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In Vivo Mercury Methylation and Demethylation in Freshwater Tilapia Quantified by Mercury Stable Isotopes

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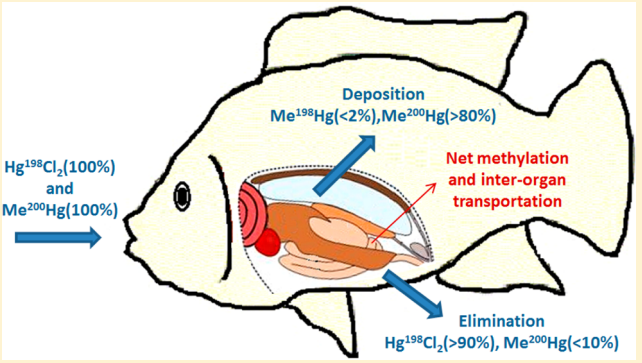
Supporting Information

ABSTRACT: In vivo methylation and demethylation processes were simultaneously investigated in freshwater tilapia after dietary exposure to mercury (^{198}Hg(II) and methyl^{200}Hg). During one month dietary exposure followed by two month depuration, both MeHg and THg increased continuously in muscle tissues but decreased in liver during depuration, indicating the inter-organ transportation of MeHg from liver toward muscle. Direct evidence of in vivo net methylation process in freshwater tilapia was observed. Specifically, 0.67−1.60% of the ingested ^{198}Hg(II) was converted into Me^{198}Hg and deposited in fish muscle at the end of depuration. The methylation potential in terms of methylated fraction was elevated at higher temperature and decreased at higher dosage. However, no direct evidence of MeHg demethylation was observed. In contrast to some previous reports of dose-dependent demethylation, the percentage of MeHg in the liver decreased significantly with increasing THg concentrations, likely due to the faster inter-organ MeHg transportation from liver toward muscle. Our study demonstrates the important role of organ- and species-specific biodynamics in understanding mercury transformation and speciation in fish. The observed in vivo methylation process in tilapia was slow, suggesting that the high %MeHg in fish should be mainly derived from MeHg ingestion instead of in vivo transformation.



INTRODUCTION

Mercury (Hg) is a global metal contaminant widely reported in various ecosystems and wildlife.[1,2] In aquatic environments, the majority of Hg in natural water is present in inorganic form (Hg[II]), while organic mercury (MeHg) contributes less than 5% of the total Hg.[3] In aquatic organisms, however, MeHg is the dominant form, e.g., 80−99% of mercury is detected in fish as MeHg.[4,5] Such intriguing findings might largely be due to the varied biodynamics of MeHg and Hg(II), in that MeHg is more bioavailable than Hg(II) and can be biomagnified through trophic transfer.[6,7] Biodynamic modeling results further demonstrate that MeHg dominates the overall mercury ingestion in fish even though MeHg contributes only a small fraction of total Hg in water.[7] Since the Hg bioaccumulation process is chemical species-specific, [8] any in vivo species transformation (via methylation and demethylation) would directly affect the final biological fate and speciation of Hg in fish, and thus in vivo methylation might contribute to the high proportion of MeHg in fish. Nevertheless, this process has seldom been investigated and still remains unclear.

Methylation and demethylation in sediment and water column have been reported in a variety of aquatic systems and are considered as two important processes regulating the Hg cycling in aquatic environments. It was widely reported that in situ methylation process could occur in sediments and surface water mainly via microbial activities (e.g., sulfur-reducing bacteria),[9−12] while mercury demethylation could occur through physical (photodemethylation[13]), chemical (selenoamino acid aided[14]), and biological processes (bacterial mediated[9]). In contrast, whether in vivo methylation occurs in animals is still a matter of controversy. Pennacchioni et al.[15] found no obvious elevation of MeHg in rainbow trout after long-term exposure to high level of $HgCl_2$, while Rudd et al.[16] found that intestinal contents of freshwater fish could convert ^{203}Hg(II) into $CH_3{}^{203}Hg^+$, and such an in vivo methylation process might be driven by the microbial activities in the gut.[17,18] The reverse process, namely, demethylation of MeHg, is suggested to occur in the liver of mammals[19,20] and water birds[21−23] and in the brain of human and monkey.[24,25] It has been suggested that selenium might be involved in the demethylation process, probably via the formation of bis-(methylmercuric) selenide and finally decomposed into inorganic HgSe(s).[26,27] Besides, several studies have shown that %MeHg decreases as the total amount of mercury (THg) in liver increases, which further suggests that there might be a

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threshold value of THg concentration in liver above which demethylation occurs.[21,22] However, in these earlier studies, there was no abundant evidence of the presence of newly formed MeHg or Hg(II), and the potential influence of inter-organ transportation was not considered.

Enriched stable mercury isotopes have long been widely used to trace in situ Hg transformation studies in sediment and water column[28−30] and has shown its power in evaluating the contribution of different exposure pathways to the overall Hg accumulation in wild-living fish.[31] To our knowledge, this technique has never been applied for studying the in vivo transformation because the concurrent biodynamic process of Hg (such as accumulation, inter-organ transportation, and elimination) in biota makes this entire process rather complicated. In this study, we hypothesize that mercury transformation might be detectable in some specific organs such as muscle and liver. First, MeHg tends to be concentrated in the edible muscle of fish[7] and has been recognized as the dominated mercury form in fish muscle.[32] We assumed that the newly formed MeHg would be finally stored in muscle and, therefore, can be used to estimate the in vivo methylation potential. Second, liver is commonly recognized as an important site of xenobiotic detoxification in wildlife; therefore, it may play a critical role in governing Hg inter-organ transportation. Some recent studies suggested liver as a potential demethylation site,[21,22] but no direct evidence has been observed.

We explored the methylation/demethylation potential in freshwater tilapia (*Oreochromis niloticus*) during a one-month feeding period by labeling the food pellets with mercury stable isotopes ^{198}Hg[II] and Me^{200}Hg. The fish were then depurated for two months, and the Hg biodynamics (accumulation and transportation) and speciation in fish muscle and liver were investigated. For the first time, we quantified the in vivo Hg transformation potential by considering the influence of organ-specific accumulation and transportation process. Two factors that might affect the transformation process were further investigated: (1) water temperature, which is related to the metabolism of fish and probably the microbial activity in fish intestinal content (a potential methylation site [12]), and (2) dose effect, to examine whether in vivo Hg transformation is a dose-dependent process, as suggested in the demethylation process.[21,22]

MATERIALS AND METHODS

Experimental Design and Sample Collection. We investigated the possibility of in vivo mercury transformation process (methylation and demethylation) occurring in fish body and the potential effects of fish metabolism (by controlling the water temperature) and mercury body burden (by adjusting the dietborne mercury dose). Five treatments were set up: 1) control treatment (CT), where fish were fed with clean food (1.05 ng g^{-1} MeHg and 3.9 ng g^{-1} THg) at 25 °C; 2) low concentration treatment (LC), where fish were fed with low-dose mercury contaminated food (0.56 μg g^{-1} MeHg and 1.1 μg g^{-1} THg) at 25 °C; 3) high concentration treatment (HC), where fish were fed with high-dose mercury contaminated food (12.2 μg g^{-1} MeHg and 25.4 μg g^{-1} THg) at 25 °C; 4) low temperature treatment (LT), where fish were fed with moderate-dose mercury contaminated food (1.9 μg g^{-1} MeHg and 4.0 μg g^{-1} THg) at 20 °C; and 5) high temperature treatment (HT), where fish were fed with moderate-dose mercury contaminated food at 30 °C.

The whole experiment was conducted under well controlled laboratory conditions. Tilapia (*Oreochromis niloticus*, 20−30 g wet weight, 2−3 months old) were collected from a local fish pond in Yuen Long, Hong Kong. For each treatment, around 30 individuals of similar sizes were first acclimatized at the corresponding water temperature for one month, in a 120 L ecologically balanced tank (by adding ceramic rings and aquarium beneficial bacteria) with continuous circulation and aeration of dechlorinated tap water. Heating rods and water-cooling machines were used to constantly adjust the water temperature at the required level. The experiments lasted for three months, including a one-month exposure period, followed by a two-month depuration period. During the entire period, the fish were fed with mercury-labeled (during exposure) or clean (during depuration) commercial food pellets at a fixed ingestion rate (0.015 g g^{-1} d^{-1}, based on fish wet weight and food dry weight). To avoid the potential recycling of food released and fish egested mercury, the water medium was half renewed every other day, and the feces of fish was removed every day. At different time points (15, 30, 45, 61, and 90 d), all individuals in each treatment were weighed to determine their growth rates, and five of them were randomly selected for dissection and were stored at −80 °C. The liver and fish muscle of the sampled individuals were freeze-dried and ground to a fine powder for further measurements. All data were thus based on dry weights.

The fish food pellets were labeled with mercury stable isotopes ^{198}Hg(II) and Me^{200}Hg. The isotopes ^{198}HgO (purity >99%) and ^{200}HgO (purity >95%) were obtained from Trace Sciences International. ^{198}Hg(II) was prepared by dissolving ^{198}HgO in trace-metal purified reagent-grade HCl, and Me^{200}Hg was synthesized from ^{200}HgO using methylcobalamin following the Rouleau and Block[33] method and stored in 0.005 M Na_2CO_3 solution. To label the food pellets, 60 g of mercury-free pellets were added in 1 L polypropene beakers and then thoroughly mixed by plastic sticks for 10 min in a fume hood after adding 20 mL of 1:1 mixed (dosage based) mercury isotope solutions (^{198}Hg[II]/ ^{200} MeHg) containing different amounts of each isotope (30 μg for LC treatment, 120 μg for LT and HT treatments, and 600 μg for HC treatment). The food pellets were naturally dried for 48 h in the fume hood and stored at 4 °C before use. Besides, to minimize the potential mercury species transformation on food pellets, the food pellets were prepared every week.

Sample Analysis. In order to investigate the overall biological fate of mercury ingested in fish during the long-term exposure and depuration periods, the total mercury (THg) and methylmercury (MeHg) concentrations in fish muscle and liver were determined in the laboratory of Hong Kong University of Science and Technology. For THg determination, approximately 0.1 g of dried samples was digested by freshly mixed analytical grade HNO_3/H_2SO_4 (3:1, v/v) at 95 °C for 3 h. A suitable volume of the digested sample was taken for further processing, including BrCl oxidation and $NH_2OH^{\bullet}HCl$ reduction. The THg concentrations were analyzed using the single gold trap amalgamation technique by CVAFS (QuickTrace M-8000, USA), with continuous flowing $SnCl_2$ reduction system. The method detection limit (MDL) was 0.18 ng L^{-1}. MeHg in fish muscle and liver were extracted by digesting 0.03−0.05 g of homogenized tissues with 25% KOH in methanol at 65 °C for 3 h. MeHg concentrations in those samples were measured using an automated analytical system (MERX, Brooks Rand), following the United States

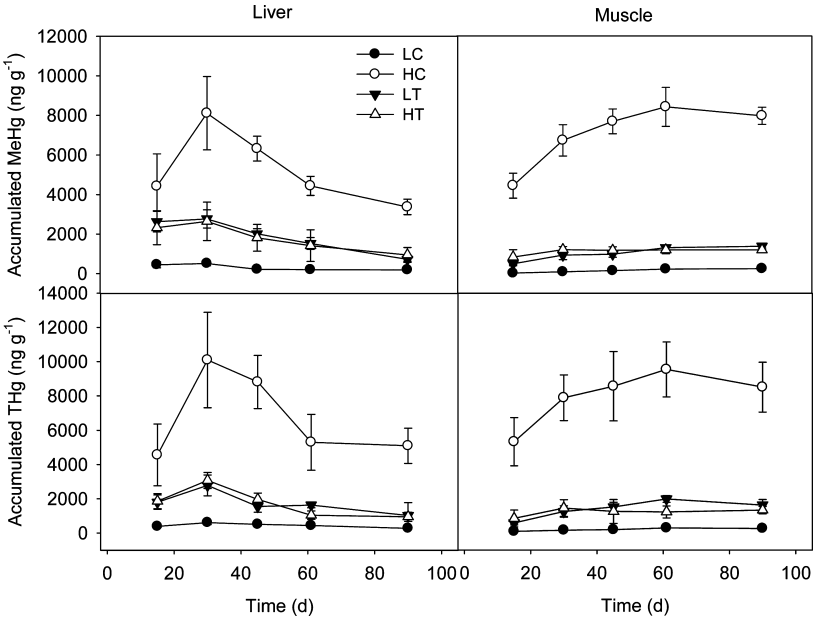

Figure 1. Bioaccumulation patterns of MeHg and THg in muscle and liver of tilapia during the entire experimental period (90 days). Data are mean ± standard deviations (n = 5). LC: low-dose mercury contaminated food +25 °C; HC: high-dose mercury contaminated food +25 °C; LT: moderate-dose mercury contaminated food +20 °C; HT: moderate-dose mercury contaminated food +30 °C.

Environmental Protection Agency (USEPA) Method 1630.[34] The extraction solution was buffered with sodium acetate at pH 4.9 and ethylated by sodium tetraethylborate ($NaBEt_4$) in a 40 mL Teflon line borate glass vial. Sample vials were filled with Milli-Q water and were capped and shaken to ensure the absence of air before analysis. The analytical accuracy of THg and MeHg determination was checked by concurrent digestion and analysis of certified reference material IAEA-142 (mussel homogenate), with a recovery of 95−102% for THg and 91−98% for MeHg.

The Hg isotopes ($T^{198}Hg$, $T^{200}Hg$, $Me^{198}Hg$, and $Me^{200}Hg$) of the liver and muscle samples were determined in the State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, China. Generally, the sample pretreatment followed the USEPA methods 1630 for MeHg[34] and 1631 for THg.[35] For THg isotopic analysis, after overnight BrCl oxidation and subsequent $NH_2OH^{\bullet}HCl$ reduction treatments, a subsample of the digested solution was spiked into the bubbler (containing 50 mL Milli-Q water and 0.06 mL $SnCl_2$ solution) of a preconcentration system which was connected to soda-lime and gold trap. After a 20 min reaction, all BrCl-oxidizable mercury forms were converted to Hg^0 and were further collected onto the gold trap by purging the solution with mercury-free N_2 (200−300 mL/min) for 30 min. Subsequently, the gold trap was connected to Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7700x, Japan) and further determined after thermal desorption (400−500 °C). For MeHg isotopic analysis, the digested samples were first reacted in the preconcentration system (containing 70 mL Milli-Q water, 200 μLHAc-NaAc buffer and 100 μL $NaBEt_4$) for 15 min, and then the converted methylethyl mercury was collected onto a Tenax-TA trap (Alltech Inc.) after 15 min of purging. Trapped mercury species were thermally desorbed and separated using a GC glass column packed with 15% OV-3 Chrom W-AW (DMCS, 80/100 mesh) by heating to 80 °C, performed on a GC-ICP-MS system after thermal desorption (80−120 °C). Mercury standard solution was added in every six samples to correct the potential drift of mechanical response. Quality control for the isotopic mercury determination in samples was conducted using duplicates, method blanks, matrix spikes, and concurrent digestion and analysis of certified reference material IAEA-142 (mussel homogenate). The precision of isotopic ratio measurement was calculated by six consecutive determinations of MeHg (150 pg), resulting in a relative standard deviation (RSD) of 0.57%. Instrumental bias was removed by correcting the ion intensity ratio of enriched $^{198}Hg(II)$ and $Me^{200}Hg$ samples (blank corrected) from the bias measured with natural abundance of Hg.[28] The detection limit for methylation and demethylation measurement were calculated from a modified method reported in a previous study[36] and is given in the Supporting Information (SI-1 and SI-2).

Quantification of in Vivo Methylation Potential. It was difficult to directly quantify the in vivo mercury methylation rate in fish, mainly because (1) the accumulation, elimination, and inter-organ transportation of mercury occur simultaneously with a potential methylation process,[7] and the newly formed MeHg followed a similar biodynamic process and was relocated in different organs; and (2) the exact methylation site in fish is still unknown. Since fish muscle is recognized as the final target organ for MeHg in fish, we considered muscle as the best site to estimate the methylation potential. We used methylated fraction (F_m, %), determined as the fraction of newly deposited MeHg derived from total ingested Hg(II), to evaluate the methylation potential of fish

$$F_m = C_{MeHg}/(IR \times C_f \times t) \quad (1)$$

where the F_m is the methylated fraction (%) from total ingested Hg(II), C_{MeHg} is the newly formed $Me^{198}Hg$ (ng g^{-1}), IR represents the fish daily ingestion rate (g g^{-1} d^{-1}), C_f is the $^{198}Hg(II)$ concentration in ingested food (ng g^{-1}), and t is the dietary exposure time (d). In this study, the dietary ingestion of inorganic mercury ($IR \times C_f \times t$) was controlled by feeding the fish with mixed stable isotope ($^{198}Hg[II]$ and $Me^{200}Hg$) labeled

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food pellet. C_{MeHg} refers to the newly formed $Me^{198}Hg$ at the end of depuration, and C_f represents the sum of original $^{198}Hg(II)$ and spiked $^{198}Hg(II)$ in the food. The impact of potential methylation from original Hg(II) was negligible, since the original Hg concentration was low in the fish (Hg[II]<5 ng g^{-1}) as compared to the accumulation from spiked ^{198}Hg (II) (0.5−13 $\mu g\ g^{-1}$).

RESULTS AND DISCUSSION

Mercury in Liver and Muscle. The mercury accumulation in fish was organ- and chemical species-specific during the three-month experimental period. Generally, MeHg and THg concentrations in fish liver increased during the 30-d exposure period and decreased in the following 60-d depuration period. In contrast, both the THg and MeHg concentrations in fish muscle increased continuously during the entire period (Figure 1), except for the control treatment in which its concentration remained constant at a low level (10.9−28.2 ng g^{-1} of THg, data not shown). At the end of the experiment, THg in fish of the HC treatment reached a very high level, i.e., 8.51 $\mu g\ g^{-1}$ in fish muscle and 5.09 $\mu g\ g^{-1}$ in fish liver, as compared to 287 ng g^{-1} in muscle and 205 ng g^{-1} in liver in the LC treatment. The MeHg and THg accumulation patterns in HT and LT treatments were similar. During the entire experimental period, no negative impact on the fish physiology (e.g., respiration) or behavior (e.g., feeding and swimming activities) was observed after Hg exposure, even in the HC treatment. The ingestion rate was well-maintained at a constant level (0.015 g g^{-1} d^{-1}), as the provided foods were adjusted every other day based on the body weight of fish and were all consumed every day. We observed that the growth rates of fish were temperature- and size-dependent, i.e., higher at lower temperature (20 °C, LT treatment, Figure 2), and decreased as fish aged (in all treatments, Figure 2), which may be related to the varied metabolic rates under different temperatures and life stages.[37]

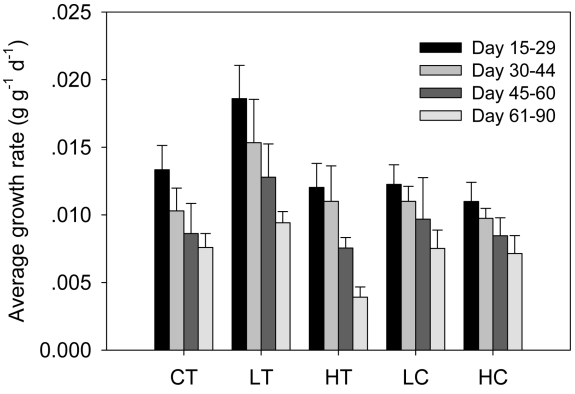

Figure 2. Average growth rate (g g^{-1} d^{-1}) of tilapia during the entire experimental period (90 days). Data are mean ± standard deviations (n = 5). CT: control, clean food +25 °C. Other acronyms are the same as in Figure 1.

At the end of the experiment, MeHg was the major form of mercury in both liver (>80%) and muscle (>90%) in all treatments (Table 1), although only 48−51% of mercury in the ingested food pellet was presented as MeHg (Table 2), indicating that MeHg dominated mercury bioaccumulation in fish tissues. Similarly, in our previous 30-d accumulation experiment,[8] the final concentration of MeHg in the whole fish body was 6.1−18.9 times higher than that of Hg(II) after feeding with radiolabeled food containing similar Hg(II) and MeHg levels in fish diet. This species-specific accumulation can be well explained by a biodynamic model, which considers both the metal uptake (from dissolved phase and dietary assimilation) and elimination processes. Another previous study[7] reported that the assimilation efficiency of MeHg (>90%) was much higher than Hg(II) (<30%) in tilapia after a pulse feeding, and more than 90% of ingested MeHg remained in the fish body after 30 days of depuration, while only around 10% of Hg(II) remained in the fish body. Such high assimilation efficiency and low elimination of MeHg resulted in high bioaccumulation in the fish body. Because the biodynamic process was Hg species-dependent, any newly formed MeHg may follow the same rule of transportation and distribution as the initially ingested MeHg in the fish body.

In Vivo Methylation Process. We observed detectable methylation in all mercury exposed treatments, i.e., newly formed $Me^{198}Hg$ derived from ingested $^{198}Hg(II)$ was found in both the fish liver and muscle. The isotopic ratio of $Me^{198}Hg/Me^{200}Hg$ in fish liver and muscle generally increased with time during the entire experimental period (Figure 3). At the end of depuration, the $Me^{198}Hg/Me^{200}Hg$ isotopic ratio increased by 1.2−2.0 times compared to that on day 15. In contrast, the $Me^{198}Hg/Me^{200}Hg$ in the control treatment was constant (42.1−43.7%), comparable to the natural $^{198}Hg/^{200}Hg$ ratio (0.433), indicating no significant ingestion of additional Hg tracers. Although the impurity of added $Me^{200}Hg$ tracer (purity >95%, containing 8.2‰ of $Me^{198}Hg$) would result in concurrent accumulation of $Me^{198}Hg$, such accompanied ingestion of $Me^{198}Hg$ could not explain the increase of the $Me^{198}Hg/Me^{200}Hg$ isotopic ratio since the ingested $Me^{198}Hg$ would follow the same biodynamic process as $Me^{200}Hg$. Therefore, the increased $Me^{198}Hg/Me^{200}Hg$ isotopic ratio suggested the formation of $Me^{198}Hg$ derived from the ingested $^{198}Hg(II)$. The detection limit of monitoring $^{198}Hg(II)$ methylation after dual tracers addition ($^{198}Hg[II]$ and $Me^{200}Hg$) using GC-ICP-MS were further quantified (calculation shown in SI-1), by considering the impurity of added $Me^{200}Hg$ tracer and original MeHg in fish. The detection limit of newly formed $Me^{198}Hg$ depended on the precision of isotope ratio measurements and the background concentration of $Me^{198}Hg$ (sum of original $Me^{198}Hg$ and accumulated $Me^{198}Hg$ tracer derived from the impurity of ingested $Me^{200}Hg$ tracer). In the present study, an increase of 1.4×10^{-4}−2.3×10^{-4} (corresponding to 0.07−1.14 ng g^{-1} for varied treatments) of the total background MeHg in fish muscle could be precisely detected (SI-1).

In fact, the $Me^{198}Hg$ concentrations in fish liver and muscle measured by GC-ICP-MS were 2−5 times higher than the background $Me^{198}Hg$ concentration in fish muscle, due to the methylation process. We further calculated the newly formed $Me^{198}Hg$ in fish liver and muscle by excluding the background $Me^{198}Hg$ concentration (sum of the concurrent accumulated $Me^{200}Hg$ from spiked tracer and the original $Me^{200}Hg$ in fish). As shown in Figure 4, the newly formed $Me^{198}Hg$ was detected in both liver and muscle in all $^{198}Hg(II)$ exposed treatments. The biodynamics of newly formed $Me^{198}Hg$ showed a similar tendency, i.e., it increased continuously with time in the muscle but decreased during the depuration period in liver. As expected, more $Me^{198}Hg$ was formed when fish ingested a larger amount of $^{198}Hg(II)$, reaching as high as 55.8 ng g^{-1} for HC compared to 2.9 ng g^{-1} for LC at the end of depuration. Under the same feeding condition, the newly formed $Me^{198}Hg$

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Table 1. Mercury Isotopic Ratio ($T^{198}Hg/T^{200}Hg$ and $Me^{198}Hg/Me^{200}Hg$), Mercury Speciation (% MeHg) in Muscle and Liver of Tilapia (*O. niloticus*), and Mercury Concentration Ratio (C_{muscle}/C_{liver}) on Day 15 (First Time Point after Exposure) and Day 90 (End of Depuration)[a]

		%MeHg		C_{muscle}/C_{liver}		$T^{198}Hg/T^{200}Hg$		$Me^{198}Hg/Me^{200}Hg$	
treatment		muscle	liver	MeHg	THg	muscle	liver	muscle	liver
CT	day 15	93.4	85.7	2.46	2.32	0.423	0.434	0.421	0.426
	day 90	92.1	82.9	2.73	2.59	0.432	0.427	0.429	0.437
LC	day 15	98.5	97.8	0.14	0.14	0.024	0.065	0.0088	0.022
	day 90	102.9	92.1	1.52	1.40	0.020	0.131	0.018	0.041
HC	day 15	101.2	90.3	0.88	0.88	0.022	0.112	0.0084	0.021
	day 90	94.7	84.1	1.86	1.67	0.026	0.191	0.012	0.029
LT	day 15	93.2	94.2	0.32	0.32	0.021	0.063	0.010	0.019
	day 90	98.9	87.6	1.59	1.39	0.022	0.140	0.012	0.032
HT	day 15	93.9	93.1	0.46	0.53	0.027	0.057	0.012	0.026
	day 90	92.6	88.2	1.36	1.40	0.025	0.139	0.020	0.047

[a]CT: control, clean food +25 °C; LC: low-dose mercury contaminated food +25 °C; HC: high-dose mercury contaminated food +25 °C; LT: moderate-dose mercury contaminated food +20 °C; HT: moderate-dose mercury contaminated food +30 °C.

Table 2. Calculation of Methylated Fraction in Tilapia after One-Month Exposure to $^{198}Hg(II)$ and $Me^{200}Hg$ Labeled Food Followed by Two-Month Depuration, Based on Newly Formed $Me^{198}Hg$ and Initial Ingested $^{198}Hg(II)$ in Fish Muscle ($C_f \times IR \times t$)[a]

	C_f (ng g^{-1})				
treatment	MeHg	THg	%MeHg	$Me^{198}Hg$ (ng g^{-1})	F_m (%)
CT	1.05 ± 0.32	3.9 ± 0.8	27.1	NA	NA
LC	564 ± 18.2	1097 ± 112	51.4	2.9 ± 0.5	1.23
HC	12175 ± 1736	25384 ± 3154	48.0	55.8 ± 14.9	0.94
LT	1935 ± 174	4025 ± 19.1	48.1	6.3 ± 1.0	0.67
HT	1935 ± 174	4025 ± 19.1	48.1	15.1 ± 2.1	1.60

[a]Acronyms are the same as in Table 1.

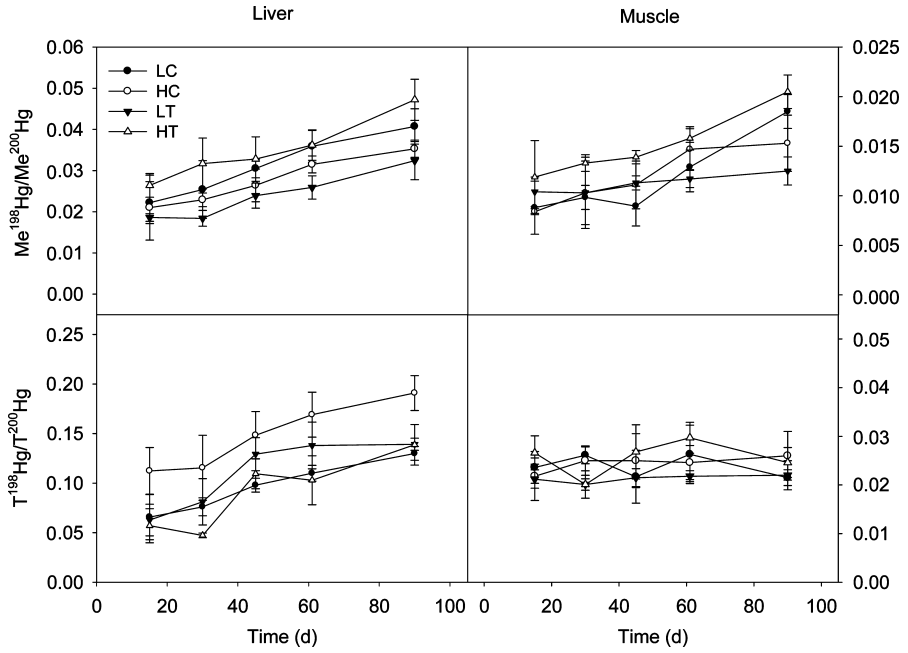

Figure 3. Mercury isotopic ratio ($T^{198}Hg/T^{200}Hg$ and $Me^{198}Hg/Me^{200}Hg$) determined in muscle and liver of tilapia during the entire experimental period. Data and acronyms are the same as in Figure 1.

concentration in fish muscle and liver was higher at 30 °C (3.4−15.1 ng g^{-1}) than at 20 °C (1.3−6.3 ng g^{-1}) during the entire experimental period, indicating higher in vivo methylation potential under warmer conditions. We then further calculated the methylated fraction (F_m) in the fish muscle (MeHg pool for fish) derived from ingested $^{198}Hg(II)$ based on eq 1, to evaluate the in vivo methylation potential (Table 2). After one month dietary exposure and two month depuration, the methylated fraction was generally low for all exposed treatments (<2%). Under our experimental conditions, the

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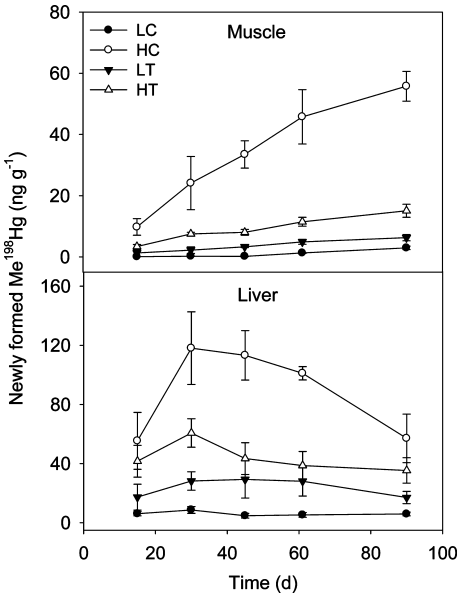

Figure 4. Newly formed $Me^{198}Hg$ in muscle and liver of tilapia, excluding the concurrent accumulation from ingested food. Data and acronyms are the same as in Figure 1.

methylated fraction in tilapia was higher at high temperature (F_m = 1.60%) than at low temperature (F_m = 0.67%), suggesting the important role of temperature in regulating in vivo methylation. Interestingly, it appeared that the methylation potential was lower for the HC treatment (F_m = 0.94%) than the LC treatment (F_m = 1.23%). It might be possible that the in vivo demethylation process (as discussed below) was promoted when fish had a high MeHg body burden, resulting in lower net methylation rate.

There were only a few early studies considering the in vivo methylation process in higher levels of organisms (such as fish, mammals), and those results are generally contradictory. Pennacchioni et al.[15] found no obvious elevation of MeHg in rainbow trout (including liver, muscle, kidney, and intestine) after 94 days of exposure to high levels of waterborne (2 $\mu g\ L^{-1}$) or dietary (0.6 $\mu g\ g^{-1}\ d^{-1}$) $HgCl_2$. In contrast, Rudd et al.[16] observed that the intestinal contents of six freshwater fish species could convert $^{203}Hg(II)$ into $CH_3{}^{203}Hg^+$ and there were large interspecies and intraspecies variations. Rudd et al.[16] suggested that the methylation process in fish was most likely driven by bacterial activity in the intestinal contents. Thus, in the Pennacchioni et al.[15] study, methylation might have been inhibited by the addition of large quantities of bacterial antibiotics.

Based on the above considerations, bacterial antibiotics were not added in the fish culturing tanks in our study. Nevertheless, in situ methylation of $^{198}Hg(II)$ due to fish elimination might occur in the water column and was then accumulated in fish via dissolved uptake. We tried to evaluate the contribution of in situ methylation to the overall newly $Me^{198}Hg$ accumulation in tilapia, based on the Hg(II) elimination rate constant (0.039 d^{-1}), MeHg dissolved uptake rate constant (0.333L $g^{-1}\ d^{-1}$) in tilapia,[7] methylation rate in water column (6% d^{-1}),[38] and bioavailability of $Me^{198}Hg$ to tilapia as controlled by dissolved organic matter.[39] For the HC treatment, which was supposed to have the highest in situ methylation potential among all treatments, the maximal concentration of daily in situ formed $Me^{198}Hg$ would be less than 10 ng L^{-1}, and the predicted dissolved uptake rate of $Me^{198}Hg$ in tilapia would be lower than 0.1 ng $g^{-1}\ d^{-1}$ considering the bioavailable speciation. As a result, the estimated in situ methylation would contribute only less than 10% of the overall newly formed $Me^{198}Hg$ in tilapia. Such contribution would be even lower when considering the adsorption process of Hg and the concurrent demethylation process. In the present study, the calculated 0.67−1.60% of ingested $^{198}Hg(II)$ was converted to $Me^{198}Hg$ and accumulated in fish muscle after one month continuous dietary exposure and two month depuration, which was comparable to the results reported by Rudd et al. (0.05‰−4‰ d^{-1}).[16]

Besides, the organ-specific accumulation patterns of newly formed[198] MeHg suggested fish muscle as the final storage site for MeHg and liver as an important transfer site. Therefore, in vivo methylation might have already occurred in the organ initially exposed to Hg (fish intestine), before whole-body transportation, and intestinal bacteria might play a critical role in governing methylation process.[16] Such a proposed mechanism can explain the higher methylation potential under warmer conditions observed in the current study. Since fish are poikilotherm, and their body temperature is essentially the same as the surrounding environment, microbial activity in their intestine might accelerate at higher temperatures, thus promoting the methylation process. In fact, it has been reported that temperature can significantly influence the bacteria-mediated in situ methylation in freshwater sediments and the water column.[40,41] Based on these observations, we suggest a possible in vivo methylation process in fish: methylation occurs in fish intestine (via bacteria activity) derived from Hg(II), and the newly formed MeHg first reaches the liver and other internal organs and is finally deposited in fish muscle. Nevertheless, such low methylation could not contribute significantly to the overall MeHg accumulation in fish, indicating that the high %MeHg observed in biota should be mainly from direct MeHg ingestion. Although the %MeHg in natural water was very low (<5%), MeHg could be highly accumulated in aquatic biota due to its high bioavailability (i.e., easy to be accumulated from water and food but difficult to be eliminated[7]).

Inter-organ Transportation of Mercury. An inter-organ transportation from liver to muscle was observed in the present study. First, the calculated C_{muscle}/C_{liver} of MeHg generally increased with time for all treatments, directly demonstrating the inter-organ transportation of MeHg from liver to muscle (Table 1). Second, the newly formed $Me^{198}Hg$ concentration decreased during depuration in liver but increased continuously in muscle (Figure 4), indicating transportation of $Me^{198}Hg$ from liver toward muscle. Third, the $T^{198}Hg/T^{200}Hg$ isotopic ratio significantly increased in all mercury exposed treatments during the entire experimental period in fish liver but not in muscle (Figure 3). The increasing isotopic ratio found in fish liver might have resulted from increasing $T^{198}Hg$ or decreasing $T^{200}Hg$. On one hand, since most of the ingested $^{198}Hg(II)$ should be eliminated (>90%) after two months of depuration,[7] only a small portion of the remaining $^{198}Hg(II)$ might be methylated and subsequently transported to fish muscle. As a result, the $T^{198}Hg$ (mainly $^{198}Hg[II]$) in fish liver increased during the first few days of exposure, reached a constant level,[8] and then decreased in the following depuration period through the inter-organ transportation. Therefore, accumulation of $^{198}Hg(II)$ can only contribute to the increase of $T^{198}Hg/T^{200}Hg$ during exposure but not during depuration. If elevated $T^{198}Hg$ was to be seen in the liver, it would be the newly formed

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$Me^{198}Hg$. On the other hand, the sharp decrease of MeHg (>96% was presented as $Me^{200}Hg$) in liver (Figure 1) during the depuration could well explain the decrease of $T^{198}Hg/T^{200}Hg$ isotopic ratio, again supporting the inter-organ transportation. In fact, the inter-organ transportation was also revealed in our previous biokinetic study using a radiotracer technique, i.e., around 80% of ingested MeHg was relocated to fish muscle after 30 days of depuration,[7] suggesting that the fish muscle was the final target organ for MeHg. The inter-organ transportation of MeHg might be explained by its high mobility (binding with sulfhydryl ligands[42]). Glutathione has been reported to be associated with MeHg transportation (via a biliary transport system[43]) and redistribution in other organs including the muscle.[44] In addition, Leaner and Mason[45] experimentally showed that blood can efficiently transfer MeHg to various fish tissues after dietborne exposure.

Inter-organ Transportation As Detoxification Mechanism. It was difficult to find direct evidence of in vivo demethylation mainly for the following reasons. First, the Hg accumulation and elimination processes were organ-specific and inter-organ transportation of MeHg occurred simultaneously. Since the site for demethylation was unknown, demethylation could not be directly calculated from the decrease of $Me^{200}Hg$ concentration. Second, the concentration of newly formed $^{200}Hg(II)$ could not be directly quantified. Finally, the detection limit of monitoring $Me^{200}Hg$ demethylation was not sufficiently low (>1.6% of background MeHg, SI-2) even if the inter-organ transportation can be ignored.

In the present study, we observed an intriguing relationship between liver %MeHg and liver THg levels, i.e, %MeHg decreased with increasing THg concentration. As shown in Figure 3, the $T^{198}Hg/T^{200}Hg$ isotopic ratio in liver was always higher when fish ingested a higher dose of Hg since day 15 (11% for HC and 6.5% for LC), while temperature showed no effect on this value (5.7% for LT and 6.2% for HT). If the small portion of converted Hg (via methylation or demethylation) was ignored, then the $T^{198}Hg/T^{200}Hg$ ratio could reflect the Hg(II)/MeHg value since fish ingested $^{198}Hg(II)$ and $Me^{200}Hg$ labeled food. Therefore, the high $T^{198}Hg/T^{200}Hg$ isotopic ratio of HC indicated low %MeHg when fish received a high dose Hg. In fact, it has been suggested that the high percentage of inorganic Hg observed in liver of marine mammals and waterbirds,[19,21] especially when THg is high, might be derived from MeHg demethylation, and there might be a threshold value of THg concentrations in liver for demethylation.[21,22] Similarly, the calculated %MeHg in our study was significantly lower for HC (90%) than LC (97%) at day 15 (Table 1), consistent with the dose−response threshold observations.[21,22] However, our study revealed that the variation of %MeHg between HC and LC was not likely caused by the elevated demethylation process as suggested in those previous studies, because Hg species transformation does not affect the THg concentration and therefore cannot explain the difference in $T^{198}Hg/T^{200}Hg$ isotopic ratio. Instead, the dose-dependent Hg inter-organ transportation seems a more reasonable explanation. As shown in Table 1, the determined C_{muscle}/C_{liver} of THg and MeHg were higher for HC (0.88) than LC (0.14) at day 15, demonstrating a higher transportation rate of MeHg from liver toward muscle in the case of high-dose Hg exposed fish. Based on these findings, we suggest that the low %MeHg in liver might be mainly due to the MeHg inter-organ transportation toward muscle rather than demethylation, and such a transportation process can be elevated when the fish receives high-dose Hg exposure.

Due to the great impact of inter-organ transportation, we could not directly quantify the demethylation from the decrease of $Me^{200}Hg$ concentration. Nevertheless, evidence of indirect detoxification was observed in our study. The determined net methylated fraction of HC (F_m = 0.94%) was lower than that of LC (F_m = 1.23%), which was possibly due to the promoted concurrent demethylation of newly formed $Me^{198}Hg$ at high Hg concentration. In previous studies, the interaction of mercury and selenium has been well-documented as toxicological antagonism and Se has been proposed to play an important role in MeHg demethylation in liver.[26,46] In a new reported chemical demethylation pathway, selenoamino acid can initiate the demethylation reaction via formation of $(CH_3Hg)_2Se$ and finally is decomposed into HgSe(s).[27]

To conclude, we found that in vivo mercury species transformation potential was low in freshwater tilapia, i.e., 0.67−1.60% of ingested $^{198}Hg(II)$ was converted into $Me^{198}Hg$ and deposited in fish muscle after one-month dietary Hg exposure followed by two-month depuration, while no direct evidence of demethylation was observed. Inter-organ transportation was clearly revealed, providing a possible explanation for the organ-specific Hg speciation in fish tissues, i.e., the high %MeHg in muscle and relatively lower %MeHg in liver were likely due to MeHg transportation from liver toward muscle. Moreover, our results supported the notion that the high %MeHg observed in fish was mainly derived from direct MeHg bioaccumulation instead of in vivo transformation. Since diet is the major pathway for MeHg accumulation in fish,[7] reducing MeHg level in fish diet would be the key solution in minimizing Hg contamination in the fishery industry.

■ ASSOCIATED CONTENT

Supporting Information

Calculation of the detection limit for newly formed $Me^{198}Hg$; calculation of the detection limit for the decrease of added $Me^{200}Hg$. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Fitzgerald, W. F.; Lamborg, C. H.; Hammerschmidt, C. R. Marine biogeochemical cycling of mercury. *Chem. Rev.* **2007**, *107*, 641−662.

(2) Wang, W.-X. Biodynamic understanding of mercury accumulation in marine and freshwater fish. *Adv. Environ. Res.* **2012**, *1*, 15−35.

(3) Watras, C. J.; Back, R. C.; Halvorsen, S.; Hudson, R. J. M.; Morrison, K. A.; Wente, S. P. Bioaccumulation of mercury in pelagic freshwater food webs. *Sci. Total Environ.* **1998**, *219*, 183−208.

(4) Grieb, T. M.; Driscoll, C. T.; Gloss, S. P.; Schofield, C. L.; Bowie, G. L.; Porcella, D. B. Factors affecting mercury accumulation in fish in upper Michigan peninsula. *Environ. Toxicol. Chem.* **1990**, *9*, 919−930.
(5) Southworth, G. R.; Turner, R. R.; Peterson, M. J.; Bogle, M. A. Form of mercury in stream fish exposed to high concentrations of dissolved inorganic mercury. *Chemosphere* **1995**, *30*, 779−787.
(6) Wang, W.-X.; Wong, R. S. K. Bioaccumulation kinetics and exposure pathways of inorganic mercury and methylmercury in a marine fish, the sweetlips *Plectorhinchus gibbosus*. *Mar. Ecol.: Prog. Ser.* **2003**, *261*, 257−268.
(7) Wang, R.; Wong, M.-H.; Wang, W.-X. Mercury exposure in the freshwater tilapia *Oreochromis niloticus*. *Environ. Pollut.* **2010**, *158*, 2694−2701.
(8) Wang, R.; Wang, W.-X. Contrasting mercury accumulation patterns in tilapia (*Oreochromis niloticus*) and implications on somatic growth dilution. *Aquat. Toxicol.* **2012**, *114*−*115*, 23−30.
(9) Pak, K.-R.; Bartha, R. Mercury methylation and demethylation in anoxic lake sediments and by strictly anaerobic bacteria. *Appl. Environ. Microbiol.* **1998**, *64*, 1013−1017.
(10) Monperrus, M.; Tessier, E.; Amouroux, D.; Leynaert, A.; Huonnic, P.; Donard, O. F. X. Mercury methylation, demethylation and reduction rates in coastal and marine surface waters of the Mediterranean Sea. *Mar. Chem.* **2007**, *107*, 49−63.
(11) Jensen, S.; Jernelöv, A. Biological methylation of mercury in aquatic organisms. *Nature* **1969**, *223*, 753−754.
(12) Lehnherr, I.; St. Louis, V. L. Importance of ultraviolet radiation in the photodemethylation of methylmercury in freshwater ecosystems. *Environ. Sci. Technol.* **2009**, *43*, 5692−5698.
(13) Lehnherr, I.; St. Louis, V. L. Importance of ultraviolet radiation in the photodemethylation of methylmercury in freshwater ecosystems. *Environ. Sci. Technol.* **2009**, *43*, 5692−5698.
(14) Mohammad, A. K. K.; Wang, F. Chemical demethylation of methylmercury by selenoamino acids. *Chem. Res. Toxicol.* **2010**, *23*, 1202−1206.
(15) Pennacchioni, A.; Marchetti, R.; Gaggion, G. F. Inability of fish to methylate mercuric chloride in vivo. *J. Environ. Qual.* **1976**, *5*, 451−454.
(16) Rudd, J. W. M.; Furutani, A.; Turner, M. A. Mercury methylation by fish intestinal contents. *Appl. Environ. Microbiol.* **1980**, *40*, 777−782.
(17) Clarkson, T. W.; Vyas, J. B.; Ballatori, N. Mechanisms of mercury disposition in the body. *Am. J. Ind. Med.* **2007**, *50*, 757−764.
(18) Rowland, I. R. Interactions of the gut microflora and the host in toxicology. *Toxicol. Pathol.* **1988**, *16*, 147−153.
(19) Palmisano, F.; Cardellicchio, N.; Zambonin, P. G. Speciation of mercury in dolphin liver: a two-stage mechanism for the demethylation accumulation process and role of selenium. *Mar. Environ. Res.* **1995**, *40*, 109−121.
(20) Wagemann, R.; Trebacz, E.; Boila, G.; Lockhart, W. L. Mercury species in the liver of ringed seals. *Sci. Total Environ.* **2000**, *261*, 21−32.
(21) Collin, A. E.; Joshua, T. A.; Julie, Y.; Terrence, L. A. Mercury demethylation in water bird livers: dose-response thresholds and differences among species. *Environ. Toxicol. Chem.* **2009**, *28*, 568−577.
(22) Henny, C. J.; Hill, E. F.; Hoffman, D. J.; Spalding, M. G.; Grove, R. A. Nineteenth century mercury: Hazard to wading birds and cormorants of the Carson River, Nevada. *Ecotoxicology* **2002**, *11*, 213−231.
(23) Dock, L.; Rissanen, R.-L.; Vahter, M. Demethylation and placental transfer of methyl mercury in the pregnant hamster. *Toxicology* **1994**, *94*, 131−142.
(24) Takeuchi, T.; Eto, K.; Hidehiro, T. Mercury levels and histochemical distribution in a human brain with Minamata disease following a long-term clinical course of twenty-six years. *Neurotoxicology* **1989**, *10*, 651−658.
(25) Vahter, M. E.; Mottet, N. K.; Friberg, L. T.; Lind, S. B.; Charleston, J. S.; Burbacher, , T.M. Demethylation of methyl mercury in different brain sites of Macaca-fascicularis monkeys during long-term subclinical methyl mercury exposure. *Toxicol. Appl. Pharmacol.* **1995**, *134*, 273−284.
(26) Khan, M. A. K.; Wang, F. Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism. *Environ. Toxicol. Chem.* **2009**, *28*, 1567−1577.
(27) Khan, M. A. K.; Wang, F. Chemical demethylation of methylmercury by selenoamino acids. *Chem. Res. Toxicol.* **2010**, *23*, 1202−1206.
(28) Hintelmann, H.; Evans, R. D.; Villeneuve, Y. Measurement of mercury methylation in sediments by using enriched stable mercury isotopes combined with methylmercury determination by gas chromatography-inductively coupled plasma mass spectrometry. *J. Anal. At. Spectrom.* **1995**, *10*, 619−624.
(29) Avramescu, M.-L.; Yumvihoze, E.; Hintelmann, H.; Ridal, J.; Fortin, D.; Lean, D. R. S. Biogeochemical factors influencing net mercury methylation in contaminated freshwater sediments from the St. Lawrence River in Cornwall, Ontario, Canada. *Sci. Total Environ.* **2011**, *409*, 968−978.
(30) Monperrus, M.; Tessier, E.; Amouroux, D.; Leynaert, A.; Huonnic, P.; Donard, O. F. X. Mercury methylation, demethylation and reduction rates in coastal and marine surface waters of the Mediterranean Sea. *Mar. Chem.* **2007**, *107*, 49−63.
(31) Hrenchuk, L. E.; Blanchfield, P. J.; Paterson, M. J.; Hintelmann, H. H. Dietary and waterborne mercury accumulation by yellow perch: A field experiment. *Environ. Sci. Technol.* **2012**, *46*, 509−516.
(32) Bloom, N. S. On the chemical form of mercury in edible fish and marine invertebrate tissue. *Can. J. Fish. Aquat. Sci.* **1992**, *49*, 1010−1017.
(33) Rouleau, C.; Block, M. Fast and high-yield synthesis of radioactive $CH_3{}^{203}Hg(II)$. *Appl. Organomet. Chem.* **1997**, *11* (9), 751−753.
(34) USEPA, 2001. Method 1630: Methyl mercury in water by distillation, aqueous ethylation, purge and trap, and CVAFS; EPA-821-R-01-020; Office of Water, U.S. Environmental Protection Agency: Washington, DC, pp 1−41.
(35) USEPA, 2002. Method 1631, Revision E: Mercury in water by oxidation, purge and trap, and Cold Vapor Atomic Fluorescence Spectrometry; EPA-821-R-02-019; Office of Water, U.S. Environmental Protection Agency: Washington, DC, pp 1−46.
(36) Hintelmann, H.; Evans, R. D. Application of stable isotopes in environmental tracer studies−Measurement of monomethylmercury (CH_3Hg^+) by isotope dilution ICP-MS and detection of species transformation. *Fresen. J. Anal. Chem.* **1997**, *358*, 378−385.
(37) Otterlei, E.; Nyhammer, G.; Folkvord, A.; Stefansson, S. O. Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. *Can. J. Fish. Aquat. Sci.* **1999**, *56*, 2099−2111.
(38) Eckley, C. S.; Hintelmann, H. Determination of mercury methylation potentials in the water column of lakes across Canada. *Sci. Total Environ.* **2006**, *368*, 111−125.
(39) Wang, R.; Wang, W.-X. Importance of speciation in understanding mercury accumulation in tilapia controlled by salinity and dissolved organic matter. *Environ. Sci. Technol.* **2010**, *44*, 7964−7969.
(40) Korthals, E. T.; Winfrey, M. R. Seasonal and spatial variations in mercury methylation and demethylation in an oligotrophic lake. *Appl. Environ. Microbiol.* **1987**, *53*, 2397−2404.
(41) Gilmour, C. C.; Henry, E. A.; Mitchell, R. Sulfate stimulation of mercury methylation in freshwater sediments. *Environ. Sci. Technol.* **1992**, *26*, 2281−2287.
(42) Rabenstein, D. L.; Evans, D. A. The mobility of methylmercury in biological systems. *Bioinorg. Chem.* **1978**, *8*, 107−114.
(43) Ballatori, N.; Clarkson, T. W. Biliary transport of glutathione and methylmercury. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **1983**, *244*, G435−441.
(44) Richardson, R. J.; Murphy, S. D. Effect of glutathione depletion on tissue deposition of methylmercury in rats. *Toxicol. Appl. Pharmacol.* **1975**, *31*, 505−519.

(45) Leaner, J. J.; Mason, R. P. Methylmercury uptake and distribution kinetics in sheepshead minnows, *Cyprinidon variegatus*, after exposure to CH_3Hg-spiked food. *Environ. Toxicol. Chem.* **2004**, *23*, 2138−2146.
(46) Sumino, K.; Yamamoto, R.; Kitamura, S. A role of selenium against methylmercury toxicity. *Nature* **1977**, *268*, 73−74.