A REPORT ON THE METHYLMERCURY SITUATION IN PAR POND

FINAL REPORT

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June 1975

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Prepared for the U. S. Energy Research and
Development Administration under contract AT(38-1)-825

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AT-(38-1)-825

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Par Pond, the 1133 Ha reservoir located on the Savannah River Plant (SRP) of the Atomic Energy Commission, contains fish with anomalously high levels of mercury (Kania, 1973; Tilley and Wilhite, 1972). One theory is that the mercury was initially introduced into Par Pond with the water pumped from the Savannah River, a known contaminated source. The mercury in the pond system has been translocated into the fish populations and the sediments, normally considered to be a mercury sink in the aquatic environment, contain only natural levels of Hg (Tilley and Wilhite, 1972). Table 1, Fig. 1. This apparent anomaly has been attributed to the unique aspects of the complex pond system, such as the thermal input which may stimulate the bacterial processes that are reported to convert Hg into easily metabolized forms (Wood, 1974), or the unusually large fish populations of this protected system which may keep trace elements, including mercury, in continuous cycle within the pond's biomass.

This report deals with several of the aspects of the mercury problem in the Par Pond environment. The specific problem that is addressed in this report concerns the methylation and accumulation of the mercury in the fish populations of the pond environment. The analysis of the methylation is essential since methylmercury is considered to be the most toxic mercury form in the biological system. At the general and theoretical level, this report contains statements about the chemical nature of the Par Pond system, makes recommendations for remedial treatments to eliminate the mercury problem in this environment and suggests several levels of investigation that must be undertaken to increase our understanding of cooling ponds.

I. The Concentration of Methylmercury in the Fish of Par Pond

Fish were collected from the Par Pond environment during the spring of 1974.

These fish were harvested by hook and line and with the aid of the Georgia Cooperative Fisheries Unit Electrofishing Boat. The fish were weighed, measured, and immediately frozen in dry ice. Muscle samples were taken from fish too large to freeze in the field ice chest.

Quantification of the methylmercury concentration was made on a Perkin Elmer Model 900 Gas Chromatograph using a KBr saturated SE-30 glass column and a Ni-63 electron capture detector. The extraction and operating conditions of the GC were similar to those in the Environmental Protection Agency (EPA) procedure published by Longbottom et al. (1972).

The fish were analyzed for total mercury by the flameless mercury technique as outlined by Uthe et al. (1970) and Kopp et al. (1972). A Perkin Elmer Model 303 Atomic Absorption Spectrophotometer was used for this analysis.

The methyl to total mercury ratios for the fish muscle samples from Par Pond are shown in Table 2. A considerable amount of individual variability in the percent methylmercury concentration can be seen in the 12 bass sampled. The percentages range from 67 to 100% with an average of 87%. (The efficiency of methylmercury extraction of muscle tissue was found to be > 90%.) The predominant form of mercury in Par Pond bass muscle was the methyl form. The average methylmercury concentration in the 12 bass was 2.31 ppm. No correlation was seen between the per cent methyl mercury concentration and weight among the bass. The 3 crappie sampled had an average of 1.05 ppm methylmercury; 3 bluegill had 0.21; 1 herring had 0.30 ppm; 1 shad had 0.19 ppm and 2 suckers had 1.16 ppm. The ratio of methylmercury to total mercury in the crappie and suckers ranged from 70-100%; the other fish had 100% methyl to total mercury. Under-sized and biased samples prevented any attempts to correlate the methylmercury concentration in these fish with other factors such as size and sex. It is interesting that the top predator in Par Pond, which was the bass, had higher

methylmercury and total mercury concentrations than all the other fish sampled. This has been noted in several other field studies and thus has been one of the points in the argument for food chain transfer being the most important pathway by which mercury is accumulated (Georgia Water Quality Control Board, 1971; Pilley et al. 1972; Zeller and Finger, 1971). However, it would be impossible to draw any conclusions on this point from these data since lower food chain organisms were not sampled adequately for this type of analysis.

This initial survey provided sufficient information to draw a certain set of conclusions and to generate a series of reasonable and testable hypotheses. These results, coupled with the detection of mercury in small fish and vegetation, suggested that the mercury within the pond is either actively cycling within the pond biomass as suggested by Tilley and Wilhite, or in spite of the restricted inputs, sufficient amounts of Hg are reaching the system (either through the water or aerosols) to load up the biotic components. In either case, the sediments of the pond are not serving as a significant sink and the mercury is ultimately present in the form of methylmercury in the flesh of the fish. The inter- and intraspecific variability of the mercury concentrations in the fish lead to the conclusion that any intensive survey of the seasonality of the mercury transport system would require extremely large sample sizes. Unfortunately, the analysis of methylmercury in fish is extremely slow and tedious and not compatible with the large-scale sampling required for this type of analysis. However it is possible to conclude that either:

1. The methylation of the mercury occurred in vivo and was a function of the physiological state of the fish. This postulate would explain the large intraspecific variability, or

2. The methylation is a function of the general environment and the

behavioral differences of trophic relationships of the fish lead to variable accumulations in the biota.

II. Tests of the in vivo Methylation by the Fish.

Biotransformation of inorganic mercury by fish was investigated by in vitro and in vivo methods. In vitro methylation was attempted first to see if the potential for methylation by fish livers existed. These methylation experiments were conducted using procedures similar to those outlined by Imura et al. (1972). Livers from two species of fish were used, bluegills (Lepomis macrochirus) and rainbow trout (Salmon gairdnei). Since some field data (unpublished) has suggested that population crowding in fish may be a factor in the extent to which methylation occurs, livers from both normal-size populations as well as overstocked populations were used. The livers were homogenized with a few ml. of ice-cold deionized water in a tissue grinder to which a very small quantity of clean sand was added. To these homogenates, 10 ppm of HgCl_2 was added and the homogenates plus control homogenates were incubated for at least 5 hours in the dark in a $32^\circ\text{--}33^\circ\text{C}$ water bath. After incubation, the homogenates were analyzed for methylmercury. Several replications of the above experiment were made with such modifications as the addition of 6.8 pH H_2PO_4 buffer, performing preliminary experimental steps under light and dark conditions (since B_{12} is light sensitive), allowing various incubation times, using fresh and frozen livers and using two different extraction procedures for the methylmercury determination (Longbottom et al. 1972 and Westoo["], 1968).

In vivo methylation experiments were also conducted since the lack of positive results in the in vitro methylation experiments did not preclude the possibility that such formation may occur in vivo.

Gambusia were periodically exposed to both Hg^0 and HgCl_2 (comparable

concentrations and conditions) for a maximum of 10 two-hour periods and held in mercury-free water for additional periods up to 38 days. At designated times, or upon death, fish were removed and the whole body extracted according to the Longbottom op cit procedure; the methylmercury concentrations of the test fish were then compared to those of extracted controls.

The various experiments on in vivo and in vitro methylation did not prove successful (Table 3). The hypothesis that methylation of inorganic mercury can occur in vitro or in vivo via the methylcobalamin mechanism is strongly suggested by the literature. Studies of the chemical methylation of mercury indicate that methylcobalamin is at least partly responsible for the methylation of mercury in nature (Imura et al., 1972; Bertilsson and Neujahr, 1971). Methylcobalamin has been identified, not only in microorganisms, but also in calf liver, blood plasma and pork liver (Wood et al., 1968; Lindstrand, 1964; Burke et al., 1970). Vitamin B₁₂, of which methylcobalamin is a derivative, is stored in fish largely in the liver (Smith, 1965). Stronger experimental evidence that suggests in vivo methylation can occur in fish is found in the work of Imura et al. (1972). He found in vitro methylation by liver homogenates of several tuna species by only to a lesser extent in amber jack, mackeral and rainbow trout. His analytical procedure and sample sizes were replicated in this study using bluegill and trout livers, but his results could not be replicated. Therefore, the inability to find in vivo or in vitro methylation can only be attributed to these factors: 1) the formation of thick emulsions in the liver extracts which resulted in low extraction efficiency for methylmercury and/or 2) the fish species used were not efficient at methylation.

A third possibility, that of the physiological condition of the fish determined the methylation capacity was also tested. In these experiments, Gambusia

was collected and placed in experimental tanks equipped with outside filters. The experimental design consisted of three ten-gallon aquaria and one twenty-gallon aquarium. Two of the ten-gallon tanks served as experimental tanks with heat (30°C) and mercury treatments, the third tank served as a thermal control with no heat additions, but with mercury treatments. The twenty-gallon tank served as a control with neither heat nor mercury and in addition was a stock tank to cover mortalities during the acclimatization of the Gambusia to the experimental temperatures.

At the time of the initiation of the experiment each of the mercury-thermal tanks contained 21 fish. The 30°C tanks were treated with 50 ppb of mercury every other day for fourteen days. Control I (21°C) contained 19 fish and was also treated with 50 ppb of mercury every other day for fourteen days. Control II (21°C) was held at ambient temperatures and received no mercury dosage. After addition of the mercury dosage, the external filters were turned off for one hour. Activation of the filters after this period effectively removed the residual mercury contained in the water system. Frequent filter changes were required to prevent contamination from one dose to the next and to insure the fish tanks remained clean.

At the end of fourteen days and a total of seven treatments, fish were removed from each tank and were analyzed for the presence of methylmercury. Other fish were tested for the accumulation of total mercury on the atomic absorption spectrophotometer as outlined above. Table 4 illustrates the results of these simple tests. The values indicate that the major effect of the elevated temperatures is the accumulation of the ionic mercury. In vivo methylation of mercury in these thermally stressed fish did not occur.

Although these results indicate that in vivo and in vitro methylation

cannot be detected by our methods, the argument that the relatively short incubation periods of these experiments did not allow sufficient time for the methylation to occur must be considered.

III. Since it appears that in vivo methylation is not a significant source for the methylmercury in the fish and it apparently cannot account for the intra-specific variability encountered in the Par Pond environment, it must be concluded that a general methylation occurs within the aquatic environment.

One possible mechanism that has been explored is the methylation of the mercury by the natural organic ligands contained within the sedimentary environment of the pond. We have already documented information on the reactivity of the organic system to Hg in terms of the evolution of elemental mercury (Hg^0) (Alberts et al., 1974; Miller et al., 1974). These results show that the mercury reaching the sedimentary environment is reduced and evolved from the system as elemental mercury. These results coupled with information on the behavior of the organic system lead to the conclusion that the humic acids (organics) were serving as an extracellular electron transport system in the reducing sedimentary environment (Schindler and Alberts, in press). Our experiments in this area show that this extracellular transport system may lead to the shift of electron flow from anionic (i.e. $\text{SO}_4^{=}$) terminal electron acceptors to reducible cations such as iron and mercury and assimilable organic material reaching the sediments and stimulating the microbial processes may result in the accelerated liberation of elemental mercury in a reduced sediment (Schindler et al., 1975). However, not all of the mercury is liberated by this mechanism; a variable fraction is retained by the organic macromolecule and is alkylated, resulting in the formation of methylmercury by the organic system. The effects of the reduction-alkylation indicate that under the appropriate conditions the majority of

the mercury entering the aquatic environment would be either liberated as elemental mercury or would be made available to the organisms as methylmercury. A small percentage of the mercury would accumulate in the organic matter and would remain in the sediments.

All of these results show that our understanding of the behavior of mercury in the environment is insufficient. Just as we can demonstrate the potential for the alkylation of mercury in the sediments of the Par Pond environment we can also show that other aquatic environments have varying potentials for electron transport, mercury reduction and alkylation. Based on these observations, it is possible to suggest a process whereby the mercury in Par Pond environment can be inactivated. The operational procedure would require the continued input and exchange of the Par Pond water with the Savannah River water for a period long enough to suppress the reduction-alkylation potential of the organic ligand system. This operation would have two possible effects: the first would be a simple flushing of the system; the second would involve some of the theoretical aspects of the organic ligands and its behavior.

The functional groups of the amorphous organic compounds found in nature are undoubtedly involved in both the electron transport and the alkylation. This reactivity may involve the behavior of the carboxyl and carbonyl functional groups and their stability during electron donation and withdrawal. Destabilization of the organic molecule could occur following electron withdrawal; this may then result in competing resonance of the electrons in the organic molecule which the rearrangement and formation of the alkylmercury compounds would satisfy. If this is a correct assumption in terms of the reactivity of the macromolecule, it is also reasonable to assume that any blocking of these functional or prosthetic sites would lead to both the loss of the electron transport capacity of

the molecule and the alkylation potential. Calcium is known to cause the coagulation and condensation of natural organic compounds (Ong and Bisque, 1968). This type of reaction, or reactions that forced the closing and condensation of the macromolecule would prevent the above process. Our observations in the laboratory show that the addition of calcium does impede the rapid transport of electrons through the organic macromolecule and reduces the amount of alkylation that may occur as a result of this process. In the same light, clays and other cations that block or cause coagulation of the organic would be expected to have the same effect. The Savannah River water contains a considerable amount of clays and coagulating cations that should effectively block the reactivity of the organic molecule.

A second-order response of this type of system would be the elimination of the organic system as an electron-acceptor that could be competing with the formation of sulfide via sulfate reduction. Since sulfide is a very important ligand in the control of heavy metals including mercury, the generation of this element would be expected to cause the precipitation and accumulation of any of the mercury that reached the sediments. With a low level of siltation, this should form a mercury benchmark in the sediments.

This is merely a theoretical means of controlling the biogeochemical relationships of the Par Pond aquatic system. At this level, there are a number of assumptions that should be tested to increase the confidence of the predicted theoretical results. However, with the correct plan of action, validation of the above theory by an empirical test at the Par Pond site would provide a major breakthrough in reactor cooling pond management, since the behavior of many of the constituents could be controlled by pumping operations.

If it is possible, the Savannah River water should be pumped into the Par Pond system during all phases of reactor operation. Coagulation and condensation

of the organic molecule should lead to the immediate reduction of the evolution of Hg^0 from the sedimentary environment. This should be detected in the fish as a reduction in the concentration of mercury in the fish livers. The amount of time required for this phase of the experiment would depend on the productivity of the system (in a sense, this creates the reactive organic material) and the amount of Ca^{++} and clays in the Savannah River waters that reach the pond environment. Elimination of the methylmercury from the fish would be slower and will depend on the half life in the fish (over a year). Maximum information will be obtained if the chemical and biotic composition of the pond environment is assayed at the same time as the biotic production. This should provide the needed information to test the effects of the chemical speciation on biotic composition. The stimulation of the sedimentary system by the autochthonous production and the loss of reductive capacity of the organic system can be monitored by the ETS methods that have already been developed (Zimmerman, in press). Generation of sulfide and burial of heavy metals as the sulfide form can be monitored by the methods of Giammetteo, M.S. Thesis). Future predictive schemes for chemical speciation can be developed by using the methods of Morel and Morgan (1973). However, their method would have to be expanded to account for the behavior of the organic ligands within the environment. A program of this magnitude would require the input and cooperation of all of the researchers at the pond site. Since it is unlikely that such a program could be developed, alternative experimentation on some of the smaller contaminated ponds might be possible.

Conclusions.

1. The results of this investigation show that the majority of the mercury in the muscles of the fish of Par Pond is in the methylmercury form. Very low

concentrations of mercury are found in the sediments of the pond environment.

2. Physiological methylation of the mercury could not be demonstrated in this study. In vivo and in vitro techniques were utilized to test this aspect of biomethylation.

3. Organic ligand methylation and reduction of the mercury was shown to be a possible mechanism to account for both the mercury distribution in the pond environment and the concentrations of methylmercury found in the fish. Field validation and correction of the mercury situation in Par Pond may be possible by an altered pumping operation.

Station Number and Sediment in Centimeters	Organic Carbon in Alkali Extract (μmoles gram ⁻¹ Dry Sediment)	Cations in Alkali Extract (μmoles gram ⁻¹ Dry Sediment)		Σ Cations in Acid Extract	Eh (milli- volts)	Total Hg nmoles g ⁻¹ Dry Sediment
		(Ca, Mg, Fe, Mn, Cr, Zn, Al)				
1A (Total-9)	52.5	10.85		120.5	- 40	0.09
1B (Total-14)	369.8	2.33		226.2	0	0.09
2A (Total-7)	63	1.68		249.5	0	0.17
3A (0-10)	279.2	16.20		255.4	- 80	0.30
(10-22)	271.8	9.93		128.6	0	0.59
3B (0-10)	762.8	37.67		1277.1	-100	1.26
(10-20)	714.0	24.75		832.7	+ 20	1.14
(20-32)	621.1	14.27		312.3	+100	0.72
4A (0-10)	701.0	34.56		700.5	-100	0.97
(10-20)	119.7	5.03		71.3	- 40	0.10
(20-25)	365.7	5.75		78.5	+ 60	0.11
4B (0-10)	864.3	27.93		1484.4	-240	2.46
(10-23)	625.3	29.75		1006.5	-160	0.85
5A (0-10)	967.2	43.56		567.3	- 80	0.73
(10-22)	1118.9	30.89		346.7	+ 50	0.38
5B (0-10)	1247.4	48.97		737.9	-140	1.06
(10-20)	1656.5	38.36		598.8	- 40	0.56
(20-30)	1220.9	20.47		583.7	0	0.46
6A (0-10)	2036.1	45.39		304.0	- 40	0.75
(10-20)	1200.4	14.30		127.6	+200	0.24
6B (0-10)	1865.3	40.57		235.9	-130	0.55
(10-20)	2134.0	30.50		240.6	+160	0.36
(20-30)	837.7	29.54		114.4	+160	0.20
7A (0-12)	597.6	20.09		241.9	-140	0.42
(12-23)	598.6	12.98		137.0	+180	0.20
7B (0-9)	851.8	35.21		227.3	+120	0.36
(9-18)	731.0	22.17		124.5	+180	0.21
8A (0-10)	1032.3	31.85		398.0	-100	0.48
(10-20)	984.8	24.14		342.7	+ 50	0.45
(20-30)	970.3	25.36		303.9	+ 50	0.30

8B (0-10)	1252.1	29.74	348.4	- 80	0.62
(10-20)	1263.8	31.60	420.9	- 50	0.47
(20-30)	1128.5	30.08	356.8	+ 80	0.47
9A (0-10)	273.0	4.70	195.7	0	0.15
(10-20)	302.4	12.72	192.5	+ 80	0.13
(20-30)	282.8	6.56	156.3	+ 20	0.10
(30-39)	360.1	12.23	282.8	+ 20	0.16
9B (0-10)	311.5	6.98	174.6	-100	0.20
(10-20)	440.4	15.09	291.7	+ 60	0.13
(20-30)	344.3	13.50	176.9	+ 40	0.14
(30-36)	240.2	14.53	136.4	+ 40	0.16
10A (0-10)	1415.8	43.10	1129.9	- 80	1.00
(10-20)	1028.9	34.48	203.7	+ 80	0.45
(20-30)	660.3	25.49	90.0	+ 80	0.20
(30-35)	501.6	20.03	31.3	+ 80	0.10
10B (0-10)	1029.3	54.96	802.5	- 80	1.30
(10-20)	1316.1	44.93	451.7	- 40	0.73
(20-25)	1064.2	24.60	66.7	+ 80	0.21
11A (0-10)	1358.3	27.11	1944.8	-120	1.86
(10-20)	810.5	30.10	1223.5	-140	1.73
(20-28)	185.3	14.59	44.5	+ 60	0.07

-- Methyl- and Total Hg Concentrations in Muscle
Tissue of Fish From Par Pond. Values are Averages of
Replicate Samples

Type	Wet Fish Weight(g.)	Length (CM.)	Sex Condition	Methyl- Hg (as Hg) µg/g=ppm	Total Hg µg/g = ppm	% Methyl to Total Hg
Bass	1359	51.2	Male Good	2.46	2.73	90
Bass	679	47.2	Male Emaciated	2.40	2.31	~100
Bass	906	45.4	Male Good	2.29	2.30	~100
Bass	679	44.8	Male Emaciated	2.40	2.47	97
Bass	566	43.2	Male Emaciated	3.12	2.97	~100
Bass	906	44.5	Male Good	1.77	2.66	67
Bass	679	43.0	Female Good	1.99	2.38	84
Bass	679	43.0	Female Good	2.37	3.50	68
Bass	906	41.0	Female Good	2.29	2.88	80
Bass	793	43.0	Male Good	1.91	1.86	~100
Bass	679	43.0	Female Good	4.18	4.31	97
Bass	113.2	25.5	? Good	0.50	0.74	68
Ave.=2.31				Ave.=2.59	Ave.=87	
Crappie	226	31.5	Female Good	0.85	0.93	91
Crappie	679	37.0	Female Good	1.83	1.82	~100
Crappie	453	32.5	Female Good	0.49	0.70	70
Ave.=1.05				Ave.=1.15	Ave.=87	
Bluegill	92			0.20	0.18	~100
Bluegill	28			0.20	0.15	~100
Bluegill	263			0.24	0.16	~100
Ave.=0.21				Ave.=0.16	Ave.=~100	

Type Fish	Wet Weight(g.)	Length (CM.)	Sex Condition	Methyl- Hg (as Hg) μg/g=ppm	Total Hg μg/g = ppm	% Methyl to Total Hg
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Herring	78			0.30	0.23	~100
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Shad	700			0.19	0.17	~100
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Sucker	447			0.19	0.24	79
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Sucker	146			0.13	0.16	81
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Ave.=0.16 Ave.=0.20 Ave.=80

. Methyl-Hg Concentrations in Gambusia in the In Vivo Methylation Experiments

Hg⁰ Exposed Fish

# of Fish	# of Exposures + # Days in Fresh-water	Wet Weight (grams)	CH ₃ -Hg (as Hg) $\mu\text{g/g}=\text{ppm}$
3	Control	1.98	0.16
1	Control	1.04	0.28
1	4 Exposures	0.63	0.25
3	@ 4 Exposures	2.90	0.14
2	9 Exposures 10 Exposures	1.92	0.17
1	10 Exposures +34 Days in Freshwater	0.94	0.36
1	10 Exposures +23 Days in Freshwater	1.07	0.27

HgCl₂ Exposed Fish

# of Fish	# of Exposures + # Days in Fresh-water	Wet Weight (grams)	CH ₃ -Hg (as Hg) $\mu\text{g/g}=\text{ppm}$
2	Control	1.82	0.16
1	Control	0.96	0.23
2	10 Exposures 14 Days Fresh-water	1.52	0.16
1	10 Exposures +13 Days Fresh-water	0.66	0.50
1	10 Exposures +32 Days Fresh-water	1.04	0.27
2	10 Exposures +33 Days in Fresh-water	1.10	0.31

	<u>Methylmercury</u> <u>X ng/g</u>	<u>Total Hg</u> <u>$\bar{X}$ μg/g</u>	<u>% Methylmercury</u>
Tank I	0.4043	5.8927	0.007
Tank II	0.4126	4.5355	0.009
Control I	0.4012	3.0597	0.013
Control II	0.4850	0.1541	0.315

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