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ORGAN-SPECIFIC ACCUMULATION, TRANSPORTATION, AND ELIMINATION OF METHYLMERCURY AND INORGANIC MERCURY IN A LOW Hg ACCUMULATING FISH

XIAOYAN PENG,† FENGJIE LIU,† and WEN-XIONG WANG*†‡
†Division of Life Science, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China
‡State Key Laboratory of Environmental Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guiyang, China

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Abstract: Low mercury (Hg) concentrations down to several nanograms Hg per gram of wet tissue are documented in certain fish species such as herbivorous fish, and the underlying mechanisms remain speculative. In the present study, bioaccumulation and depuration patterns of inorganic Hg(II) and methylmercury (MeHg) in a herbivorous rabbitfish *Siganus canaliculatus* were investigated at organ and subcellular levels following waterborne or dietary exposures. The results showed that the efflux rate constants of Hg(II) and MeHg were $0.104\,d^{-1}$ and $0.024\,d^{-1}$, respectively, and are probably the highest rate constants recorded in fish thus far. The dietary MeHg assimilation efficiency (68%) was much lower than those in other fish species (~90%). The predominant distribution of MeHg in fish muscle was attributable to negligible elimination of MeHg from muscle (< 0) and efficient elimination of MeHg from gills ($0.12\,d^{-1}$), liver ($0.17\,d^{-1}$), and intestine ($0.20\,d^{-1}$), as well as efficient transportation of MeHg from other organs into muscle. In contrast, Hg(II) was much more slowly distributed into muscle but was efficiently eliminated by the intestine ($0.13\,d^{-1}$). Subcellular distribution indicated that some specific membrane proteins in muscle were the primary binding pools for MeHg, and both metallothionein-like proteins and Hg-rich granules were the important components in eliminating both MeHg and Hg(II). Overall, the present study's results suggest that the low tissue Hg concentration in the rabbitfish was partly explained by its unique biokinetics. *Environ Toxicol Chem* 2016;35:2074–2083. © 2016 SETAC

Keywords: Hg MeHg Fish Biokinetics Subcellular distribution

INTRODUCTION

Mercury (Hg) is among the most hazardous metals to human and ecosystem health, and its risk has been recognized for decades [1–3]. Fish consumption is a major source of human Hg exposure, and considerable efforts have been made in the measurements of concentration and speciation of Hg in fish [4–10]. A wide range of Hg concentration is documented in different fish species, and notably very low Hg (0.5–34 ng/g wet tissue) is determined in certain species such as herbivorous rabbitfish [11–12]. However, the underlying mechanisms for the low Hg bioaccumulation are unknown.

Most Hg in fish, including herbivorous species, is in the form of MeHg [13], although MeHg is typically less than 10% of total Hg in all environmental matrices [14]. Dietary exposure is a primary exposure pathway in fish [12]. Compared with inorganic Hg, MeHg is effectively assimilated and difficult to be depurated out of the fish body [12]. Muscle is a primary MeHg storage site [13]; however, it is unclear why MeHg is primarily stored here.

Fish must detoxify accumulated Hg, and the toxic metal can perturb biological functions of important proteins by binding their functional groups [15,16]. Induction of metallothionein-like proteins [17–20] and forming insoluble Hg–Se granules [21–23] are the 2 documented mechanisms to counteract the toxic effects elicited by Hg. Subcellular metal distribution can shed light on metal elimination other than its toxicity. For example, Cu-rich granules in amphipod crustaceans [24] and Cd-phytochelatin complex [25,26] in marine algae are important exporting forms. The synthesis of metallothionein-like protein and its remobilizing Cd from other subcellular compartments effectively reduces its elimination [27,28]. However, at present, little information is available on potential relationships between subcellular Hg distribution and its elimination.

In the present study, uptake, assimilation, and elimination of MeHg and inorganic Hg(II) was quantified in 1 rabbitfish *Siganus canaliculatus* (also known as *Siganus oramin*) to investigate the underlying mechanisms for the low Hg bioaccumulation. In our earlier studies, total Hg concentrations (wet wt) in muscle tissue of rabbitfish (*S. canaliculatus* and *Siganus fuscescens*) collected from different regions of Southern China were 10 ng/g to 30 ng/g from the Fujian Province [10], 4 ng/g to 21 ng/g from the Pearl River Estuary [11], 5 ng/g to 34 ng/g from Dapeng Bay [11], and 0.5 ng/g to 4 ng/g (for MeHg only) from Hong Kong coastal waters [12]. These concentrations are among the lowest Hg concentrations measured in any marine fish in the literature. Simultaneously, redistribution of Hg in different fish organs during uptake, assimilation, and efflux periods were monitored to better understand why muscle is a primary organ for MeHg storage. Finally, subcellular Hg distribution was determined to investigate its potential association with Hg elimination.

MATERIALS AND METHODS

Organism

Wild rabbitfish (*Siganus canaliculatus*; 90.8 ± 16.0 g body wet wt and the ratio of wet wt to dry wt = 3.4) were collected from Sai Kung, Hong Kong. They were immediately transported to the Coastal Marine Laboratory of the University in Sai Kung and acclimated in an aquarium of 120 L recirculating natural sand-filtered seawater (pH = 8.0, salinity = 30 psu, and total dissolved Hg = 0.20 ng/L) at 23 ± 0.5 °C for at least 1 mo prior to conducting the following exposure experiments.

* Address correspondence to wwang@ust.hk
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Background concentrations of MeHg and total Hg in the whole fish body were 5.5 ± 1.4 ng/g and 7.0 ± 3.1 ng/g ($n = 3$, mean $\pm$ standard deviation [SD]) on a wet weight basis, respectively. During the acclimatization period, fish were fed with food pellets 5 times each day at 2.5 g dry diet per day per fish. During the feeding period, fish could eat the food provided within 1 min. The food pellets were purchased from Xiamen, China, and the determined background concentrations of total Hg and MeHg in the pellets were 73.5 ± 1.8 ng/g and 20.2 ± 2.7 ng/g ($n = 3$, mean $\pm$ SD) dry weight, respectively.

Waterborne exposure of Hg(II) and MeHg

The goal of waterborne exposure of Hg(II) and MeHg experiment was to measure the accumulation and organ distribution of Hg(II) and MeHg taken up from seawater. To eliminate possible effects of background Hg on the measurements of organ Hg distribution, the γ-emitting radioisotope ^{203}Hg was used as a tracer. We purchased ^{203}Hg(II), (in 0.1 M HCl), from the Risø National Laboratory. Using a well-established method [29] Me^{203}Hg was synthesized from ^{203}Hg(II) and was stored at 4 °C in the dark in dilute HCl (Optima, Fisher Scientific).

The exposure media were prepared 24 h before the exposure to allow the Hg radioisotopes to equilibrate with 0.22 μm-filtered seawater, and nominal concentrations of waterborne Hg(II) and MeHg were 0.4 μg/L and 0.1 μg/L, respectively. These levels are much higher than those determined in natural waters, but they were necessary for the detection requirement. Meanwhile, fish were acclimated in the same media, but without adding ^{203}Hg, 1 night before initiating the exposure to evacuate their gut contents. To minimize the potential adsorption of Hg onto exposure beakers, Teflon beakers were used for the Hg(II) exposure experiment, and polypropene beakers were used for the MeHg experiment. Four fish were then added randomly to the beakers containing 4 L exposure medium and were exposed for 6 h in the absence of food. During the 6 h period, the media were gently aerated and the variation in the radioactivity of exposure media (i.e., total Hg concentration) was less than 10%. One fish was removed from each of the 4 replicated beakers at 1 h, 2 h, 4 h, and 6 h and immediately rinsed with 0.22 μm-filtered, nonradioactive seawater. After measuring the whole-body wet weight, fish were immediately dissected into gill, liver, intestine, muscle, and remaining carcass, and the wet weight and radioactivity of these organs were measured. Mercury concentration in the whole body was calculated as the sum of all body parts, because the detector vial is not big enough for a whole fish. Waterborne uptake rates (and rate constants) of Hg(II) and MeHg were estimated based on the fact that the short-term uptake was linear within 6 h. Furthermore, the uptake rate was linearly correlated with waterborne Hg concentration in the range of 1 ng/L to 0.2 μg/L for tilapia and black seabream [30,31].

Dietary exposure of Hg(II) and MeHg

The goal of the dietary exposure of Hg(II) and MeHg experiment was to quantify the accumulation and organ distribution of Hg(II) and MeHg during 21 d dietary exposure. To obtain Hg(II) or MeHg-contaminated fish diets, the food pellets were spiked with stable Hg(II) or MeHg solution (chlorocomplex). Radioisotopes of Hg were not used in the present experiment because in practice it was impossible to keep 8 large exposure tanks in the radioactive laboratory for 1 mo. The pellets were air dried in a fume cupboard at 20 °C for at least 2 d, and then kept at –20 °C. The pellets were prepared weekly to minimize possible methylation or demethylation of Hg during the storage period. The determined concentration of MeHg in the MeHg-contaminated pellets was 50.9 ± 3.2 ng/g dry weight, whereas it was 20.2 ± 2.7 ng/g dry weight in the uncontaminated pellets ($n = 3$, mean $\pm$ SD). The determined concentration of total Hg in the Hg(II)-contaminated pellets was 552.6 ± 1.5 ng/g dry weight, whereas it was 73.5 ± 1.8 ng/g dry weight in the uncontaminated diet ($n = 3$, mean $\pm$ SD). The Hg levels in the contaminated pellets are comparable with those observed in natural water settings [14].

Fish were randomly grouped into 8 tanks (60 cm × 30 cm × 45 cm, 4 fish per tank and 4 replicates), and acclimated for at least 2 wk before initiating the dietary exposure. One control group fed with uncontaminated pellets was also prepared. During the exposure period, fish were fed the same food dosage as the acclimation period. The average growth in body weight was not significant during the whole exposure period; thus, the amount of food pellets was not adjusted. Feeding activity was relatively constant, and the pellets could be eaten up within 1 min. After finishing a meal, the tanks were immediately and continuously supplied with fresh clean seawater. During the exposure period, samples of seawater were also regularly taken from the tanks. Measurement of waterborne concentrations of Hg(II) or MeHg in seawater showed that there was no significant increase after the feedings. On the exposure day 0, day 7, day 14, and day 21, 1 individual was sampled from each tank, and the fish were kept in clean seawater overnight to empty the undigested diet in the gut. Then, fish were wet weighted and dissected as in the *Waterborne exposure of Hg(II) and MeHg* experiment. Individual organ samples were further wet weighted and homogenized, and 1 portion of the homogenized samples was used to determine the total Hg and MeHg concentration. Tissue concentration (ng/g) is expressed based on wet weight, and the Hg concentration in whole body is calculated as the sum of Hg content in all parts divided by its total body weight.

Assimilation of dietary Hg(II) and MeHg

The pulse-chase technique was used to estimate the assimilation efficiency of Hg(II) and MeHg from the pellets by the fish [32]. Briefly, the pellets were radiolabeled with aliquots of ^{203}Hg(II) or Me^{203}Hg solution and then air dried overnight at 20 °C in a fume cupboard. Total radioactivity of 0.5 g ^{203}Hg(II) or Me^{203}Hg spiked pellets was determined. Preliminary experiments showed that less than 1% of the radioisotopes were released into seawater within 30 min. The pellets were fed to individual fish that had been starved during the previous night in each of the 4 replicated individual beakers, and the 0.5 g pellets were eaten within 1 min. Thus, the initial radioactivity of each fish was equal to the radioactivity of the 0.5 g diet. The fish were then transferred into clean seawater and further depurated for 39 h; assimilation was considered to be complete at 39 h [33]. The seawater was renewed every 6 h to 10 h to minimize the external re-uptake of radioisotopes from fish excretion. After 39 h, the fish were removed and dissected as mentioned in *Waterborne exposure of Hg(II) and MeHg*, and the radioactivity of tissue samples was measured. Total radioactivity of whole fish at 39 h was calculated as the sum of all the parts, and assimilation efficiency was calculated as the total fish radioactivity at 39 h divided by the initial fish radioactivity. There are 4 replicates in the present experiment.

Depuration of Hg(II) and MeHg and subcellular distribution

The purpose of the depuration of Hg(II) and MeHg and subcellular distribution experiment was to quantify elimination, organ distribution and subcellular distribution of Hg(II) and

MeHg during a 9-d depuration period, although a long time frame is generally needed to calculate the efflux rate constant of MeHg in most fish. Thus, the radioisotopes ^{203}Hg(II) and Me^{203}Hg were used to monitor the small but significant elimination precisely.

Fish were placed individually into 10-L plastic containers and were acclimated for at least 1 wk. Each fish was supplied with freshly prepared ^{203}Hg(II)-spiked or Me^{203}Hg-spiked pellets, and there were 5 feedings each day for each fish as described in the section *Assimilation of dietary Hg(II) and MeHg*. Furthermore, based on the radioactivity of seawater and pellets, <1% of ^{203}Hg(II) or Me^{203}Hg was released from the pellets into seawater during the feeding. After 7 d of radiolabelled diet feeding, fish were then gut emptied for 39 h in clean seawater, and then 4 ^{203}Hg(II) or Me^{203}Hg radiolabeled individuals (i.e., 4 replicates) were collected. We radiolabeled the fish for 7 d, allowing the radioisotopes to be sufficiently partitioned into the slowly exchanging compartments; thus, the efflux can be realistically quantified. The remaining fish were further depurated in clean seawater for 9 d, and were sampled at day 3, day 6, and day 9. During the sampling period, the fish were fed with unlabeled food pellets, and the seawater was renewed daily.

Fish samples were first rinsed in clean seawater several times to remove surface absorbed Hg. After the wet weight measurement, they were dissected as described above in *Waterborne exposure of Hg(II) and MeHg* and then the wet weight and radioactivity of each part were measured. Then, fish samples were stored in individual bags at –80 °C until the subcellular fractionation analyses. The efflux rate constant (d^{-1}) is calculated as the absolute value of the slope of linear regression of the natural log of the percentage of ^{203}Hg retained in the whole fish body against depuration time.

Subcellular distribution of Hg(II) and MeHg in the gill, liver, intestine, and muscle were measured using a widely used method [34–36], quantifying Hg in 5 operationally defined pools (i.e., cellular debris, metal-rich granules, organelles, heat-denatured proteins, and metallothionein-like proteins). Briefly, samples were individually homogenized (Ultra-Turrax T25 basic, IKA) in a Tris-buffer placed in a water–ice bath [36]. Then, the homogenized samples were centrifuged at 1500 *g* for 15 min at 4 °C, and the yielded pellets were digested in 1 N NaOH at 80 °C for 1 h. After digestion, the samples were centrifuged at 10 000 *g* for 10 min at 20 °C to separate the metal-rich granules (as the pellets) and cellular debris (as the supernatant) fractions. Meanwhile, the supernatant was ultracentrifuged at 100 000 *g* for 1 h at 4 °C to separate the organelles (as the pellets) and the cytosolic fractions (as the supernatant). The cytosolic samples were heated in a water bath at 80 °C for 10 min and then cooled at 4 °C for 1 h. The samples were centrifuged again at 50 000 *g* for 10 min at 4 °C to yield the heat-denatured proteins (as the pellets) and metallothionein-like protein (as the supernatant). Finally, the radioactivity of each subcellular sample was measured. The preliminary test with muscle from Hg(II)-exposed fish indicated that the maximal percentage of unbroken cells following the homogenization step was 3%, given that 3% of Hg(II) was associated with cellular debris in this sample.

Measurements of radioactive ^{203}Hg and stable Hg

The radioactivity of ^{203}Hg(II) and Me^{203}Hg were determined by a Wallac 1480 NaI (T1) gamma counter. The gamma emission of ^{203}Hg was determined at 232 keV. All data were calibrated for radioisotope decay and counting efficiency by measuring appropriate standards periodically. Counting times were adjusted to acceptable counting errors, generally <5%.

Concentration of total Hg in the samples was analyzed using the United States Environmental Protection Agency method 7474 with a few modifications [36]. Specifically, the samples were digested at 60 °C to 80 °C in concentrated nitric acid (68%, analytical reagent, VWR), and then aliquots of the digested samples were cold digested with a hydrocholoride/bromate/bromide mixture. Tin(II) chloride ($SnCl_2$) was added as a reducing agent to produce elemental Hg, and the elemental Hg vapor was carried to the fluorescence detector of cold vapor atomic fluorescence spectrometry (CVAFS, QuickTrace M-8000). To measure MeHg, the samples were first digested in 25% potassium hydroxide KOH to methanol solution in an oven at 85 °C for 4 h, and then citrate buffer was added to the digested samples. Aliquots of the digested samples were ethylated by sodium tetraethylborate (NaBEt4), and MeHg concentrations in the samples were measured using an automated analytical system (MERX, Brooks Rand). Quality controls for the analyses were implemented by using method blanks and the certified reference materials (Tuna Fish Flesh Homogenate-IAEA 436 and Oyster Tissue-1566b), and recoveries of the standards were 95 ± 6% for total mercury and 90 ± 8% for MeHg. There were 4 replicates for the samples. The concentration of inorganic Hg(II) presented in the tissues was calculated as the difference between total Hg and MeHg.

Data analyses

SigmaPlot 12.5 (SigmaPlot) and Microsoft Excel 2010 for Windows were used for data analyses. Linear regressions were done with SigmaPlot, and the significance level for the regressions was set at $p < 0.05$. The means of accumulation rates and efflux rate constants in the organs were compared with the Student–Newman–Keuls test following one-way analysis of variance, and significance level was set at $p < 0.05$ (SPSS 16.0).

RESULTS

Waterborne exposure of Hg(II) and MeHg

Whole body accumulation of MeHg or Hg(II) and organ distribution are shown in Figure 1. Whole body concentrations of both Hg species increased linearly over the 6 h exposure period. The calculated uptake rate constants of MeHg or Hg(II) on a dry weight basis were $0.830\ ng\ g^{-1}\ d^{-1}\ (ng\ L^{-1})^{-1}$ and $0.005\ ng\ g^{-1}\ d^{-1}\ (ng\ L^{-1})^{-1}$, respectively, in the rabbitfish under the present exposure conditions (Table 1).

The majority of MeHg and Hg(II) were stored in gill, and specifically 46.9% to 59.5% of body MeHg and 42.3% to 64.9% of body Hg(II) was accumulated in gill. The percentage of intestine Hg(II) increased linearly with exposure time, whereas that of Hg(II) in gill decreased progressively. Up to 41.9% of body Hg(II) was found in the intestine at 6 h of exposure. A small portion of body MeHg (6.5–11.3%) and Hg(II), (0.6–2.5%) was stored in the muscle. The portions were relatively constant during the 6-h exposure.

Organ accumulation rates from the waterborne phase were also estimated based on linear regression of tissue Hg concentration against time (1–6 h; Table 2). The average accumulation rates of MeHg followed an order of gill > liver > intestine > muscle, and the differences among organs were significant ($p < 0.05$). Similarly, the average accumulation rates of Hg(II) followed the order of gill ≈ intestine > liver > muscle, although the linear regressions were not significant ($p > 0.05$).

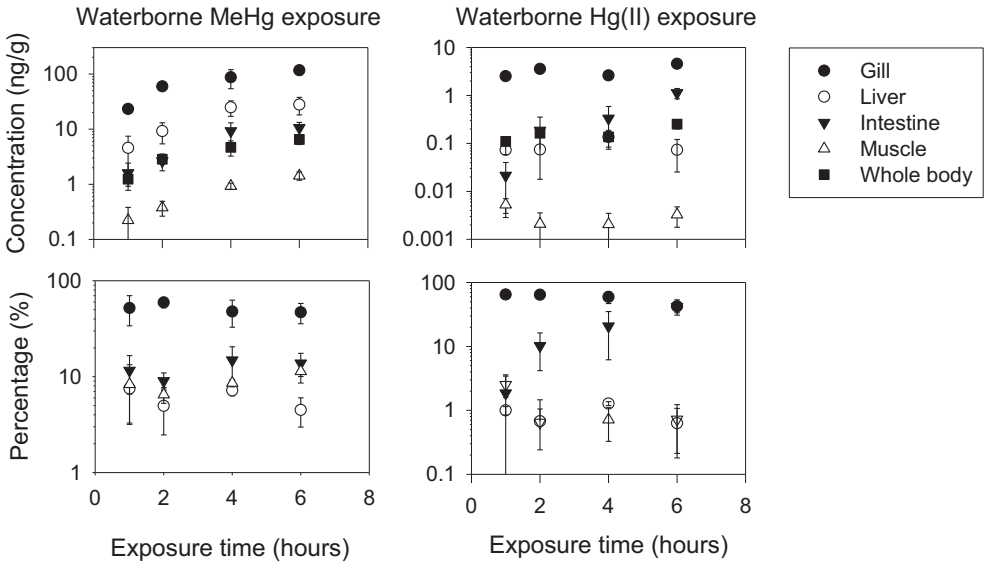

Figure 1. Accumulation (ng/g wet wt) and organ distribution of MeHg or Hg(II) in the rabbitfish *Siganus canaliculatus* during waterborne exposure. Total waterborne MeHg = 0.1 μg/L and total waterborne Hg(II) = 0.4 μg/L. Mean ± standard deviation, (n = 3–4).

Table 1. Biokinetics (uptake, assimilation, and efflux) of MeHg and Hg(II) in whole body of fish[a]

	MeHg			Hg(II)			
	k_u (L g^{-1} d^{-1})	AE (%)	k_e (d^{-1})	k_u (L g^{-1} d^{-1})	AE (%)	k_e (d^{-1})	References
Rabbitfish (*Siganus canaliculatus*)	0.830 ± 0.101	68 ± 1	0.024 ± 0.003	0.005 ± 0.001	36 ± 8	0.104 ± 0.023	The present study
Tilapia (*Oreochromis niloticus*)	0.333	90–99	0.006	0.086	9–32	0.039	[30]
Mosquitofish (*Gambusia affinis*)	0.185–0.338	90–94	0.016–0.019	0.052–0.078	42–51	0.021–0.042	[37]
Redear sunfish (*Lepomis microlophus*)	0.454–1.28	86–91	0.015–0.021	0.038–0.051	8–10	0.003–0.035	[37]
Sweetlips (*Plectorhinchus gibbosus*)	4.52	56–95	0.010–0.013	0.195	10–27	0.029–0.055	[55]
Killifish (*Fundulus heteroclitus*)		80–99	0.010–0.019				[56]
Killifish (*Fundulus heteroclitus*)	0.370–0.781		0.006–0.009	0.026–0.215		0.023–0.046	[57]
Atlantic silverside (*Menidia menidia*)	1.15–4.38	82–89	0.006–0.014	0.015–0.017	8–15	0.071–0.075	[58]
Tiger Bass (*Terapon jurbua*)	1.9	93	0.0018	0.08	38	0.026	[38]
Black seabream (*Acanthopagrus schlegeli*)	0.36W$^{-0.54}$	80–100	0.0062W$^{-0.40}$	0.24W$^{-0.68}$	25.6W$^{-0.68}$	0.050W$^{-0.36}$	[31]
Goldfish (*Carassius auratus*)		98	0.010				[59]
Atlantic cod (*Gadus morhua*)			0.002				[60]

[a]Uptake rate constants k_u (from waterborne exposure) are on a dry weight basis; k_e is efflux rate constant (from depuration experiment). Data for *S. canaliculatus* represent mean ± standard error, n = 4. The data for other fish are collected as of September 22, 2015, and a part of them is originally summarized by Wang [12].
AE = assimilation efficiency (from the pulse-chase experiment).
W = dry wt. of fish (g).

Table 2. Organ-specific accumulation and efflux of MeHg and Hg(II) in fish *Siganus canaliculatus*[a]

	Waterborne exposure		Dietary exposure		Efflux rate constant	
	MeHg accu. (ng/g/h)	Hg(II) accu. (ng/g/h)	MeHg accu. (ng/g/d)	Hg(II) accu. (ng/g/d)	MeHg (d^{-1})	Hg(II) (d^{-1})
Gill	17.61 e	0.30[b]	2.34 b	1.30 b	0.12 b	< 0
Liver	4.98 d	0.003[b]	3.91 b	3.62 c	0.17 c	< 0
Intestine	1.97 c	0.21[b]	3.71 b	16.75 d	0.20 c	0.13
Muscle	0.25 b	< 0	2.19 b	1.58 b	< 0	< 0

[a]Accumulation rate (accu.) is calculated from the linear regression of organ mercury concentration (wet wt) as a function of the exposure time (the first 6 h for waterborne exposure and the first 7 d for dietary exposure). $p < 0.05$ for all regressions except for the numbers with 'f'. Waterborne concentrations are 0.1 μg/L for MeHg and 0.4 μg/L for Hg(II) exposure, and dietary concentrations are 51 ng/g for MeHg and 479 ng/g for Hg(II) exposure. During the depuration, "< 0" indicates a net increase in Hg accumulation. Letters B to E indicate that the numbers in the same column sharing the same letters are not significantly different.
[b]The linear regression is not significant (i.e., $p > 0.05$)

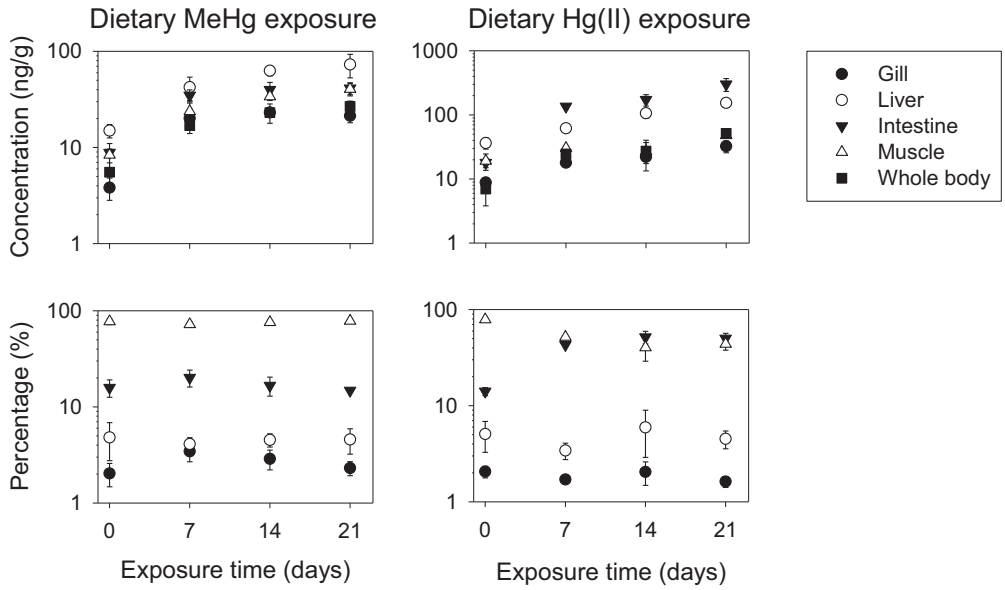

Figure 2. Accumulation (ng/g wet wt) and organ distribution of MeHg or Hg(II) in the rabbitfish *Siganus canaliculatus* during dietary exposure. Dietary MeHg = 51 ng/g and dietary Hg(II) = 479 ng/g dry weight. Mean ± standard deviation, ($n = 3$–4).

Notably, there was no increase in muscle Hg(II) concentration after 1 h exposure.

Dietary exposure of Hg(II) and MeHg

Assimilation efficiency of dietary MeHg and Hg(II) by the fish were 68% and 36%, respectively (Table 1). The bioaccumulation pattern and organ distribution following the 21-d dietary exposure of MeHg or Hg(II) are shown in Figure 2. During the exposure, the concentrations of both MeHg and Hg(II) in whole body increased progressively. The accumulation of MeHg in whole-body started to level off after 21 d (i.e., 26.7 ± 3.7 ng/g on day 21). Its whole-body accumulation appeared to reach a steady state on day 14 (i.e., 23.0 ± 1.0 ng/g). In contrast, the whole-body Hg(II) accumulation increased linearly over the period.

For MeHg, muscle was the dominant storage site, and 72.3% to 78.3% of body MeHg was associated with this organ. Intestine also stored a substantial amount of body MeHg (14.8–20.1%), and a small portion of MeHg was found in gill (2.0–3.4%) and liver (4.1–4.8%). In the dietary Hg(II) exposed fish, intestine (43.0–51.8%) and muscle (40.2–51.9%) contributed equally to Hg storage, whereas only a small fraction was found in liver (3.4–5.9%) and gills (1.6–2.1%). Average accumulation rates from diet among the organs could be compared based on the first 7-d accumulation (Table 2), although the exact rates can be likely overestimated by assuming the linear accumulation. The accumulation rates of MeHg were comparable among the organs whereas the accumulation rate of Hg(II) in intestine was much higher than those in other organs ($p < 0.05$).

Depuration of Hg(II) and MeHg

During the 9-d depuration period, the concentration and percentage of MeHg and Hg(II) in the organs are shown in

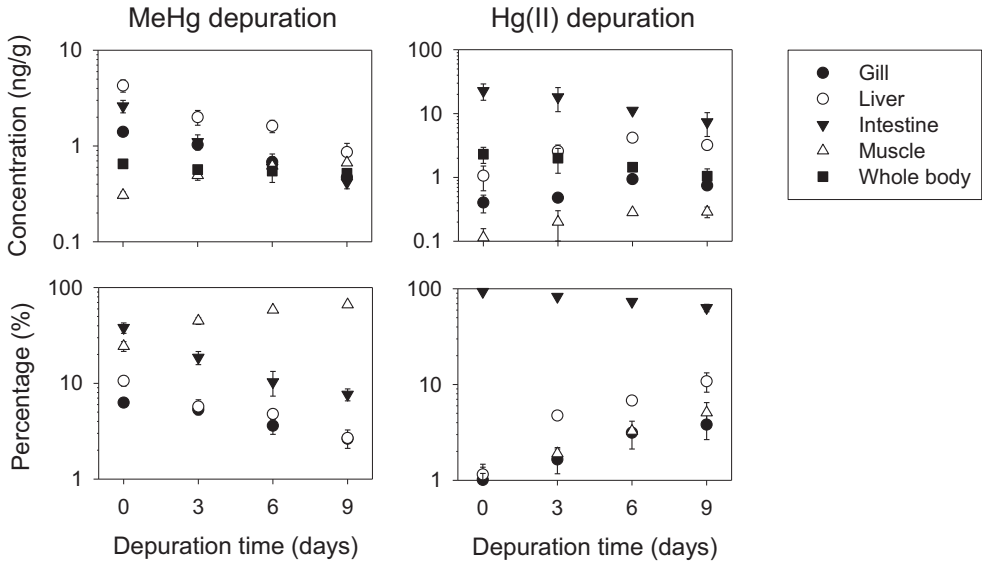

Figure 3. Depuration (ng/g wet wt) and organ distribution of MeHg or Hg(II) in the rabbitfish *Siganus canaliculatus*. Mean ± standard deviation, ($n = 3$–4).

Figure 3. The calculated k_e of MeHg ($0.024\ d^{-1}$) was significantly slower than that of Hg(II), ($0.104\ d^{-1}$). Moreover, the k_e of MeHg for gill, liver, and intestine were comparable, and the k_e of Hg(II) was positive for intestine and negative for the other organs (Table 2). Correspondingly, concentration of MeHg in gill, liver, and intestine decreased, whereas concentration of MeHg in muscle increased by 2.2-fold. For Hg(II), its concentration in the intestine showed the greatest decrease, whereas the concentrations in the other 3 organs increased.

The percentage of MeHg in muscle increased but in the other organs decreased with time. By the end of the 9-d depuration, the percentage of whole body MeHg in muscle, intestine, liver and gills were 66.5%, 7.6%, 2.7%, and 2.6%, respectively. In contrast to MeHg, only the concentration of Hg(II) in intestine decreased sharply with the depuration time (i.e., from an initial value of 93.4% to 63.3% after 9 d of depuration), meanwhile the percentage of Hg(II) in gill, liver, and muscle increased significantly.

Subcellular concentration of MeHg in gills, liver, and muscle decreased during the depuration time (Figure 4). Specifically, the percentages of the depurated MeHg in gills from each fraction were 32.8% by heat-denatured proteins, 28.6% by organelles, 26.5% by metallothionein-like proteins, 12.8% by cellular debris, and 0% by metal-rich granules; these in liver were 30.5% by heat-denatured proteins, 26.9% by cellular debris, 18.0% by organelles, 15.0% by metallothionein-like proteins, and 9.6% by metal-rich granules. Those in intestine were 37.0% by metallothionein-like proteins, 20.6% by metal-rich granules, 19.9% by cellular debris, 16.1% by organelles, and 6.6% by heat-denatured proteins. The concentration of subcellular Hg(II) in intestine decreased as well but not in other organs; indeed, the net increase of Hg(II) in gills, liver, and muscle was observed during the depuration. The percentage of the depurated Hg(II) in intestine from each fraction was 48.9% by cellular debris, 22.0% by metal-rich granules, 17.2% by organelles, 10.2% by metallothionein-like proteins, and 1.8%

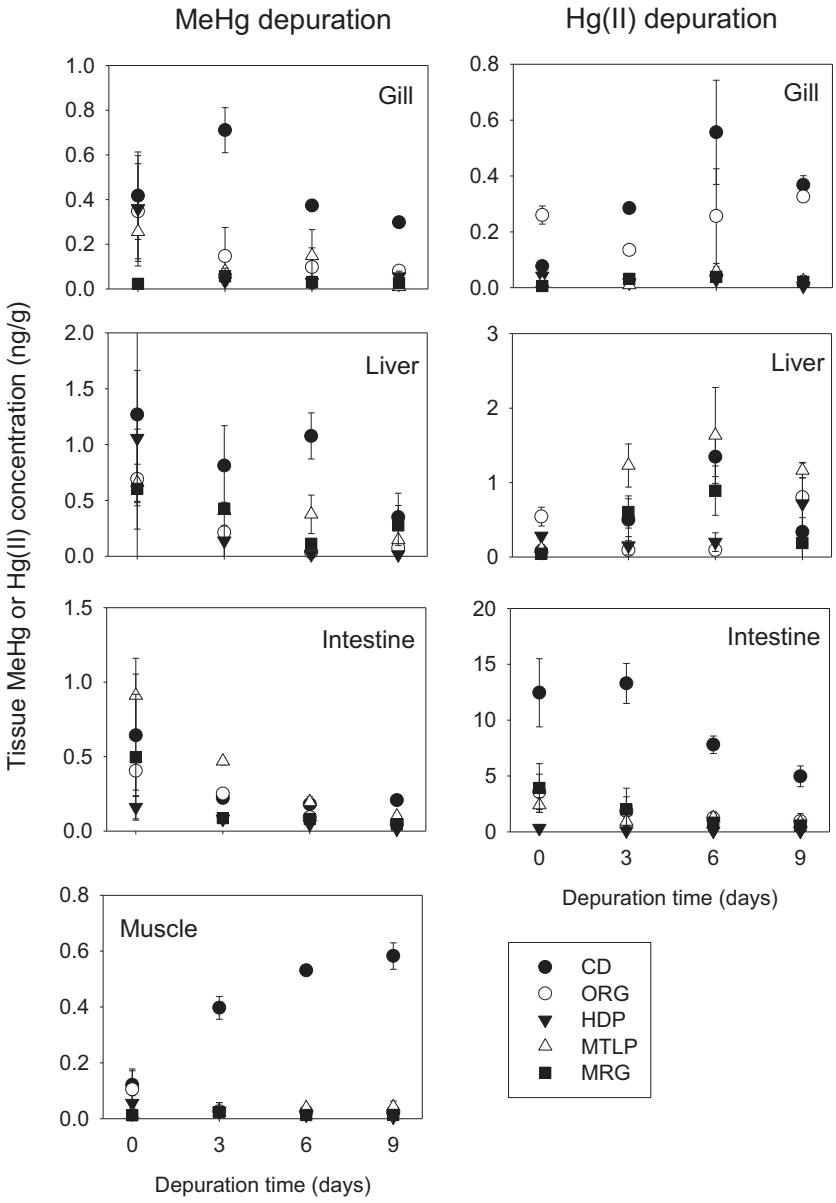

Figure 4. Subcellular depuration (ng/g wet wt) of MeHg or Hg(II) from gill, liver, intestine, and muscle in the rabbitfish *Siganus canaliculatus*. CD = cellular debris; MRG = metal-rich granules; ORG = organelles; HDP = heat-denatured proteins; MTLP = metallothionein-like proteins. Mean ± standard deviation, ($n = 3$–4).

by heat-denatured proteins. The subcellular concentrations of Hg(II) in muscle were not available due to the undetectable low Hg(II) concentrations.

DISCUSSION

Low Hg bioaccumulation by rabbitfish

The background concentrations of muscle MeHg in the rabbitfish were 5.7 ng/g to 11.0 ng/g wet weight in the present study, which was consistent with previous measurements of 0.5 ng to 30.4 ng MeHg/g wet muscle of the same fish species [10–12]. Remarkably, these concentrations in *S. canaliculatus* are much lower than those reported in the majority of other field-collected fish species [5–7,13]. Several mechanisms may account for the low concentration of Hg observed in the rabbitfish: unique biokinetics of Hg bioaccumulation, fast growth rate, and herbivorous feeding habit.

Biokinetics of Hg(II) and MeHg in 12 fish species are compiled in Table 1. First, for a given species, the k_e is highly influential in determining bioaccumulation. It appears to be a definable trait for each metal and little affected by geochemical conditions [14]. The efflux rate constants (i.e., k_e) of Hg(II) and MeHg of *S. canaliculatus* in the present study were $0.104\,d^{-1}$ and $0.024\,d^{-1}$, respectively, and the rates are probably the highest reported in fish thus far. Similar to the rabbitfish, mosquitofish, *Gambusia affinis*, and redear sunfish, *Lepomis microlophus*, are characterized by high MeHg efflux rate, and correspondingly low Hg concentrations in their bodies are observed in field-collected samples [37]. On the other hand, carnivorous fish such as tiger bass [38] and black seabream [31] residing in the same environmental setting as the rabbitfish have much lower efflux rate, highlighting that high k_e might be specific in herbivorous fish. Overall, the relatively high k_e in herbivorous fish point out the important role of high physiological Hg loss in their low Hg bioaccumulation. More studies are required to ascertain whether or not high k_e is ubiquitous in herbivorous fish.

Second, dietary MeHg uptake is considered as the dominant Hg accumulation pathway in fish, and generally approximately 90% of dietary MeHg can be assimilated regardless of food type and fish species (Table 1). However, the rabbitfish only assimilated 68% of dietary MeHg, and the value would increase little even if taking into account the physiological loss during the 39 h digestion period. Moreover, another marine fish *Terapon jurbua* feeding on the similar type of food pellets assimilates up to 93% of dietary MeHg [38], highlighting the unique low-MeHg assimilation by the rabbitfish. Consistent with other studies, dietary Hg(II) assimilation (36%) by the rabbitfish is much lower than MeHg. The ubiquitously low-Hg(II) assimilation in fish is primarily due to the limited transportation of Hg(II) across the intestinal epithelium to the blood and other body organs and is partly associated with the high affinity of gut mucosal membrane for Hg(II), (i.e., functioning as an effective barrier for Hg(II)) [39].

Third, uptake of waterborne Hg plays a relatively small role in overall bioaccumulation by fish in most conditions, although its importance can be significant when there are low feeding rates and low Hg concentration factors in prey [12]. Compared with other fish, waterborne uptake rate constant (i.e., k_u) for Hg(II) is the lowest in the rabbitfish, but not for MeHg. It should be noted that k_u varies much with water chemistry (pH, dissolved organic carbon, other cations), and thus it would be incomparable without knowing Hg speciation and water chemistry.

Last but not least, the low trophic level and fast growth of the rabbitfish are also important in determining its low Hg concentration. Specifically, Hg in biota is positively related to trophic position of the biota, and fish of high trophic levels (e.g., fish-eating fish) tend to accumulate more Hg than those sitting at low positions of a food web (e.g., herbivorous fish). Thus, the low Hg accumulation in the rabbitfish is likely related to its herbivorous feeding habit (i.e., the low trophic level). Moreover, benthic macroalgae and plankton as the primary diet of *S. canaliculatus* [40] have relatively low concentrations of Hg than prey at upper trophic levels. On the other hand, the fish grows to 80% of its adult size in 6 mo, and the fast growth may effectively dilute accumulated Hg in its body. Consistent with this hypothesis, there is little or a small increase in muscle Hg, even over a large increase in its body size [11].

Muscle as a primary organ in MeHg storage

The present study's results suggest that muscle is a major sink in Hg bioaccumulation. First, on dietary exposure, either MeHg or Hg(II) is assimilated in the digestive system and then redistributed into other body parts via blood at distinct rates. Consistent with the higher assimilation efficiency of MeHg than Hg(II), 2-fold higher MeHg absorption in the intestine is also documented based on its high accumulation capacity (i.e., 0.073 ng MeHg/g fish/d per 1 ng MeHg/g prey vs 0.035 ng Hg(II)/g fish/d per 1 ng Hg(II)/g prey). More important, redistribution of intestine MeHg through whole body is very efficient, based on the similar dietary MeHg accumulation rates among the organs in the rabbitfish. The underlying mechanisms for effective MeHg moving around the fish body are unknown, but are probably similar to rats in which the MeHg-cysteine complex is mistakenly taken up by the common methionine (structurally similar to MeHg-cysteine) transporters found in cell membranes [41]. As expected, the transportation of intestine Hg(II) into other tissues is much less efficient as evidenced by the much lower dietary Hg(II) accumulation rates in other organs. Furthermore, the Hg(II) accumulation rate in muscle is only 10% of that in the intestine. Similarly, in fish *T. jarbua*, the modeled rate constant of transporting Hg(II) from intestine to blood is much lower than that of MeHg [42]. Overall, the results of dietary exposure suggest that the intestine is the primary pool for Hg(II) accumulation, whereas MeHg can be effectively transported from the intestine into muscle.

With waterborne exposure, as expected, the majority of either MeHg or Hg(II) was accumulated in the gills of the rabbitfish. However, the MeHg absorption by the gills was much faster than Hg(II) as indicated by the much higher k_u for MeHg. It is unclear whether the MeHg is transported across the gill cells in the MeHg-cysteine form by the methionine transporters as in the intestine, because the concentration of cysteine in sequestrating MeHg nearby gills is unknown. More likely, the MeHgCl complexes of lipid solubility would have diffused across cell membranes [43], and other MeHg species would have gone into gills via unknown metabolically controlled pathways [44]. For the Hg(II) transportation pathway, both passive diffusion of neutrally uncharged $HgCl_2$ [43,45] and accidental Hg(II) cation transport through metal transporters [46,47] may play a role in Hg(II) bioaccumulation in the gill.

On the other hand, the importance of intestine in waterborne Hg(II) uptake is observed in the rabbitfish, although the gills are generally expected to be the primary targets for waterborne metal accumulation. The fast increase in Hg(II)-intestine may be linked to seawater drinking for osmoregulation, resulting in constant waterborne Hg(II) exposure of the intestine [48,49].

It is impossible that Hg(II)-gills would have been quickly transferred into the intestine via blood [42].

The accumulation pattern during waterborne exposure also sheds light on the distinct interorgan transportation efficiency between MeHg and Hg(II). The MeHg accumulated in gills was more easily distributed to other tissues than Hg(II). Only 1% of body Hg(II) was distributed to muscle, whereas approximately 10% of body MeHg accumulated in this tissue after 6 h of exposure. Moreover, other than muscle, both the liver and the intestine were apparently the important sinks for waterborne MeHg in the short-term exposure, and their accumulation rates were higher than that of muscle. Similar to the observation in white sturgeon [50,51], the depuration pattern in rabbitfish also suggests that both liver and intestine serve as transit sites in distributing MeHg. In contrast, Hg(II) accumulated in either the gills or intestine is very difficult to be transported out, and even a short-term waterborne Hg(II) exposure could result in a saturated accumulation in muscle.

The depuration pattern in the rabbitfish strongly suggests that muscle is the major organ in MeHg storage, and the intestine is the only and primary organ in Hg(II) elimination. Specifically, MeHg in all tissues except in muscle is depurated by either sinking into muscle or eliminating out of the body. Based on the mass balance calculation, 58.6% of MeHg in the remaining body finally settled down in muscle, whereas the other 41.4% was eliminated out of its whole body by the end of the 9 d depuration. Over a long time frame, it is expected that most of MeHg would go into muscle as the final sink. Moreover, the similar efflux rate constants among the MeHg depurating organs (i.e., gill, liver, and intestine) further indicate the common elimination pathway of MeHg across membranes. In the same way, the calculation shows that 86.6% of Hg(II) in intestine was eliminated out of fish body, and the other 13.4% was redistributed into the remaining body. The depurated amount of Hg(II) from the intestine accounted for nearly 100% of the depurated Hg(II) from the whole body, highlighting the importance of the intestine in eliminating Hg(II) [50]. The very similar depuration patterns by the organs are also observed in the fish *T. jarbua* [42].

Association between Hg depuration and subcellular distribution

Fish muscle is much more efficient in MeHg sequestration than other organs; conversely, other organs are much more efficient in MeHg depuration than muscle. Subcellular distribution patterns (i.e., intracellular Hg speciation) may help explain these interesting observations, although the distribution is operationally defined and the biochemical nature is unclear at this time.

First, during the depuration, the primary increase in muscle was the MeHg associated with cellular debris. The increased amount of MeHg in cellular debris (0.46 ng/g muscle) was even slightly higher than the total increase of MeHg in muscle (0.36 ng/g muscle). This was due to a corresponding decrease of MeHg (0.13 ng/g muscle) associated with organelles and heat-denatured proteins and a small increase of MeHg (0.03 ng/g muscle) associated with metallothionein-like proteins and metal-rich granules. In other words, cellular debris was the ultimate pool in MeHg storage, and a part of intracellular MeHg associated with biologically important components (i.e., organelles and heat-denatured proteins) was redistributed into and detoxified by metallothionein-like proteins and metal-rich granules. Cellular debris consisted primarily of membranes, nuclei, and some connective tissues, and the intracellular distribution indicates that MeHg is trapped on membranes instead of nuclei. We speculate that some unknown membrane proteins of high affinity for MeHg in muscle are probably the major MeHg-binding pools, because MeHg is coordinated by an aliphatic thiol within MeHg-protein complexes [15,52,53]. Mason et al. [43] also noted that MeHg in fish resides in proteins rather than fat.

In contrast to the MeHg-cellular debris in muscle, the concentrations of MeHg-cellular debris in the gills, liver, and intestine decreased significantly over time; thus, it is hypothesized that the unknown membrane proteins in MeHg sequestration may be specific in muscle. On the other hand, metallothionein-like proteins, is important in MeHg elimination from the gills and intestine instead of its well-known detoxification role, as evidenced by the fact that approximately one-third of total tissue MeHg was depurated from these proteins. Metal-rich granules play a less important role than metallothionein-like proteins in MeHg elimination in liver and intestine, and no granule formation was observed in gills. The granules could be the insoluble Hg-Se form following a demethylation of MeHg as in other organisms [21,54]. In all organs except for muscle, MeHg in each of the 5 subcellular fractions was eliminated to different extents, which may be responsible for the observed efflux of MeHg in gills, liver, and intestine.

Because the intestine is the only important organ in Hg(II) depuration, it is interesting to examine the subcellular distribution during its depuration. Notably, half of the intestine Hg(II) was depurated out from cellular debris, another 22% from Hg-rich granules and 10% from metallothionein-like proteins. Little is known about the eliminating form of Hg(II) and the depurating process across membranes. It is possible that a part of Hg(II) may desorb from surface of digestive cells and then incorporated into feces, and some are excreted out of the intestine cells in a form of the Hg-rich granules.

In conclusion, the very low Hg concentration in the rabbitfish is attributed to high physiological loss of both MeHg and Hg(II), the relatively low dietary MeHg assimilation efficiency, its fast growth rate, and herbivorous habit. Instead of other organs, the dominant role of muscle in storing MeHg is explained by the negligible elimination of MeHg, efficient redistribution of MeHg from other organs into muscle and the very slow transportation of Hg(II) into muscle, as well as the highly efficient Hg(II) elimination by the intestine. Moreover, some specific membrane proteins in muscle are probably the ultimate sink for MeHg in fish. The present study highlights the importance of biokinetics and subcellular speciation in understanding Hg bioaccumulation in aquatic organisms.

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Data availability—Data, associated metadata, and calculation tools are available on request to the corresponding author (wwang@ust.hk).

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