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Uptake, Toxicity, and Trophic Transfer of Mercury in a Coastal Diatom

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The primary mechanisms controlling the accumulation of methylmercury and inorganic mercury in aquatic food chains are not sufficiently understood. Differences in lipid solubility alone cannot account for the predominance of methylmercury in fish because inorganic mercury complexes (e.g., $HgCl_2$), which are not bioaccumulated in fish, are as lipid soluble as their methylmercury analogs (e.g., CH_3HgCl). Mercury concentrations in fish are ultimately determined by methylmercury accumulation at the base of the food chain, which is governed by water chemistry, primarily pH and chloride concentration. Our studies of mercury speciation, toxicity, and phytoplankton uptake demonstrate that passive uptake of uncharged, lipophilic chloride complexes is the principal accumulation route of both methylmercury and inorganic mercury in phytoplankton. The predominance of methylmercury in fish, however, is a consequence of the greater trophic transfer efficiency of methylmercury than inorganic mercury. In particular, methylmercury in phytoplankton, which accumulates in the cell cytoplasm, is assimilated by zooplankton four times more efficiently than inorganic mercury, which is principally bound in phytoplankton membranes. On the basis of these results, we constructed a simple model of mercury accumulation in fish as a function of the overall octanol−water partition coefficient of methylmercury. Our model can explain the variability of mercury concentrations in fish within, but not among, different lake regions.

Introduction

Unlike other trace metals, mercury is "biomagnified" at all levels of aquatic food chains (*1*) making its bioaccumulation from water more akin to that of hydrophobic organic compounds than inorganic metal ions. Mercury is concentrated roughly a million times between water and piscivorous fish (*1, 2*). Mercury levels in fish have been correlated with lakewater chemistry (*3*−*11*) and mercury concentrations in zooplankton (*1, 2*), but we have no mechanistic understanding of the various bioconcentration steps for mercury in aquatic food chains or an explanation for the predominance of methylmercury over inorganic mercury in fish.

Mercury bioaccumulation in aquatic organisms is sometimes attributed to the lipophilic character of organic mercury, while the accumulation of inorganic mercury is typically regarded as of secondary importance. This is paradoxical since it has been shown that the uncharged chloride complexes of both methylmercury (CH_3HgCl) and inorganic mercury ($HgCl_2$) are hydrophobic and readily cross artificial membranes (*12*). If lipid solubility is the explanation for methylmercury bioaccumulation, and if both CH_3HgCl and $HgCl_2$ are equally hydrophobic, then should not both forms of mercury accumulate to the same extent in the aquatic biota? This is contrary to the data which show an overwhelming predominance of methylmercury over inorganic mercury in fish.

Here we examine the mechanisms controlling the accumulation and toxicity of inorganic mercury and methylmercury in phytoplankton and the efficiency of their assimilation by herbivorous zooplankton. In particular, we test the hypothesis that the concentration and form of mercury in aquatic organisms is controlled by the accumulation of hydrophobic, neutrally charged mercury and methylmercury complexes by these organisms. We also test the hypothesis that the intracellular distribution of inorganic mercury and methylmercury in phytoplankton (as determined by their binding to phytoplankton cell constituents) controls their assimilation efficiencies in planktonic herbivores and, hence, their accumulation by fish and other secondary consumers. Finally, we present a bioaccumulation model to predict mercury concentrations in fish from inorganic mercury and methylmercury speciation in the water.

Experimental Section

Partition Coefficients. The overall octanol−water partition coefficients of mercury and methyl mercury (D_{ow} = $[Hg]_{octanol}/[Hg]_{water}$) were measured over a range of pH and chloride concentrations. Extractions were performed with equal volumes (50 mL) of octanol and artificial lake water (Fraquil; *13*) containing 7.4 nM mercuric chloride. The pH was adjusted by adding the appropriate phosphate buffers, and the chloride concentration was determined by the amount of added calcium chloride. After the extraction (equilibration) step, the water layer was separated and analyzed for its mercury or methylmercury concentration. The overall octanol−water partition coefficient (D_{ow}) is related to the coefficients (K_{ow}) of individual mercury and methylmercury species (e.g., $[HgCl_2^0]_{octanol}/[HgCl_2^0]_{water}$) by the expression:

$$D_{ow} = \sum f_i (K_{ow})_i \qquad (1)$$

where f_i is the mole fraction of the total mercury present as the species i. The K_{ow} values of $HgCl_2$ and $Hg(OH)_2$ are equal to the D_{ow} values measured in experiments in which

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TABLE 1

Experimentally Determined and Model-Estimated Overall Octanol−Water Partition Coefficients (D_{ow}) for Inorganic Mercury at Various Chloride Concentrations and pH[a]

pCl	pH	% $HgCl_2$	% $Hg(OH)_2$	% HgOHCl	% other	exp D_{ow}	model D_{ow}
3.3	4.77	95.9	0.2	4.0	0.3	3.3	3.24
3.3	5.52	89.6	2.5	7.5	0.3	2.7	3.07
3.3	6.00	51.4	29.0	19.3	0.3	1.8	1.96
3.3	6.67	5.4	82.9	12.0	0	0.46	0.37
3.3	7.25	9.7	57.9	32.4	0.1	0.84	0.74
4.3	6.72	0.2	98.0	0.8	0	0.05	0.07
5.3	6.71	0	98.5	1.5	0	0.13	0.07

[a] Model: $D_{ow} = f_{HgCl_2}K_{ow\text{-}HgCl_2} + f_{Hg(OH)_2}K_{ow\text{-}Hg(OH)_2} + f_{HgOHCl}K_{ow\text{-}HgOHCl}$. Best fit octanol−water partition coefficients (K_{ow}) for each neutral charge complex: $K_{ow-HgCl_2} = 3.33$; $K_{ow\text{-}Hg(OH)_2} = 1.20$; $K_{ow\text{-}HgOHCl} = 0.05$.

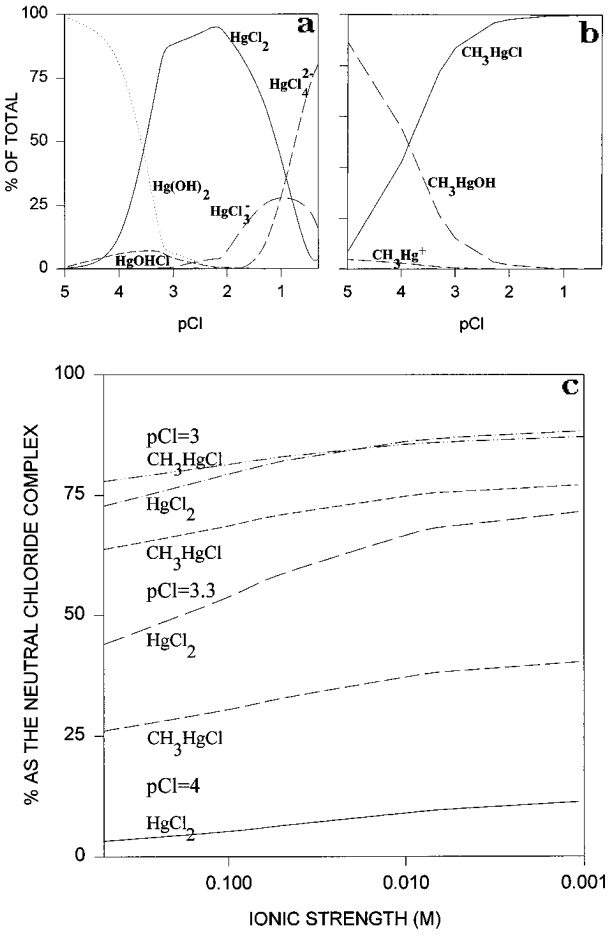


FIGURE 1. Speciation of inorganic (a) and methylmercury (b) at pH 6 as a function of chloride concentration (pCl = −log[Cl⁻]; *I* = [NaCl]) and the percentages of $HgCl_2$ and CH_3HgCl as a function of ionic strength at three chloride concentrations (c).

each of these species is dominant (see Table 1). Octanol−water partition coefficients (K_{ow}) of other mercury and methylmercury species were estimated based on the simultaneous solution of eq 1 for a number of experiments using measured D_{ow} values and the mole fractions (Figure 1) determined with MINEQL (*13*). Activity coefficients used in MINEQL calculations are determined with the Davies equation

$$\ln \gamma_i = -AZ_i^2[I^{1/2}/(1 + I^{1/2}) - bI] \qquad (2)$$

in which γ is the activity coefficient of each species, Z_i is the charge, I is the ionic strength, $A = 1.17$ mol$^{-1/2}$ L$^{1/2}$, and $b = 0.2$. The effect of ionic strength on mercury speciation at three chloride concentrations is shown in Figure 1c.

Cultures. Cultures of the marine diatom *Thalassiosira weissflogii* were maintained in acid-cleaned polycarbonate bottles containing artificial seawater (Aquil) with added vitamins, trace metals, and EDTA (*14*). All cultures were maintained under constant fluorescent light (150 μEinstein m^{-2} s^{-1}; *14*) at 20 °C.

Mercury Uptake Experiments. The accumulation of mercury and methylmercury in *T. weissflogii* was measured in short-term (4-h) uptake experiments over a wide range of pH (range 4−8) and chloride concentrations (10^{-5} to 0.32 M). Microscopic examination of cells showed that the diatom's plasma membranes remained intact under these conditions (i.e., no broken or deformed cells were observed). Membrane integrity at low ionic strength was further confirmed in mercury uptake experiments which, at pH 6.7, showed constant accumulation rates over a wide range of chloride concentrations (Figure 3). Diatoms from mercury-free cultures were collected on 3-μm polycarbonate filters and resuspended into 500 mL of water (Fraquil or artificial seawater) of various pH, pCl (= −log[Cl⁻]), and mercury concentrations. Cell densities in the resuspensions were 1−2 × 10^4 cells mL^{-1} (equivalent to 5−10 μg dry wt mL^{-1}). Mercury was added at the required concentration from 5 μM $HgCl_2$ and CH_3HgCl stocks. Mercury speciation was controlled by changing the chloride concentration and pH (with phosphate buffers) of the experimental media and calculated using MINEQL, taking into account the effect of ionic strength. The decrease in activity coefficients with increasing ionic strength is taken into account by MINEQL. Exposure concentrations ranged from 0.3 pM (no added Hg) to 240 pM (5 nM Hg at 17.5‰) for $HgCl_2$ and up to 150 pM for CH_3HgCl. After 4 h, 5 mL of the water was decanted for a cell count, and the rest was filtered through a 3-μm polycarbonate filter that was rinsed with water of identical ionic strength and stored frozen. The average concentration per cell was determined from cell counts and total Hg per filter. Filtrates were analyzed for pH, for pCl (ion chromatography), and, in the second set of experiments, for mercury concentration. For dimethylmercury, exposure concentrations up to 10 nM were used while experiments with elemental mercury used exposure concentrations of 1 nM or less.

The uptake rates of mercury and methylmercury in *T. weissflogii* were also estimated from long-term experiments. Cultures exposed to inorganic mercury, methylmercury, dimethylmercury, and elemental mercury at various salinities were sampled periodically during exponential growth. Growth rates were calculated as the slopes of semi-log plots of cell density (determined by Coulter Counter) vs time. Relative growth rates were determined as the ratio of experimental growth rates to the growth rates of control cultures grown under the same conditions but in the absence of mercury. This was necessary since it was noted that there was a 10% decrease in growth rate as the salinity was decreased from 35‰ to 10‰. In some instances, phytoplankton uptake led to a decrease in mercury concentration in the medium with time resulting in a decrease in Hg toxicity (i.e., an increase in growth rate) in the latter stages of the batch culture. For these cultures, initial growth rates are reported.

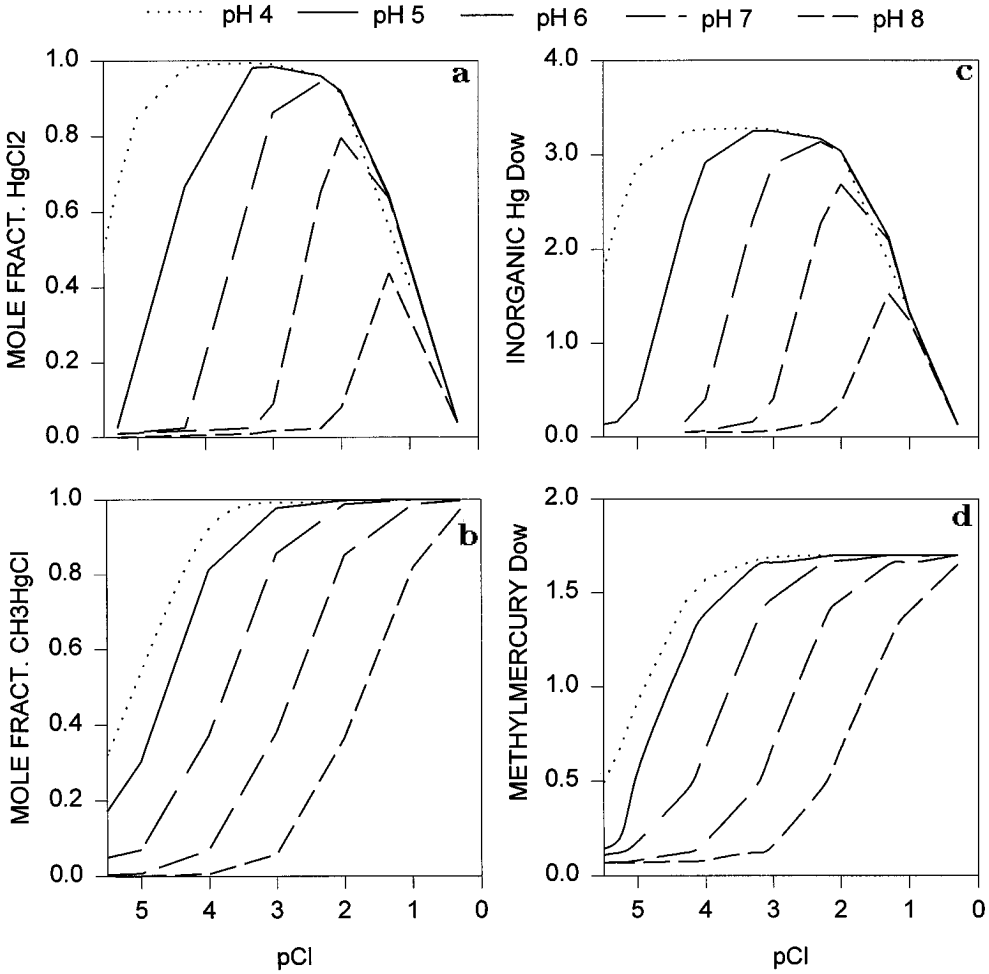


FIGURE 2. Percent $HgCl_2$ (a) and CH_3HgCl (b) and the overall octanol–water partition coefficients (D_{ow}) of inorganic mercury (c) and methylmercury (d) as a function of chloride concentration (pCl = −log[Cl^-]) for pH 4–8. Ionic strength = 0.01 M plus [NaCl].

Cellular Reactivity of Mercury and Methylmercury. The reactivities of mercury species with phytoplankton cell material were determined with 5–10-mL solutions containing 10^7 homogenized cells in phosphate buffer (pH 4.5) containing 10^{-3} M Cl^-. These conditions were chosen so that chloride speciation would dominate for inorganic mercury and methylmercury. Prior to cell sonication, the solution was bubbled with argon to remove oxygen, and 0.5 mL of 10% hydroxylamine solution was added to help maintain reducing conditions, thus preventing the oxidation of thiol groups. In separate experiments (also in phosphate buffer at pH 4.5), cell homogenates were exposed to 5 μM inorganic mercury, 5 μM methylmercury, 0.15 μM Hg^0, and 0.75 μM $(CH_3)_2Hg$. In the combined inorganic mercury and methylmercury treatment, 3.3 μM of each form of the metal was added to the experimental vessel. Aliquots were removed with time after the addition of mercury, filtered using a 0.4-μm cartridge filter and analyzed for mercury content of the solution.

Trophic Transfer. To assess the effect of trophic transfer on mercury accumulation in aquatic food chains, we determined the assimilation efficiencies of each form of mercury in copepods and the cellular fractionation of mercury and methylmercury in the diatoms fed to the copepods.

Adult calanoid copepods (*Acartia tonsa*, *Temora longicornis*, *Centropages* sp.) collected from Massachusetts Bay were maintained in the laboratory at 20 °C in 35‰ glass fiber filtered seawater for 48 h and fed *Thalassiosira weissflogii* at a cell density of 10^4 cells mL^{-1}. For the mercury assimilation experiments, copepods were fed Hg containing diatoms (10^4 cells mL^{-1}) for 5 h, after which time they were allowed to clear their guts for 4 h in the absence of food. The copepods, fecal pellets, and diatoms were sampled at the end of each experiment. The assimilation efficiency (AE) was determined by the inert tracer ratio method (*15*), i.e.

$$AE = [Hg/Si)_{food} - (Hg/Si)_{feces}]/(Hg/Si)_{food} \quad (3)$$

where Si was used as an unassimilated tracer. Mass balance calculations confirmed the results of the tracer method. A portion of the cells used in the assimilation experiments were subjected to cellular fractionation. Five milliliters of cell concentrate was gound for 3 min with a glass fiber filter and a Teflon pestle in a glass grinding tube and then centrifuged at 1000*g* for 10 min to separate cell membranes from cytoplasm. The concentration of methylmercury and inorganic mercury was determined in whole cells and the supernatant (cytoplasmic) fraction of ground cells.

Chemical Analyses. Mercury analyses relied on atomic fluorescence quantification (*16*). For total mercury, water and filter samples were oxidized using bromine monochloride solution, excess oxidant being neutralized with hydroxylamine (*17*). The mercury was then reduced with tin chloride, purged from solution, and trapped on gold (*18*).

TABLE 2

Octanol–Water Partition Coefficients (K_{ow}) of Neutral Charge Mercury Complexes and Compounds and Cadmium Chloride

neutral charge complex	K_{ow}
$HgCl_2$	3.33[a]
$Hg(OH)_2$	0.05[a]
CH_3HgCl	1.7[a,b]
CH_3HgOH	0.07[b]
$(CH_3)_2Hg$	182[c]
Hg^0	4.15[d]
$CdCl_2$	0.21[a]

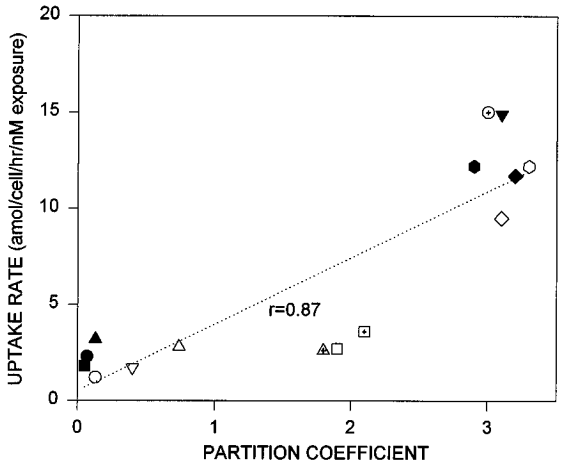
[a] This paper. [b] Ref 20. [c] Ref 21. [d] Ref 4.

To determine reactive mercury, samples were not oxidized prior to $SnCl_2$ reduction. Methylmercury was determined by aqueous-phase ethylation of water samples or alkaline digestates of filters, followed by cryogenic gas chromatography with atomic fluorescence detection (*19*). For Hg^0 and $(CH_3)_2Hg$, the actual concentration in solution was measured by purge-and-trap cryogenic GC techniques (*16*, *19*). All sample manipulation and degassing was done under clean room conditions.

Results

Mercury Speciation and Accumulation. To test the hypothesis that the accumulation of mercury and methylmercury in aquatic microbiota occurs through the passive diffusion of uncharged chloride complexes across the cell membrane, we first examined the chemical speciation and hydrophobicity of mercury under various chemical conditions typical of natural waters. The proportions of inorganic mercury and methylmercury present as various uncharged chloride and hydroxide complexes depend on both chloride concentration and pH. The effect of chloride concentration on mercury speciation at pH 6 is shown in Figure 1. At low chloride concentrations ($<10^{-3.5}$ M), the speciation of inorganic mercury is dominated by three uncharged complexes, $HgCl_2$, HgOHCl, and $Hg(OH)_2$, with $Hg(OH)_2$ being the most abundant. As the chloride concentration increases to about $10^{-2.3}$ M, a value typical of high chloride lake water, the percent of $HgCl_2$ rises to its maximum while concentrations of HgOHCl and $Hg(OH)_2$ decrease to only a few percent of the total inorganic mercury. At higher $[Cl^-]$, the concentration of $HgCl_2$ decreases as that of $HgCl_4^{2-}$ increases (Figure 1a). Methylmercury forms two complexes in solution, CH_3HgOH and CH_3HgCl (Figure 1b). Methylmercury hydroxide is dominant at low chloride concentrations while methylmercury chloride increases sigmoidally with increasing $[Cl^-]$. The stabilities of $HgCl_2$ and CH_3HgCl are both inversely related to ionic strength (Figure 1c). As pH increases from 4 to 8, the maximum percent of $HgCl_2$ shifts to higher $[Cl^-]$ as a result of increasing hydroxide complexation of Hg^{2+} (Figure 2a). Similarly, the sigmoidal increase in percent CH_3HgCl is offset toward higher chloride concentrations at higher pH (Figure 2b).

The overall octanol–water partition coefficients (D_{ow}) of inorganic mercury at various chloride concentrations and pH vary from 0.05 to 3.33 (Table 1). Based on these results and the results of the speciation calculations, we estimated the K_{ow} of $HgCl_2$ as 3.33, that of HgClOH as 1.20, and that of $Hg(OH)_2$ as 0.05 (Table 2). The D_{ow} of methylmercury we measured in seawater (94% methylmercury present as CH_3HgCl) is 1.7 (Table 2). This is in

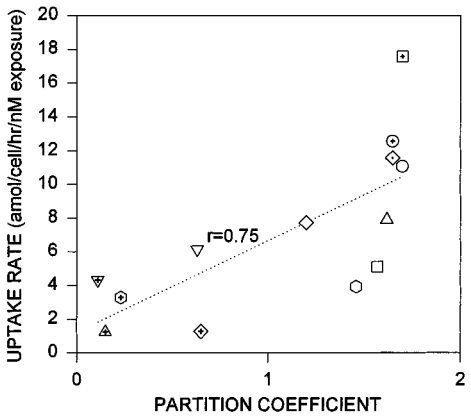


SYMBOL	pH	pCl	I
▲	8.1	0.5	0.7
○	8.1	0.5	0.7
△	7.3	3.3	1.5E-3
▽	6.7	3.3	0.08
●	6.7	4.3	0.08
■	6.7	5.3	0.08
□	6.0	3.3	0.01
▼	5.6	2.2	0.1
◬	5.5	3.8	0.1
◇	5.5	3.3	1.5E-3
⊡	5.3	3.7	0.1
⬡	4.8	3.3	0.1
⬢	4.3	2.9	0.1
◆	4.2	2.4	0.1
⊙	4.0	2.9	0.1

FIGURE 3. Uptake rate of inorganic mercury in the diatom *Thassiosira weissflogii* as a function of the overall inorganic mercury octanol–water partition coefficient (D_{ow}) measured in short-term (4-h) experiments.

accord with the results of Faust (*20*), who using the data of Major et al. (*21*) for the octanol–water partitioning of methylmercury at various Cl^- concentrations and pH estimated the K_{ow} values for CH_3HgCl and CH_3HgOH to be 1.7 and 0.07, respectively. Combining our K_{ow} values and those of Faust (*20*) with MINEQL speciation calculations, we modeled the relationship between pCl, pH and the D_{ow} of inorganic mercury and methylmercury (Figure 2c,d). The similarity between the speciation and D_{ow} curves in Figure 2 demonstrates the importance of the neutrally charged chloride complexes (i.e., $HgCl_2$ and CH_3HgCl) in determining the overall hydrophobicity of mercury in solution. Although hydroxide complexes of both inorganic and methylmercury dominate below a $[Cl^-]$ concentration of about $10^{-3.3}$ M, their K_{ow} values are both more than an order of magnitude lower than those of the $HgCl_2$ and CH_3HgCl, respectively. As a result, while hydroxide complexes acccount for most of the hydrophobicity of inorganic and methylmercury in low $[Cl^-]$ waters, the overall hydrophobicities of mercury and methylmercury in such waters, relative to their values in high $[Cl^-]$ waters, is reduced.

Short-term uptake rates of mercury and methylmercury in the diatom *T. weissflogii* at various Cl^- concentrations and pH are positively correlated with calculated D_{ow} values (Figures 3 and 4). For example, inorganic mercury uptake rates are low in seawater (pH 8.1, pCl = 0.5) and in neutral pH (pH 6.7) freshwaters that have chloride concentrations less than $10^{-3.3}$ M (Figure 3), both conditions at which the D_{ow} of inorganic mercury is also low (Figure 2c). Accumulation rates at pCl = 3.3 increase as pH decreases from 6.7 to 4.8 (Figure 3) following the increase in the proportion of $HgCl_2$ (Figure 2a). The very low ionic strength



SYMBOL	pH	pCl	I
○	8.1	0.5	0.7
□	5.8	2.3	0.1
△	4.5	2.8	0.1
▽	6.6	3.3	0.12
◇	6.2	3.3	1.2E-3
⬡	5.8	3.3	0.04
⊙	4.9	3.3	1.2E-3
⊡	4.3	3.3	0.2
⧋	6.6	4.3	0.12
⧩	6.6	5.3	0.12
◈	5.6	3.9	0.1
⊕	4.2	4.3	0.1
◈	3.1	4.3	0.1

FIGURE 4. Uptake rate of methylmercury in the diatom *Thassiosira weissflogii* as a function of the overall methylmercury octanol−water partition coefficient (D_{ow}) measured in short-term (4-h) experiments.

water used in the uptake experiments conducted at pCl = 3.3 and pH 7.3 resulted in an increase in the mole fraction of $HgCl_2$ (Figure 1c) and, hence, an increase in D_{ow} and the diatom uptake rate (Figure 3). Inorganic mercury accumulation in diatoms is also sensitive to the chloride concentration. For example, at pH 5.5, the decrease in the $HgCl_2$ concentration as chloride concentrations decrease from $10^{-3.3}$ to $10^{-3.8}$ M (Figure 2a) results in a factor of 3 decrease in inorganic mercury uptake rates.

The diatoms exhibit high methylmercury uptake rates in high chloride waters regardless of pH (Figure 4). Low methylmercury uptake rates are only observed at low chloride concentrations (pCl ≥ 3.9) when CH_3HgCl levels are also low (Figure 2b).

The relationships between short-term uptake rates and D_{ow} values for mercury and methylmercury both have positive intercepts (Figures 3 and 4). The corresponding accumulation likely reflects an uptake rate of around 0.5−1.0 amol $cell^{-1}$ h^{-1} nM^{-1} of the ionic forms of inorganic and organic mercury.

Very little mercury was accumulated in diatom cells during 18 h of exposure to 1 nM elemental Hg^0. Cell quotas were less than 20 amol $cell^{-1}$ for the exposed diatoms, equivalent to an uptake rate of less than 1 amol $cell^{-1}$ h^{-1} nM^{-1}. For $(CH_3)_2Hg$, there was no significant change in cell Hg concentration after 16-h exposure to concentrations ranging from 10 to 100 pM. Cell quotas were within a factor of 2 of the control (<10 amol $cell^{-1}$), and the resulting accumulation rates were less than 1 amol $cell^{-1}$ h^{-1} nM^{-1}.

Passive Diffusion and Mercury Toxicity. If mercury and methylmercury accumulate in unicellular algae by diffusion of their uncharged chloride complexes, the toxicity of both forms of the metal in aquatic microbes should depend on the chloride concentration and pH to which the organisms are exposed. We measured the inhibition of

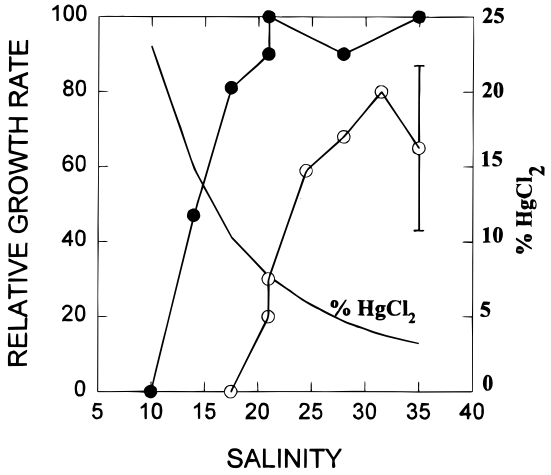


FIGURE 5. Percent maximum growth rate of the diatom *Thassiosira weissflogii* and percent $HgCl_2$ over a salinity range of 10−35‰: (●) 2 nM; (○) 5 nM.

growth in *T. weissflogii* cells exposed to mercury and methylmercury over the range of salinities that are suitable for this euryhaline species. Mercury toxicity in *T. weissflogii* decreases with increasing salinity such that at a salinity of 10‰ (pCl = 0.83) the growth of phytoplankton exposed to 2 nM total Hg is inhibited 100% with respect to controls (no mercury) and not at all above 20‰ (pCl = 0.53; Figure 5). Since the proportion of $HgCl_2$, like toxicity, decreases from 23% to 7.5% between salinities of 10‰ and 20‰, growth rate inhibition in *T. weissflogii* appears to be directly related to the concentration of $HgCl_2$.

One way to test that mercury toxicity in *T. weissflogii* is controlled by the concentration of $HgCl_2$ in the medium rather than by the total or the free ion concentration of mercury is to compare all the growth data as a function of each of these three parameters (Figure 6a−c). The relationships between relative growth rates and total and free mercury concentrations (Figure 6a,b) are poor. There are, for example, total mercury and free mercury ion concentrations at which there is both no apparent toxicity (100% relative growth rate) and complete inhibition of growth. When relative growth rates are plotted as a function of the concentration of $HgCl_2$ (Figure 6c), the data are much tighter and the variability among salinities is similar to that among replicates in each salinity treatment. For example, at a salinity of 14‰, similar growth rates are obtained over about a 2-fold (0.3 log unit) range in $HgCl_2$ concentration, the same spread as observed in all the data of Figure 6c.

Over the salinity range used in the methylmercury toxicity experiments (14−35‰, equivalent to pCl = 0.3−0.7), almost all the methylmercury is present as CH_3HgCl (87−94%; Figure 2b). There is more variability in these data than in those for inorganic mercury, perhaps as a result of lower exposure concentrations (0.01−0.4 nM). Nonetheless a clear relationship between relative growth rates and CH_3HgCl concentration is evident (Figure 6d). Furthermore, 50% growth inhibition occurs at roughly the same concentration of both CH_3HgCl and $HgCl_2$ (Figure 6d).

Not all neutrally charged mercury compounds or complexes cause toxicity at the exposure concentrations tested. For example, we found that 10 nM cysteine, which forms neutrally charged, 1:1 chelates with inorganic mercury, completely alleviates the growth inhibition of *T. weissflogii* caused by 5 nM inorganic mercury. Dimethylmercury, $(CH_3)_2Hg$, a neutrally charged compound that

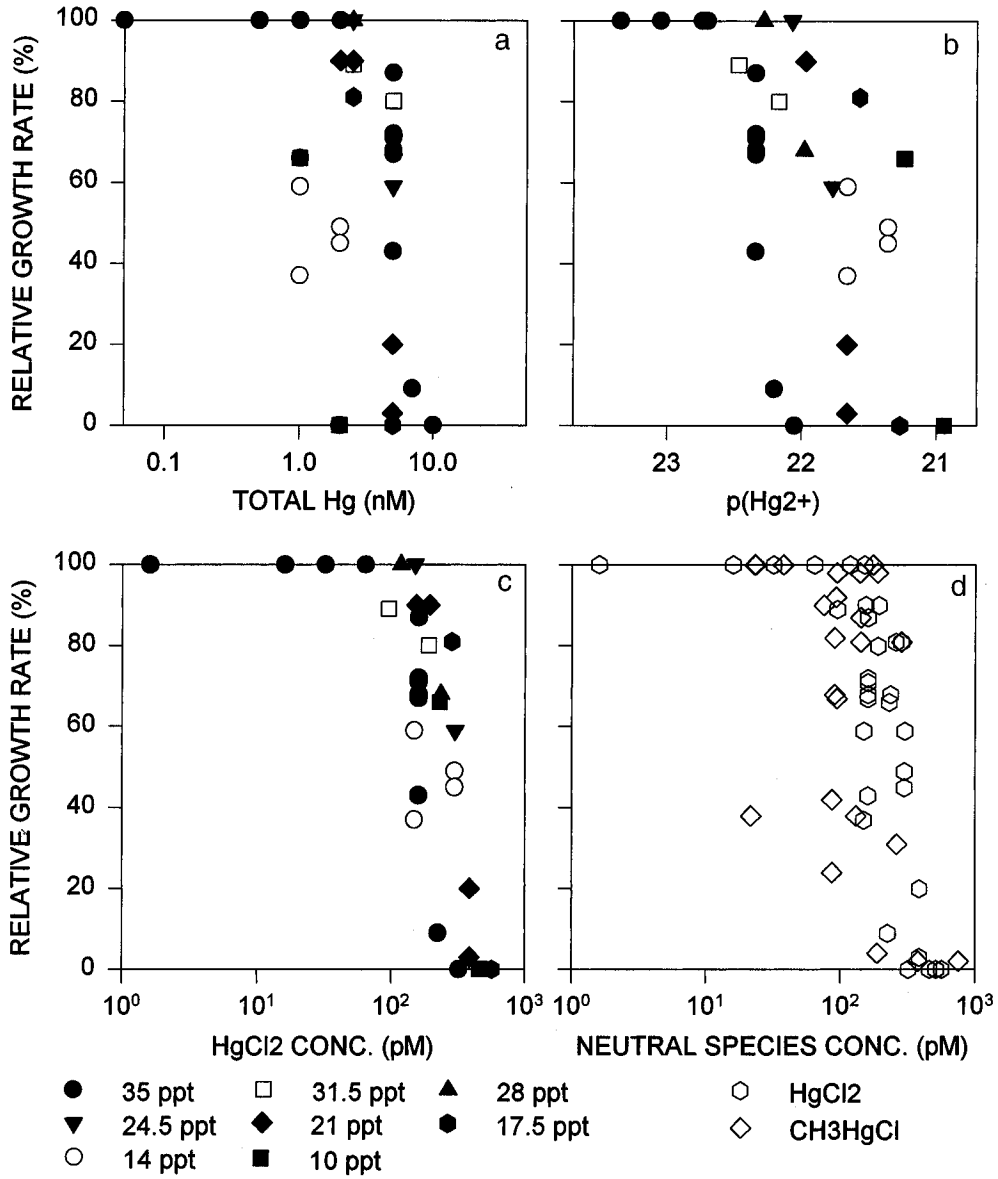


FIGURE 6. Percent maximum growth rate of the diatom *Thassiosira weissflogii* as a function of the concentration of total Hg (a), $pHg^{2+} = -\log[Hg^{2+}]$ (b), $[HgCl_2]$ (c), and both $[HgCl_2]$ and $[CH_3HgCl]$ (d).

is substantially more hydrophobic than CH_3HgCl (K_{ow} = 182 vs 1.7) does not induce toxicity even at concentrations of 10 nM. Similarly, elemental mercury (Hg^0), which is slightly more hydrophobic than $HgCl_2$ (K_{ow} = 4.14 vs 3.33), does not cause toxicity at nM exposure concentrations, well above the toxic concentration of $HgCl_2$. Thus, hydrophobicity is a necessary but not sufficient condition for toxicity. The results from these toxicity experiments are consistent with those from $(CH_3)_2Hg$ and Hg^0 accumulation experiments: clearly, both Hg^0 and $(CH_3)_2Hg$ are neither significantly accumulated by nor toxic to *T. weissflogii*.

We hypothesized that mercury accumulation and toxicity depend on the reactivity of each form of mercury with intracellular ligands in addition to passive diffusion of hydrophobic species across the cell membrane. To test this hypothesis, we exposed broken cells to various mercury species and followed the binding of dissolved mercury with particulate cellular constituents over time. Cells were broken to ensure that membrane transport would not limit the reactivity of dissolved mercury with cellular binding sites. Inorganic mercury was removed rapidly from solution and saturated potential binding sites in 7 min (Figure 7). Methylmercury was removed from solution at a slower rate than inorganic mercury, and the amount of methylmercury bound per cell continued to rise even after 25 min of exposure. When inorganic mercury and methylmercury were added simultaneously to broken cells (3.3 μM of each), the extent of inorganic mercury accumulation decreased slightly from 1.8 to 1.7 fmol $cell^{-1}$, while the amount of methylmercury bound reached a maximum of only 0.42 fmol $cell^{-1}$ (Figure 7). As expected, $(CH_3)_2Hg$ and Hg^0 were not removed from solution to any measurable extent (<0.01 fmol $cell^{-1}$) during the course of the experiment (45 min; Figure 7), indicating their lack of reactivity with cellular material.

Cell Fractionation and Trophic Transfer. A direct relationship between the dissolved and particulate fractionation of an element in phytoplankton and the efficiency of its assimilation by herbivorous zooplankton has been previously shown for both trace elements and major cellular components such as carbon, phosphorus, and sulfur (*22*). Upon feeding Hg-laden *T. wessflogii* to copepods, we

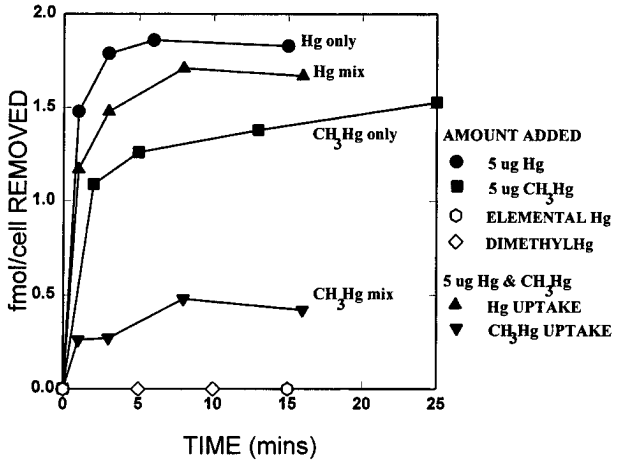


FIGURE 7. Reactivity of mercury species with broken diatom cells (*Thassiosira weissflogii*). Cells were resuspended in phosphate buffer at pH 4.5 containing 10^{-3} M chloride. Prior to breaking the cells by sonication, the solution was bubbled with argon to remove oxygen, and hydroxylamine (final concentration 1−2%) was added to help maintain reducing conditions.

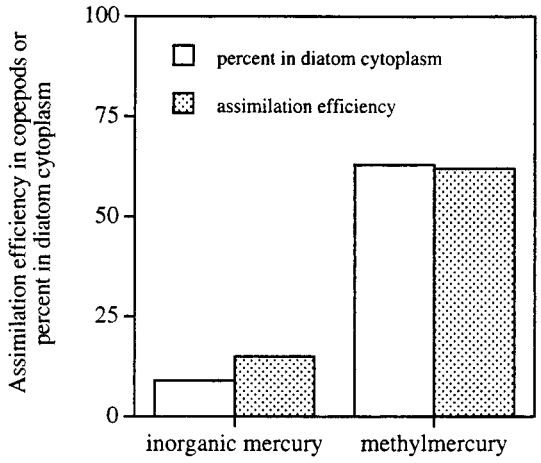


FIGURE 8. Assimilation efficiencies of inorganic mercury and methylmercury in Massachusetts Bay copepods (*Acartia tonsa*, *Temora longicornis*, *Centropages* sp.) fed the diatom *Thassiosira weissflogii*. Also shown are the precentages of inorganic mercury and methylmercury in the cytoplasmic fraction of the diatom food.

observed that methylmercury and inorganic mercury also fit this relationship (Figure 8). For methylmercury, 63% was in the cytoplasmic fraction of the diatoms and 62% was assimilated by the copepods; for inorganic mercury, only 9% was in the diatom cytoplasm and 15% was assimilated. Consistent with our observation that reactivity with particulate cellular material is greater for inorganic mercury than methylmercury, inorganic mercury is concentrated predominantly (91%) in diatom cell membranes while only 37% of accumulated methylmercury is membrane bound (Figure 8). These results show that methylmercury is transferred more efficiently from phytoplankton to herbivores than inorganic mercury and provide an explanation for this difference.

Discussion

Hydrophobicity of Inorganic Mercury and Methylmercury. Mercury differs from the other Group IIb metals, Zn and Cd, in that it tends to form nonpolar complexes. The Hg^{2+} cation, polarized due to the inefficient shielding of the nucleus by the 4f and 5d shells, only forms polar coordination bonds with highly electronegative anions such as fluoride and nitrate. As demonstrated by their low boiling points and their high solubilities in nonpolar solvents, mercury halide (except F) compounds contain essentially covalent Hg−X bonds, while Cd halides are much less covalent in character and Zn halides and chiefly ionic. The covalent nature of $HgCl_2$ results in it having a 16-fold higher K_{ow} than that of $CdCl_2$ (Table 2). Thus, passive uptake by diffusion across lipid cell membranes is likely more important for $HgCl_2$ than for the other Group IIb metals or the transition metals.

We found that the overall octanol−water partition coefficient (D_{ow}) of inorganic mercury is dominated by the fraction present as $HgCl_2$ above a $[Cl^-]$ of $10^{-3.3}$ M (Table 1). Previous investigations, while noting the effect of pH on mercury partitioning, have ignored the importance of mercury speciation. Nonetheless, the D_{ow} values of inorganic mercury previously reported [$D_{ow} = 2.5$ in a 0.1 M chloride solution (*23*) and in a low chloride solution, $D_{ow} = 0.16$ at pH 9.5 and 0.65 at pH 5 (*24*)] are consistent with our results (Figure 2c). The hydrophobicity of methylmercury is dominated by CH_3HgCl above a $[Cl^-]$ of $10^{-3.3}$ M (Table 1). In water bodies that have a $[Cl^-]$ less than $10^{-3.3}$ M (approximately half of all terrestrial waters; *25*), the D_{ow} of inorganic mercury is dominated by $Hg(OH)_2$ and that of methylmercury is dominated by CH_3HgOH.

Mercury Accumulation in Microalgae by Passive Diffusion. Our octanol−water partitioning and phytoplankton uptake data strongly suggest that in waters with $[Cl^-] \geq 10^{-3.3}$ M the intracellular accumulation of inorganic mercury and methylmercury in unicellular algae does indeed occur by passive diffusion of the neutrally charged chloride complexes $HgCl_2$ and CH_3HgCl across the cell's lipid bilayer. This supports the conclusion of Davies (*26*) that the uptake of inorganic mercury in the marine microalgae *Dunaliella tertiolecta* and *Isochrysis galbana* occurs by diffusion. As a further test, we compared the membrane permeabilities and K_{ow} values of $HgCl_2$ and CH_3HgCl with the general relationship between membrane permeabilities and K_{ow} values described by Stein (*27*) for low molecular weight neutrally charged compounds diffusing through red blood cell membranes. This relationship is described by

$$P^* = \frac{K_{ow} D_{mem}}{l} \tag{4}$$

where P^* is the corrected membrane permeability in cm s^{-1}, D_{mem} is the molecular diffusion coefficient in the lipid bilayer in $cm^2\ s^{-1}$, and l is the membrane thickness in cm (*27*). An empirical relationship corrects the permeability coefficient P for the effects of molecular size on diffusion rate through the membrane:

$$\log P^* = \log P + mv \tag{5}$$

where m is a proportionality constant ($m = 0.0546$ mol cm^{-3}) and v is the van der Waals volume (in $cm^3\ mol^{-1}$) of each species.

Membrane permeabilities of $HgCl_2$, $Hg(OH)_2$, CH_3HgCl, and CH_3HgOH were estimated from the following equation:

$$V = 4\pi R^2 PC \tag{6}$$

(where V is the uptake rate in mol $cell^{-1}\ s^{-1}$, R is the cell radius in cm, P is the membrane permeability, and C is the external concentration of mercury in mol cm^{-3}) using short-term uptake rates (Figures 3 and 4) measured at pH and

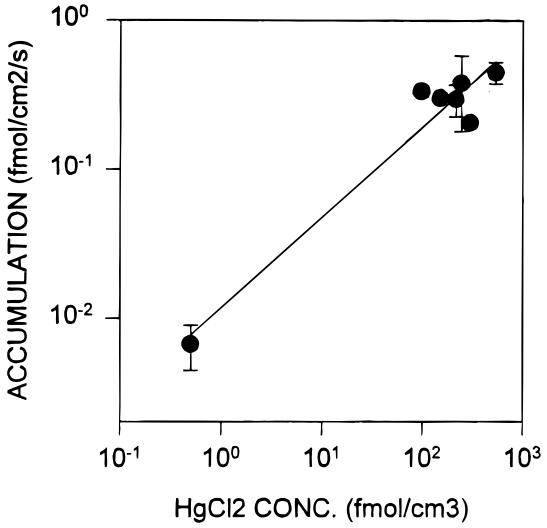


FIGURE 9. Relationship between the long-term, surface area-specific uptake rate ($Q\mu/4\pi R^2$, where Q = mercury quota per cell, μ is the specific growth rate, and R is the cell radius; see eq 7) of inorganic mercury in the diatom *Thassiosira weissflogii* and the concentration of $HgCl_2$. The slope of the line is the cell membrane permeability (P).

chloride concentrations where each of the four species is dominant and a constant diatom cell surface area based on a cell radius of 5.6 μm.

In long-term steady-state experiments, the diffusive mercury accumulation rate is described by

$$V = Q\mu = 4\pi R^2 PC \qquad (7)$$

where Q is the mercury cell quota (mol cell^{-1}) and μ is the specific growth rate (s^{-1}). Here, the membrane permeability is equal to the slope of a plot of cell surface-specific uptake rates ($Q\mu/4\pi R^2$) vs mercury concentrations (Figure 9). The resulting permeability estimates from long- and short-term experiments are comparable, suggesting that the uptake mechanisms involved in mercury and methylmercury accumulation are independent of the physiological state of the organism.

For all four neutrally charged mercury complexes studied, the calculated (and corrected) membrane permeabilities follow closely Stein's relationship with the corresponding K_{ow} values (Figure 10), confirming that similar mechanisms, namely, diffusion of lipid soluble species through the lipid bilayer, control the accumulation of mercury compounds in *T. weissflogii* and of the organic compounds in red blood cells.

The permeabilities measured here for $HgCl_2$ (7.4×10^{-4} cm s^{-1}) and CH_3HgCl (7.2×10^{-4} cm s^{-1}) are both lower than those previously reported; 1.3×10^{-2} cm s^{-1} for $HgCl_2$ (*12*) and 2.6×10^{-3} cm s^{-1} for CH_3HgCl (*24*) both determined using artificial membranes in organic solvents. Our permeability calculations assume that the whole surface of the cell membrane consists of a lipid bilayer that is available for diffusion. The lipid concentration of plasma membranes varies widely, from about 20 to 75% (*28*), and no value has been reported for *T. weissflogii*. Thus, it is likely that our values, which represent the overall permeability of the plasmalemma, underestimate to some extent the actual permeability of the lipid portion of the cell membrane.

The K_{ow} we measured for $CdCl_2$ is about 16-fold lower than that of $HgCl_2$ (Table 2). Gutknecht (*29*) reported a lipid bilayer permeability for $CdCl_2$ of 4.1×10^{-8} cm s^{-1},

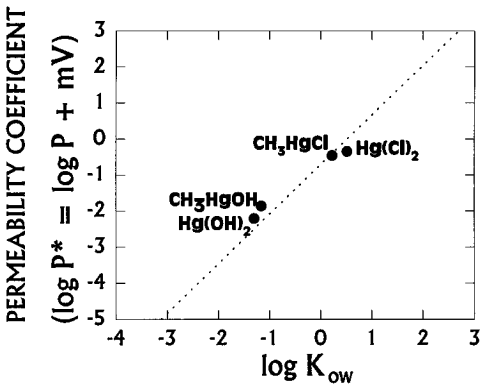


FIGURE 10. Relationship between the molecular size-corrected membrane permeabilities (P^*) of uncharged mercury species and their octanol–water partition coefficients (K_{ow}). The dotted line is Stein's (*25*) empirical relationship for various organic compounds. The van der Waals volumes of the four mercury species are 51 cm^3 mol^{-1} for $HgCl_2$, 43 cm^3 mol^{-1} for $Hg(OH)_2$, 53 cm^3 mol^{-1} for CH_3HgCl, and 49 cm^3 mol^{-1} for CH_3HgOH (*51*).

more than a factor of 10^4 lower than the permeability we measured for $HgCl_2$. Because of the non-covalent nature of the bonds in $CdCl_2$, it is difficult to estimate the molecular volume of $CdCl_2$ and to use eqs 4 and 5 to relate K_{ow} and P. However, using Gutknecht's permeability and our K_{ow} yields a molecular volume for $CdCl_2$ of about 100 cm^3 mol^{-1}, not inconsistent with the volume of $CdCl_2$ (45 cm^3 mol^{-1}) plus two water molecules (65 cm^3 mol^{-1}). Thus our $CdCl_2$ K_{ow} appears to be consistent with Gutknecht's permeability within a factor of 2.

Reactivity of Mercury Species with Intracellular Ligands. While high compared to ionic solutes, the K_{ow} values of inorganic mercury and methylmercury are low relative to those of organic compounds such as polychlorinated biphenyls ($K_{ow} > 10^4$). Yet the phytoplankton bioconcentration factors of the mercury species ($2-5 \times 10^4$; *2*) are comparable to those of polychlorinated biphenyls (ca 10^5; *30*). The high accumulation of mercury compounds despite their low hydrophobicity likely results from significant reaction of these mercury compounds with intracellular ligands. Phinney and Bruland (*31*) showed that the accumulation of uncharged organic Cu, Cd, and Pb complexes depends on the dissociation of the organic metal complexes and the reaction of free metal with intracellular ligands. The results of our reactivity experiments show that both inorganic mercury and methylmercury react quickly and to a significant extent with algal cell ligands. In contrast, the lack of such reaction by dimethylmercury and elemental mercury, which would require an unfavorable redox reaction or formation of a carbanion, clearly explains why they are not accumulated by the algae.

The reactivity experiments also demonstrate that inorganic mercury binds more strongly than methylmercury with cellular particulate binding sites. We may speculate that inorganic mercury is sequestered within the membrane because its rate of reaction with cellular thiol ligands is faster and its extent of reaction is greater than that of methylmercury, which is sequestered in the cytoplasmic fraction.

A number of earlier studies (*32–34*) with red blood cells provide corroborative evidence to the results presented here. Suzuki (*34*) notes that while mercury vapor easily penetrates red cell membranes, retention depends on its oxidation, and various workers have suggested that catalase

is the primary Hg^0 oxidizer in red blood cells. It is possible that oxidation of Hg^0 could occur by a similar mechanism in *T. weissflogii*, but our results suggest that this oxidation is small as there is little accumulation of Hg^0 even at nanomolar exposure concentrations.

On reaction of inorganic mercury and large organomercurials (chloromerodrin, parachloromercuribenzoate, PCMB, and its sulfonated derivative, PCMBS) with hemolyzed red blood cells (*32*), it was found that about 4 fmol $cell^{-1}$ of inorganic mercury was bound and less of the larger compounds (1 fmol $cell^{-1}$ for PCMB and PCMBS; 3 fmol $cell^{-1}$ for chloromerodrin). The total binding capacity of red blood cells for inorganic mercury and large organomercurials (*32*) is of the same order as that estimated for *T. weissflogii* (1.8 fmol $cell^{-1}$). The mercury binding capacity of *T. weissflogii* (which at a volume $V = 900\ \mu m^3$) is within the range estimated for a variety of phytoplankton by Fisher et al. (*35*): 1 fmol $cell^{-1}$ for *T. pseudonana* ($V = 61\ \mu m^3$) to 10 fmol $cell^{-1}$ for *Oscillatoria woronichinii* ($V = 2445\ \mu m^3$). In contrast to other mercury species, PCMBS, which is charged, is not taken up to any significant extent by whole blood cells. The results of the red blood cell studies suggest that diffusion into the membrane is required for mercury binding and that less than 2% of the mercury binding sites are on the cell surface in direct contact with the medium.

Mercury Toxicity. The total inorganic mercury concentration that caused reduced growth in *T. weissflogii* in our experiments at 35‰ salinity is similar to concentrations found to inhibit phytoplankton (eukaryotes and cyanobacteria) growth in previous investigations (*35, 36*). In our experiments, exposure to a total concentration of 10 nM completely stopped growth while cells grew normally at 2 nM total mercury (Figures 5 and 6). For methylmercury, concentrations above about 80 pM caused a reduction in growth rate. These concentrations are 2−3 orders of magnitude higher than concentrations of reactive mercury and methylmercury found in natural waters. Concentrations of reactive mercury are typically less than 10 pM, although total mercury concentrations as high as 100 pM have only been reported for some highly anoxic waters (*37, 38*). For methylmercury, concentrations in oxic waters are generally below 0.5 pM and higher concentrations (>5 pM) have also been measured only in low oxygen or anoxic waters (*37, 39*). Thus, toxicity of inorganic or methylmercury to phytoplankton in natural waters is very unlikely.

For *T. weissflogii*, the toxic concentration range for the neutral chloride complexes of inorganic mercury and methylmercury is of the same order (Figure 6). This is reasonable since both species cross the membrane at similar rates and are highly reactive with cellular constituents. The higher toxicity of methylmercury than inorganic mercury reported by many investigators therefore likely reflects the fact that, under the experimental conditons used, more of the methylmercury is in the form of a neutral permeable complex. For example, only 3.3% of the inorganic mercury is present as $HgCl_2$ in seawater while all of the methylmercury is CH_3HgCl, and thus the toxicity, when expressed on the basis of total concentration, is 30 times higher for methylmercury.

Organic complexation directly reduces toxicity by reducing the concentration of the highly permeable inorganic neutral species. For example, studies by Babich and Stotzky (*40*) showed that the addition of cysteine reduced the toxicity of mercury to bacteria, while Nuzzi (*41*) showed that the addition of glutathione reduced the toxicity of mercury compounds to phytoplankton. Farrell et al. (*42*) indicated that amino acids influence mercury toxicity by controlling the concentration and relative distribution of neutral mercury complexes. Similarly, our results show the ameliorating effect of cysteine addition. In natural waters, the complexation of Hg by DOC would likely also decrease the uptake and toxicity of Hg by phytoplankton in view of the highly charged nature of DOC (10−20 mequiv of titratable acid groups g of C^{-1} at neutral pH; *43*) and of the results obtained with humic acids and copper (*44*).

Food Chain Transfer and Mercury in Fish. The results of the assimilation experiments show that methylmercury is more efficiently transferred than inorganic mercury from diatoms to copepods during zooplankton grazing. Approximately four times as much of the methylmercury is assimilated (62%) compared to inorganic mercury (15%; Figure 8). This difference corresponds roughly to differences in the fraction of the mercury complexes found in the cytoplasm of the diatoms: 63% of the methylmercury and 9% of the inorganic mercury. Similarly, the ratio of methylmercury to inorganic mercury in zooplankton was measured to be about four times greater than that in phytoplankton in field experiments at Little Rock Lake, WI (*2*): a factor of 4.5 greater in one basin and 3.6 in the other, as calculated from the reported bioconcentration factors. In other trophic transfer studies, Fisher et al. (*45*) determined the assimilation efficiency of inorganic mercury in the copepod *Anomalocera patersoni* fed the prymnesiophyte alga *Isochrysis galbana* to be 21%, comparable to what we measured (15%). To our knowledge, there are no published data on methylmercury assimilation efficiencies in copepods.

Although greater trophic transfer of methylmercury than inorganic mercury in aquatic consumers has been frequently observed (*46, 47*), a mechanistic explanation for such observations is lacking. One explanation is provided by our finding that the assimilation efficiencies of methylmercury and inorganic mercury in copepods are determined by mercury accumulation in the cytoplasmic fraction of phytolankton prey and that methylmercury tends to be enriched in this fraction as a result of its lower reactivity for cellular binding sites than inorganic mercury (vide supra; Figure 7). The general applicability of this mechanism among suspension feeding herbivores requires further examination. Slower efflux rates of methylmercury than inorganic mercury, which have been observed in some aquatic consumers (*48*), will also contribute to the enrichment of methylmercury in fish, but very slow efflux rates of both inorganic mercury and methylmercury, as were found in mussels (*49*), will attenuate this effect. It is likely that differential trophic transfer of inorganic mercury and methylmercury at all levels of aquatic food webs, particularly in primary consumers, can account for the predominance of methylmercury in fish.

To demonstrate the importance of mercury accumulation at the base of aquatic food chains to the accumulation of mercury in fish, we have constructed a model to describe the relationship between dissolved mercury and mercury concentrations in fish. Because of the greater efficiency in food chain transfer, in a first approximation we need consider only methylmercury in our model. Methylmercury concentrations in phytoplankton are proportional to the methylmercury concentration in the water and the overall octanol−water partition coefficient (D_{ow}) as determined by chemical parameters. We can therefore estimate a

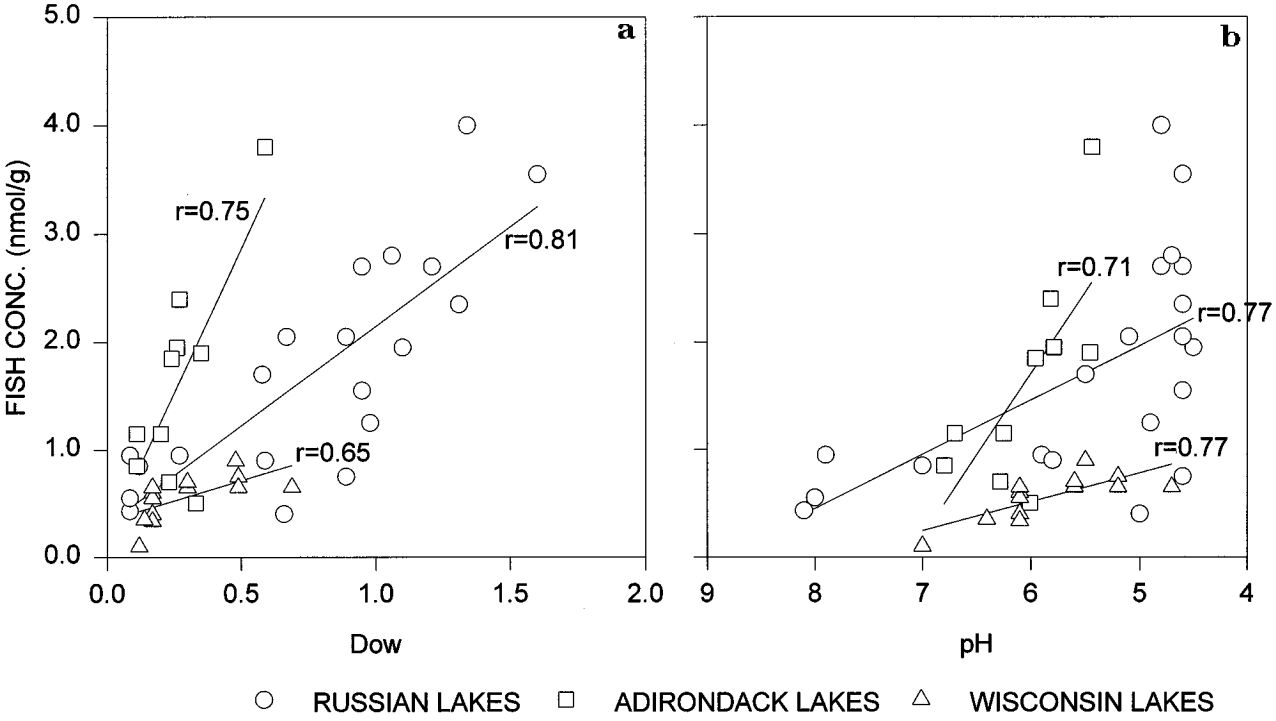


FIGURE 11. Mercury concentrations in fish from lakes in Wisconsin (*45*), the Adirondack Mountains (*10*), and Russia (*6*) as a function of the overall methylmercury octanol–water partition coefficient (D_{ow}; a) and pH (b).

coefficient of proportionality between phytoplankton and lakewater methylmercury concentrations from our laboratory data taking into account the average size of the algae and their growth rate. The concentration of methylmercury in phytoplankton is given by

$$[Hg_{pp}] = \frac{D_{ow}U[CH_3Hg]4\pi R^2}{4/3\pi R^3\rho\cdot\mu} \quad (8)$$

in which $[Hg_{pp}]$ is the methylmercury concentration in phytoplankton (nmol g^{-1}), U is the algal surface area-specific methylmercury uptake rate (nmol μm^{-2} d^{-1} nM^{-1}), $[CH_3Hg]$ is the concentration of methylmercury in the water (nM), R is the phytoplankton cell radius (μm), ρ is the phytoplankton cell density (10^{-12} g μm^{-3}), and μ is the phytoplankton growth rate (d^{-1}). The average uptake rate we measured in *T. weissflogii* (surface area 400 μm^2) is 3.6 $\times 10^{-10}$ nmol μm^{-2} d^{-1} nM^{-1}. Equation 8 therefore reduces to

$$[Hg_{pp}] = 1080D_{ow}\cdot[CH_3Hg]/R\mu \quad (9)$$

The concentration in fish should then be proportional to the product of the phytoplankton concentration and a bioconcentration factor that reflects the average concentration increase between phytoplankton and planktivorous fish (two trophic levels). It is estimated that methylmercury concentrations in planktivorous fish are a factor of 9–16 (*1*, *2*) greater than those in phytoplankton. We therefore used a geometric mean enrichment factor of 12 to relate methylmercury concentrations in phytoplankton and planktivorous fish. The concentration of mercury in fish is then given by

$$[Hg_{fish}] = 1.3 \times 10^4 D_{ow}[CH_3Hg]/R\mu \quad (10)$$

We can test the applicability of eq 10 to natural conditions by comparing mercury concentrations in freshwater fish with overall partition coefficients (D_{ow}) calculated from the pH values and chloride concentrations of the lakes from which the fish were collected. Mercury concentration in fish from lakes in Wisconsin (*50*), the Adirondack Mountains (chloride data were not available for all the lakes; *10*), and Russia (*6*) are plotted in Figure 11 as a function of D_{ow} and pH. Mercury levels in fish from each of these three regions are well correlated with calculated D_{ow} values. The slopes of the three lines in Figure 11a ranged from 0.15 to 1.1, compared with 0.74 for a theoretical lake having an average methylmercury concentration of 0.5 pM and phytoplankton with R = 2.5 μm and μ = 0.7 d^{-1}. The regression lines in Figure 11a for all three lake regions converge at the same positive intercept, apparently reflecting the basal level of mercury in fish (55 ppb) originally accumulated from the water to the first level of the food chain by a mechanism other than passive diffusion of lipophilic species. The corresponding uptake rate of CH_3Hg^+ in *T. weissflogii* necessary to result in a fish concentration of 55 ppb would be ca. 1 amol $cell^{-1}$ h^{-1} nM^{-1}, which is well within the large uncertainty of the positive intercept we observed in the relationship between methylmercury uptake in diatoms and D_{ow} (Figure 4).

According to the results of Figure 11a, methylmercury hydrophobicity as characterized by D_{ow} appears to account for a large portion of the variability of mercury levels in fish within a particular lake region. Hydrophobicity by itself cannot of course account for all the variability in fish mercury concentrations and particularly does not account for differences among various lake regions. Differences among the slopes of the lines for the different lake regions in Figure 11a could result from differences in dissolved methylmercury concentrations, phytoplankton size, and growth rates or other parameters such as food web structure, DOC concentrations, etc. While no information is available on the methylmercury concentrations in the Russian lakes, the total methylmercury concentrations in Wisconsin and

the Adirondacks are similar, and we conclude that differences in methylmercury concentrations are not the primary reason for the higher accumulation in Adirondack lake fish. Fish age is probably not the primary reason either since we selected values for less than 2-year-old fish for the Adirondack and Wisconsin data and the mercury concentration varies only by a factor of 2 between 1-year-old and 3-year-old fish.

From the considerations above, we hypothesize that differences in phytoplankton species composition and/or in food web structure may contribute significantly to the inter-region differences in the relationship between mercury levels in fish and D_{ow}. As is apparent in eq 10, phytoplankton cell size could have an important effect on methylmercury concentrations in fish. As cell radius (R) increases, mercury levels in fish decline such that as R increases from 1 to 12 μm (the range of values found in the three lake regions used in this analysis), the slope of the fish concentration−D_{ow} line decreases from 1.9 to 0.15, assuming constant specific growth rate. Thus, the dominance of larger species in a lake's phytoplankton population results in lower methylmercury accumulation in fish. It has been noted that mercury concentrations are higher in fish from oligotrophic lakes than those in fish from other regions (*1*), and these have been attributed to a lower "biodilution" effect. Since there is a well-known correlation between phytoplankton size and lake productivity (small species being found in more oligotropic waters), we suggest that greater accumulation (per unit mass) of mercury in small than in large phytoplankton may lead to high mercury concentrations in fish from oligotrophic lakes.

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