ are volatile and, under suitable conditions, can readily be lost to the atmosphere. Based on actual experimental data, Baughman et al. (1973) have calculated half-lives of

about 5.2 and 12 hours for Hg^{o} and diMeHg respectively in a moderately turbulent river.

Chemical Mobilisation

In general, the chemical state of Hg is the most important factor in controlling its location and flux. Oxidation of Hg^{0} to Hg^{2+} will be given by the equation:

$$E = 850 + 30 \log Hg^{2+}/k$$

where E = minimum potential (mV) for conversion to Hg2+ and k = binding constant between inorganic Hg and available complexing agents. Hence, the proportion and variety of chelators present will affect the potential necessary for oxidation of Hg^{o} and its combination with available ligands. Values of k for interaction of Hg with microbial growth media and Ottawa River water have been shown to be very high (Ramamoorthy & Kushner, 1975a; 1975b).

In many fresh waters and interstitial sediment waters the most influential chelators will probably be the soluble organics, whether they are naturally occurring substances, such as humic and fulvic acids, or pollutants. Studies of Ottawa River water taken from the OREL site show a metal holding capacity with respect to Hg of 15 mg/L (Ramamoorthy & Kushner, 1975a). Filtration of the samples through ultrafilters with an exclusion limit down to 1400 MW shows little loss in binding capacity. This binding capacity was destroyed by ashing, indicating that the Hg was bound to low MW ligands which were organic in nature (Ramamoorthy & Kushner, 1975a). Inorganic chelators cannot be disregarded; in another local waterway (the Rideau Canal) these same authors showed a significant contribution from bicarbonate or carbonate binding.

The nature of the organic molecules binding strongly to Hg is unknown but Ramamoorthy and Kushner (1975a) suggest fulvic acid as a possible candidate. Other work (Peng et al., 1982) has shown strong complexing of Hg by both fulvic acid and humic acid, but the greatest complexing was between Hg and the low MW fulvic acid: the overall complexing capacity of the fulvic and humic acids in water was greater than in sediment.

It is probable that a wide range of low molecular weight substances would be available for this role in natural waters. Hama & Handa (1980) studied the molecular weight distribution and characterisation of dissolved organic matter in lakes and found that carbohydrates occurred exclusively as polysaccharides, while free and combined amino acids with >1400, 600-1400, 200-500 and <200 molecular weight accounted for about 40, 5, 20 and 35% of total proteins and amino acids respectively. Organic acids, a large proportion of which was acetic acid, were found exclusively at <200 MW. These 3 fractions constituted only 20% of the organic carbon; the remainder was uncharacterised. Of these, the compounds with the highest affinity for Hg are the sulphur containing amino acids. It is rather surprising that no one has tried to link Hg to the relatively high concentrations of simple, biogenic organic thiols and sulphides that are known to occur in natural waters (Jenkins et al., 1967; Alexander, 1974) and are now believed to play a vital role in natural sulphur cycles (Lovelock et al., 1972; Nguyen et al., 1978; Andreae et al., 1983). Further understanding of Hg translocation processes may be possible if the nature of the most prevalent Hg-binding molecules

in water could be determined.

Low pH values increase the adsorption of Hg to sediment (Matsumura et al., 1972), and increase the uptake of Hg by fish (Tsai et al., 1975). Miller & Akagi (1979) concluded that at low pH the distribution of MeHg between the sediment and the overlying water is sufficiently altered to account for all the increased uptake observed in fish.

In waters that are high in Cl^- ions, such as estuarine or sea water, the solubility of Hg may be greatly increased by the formation of uncharged $HgCl_2$ and soluble anionic complexes such as $HgCl_3^-$ and $HgCl_4^{2-}$. Jewett et al. (1975) have shown that large pH changes have little effect on the $HgCl_n^{2-n}$ species present, whereas small changes in chloride concentration produced significant changes in the relative abundances of the chloromercury species present.

Biological Mobilisation

Alberts et al. (1974) describe the evolution of Hg^o from humic acid without apparent microbial involvement. Loss of Hg occurred at a slow but significant rate and the total amount, but not the rate limiting step, was influenced by pH. Other workers (Ramamoorthy et al, 1983) report that both living and dead bacteria transport Hg^o from water into air in equivalent amounts. But most reports on Hg reduction to Hg^o involve microbial detoxification mechanisms for Hg or MeHg or both, and there is abundant evidence that production of Hg^o by microorganisms correlates well with their resistance to mercurials. It is not known, however, what proportion of the Hg volatilised from the earth's surface is due to physical, chemical or biological action.

The present state of knowledge on transformation of inorganic and organic mercurials to Hg^{o} has been recently reviewed (Summers & Silver, 1978; Pan Hou & Imura, 1983). The mechanism of bacterial resistance to Hg has been characterised to plasmid-borne genes in all bacterial species studied (Summers & Silver, 1978), though the spectrum of mercurials reduced varies from plasmid to plasmid, and resistance is always inducible. The frequent association of Hg resistance with multiple antibiotic resistance (Nakahara et al., 1977; Summers & Silver, 1978) has led to the proposal by Tanaka et al. (1983) that transposons encoding multiple drug resistance might have arisen from an ancestral Hg transposon. Seasonal fluctuations in the percent of Hg-resistant bacteria in sediments have been observed (Colwell et al., 1975).

Perhaps the most important finding from the point of view of this study is that bacterial degradation of MeHg could occur in lake sediments (Spangler et al., 1973a) and river sediments (Billen et al., 1974). Spangler et al. (1973b) also reported that of 207 isolates from environmental samples, 30 were positive for aerobic demethylation and most of those also degraded MeHg anaerobically: resistant isolates could all tolerate 0.5ppm MeHg. Shariat et al.(1979) found that most common laboratory strains could demethylate 20-80% of added MeHgCl. Further studies (Mason et al., 1979) concluded that demethylating ability would be fairly rapid at the range of pH values likely to be encountered in the environment.

Mercury and MeHg resistant fungi also volatilise Hg^{o}

(Brunker & Bott, 1974); but the genetic basis for Hg resistance in fungi has not been elucidated, though it appears to be related to MET metabolism in yeast. Singh & Sherman (1974), using MeHg resistant yeasts, have observed that about 50% of the mutants obtained as MeHg resistant also demonstrate a requirement for methionine (met$^-$). They suggest from the growth response of these met2 and met15 strains on double gradient plates of MET and either MeHgCl or $HgCl_2$ that, at a critical concentration, MeHg or Hg may, at least partially, alleviate the MET requirement of their mutants. However, they also observed an apparent contradiction: these met2 and met15 mutants were recovered at a much higher frequency on methionineless media. The authors offer a possible explanation for their observations based on build-up of a hypothetical compound which inhibits MET biosynthesis: this compound is then destroyed by MeHg which not only detoxifies MeHg, but also relieves the MET requirement. Formation of this detoxifying compound, which the authors suggest might be a SH-containing compound, would then be inhibited by the presence of MET in the growth medium, possibly by a feedback process.

It can be seen from the aforegoing discussion that both Hg and MeHg resistant microorganisms are extremely widespread, can carry out the reduction of Hg under both aerobic and anaerobic conditions and have the potential to play a large role in Hg mobilisation and interconversion. Demethylating action by bacteria may also be responsible for the very low levels of MeHg found in many environmental samples by preventing MeHg build-up.

Though the production of Hg^o is well known, its subsequent fate is much less certain. In areas of high Eh, and with high

organic or inorganic content it is likely that at least some of it will be reoxidised to Hg^{2+}, and thus recycled within the aquatic environment. However, much of it may be protected in reduced form, possibly by lipids or a microenvironment with a low Eh, and then lost to the air.

In addition to the volatilisation of Hg, microorganisms of all types may play a part in the mobilisation of Hg from aquatic sediments by producing extracellular sequestering agents which would bind the Hg. Such compounds include organic thiols and sulphides, proteins and amino acids, organic acids, biogenic amines and mucopolysaccharides. Most of these are known to be found in measurable concentrations in natural waters (Alexander, 1974; Hama & Handa, 1980; Andreae & Raemdonck, 1983). However, since Hg is found mainly in association with compounds of low (< 1400) MW (Ramaoorthy & Kushner, 1975a; Peng et al., 1982) the amino acids, organic acids and organic thiols and sulphides may be the most important of these. In addition, humic and fulvic acids formed by degradation of organic matter are known to bind Hg strongly; it has been shown that the total humic and fulvic acids present in the water column may exceed those present in the sediment (Peng et al., 1982).

Formation of MeHg may facilitate movement of Hg into biota, and is discussed fully in the next section. However, the production of the more volatile diMeHg species would, if shown to occur in situ, play an important role in mercury mobilisation.

FORMATION OF METHYL MERCURY IN THE ENVIRONMENT

The literature on MeHg formation is abundant, but rather confused: about the only incontrovertible fact is that it is produced. Perhaps the best way to examine this topic is to ask a number of questions:

1) Where is MeHg formed?

2) When is it formed?

3) Under what conditions is it formed?

4) Is it formed biologically, abiologically or both?

5) What is the Hg source?

6) What is the source of the methyl groups?

7) How stable is the MeHg product?

8) How are the factors favouring its formation and breakdown balanced?

WHERE IS METHYL MERCURY FORMED?

Since the first report of MeHg formation in aquaria sediments and rotting fish (Jensen & Jernelov, 1969), environmental Hg methylation has been widely assumed to be due to the activity of bacteria in Hg-containing sediments (Bishop, 1972; Alexander, 1974; Hamdy & Noyes, 1975). The MeHg was then supposed to be released to the overlying water from where it could be accumulated by fish (Langley, 1973; Philips & Medvick, 1981). The evidence for this theory is incomplete and mostly circumstantial, but it has persisted widely because of the observation that fish swimming above sediments which contain inorganic Hg accumulate MeHg in their tissues (Gillespie & Scott, 1971; Langley, 1973; Kudo, 1976; Miller & Akagi, 1979). In this type of system, however, methylation occurring in the sediment, in the water column

or on and in fish cannot be distinguished.

It can be argued that the small quantities of MeHg found in sediments may not be readily released because of the high concentration of sulphides and thiols which are also present. This would be supported by the higher concentrations of MeHg found in sediments with a high organic content (Bartlett & Craig, 1981). In addition, if the sediment were the principal source of MeHg in fish, then bottom feeders would be expected to contain the highest levels of MeHg this is not the case (A. DeFreitas, personal communication).

Since MeHg is found ubiquitously, it would not be unreasonable to assume that MeHg formation is also widespread. It has been shown to occur in fluvial (Langley, 1973; Craig & Bartlett, 1981), lacustrine (Rissanen et al., 1970; Furutani & Rudd, 1980), estuarine (Olsen & Cooper, 1974; Blum & Bartha, 1980) and marine sediments (Rissanen et al., 1970; Berdicevsky et al., 1979), as well as terrestrial soils (Rogers, 1975; 1977). In many respects the surface layer of water, and other surfaces and interfaces within the water column are analogous to sediment. Nutrient and microorganism accumulation at surfaces and interfaces is very well recognised (Bitton & Marshall, 1980; Norkrans, 1980). Since Hg binds very strongly to organic matter, it will also tend to accumulate at surfaces and interfaces, and if the only source of Hg to a water body is in rainfall, then the surface and subsurface layers of water may play a major role in MeHg production. Methyl Hg production has recently been reported in both fresh (Furutani & Rudd, 1980) and marine (Topping & Davies, 1981) water

columns. Nothing is presently known about aerial formation of MeHg but it would seem less likely to occur, unless it is as a result of concentrated industrial emissions. However, Soldano et al. (1975) have reported an increase in airborne organic Hg and a decrease in Hg^{o} within several miles of sewage treatment plants; it is not known if this corresponds to a formation of MeHg.

There is still controversy about the in vivo biosynthesis of MeHg; it has been reported to occur in some cases but not in others. In view of the high levels of MeHg in fish, some investigations have been conducted on endogenous methylation using in vitro liver homogenates (Imura et al., 1972; Matsumura et al., 1975). Both groups have reported endogenous methylation (0.1-0.3% conversion) from inorganic Hg. The factor/s responsible were not destroyed by autoclaving and or HCl treatment, could not be separated by centrifugation and reacted very rapidly. In addition, in vitro methylation was only slightly inhibited by UV or visible radiation but was markedly inhibited by mersalyl. Matsumura et al. (1975) also found much reduced levels of MeHg when fresh livers were used instead of frozen ones. The observation of endogenous methylation in vitro does not, however, guarantee that the same process will be operative in vivo.

Recently, Rudd et al. (1980) reported Hg methylation, under strictly anaerobic conditions, by fish intestinal contents from 6 species of fish and a number of different lakes, both polluted and non-polluted, but with considerable fish to fish variation. However, Pan Hou & Imura (1981a), Pennachioni et al. (1976), Huckabee et al. (1978) and ourselves (see below) have been unable to observe this. This apparent conflict may be due to differences

in sample handling and conditions of incubation as well as to the predominance of demethylating activity under aerobic conditions.

Methyl Hg formation by human feces has been reported under strictly anaerobic conditions (Edwards & McBride, 1975) and by isolates from human and rat (Rowland et al., 1975; 1977) intestinal flora under both aerobic and anaerobic conditions as well as isolates of human buccal flora (Heintze et al., 1983). However, none of 11 species of anaerobes isolated from strained rumen fluid was able to produce MeHg (Kozak & Forsberg, 1979).

Conversion rates of Hg to MeHg have been reported to be very high in sewage treatment plants (Jernelov, 1969). Although there are large bacterial populations in such settings, it is not known if methylation occurs there by a biological or abiological mechanism. Decaying matter in general appears to be a good source of MeHg formation in the environment, however, in most reports, it cannot be ascertained if there was direct microbial participation in the methylation process. The best known example of this is the original report of MeHg production in decaying fish (Jensen & Jernelov, 1969). There is some indication that wood chip sediments in the Ottawa River (Kudo, 1977) and elsewhere (Jackson & Woychuk, 1980) are particularly suited to MeHg production. It is not known if this could be due to liberation of the known labile methyl groups from lignin during wood decomposition. Also, the observation that MeHg formation can occur abiologically with fulvic and humic acids derived from leaf litter (Nagase et al., 1982) implies that decaying leaves may provide another source of MeHg in both the aquatic and terrestrial environment.

WHEN DOES ENVIRONMENTAL MERCURY METHYLATION OCCUR?

There is considerably less information available on this topic, partly because of the difficulties of doing *in situ* studies. It would seem probable that some MeHg production occurs continually, but seasonal fluctuations have been reported in fresh water and sediment (Furutani & Rudd, 1980) and in estuarine sediment (Olson 1978). Furutani & Rudd (1980) showed peaks of MeHg production in the sediment from a polluted Canadian lake to occur in May and to a lesser extent at the end of June and August. Peaks in the water column were coincident with the June peak and slightly preceded the peak in August. The authors correlate the methylation with overall community respiration. It would not be unreasonable, however, to speculate that the May peak in the sediment was due to increased bacterial degradation of detritus with other added nutrients resulting from spring run-off. Water levels were not tested at this time. The other peaks could possibly be related to high phytoplankton productivity and later decay during the summer months. Similar explanations could be made for the work of Olson (1978), but here, because of the climate, it is possible that both peaks observed in the estuarine sediment (Dec-Jan & April-July) were associated with algal blooms, possibly of different species.

UNDER WHAT CONDITIONS DOES ENVIRONMENTAL METHYL MERCURY PRODUCTION OCCUR?

a) Aerobic or anaerobic?

Both aerobic and anaerobic methylation has been reported in whole sediments (Rissanen *et al.*, 1970; Berdicevsky *et al.*, 1979), bacterial cultures from sediments (Hamdy & Noyes, 1975;

Bisogni & Lawrence, 1973; Bishop, 1972) and feces (Rowland et al., 1975; 1977). Some workers claim that methylation takes place only under one condition or the other, but it would seem to depend on the type of organisms used, and it is now widely accepted that methylation can occur under either aerobic or anaerobic conditions. Eh may play an important role both in controlling the available Hg species (discussed earlier) and in the metabolic state of the organisms present.

b) pH?

Likewise, pH is a very important factor, though it is probable that some organisms can methylate at most of the pH values usually found in natural waters. Most workers with microbial cultures have used buffered media and not examined the effect of pH (Bisogni & Lawrence, 1973; Hamdy & Noyes, 1975; Vonk & Sijpesteijn, 1973). It has been reported for some systems that methylation of Hg has an optimum pH, of 7 (Yamada & Tonomura, 1971a) or 4 (Bertilsson & Neujahr, 1971; Rogers, 1977; Nagase, 1982), but this may vary depending on the mechanism of methylation and indeed the same mechanism could also be operative biologically and abiologically, but at different pH levels: such may be the case with methylcobalamin (Wood et al., 1968; Bertilsson & Neujahr, 1971). Baker et al. (1981), measuring Hg methylation in the acidic aquatic environment, found that it could only take place between pH 5.5-6.5. Miller & Akagi (1979) have found that pH did not affect the total amount of MeHg produced, but did affect its distribution between water and sediment.

c) Cl^- ?

Because of the large quantity of chloride ion in sea water there has been some speculation that this may affect the Hg methylation process in estuarine and sea water. Jewett et al. (1975) have shown that although pH has very little effect on the Hg species in solution in salt water, a small change in concentration of chloride ions can significantly change the chloromercury species present. In the abiotic transmethylation of Hg from trimethyl tin, the maximum methylation occurred with a Cl to Hg ratio of 4. The effect of sea salt anions has been shown to reduce the availablity of Hg for methylation (Blum & Bartha, 1980; Compeau & Bartha, 1983). A maximum methylation rate was observed at a salinity of 0.1% (2.3% conversion) compared with 2-3% (0.05% conversion) (Blum & Bartha, 1980). The HCO_3^- component of sea salts was also shown to slow methylation (Compeau & Bartha, 1983). In a study of Hg in one river system and its estuary (Airey & Jones, 1982) it has been shown that both the dissolved Hg and the Hg load on suspended particulate matter is always greater in fresh than in tidal waters. It might, therefore, be implied that MeHg production in fresh water would be greater than in seawater, but this is not known to be the case.

d) S^- , SH^- ?

The role of sulphur in Hg and MeHg chemistry has already been discussed above. Numerous investigators have shown a reduction in MeHg formation in the presence of either high sulphide or thiol concentrations (Bisogni & Lawrence, 1973; Yamada & Tonomura, 1972a; 1972b; 1972c) It is thought that the former may reduce the availability of Hg for methylation, whereas, in some

cases there is evidence that thiols such as homocysteine and cysteine may cause more methylation initially with a subsequent decline (Yamada & Tonomura, 1972a). The yield of MeHg has also been reported to increase in the presence of excess homocysteine or cysteine over Hg present (Landner, 1971). As this did not happen in the presence of other thiols, the author attributes MeHg formation to an incorrect synthesis of methionine (MET); his explanation is however unclear. From his data, an equally plausible alternative would be the methylation of Hg from the methyl group of MET, where the function of homocysteine is not only to provide a carbon skeleton for MET, but also to bind and detoxify the MeHg product. Landner (1971) also reported an increase in toxicity of Hg on the addition of MET: this could be accounted for if MET was directly involved in the methylation reaction.

Several workers (Landner, 1971; Hamdy & Noyes, 1975; Pan Hou & Imura, 1982) have suggested that Hg methylation might be a detoxification mechanism. However, this is logistically unlikely as the tolerance for MeHg on a molar basis is always less than for inorganic Hg. It is more likely, since each of these groups was working with Hg resistant strains, that in the presence of a high concentration of Hg there is an accidental methylation of Hg by a normal cellular methylating system.

Even HgS can be made available for methylation, presumably by oxidation (Fagerstrom & Jernelov, 1971), and photochemical production of MeHg from pure mercuric sulphide has been shown (Akagi et al., 1977; Okouchi & Sasaki, 1983; and ouselves - see below). It is believed that the HgS is dissociated by photolysis,

with the oxidation of the S to SO_4 via a polythionic acid and the reduction of Hg^{2+} to Hg^{o} (Okouchi & Sasaki, 1983). It is possible that this could increase the MeHg production in certain waters, and may be important in dredging, but the environmental significance of such a process is difficult to assess. However, Hg bound to particulate matter suspended in surface waters may be present as an organic sulphide complex. Nothing is known about the photochemical production of MeHg from such material.

e) Other factors?

Increased heavy metal pollution raises the possibility of methyl group transfer from one metal to another (Craig, 1981): this has been shown in the laboratory (Huey et al., 1975).

IS MERCURY METHYLATION BIOLOGICAL OR ABIOLOGICAL?

One of the major controversies of Hg methylation has been whether it occurs biologically or abiologically. It would now seem that both modes are likely to occur.

Even though the exact role of microorganisms in Hg methylation remains to be elucidated, their importance in the overall process is widely accepted. Biological involvement in MeHg production can be of 2 types:

a) active biological methylation by an enzymatic process

or b) chemical methylation in which the function of the organism is to provide the methyl donor molecule and or catalyst.

There is little firm evidence to support either mechanism, but more circumstantial evidence for the latter. In experiments with both pure and mixed cultures of microorganisms, it has universally been observed that, as the quantity of added Hg is increased, the total amount of MeHg found also increased, but the

percentage yield decreased: 1-0.001% for 1-1000 ppm (Jensen & Jernelov, 1969); 5.9-0.1% for 0.2-53 ppm (Bisógni & Lawrence, 1973); 0.5-0.3% for 0.1-1.4 ppm (Edwards & McBride, 1975); 98-0.024% for 0.1-10 ppm (Berdicevsky et al., 1979); 1.2-0.02% for 10-40 ppm (Yamada & Tonomura, 1972a). This could imply that either the methyl donor concentration was the limiting factor, or that the most actively methylating organisms are also among the most sensitive to MeHg and are preferentially killed as the MeHg concentration rises. The small quantity of MeHg produced even in rich cultures and after a long time do not support an active enzyme role, particularly since many enzymes are known to be inhibited by mercurials (Clarkson, 1972; Thrasher, 1973).

Of the known biological methylating agents, only the methyl corrinoids are capable of transferring methyl groups to Hg (as CH_3^-) (Wood, 1968; Wood, 1974). They showed MeHg production in cell-free extracts of a methanogenic bacterium which was able to use methylcobalamin as the source of the methyl group in methane formation. Mercuric ions strongly inhibited this reaction and methylated Hg products were identified. Transfer of alkyl groups to Hg was also shown to occur non-enzymatically. Since this first investigation, alkylation of Hg by methyl cobalamin has been widely studied (Bertilsson & Neujahr, 1971; Agnes et al., 1971; Imura et al., 1971 De Simone et al., 1973). DeSimone et al. (1973) chose mercuric acetate (HgAc) as the attacking agent because they thought it might be one of the Hg compounds found in natural conditions. This may be true, but HgAc itself has been shown to produce MeHg at a significant rate under exposure to UV

or sunlight (Akagi & Takabatake, 1973; ourselves - see below).

Methyl cobalamin can react chemically with inorganic Hg to give MeHg: 80% yield in 2 hours (Imura et al., 1971), which is more than any other environmentally feasible mechanism yet known. Although this is widely accepted as the means of producing MeHg in most bacterial cultures (Yamada & Tonomura, 1972 a & b; Hamdy & Noyes, 1975; Pan Hou & Imura, 1982), the levels of methylcobalamin in natural systems are unknown. Since it is a photolabile compound, it is unlikely to accumulate in a shallow water column, but it can probably be reformed rapidly if a suitable methyl donor is available. It has been reported in pelagic fish liver (Matsumura et al., 1975) and algal cultures (Neujahr & Fries, 1966). The initial report on the involvement of methyl cobalamin in Hg methylation (Wood et al., 1969) used an extract of a methanogenic bacterium because methylcobalamin was thought to be an important intermediate in methane biosynthesis. Although Hg methylation has been reported from methanogenic enrichment cultures from pond sediment, but not autoclaved controls, (Bishop, 1972), no MeHg was found in semicontinuous methane fermentations (Yamada & Tonomura, 1972c), or in cultures of bacterial isolates from rumen fluid (Kozak & Forsberg, 1979).

There is controversy over whether the natural product is mono- or diMeHg. Imura et al. (1971), Jensen & Jernelov (1969) and Wood et al. (1968) all argue that the dimethyl product is the natural one and the monomethyl Hg found is an artefact of the extraction procedure. However, most workers with laboratory bacterial cultures were unable to find diMeHg in reasonable quantities (Bisogni & Lawrence, 1973; Bishop, 1972) and this was put

down to stripping from the system. However it is possible that the dimethyl product is only produced if the methyl groups are in excess. This might be the case in natural waters, but in experiments in Hg methylation the Hg concentrations tend to be much higher and the methyl groups may be limiting the formation of both mono- and diMeHg.

One of the important points raised by Bertilsson & Neujahr (1971) was that transfer of the methyl group from methyl cobalamin to Hg was markedly inhibited by thiol groups and abolished completely by cell extracts of E.coli at a protein concentration of 1 mg/mL. This might lead to the conclusion that the inside of a cell would be an unlikely place for any non-enzymatic reaction. However, since -SH groups have been implicated in reactions catalysed by B_{12}-dependent enzymes, including homocysteine thiolate anion in methionine synthetase (Frick et al., 1976), and ethane thiol sulphonic acid as an intermediate methyl group acceptor in methane biosynthesis, the enzymatic transfer of a methyl group from methylcobalamin to Hg should be able to occur in vivo. However, in spite of the general acceptance of methylcobalamin involvement in Hg methylation, no conclusive proof of enzyme involvement is available. Kinoshita et al. (1978) investigated the possibility that oral administration of methylcobalamin might enhance biological methylation in vivo, but observed no effect.

A further complication is introduced into this puzzle by the observation that cultures of some bacteria which produce MeHg, do so only after they have been allowed to sit for prolonged periods

before analysis (W. P. Iverson, personal communication); presumably some cellular decay is in process at that time. Still other workers (Ramamoorthy et al., 1982) report production of MeHg by killed bacterial cultures, but not by living ones. These last results implicate release of a methylating factor during the decay process, and would suggest abiological methylation of Hg in the natural environment. This is consistent with the original observation by Jensen & Jernelov (1969) of MeHg production by rotting fish.

Some of the work on abiological methylation has already been mentioned but is worth restating. Methylation of Hg can occur non-enzymatically at a pH of 4 (De Simone et al., 1973).

Abiotic methylation has been reported in soil (Rogers, 1975; 1977) and from humic and fulvic acids extracted from leaf litter and sediment (Nagase et al., 1982). In the first case the methylating factor was: soluble in NaOH, heat stable (121 °C), UV labile and appeared to be low MW (<12,000) fulvic acid (FA). No methylation was observed with commercial samples of humic acid (HA). Nagase and coworkers (1982) observed that all humic and fulvic acid samples methylated Hg under lab conditions in the dark and in the absence of bacteria. The production of MeHg was influenced by temperature, Hg concentration, pH and concentration of HA and FA. All FA methylated at both 4 °C and 20 °C, but HA only methylated at 20 °C. Low FA (MW <200) was by far the most active methylating fraction, with a pH optimum of 4. Although no characterisation of the actively methylating fraction was done, it is quite possible that organic sulphur compounds or organic acids are involved here. These studies not only suggest that Hg methyl-

ation may take place abiotically in the natural environment, especially in acid waters, but also that leaf decomposition in natural waters might be one factor which influences the periodicity of MeHg production. A further implication might be that, in many natural environments, it is the Hg which is limiting the methylation process rather than the methyl group availability. In this case, even subsequent demethylation of MeHg formed would not ensure Hg removal from the system because continual provision of available methyl groups would remethylate it in a cyclic fashion.

In addition to the above, photochemical studies using irradiation of defined chemical systems have shown MeHg production from: a) pure mercuric sulphide in the presence of acetate ions (Akagi et al., 1975; 1977). Along with the methylation, which was considerably greater under aerobic conditions than in a nitrogen atmosphere, they report significant amounts of "photo-sulphur" which they believe acts as a photosensitizer for the methylation reaction. b) mercuric chloride and one of the following methyl donors, methanol, ethanol, acetic acid, propionic acid (Akagi & Sakagami, 1971a; 1972b). These are all metabolic end products of bacterial metabolism and likely to be present, at least transiently, in the natural environment. The yield was highest with acetic acid (0.1% in 20 hrs.). Formation of the alkyl product was slow over the first 24 hours and then increased up to 96 hours. c) mercuric acetate alone (Akagi & Takabatake, 1973). This reaction appeared to follow first order kinetics for 168 hours and give a yield of about 10%. d) $HgCl_2$ and one of the following aliphatic amino acids, glycine, alanine, valine, leucine and

isoleucine (Hayashi et al., 1979). The Hg product was almost exclusively MeHg for all amino acids except glycine, which produced no MeHg, and leucine which produced MeHg and an additional unidentified Hg compound. An interesting difference was observed between blacklight irradiation and the Hg vapour lamp; the first showed ILEU > LEU > VAL > ALA, whereas the second was ALA > LEU > VAL > ILEU. Since no MeHg was formed from glycine, the authors concluded that MeHg formation was due to the fragmentation of the aliphatic side chains during photolysis.

The significance of photochemical methylation is difficult to assess from these experiments, because they were all done under chemically defined conditions. The reaction itself is much slower than that between methyl cobalamin and Hg, producing a maximum yield of 10% after several days (Akagi & Takabatake, 1973). However, it is possible that the most natural or sensitive Hg substrates or methyl donors have not yet been tried, and photochemical reactions could have considerable importance in shallow waters or the surface layers of deeper ones.

From the above, it would appear that Hg methylation in the aquatic environment could occur by a variety of different but interrelated mechanisms. It may therefore be impossible to estimate the relative contributions of each to the environmental Hg load.

HOW STABLE IS THE METHYL MERCURY PRODUCT?

The simple answer to this is that it is stable enough to be measured. There are several studies that indicate photolysis of organomercurials. Baughman (1973) has studied MeHg thiols and sulphide and determined that the photochemical half-lives of

these compounds are 46-120 hrs.(thiols) and >0.43 hrs.(sulphide). He also suggests that photodecomposition of the MeHg halides, particularly the iodide, used for analysis could introduce significant errors into analytical measurements. Organomercurials are also subject to ozonolysis (Waters et al., 1972) which may be an important method for their destruction in the atmosphere.

In addition to their photochemical instability and their reaction with ozone, MeHg compounds are readily demethylated by a number of species of bacteria (Spangler et al., 1973b; Billen et al., 1974; Shariat et al., 1979) and this process is likely to be fairly rapid at normal environmental pH values (Mason et al., 1979). It is interesting to note that although no Hg methylation or volatilisation of Hg^{2+} by rumen bacteria was observed (Kozak & Forsberg, 1979) a significant proportion of isolates demethylated MeHg, and the rate was proportional to added MeHg up to 100 ppm.

WHAT IS THE SOURCE OF MERCURY FOR METHYLATION?

It is clear from the majority of the work on this topic that inorganic Hg added to a methylating system as a chloride, nitrate, acetate or even sulphide can form MeHg. DeSimone et al. (1973) suggested HgAc could be analogous to the Hg compounds found in sediments, and certainly, if Hg in natural waters occurs primarily as $Hg(OH)_2$, then some HgAc might be expected. However, the universal observation among workers in this field that methylation is greatest in where the organic concentration is greatest may imply that an organic complex of Hg would enhance methylation in some way. Also, when mercuric ions are added to organic material they are rapidly bound (Ramamoorthy & Kushner, 1975a; 1975b).

It therefore seems probable that the natural, though not necessarily the best, substrate for methylation would be a Hg thiol or Hg organic sulphide. One other possiblity which is very attractive, is that a Hg complex with the thioether moiety of methionine, or other thioethers, would make a suitable substrate. Birgersson et al. (1973) have shown that the methionine complex with Hg acetate is stoichiometric (2:1) and is readily isolated; the Hg is localised to the sulphur of methionine. This contrasts with the polymeric ionic complex between $HgCl_2$ and methionine. The strong thioether-Hg bond is also supported by Carty & Taylor (1976). Previously, the Hg had been thought to interact with methionine only through the amine or carboxylate sites. Since both acetic acid and methionine are present in measurable quantities in surface waters, this complex may exist in naturally.

The question also arises as to whether Hg^o is available for methylation: here again there is apparent conflict. Holm & Cox (1975), using a system where Hg could only reach the bacterial culture as Hg^o, showed no Hg methylation, though considerable Hg was bound to the cells. In this system (glucose-minimal salts, sterile) Hg^o was stable for 48 hours. Jernelov (1972) reported Hg methylation at a rate comparable to $HgCl_2$. No experimental details are reported, but it is possible that the Hg was oxidised in this case. If Hg^o is oxidised in the presence of acetic acid then it is likely that HgAc could be found in natural waters.

WHAT IS THE SOURCE OF THE METHYL GROUPS?

Some discussion of this has already been done above. It is clear that methylcobalamin can act as the methyl donor if present in sufficent concentrations and under the right conditions. How-

ever, even methylcobalamin must get its methyl groups from somewhere, and it would seem more likely that this sytem could operate by transmethylation if a continuous supply of methyl groups was available. The key question is then, what is the source of the methyl groups? This question has not been addressed by any of the workers in the field. It would be particularly interesting to know what factors are responsible for the abiological Hg methylation observed by Nagase et al. (1982).

HOW ARE THE FACTORS FAVOURING THE FORMATION AND BREAKDOWN OF METHYL MERCURY BALANCED?

It would seem that MeHg can be produced chemically and biologically and is also degraded biologically. In many systems this leads to cyclic fluctuations in the levels of methyl Hg which builds up until the demethylating bacteria are induced (Jernelov, 1975). Similar fluctuations have been reported in pure culture (Hamdy & Noyes, 1975), but it is not yet clear if a single organism will both form and degrade methyl Hg. Such a situation has however been reported for Clostridium cochlearium (Pan Hou & Imura, 1981) where demethylation appears to depend on the presence of a transferrable plasmid. Also, in a mixed culture such as intestinal flora of fish, one would expect to find both methylation and demethylation. Here, however, the rapid uptake of MeHg in the gut may ensure MeHg production for prolonged periods. In the surface layers of water the additional formation and destruction of organic Hg due to sunlight must be considered. Little is known about the relative rates of formation and degradation of MeHg in natural systems.

INTRODUCTION TO THE EXPERIMENTAL SECTION

The experimental work reported here was undertaken in an attempt to evaluate the role of microorganisms in the formation of MeHg in the aquatic environment. The study was divided into 4 sections:

i) Estimation of the relative numbers of bacteria in the freshwater environment which were able to grow in the absence and presence of Hg.

ii) Competition studies between bacteria and sediment, or bacteria and water for Hg in a third compartment of a 3-compartment model.

iii) Experiments on the formation of MeHg by microbial cultures.

iv) Experiments on the photochemical formation of MeHg.

These sections were aimed at investigating the following questions respectively:

i) If Hg methylation is effected by a wide variety of naturally occuring organisms, how does the presence of Hg in culture media affect the numbers of organisms capable of growth?

ii) Can microrganisms have direct access to Hg in the natural environment?

iii) Do cultures of microorganisms from the Ottawa River, or elsewhere, methylate Hg?

iv) Is there an alternate abiological route by which significant quantities of MeHg can be formed in the environment?

MATERIALS & METHODS

Microbial Cultures

Mixed cultures of microorganisms were obtained from water, sediment or fish taken from the Ottawa River in the section under study by the Ottawa River Project (Ottawa River Project, 1977). Pure cultures of bacteria were isolated from the above and stored at 4°C on agar slants.

Three bacterial strains with the reported ability to methylate Hg were obtained from Dr. W. P. Iverson, National Bureau of Standards, Washington, D.C. :

a) 3-CD, a facultative organism which produced CH_3Hg under anaerobic conditions (W. P. Iverson, personal communication).

b) J-53-1, a plasmid-bearing substrain of *E. coli* K12 which produced both CH_3Hg and Hg^{o} (W. P. Iverson, personal communication).

c) *Pseudomonas* strain #244, an isolate from Chesapeake Bay which was used in studies on the methylation of tin and cadmium (Huey *et al.*, 1975).

Fungal cultures were obtained from Dr. M. Wellman at the University of Western Ontario (*Scopulariopsis brevicaulis*); Dr. I. B. Heath at York University (*Saprolegnia ferax*); and the National Research Council culture collection (*Aspergillus niger*, NRC #401121; Neurospora crassa, NRC#407002; *Myrothecium verrucaria*, NRC#413005; and *Penicillium notatum*, NRC#408010).

A laboratory strain of *Pseudomonas fluorescens* was used for experiments on the transfer of Hg to bacteria.

Microbial Growth Media

Ottawa River bacterial isolates and mixed bacterial cultures

used in methylation experiments were grown on Foote and Taylor (F & T) medium (Foote & Taylor, 1949: 0.05% peptone, 0.02% K_2HPO_4, 0.005% $MgSO_4.7H_2O$, trace of $FeCl_3$ & 1.5% agar for solid media only), or the same medium supplemented with 0.05% yeast extract and 0.1% glucose (F & T+). The other microbial cultures were grown in: 3-CD - Nelson's medium (Nelson et al., 1972: estuarine salts solution comprised of 1% NaCl, 0.23% $MgCl_2.6H_2O$ & 0.03% KCl and containing 0.2% glucose, 0.5% casamino acids, 0.1% yeast extract; pH adjusted to 7.3); J-53-1 - either glucose mineral salts medium (0.5%glucose, 0.1%$NH_4H_2PO_4$, 0.1%KCl, 0.1% $MgSO_4.7H_2O$) or trypticase soy broth (BBL: 1.7% trypticase peptone, 0.3% phytone peptone, 0.5% NaCl, 0.25% K_2HPO_4 & 0.25% glucose; pH adjusted to 7.3); Pseudomonas strains - nutrient broth (0.5% peptone & 0.3% beef extract) and fungal cultures - Sabouraud maltose or Sabouraud dextrose medium (1% Neopeptone, 4% maltose or 4% dextrose).

Enumeration of Bacteria in Compartments of the Ottawa River

Sediment samples were collected from Ottawa River sediments in mid-summer by the Geology Department, University of Ottawa and were variously described according to their location as clay-type (near south shore), sand-type (mid-stream) and wood-chip (near pulp mill on the north shore). These samples reached our laboratory for testing 1-2 days after collection. Additional sediment samples were obtained from a shallow polluted arm of the Ottawa River, Brewery Creek. Mid-stream water samples were freshly collected by dipping a sterile plastic container from a boat.

Fish samples were obtained from fresh specimens caught in late summer. Slime samples were obtained by scraping the living

fish with sterile glass slides. Total volume of slime collected was approximately 1-2 mL per fish for the skin surface and about 0.2 mL per fish for the gill surface. Gut contents were removed immediately after the fish were killed.

Bacterial samples from the different sediment types and the intestinal and external flora of fish were suspended in sterile diluting medium containing 0.1% pyrophoshate and 0.1% Tween 80 as an aid to dispersion of bacterial clumps. Actidione (cyclohexamide) was added to the enumeration medium at 50 µg/mL to suppress the growth of eukaryotic organisms. Sediment samples (10g) were ground with 100 mL. of diluting medium in a large mortar. Fish samples were homogenised with 15 mL of diluting medium. Serial 10-fold dilutions were made in the same medium. Pyrophosphate and Tween 80 were added to the water sample to give 0.1% of each; subsequent dilutions were made as before.

The Foote and Taylor medium was developed for the enumeration of bacteria in natural waters (Foote & Taylor, 1949) and is designed to allow slow growing organisms to be enumerated as well as the more rapidly growing species which are retarded by lack of nutrients. Appropriate concentrations of $HgCl_2$ or $Hg(NO_3)_2$ were added to Foote and Taylor agar immediately before pouring plates which were used the following day. Replicate 0.1 mL aliquots (at least 3 per dilution and set of experimental conditions) of the bacterial suspensions were spread on the agar plates which were then incubated at 20 °C for 8 days under aerobic or anaerobic conditions. Anaerobic conditions were those pertaining when the culture was held under commercial nitrogen. No attempt was made

to grow strict anaerobes as our work was mainly concerned with those bacteria found in the more aerobic part of the river: water, upper layers of sediment and fish surfaces. In the Ottawa River total vertical mixing has been reported (A. deFreitas, personal communication) and strict anaerobes are unlikely to be found in many places. Plates were examined and the number of colony forming units was counted. These experiments were repeated at least twice with different samples of each sediment type or fish surface.

Growth of Microbial Cultures for Methylation Experiments

Mixed cultures of sediment bacteria were obtained from either enrichment cultures of Ottawa River sediment in Foote and Taylor medium containing 10 ppm $HgCl_2$ or aquaria sediments at the National Research Council. Pure cultures of sediment bacteria were obtained either from plating out the above enrichment cultures or from the enumeration plates (10 ppm Hg). Mixed cultures of bacteria associated with fish were obtained as described above; pure cultures of these organisms were also isolated. All isolates were checked for purity by replating twice on Foote and Taylor agar before storage at 4 °C on agar slants.

Cultures were grown either with 1 or 10 ppm added Hg or with no Hg; Hg was always added last to minimise loss from the system. Details of experimental conditions and cultures used are given in the results section. Controls were autoclave-killed inocula. Bacterial growth was followed by measuring the increase in optical density at 650 nm. Aerobic cultures were shaken slowly on an orbital or reciprocating shaker; anaerobic cultures were either gassed with commercial nitrogen or completely filled with medium,

sealed and left stationary. No attempt was made to culture strict anaerobes. During growth all cultures were held at 20-22 °C. When the stationary phase was reached, the cultures and controls were terminated by the addition of concentrated HCl (1/4 vol) and NaCl (10g/50 mL) prior to extraction for organic Hg. A mass balance was performed on all experiments where ^{203}Hg was used.

Due to the nature of fungal growth in liquid medium, growth could not be followed readily by optical density measurements. Growth was terminated when formation of the mycelial mat seemed complete; about 10 days. To facilitate the extraction of fungal cells, the cultures were harvested by filtration and freeze-dried prior to extraction for organic Hg.

Dry Weight Determination

Dry weights on bacteria, fungi or sediments were determined by oven drying (120 °C) of triplicate samples to constant weight.

Intercompartmental Transfer of Mercury

We used a simple model system to study the exchange between water, sediment and bacteria. In this, a dialyser containing 5.5 L of river water served as the body of water, and spatial separation of the bacteria and sediment was achieved by means of dialysis tubing. Equal numbers of dialysis sacs containing sediment suspension (ca. 0.5 g dry weight in 6 mL distilled water) and bacterial suspension (ca. 0.15-0.25 g dry weight in 6 mL distilled water) were held vertically in the dialyser (Pope Scientific Co., U.S.A.) by the addition of a glass marble to each sac. Control sacs containing distilled water only were also included. Sediment and bacteria containing sacs were suspended

alternately and rotated mechanically in the river water which was also magnetically stirred. The dialyser, river water and sediment were autoclave sterilised, but it was thought that autoclaving the bacterial compartment might totally change its binding characteristics. Chloramphenicol (100 µg/mL) was added to the river water to minimise bacterial growth. It was previously determined, using a Hg-specific electrode, that chloramphenicol did not bind Hg^{2+}. Dialysis sacs and aliquots of river water were removed at specific time intervals and analysed for total and inorganic Hg by flameless atomic absorption spectroscopy. Results were related to dry weights of sediment and bacteria and to weight of water. Background levels of the endogenous Hg content were obtained for all compartments and the dialysis tubing.

Characterisation of compartments:

River water - samples were collected from the Ottawa River (mid-channel) at the flow-through access in the Ottawa River Ecology Laboratory (OREL). The following measurements were obtained from continuous monitors at the collection site: Eh +345-+430 mV; pH 7.70-7.85; pCl 3.68-3.8; conductivity 21-22 µ mhos; turbidity 8-10 FTU. Binding characteristics of the river water collected from this site with respect to Hg have been determined (Ramamoorthy & Kushner, 1975a).

Sediment - samples were a clay-type sediment from the Ottawa River which was shown by X-ray diffraction, scanning and transmission electron microscopy, and differential thermal analysis to be composed of poorly crystallised kaolinite and illite (Ramamoorthy et al., 1977). Surface area measurements by the method of Brunauer, Emmett and Teller (1938) gave a value of 25.5 m^2/g

dry weight. Organic content was found to be 10.1% on a dry weight basis by dry ashing samples at 700°C for 16 hours: mean grain size was 0.35 mm. Cation exchange capacity, using the ammonium acetate method(Chapman & Pratt, 1961), was determined as 18.58 m equivalents /100 g dry weight .

Bacteria - used were Pseudomonas fluorescens which had been grown in nutrient broth at 30°C for 72 hours. This stationary phase culture was harvested and washed thoroughly to remove growth medium. The pelleted washed cells were used in the experiments and separate dry weight determinations were performed for each batch of cells used. Pseudomonads were chosen because they are common river organisms and they have also been shown to predominate amongst the organisms capable of growing in Hg-polluted waters (Nelson et al.,1972) and amongst those capable of volatilising Hg° (Nelson et al., 1972; Nakahara et al., 1977).

Microscopic examination of the cultures at time of harvesting revealed some cells with aberrant growth and quite a wide range of sizes was observed (2-12 μm in length). This complicated surface area measurements, which were performed by counting numbers with a Coulter counter and multiplying by the average surface area of one bacterium. For purposes of calculation the bacteria were assumed to be cylinders with hemispherical ends. All estimates obtained were however of the same order of magnitude (40±20 m^2 /g dry weight) which corresponds to 1-3 times the surface area of the same weight of the sediment used.

Photochemical Production of Methyl Mercury

a) Specific compounds

Although <5% of the intensity of sunlight radiation is in the UV region, there is still considerable activity at the longer UV wavelengths, and >90% of this occurs in the UV spectral band commonly referred to as UV-A (3150 - 4000Å) (Caldwell, 1972). Blacklight lamps, with a peak intensity at about 3500Å, are commonly used to irradiate at this range of wavelengths (Akagi & Sakagami, 1972). Sunlight long-wave UV radiation was measured using a UV Products Blak-Ray meter (Model #J221) on a sunny day on the roof of the laboratory. Four blacklight UV lamps (15W) were then adjusted in height above a surface to give the same intensity of radiation, measured on the same meter, approx.-20$\mu W/cm^2$. Disposable, polystyrene tissue culture flasks (Corning) were chosen as closed parallel-sided vessels to use for exposure of reaction mixtures. No measureable decrease in intensity was observed when empty or water-filled flasks were irradiated with either source, so the plastic was considered suitable for irradiation experiments with this range of wavelengths.

Both distilled water and river water were filter sterilised through 0.22 µm membrane filters (Nalgene). Acetic acid was made from glacial acetic acid and sterile distilled water. Mercury salts were weighed out under an orange photographic safelight; they could not be sterilised but were present in such high concentrations that any bacteria they carried with them would be unlikely to grow. By careful preparation of constituents, the systems were assembled in total darkness.

Sunlight-exposed reaction mixtures were placed on the roof of the laboratory and inverted daily to remove condensation from the top surface of the flask. UV-exposed reaction mixtues were

continuously illuminated by blacklight in an otherwise darkened enclosure. All chemical systems were incubated for periods of 3 days and 6 days prior to extraction for organic Hg (Stevens & Robertson prcoedure - see below) and analysis by GLC. Flasks designed to measure initial (T_o) values were, in fact, necessarily exposed briefly to normal room lighting, for the period required to add the chemicals for organic Hg extraction. After addition of these chemicals the flasks were returned to the dark to await extraction. Extraction was carried out in normal room lighting using the Stevens & Robertson (1974) procedure.

b) Water-Sediment Systems

Sterile clay sediment (autoclaved 1 hour) was loaded with $HgCl_2$ to give a final concentration of 10 ppm. It was then washed thoroughly to remove any unbound Hg and 100g was transferred to each of 2 sterile glass columns, one of which was covered with black plastic. To each column, either under blacklight or in the dark, was added 3.5 L of either autoclaved deionised water or autoclaved river water containing 1 ppm acetic acid. Columns were kept under the same conditions, unstirred, for 7 days. The clay was then centrifuged off (1000 x g for 10 min.) and both clay and water were analysed for CH_3Hg (Westoo extraction and GLC).

Similar procedures were used for blacklight and dark exposure of sand sediment (250 g) containing 10-20 ppm $HgCl_2$ but using sterile dechlorinated tap water with fish as potential bioaccumulators of any CH_3Hg formed. Each column contained 4 fish (Brachydanio rerio or zebra fish, weighing 250-350 mg each): 2 of which were sacrificed at 3 days and 2 at 8 days. Fish and water

were analysed by Westoo extraction and GLC.

Chemical and Chromatographic Supplies

Mercury salts used throughout this study were analytical grade (Fisher Scientific). Radioactive Hg^{2+} and CH_3Hg^{+} salts were obtained from New England Nuclear Canada Ltd. Pesticide grade solvents and high purity acids were necessary to minimise Hg impurities introduced unwittingly; with one exception, solvents and acids were purchased from Fisher Scientific. High purity, glass-distilled benzene was purchased from Caledon Laboratories, Georgetown, Ontario. This product has a very low chlorinated hydrocarbon residue and is particularly suited to use with an electron capture detector. The concentrated HCl used in extracts for GLC analysis was preextracted twice with the glass-distilled benzene to remove three large interfering peaks caused by benzene-soluble impurities.

Glass columns, septa, Chromosorb G, diethylene glycol succinate (DEGS) and other accessories for the gas-liquid chromatograph were purchased from Chromatographic Specialties, Brockville, Ontario.

Handling of Mercury Compounds

Inorganic Hg compounds were handled with caution to avoid inhalation. Mercuric nitrate is deliquescent and was stored in a dessicator. The true weight was extrapolated from a series of weighings over several minutes. Methyl Hg compounds were stored and handled only in the fume hood. Radioactive Hg compounds were stored in lead-shielded containers.

It was not considered desirable or safe to autoclave sterilise Hg solutions. Concentrated Hg solutions were filter steril-

ised (0.22 μm pore size membranes) with relatively little loss. Dilute Hg solutions (1 ppm or less) were irradiated with an intense source of short wavelength radiation (Co^{60}) in order to avoid Hg loss on passage through a microporous filter.

To minimise volatile Hg compounds in the laboratory air, culture vessels were fitted with devices containing finely divided cocoanut charcoal (200-500 mesh) to adsorb any Hg vapours (A. deFreitas, personal communication).

Analytical Methods

During the early part of this study, radiochemical analysis, using Hg^{203}, was the only technique available for Hg determination in our laboratory. Extraction of samples by one of the standard methods (see below) gave estimates of organic and inorganic Hg. No further speciation of Hg was possible with this technique, although a thin-layer chromatographic (TLC) method was developed as a confirmatory test for MeHg.

Subsequently, a gas-liquid chromatograph with an electron capture detector was made available to us from the National Resarch Council of Canada. This was set up to measure MeHg and used in further experiments.

Extraction of Samples for Organic Mercury

Extraction of samples for MeHg was performed either by the method of Westoo (1968) or an analogous procedure (Stevens & Robertson, 1974). Figure 3 shows a schematic summary of the two methods which both rely on the extraction of MeHg as a halide. The chloride (Westoo, 1968) is the most frequently used in spite of the fact that, even under optimum conditions, the partition

FIGURE 3

EXTRACTION OF ORGANIC MERCURY COMPOUNDS

WESTOO (1968) developed for foodstuffs and biological materials	STEVENS & ROBERTSON (1974) developed for water analysis
HOMOGENISE SAMPLE WITH WATER IF NECESSARY	
acidify with HCl (1/4 vol) in presence of high NaCl (10 g/70mL).	acidify with HBr (1/10 vol) in presence of high KBr (10 g/50 mL)
EXTRACT 3 TIMES WITH EQUAL VOLUME OF BENZENE & POOL EXTRACTS	
discard aqueous homogenate, dry benzene extract with anhydrous sodium sulphate	discard aqueous homogenate, dry benzene extract with anhydrous sodium sulphate
BACK-EXTRACT WITH 1% CYSTEINE ACETATE (6.0 mL/50 mL benzene extract)	
discard first benzene extract, acidify with HCl (0.6 mL/2 mL of cysteine acetate extract)	discard first benzene extract, acidify with HI (0.1 mL/4 mL cysteine acetate extract)
EXTRACT 3 TIMES WITH EQUAL VOLUME OF BENZENE & POOL EXTRACTS	
discard cysteine acetate and dry final benzene extract with anhydrous sodium sulphate	discard cysteine acetate and dry final benzene extract with anhydrous sodium sulphate

coefficient of MeHgCl from water into benzene is not more than 70%. A more favourable partition coefficient for the extraction of MeHgBr and MeHgI (Stevens & Robertson, 1974) leads to a higher recovery. The presence of excess halide ensures that Hg^{2+} present is complexed as $HgCl_4^{2-}$ or $HgBr_4^{2-}$ which partition almost entirely to the aqueous phase.

The following points not mentioned in Figure 3 should be stated about the extraction procedure:

a) Each extraction involved shaking vigourously for 5 minutes in a separating funnel with periodic release of pressure.

b) Benzene extracts greater than 100 mL were concentrated under reduced pressure in a rotary evaporator.

c) A few drops of mineral oil was added to the benzene extract to minimise loss of MeHg (A. deFreitas, personal communication).

d) Cysteine acetate solution was made fresh weekly according to Westoo (1968).

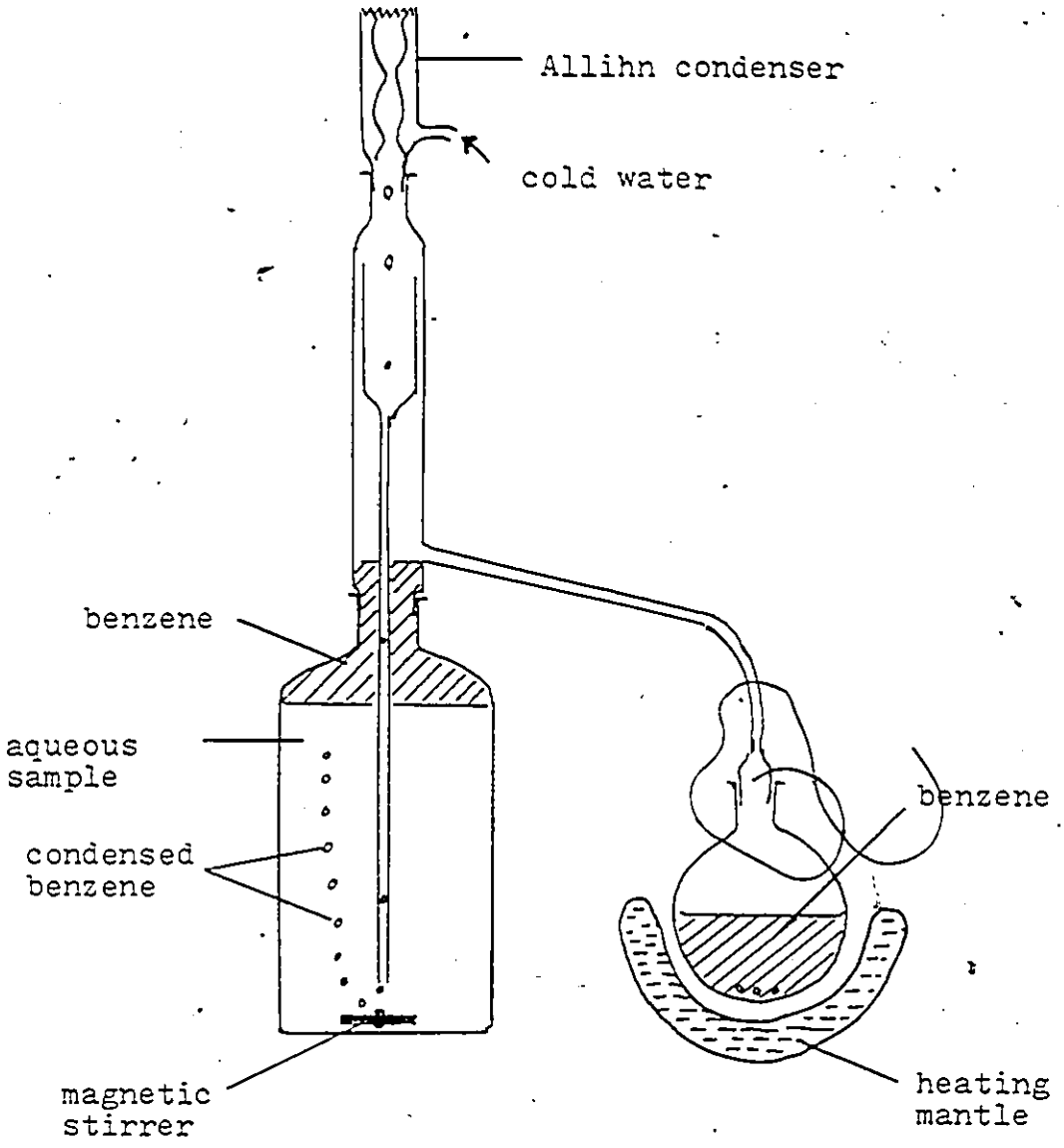
e) Final extracts were stored at room temperature in the dark before analysis.

Large Scale Extraction of Organic Mercury

Investigations of MeHg production frequently require the extraction of large volumes of microbial cultures or other liquids with organic solvents. For ease and safety of handling large volumes of benzene a continuous liquid-liquid extractor was set up which had a maximum capacity of 3.5 L (Fig. 4). Continuous extraction of microbial cultures for 48 hrs. gave the first benzene extract which was evaporated to 10 mL under reduced pressure before proceeding as above. An additional advantage of this apparatus is that its use avoids the difficulties of phase

FIGURE 4

APPARATUS FOR THE CONTINUOUS EXTRACTION OF LARGE VOLUMES OF AQUEOUS SAMPLES BY SOLVENTS LIGHTER THAN WATER



separation due to the accumulation of material at the benzene water interface.

Radiochemical Determination of Mercury

A number of radioactive isotopes of Hg are available, but the only one suited to biological experimentation is ^{203}Hg with a half-life ($T_{1/2}$) of 47 days. For every disintegration ^{203}Hg produces 1 electron and 1 gamma ray with an energy of 0.279 Mev. Radioactive decay was monitored on a Nuclear Chicago Gamma Well Counting System, Model # 4216, using thallium-doped sodium iodide as an external fluor. Whenever possible, 1 mL sample volumes were counted; a correction curve was, however, prepared for counting larger volumes where the geometry, and therefore efficiency, of counting was changed.

The following corrections were made to the counts obtained:

a) background subtracted

b) correction for volume of sample, if necessary

c) correction for ^{203}Hg decay ($T_{1/2}$ = 47 days)

Results for some experiments were obtained on a similar instrument at the National Research Council. The counting efficiency of both machines for ^{203}Hg standards was approximately 40%.

TLC Confirmatory Test for Methyl Mercury Compounds

The small quantity of MeHg usually present in natural or experimental systems makes direct colourimetric determination impossible. This can be overcome by using ^{203}Hg substrate and looking for areas of high radioactivity on the TLC plate. Detection of specific compounds can be simplified by using non-radioactive compounds as carriers which can then be detected colourimetrically using dithizone as a spray reagent (Hg dithizonates

are orange) and counted to determine associated radioactivity.

Trials with many of the solvent systems reported in the literature (Fishbein, 1971 & 1972) gave either somewhat variable results, or left the inorganic Hg on the baseline. The best system found was one developed in this study using silica gel H plates (0.25 mm) and 90% toluene - 10% methanol as the developing solvent. At room temperature and using a saturated developing tank, this system gave R_f values of 0.90 for CH_3HgCl and 0.41 for $HgCl_2$.

Determination of Mercury by Flameless Atomic Absorption Spectroscopy

Total Hg analysis by this method was performed for us at a National Research Council laboratory also involved in the Ottawa River Project. Details of the analytical procedure are available in the Final Report of the Ottawa River Project (Norstrom, 1977).

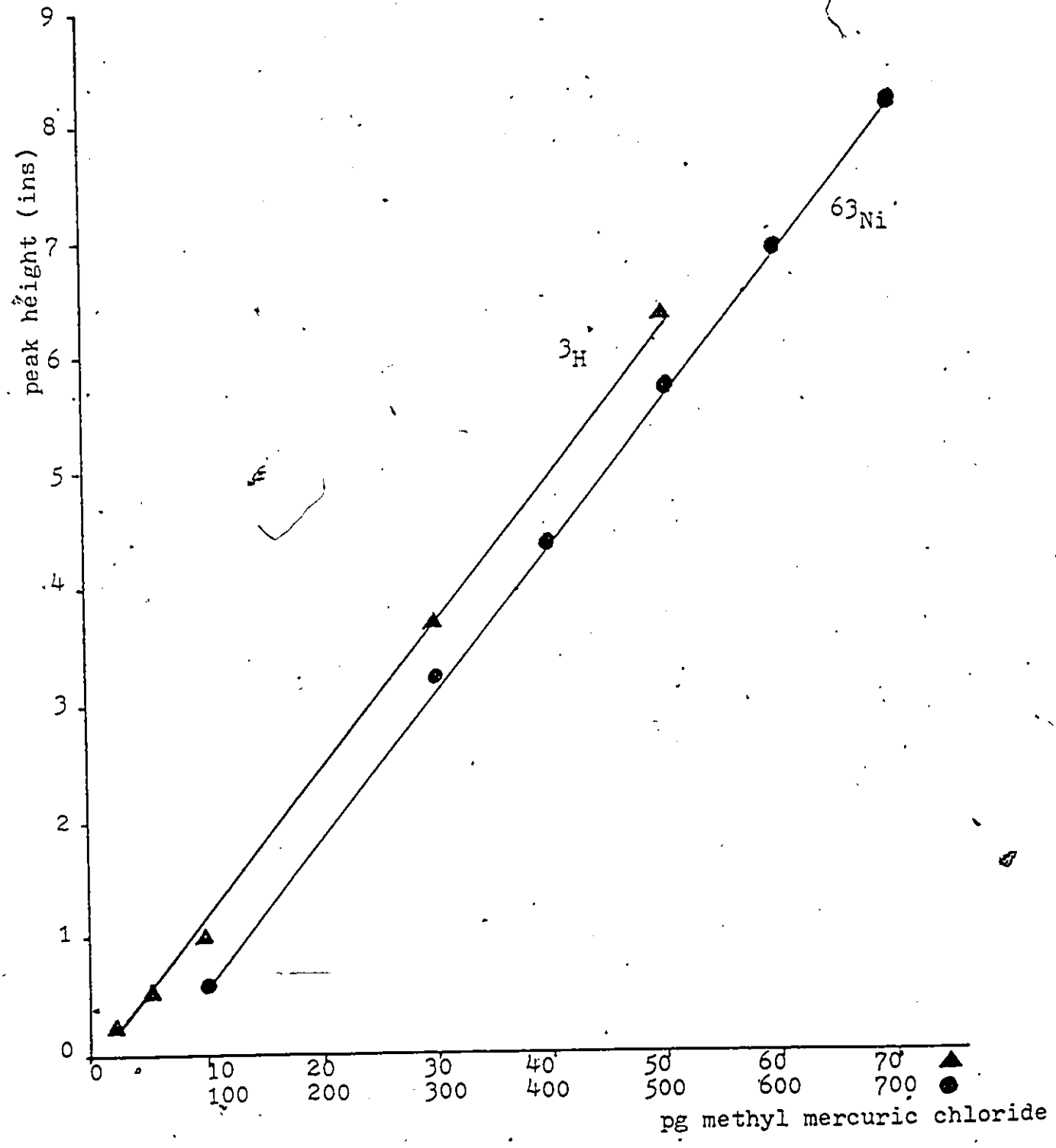
Gas-Liquid Chromatography

Since extracts of biological material frequently contain a very large number of compounds, use of a selective detector is advisable. An electron capture detector was used which is particularly sensitive to Hg compounds extracted as their halides. Unfortunately there is no simple method to predict response with this system and hence no quantitative relationship between peaks of different compounds. Quantitation is obtained by comparison with known standards of different concentrations.

GLC analysis was performed on a Hewlett Packard 5750B gas chromatograph fitted with both ^{63}Ni and ^{3}H detectors. Figure 5 shows a calibration for both detectors using MeHgCl standards.

FIGURE 5

CALIBRATION OF ELECTRON CAPTURE DETECTOR



The ^{3}H foil was found to have the greater sensitivity (minimum detectable CH_3Hg approx 2 pg), but became easily poisoned with Hg (Fishbein, 1970) and was subject to small in-line pressure changes which gave rise to false peaks. The ^{63}Ni detector was considerably more stable, but less sensitive (minimum detectable MeHg approx. 50 pg). It was used in the pulsed mode with a 150 μ sec pulse interval, using 90% argon - 10% methane as the carrier gas at a flow rate of approx. 30 mL/min. Detector temperature was 240 °C, oven temperature 140 °C and injection port temperature 180 °C. The glass column was a 6 ft. x 2 mm, packed with acid-washed, DMCS treated Chromosorb W as the solid support and coated with 5% diethylene glycol succinate (DEGS) as the stationary phase.

Carrier gas flow rate was decreased overnight and consequently varied slightly from day to day after readjustment. This resulted in slightly varying retention times (approx. 3.5 min.) for MeHgCl which were monitored from standard solutions chromatographed immediately before or after unknown samples. To avoid the possibility that MeHg compounds might be concealed by changing retention times in complex mixtures, samples of the extracts were chromatographed at each stage of the extraction procedure. In particular, after back-extraction by cysteine acetate, the benzene layer was rechromatographed to check for possible disappearence of peaks.

One set of experimental results was obtained using a gas chromatograph at the Canada Centre for Inland Waters, Burlington, Ontario. This machine used a ^{63}Ni detector of a more modern design with greater sensitivity, and was capable of detecting 1-2

pg of MeHg halide. The detector was operated in the pulsed mode with 150 μ sec. pulse interval and high purity nitrogen as the carrier gas with a flow rate of 70 mL/min. The oven temperature was 130 °C, the injection port 150 °C and the detector was at 230 °C. The column material was acid-washed DMCS treated Chromosorb W (precoated with KBr) and 5% DEGS as the stationary phase. The retention time for CH_3HgBr under these conditions was about 2.7 min.

Sample and standard volumes injected onto the GLC columns were mostly 5 μL or less, but never more than 25 μL. A standard CH_3Hg solution was chromatographed between each experimental sample.

Glassware Preparation

All glassware used in this study was cleaned with NoChromix-H_2SO_4 acid (Godax Laboratories, N.Y.) and rinsed with large quantities of glass-distilled water before use.

EXPERIMENTAL & RESULTS

i) Enumeration of Bacteria in Various Compartments of the Ottawa River in the Absence and Presence of Mercury.

Table 1 shows the results obtained for one set of experiments on 3 types of sediment and water from the Ottawa River. Viable bacteria were reduced slightly but somewhat variably by 1 ppm Hg in the medium, with river water showing the greatest loss. However, when the Hg concentration was 10 ppm, <2% of the bacteria were able to form colonies. There is no clear indication of differences between aerobic and anaerobic conditions. Figure 6 shows compiled data for water and sediment samples, and further illustrates the same points. Up to and including 1 ppm added Hg, a slight and variable reduction is observed of $<1 \log_{10}$. At 2 ppm only 1-10% of bacteria form colonies and at 10 ppm this decreases even further.

A summary of the results on bacteria associated with some fish from the Ottawa River is given in Table 2. It can be seen that in the absence of added Hg, large numbers of viable bacteria were found. In the presence of Hg (1ppm) the reduction in colony numbers was varied, but always $<1 \log_{10}$: interestingly there was no reduction in the large numbers of viable bacteria in samples from the perch gut. However, at 10 ppm Hg, in all fresh samples, less than 1% of bacteria remained viable. For the perch samples that were collected and then refrigerated for 3 days before plating, the total viable count in the absence of added Hg was lower by about $1 \log_{10}$, but a greater proportion of bacteria remained viable in the presence of both 1 and 10 ppm Hg than was the case for fresh samples.

TABLE 1

COMPARISON OF THE VIABLE AEROBIC AND ANAEROBIC BACTERIA IN WATER AND SEDIMENTS FROM THE OTTAWA RIVER IN THE PRESENCE AND ABSENCE OF ADDED MERCURY

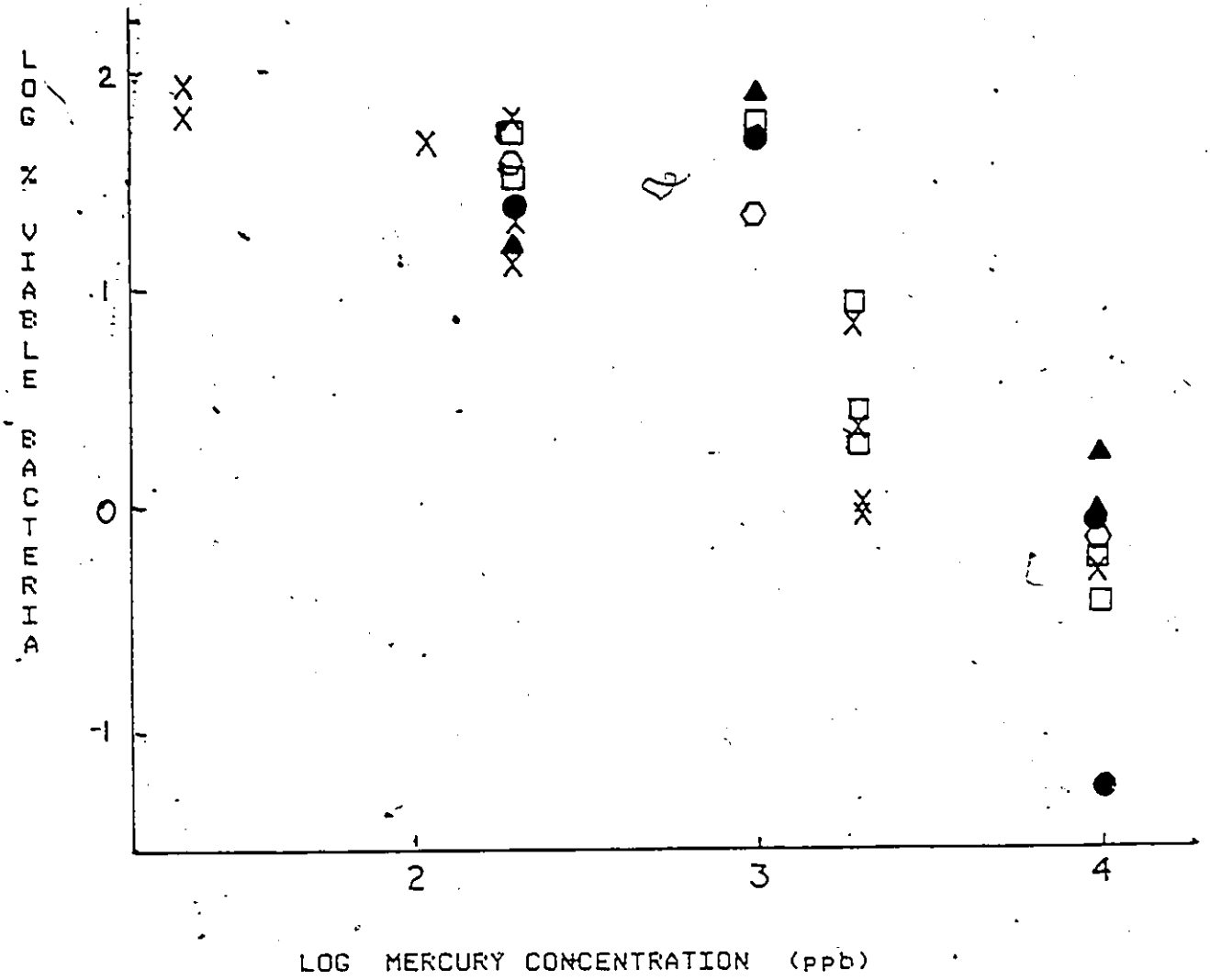
Source of Bacteria	Conditions of growth	Added Hg (ppm)	Bacteria /g or /mL	% Viable Bacteria
clay-type	aerobic	0	$(1.5\pm0.3).10^{5}$	100.0
sediment	"	1	$(1.2\pm0.4).10^{5}$	80.0
"	"	10	$(2.6\pm0.1).10^{3}$	1.7
"	anaerobic	0	$(1.7\pm0.2).10^{5}$	100.0
"	"	1	$(9.3\pm0.8).10^{4}$	54.7
"	"	10	$(1.7\pm1.0).10^{3}$	1.0
sand-type	aerobic	0	$(2.5\pm0.7).10^{4}$	100.0
sediment	"	1	$(2.2\pm0.3).10^{4}$	88.0
"	"	10	$(2.3\pm0.5).10^{2}$	0.9
"	anaerobic	0	$(1.9\pm0.6).10^{4}$	100.0
"	"	1	$(9.3\pm0.6).10^{3}$	48.9
"	"	10	$(9.3\pm1.2).10$	0.05
wood chip	aerobic	0	$(6.3\pm0.4).10^{5}$	100.0
sediment	"	1	$(3.3\pm0.5).10^{5}$	52.3
"	"	10	$(2.4\pm1.3).10^{3}$	0.3
"	anaerobic	0	$(5.6\pm0.4).10^{5}$	100.0
"	"	1	$(3.8\pm1.3).10^{5}$	67.8
"	"	10	$(3.6\pm0.2).10^{3}$	0.6
mid stream	aerobic	0	$(2.0\pm0.5).10^{4}$	100.0
water	"	1	$(3.4\pm0.9).10^{3}$	17.0
"	"	10	$(1.0\pm0.1).10^{2}$	0.5

Bacterial counts are given as bacteria/g dry weight for sediment samples and as bacteria per ml for water. Incubation was in Foote & Taylor medium at 20°C for 8 days. Results shown are mean and standard deviation for quintuplate samples.

FIGURE 6

% VIABLE BACTERIA IN WATER AND SEDIMENTS

AS A FUNCTION OF MERCURY CONCENTRATION



Results are shown as the mean of at least triplicate samples for Ottawa River water (▲) and for sediment samples from both the Ottawa River (X, ○, ●) and Brewery Creek (□). Ottawa River sediments were collected from the south shore (clay-type; X), midstream (sand-type; ○) and the north shore near a pulp mill (woodchip; ●).

TABLE 2

VIABLE COUNTS OF AEROBIC BACTERIA ASSOCIATED WITH FISH IN THE ABSENCE AND PRESENCE OF ADDED MERCURY

Source of Bacteria	Hg added (ppm)	Bacteria per ml	% Viable Bacteria
FRESH SAMPLES			
Perch Gill	0	$(1.8\pm0.4).10^{5}$	100
"	1	$(4.5\pm0.8).10^{3}$	2.5
"	10	0.0	0
Perch Skin	0	$(3.7\pm1.7).10^{6}$	100
"	1	$(1.9\pm0.4).10^{6}$	51
"	10	$(1.2\pm0.7).10^{4}$	0.3
Black Crappie Gill	0	$(1.2\pm0.1).10^{6}$	100
"	1	$(2.1\pm0.3).10^{5}$	18
"	10	0.0	0
Black Crappie Skin	0	$(2.2\pm0.8).10^{6}$	100
"	1	$(4.2\pm1.1).10^{6}$	19
"	10	$(4.4\pm1.6).10^{3}$	0.2
Perch Cut	0	$(2.5\pm0.8).10^{8}$	100
"	1	$(2.6\pm0.5).10^{8}$	104
"	10	$(6.9\pm0.2).10^{5}$	0.3
REFRIGERATED SAMPLES (3 days)			
Perch Gill	0	$(1.8\pm0.7).10^{4}$	100
"	1	$(1.9\pm0.7).10^{4}$	106
"	10	$(0.7\pm0.5).10^{3}$	4
Perch Skin	0	$(7.5\pm0.2).10^{5}$	100
"	1	$(8.4\pm1.9).10^{5}$	112
"	10	$(1.8\pm0.3).10^{5}$	24
Pumpkin Seed Skin	0	$(2.2\pm0.8).10^{5}$	100
"	1	$(1.3\pm0.3).10^{5}$	54
"	10	$(3.6\pm0.8).10^{4}$	16

Fish were obtained in late summer. Cultures were grown on Foote & Taylor medium under aerobic conditions at 20 °C for 8 days. Results are mean and standard deviation of quintuplates for fresh samples and triplicates for refrigerated samples.

ii) Intercompartmental Transfer of Mercury

Although biological material binds Hg very strongly, it was not known if bacteria would be able to compete successfully with sediment for Hg added to the water column, or if they were capable of accumulating, via the water column, Hg already bound to the sediment. However, if bacteria are to play a significant role in Hg methylation in a polluted aquatic environment, one or both of these should be true since the majority of the Hg is usually bound to the sediment fraction.

Very little is known about Hg exchanges in multicompartment systems, though the 2-compartment sediment-water system has been extensively studied (Ramamoorthy & Rust, 1976; 1978). Mercury levels used in these experiments were much greater than those normally found in the Ottawa River, but were not inconsistent with levels that have been found in highly polluted waters.

Competition for Mercury Between River Sediment and Bacteria

Figure 7 shows the results of Hg accumulation by sediment and bacteria as a function of time, when river water, containing approx. 2 ppm $Hg(NO_3)_2$ is used as the source compartment. The bacteria accumulated nearly 20 times the amount of Hg accumulated by the sediment, on a dry weight basis, over a 72 hour period. Since the surface area of the bacteria was found to be 1-3 times that of the sediment on a dry weight basis, bacteria also had a higher affinity for Hg per unit surface area than did sediment particles. Table 3 shows the total of Hg bound to both clay and bacteria as a percentage of the added Hg and the ratio of the Hg bound in the 2 compartments. After 24 hrs. a steady state appears

FIGURE 7

MERCURY ACCUMULATED FROM WATER

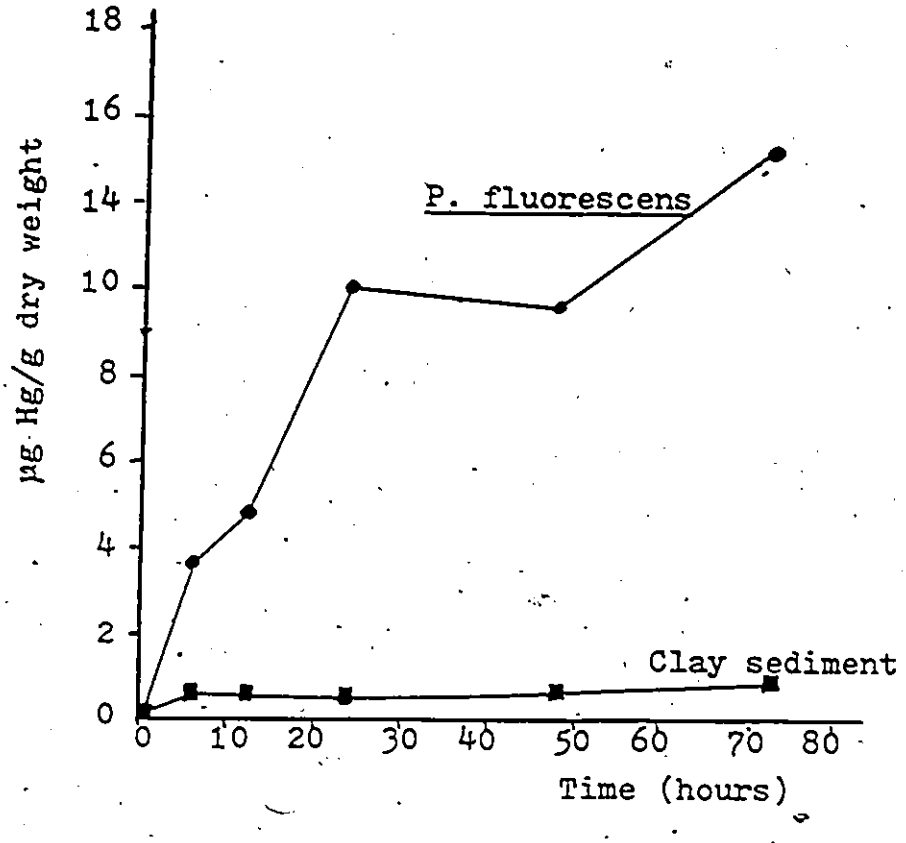


TABLE 3

MERCURY ACCUMULATED FROM WATER

TIME (hrs.)	RECOVERY OF Hg IN BACTERIAL + CLAY COMPARTMENTS AS % OF ADDED Hg	$\frac{Hg^{++}(Ps.\ fl.)}{Hg^{++}(Clay)}$
0	0.04	0.08
1	0.37	1.10
6	0.52	3.20
12	0.58	3.50
24	0.79	5.80
48	0.85	5.80
72	0.92	5.75

to be reached in which the bacterial compartment binds about 5.8 times the quantity of Hg that the sediment binds. Initially the clay compartment bound more than the bacteria, but this was quickly reversed. This could indicate that the sediment had a small number of very high affinity binding sites, but that in general binding sites on the bacteria were of higher affinity than those on the clay.

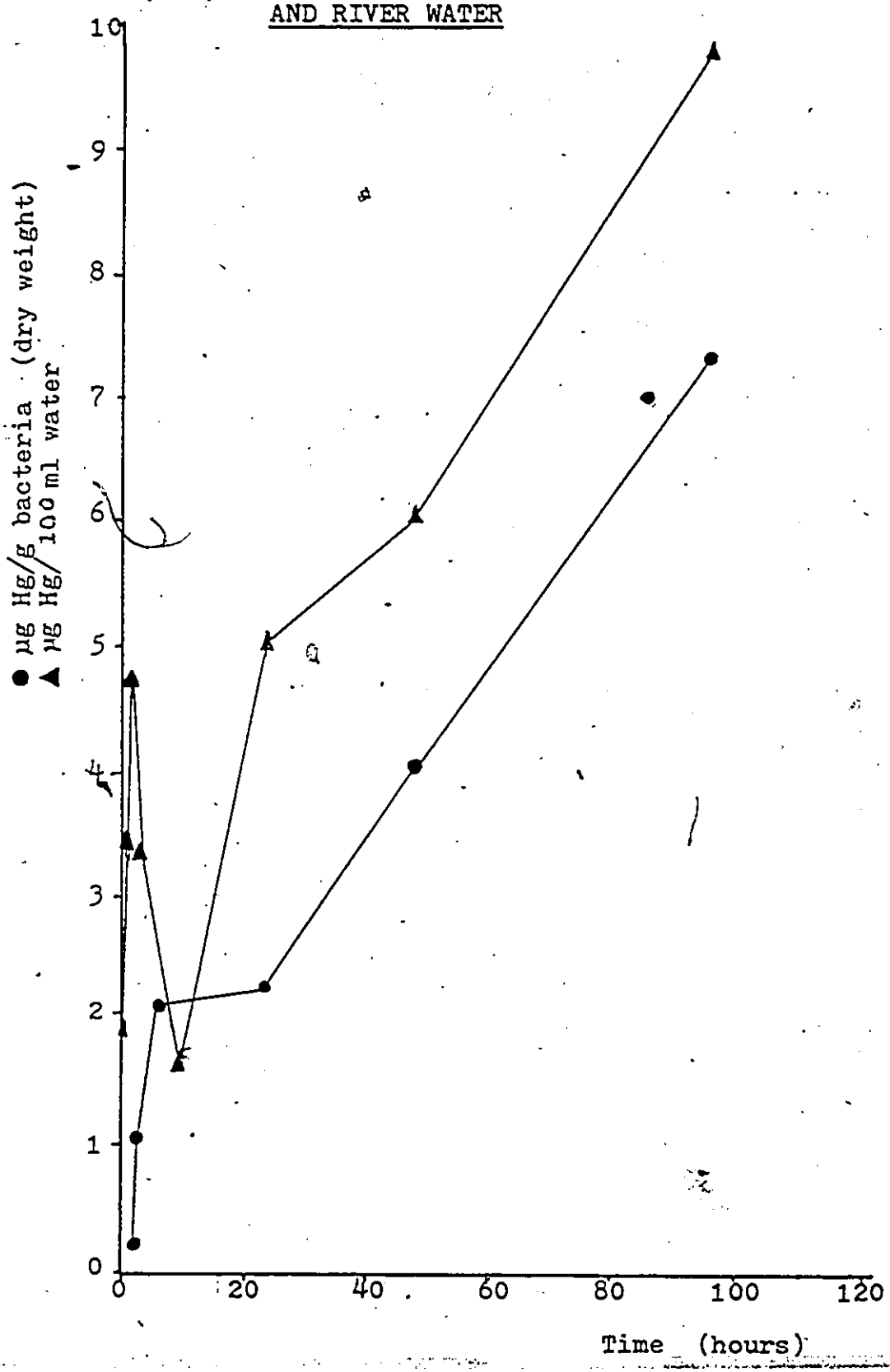
The total amount of Hg in both clay and bacteria was very low, amounting to <1% of the added Hg even after 72 hours. Of the 3.4 mg (28%) recovered at 72 hours, 67% remained in the water, 28% was attached to the bacteria and 5% was bound to the sediment. The loss of Hg, which is a well recognised phenomenon, was presumed due to its volatilisation from the system. In other experiments volatile ^{203}Hg trapped in charcoal plugs has been shown to account for the mercury loss. The clay sediment used in this study had the highest binding capacity of a number that were tested (Ramamoorthy et al., 1977). In spite of this, the results obtained here show that, under the experimental conditions of Eh (+216-+323) and pH (7.4-7.5), bacteria can compete very successfully with sediment for Hg added to the water column.

Accumulation of Hg, from Hg Containing Sediments, by River Water and Bacteria in the Absence and Presence of a Chelation Mixture.

Similar experiments in which clay was the Hg source compartment, showed that bacteria can accumulate Hg from the sediment even when there are 2 dialysis membranes between them. The sediment was preloaded with Hg by stirring with an $HgCl_2$ solution overnight, and washed extensively to remove any unadsorbed Hg. Figure 8 shows accumulation of Hg by Pseudomonas and water from

FIGURE 8

ACCUMULATION OF MERCURY ADDED TO SEDIMENT BY BACTERIA AND RIVER WATER



sediments containing approx. 500 ppm Hg in the absence of an added chelation mixture.

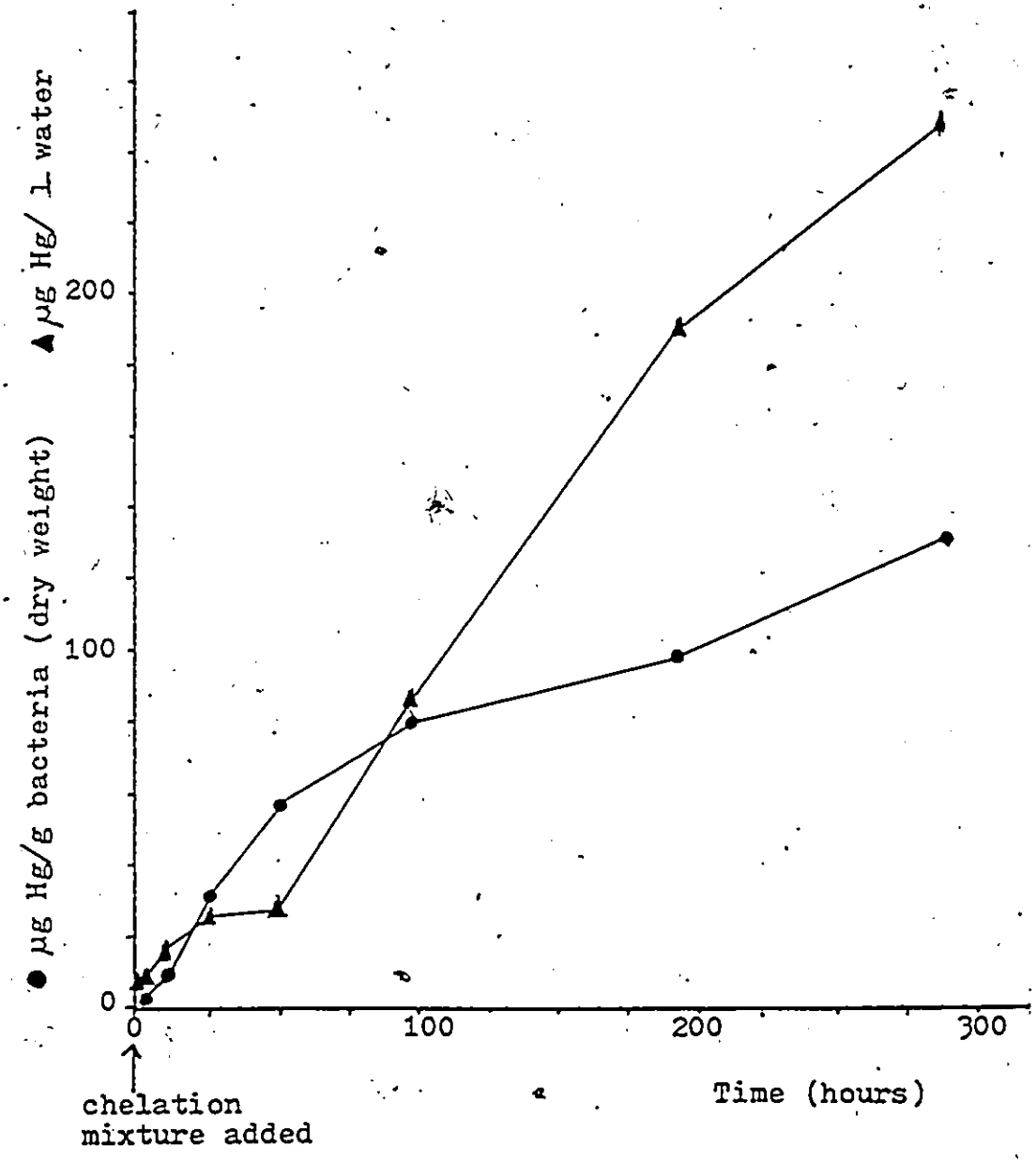
Initially Hg in water rose rapidly to about 4.8 ppb, but by 10 hours it had dropped back to about 1.5 ppb. This may have been due to a small quantity of unbound Hg not washed from the sediment but rapidly released from the interstitial suspending medium to the water column and thence accumulated by the bacteria. Changes in Hg levels in the bacteria followed changes in the water column but both compartments accumulated increasing amounts of Hg over 96 hours. Although levels of Hg in the bacterial compartment were 3 orders of magnitude higher than in the water on a weight basis, no comparison between Hg levels in bacteria and water can be mde since bacteria acquire their Hg only through the water, thus reducing the Hg concentration in the water. The final concentration of Hg in water was 98 ppb and in bacteria was 7.3 ppm. The total Hg recovered in the water (491.5 ug) and bacterial (17.2 ug) compartments at 96 hours was 21.3% of the Hg added to the system.

Figure 9 shows the results obtained when 50 ml. of the following chelation mixture was added to the water compartment at zero time. Chelation mixture: citric acid (5 ppm); NTA (nitrilo triacetic acid - 1 ppm); fulvic acid (5 ppm); Cl^- (3 ppm as NaCl); PO_4^{2-} (1 ppm as Na_3PO_4); and CO_3^{2-} (5 ppm as Na_2CO_3).

At 96 hours the levels of Hg in water are similar to those found previously even though there was a higher concentration of Hg in the sediment (approx. 1400 ppm). Bacteria on the other hand accumulated much more Hg in the presence of this chelation

FIGURE 9

ACCUMULATION OF MERCURY ADDED TO SEDIMENT BY BACTERA & RIVER WATER



mixture than they had done in river water alone. Table 4 shows a comparison of results abstracted from data obtained in these experiments. Over the first 96 hours, Hg found in the water was lower in the presence of chelators than in their absence. However, the bacteria accumulated up to 20 times as much Hg in the presnce of the chelation mixture as with river water alone. Since Hg can only reach the bacteria through the water, this implies that Hg in the water column was being more rapidly removed by the bacteria in the presence of a chelation mixture.

The results of these experiments clearly show that, under the experimental conditions of Eh (+390-+430 mV) and pH (6.9-7.6), bacteria can accumulate Hg previously bound to the sediment even though there is no direct contact between the two compartments. Increased levels of organic chelators in the water appear to enhance the levels of Hg associated with the bacteria.

TABLE 4

ACCUMULATION OF MERCURY, FROM MERCURY CONTAINING SEDIMENTS THROUGH RIVER WATER, BY BACTERIA IN THE ABSENCE AND PRESENCE OF AN ADDED CHELATION MIXTURE

Time (hrs)	WITHOUT CHELATORS			WITH CHELATORS		
	Total Hg in bact. (ug)	Total Hg in water (ug)	Hg bound by bact. as % of total in water	Total Hg in bact. (ug)	Total Hg in water (ug)	Hg bound by bact. as % of total in water
0	0.14	96	0.1	3.4	15	22.7
2	1.5	242	0.6	7.3	24	30.4
3	5.8	167	3.5	10.5	29.5	35.6
10	9.9	84	11.8	37.8	77.5	48.8
24	10.3	254	4.1	133.0	127.0	105.0
48	13.9	304	4.6	240.6	135.0	178.2
96	17.2	492	3.5	352.5	427.0	83.0
192	-	-	-	423.1	950.0	44.5
288	-	-	-	561.6	1240.0	45.3

iii) - Experiments on the Formation of Methyl Mercury by Microbial Cultures

Since the results of these experiments depend on the ability to extract MeHg for analysis, it was considered necessary to test the extraction procedures used with standard Hg and MeHg salts. Test extractions (Westoo) of killed bacterial cultures spiked with 1, 2 and 10 ppb of MeHgCl gave recoveries of 64, 62 and 69% respectively. The spiked samples were deliberately left overnight to simulate in use conditions. Extraction of $CH_3^{203}HgCl$ (1 ppb) from distilled water (1 L) (Stevens & Robertson) gave a recovery of 82% which is in good agreement with the values given by the authors (Stevens & Robertson, 1974). Using the continuous liquid-liquid extractor (Fig 4) MeHgCl (10 ppb) was extracted with 100% efficiency from 3.5 L of a rich culture medium using the Westoo procedure.

When $^{203}HgCl_2$ was extracted from water, culture media or killed bacterial cultures, it was shown (Westoo extraction) that a small proportion (0.02-0.12%) of the inorganic Hg carried over into the organic phase. In distilled water (3.5 L) 0.05% of 20 ppb $HgCl_2$ was detected in the final benzene extract though GLC analysis did not indicate the presence of MeHg. Thus, appearance of radioactivity in the final benzene extract does not necessarily show that MeHg was formed. If MeHg is suspected, then it must be confirmed by alternate methods of analysis. Results claiming MeHg production based solely on radioactive counts in an organic extract should be viewed with suspicion.

The experiments on bacterial methylation of Hg using cultures from the Ottawa River (OR) are summarised in Table 5.

TABLE 5

SUMMARY OF EXPERIMENTS PERFORMED ON THE BACTERIAL METHYLATION OF MERCURY USING MIXED OR PURE CULTURES OF BACTERIA FROM THE OTTAWA RIVER (OR) & NATIONAL RESEARCH COUNCIL (NRC) AQUARIUM SEDIMENT

Source & Nature of culture	#	Medium	Vol (mL)	* Hg (ppm)	# Growth	@ Volatile Hg	Time (hrs)
OR SEDIMENT - (mix, aerobic)	3	F & T	200	10	+++	+++	24
(mix, anaerobic)	3	F & T	500	10	+++	+++	24
(mix, aerobic)	2	F & T	200	1	+++	+++	48
(mix, anaerobic)	2	F & T	500	1	+++	++	48
(mix, aerobic)	2	F & T +	200	10	+++	+++	24
(mix, anaerobic)	2	F & T +	200	10	+++	+++	24
(mix, aerobic)	2	F & T +	200	1	+++	++	24
(mix, anaerobic)	2	F & T +	200	1	+++	++	24
(pure, aerobic)	2	F & T	200	10	++	+++	48
(pure, anaerobic)	2	F & T	500	10	++	++/+	48
(pure, aerobic)	2	F & T	200	10	++	+++	264
(pure, anaerobic)	2	F & T	500	10	++	++	264
OR FISH SKIN SLIME - (mix, aerobic)	2	F & T	200	1	++	+++	116
(mix, aerobic)	1	F & T +	200	10	+++	+++	24
(mix, aerobic)	1	F & T +	200	1	+++	++	24
(pure, aerobic)	2	F & T	200	10	+	+	264
OR FISH GILL SLIME - (mix, aerobic)	2	F & T	200	1	++	++	116
(mix, aerobic)	1	F & T +	200	10	+++	+++	24
(mix, aerobic)	1	F & T +	200	1	+++	+++	24
(pure, aerobic)	1	F & T	200	10	+	+	264

TABLE 5 (continued)

OR FISH INTESTINAL FLORA -							
(mix, anaerobic)	2	F & T +	300	10	+++	+++	24
(mix, anaerobic)	2	F & T +	300	1	+++	++	24
NRC AQUARIUM SEDIMENT -							
(mix, aerobic)	2	F & T	200	10	+++	++	24
(mix, anaerobic)	2	F & T	500	10	+++	+++	24

-x $HgCl_2$ or $Hg(NO_3)_2$ with ^{203}Hg tracer.

-# growth is indicated qualitatively: +++ reached stationary phase within 48 hrs.; ++ reached stationary phase during longer incubation; + grew poorly, did not reach stationary phase.

-@ volatilisation is indicated qualitatively: +++ indicates that >60 of Hg was lost through volatilisation; ++ indicates that more Hg was lost by volatilisation than in the control cultures; + indicates that Hg loss by volatilisation was less than or equal to control values. Volatilised Hg was trapped on charcoal containing devices fitted into the neck of the culture vessels.

-F & T is Foote & Taylor medium; F & T + is Foote & Taylor medium supplemented with 0.05% yeast extract and 0.1% glucose.

-mix = mixed culture, pure = pure culture

-These experiments were not given in detail because the results were all negative. Controls for each experiment contained autoclave-killed inocula, and were extracted and analysed in the same way as the live cultures. No attempts were made to characterise pure cultures; they were selected from colonies able to grow on F & T medium with 10 ppm added Hg and were all morphologically distinct. Had any of these cultures produced MeHg, they would then have been identified.

At the end of the incubation period cells were pelleted (5,000 × g for 15 min.) and both cells and culture liquor were extracted for MeHg (Westoo). Control cultures containing autoclave-killed inocula were treated similarly. In no case was there a significant difference in radioactivity found in the final benzene extracts of live cultures when compared with either killed controls or sterile culture medium: the range of organic extractable Hg found was in the range of 0.05-0.12%. This implied that either no MeHg was formed or that any formed was rapidly removed in all growing cultures.

After consistent failure to find increased organic Hg in microbial cultures, the possibility arose that we were not giving the correct Hg substrate. We therefore chose mercuric acetate (HgAc) as the substrate for the next experiment with a mixed culture of sediment bacteria from an aquarium at the National Research Council. Live cultures and autoclave-killed controls were incubated for five intervals from 6 to 192 hours with 1 ppm ^{203}HgAc. Other cultures were grown for 240 hr. and one was autoclave-killed before the addition of Hg to both; they were then reincubated for a further 24 hr. One set of flasks was included where live cultures and killed controls were incubated with $^{203}CH_3Hg^+$ (<1 ppb) only, in order to test the survival and recovery of MeHg under the conditions of the experiment. Volatile Hg was trapped in tightly fitting charcoal plugs. The recovery and partitioning of Hg in this experiment is shown in Table 6.

Surprisingly for a mixed culture of sediment bacteria, growth was hardly apparent during the first 48 hours of

TABLE 6

PARTITIONING OF MERCURY AFTER INCUBATION WITH SEDIMENT BACTERIA

		% recovery			
		culture	charcoal	organic	total
6 hr.	live culture	98.08	0.52	1.15	99.75
6 hr.	killed control	98.00	0.76	0.99	99.85
24 hr.	live culture	98.05	2.54	1.49	102.08
24 hr.	killed control	88.58	1.86	1.31	91.75
48 hr.	live culture	88.32	2.73	1.20	92.25
48 hr.	killed control	82.93	4.09	1.15	88.17
96 hr.	live culture	59.21	3.59	1.33	64.13
96 hr.	killed control	71.77	4.23	0.85	76.85
192 hr.	live culture	36.73	9.21	1.87	47.81
192 hr.	killed control	58.37	8.95	0.59	67.91
240 hr.	without and 24 hr. with Hg				
	live culture	107.78	0.77	0.35	108.21
	killed control	95.73	0.78	0.32	96.83
216 hr.	with CH_3Hg				
	live culture	58.27	0.83	41.90	101.00
	killed control	35.14	0.46	64.40	100.00

200 mL cultures were grown aerobically at 10 °C in Foote & Taylor medium supplemented with yeast extract (0.05%) and glucose (0.1%)

culture = cells + culture medium

charcoal = plug fitted to culture flask to adsorb volatile Hg

incubation, though stationary phase was reached by 192 hrs. The quantity of organic ^{203}Hg obtained after extraction of both live and killed cultures at all time periods was about 10 times more than had been obtained for previous experiments. Because the increased organic ^{203}Hg occurred even at short time periods, MeHg was not suspected at first, and it was assumed that high radioactivity resulted from increased carry over of the HgAc into the benzene extracts. Since that time we have shown that HgAc solutions can contain MeHg (see below): this is believed to be the case here. If approximately 1% is the proportion of MeHg in the HgAc, then the cultures were exposed to 10 ppb MeHg.

There was a slow but steady loss of Hg from the killed cultures (Table 6) which is reflected in the totals recovered. Loss of Hg from the live cultures was negligable during the first 48 hours, but then increased in parallel with the growth of the cultures. This is in contrast to the cultures containing MeHg alone (Table 6), which lost no Hg from either live culture or killed control during 9 days. When the cultures were grown for 10 days before addition of Hg, no significant loss of Hg was observed during the subsequent 24 hours and, rather surprisingly, less organic Hg was recovered in the final benzene extract (Table 6) than for cultures where Hg was added at zero time. It is not known whether the reduced MeHg results from a) reductive demethylation and reoxidation of Hg which effectively converts organic soluble Hg to water soluble Hg, b) reduced formation of MeHg, or c) binding of the Hg to the increased biomass in these cultures which has impaired the extraction process.

The cultures used to test the survival and extraction of

MeHg were subjected to a lower concentration of MeHg than those with added HgAc. The recovery of 64% of the added MeHg in the killed control is similar to the recoveries obtained in test extractions with the Westoo procedure. If demethylation caused the lower recovery of organic Hg in the live culture (Table 6), then the Hg^{o} must have been reoxidised to Hg^{2+} because of the excellent total recovery of Hg.

Cultures with the Reported Ability to Methylate Mercury

After failure to show MeHg production by any of the mixed or pure cultures obtained from the Ottawa River, it was decided to look at some cultures which had already been reported to produce MeHg: 3 such cultures were obtained.

The first experiments were run with isolate 3-CD. Live and killed samples of 3-CD were inoculated into 100 mL aliquots of Nelson's medium and incubated under both aerobic (24 hrs) and anaerobic (48 hrs) conditions in the absence or presence of ^{203}HgAc. The stationary phase cultures were extracted for organic Hg (Westoo). Considerable radioactivity was found in the final benzene extracts of all cultures (both live and killed) containing ^{203}Hg (Table 7a). GLC analysis of these final benzene extracts revealed the presence of a compound with the same retention time as MeHg. No similar peak was observed in the absence of HgAc.

A subsequent time course of triplicate 50 mL anaerobic cultures of 3-CD was performed in Nelson's medium containing 1 ppm HgAc. Controls containing killed culture (here the cultures were incubated for the set time, killed and reincubated with HgAc

TABLE 7

METHYL MERCURY PRODUCTION WITH 3-CD AND MERCURIC ACETATE

a)

	% recovery			
	liquor	charcoal	benzene	total
live aerobic	57.5	31.2	0.4	89.1
killed aerobic	100.6	0.7	0.2	101.5
live anaerobic	95.9	6.1	0.3	102.3
killed anaerobic	100.9	2.5	0.04	103.4

100 mL cultures were incubated in Nelson's broth at 20 °C for 24 hrs. (aerobic) and 48 hrs. (anaerobic)

b)

		% MeHg detected in final benzene extract				
		Time (hrs.)				
		0	24	48	72	120
sterile control		2.5	3.1	1.2	2.7	2.2
mercury control		0.0	0.0	0.0	0.0	0.0
killed culture		-	2.9	3.2	2.3	1.9
live cultures	1	1.0	2.2	3.0	2.6	2.8
(3 separate	2	3.4	3.7	2.1	1.3	-
experiments)	3	3.6	3.0	3.3	2.4	2.7

50 mL cultures were grown in Nelson's broth at 20 °C under anaerobic conditions

for 48 hrs.) and controls containing no Hg were also included. Cultures were extracted by the Westoo procedure and analysed by GLC. The results for recovery of MeHg are shown in Table 7b.

Methyl Hg was found in sterile controls and all cultures where HgAc was present regardless of the incubation time or the presence or absence of bacterial mass. No MeHg has been found in the microbial methylation experiments using $HgCl_2$ or $Hg(NO_3)_2$ but when HgAc is used as the Hg substrate, MeHg has been detected in live cultures, killed controls and even sterile culture media. Photochemical production of MeHg from HgAc has been shown to occur under fluorescent lighting (Jewett et al., 1975) and it is possible that the inadvertant exposure of HgAc to a light source resulted in contaminating amounts of MeHg before the experiments. It is also possible that MeHg was formed by a reaction between HgAc and the microbial growth medium. Whatever the reason, HgAc is not a suitable substrate for the laboratory investigation of MeHg production.

When it was realised that HgAc could not be used, attempts were made to produce MeHg from bacterial strains 3-CD and J53-1 (unfortunately Pseudomonas strain #244 had died) in the presence of $HgCl_2$. J53-1 has been reported to produce more CH_3Hg under aerobic conditions than 3-CD does under anaerobic conditions (W. P. Iverson, personal communication). 3-CD was grown anaerobically in 1 L of Nelson's broth with and without 1 ppm $HgCl_2$. J53-1 was grown aerobically in 1 L mineral medium plus glucose and in 1 L of trypticase soy broth, both of these with and without 1 ppm $HgCl_2$. When the cultures reached stationary phase they were

extracted for CH_3Hg using the Westoo procedure and analysed by GLC. There was no indication of any compound with the same retention time as a standard MeHgCl solution in any of the cultures. As this did not agree with the results obtained by Dr. Iverson, the experiment was repeated in duplicate. One set of cultures was extracted and analysed whole, cells from the second set of cultures were harvested by centrifugation and analysed separately from the supernatant liquor. Again no CH_3Hg was detected.

It was subsequently learned (W. P. Iverson, personal communication) that MeHg was only produced by these cultures if the cultures were kept about 10 days before extraction. This implies that the formation of MeHg was not occuring during growth and probably resulted from breakdown of some bacterial cells, although the presence of a very slow growing contaminant which produces CH_3Hg could not be ruled out entirely.

Fungal Cultures

The rationale behind examining fungi as potentially active methylators of Hg is that the active methylators of arsenic, selenium and tellurium were all shown to be fungi not bacteria (Challenger, 1945). In addition other workers have reported the ability of fungi to methylate Hg (Landner, 1971; Vonk & Sijpestiejn, 1973). The choice of fungal cultures was partly reasoned and partly random: S. brevicaulis was shown to be particularly active in producing trimethyl arsine, S. ferax is widespread in natural waters and forms part of the normal gill flora of freshwater fish, N. crassa and A. niger have both been shown by other workers to produce MeHg.

Cultures (1 L) of A. niger, N. crassa and M. verrucaria

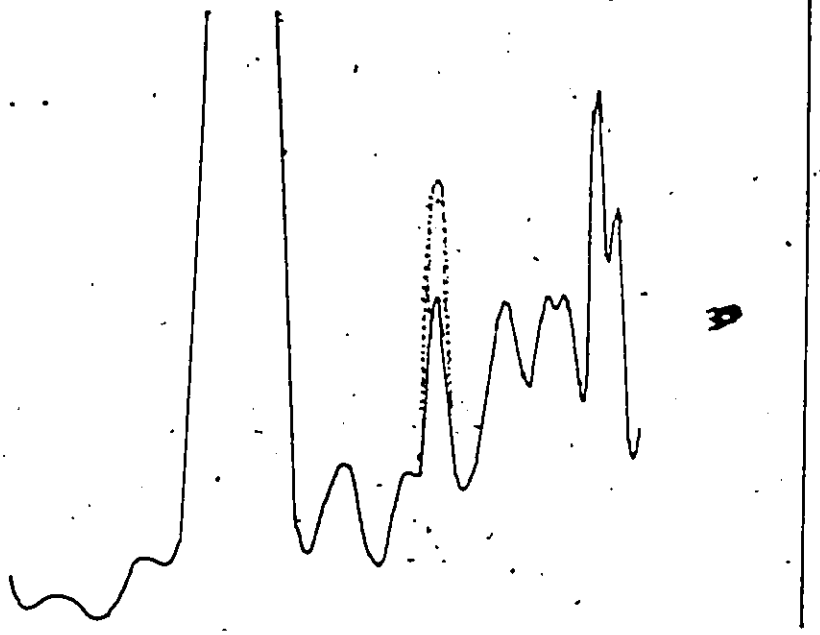
were grown with and without 0.1 ppm $HgCl_2$ at 20°C on a reciprocating shaker in the fume hood. Similar cultures of S. brevicaulis, S. ferax, and P. notatum were grown with and without 1.0 ppm $HgCl_2$. With the exception of S. ferax in the culture containing Hg, all cultures grew well and growth (formation of the mycelial mat) seemed complete in 10 days. Cultures were harvested by filtration under suction and the mycelial mat was freeze dried. Separate extractions of the cells and culture liquor were carried out by the Westoo procedure and extracts were analysed for the presence of MeHg by GLC. A small fraction of the harvested culture was used to determine dry weight.

In all cases, extracts of both fungal cells and culture liquor produced very complex chromatograms. No clear evidence was obtained of peaks corresponding to MeHgCl. A peak in the first benzene extract of M. verrucaria culture broth which co-chromatographed exactly with MeHgCl (Fig 10a) did not disappear on back-extraction with cysteine acetate and was absent in the final benzene extract (Fig 10b). It was therefore assumed not to be MeHg but was believed to be an unknown compound that, by coincidence, showed the same retention time in the chromatographic system used. However, the possibility that it was bis-sulphur diMeHg (MeHg-S-S-HgMe) remains: after completion of this study I was made aware of a paper, in Japanese with no English abstract (Fujiki, 1970) which indicates that MeHg-S-S-HgMe does not react with cysteine acetate and will only be extracted by the Westoo procedure as MeHgCl if equimolar CuCl is present. Thus, if MeHg-S-S-HgMe had been formed, it would not have been detected here.

FIGURE 10

CHROMATOGRAPHS FROM M. VERUCCARIA CULTURE BROTH

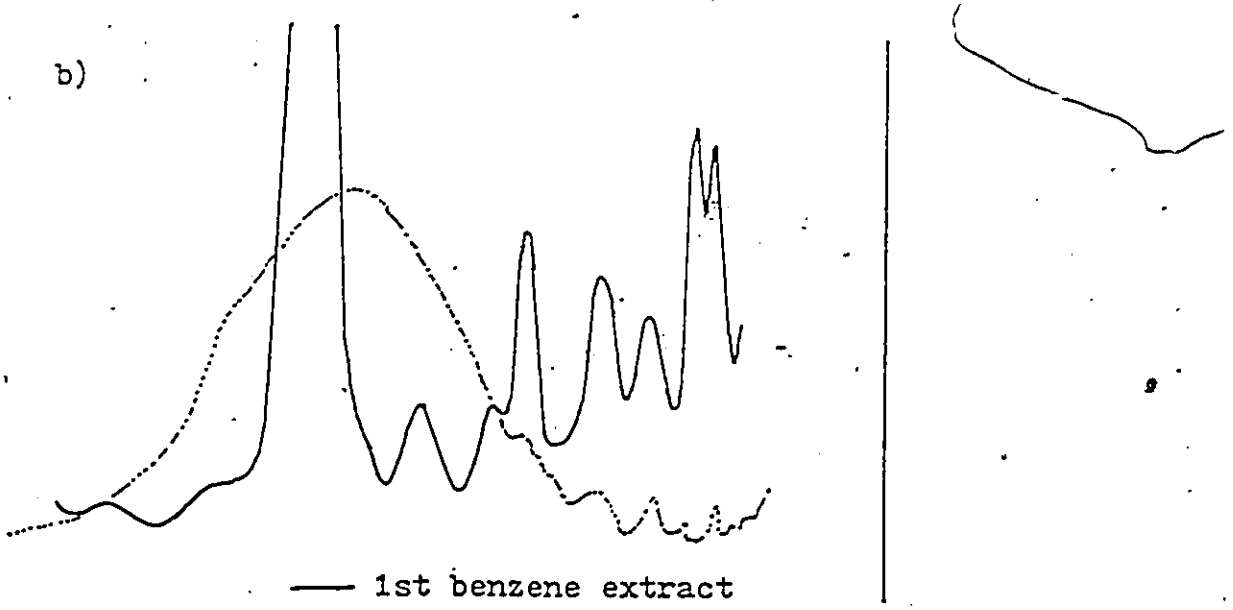
a)



—— 1st benzene extract

---- CH_3HgCl standard

b)



—— 1st benzene extract
after back-extraction with cysteine acetate

—·— final benzene extract

Mercury Binding and Volatilisation by Microbial Cultures

It should be noted that 14-36% of the total Hg recovered in mixed cultures in Foote & Taylor medium was cell associated. Also, in all mixed cultures, and some pure ones, Hg was lost to varying degrees in a volatile form. This may be partially due to specific inducible reduction systems found in some bacteria, as has been observed by others (Pan Hou & Imura, 1983) however, there is also the possibility of physicochemical loss of Hg, as was discussed in the review.

iv) Photochemical Production of Methyl Mercury

a) Specific Compounds

After finding MeHg in the microbial cultures to which HgAc had been added, we decided to investigate the possibility of abiological formation of MeHg in the environment from HgAc. Some of the work of Akagi and his coworkers (Akagi & Sakagami, 1972; Akagi et al., 1972; Akagi & Takabatake, 1973) was repeated and extended to include more natural conditions; sunlight and river water. Three different chemical systems which included HgAc or acetic acid and Hg were used to test for methyl mercury production. These were:

UV EXPOSURE

0.1 m mole HgAc - 10 mL distilled water

0.1 m mole HgO + 0.2 m mole CH_3COOH - 10 mL distilled water

0.1 m mole HgS + 0.2 m mole CH_3COOH - 10 mL distilled water

SUN EXPOSURE

as above, with either distilled water or river water.

The results are shown in Table 8, and typical chromatograms in Figure 11. Although the radiation intensity was approximately the same for UV and sun exposure, the length of exposure was very

TABLE 8

PHOTOCHEMICAL PRODUCTION OF METHYL MERCURY

SYSTEM	TIME (hr)	METHYL MERCURY PRODUCED (ppm)		
		DISTILLED WATER		RIVER WATER
		UV	SUN	SUN
	0	0.004	0.004	0.088
HgAc	72	27.6	0.075	0.412
	144	159.0	95.6	0.572
	0	0.004	0.004	0.003
$HgO + CH_3COOH$	72	82.8	16.6	0.002
	144	177.0	33.6	0.015
	0	0.001	0.001	0.002
$HgS + CH_3COOH$	72	<0.001	<0.001	<0.001
	144	0.008	0.116	0.159

FIGURE 11

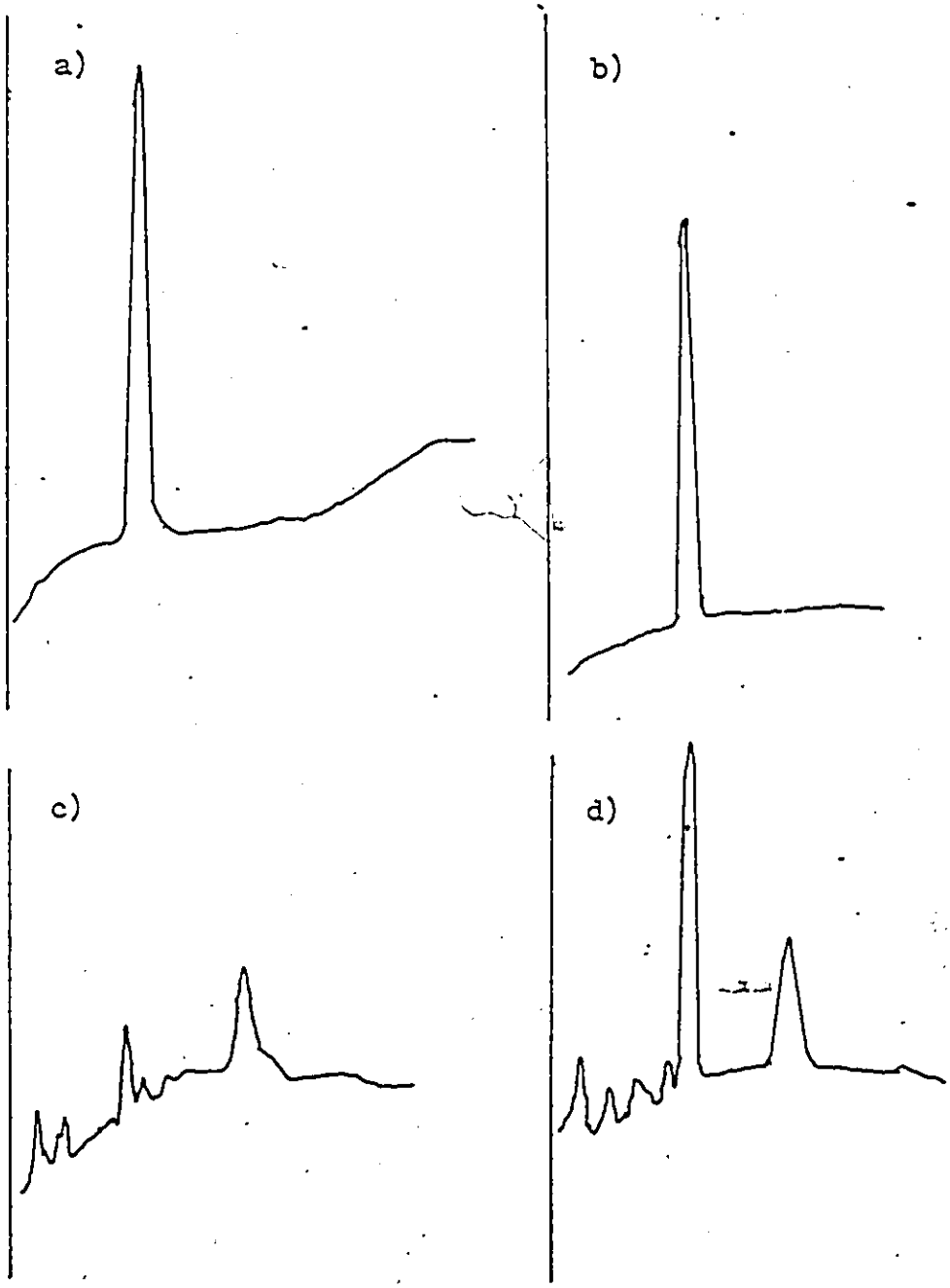
TYPICAL CHROMATOGRAMS FROM PHOTOCHEMICAL PRODUCTION OF CH_3Hg

a) 200 pg standard methyl mercuric chloride solution

b) 2 µl $Hg(CH_3COO)_2$ dil. x 10^4, 6 days in dist. aq., UV

c) 2 µl HgS undiluted, 3 days in dist. aq., UV

d) 2 µl HgS undiluted, 6 days in dist. aq., UV



different because of normal daylight variations: UV exposure being proportionately longer.

A small quantity of MeHg was observed in all the T_0 samples, but the value for HgAc in river water was much higher than for any other combination tested. This could possibly be due to a substance in river water which rapidly attacks HgAc, probably not photochemically, with the formation of CH_3Hg. However, by 144 hrs. there was much less CH_3Hg in the river water sun-exposed sample than in the corresponding distilled water sample. One possible explanation for this is that there is more than one mechanism for producing CH_3Hg, and if a free radical mechanism is involved, then there would probably be large numbers of terminators in river water which would limit its effectiveness in that setting. The difference in methyl mercury levels between sun and UV exposed HgAc solutions at 144 hours may be accounted for by the different lengths of exposure.

The HgO and CH_3COOH system reacted somewhat similarly to HgAc in distilled water, but produced very little MeHg in river water. The HgS and CH_3COOH system essentially produced no MeHg over the first 72 hrs. By 144 hrs. CH_3Hg was found to have increased in all samples, but in UV-exposed samples the level was <1/10 that in sun-exposed samples. This suggests that the different spectral composition (either higher or lower wavelengths) of sunlight may be important in the photochemical production of CH_3Hg from HgS.

Nearly all the reaction mixtures contained precipitates, although, initially, only the added insoluble HgS could be seen. After 3 days, the HgAc samples all contained white precipitates,

but, in addition, the UV sample contained a black precitate, presumably HgS. The dark control for HgAc samples also contained a slight white precipitate, but in very small quantity. The HgO/acetic acid system contained black precipitates in distilled water, a heavy white precipitate in river-water, and a very slight white precipitate in the dark control. All HgS systems appeared as they had done initially, a clear solution over insoluble HgS, with the exception of the control, where the solution was an opalescent yellow, and presumably contained free sulphur. The nature of the white precipitate was not known, but all precipitates were soluble in HCl and presumably inorganic. It is not known if the pH difference between the distilled water (5.6) and the river water (7.1) would have an effect here. Akagi & Takabatake (1973) observed yellow precipitates in solutions of HgAc: differences here could reflect differences in water composition, or purity of the chemicals used.

b) Water Sediment Systems

We decided to look at sterile water sediment systems to try and evaluate whether UV stimulated production of MeHg was likely to be a factor in the aquatic environment. The experimental procedure used here has already been described. As there is some question as to how far sunlight long wave UV can penetrate in water, we tested this with the 18" water columns used in these experiments and the long wave UV meter. No apparent decrease in intensity was observed for an 18" depth.

No methyl mercury was detected in any of the water or sediment samples analysed. In one experiment fish were used as bio-

accumulators of MeHg, and were also analysed for MeHg content. Figure 14(a-d) shows some of the chromatograms obtained with fish analysis. Peaks were observed (a-c) with the approximate retention time of MeHgCl but were not exactly coincident (d). It is not known if these peaks represent MeHg; but if they do, they occur in both dark and blacklight exposed fish, which would seem to eliminate a stimulative effect of UV radiation under these circumstances, but does not distinguish between a chemical or biological mechanism for MeHg production. Although the sediment and water were originally sterile, the fish were not. In view of reports of endogenous methylation in fish or methylation by their intestinal flora (see review) it would seem unwise to base conclusions about environmental methylation in water and sediments on analysis of fish.

FIGURE 12

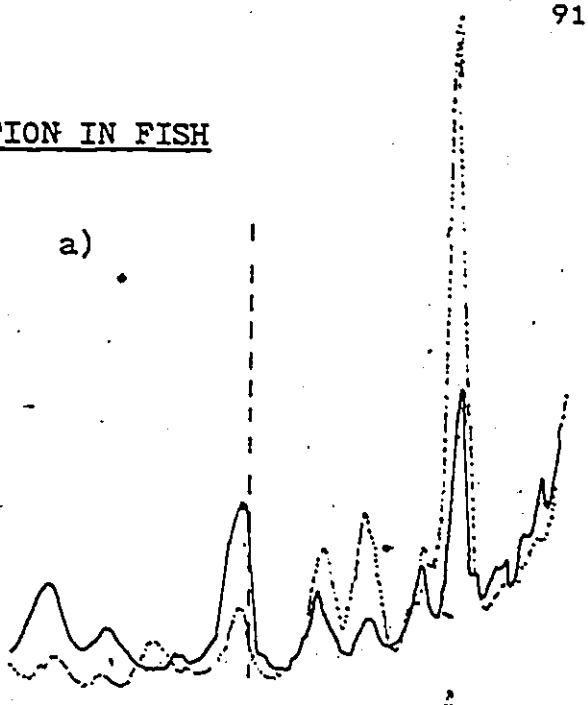
METHYL MERCURY ACCUMULATION IN FISH

5 ul 1st C_6H_6 extract from UV exposed fish and dark exposed fish at 3 days

——— = UV exposed fish

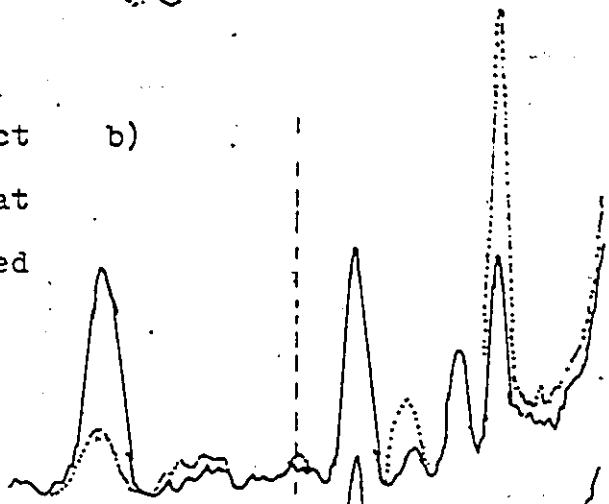
..... = dark exposed fish

a)



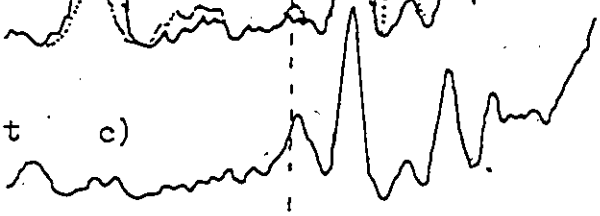
5 ul final C_6H_6 extract from UV exposed fish at 3 days and dark exposed fish at 3 days

b)



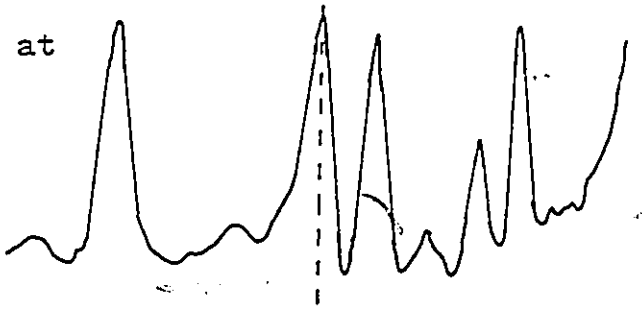
5 ul final C_6H_6 extract from UV and dark exposed fish at 8 days

c)



5 ul final C_6H_6 extract from UV exposed fish at 3 days + 100 pg MMC standard

d)



-----indicates the position of CH_3HgCl (MMC) standard

DISCUSSION

Estimation of the relative numbers of bacteria in the freshwater environment which were able to grow in the absence and presence of inorganic Hg.

These experiments did not attempt to give a complete picture of the numbers of bacteria present or of the tolerance of the bacterial population for Hg. It was hoped, however, to estimate the degree by which bacterial populations in various river compartments might be reduced under conditions of Hg contamination and low nutrient (organic matter) levels such as are found in many aquatic ecosystems, and, most importantly, to obtain some cultures from these samples which might have the ability to methylate Hg.

Hamdy and Noyes (1975), when screening for isolates highly resistant to Hg, noted that much better growth was obtained on their Hg containing plates if they were left 48 hrs. before use. This may be because some loss of Hg occurs and a concentration gradient is set up in the plates which allows organisms to grow on the lower Hg concentrations in the surface layers. Our plates were not stored longer than 18 hours before use, but the prolonged incubation periods may have had the same effect as some colonies appeared late in incubation and obviously grew poorly on the Foote & Taylor medium containing Hg.

Colony counts obtained on sediment samples taken from shallow waters (Brewery Creek, Ottawa River north and south shores) did not differ by more than an order of magnitude, but later mid-stream samples showed lower counts of viable bacteria. It is possible that this difference is related to the organic

content of the sediment and the scouring of the sediment surface in a fast flowing river. In the mid-stream the sediment is comprised largely of sandy material. In spite of this, the proportion of bacteria which tolerated the Hg levels used was quite similar in all the sediment samples tested. Some site to site variation was, however, observed; as has been reported for sites in the Chesapeake Bay area (Nelson et-al. 1972). It is also probable that the water quality and composition will affect the bacterial populations at different locales. For example, the clay and sand sediments show similar proportions of tolerant bacteria under aerobic and anaerobic conditions; however, aerobic bacteria from wood chip sediment show slightly increased sensitivity to Hg. It is not known if this could be related to the nature of the sediment and therefore a difference in the composition of the bacterial population that was found.

Handling and storage of sediment samples prior to bacterial isolation could also affect the total count obtained, as well as the proportion of Hg tolerant organisms detected. We have observed this for Perch skin and gill slime (Table 2), where the total viable counts in the absence of Hg were reduced by about 90% after 3 days refrigeration. It seems logical that the bacteria which do not survive storage would be among the most sensitive to a change in conditions. Since these bacteria were from the highly oxygenated surfaces of fish, it is possible that a drop in Eh on standing of the sample would have caused their loss. Coincidentally, these same bacteria may be among those most sensitive to Hg because the stored samples showed an apparent increase in per-

centage tolerance when compared with the fresh samples.

It is particularly interesting to note the maximal survival of bacteria from perch gut in the presence of low concentrations of added Hg (1 ppm), though it is not known whether this is a real phenomenon which may possibly have significance in vivo. It may be due merely to the presence of an increased amount of organic material in the intestinal contents, which is binding the Hg and making it unavailable to the bacteria. At 10 ppm, the sensitivity of the bacteria was approximately the same as for the other fresh fish samples. Another point of interest is that no bacteria were detected from fresh fish gill in the presence of 10 ppm Hg, although the numbers obtained at 1 ppm Hg were not much different from the skin samples, and stored perch gill showed quite a few viable colonies. One possible explanation for this is that, during the 3 days refrigeration, there had been a change in the bacterial population which allowed detection of some more tolerant bacteria. These observations emphasise the need for immediate analysis of environmental samples if it is hoped to look at a realistic cross-section of the bacteria present in the natural environment.

It is clear from the results obtained that, in Foote & Taylor medium, at about 200 ppb, Hg does have a profound effect on the numbers of viable bacteria, and at 2-10 ppm added Hg very few of the total number of colonies remain viable (approx. $2 \log_{10}$ reduction). Thus it may be assumed that, although all the Hg added to the Foote & Taylor medium is "bound" (Ramamoorthy & Kushner, 1975b) to the media constituents, at least some of it is "available" to the bacteria. If sediment is to be the main source

of MeHg for fish, and bacterial methylation is the main method by which it is produced in the sediment, then, in polluted waters, the remaining bacteria in the sediment must be the ones which are capable of producing MeHg. On the other hand, a wider spectrum of bacteria may be able to methylate Hg in the water column, because even in moderately polluted waters there is usually a lower Hg content than in the sediment. It is possible that most bacteria produce small quantites of MeHg but that some are rapidly killed: this would mean that the largest conversion of inorganic Hg to MeHg might be expected in conditions of low Hg concentration where the contributions of all methylating species would be additive.

As far as is known, no systematic studies of in situ Hg tolerance have been reported for fresh water. For estuarine sediment, in the Chesapeake Bay area, it has been shown (Nelson et al., 1972) that 0.1-46% of the bacteria are tolerant of 6 ppm added $HgCl_2$: the majority fell into the lower part of that range (<10% tolerant strains). This is consistent with the figures which we have obtained for similar Hg concentrations and bacterial populations. Fujiki and Tajima (1973) suggest that bacteria were not responsible for the Hg methylation in the Minamata Bay because there were very few isolated from the mud which contained meHg. It should also be mentioned that we obtained no bacterial colonies on plates containing 100 ppm added Hg. Bisogni & Lawrence (1973), using a mixed sewage sludge bacterial population in a continuous broth culture in rich medium found 100 ppm Hg to be necessary before there was a marked effect on bacterial metab-

olism. However, there are two possible reasons for this: the rich culture medium they used which may have made the Hg less available, and their system was stripped of Hg fairly rapidly (Bisogni & Lawrence, 1973). Sewage sludge may also be expected to be enriched for Hg resistant bacteria since sewage is known to contain relatively high Hg levels (Van Loon, 1973).

There is no published work on the proportion of Hg tolerant bacteria associated with fish, but all samples tested showed appreciable numbers at 1 ppm added Hg. This could be important when considering the route and form of Hg which accumulates in fish from the surrounding waters. From the large proportion tolerant to 1 ppm in fish gut, and considering the dilution factor of the sample tested, it may be estimated that in situ populations of bacteria in fish gut, which are viable at 1 ppm Hg, may exceed 10^9 per mL of gut contents. Since this is several orders of magnitude above the numbers of bacteria found per gram of sediment at the same Hg concentration, bacteria on or in fish are likely to be far more important in determining the quantity and nature of Hg accumulated by the fish than are sediment bacteria. The demonstration of Hg methylation by fish intestinal contents (Rudd et al., 1980) appears to confirm this, but direct bacterial involvement was not proved in this case, and the presence of decaying matter in fish gut may be equally important.

Competition Between Bacteria and Sediment, or Bacteria and Water for Hg in a third compartment of a 3-compartment model.

Mercury transfer has been studied extensively in 2-compartment systems (sediment-water) from the Ottawa River (Ramamoorthy & Rust, 1976;1978) and desorption was shown to be slow even in

the presence of chelators. As far as is known, this is the only attempt to study Hg interchanges in a simulated 3-compartment model of a natural system. As was established in the review, water bodies in non-polluted areas would receive nearly all their Hg as rain. The results obtained when water is used as the Hg source compartment led us to believe that bacteria can compete very successfully with sediment for Hg added to the water column. In fact, after 72 hours, 67% of the remaining Hg was still in the water compartment, being bound to neither the bacteria nor the sediment, even though the system was stirred. Since the principal binding sites for Hg in Ottawa River water have been shown to be <1400 MW (Ramamoorthy & Kushner, 1975) it seems unlikely that the dialysis tubing (MW cut-off approx. 12,000) would restrict the availability of Hg to the enclosed compartments for more than a very short period of time. The river water was autoclaved, but it is probable that some suspended organic matter remained, which could account for the Hg remaining in the water compartment. It is very important in such experiments, and when considering the natural situation, to take into account the order in which the components are added. Therefore, it could be argued that any Hg falling into a body of water as rain would probably not only be bound by bacterial or other biomass before it reached the sediment, but also may remain in the water for some time if the system is not too turbulent.

If wasting through an underwater pipe is the main source of Hg contamination, the sediment Hg concentrations might be expected to be high. When the sediment was used as the source compart-

ment the bacteria were also able to accumulate Hg. This would tend to indicate that Hg was indeed available to bacteria in sediment interstitial waters and at the sediment water interface. In a system with total vertical mixing, such as the Ottawa River, it could also indicate that Hg from the sediment was available to all parts of the aquatic system. Because the bacteria are binding the Hg, it is likely that more will be released from the sediment to maintain the equilibrium until a new equilibrium state involving the 3 compartments is established, or all the Hg is lost. The addition of a chelation mixture to the system greatly increased the binding of Hg by the bacteria. This would imply that, in waters enriched with organic matter, not only would the sediment Hg be more available to biota, but the water Hg would also. It is not known which component(s) of the chelation mixture used were most responsible for the Hg binding to bacteria, but it seems likely that it would have been the fulvic acid (Ramamoorthy & Kushner, 1975). This is supported by other work (Peng et al., 1983) which indicates not only that Hg is bound most strongly to the low MW components of fulvic acid (FA), but that the overall complexing ability in the water can be greater than in the sediment. It is interesting to note here that two principal publications on the abiotic methylation of Hg (Rogers, 1977; Nagase et al., 1982) also implicate low MW components of FA in Hg methylation. It is therefore possible that, either such components are bacterial end products or products of bacterial degradation of organic matter, or that the binding of Hg to bacteria through these agents gives the impression that bacteria themselves carry out methylation, when in fact it occurs abiologically.

Many microorganisms are known to secrete or excrete large quantities of organic matter, and it has been suggested that one of the functions of this material may be to chelate essential metals from the environment (Ittekkot, 1981). The exact nature of the organic material is unknown in many cases, but aliphatic organic acids, amino acids, low molecular weight organic sulphides and complex mucopolysaccharides are among the most prominent components which have been identifed. Of these, the compounds most likely to be important for Hg binding are the sulphur containing amino acids and the organic sulphides, which both show a high affinity for Hg due to their sulphur content. It is also possible that these are among the components in the water body which comprise the binding sites for Hg observed by Ramamoorthy and Kushner (1975), and in the low molecular weight FA which has been shown by Nagase et al. (1982) to methylate Hg.

Since the experimental system used to study these exchanges could not readily be enclosed, some loss of Hg occurred from the system. It is thought that this loss may have had 2 components. When Hg was added to the water, approx 25% of it was lost immediately, presumably due to the physicochemical reduction of Hg^{2+} to Hg^{o}. The level of Hg in the system then remained constant until about 48 hrs. when it started to decline. This loss is consistent with volatilisation of Hg by the bacterial compartment, but whether this is an induced reductive volatilisation as has been observed for many pseudomonads (Nelson et al.,1972), or whether it is merely due to the presence of large numbers of dead and dying bacteria (Ramamoorthy et al., 1983) is unclear.

A similar experimental set-up was used by Laube et al. (1979) to study cadmium and copper accumulation by algae overlying metal-contaminated sediment. They showed the movement of these metals from the sediment compartment to algae in the water compartment above. The addition of NTA to their cultures in some cases increased the toxicity of these metals for the algae (Laube et al., 1980). Mercury was not used because it was found too difficult to work with (D. J. Kushner, personal communication).

The Formation of Methyl Mercury by Microbial Cultures.

Failure to find any detectable levels of MeHg production by the microbial cultures used was, to say the least, disappointing. This is contrary to the results observed by many other workers (see review). There are a number of possible explanations for this:

1) Methyl Hg was not formed because the cultures used were not in the right physiological state. There is an indication (Ramamoorthy et al., 1982; W. F. Iverson, unpublished) that methyl Hg formation may only occur as a result of dead and decaying bacteria. If this is the case, then no methyl Hg would be likely to be found, because the cultures used in these experiments were 'terminated' by concentrated HCl when they reached stationary phase and were not allowed to sit more than a few hours. Most workers observing microbial methylation of Hg have not reported viable counts on their cultures. In some cases (Bisogni & Lawrence, 1973; Vonk & Sijpestiejn, 1973) they have used rich culture media and/or long incubations. Even when stationary phase is reached quite rapidly (< 12hrs.), some cultures were analysed only after 3 days.

Yamada & Tonomura (1972a) show the production of MeHg to occur only up to mid-log phase of growth. Very little was found in the first 24 hours during which time the Eh of the culture was very low. When the Eh of the culture rose, so did the methyl Hg, over a period of a few hours. It is difficult to confirm from their publication that this was a steady rise, since only one point is plotted in that period, so it is not inconsistent with a chemical production of MeHg which is Eh limited. They also noticed a correlation between MeHg production and sporulation in the Clostridium used in their experiments (Yamada & Tonomura, 1972b). It is possible therefore that, even in the case of that resistant organism, it was the decay of some of the population which gave rise to the presence of MeHg.

2) Methyl Hg was not formed because the correct methyl donors were not provided in the growth media, or made by the cultures used. In an attempt to simulate the low levels of nutrients found in most aquatic ecosystems we used a medium low in organic nutrients (0.05% peptone). It is possible that this medium did not furnish the required components necessary for MeHg production by the microorganisms. Influence of the culture medium has been studied by Yamada & Tonomura (1972a), who observed MeHg production in a medium containing meat extract and peptone, but not when casamino acids or tripticase were used. It is possible that these more highly digested substrates may have substantially different quantities of potential methyl donors, such as methylated amino acids. Rowland et al. (1977) report that the ability of rat cecal contents to methylate Hg decreases with storage, and that the rat

cecal contents were more efficient at methylating Hg than the rat intestinal contents. It is not known if this is due to different bacterial populations, or whether the presence of methyl donors may have been enhanced by better or longer digestion in the cecum. Other workers also have reported the increased levels of methyl Hg production in the preence of vitamin B_{12}. However, as discussed earlier, methyl transfer through methylcobalamin can occur chemically, as well as enzymatically which introduces another complication, and it was not added in our study.

3) Methyl Hg was formed but subsequently demethylated and volatilised. This would seem to be a more likely explanation in mixed cultures than in pure ones, because the same organism would have to both methylate and demethylate Hg: however, such a phenomenon has been described (Pan Hou & Imura, 1981b). Although cyclic levels of methyl Hg have been observed in culture (Edwards & McBride, 1975; Berdicevsky et al., 1979), and demethylation is a commonly observed phenomenon, it would seem unlikely that I could have missed all the peaks of methyl Hg production and only sampled in the troughs unless methyl Hg is only formed after bacterial death, as observed by Ramamoorthy et al. (1982).

Another possibility is that the Hg was all lost from the cultures, and there was nothing left to methylate. This can be discounted because although some losses were observed, there was still a considerable amount of Hg remaining bound to the cells in all the cultures used.

4) MeHg was formed and volatilised from the culture as diMeHg. This seems a highly unlikely possibility for a number of reasons. No other observers of Hg methylation have found significant

quantities of diMeHg in their cultures, though small quantities have been detected (Bisogni & Lawrence, 1973; Bishop, 1972). Also, since it is likely that the availability of the methyl groups is the limiting factor in Hg methylation in culture, the dimethyl compound is much less likely to be formed; and the rate constant has been reported to be much slower for addition of the second methyl group.

5) MeHg was in fact formed but was not well extracted, or was present in too small a quantity to be detected by the analytical methods used. This seems unlikely, because the test extractions indicated a good recovery of spiked MeHg and it is a standard method used by many workers in the field. Also, no problem was encountered in extracting and detecting MeHg from the cultures where HgAc was the Hg source. The GLC detection limit was about 50 pg (10 ng in a 1 mL sample), which corresponded to about 0.005% conversion from 200 mL of 1 ppm Hg. Some decomposition of ionic Hg compounds has been reported (Baughman et al., 1973) and the rate of decomposition was inversely related to sample size, so it is possible that there was some loss by this route. Hamdy & Noyes (1975) have reported lower values obtained for GLC analysis than for TLC analysis on the same samples.

Some, but not all, of our methylation experiments were conducted with radiochemical analysis only, because the GLC was not available at that time. It is just possible that the observed "carry-over" of inorganic Hg during the extraction procedure (0.05-0.12%) was in fact due to MeHg, but because no differences were observed between the inoculated samples and controls anal-

ysed either at the beginning or the end of the incubation period, we had not attributed this to methyl Hg production. It sems unlikely that MeHg was produced because the radioactive samples which were also analysed by TLC, did not indicate the presence of MeHg. None of the other workers have reported either using or extracting control cultures for their experiments. That is, they did not adequately control for abiological MeHg production. Vonk & Sijpesteijn (1973) do report that no methyl Hg was found in the media constituents, but that was before they added Hg.

It is not known exactly why no MeHg production was observed in our cultures, except when HgAc was used as the Hg substrate. The most probable explanations are, however, that either the physiological state of the organisms was not suitable (i.e. the bacteria were intact) or that we had not provided the right methyl donors. In spite of this, the possibility that MeHg was formed and then destroyed cannot be discounted.

Photochemical Methylation

The results of our experiments have confirmed those of Akagi & Takabatake (1973) for the photochemical production of MeHg from HgAc in distilled water. The yield was approximately 8% in 144 hrs. whereas Akagi & Takabatake (1973) found a 10% conversion in 168 hrs.; they do not report the intensity of radiation used. The most interesting observation with HgAc, however, is the amount of MeHg detected at T_0 in river water. This may not be due to a photochemical reaction, but could imply that a direct chemical reaction was taking place between the river water and the HgAc to produce MeHg. One could speculate that a low MW component, similar to the methylating agents in fulvic acid (Nagase et al.,

1982), might be involved here. If this was the case, then it was probably present in limited quantities, but may have given a good yield of MeHg. The overall yield in river water was however decreased when compared with distilled water under either UV or SUN irradiation, and it is likely that this may be due to the scavenging of methyl radicals by compounds which are present in natural waters.

Prolonged irradiation of either HgAc or HgO + acetic acid resulted in quite high concentrations of MeHg. Differences between UV and sun-exposed samples may, to some extent, reflect differences in total exposure times. It is interesting to note that sunlight irradiation of HgS in the presence of acetic acid gave more MeHg than did the UV exposure. This may reflect a different absorption optimum for HgS, or some of its photochemically induced products.

It must be emphasised that quantities of MeHg detected were a minimum, because of the probable photodecomposition of the MeHg product (Baughman et al., 1973).

One interesting observation may be made if the level of MeHg in the T_0 samples here is compared with the levels of MeHg found in the microbial methylation experiments. If the MeHg was present as an impurity in HgAc, then, because of the much higher HgAc used here, it might be expected that more MeHg would be found. This is not the case: a much higher proportion of the HgAc was found as MeHg in the experiments with microbial growth media (approx. 2%) than is found in either the distilled water (approx 1.25×10^{-4} %) or river water (approx. 2.8×10^{-3} %) at zero time

in the photochemical experiments. Therefore, it seems likely that there was an interaction between the media constituents and HgAc to produce MeHg in that case. The same lot of HgAc was used for all the experiments.

It is clear both from our results, and those of others, that simple molecules can act as methyl donors for Hg. Although it is not known in natural systems how far sunlight will penetrate into surface waters, it is possible that some suitable methyl donors could be found in the top layers of water where the microbial growth and nutrient levels are highest. Thus, with the input of Hg from rainfall or from solubilisation or volatilisation from deeper water, the surface layers of a body of water may be particularly suited to chemical or photochemical formation of MeHg. Once formed, it is likely to be photolysed, unless it is incorporated into biological sinks. The most suitable methyl donors in this process are as yet unknown.

ALTERNATIVE MECHANISMS FOR ENVIRONMENTAL MERCURY METHYLATION

It was considered a part of this study to look at what might be an environmentally feasible explanation for methyl mercury production. Since completion of this study, the author has thought a good deal, not only about her own results, but also about the anomalies that have appeared in work in this area. It seems probable that the pieces of this puzzle would begin to fall into place if it was possible to determine the form of the Hg substrate in natural waters, and the source of the methyl groups for the methylation reaction.

A hypothesis is suggested here which appears to offer some alternative, as yet untested, mechanisms for environmental MeHg

production. If any of these mechanisms are subsequently shown to be valid, then they may offer explanations for some of the results obtained here as well as for those of other workers. It should however be stated that no single mechanism is likely to account for all the Hg methylation observed in the environment.

1) Formation of MeHg in the environment may be closely linked with the biological or abiological breakdown of methionine or methylated amino acids.

2) Formation of MeHg in the environment may also occur by biological, chemical or photochemical reaction of Hg with low molecular weight alkyl thiols and/or sulphides such as methyl mercaptan (CH_3SH), dimethyl sulphide ($(CH_3)_2S$) or dimethyl disulphide ($(CH_3)_2S_2$).

3) Formation of MeHg may also occur in fresh surface waters by the photochemical activation of acetic acid in the presence of mercurials, or HgAc if present.

4) In most natural environments, at the times of peak methylation, it is the concentration of available Hg which limits the rate and extent of Hg methylation.

The following background information is useful in considering this proposal:

1) Methionine was one of the first compounds implicated in Hg methylation (Landner, 1971), but it was assumed to be in the biosynthetic pathway, with MeHg formed as an incorrect methionine.

2) Both Landner (1971) and Singh & Sherman (1974) have reported increased toxicity of Hg for their cultures in the presence of

methionine. These authors explain their results on the basis of methionine biosynthesis; but, methionine breakdown, with methionine acting as the methyl group donor in the synthesis of MeHg, would seem to be an equally plausible explanation. These papers cannot be examined here in detail, but some information is given in the review section.

3) Methionine is known to be important in transmethylation reactions and can be demethylated by 2 different pathways: in one the sulphur leaves with the methyl group, and in the other it stays with the 4-C skeleton. It was suggested as early as 1940 (Toennies, 1940) that although in vitro the methyl group tends to leave methionine alone, in vivo probably both routes are possible. This has been shown to occur: recycling of the methionine sulphur in duckweed ocurrs both with the methyl group and with the carbon skeleton (Giovanelli et al., 1981).

4) Methionine may be able to function as a methyl donor both non-enzymatically, and enzymatically through s-adenosyl methionine (SAM). Imura et al.(1971) and Jernelov (1972) both report lack of involvement of SAM in Hg methylation with in vitro systems. Gay (1975) used methionine as a potential methyl donor in synthesis of MeHg by extracts of a pea plant. No MeHg was formed with SAM alone, or with SAM and a variety of cofactors. However, when an extract of ground pea tissues was added to a solution containing methionine, $HgNO_3$ and ATP, MeHg formation was observed. The results of this study suggest, but do not prove the involvement of SAM in Hg methylation. It is, however, possible that methionine may act as a methyl donor directly, if the right

attacking Hg species is presented, or indirectly through an intermediate such as vitamin B_{12}.

If one considers the microbial system, and the possibility that MeHg is formed only after death of the organism, then the following may be a reasonable scenario. Inorganic Hg has limited access to living cells and may be detoxified as fast as it can permeate the cell wall. However, when the cell dies, as may happen in microbial cultures with high added Hg, inorganic Hg may be able to enter more freely. Breakdown of the internal structure of the cell may allow direct access of Hg to enzymatic or non-enzymatic methylating systems normally present in the cell. Such methylating systems may have a limited period of activity before they are destroyed. The possibility of methionine or SAM acting as methyl group donors for Hg methylation has not been adequately investigated.

5) Alkyl thiols and sulphides are known to be involved in the alkylation of some elements other than Hg (Challenger, 1945; 1951). The breakdown of methionine to produce alkyl thiols and sulphides has been described in blue-green algal blooms and bacterial degradation of blue-green algal blooms (Jenkins et al., 1967), other bacteria and fungi (Alexander, 1974 and refs therein), bacterial putrefaction of fish muscle (Miller et al., 1973; 1976), man (Kaji et al., 1981 and refs. therein) and industrial processes (Sheraton & Murray, 1981). Dimethyl sulphide and methyl mercaptan are usual byproducts in the pulping of wood, presumably the methoxy groups of lignin, originally derived from methionine, are the source of the methyl groups here. Some micro-organisms are also capable of producing dimethyl sulphide from

inorganic sulphate (Challenger, 1951).

If Hg is already attached to the thioether group of methionine, then what happens when methionine is demethylated? Could MeHg be produced directly?

6) Jernelov (1969) has briefly mentioned the high capacity of sewage treatment plants to generate MeHg. Sewage is also known to contain large quantities of organic sulphur compounds.

7) It is known that alkyl thiol and alkyl sulphide compounds react with Hg (Challenger, 1951; Wood et al., 1975). They have also been shown to be photolysed to produce highly reative species (Sheraton & Murray, 1981). Of interest, perhaps, in this regard, whether photolysis or other biological or chemical mechanisms are involved, is that both the Hg and the methyl group are simultaneously present.

8) Low MW alkyl sulphides, and diMeS in particular, have been implicated as the major volatile sulphur products in the global sulphur cycle (Lovelock et al., 1972; Nguyen et al., 1978). The concentration of diMeS in seawater has been extensively studied and found to correlate with indicators of algal activity (Andreae & Raemdonck, 1983; Andreae et al., 1983). Levels in surface layers of marine waters are approx 50ng/L. Freshwater algae also produce volatile organic sulphides (Berchard, 1974). Emission of dimethyl sulphide and dimethyldisulphide from land has been estimated at 4-8 mgS/m^2/yr and 1-4 mgS/m^2/yr, respectively (Adams et al. 1980).

9) Furutani & Rudd (1980) observed in situ peaks of MeHg production in the water column in June and late August. It is known

that blue-green algae produce extracellular peptides and amino acids in the early stages of bloom formation (Fogg, 1969); levels of these have been measured (Ittekkot, 1981) and it has been suggested that these may function as ligands to remove metals from the environment. The bacterial community could also degrade most of these compounds; and bacteria are always found associated with blue-green algal blooms. If methionine, or other amino acids, were able to act as a methyl donor for Hg methylation, then a rise in MeHg levels might be expected at that time. In addition blue-green algal blooms also produce large amounts of low molecular weight organic sulphur compounds such as methyl mercaptan and dimethyl sulphide (Jenkins et al., 1967); either directly by the alga or indirectly from the associated bacteria. These compounds are almost certainly able to bind inorganic Hg and if they can also act as methyl donors either biologically or abiologically, possibly by transmethylation via cobalamin, then a peak in MeHg would result. It is interesting to note that although methyl cobalamin is known to be photolabile, it has been found in unicellular algal cultures (Neujahr & Fries, 1966). Also, the August peak of MeHg in the water occurred prior to the peak in the sediment. This peak may coincide with algal death and degradation, and may be associated with bacterial decay of blue-green algae which is known to produce simple organic sulphur compounds (Jenkins et al., 1967; Andreae & Raemdonck, 1983). Such a process would be likely to start in the water column before the algal bloom sinks to the sediment. It is also possible that bacterial or fungal degradation of leaf litter would produce the same low molecular weight organic thiols and sulphides.

10) A similar explanation could account for the observations of high levels of Hg in fish from lakes where there is no direct source of Hg input and sediment Hg levels are low. Philips & Medvick (1981) felt instinctively that the Tongue River Reservoir was particularly suited to methylation: they noted a high algal productivity, high water flow and thermal stratification. If the main source of Hg to a water body is in rain, then such Hg would be unlikely to be found in large concentrations in the sediment, but would probably contribute significantly to the morbidity and mortality of the primary producers some at least of which are known to be extremely sensitive to Hg (Hannan & Patouillet, 1972; Harriss et al., 1970; Jeffries & Butler, 1972; Springthorpe & Kushner, unpublished results). It is even possible that this observed sensitivity is correlated with MeHg in rain or MeHg produced from inorganic sources reaching the water. Some algae have been shown to have a great fixation capacity with respect to Hg (Ribeyre et al., 1981). It is also worth noting here that the concentration of dissolved amino acids in natural waters has been observed to increase with the degree of eutrofication (Feierabend, 1978) and exhibit diurnal and annual fluctuations.

11) It is interesting that Nagase et al. (1982) used leaf mould for their studies, because methyl mercaptan and dimethyl sulphide are known to be products of wood pulping released in waste gases (Sheraton & Murray, 1981) and the proportion of methyl mercaptan increases with the proportion of leafy species used (Kharitonovich et al., 1983). Also, if MeHg is produced in or from pulp mill waste gases, then it could help explain two other

observations:

a) that fish in Sweden have higher levels of MeHg than those in Japan (Kazantzis, 1971),

b) Levels of MeHg in fish in the English-Wabigoon River System have not declined as rapidly as those in the Detroit River (Hanlon, 1976).

A paper that I have only been able to obtain as an abstract (Jackson & Woychuk, 1980) reports that MeHg content in wood-chip deposits below the pulp-mill on the Wabigoon River system can be correlated with methionine biosynthesis in the wood-chips. Kudo et al. (1977) also report more MeHg produced in wood-chip sediments in the Ottawa River than in other types of sediment from the same area of the river.

It is possible, therefore, that the high levels of MeHg which have been found in the vicinity of pulp mills, are there, not just because of the Hg-containing wastes from the pulp-mills and often-associated chloralkali plants, but also because of the production of suitable methyl donors. Thus, it would be possible to account for the continuing high levels of Hg and MeHg in the Wabigoon River system if the continual supply of fresh methyl donors remethylated Hg in a cyclic fashion. Such a phenomenon would make pulp-producing countries such as Canada and Sweden particularly vulnerable to MeHg pollution.

12) If volatile organic sulphur compounds can act as methyl donors in the formation of MeHg, then an additional implication would be that Hg, and possibly other metals, could be methylated in the atmosphere. The organometalic sulphur compounds formed may then contribute to the deleterious effects of acid rain.

Coals are known to contain Hg (see review). In a study of coal fly ash, Natusch et al. (1975) estimated that sulphur concentrations in the ash surface were greater than any other element. Also, organic S(IV) compounds have been shown to be present in the paticulate matter from the plume of a coal-fired power plant (Eatough & Hansen, 1983). Although these organic sulphur compounds have not been fully characterised, the authors suggest that they may be similar to known alkylating agents such as methyl sulphate and dimethylsulphate. The possibility exists, therefore, of MeHg formation in the vicinity of coal-fired power plants. Although MeHg and acid rain have not been directly linked, Johnson (1982) suggests that accelerated Hg contamination of fish is one effect of acid rain.

13) The concentration of acetic acid in both fresh surface waters and waste waters is considerable: it is the major identified organic acid, with approx. 50-130 µg/L for lake water (Hama & Handa, 1980), and 6-37 µg/L for waste water (Painter, 1971).

14) There has been some question as to the exact nature of the organic Hg found in fish. In spite of reports from Sweden that the majority is in the methyl form, in Japanese waters only 10-80% of total Hg was in the methyl form, and it has been suggested (Neville & Berlin, 1974) that this may be related to either the feeding habits of the fish, or differences in the analytical techniques used for detection of the organic Hg. In addition, it has been reported that the Hg compound in shellfish which was partly responsible for the Minamata poisoning cases was methyl-(methylthio)mercury (CH_3-S-Hg-CH_3) (Uchida et al., 1961).

Jernelov (1969) has made only passing reference to the possibility of other organic Hg compounds including both $CH_3-S-Hg-CH_3$ and $(CH_3-S)_2Hg$, but nothing more is said, other than the fact that sulphur compounds interfere with the determination of MeHg. I think this point deserves further investigation, particularly because Wood et al. (1975) implicate methyl mercaptan in the formation of methyl-(methyl thio)mercury.

There are many experiments which could be done to test this hypothesis and I do not propose to go into great detail here. However, it would seem reasonable to start by using ^{14}C-methionine, which is specifically labelled in the C5 methyl group, in strictly chemical reactions with Hg compounds which are likely to be found in the natural environment. Photochemical production of MeHg should also be studied using some of the compounds mentioned above (e.g. alkyl thiols and sulphides, methylated amino acids, including methyl methionine): these should also be specifically labelled with ^{14}C. Depending on the results of the chemical and photochemical studies, bacteria and algae, or their extracts, would be introduced to assess their role in MeHg production. It would also be ideal to have a simulated lake system, with algae and bacteria, where Hg was supplied in low concentrations as "rain". It would be an essential requirement of such a study to have excellent analytical facilities available.

CONCLUSIONS

1. The presence of 1-10 ppm added inorganic mercury will significantly alter the numbers of bacteria that can form colonies, in populations recovered from the Ottawa River water, sediment and fish slime and gut, often resulting in a >2 log$_{10}$ reduction in their numbers.

2. Bacteria bind mercury very strongly and are capable of competing successfully with sediment binding sites both when mercury is added to the water and when it is already bound to the sediment. Presence of a chelation mixture greatly enhanced mercury binding to the bacteria.

3. No evidence was obtained for the microbial production of methyl mercury; the reasons for this are discussed.

4. Methyl mercury can be formed by UV stimulated reactions from mercuric acetate, and from mercuric oxide, and mercuric sulphide in the presence of acetic acid.

5. A hypothesis has been suggested for possible alternate mechanisms of mercury methylation in the environment.

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