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Metallothionein induction and bioaccumulation kinetics of Cd and Ag in the marine fish *Terapon jarbua* challenged with dietary or waterborne Ag and Cu

Aimin Long[1,2], Wen-Xiong Wang[1,*]

[1]Department of Biology, The Hong Kong University of Science and Technology (HKUST), Clear Water Bay, Kowloon, Hong Kong

[2]*Present address:* South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China

ABSTRACT: A series of experiments were conducted to determine the effects of sublethal pre-exposure of dissolved and dietary silver (Ag) and copper (Cu) on the subsequent Ag and cadmium (Cd) bioaccumulation by the marine 3-line theraponids *Terapon jarbua*. Parameters measured in this study included the accumulated metal concentration and metallothionein (MT) induction in different fish tissues, and the dietary assimilation efficiency (AE) and dissolved uptake of Ag and Cd. An obvious increase in the Ag (up to 4-fold) or Cu (up to 2-fold) body concentration and MT induction (up to 2-fold) in fish was observed following 1 wk waterborne or dietary exposure. However, MT induction was not sufficient to bind with the accumulated metals, and the accumulated Cu and Ag were also bound with ligands other than MT. There was a significant correlation between the accumulated Ag or Cu concentration and the MT induction. However, MT induction in the muscle was more responsive to dietary Ag exposure than to waterborne Ag exposure. The AE and the dissolved uptake rate constant of Ag and Cd both increased in response to waterborne and dietary Cu or Ag pre-exposure. In contrast, the efflux rate constant for Ag and Cd decreased in response to Cu or Ag pre-exposure. The quantified biokinetic parameters of Ag or Cd were significantly correlated with the accumulated body burdens of Ag and Cu, and the induced MT concentration. Such a relationship may also be dependent on the route of metal pre-exposure. For Ag, the biokinetic modification was obvious when Ag exceeded 1.5 μg g^{-1} dry weight. Our study also demonstrated that simultaneous exposure to dissolved Ag and Cu had an antagonistic effect on Cu and Ag bioaccumulation, probably as a result of competition between Cu^+ and Ag^+ for binding sites.

KEY WORDS: Pre-exposure · Bioaccumulation kinetics · Metal body concentration · Metallothionein

INTRODUCTION

Marine fish accumulate metals from both the waterborne and dietary phases, but the relative importance of these 2 potential sources remains less well understood (Willis & Sunda 1984, Dallinger et al. 1987, Xu & Wang 2002). Previously, many studies have quantified the effects of metal pre-exposure, via either a dietary or waterborne phase, on the subsequent bioaccumulation by freshwater fish, especially using the rainbow trout as the model organism (Handy 1992, Hollis et al. 2001, Kamunde et al. 2002a,b, Niyogi & Wood 2003). These studies have shown that some critical physiological changes may occur in fish after exposure or acclimation in metal-rich environments, which may lead to subsequent changes in metal bioaccumulation from the ambient environments. For example, concentrations of metallothionein (MT), a class of low-molecular weight, cysteine-rich metal-binding proteins (Kägi & Kojima 1987) that can sequester metals and protect from cellular metal toxicity (Roesijadi 1992), increased in the gills, kidney, liver and other organs of fish after metal exposure (McCarter & Roch 1983, Hollis et al. 2001, Mayer et al. 2003). In addition,

*Corresponding author. Email: wwang@ust.hk

the fish gill metal-binding affinity and density of binding sites underwent substantial changes in response to sublethal exposure to waterborne or dietary metals (Szebedinszky et al. 2001, Niyogi & Wood 2003). Kinetics of metal bioaccumulation is then altered as a result of these physiological changes or of acclimation. These results suggest that the exposure history of fish should be taken into consideration when evaluating their susceptibility to metal pollution.

Despite these extensive studies on freshwater fish, metal bioaccumulation by marine fish has been measured less frequently (Pentreath 1977, Willis & Sunda 1984, Burgos & Rainbow 2001, Baines et al. 2002, Xu & Wang 2002). The efficiency of trophic transfer in marine fish has been measured in a few marine fish species (Reinfelder & Fisher 1994, Ni et al. 2000, Xu & Wang 2002). Xu & Wang (2002) modeled the exposure of a few metals (Cd, Se and Zn) in the mangrove snapper *Lutjanus argentimaculatus*, and suggested that the accumulation of these metals was mainly achieved by trophic transfer, as a result of the very slow uptake from the aqueous phase. However, mechanistic understanding of the processes controlling metal bioaccumulation in marine fish is still limited, and there is a need to further elucidate the physiological and biochemical controls of metal bioaccumulation in marine fish. Among the many environmental factors, the effects of metal pre-exposure on the bioaccumulation kinetics in marine species of fish are not clear, although their toxicities have been measured in a few previous studies (Baker et al. 1998, Clearwater et al. 2002).

In the present study, we tested the influence of Ag and Cu exposure on subsequent Cd and Ag accumulation in the 3-line theraponid *Terapon jarbua*. Cu and Ag are both ubiquitous in the environment, due to the natural or anthropogenic activities. Ag is not essential to marine fish, and its toxicity to fish has been demonstrated (Wood et al. 1996), whereas Cu is essential in cellular metabolism (Cousins 1985), but can also be toxic to fish (Clearwater et al. 2002). After the waterborne or dietary Cu or Ag exposure for 1 wk, the concentrations of MT and metals in different fish tissues were concurrently determined. We then quantified the assimilation and dissolved uptake of Ag and Cd by the theraponids. We did not quantify the biokinetics of Cu, since its radiotracer was not readily available in our study. Instead, we measured the biokinetics of Cd, in addition to Ag, mainly because its radiotracer (^{109}Cd) was readily available and these metals share similar chemical properties in preferentially binding with sulfur ligands. Thus, we tested whether the exposure to one metal (e.g. Cu) would cause modification of the biokinetics of other metals (e.g. Cd and Ag) in the marine fish. Furthermore, the dietary and waterborne exposures were separated in our experiments such that their respective influences on metal accumulation in fish could be evaluated. Finally, we examined the interaction of Cu- and Ag-combined pre-exposure and its influence on metal accumulation in fish.

MATERIALS AND METHODS

Fish and metals. Juveniles of the marine three-line theraponid *Terapon jarbua* (3 to 5 cm long, dry weight of 0.03 to 0.44 g for different batches of experiments, dry weight to wet weight ratio of 1:5.2), which is an important commercial fish consumed in Hong Kong, were purchased from a fish hatchery farm in Hong Kong. Upon arrival in the laboratory, the fish were acclimated in aerated natural seawater (25°C, 30 psu) and fed frozen shrimps (obtained from a local supermarket) on a daily basis at a ration of 2% of body wet weight. The fish were acclimated for 5 to 7 d immediately prior to the exposure experiments. All experiments were performed in 0.22 µm-filtered natural seawater at the temperature and salinity mentioned above. The metals used in the pre-exposure were Ag (as $AgNO_3$) and Cu (as $CuCl_2$), and the biokinetics of Ag and Cd were studied using their respective radiotracers: ^{110m}Ag ($t_{1/2}$ = 249 d, in 0.1 N HNO_3, from Riso National Laboratory, Denmark) and ^{109}Cd ($t_{1/2}$ = 462 d, in 0.1 N HCl, from New England Nuclear, USA).

Metal pre-exposure. Ag (as $AgNO_3$) or Cu (as $CuCl_2$) was used as waterborne or dietary spiking metals during the pre-exposure. A total of 3 different experiments were conducted. The exposure treatments and the nominal concentrations of Ag or Cu spiked into the seawater or in the diets in each experiment are listed in Table 1. Expt 1 tested the influences of 1 wk of aqueous or dietary Cu pre-exposure on the subsequent uptake of Ag and Cd. The lowest dissolved Cu concentration (1 µg l^{-1}) was about twice the background dissolved Cu concentration measured in Hong Kong coastal waters (W.-X. Wang unpubl. data). The current criterion continuous concentration (CCC) and criteria maximum concentration (CMC) for Cu for marine waters proposed by the US Environmental Protection Agency are 3.1 and 4.8 µg l^{-1}, respectively (US EPA 2002). Expt 2 tested the influences of 1 wk of aqueous or dietary Ag pre-exposure on the subsequent uptake of Ag and Cd. In this experiment, dissolved exposure and dietary exposure were conducted at different times. The lowest dissolved Ag concentration used in the pre-exposure (0.2 µg l^{-1}) was about 30 times the background concentration of dissolved Ag measured in Hong Kong coastal waters (W.-X. Wang unpubl. data). The current CMC for Ag for marine waters proposed by the US Environmental Protection Agency is

1.9 µg l^{-1} (US EPA 2002). In Expt 3, the fish were exposed to different combined concentrations of dissolved Ag and Cu for 1 wk, and their interactive effects on subsequent Ag and Cd uptake were measured.

In each exposure experiment, there was a control treatment that did not receive any spike of metals in the water or diet.

During the 1 wk pre-exposure, about 35 individual fishes were placed in 100 l of sand-filtered seawater with aeration. In the waterborne metal-exposure treatments, the fish were exposed to metal-spiked seawater and fed with the clean frozen shrimp meat twice daily. In the dietary pre-exposure experiments, the fish were maintained in clean seawater and fed with metal-enriched shrimp tissues (Waltham Aquacenter) for 4 h each day. The shrimp tissues had been previously soaked in metal ($AgNO_3$ or $CuCl_2$)-enriched seawater for 1 d, and metal concentrations in the diets were measured by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) (Perkin-Elmer, Elan 6000) following acid digestion (Table 1). It was assumed that leaking of Ag or Cu from the shrimp tissues into the seawater followed by fish uptake from the water was negligible during the short feeding period (4 h). Seawater for all groups was regularly replaced (10% daily), and the feces were siphoned from the bottom of the aquarium every day.

No apparent toxic effect on the fish at the tested metal concentrations in all pre-exposure experiments was found. By the end of 1 wk pre-exposure, the body sizes of fish in all experimental treatments were comparable to the control fish. Clearwater et al. (2002) reviewed the critical toxic Cu dosage to different species of freshwater fish. For the laboratory-prepared diets, dietborne Cu toxicity occurred at daily doses of 1 to 45 µg g^{-1} d^{-1}. The daily dosage of Cu in our experimental fish was 0.8 to 2.9 µg g^{-1} d^{-1} for the 2 dietary Cu treatments. In addition, it is also possible to calculate the dosage of Cu as a result of drinking seawater during the aqueous exposure. Assuming a drinking rate of 12 ml g^{-1} h^{-1} for the fish (Skadhauge & Lotan 1974, Clearwater et al. 2002), the daily dosage of Cu would be 0.3 and 2.9 µg g^{-1} d^{-1} at dissolved Cu concentrations of 1 and 10 µg l^{-1}, respectively. These aqueous dosages were thus comparable to the dietary dosages in the dietary exposure experiments.

In all experiments, the assimilation efficiency (AE), the dissolved uptake of metals, the metal concentrations in different fish tissues and the metallothioneins (MT) in fish were measured following 1wk pre-exposure.

Metal tissue concentrations and MT quantification. At the end of pre-exposure, a subsample of fish from each treatment was dissected into gills, viscera (including the liver and gall bladders) and the muscle. For the dietary-exposure treatments, the fish evacuated their guts for 12 h before dissection. Any remaining undigested food was not included in the measurement. Dissected fish tissues were first rinsed with filtered seawater and dried at 80°C to a constant weight and then digested in concentrated nitric acid (HNO_3, Aristar grade, BDH). Metal tissue concentrations were quantified by ICP-MS and expressed as micrograms per gram dry weight. A certified reference material (Standard Reference Material 1566a oyster tissue, National Institute of Standards and Technology, Gaitherburg, MD, USA) was also included in the analysis, and agreement was within 10%.

Another subsample of pre-exposed fish was dissected into 3 parts (gills, viscera and muscle), and the metallothionein concentrations were measured using a modified silver saturation method (Scheuhammer & Cherian 1991, Shi & Wang 2004). Briefly, the tissues were homogenized in cold 0.25 M sucrose and ultrasonicated. After centrifugation at 16000 × *g* for 20 min, the supernatants were incubated with 0.5 M glycine buffer, 20 µg Ag ml^{-1} and 3.7 kBq ml^{-1} ^{110m}Ag for 10 min. The excess Ag was then removed using rabbit blood cell hemolysate by heating (5 min at 100°C) and centrifugation (5 min at 5400 × *g*). After further centrifugation at 19000 × *g* for 20 min, the ^{110m}Ag in the final supernatant was radioassayed. The MT concentrations were calculated as 3.55 times the Ag concentrations and expressed as micrograms per gram wet weight (Scheuhammer & Cherian 1991). The MT concentration of the whole fish was calculated as the weighed average of MT levels in all 3 parts of the fish.

Metal assimilation in fish. Assimilation efficiencies of Ag and Cd were measured as described in Xu & Wang (2002). Cysts of the prey brine shrimp *Artemia* were hatched and grown in

Table 1. Concentrations of Cu and Ag used in different pre-exposure experiments. The exposure time in all experiments was 1 wk. Metal concentrations in the waterborne control treatments and in the food treatments are the actual measurements, and in the waterborne spiked treatments are the nominal concentrations

Experiment	Metal	Pre-exposure treatments	
		Waterborne (µg l^{-1}, nominal)	Food (µg g^{-1} dry weight)
1	Cu	Control (0.5), 1, 10	Control (7), 43, 146
2	Ag	Control (0.007), 0.2, 2, 10, 20	Control (0.1), 0.3, 5.2
3	Cu + Ag	Control (0.5 + 0.007), 1 + 0.2, 1 + 10, 10 + 0.2, 10 + 10	

the laboratory for 2d before they were radiolabeled with the radioisotopes ^{110m}Ag (111 kBq l^{-1}) and ^{109}Cd (148 kBq l^{-1}) for 2d. The radiolabeled brine shrimp larvae were then collected and fed to 5 fish from each treatment for about 1 h (added every 10 min to maintain a constant prey density). After radioactive feeding treatments, the fish were rinsed by placing them in nonradioactive seawater for 2 to 3 min, afterwards the radioactivity of each fish was immediately measured. The fish were then depurated of their ingested prey individually in nonradioactive water (5 l) for 48 h. The water was renewed every 12 h, and the radioactivity retained in fish was measured at intervals from 3 to 12 h. Any feces were also removed when they were produced, to minimize radioisotope recycling in the water. The AE was calculated as the percentage of metals remaining in the fish after 48 h of depuration.

Metal uptake from the dissolved phase. The radioisotopes (17.5 kBq l^{-1} for ^{110m}Ag, corresponding to 1.6 nM, and 5.1 kBq l^{-1} for ^{109}Cd, corresponding to 1.0 nM) were first equilibrated with the 0.22 µm-filtered seawater (5 l) for 12 h. Individual fish were then placed into the seawater containing the radioactive metals. At different time points over a period of 4 h, 3 or 4 fish were removed, and the radioactivity was counted after the fish was rinsed by placing it in nonradioactive seawater for 2 to 3 min. The fish were subsequently dissected into different body parts (gills, viscera and muscle), and their radioactivity was further measured. Samples of seawater were also removed to measure the radioactivity in the seawater. Decrease of radioactivity in the water during the exposure period was < 5% of the initial radioactivity. The newly accumulated metal concentrations in the fish were then calculated using the specific activity of the radioisotope.

Since the dissolved uptake exhibited a hyperbolic function in this study, the accumulation was simulated using the simple first-order kinetic model:

$$dC/dt = k_1 \times C_w - k_2 \times C \quad (1)$$

where C is accumulated metal concentration in the fish, C_w is the metal-exposure concentration, k_1 is the uptake rate constant, and k_2 is the efflux rate constant. This equation can be solved to calculate the accumulated metal concentration (C_t) at time t:

$$C_t = \frac{k_1 \times C_w [1 - e^{-k_2 \times t}]}{k_2} \quad (2)$$

Data were fitted into this equation, and model parameters were estimated simultaneously using the nonlinear iterative least squares method (Sigmaplot Software). Values of k_1 and k_2 were expressed as ml g^{-1} d^{-1} and d^{-1}, respectively.

Radioactivity measurements and statistical analysis. Radioactivity was measured by the Wallac 1480 NaI (T1) gamma counter (Wallac Turku). All counts were related to appropriate standards. The gamma emission of ^{110m}Ag was determined at 658 keV, and ^{109}Cd, at 88 keV. Counting times were adjusted to yield a propagated counting error of <5%. Statistical analysis was carried out by *t*-test. All the percentage data were arcsine-transformed, tested for homogeneity of variance and normal distribution before statistical analysis. The level of significance for all tests was $\alpha = 0.05$. Regression analysis was also conducted to test for the correlation between the biokinetics of metals and the metal tissue concentration or MT concentration.

RESULTS

Fish exposed to dietary and waterborne Cu

Following either dietary or waterborne Cu exposure, the measured Cu and MT concentrations in the gills, viscera and muscle tissues of *Terapon jarbua* generally increased (Table 2). Exposure at the low concentration of dissolved Cu (1 µg l^{-1}) did not result in a significant accumulation of Cu and MT (except for the MT in the viscera). At 10 µg l^{-1}, Cu and MT concentrations in all 3 different tissues were 1.6 to 2.4 times those of the control treatments. In the low dietary Cu treatment (43.4 µg g^{-1}, with a dosage of about 0.8 µg g^{-1} d^{-1}), MT induction was more responsive to Cu exposure than Cu accumulation. Significant induction of MT in the gills and viscera was found in this treatment, whereas their body Cu concentrations were comparable to the control treatment. In the high dietary Cu treatment (146 µg g^{-1} , with a dosage of about 2.9 µg g^{-1} d^{-1}), Cu and MT concentrations in all 3 different body parts were 1.8 to 2.8 times those of the controls, except for the Cu concentration in the gills. Typically, the viscera had the highest concentrations of Cu and MT among the 3 different parts of fish. Following the dietary Cu exposure, the muscle tissues had higher concentrations of Cu and MT than the gills did, but such trends were reversed following waterborne Cu exposure.

Uptake of Ag and Cd by the Cu-exposed fish exhibited a hyperbolic pattern over the 4 h exposure period (Fig. 1). The uptake was therefore simulated using a simple kinetic model, and the calculated k_1 and k_2 values are shown in Table 2. Since different individual fish were measured at different time points, only mean uptake was used in fitting the curve. There was a general increase in k_1 (for Ag) and a decrease in k_2 (for both Ag and Cd) with increasing dietary or waterborne Cu exposure. The depuration of both ingested Ag and Cd by the fish leveled off by 36 h, following an initially

Table 2. *Terapon jarbua*. Concentrations of Cu and metallothionein (MT) in different body parts of fish following 1 wk pre-exposure to different concentrations of dietary and waterborne Cu, and the calculated dissolved uptake rate constant (k_1), efflux rate constant (k_2) and dietary assimilation efficiency (AE) of Ag and Cd in the fish. The numeric under 'Treatment' is the Cu concentration used during the pre-exposure (units: µg l^{-1} for waterborne Cu, µg g^{-1} for dietary Cu). Data are means ± SD (n = 3 to 5). Asterisks denote a significant difference of the pre-exposed treatment from the control ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

Treatment	Tissue concentration (µg g^{-1} dry weight)			MT (µg g^{-1} wet weight)			k_1 (ml g^{-1} d^{-1})		k_2 (d^{-1})		AE (%)	
	Gills	Viscera	Muscle	Gills	Viscera	Muscle	Ag	Cd	Ag	Cd	Ag	Cd
Waterborne												
Control	14.7 ± 4.2	30.4 ± 3.8	10.9 ± 2.7	5.3 ± 0.4	21.5 ± 2.8	4.9 ± 0.4	1.688	0.327	0.035	0.034	19.9 ± 4.1	11.6 ± 2.2
Cu1	17.4 ± 1.9	36.9 ± 5.1	11.6 ± 2.5	6.8 ± 1.0	29.5 ± 3.1*	5.9 ± 1.3	1.830	0.300	0.032	0.030	20.9 ± 8.9	15.8 ± 4.1
Cu10	34.6 ± 4.9**	60.2 ± 5.2**	17.7 ± 2.9	11.2 ± 2.4**	45.8 ± 3.5**	9.9 ± 2.9*	2.001	0.370	0.028	0.024	29.1 ± 3.2*	22.1 ± 2.7**
Dietary												
Cu43	15.5 ± 3.56	39.8 ± 5.0	17.8 ± 0.9*	7.1 ± 1.2*	32.6 ± 4.2*	12.3 ± 2.9**	1.744	0.333	0.030	0.026	24.4 ± 4.8	16.1 ± 1.4*
Cu146	18.9 ± 3.12	55.0 ± 4.7**	25.6 ± 2.2***	9.0 ± 1.0*	40.0 ± 4.9**	13.9 ± 2.9**	1.972	0.352	0.029	0.024	27.3 ± 6.9	20.1 ± 3.7*

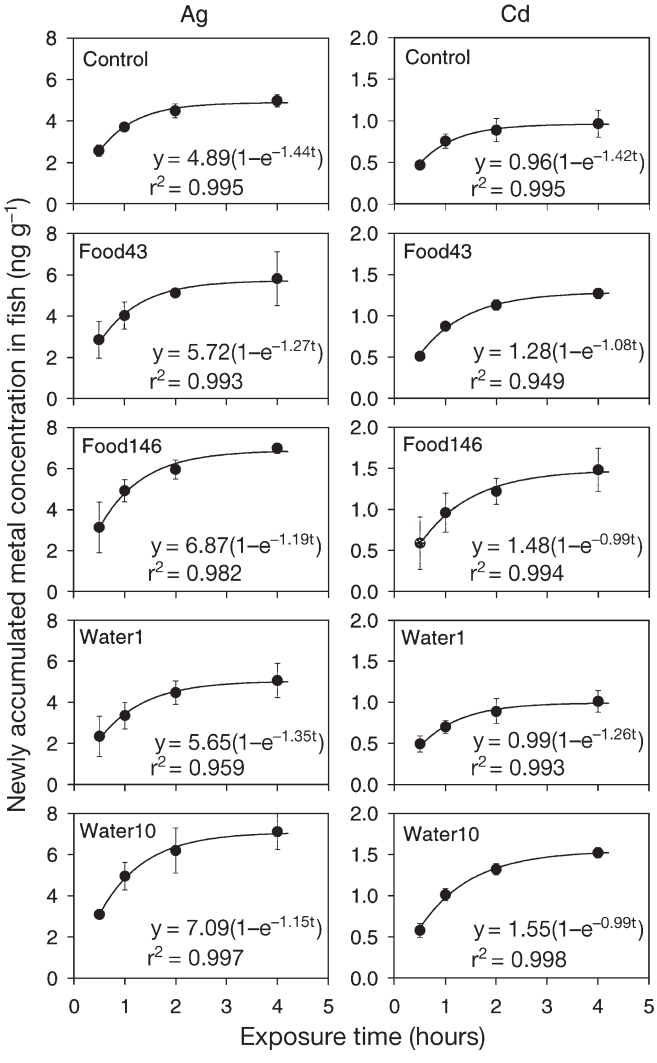

Fig. 1. *Terapon jarbua*. Calculated newly accumulated concentrations of Ag (left-hand panels) and Cd (right-hand panels) in fish following pre-exposure to different dietary or waterborne Cu levels for 1 wk. The uptake curve was fitted by a kinetic equation. The legends show the nominal Cu concentrations used during pre-exposure (µg g^{-1} in food or µg l^{-1} in waterborne exposure). Mean ± SD (n = 4)

intense digestion period (Fig. 2). The measured AEs for Ag and Cd in the control treatment were 19.9 and 11.6%, respectively (Table 2). The AEs of Ag and Cd generally increased with increasing Cu exposure, and such differences were statistically significant in several experimental treatments (e.g. Ag and Cd AEs at 10 µg Cu l^{-1}, and Cd AE in the 2 dietary Cu treatments).

Fish exposed to waterborne or dietary Ag

After exposure to dissolved Ag, the Ag concentrations in the gills, viscera and muscle tissues were significantly higher than in the controls ($p < 0.05$), except

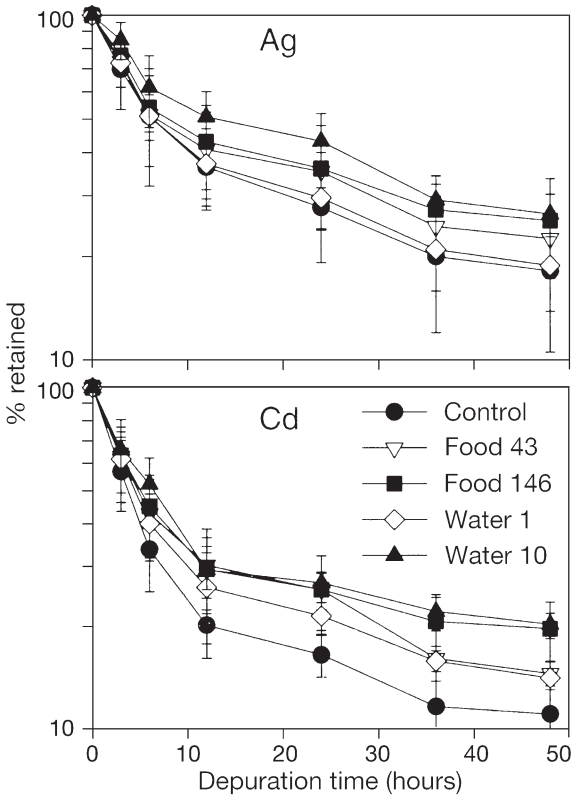

Fig. 2. *Terapon jarbua*. Retention of Ag and Cd in the pre-exposed fish following a pulse ingestion of radiolabeled brine shrimps. The fish were pre-exposed to different dietary or waterborne Cu concentrations for 1 wk. The key shows the Cu concentrations used during pre-exposure (μg g^{-1} in food or μg l^{-1} in waterborne exposure). Mean ± SD (n = 5)

at the lowest Ag concentration tested (0.2 μg l^{-1}) (Table 3). The degree to which the body concentration was elevated was comparable among the 3 different body tissues. Ag concentration in the viscera was surprisingly high (10 μg g^{-1}) in the control treatment. Similar trends were also observed for MT induction in different fish tissues (Table 3). The highest MT concentrations were found in the viscera and the order viscera > gill > muscle tissues.

The uptake of dissolved Ag and Cd by fish followed a hyperbolic pattern model (data not shown), and the calculated k_1 and k_2 values are listed in Table 3. Consistently, k_1 of Ag and Cd increased, whereas k_2 decreased with the increasing pre-exposed Ag concentration. The AEs of Ag and Cd from the ingested prey also increased with increasing aqueous Ag pre-exposure concentration. However, the AEs were significantly different from the control fish only when the fish were pre-exposed to high dissolved Ag concentrations.

Only 2 Ag concentrations were considered in experiments with dietary Ag exposure. After being fed with dietary Ag for 1 wk, the 3-line theraponids had signifi-

Table 3. *Terapon jarbua*. Concentrations of Ag and metallothionein (MT) in different body parts of fish following 1 wk pre-exposure to different concentrations of waterborne and dietary Ag, and the calculated dissolved uptake rate constant (k_1), efflux rate constant (k_2) and dietary assimilation efficiency (AE) of Ag and Cd in the fish. The numeric under 'Treatment' is the Ag concentration used during the pre-exposure (units: μg l^{-1} for waterborne Ag, μg g^{-1} for dietary Ag). Waterborne and dietary pre-exposures were conducted at 2 different times. Data are means ± SD (n = 3 to 5). Asterisks denote a significant difference of the pre-exposed treatment from the control (* $p < 0.05$; ** $p < 0.01$)

Treatment	Tissue concentration (μg g^{-1} dry weight)			MT (μg g^{-1} wet weight)			k_1 (ml g^{-1} d^{-1})		k_2 (d^{-1})		AE (%)	
	Gills	Viscera	Muscle	Gills	Viscera	Muscle	Ag	Cd	Ag	Cd	Ag	Cd
Waterborne												
Control	3.0 ± 0.9	10.0 ± 4.3	0.9 ± 0.0	5.7 ± 1.4	20.6 ± 5.5	4.3 ± 0.7	0.141	0.025	0.025	0.026	29.5 ± 3.2	16.7 ± 5.1
Ag0.2	3.8 ± 1.9	14.8 ± 5.9	1.4 ± 0.5	6.4 ± 0.4	32.1 ± 9.5	4.8 ± 0.4	0.149	0.025	0.022	0.024	30.4 ± 1.9	22.1 ± 7.0
Ag2	5.1 ± 0.4*	21.3 ± 4.4*	1.6 ± 0.2*	7.5 ± 1.1	33.8 ± 6.9*	5. 6 ± 1.8	0.156	0.027	0.019	0.020	31.6 ± 4.4	23.6 ± 5.3
Ag10	7.2 ± 1.3**	26.0 ± 1.9**	1.8± 0.1*	8.5 ± 0.6*	36.4 ± 8.6*	7.6 ± 0.2**	0.162	0.029	0.017	0.018	34.5 ± 5.1	25.0 ± 2.9*
Ag20	7.8 ± 1.7**	28.3 ± 1.6**	1.9 ± 0.4*	9.6 ± 1.3*	41.5 ± 7.7**	9.2 ± 2.6**	0.220	0.030	0.016	0.015	37.5 ± 2.9*	29.6 ± 6.4*
Dietary												
Control	1.7 ± 0.6	6.2 ± 0.3	0.2 ± 0.1	3.9 ± 0.4	22.5 ± 1.4	3.9 ± 0.6	0.754	0.091	0.077	0.112	21.1 ± 0.9	13.7 ± 1.9
Ag0.3	2.6 ± 0.6	15.2 ± 2.4**	0.6 ± 0.2*	4.2 ± 0.6	27.9 ± 8.7	6.4 ± 0.9**	0.763	0.095	0.076	0.110	21.8 ± 3.7	15.7 ± 8.1
Ag5.2	2.8 ± 1.3	17.0 ± 3.7**	0.8 ± 0.2**	4.7 ± 0.3*	34.6 ± 8.7*	8.6 ± 0.7**	0.869	0.140	0.070	0.110	24.4 ± 1.7	19.6 ± 2.8

cantly higher Ag concentrations in the viscera and muscle tissues, but there was no significant increase of Ag concentration in the gills (Table 3). Significant induction of MT was observed in the muscle tissues for both dietary Ag-exposed treatments and in the viscera for the highest Ag-exposed treatment. The viscera had the highest Ag body concentration, followed by gills and muscle tissues. However, the muscle tissue had a higher MT concentration compared to the gills in the Ag-exposed fish.

Uptake of dissolved Ag and Cd after dietary Ag pre-exposure similarly followed a hyperbolic pattern during the 4 h exposure period (data not shown). The pre-exposed fish had a higher uptake of Ag and Cd than that of the control fish (Table 3). For both Ag and Cd, an increasing trend for k_1 and a decreasing trend for k_2 with increasing Ag dietary concentration were found, but such a trend was less obvious for k_2 in the other experiments. Similar trends of Ag and Cd retention in the fish following ingestion of radiolabeled prey were found in this experiment (data not shown). However, there was no statistically significant difference between the Ag and Cd AEs in the dietary Ag-exposed groups and the control treatment. For example, the AEs for Ag and Cd in the control group were 21 and 14 %, respectively, and were 22 to 24 % and 16 to 20 % for both dietary Ag pre-treatments.

Fish exposed to a waterborne Cu and Ag mixture

Since the sizes of fish used in the Ag + Cu mixture experiments were small, only the whole individual fish was quantified for their accumulated Cu and Ag concentrations and the induced MT concentrations (Table 4). The accumulated Cu and Ag concentrations were much higher than those in the control treatments. Fish exposed to a higher Ag concentration had a lower body Cu concentration, suggesting that Ag competitively reduced the Cu uptake in the fish. A similar trend was also observed for Ag body concentration at different Cu concentrations (e.g. a lower Ag body burden at a higher Cu-exposure concentration). Obvious MT induction was observed in fish exposed to higher concentrations of ambient Cu and Ag mixtures. At the lowest Cu + Ag concentration (1 µg Cu l^{-1} + 0.2 µg Ag l^{-1}), the MT concentration was comparable to that in the control treatment.

Uptake of Ag and Cd by the fish leveled off at about 2 h, after an initially rapid accumulation (data not shown), similar to Expt 1 (Fig. 1). There was a general trend of increasing k_1 and decreasing k_2 for both Ag and Cd with increasing dosage of Ag and Cu (Table 4). For example, k_1 for Ag and Cd at the highest dosages of Cu + Ag (10 µg l^{-1} for both metals) was 1.5 and 2.4 times, respectively, those of the control fish, while k_2 decreased by 84 and 83 %, respectively. A similar trend for the AEs of Ag and Cd was also observed in this experiment (Table 4). The quantified AEs of Ag and Cd in the Cu + Ag-exposed fish were higher than those in the control group. At the highest Cu + Ag concentrations, the AEs of Ag and Cd were as high as 35 and 29 %, respectively, compared to 22 and 14 % in the control fish.

Correlations between parameters measured in experiments

In all single-metal-exposure experiments (Expts 1 and 2), the MT contents in whole individual fish and different parts (gills, viscera and muscle tissues) increased linearly with increasing accumulated metals in the theraponids exposed to Cu and Ag (Fig. 3). There was no notable difference in the relationship between MT induction and metal accumulation following waterborne or dietary metal exposure, except for Ag accumulation in the muscle tissues of fish, in which the dietary Ag pre-exposure resulted in higher MT concentrations relative to that following the waterborne Ag pre-exposure.

Table 4. *Terapon jarbua*. Concentrations of Ag, Cu and metallothionein (MT) in the fish body following 1 wk pre-exposure to different combined dissolved concentrations of Ag and Cu, and the calculated dissolved uptake rate constant (k_1), efflux rate constant (k_2) and dietary assimilation efficiency (AE) of Ag and Cd in the fish. The numeric under 'Treatment' is the Cu and Ag concentration used during the exposure (µg l^{-1}). Data are means ± SD (n = 3 to 6). Asterisks denote a significant difference of the pre-exposed treatment from the control ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

Treatment	Body burden (µg g^{-1} dry weight)		MT (µg g^{-1} wet weight)	k_1 (ml g^{-1} d^{-1})		k_2 (d^{-1})		AE (%)	
	Cu	Ag		Ag	Cd	Ag	Cd	Ag	Cd
Control	7.7 ± 1.6	1.1 ± 0.5	12.5 ± 2.5	0.871	0.099	0.050	0.032	22.3 ± 5.9	13.6 ± 5.4
Cu1 + Ag0.2	17.2 ± 2.1*	2.2 ± 0.3	13.2 ±1.1	0.900	0.131	0.034	0.028	26.6 ± 3.8	18.9 ± 2.4
Cu1 + Ag10	11.8 ± 0.9*	10.1 ± 0.7***	22.1 ± 3.7*	1.042	0.174	0.030	0.028	27.4 ± 6.9	21.5 ± 1.4
Cu10 + Ag0.2	28.1 ± 2.1**	1.3 ± 0.1	29.7 ± 3.9**	1.097	0.215	0.027	0.026	33.3 ± 6.7	24.0 ± 3.1*
Cu10 + Ag10	21.8 ± 1.8**	6.8 ± 0.5**	43.9 ± 7.0***	1.294	0.236	0.027	0.017	35.3 ± 6.1	28.8 ± 1.3**

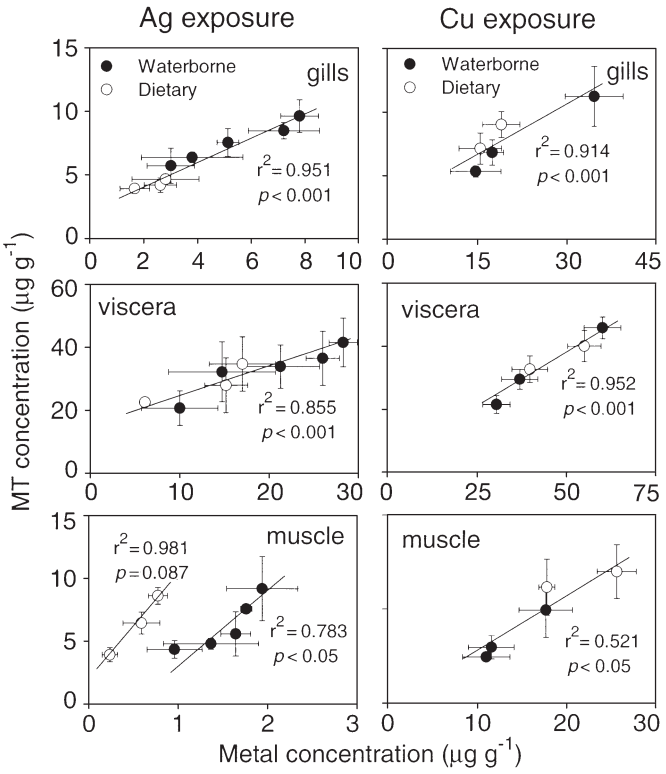

Fig. 3. *Terapon jarbua*. Relationships between the metal concentration and the metallothionein induction in different body parts (gills, viscera and muscle) of 3-line theraponids after being exposed to waterborne or dietary Ag (left-hand panels) and Cu (right-hand panels) for 1 wk. Mean ± SD (n = 3 to 4)

The relationships between metal accumulation, MT induction and biokinetic parameters for Ag or Cd in the fish after being exposed to waterborne or dietary Ag or Cu are shown in Figs. 4 to 6. The biokinetic parameters (k_1, k_2 and AE) were calculated as an index (ratio) by comparing their mean values with their respective control values. In the Cu-exposure experiment, k_1 and AE of both Ag and Cd increased linearly with increasing Cu body concentration in the fish, whereas k_2 decreased (Fig. 4). In the Ag-exposure experiment, it appeared that the biokinetic parameters remained rather constant, until the tissue Ag concentration exceeded 1.5 µg g^{-1} (Fig. 5). Above these threshold concentrations, k_1 and AE of both Ag and Cd increased, whereas k_2 decreased. The dietary Ag pre-exposure resulted in a much lower Ag body burden than the aqueous Ag pre-exposure; thus, it was difficult to compare the biokinetics at high Ag body burden between these 2 pre-exposure routes. For both Cu- and Ag-exposure experiments, all the 3 biokinetic parameters were significantly correlated with the MT concentrations in the theraponids (Fig. 6), although the correlation with MT only explained 35 to 60 % of the relationship for most of the combinations (and 85 % in 1 case).

DISCUSSION

Metal accumulation and metallothionein induction

The accumulated levels of Ag and Cu in the 3-line theraponids *Terapon jarbua* measured in this study were comparable to those in field-collected fish or laboratory exposure studies (Wood et al. 1996, Kamunde et al. 2002a,b). The theraponids accumulated Ag and Cu following waterborne or dietary exposure. The highest accumulated Cu and Ag concentrations in different tissues during the 1 wk exposure period were about 3 and 5 times the concentrations in the control fish, respectively. However, a notable difference in the relative distribution of metals in different body parts was documented after waterborne and dietary exposures. When the fish were exposed to waterborne Ag and Cu, the viscera had the highest accumulated concentration, and the muscle tissue had the lowest concentrations. An underlying mechanism is that the viscera (intestinal) rather than the gills of marine fish was the primary uptake site for several waterborne metals (Hogstrand & Wood 1998), because marine fish drink water to prevent dehydration in the hyper-

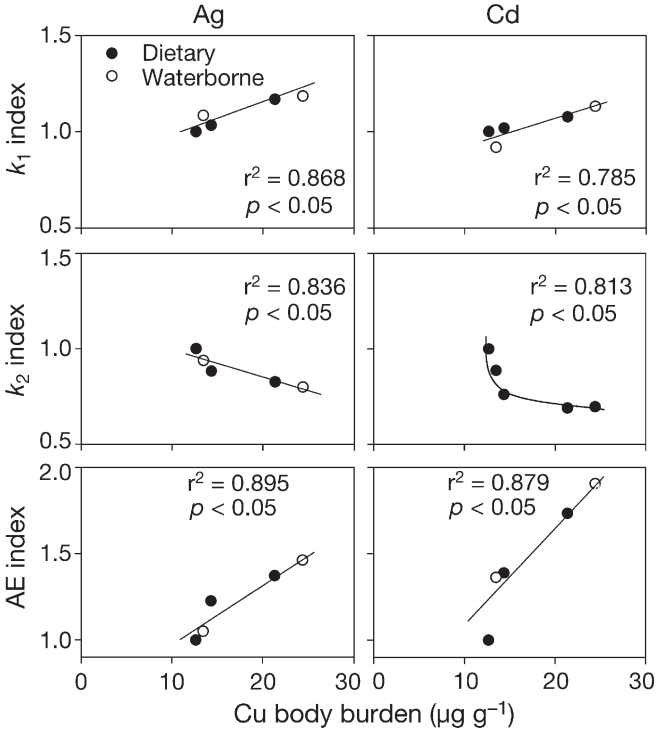

Fig. 4. *Terapon jarbua*. Relationships between the indexes of dissolved uptake rate constant k_1, efflux rate constant k_2 and dietary assimilation efficiency (AE) of Ag (left-hand panels) and Cd (right-hand panels) and the accumulated Cu whole body concentrations in fish after being pre-exposed to waterborne or dietary Cu for 1 wk. Indexes were calculated as the ratios to their respective control mean values

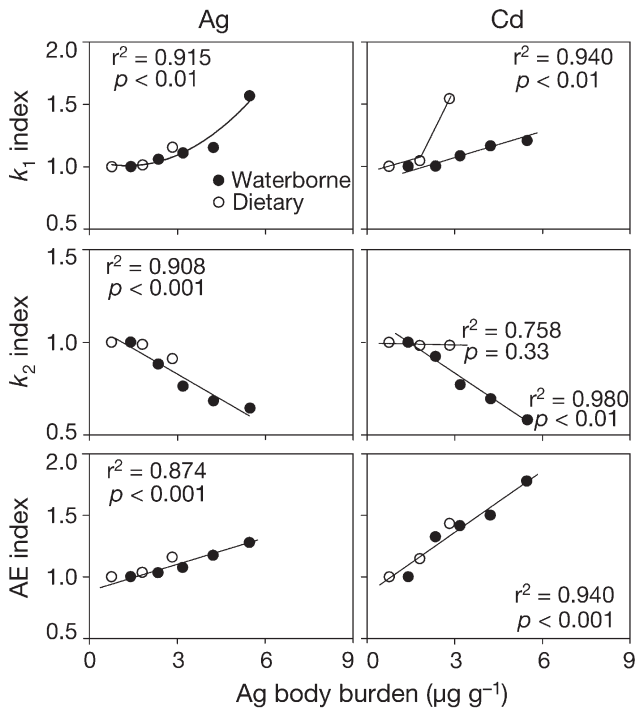

Fig. 5. *Terapon jarbua*. Relationships between the indexes of dissolved uptake rate constant k_1, efflux rate constant k_2 and dietary assimilation efficiency (AE) of Ag (left-hand panels) and Cd (right-hand panels) and the accumulated Ag whole body concentrations in fish after being pre-exposed to waterborne or dietary Ag for 1 wk. Indexes were calculated as the ratios to their respective control mean values

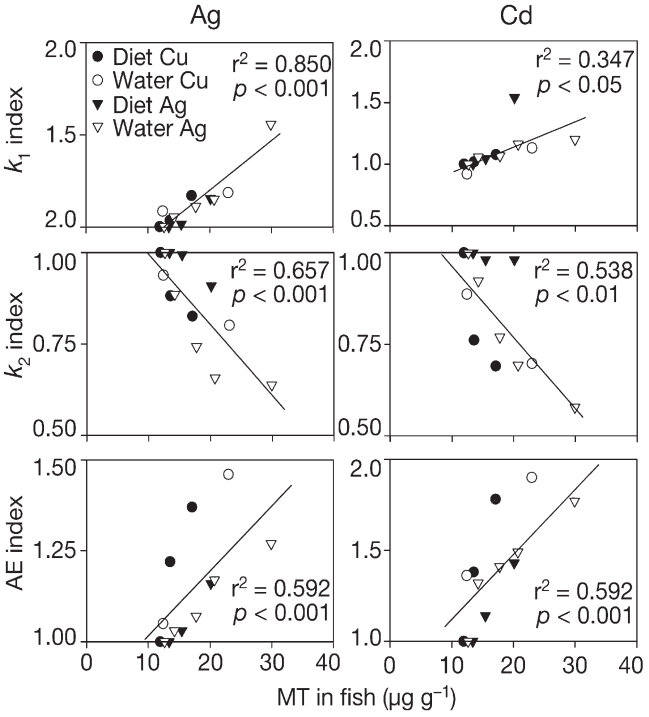

Fig. 6. *Terapon jarbua*. Relationships between the indexes of dissolved uptake rate constant k_1, efflux rate constant k_2 and dietary assimilation efficiency (AE) of Ag (left-hand panels) and Cd (right-hand panels) and the metallothionein (MT) whole body concentrations in fish after being pre-exposed to waterborne or dietary Ag and Cu for 1 wk. Indexes were calculated as the ratio to their respective control mean values

osmotic environment. The muscle tissues were the largest body fraction, and their concentrations were correspondingly low. In freshwater fish, the gut tends to accumulate the highest concentration of metals after dietary exposure, whereas the gills tend to accumulate the highest concentration of metals after dissolved metal exposure (Harrison & Klaverkamp 1989, Clearwater et al. 2000).

Ag and Cu accumulation in the theraponids was accompanied by metallothionein induction. MT can bind the metals at a relatively stable ratio, e.g. 1 M MT bound with 12 M Cu when saturated (Hollis et al. 2001). For Ag, 1 μg Ag can bind with 3.55 μg MT (Scheuhammer & Cherian 1991), and the mole ratio of MT to Ag was about 1:15.4 using a molecular weight of 6000 Da for MT (Hollis et al. 2001). In all of our 3 independent exposure experiments, the ratios of MT:Ag and MT:Cu for gills, viscera and muscle tissues were far lower than the theoretical ratios of 1:15.4 and 1:12, respectively. Thus, the induced MT during pre-exposure probably did not bind up all of the Ag and Cu in the fish tissues. Other ligands may also be responsible for the sequestration of these metals in the fish. Galvez & Wood (1999) found that only a small portion (<20%) of Ag was indeed bound with MT. Cu is essential for survival (Satoh et al. 1983) and a cofactor for several proteins that carry out fundamental functions in growth and development (Linder & Hazegh-Azam 1996). When the accumulated Cu is in excess of cellular requirements, Cu may become toxic and can be bound by MT for detoxification (Pena et al. 1999). There was an obvious increase of MT in all tissues with increasing waterborne or dietary Ag and Cu concentrations.

Because MT induction varies with species, reproductive condition, age and diet (Filipovic & Raspor 2003), the relationship between MT and metal concentration in fish is organ-specific. To our knowledge, there has been no report concerning the organ-specific relationship of metal concentration and MT induction. Positive linear correlation of MT and Ag or Cu concentration in gills, viscera and muscle tissues was observed in our study, implying that the accumulated Ag or Cu was sequestered by MT. Regardless of the exposure route, the MT levels in whole fish, gills and viscera increased with increasing Ag concentration via either waterborne or dietary exposure. A similar trend for Cu concentration and MT induction in whole body, viscera

and muscle was also observed. However, the relationship between Ag concentration and MT concentration in the muscle was somewhat different. The slope for MT induction was greater after dietary Ag exposure than after waterborne Ag exposure, suggesting that MT induction was more sensitive to dietary Ag than waterborne Ag. For marine fish, Na/KATPase is the target for Ag toxicity. Two possible sites of Ag toxicity are the intestinal Na^+ and Cl^- uptake and the branchial Na^+ and Cl^- extrusion (Grosell & Wood 2001), both of which could be affected by waterborne Ag exposure in marine fish. Given the greater induction of MT in the muscle tissues, it may be possible that dietary Ag offered less potential toxicity to the fish than waterborne exposure, but it is also possible that the potentially higher uptake resulted in a higher toxicity to the fish.

Metal uptake kinetics after pre-exposure

The uptake rate constant k_1 and efflux rate constant k_2 measured in the present study varied greatly among different experiments using different batches of fish, and were generally lower than the pervious measurements in other species of marine fish. In the control fish, k_1 ranged from 0.141 to 1.688 ml g^{-1} d^{-1} for Ag and from 0.025 to 0.327 ml g^{-1} d^{-1} for Cd, and k_2 ranged from 0.025 to 0.077 d^{-1} for Ag and from 0.032 to 0.112 d^{-1} for Cd. Xu & Wang (2002) measured the k_1 and k_2 values for Cd in the mangrove snapper *Lutjanus argentimaculatus* at 5.10 ml g^{-1} d^{-1} and 0.025 to 0.047 d^{-1}, respectively. Differences may be due to the different kinetic models as well as the fish species and sizes used in different studies. In the study of Xu & Wang (2002), the values of k_1 were obtained by linear regression based on the measured uptake rates as a function of different ambient Cd concentrations. In our study, the uptake was quantified at a fixed Cd concentration, and both k_1 and k_2 were simulated using the first-order kinetic model.

Both k_1 and k_2 responded significantly in the theraponids to the waterborne and dietary Ag or Cu exposure, showing that exposed fish had different uptake and depuration kinetics. There was an increase in k_1 and AE and a decrease in k_2 with increasing Ag or Cu body burden and MT induction for the theraponids. However, the response to Cu exposure was probably instant, whereas the response to Ag exposure occurred when the tissue Ag concentration exceeded a certain level (1.5 µg g^{-1}). Thus, the overall bioaccumulation was facilitated after the exposure to Cu and Ag. These findings were in agreement with several other studies (McCarter & Roch 1984, Grosell et al. 1996) of the freshwater rainbow trout, and suggest that fish pre-exposed to metals, via either a waterborne or dietary phase, facilitated their subsequent uptake from their aqueous environments. However, it should also be noted that such a response may be dependent on the daily dosage of metals to the fish (Clearwater et al. 2000).

With higher k_1 and AE and a lower k_2, any pre-exposure to Ag and Cu may increase the potential of Ag and Cd bioaccumulation. McDonald & Wood (1993) postulated that the increased tolerance in fish to metals was obtained through a 'damage-repair mechanism'. With the accumulation of Ag and Cu during pre-exposure, more metal-binding complexes such as MT are induced, enabling the fish to accumulate further Ag and Cd from their ambient environments. The internal mobilization of metal-binding proteins such as MT may thus be an important factor in the restoration phase through detoxification and storage of metals. Binding with MT in the gills may abolish the inhibition of Na^+/K^+ ATPase to repair the immediate damage to fish gills resulting from metal exposure (Hogstrand & Haux 1991). At the same time, the gill metal-binding characteristics may undergo significant changes, as an adaptive response to sublethal exposure to waterborne or dietary metals (Niyogi & Wood 2003). Therefore, routes of gill metal loading may result in differences in metal uptake kinetics from the water.

The exposure to Cu and Ag both affected Ag and Cd uptake, suggesting that MT was not specific in binding with a particular metal. Our study, therefore, suggests that the exposure of one metal may potentially bring major changes in the bioaccumulation kinetics of other metals as a result of their chemical similarity in preferentially binding with a specific ligand. As discussed above, the metal detoxification by MT induction was not complete, and a large fraction of metals was also bound with other ligands. It is still possible that the MT induced by Cu or Ag pre-exposure can bind with the Cd and Ag accumulated from ambient environments.

The doses of dissolved Ag and Cu used in our experiments ranged from 'nontoxic' (i.e. less than the CCC and CMC values) to chronically toxic. In addition, the dietary Cu doses (0.8 to 2.9 µg g^{-1} d^{-1}), as well as dietary Cu concentrations were probably below the toxic Cu doses (1 to 45 µg g^{-1} d^{-1}, Clearwater et al. 2002). For Ag, the dietary Ag doses/concentrations were probably chronically toxic to the fish, since Ag was not an essential metal. In our study, it is difficult to relate the changes in metal biokinetics with whether the dosages were chronically toxic to the fish. This may be primarily due to the relatively short period of exposure (1 wk) used in our study, and there was no evidence of a toxic effect on fish growth within such a short exposure period. In a recent study, Guan & Wang (2004) demonstrated that at a high dosage of Cd to a freshwater cladoceran (*Daphnia magna*), the Cd dietary AE decreased significantly.

Cu and Ag interaction on fish

Many studies on metal toxicity to fish have been based on exposure to a single metal, and there have only been a few studies on the effects of metal mixtures on the physiological status and their subsequent metal uptake in marine fish (Buhl & Hamilton 1990). In the present study of combined exposure to Cu + Ag, the Cu body burden decreased with increasing ambient Ag concentration, and, conversely, the Ag body burden decreased with increasing Cu concentrations. Thus, there was antagonistic interaction between Cu and Ag bioaccumulation, presumably as a result of competition for binding sites of Cu^+ with Ag^+. Although Cu predominantly exists in a divalent form (Cu^{2+}) in bulk water, it is probably reduced to Cu^+ via reductases on the gill surface or in gill microenvironments through the branchial epithelium (Grosell & Wood 2002, Handy et al. 2002). Some transport channels in fish gills are also metal-dependent. For the monovalent metals, including Cu^+ and Ag^+, their uptake occurs both via H^+-coupled Na^+ channels (or Na^+/H^+ exchange) on the apical membranes and via Na^+/K^+ ATPase on the basolateral membranes of the gill ionocytes. Therefore, cationic metal ions such as Cu^+ and Ag^+ will compete with Na^+ for these specific binding sites. The divalent metal uptake probably occurs via apical voltage-insensitive Ca^{2+} channels and via basolateral high-affinity Ca^{2+} ATPase (Wood 2001, Niyogi & Wood 2003).

The antagonistic effect of Cu on Ag bioaccumulation observed in our study may also be caused by the morphological changes induced by Cu in fish tissue, including defense (inflammatory) and compensatory (cell proliferation, mucus secretion) responses (Mallatt 1985). Both responses can bar the entry of toxicants and prevent them from reaching the blood stream. There have been many studies on the disruption of osmotic and ionic functions of fish gills exposed to Cu as a result of direct Cu inhibition on Na^+/K^+ ATPase (Li et al. 1996). Since Na^+/K^+ ATPase is mainly responsible for Ag gill uptake by fish (Buhl & Hamilton 1990), the reduced Ag uptake may be caused by the decreasing Na^+/K^+ ATPase activity as a result of Cu exposure.

In conclusion, following sublethal exposure to waterborne or dietary Ag and Cu, the theraponids modified their bioaccumulation kinetics for subsequent Ag and Cd uptake. The fish increased their uptake rate constant (k_1) and assimilation efficiency (AE), and decreased their efflux rate constant (k_2) for Ag and Cd. Consequently, the potential of metal bioaccumulation was facilitated as a result of metal pre-exposure. There was and antagonistic effect between Cu and Ag bioaccumulation, presumably because of the competition for binding sites. Pre-exposure to one specific metal may potentially affect the bioaccumulation of other metals in the fish. It appears that the induced MT plays a major role in the modification of trace-metal accumulation in fish. Such responses may also be dependent on the doses of metals to the fish received.

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