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Detoxification and effects of Ag, Cd, and Zn pre-exposure on metal uptake kinetics in the clam *Ruditapes philippinarum*

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ABSTRACT: There is increasing evidence to indicate that metal pre-exposure may mediate the subsequent metal uptake from the dietary or dissolved phase by marine invertebrates. In this study, we examined the detoxification and the effects of Ag, Cd, or Zn pre-exposure on the metal uptake of the clam *Ruditapes philippinarum*. The relationships between metal body burden and metal uptake of Ag, Cd, Hg, and Zn were also concurrently examined. Radiotracers ^{110m}Ag, ^{109}Cd, ^{203}Hg, and ^{65}Zn were used to chase the metal uptake in the clams. The clams accumulated Ag and Cd over time, but there was not a significant Zn accumulation. Metallothionein played a role in the detoxification of Ag and Cd, but the insoluble fraction was also the detoxification pool for Cd and Zn. Generally, metal pre-exposure did not greatly affect metal uptake. Ag pre-exposure had no effect on dietary Ag uptake. Cd and Zn pre-exposure increased Zn dietary uptake by 10%. After 2 wk Ag exposure and 3 wk of Cd or Zn exposure, Cd and Hg dissolved uptake was reduced by 2 to 6 times. However, these effects mainly appeared during the earlier part of the exposure period (i.e. up to Weeks 2 or 3). The effects of metal exposure on uptake of dissolved Cd and Hg were related to the coupling relationships of dissolved metal uptake between the 2 metals and other metals (Ag and Cd or Hg; Cd and Hg; Zn and Hg). The effects on dietary and dissolved uptake of all metals were independent of Ag, Cd or Zn body burden; thus, metal body burdens may not necessarily indicate the potential metal uptake of marine clams in the environment. Our data have important implications for kinetic modeling predicting metal uptake by marine clams.

KEY WORDS: Silver · Cadmium · Mercury · Zinc · Pre-exposure · Uptake · Clams

INTRODUCTION

Marine invertebrates have developed diverse strategies to accumulate and detoxify metals from the environment (see review by Rainbow 2002). Metal accumulation in bivalves has been extensively studied over the past few decades, and is now known to be affected by different environmental and physiological factors such as food (e.g. Wang & Fisher 1996), salinity (e.g. Mouneyrac et al. 1998, Blackmore & Wang 2003), body size (e.g. Mouneyrac et al. 1998, Chong & Wang 2001), and gut-passage time (e.g. Amiard-Triquet et al. 1998, Griscom et al. 2002). There is increasing evidence to show that past exposure to metals may induce physiological or biochemical changes, which subsequently affect metal uptake in a few marine invertebrates. For example, clams *Macoma balthica* collected from a contaminated estuary had a lower dissolved Ag and Hg uptake than clams from an unpolluted estuary (Boisson et al. 1998). Cd assimilation increased in the green mussel *Perna viridis* pre-exposed to Cd, accompanied by the induction of metallothionein-like protein (MTLP) (Blackmore & Wang 2002). Furthermore, uptake of Cd or Zn by *P. viridis* in dissolved form was reduced after pre-exposure to Zn. An increase in Ag assimilation was similarly found in *P. viridis* following dissolved and dietary Ag exposure, and was correlated with the possible binding of Ag by the sulphide complex (Shi et al. 2003). Uptake of dissolved Ag was also differentially affected by pre-exposure to Ag. The

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clams *Mactra veneriformis* and *Ruditapes philippinarum* collected from Cd-polluted sites in northern China had a higher Cd assimilation efficiency (AE) than clams from a control site in Hong Kong (Shi & Wang in press). Besides bivalves, assimilation of Cd and Hg increased significantly in the whelk *Thais clavigera* at high metallothionein (MT) concentrations, in both field-collected specimens and following MT induction by laboratory exposure to Cd (G. Blackmore & W.-X. Wang unpubl. data). The polychaete *Capitella I* species I also had a lower Cd AE following Cd exposure (Selck et al. 1999). These recent studies suggest that it is important to consider the history of metal contamination when employing marine invertebrates in biomonitoring studies.

Trace metals may interact and thus affect their uptake, accumulation and toxicity by aquatic biota. For example, Ag concentrations increased in the soft tissue of oysters following Cu exposure (Amiard-Triquet & Amiard 1998), and Se accumulation caused lower Hg uptake in *Mytilus edulis* (Fowler et al. 1975). Amiard-Triquet & Amiard (1998) suggested that such interaction was possibly due to the similar uptake pathways of metals. Metal ions of similar size and charge, such as Ca and Cd, may compete for the same binding sites and be taken up via the same transport pathways (Hinkle et al. 1987, Blazka & Shaikh 1991, Roesijadi & Unger 1993). Among the trace metals, Cd and Zn interact in their bioavailability to marine animals. Blackmore & Wang (2002) showed that pre-exposure to high Zn concentration (250 µg l^{-1}) decreased uptake of the dissolved phase of Cd in *Perna viridis*. Cd AE of *P. viridis* also increased following 7 d exposure to 100 µg Zn l^{-1} (Blackmore & Wang 2002). Coupled assimilation of Cd and Zn has recently been found in *P. viridis* (Chong & Wang 2000), barnacles (Wang et al. 1999), and fishes (Ni et al. 2000). The interaction in the bioavailability of other metals has been less well studied than that of Cd and Zn.

The green mussel *Perna viridis* and the clam *Ruditapes philippinarum* are suitable biomonitors of metal contamination in subtropical and tropical waters because they are widely distributed and can accumulate metals to levels higher than ambient concentrations (Phillips 1985, Rainbow 1993, Cha 1994). However, the biokinetics of metal uptake by bivalves are both metal- and species-specific. The AEs of Cd and Zn of the clam *R. philippinarum* were 1.8 to 4.7 and 1.1 to 1.9 times higher, respectively, than the AEs of the mussel *P. viridis*. Factors controlling differences in metal uptake by bivalves are still unclear. The effects of pre-exposure to Ag, Cd and Zn on metal uptake of the mussel *P. viridis* have been recently determined (Blackmore & Wang 2002, Shi et al. 2003), but there is no such data for the clam, which is an important biomonitor, with different metal biokinetics than mussels. In this study we sought to extend the data on the clam *R. philippinarum*. Our objectives were to examine (1) the metal accumulation, detoxification and the physiological effects of Ag, Cd or Zn exposure; (2) the effects of pre-exposure on metal uptake from the food and in dissolved forms; (3) the relationship between the metal body burden and metal uptake; and (4) the effects of exposure of one metal on the uptake of other metals. We examined metal uptake from both food and in dissolved form for 4 metals, Ag, Cd, Hg, and Zn, primarily because these metals exhibit a similar tendency in binding with sulfur-containing ligands in the bivalves.

MATERIALS AND METHODS

Collection of clams and metal exposure. Clams *Ruditapes philippinarum* with a shell length of 2.5 to 3 cm were collected from Yung Shu Au, Tolo Harbour, Hong Kong, an area considered relatively unpolluted by metals (Blackmore & Rainbow 2001, Rainbow & Blackmore 2001). The clams were maintained at a constant temperature of 24°C in aerated seawater and fed the diatom *Thalassiosira pseudonana* (Clone 3H) at a ration of about 1 to 2% of their body tissue dry weight d^{-1}. We placed about 100 clams (0.1 g dry weight each) in each exposure container with 10 l seawater (salinity = 33 psu, pH = 7.8). They were acclimated for 1 wk before the experiments began.

Metal exposure experiments were conducted separately for Ag, Cd and Zn. For the Ag exposure experiment, Ag was spiked into the seawater at the time of food addition (diatom *Thalassiosira pseudonana*) each day, and the seawater was changed every 2 d. There were 3 treatments: Ctl (control without Ag spike), Ag0.1 (0.1 µgAg l^{-1}) and Ag5 (5 µgAg l^{-1}). Different concentrations of Ag were prepared from dilutions of $AgNO_3$ (analytical grade, 1 mg ml^{-1}). Exposure lasted for a total of 4 wk. For the Cd and Zn exposure experiments, the clams were exposed to Cd or Zn for 16 h without food each day; the water was then changed, and the clams were fed *T. pseudonana* for 8 h. There were 5 treatments: Ctl (control without metal spike), Cd2 (2 µgCd l^{-1}), Cd20 (20 µgCd l^{-1}), Zn10 (10 µgZn l^{-1}), and Zn100 (100 µgZn l^{-1}). The different concentrations of Cd or Zn were diluted from $CdCl_2$ or $ZnCl_2$ (analytical grade, 1 mg ml^{-1}). Exposure lasted for a total of 6 wk. The exposure pathway and period differed between the 2 experiments in order to allow a significant increase in the body burden of the clams. In addition, uptake of Cd and Zn in dissolved form may comprise the main bioaccumulation pathway in the clams (Chong & Wang 2001). Since Ag is a highly particle-reactive metal, the clams were exposed to Ag

in both aqueous and food forms. Previous studies indicate that the route of metal pre-exposure does not affect subsequent metal accumulation in bivalves (Shi et al. 2003, D. Shi & W.-X. Wang, unpubl. data). Exposures were all conducted at 24°C. Since the clams close their valves in response to light disturbance (T. Y.-T. Ng pers. obs.), the exposure containers were covered with lids to avoid light disturbance of their pumping activity.

Metal concentration in soft tissue. We sampled 5 clams from each treatment for metal analysis at Weeks 2 and 4 (Ag exposure) and Weeks 3 and 6 (Cd or Zn exposure). They were dissected and the soft tissue was digested for metal analysis by inductively coupled plasma-mass spectroscopy (ICP-MS, Perkin-Elmer, Elan 6000). The methods for digestion were similar to those described in Blackmore & Wang (2002). Random checks with standard reference material (Oyster Tissue 1566a, National Institute of Standards and Technology) were made. Agreement was within 10%. The soft-tissue metal concentration was expressed as µg g^{-1} dry weight.

Subcellular Cd and Zn distribution. We sampled and dissected 5 clams from each treatment in the Cd or Zn exposure experiment and measured their wet weight. Different fractions of the tissue were obtained by a modified method of Blackmore & Wang (2002); i.e. the stable metal distribution was determined in our experiment, and although the tissue was homogenized and centrifuged by the same methods, the tissue fragments, cellular debris and organelles were combined to comprise the insoluble fraction. The soluble fractions were separated into heat-sensitive protein (HSP) and metallothionein-like protein (MTLP). Thus, the tissues were divided into 3 fractions: insoluble fraction, HSP and MTLP. They were then digested and analyzed for metal concentrations as described above. The subcellular metal concentration was expressed as µg g^{-1} wet weight of the tissue. Subcellular Ag distribution was not measured in this study.

Metallothionein (MT) determination. The silver saturation assay (Scheuhammer & Cherian 1991) was modified for determining MT levels in the digestive glands of the clams; 5 clams were dissected to obtain the digestive glands. The wet weight of the digestive glands was determined, and they were homogenized by ultrasonication in sucrose buffer (0.25 M). The homogenate was centrifuged at 20 000 × *g* for 20 min at 4°C and the supernatant obtained was kept at −80°C until MT analysis. During the MT analysis, the supernatant from each replicate was mixed with 0.5 M glycine buffer containing 20 µg ml^{-1} stable Ag and radioisotope ^{110m}Ag (2.85 kBq ml^{-1}). The mixture was allowed to incubate at room temperature for 10 min. After MT binding sites were saturated with Ag, excess Ag were removed by addition of rabbit red blood cell hemolysate, heating (5 min, 100°C) and centrifugation (5 min, 1200 × *g*) in 3 cycles. After the last cycle, the supernatant was centrifuged at 20 000 × *g* for 20 min. The final supernatant was radioanalyzed for ^{110m}Ag. The MT concentration was calculated as 3.55 × Ag concentration and expressed as µg g^{-1} wet weight of the digestive gland.

Clearance rate. Clearance rate or pumping activity of the clams was quantified using methods described previously (Blackmore & Wang 2002). The diatom *Thalassiosira pseudonana* was used to feed the clams (10 000 cells ml^{-1}), and samples of water were taken at 30 and 60 min to measure cell concentration. All the experimental beakers were covered over the experimental period to prevent the effects of light on the pumping activity of clams. The weight-specific clearance rate was calculated by the equation of Widdows et al. (1997). To avoid interference by behavioral changes, we used the maximum clearance rate to compare differences among the different treatments.

Metal assimilation efficiency (AE). The diatom *Thalassiosira pseudonana* was radiolabeled with ^{109}Cd (185 kBq l^{-1}), ^{65}Zn (370 kBq l^{-1}), ^{110m}Ag (370 kBq l^{-1}), and ^{203}Hg (64.8 kBq l^{-1}) before the experiments. After radiolabeling, the diatoms were collected by filtration and fed to 10 individual pre-exposed clams in 2 additions (0 and 20 min). After 40 min of radioactive feeding, we measured the radioactivity of the clams (which had ingested the radiolabeled diatoms) and then placed them in a 15 l enclosed recirculating aquarium for depuration. The radioactivity of the clams was counted at 3, 6, 12, 24, 36, and 48 h during the depuration period. Fecal pellets were collected frequently during the experiment to minimize desorption of the radiotracers from the fecal pellets to the seawater and subsequent accumulation by the clams. The aquarium was covered during the experimental period to prevent light disturbance of the clams. AE was determined as the percentage of initial radioactivity retained in the clams after 36 h depuration, using the methods established for bivalves (Chong & Wang 2000, Blackmore & Wang 2002, Shi et al. 2003).

Dissolved metal uptake. The radioisotopes ^{109}Cd (14.8 kBq l^{-1}, corresponding to 1.2 nM), ^{203}Hg (1.48 kBq l^{-1} corresponding to 0.3 nM), ^{110m}Ag (3.7 kBq l^{-1} corresponding to 0.3 nM), and ^{65}Zn (14.8 kBq l^{-1} corresponding to 0.1 nM), stable Cd ($CdCl_2$: 18 nM Cd) and Zn ($ZnCl_2$: 77 nM Zn) were spiked into 0.22 µm-filtered seawater overnight before the experiments to allow equilibrium of radiotracers and stable metals. No stable Hg or Ag was spiked into the seawater because of the relatively lower specific activities of their radioisotopes. We placed 8 clams from each treatment individually into 200 ml of 0.22 µm radiolabeled filtered seawater for

2 h, covering the exposure containers to avoid light disturbance. After exposure, the clams were rinsed with 0.22 µm-filtered seawater to remove radiolabelled seawater, dissected, and their soft tissue was radioanalyzed. After the radioactivity counts, the tissue was dried at 90°C overnight and its dry weight quantified. The uptake rate was calculated as the amount of metals taken up by the soft tissue per hour (ng g^{-1} h^{-1}). The absorption efficiency (%), which is the fraction of metals absorbed from the volume of water pumped, was also calculated by dividing the uptake rate by the ambient metal concentration and the clearance rate of the same individual clam. Absorption efficiency gives a more realistic comparison of metal uptake because it corrects for variability in clearance rate.

Measurement of radioactivity and statistical analysis. Radioactivity was measured by a gamma counter (Wallac 1480 Wizard 3″, Perkin Elmer). The spillover of radioisotopes was corrected and the counts were corrected for radioactivity decay. The radioactivity of ^{110m}Ag, ^{109}Cd, ^{203}Hg, and ^{65}Zn was counted at energy levels of 643, 88, 279, and 1115 keV, respectively. Counting time was adjusted to a counting error <5%.

The data distribution was checked for normality and homogeneity of variance before analyzes. The percentage data was arcsine-transformed before statistical analysis. For the AE experiments, repeated-measures analysis of variance was used to test for significant differences among treatments over 48 h. The compound symmetry assumptions of repeated-measures tests were checked by Huyn-Feldt ε values from the analyses. Huyn-Feldt corrected probability was adopted when the ε value was less or equal to 0.5; 1-way analysis of variance was also used to test for significant differences among treatments in the other experiments. When there were statistical differences, a Tukey's HSD multiple-comparison test was used to identify the differences. The level of significance for the tests was $\alpha = 0.05$. The correlation between different parameters was checked by linear regression analysis.

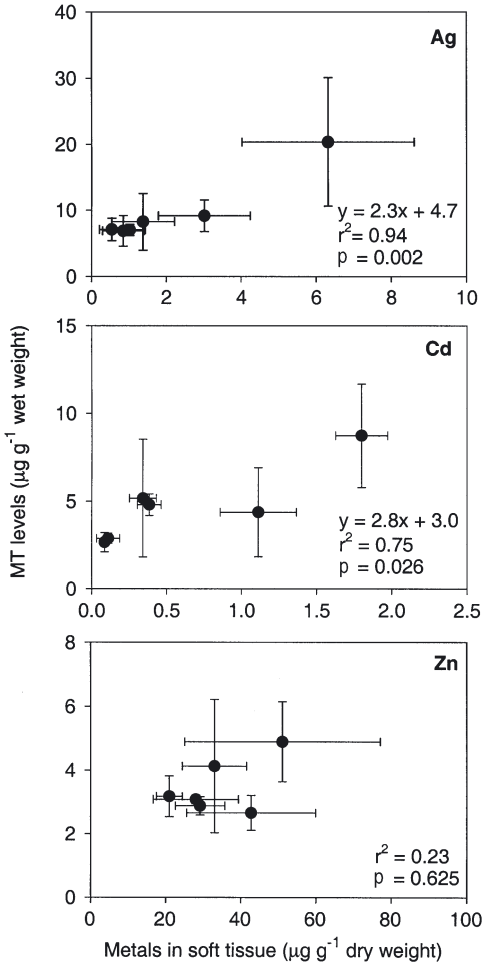

Fig. 1. *Ruditapes philippinarum*. Correlation between metallothionein (MT) levels (µg g^{-1} wet wt) and metal concentrations (µg g^{-1} dry wt) in soft tissue after pre-exposure to Ag, Cd or Zn (mean ± SD, n = 5). Equations describe linear regression relationship between soft-tissue metal concentrations and MT; r^2 is proportion of data that fits linear regression lines

Table 1. *Ruditapes philippinarum*. Metal concentration in soft tissue and metallothionein (MT) levels in digestive glands of clams after pre-exposure to Ag, Cd or Zn. Mean ± SD (n = 5). Ctl, Ag0.1, Ag5, Cd2, Cd20, Zn10, Zn100 = control, 0.1, 5, 2, 20, 10 and 100 µg l^{-1} of the corresponding metals, respectively. *p < 0.05 compared to control (Tukey's HSD multiple comparison test)

Expt	Metals in soft tissue (µg g^{-1} dry wt)		MT (µg g^{-1} wet wt)
Ag exposure			
2 wk			
Ctl	1.0 ± 0.4		7.0 ± 0.8
Ag0.1	1.4 ± 0.8		8.2 ± 4.3
Ag5	3.0 ± 1.2		9.1 ± 2.4
4 wk			
Ctl	0.9 ± 0.6		6.8 ± 2.3
Ag0.1	0.5 ± 0.3		7.1 ± 1.7
Ag5	6.3 ± 2.3*		20.3 ± 9.7*
Cd or Zn exposure			
3 wk	**Cd**	**Zn**	
Ctl	0.1 ± 0.1	29.2 ± 6.6	2.9 ± 0.3
Cd2	0.3 ± 0.1	–	5.2 ± 3.4
Cd20	1.1 ± 0.3	–	4.4 ± 2.5
Zn10	–	21.0 ± 3.5	3.2 ± 0.6
Zn100	–	33.0 ± 8.6	4.1 ± 2.1
6 wk			
Ctl	0.1 ± 0.0	42.8 ± 17.1	2.7 ± 0.6
Cd2	0.4 ± 0.1	–	4.8 ± 0.6
Cd20	1.8 ± 0.2*	–	8.7 ± 2.9*
Zn10	–	28.1 ± 11.4	3.1 ± 0.1
Zn100	–	51.1 ± 26.0	4.9 ± 1.3

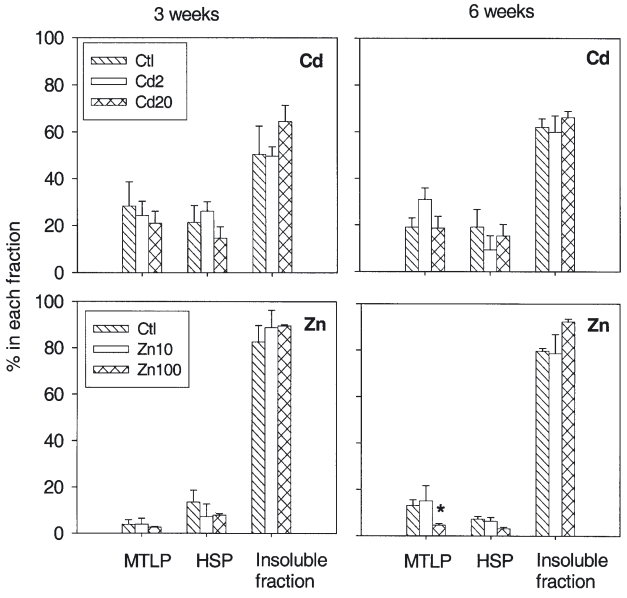

Fig. 2. *Ruditapes philippinarum*. Subcellular metal concentrations of Cd and Zn in clams after Cd or Zn exposure (mean ± SD, n = 5). Ctl, Cd2, Cd20, Zn10 and Zn100 = control, 2, 20, 10 and 100 µg l^{-1} of corresponding metals, respectively. MTLP (metallothionein-like protein), HSP (heat-sensitive protein) and insoluble fraction are different fractions of tissue after homogenization and differential centrifugation. ⋆:Significant difference at $p < 0.010$ compared to control (Tukey's HSD multiple comparison test)

RESULTS

Ag exposure increased the body burden and MT levels in clams at 5 µg l^{-1}, after 4 wk of exposure (Table 1). The Ag concentration in the clams (6.3 µg g^{-1}) in this treatment was about 6 times ($p < 0.001$) higher than in the control (0.9 µg g^{-1}) and the MT levels were about 3 times higher ($p < 0.01$). At a lower Ag concentration (0.1 µg l^{-1}), the body burden and the level of MT were not significantly different from controls (Table 1, $p > 0.05$). In general, an increase of Ag in the soft tissue of the clams was correlated with higher MT levels (Fig. 1, $r^2 = 0.94$, $p = 0.002$). Cd and Zn concentrations in the control clams were exceedingly low (0.1 µg g^{-1} for Cd, and 29–43 µg g^{-1} for Zn). The clams accumulated a higher Cd concentration (1.8 µg g^{-1}) in the 20 µg l^{-1} treatment after 6 wk of exposure than in the control treatment ($p < 0.001$) and MT also increased (8.7 µg g^{-1} at 20 µg l^{-1}, compared to 2.7 µg g^{-1} in the control; Table 1, $p < 0.01$). Cd exposure at 2 µg l^{-1} did not have similar effects (Table 1, $p > 0.05$). There was a similar correlation between Cd concentration in the tissue and MT levels (Fig. 1). The concentrations of Zn in all of the clams were fairly constant over the 6 wk ($p > 0.05$), and Zn exposure did not affect MT levels (Table 1, $p > 0.05$). The Zn body burden was not related to MT levels in the clams (Fig. 1).

The insoluble fraction contained 50 to 90% of the Cd, whereas MTLP and HSP contained 10 to 30% each (Fig. 2). The distribution of Cd in each treatment varied with week of exposure. There was a higher percentage of Cd in the insoluble fraction and less in the MTLP and HSP fractions at Week 6 compared to Week 3. Clams exposed to 20 µgCd l^{-1} accumulated 5 to 10 more Cd in all 3 fractions after exposure for 3 and 6 wk than the controls ($p < 0.01$). However, the percentage of Cd in each fraction remained unchanged, suggesting that there was no shift in Cd from one fraction to another after exposure ($p > 0.05$). Over 60% of Zn accumulated in the insoluble fraction and about 20% in MTLP or HSP. There was a comparatively smaller percentage of Zn in MTLP in 100 µgZn l^{-1} at Week 6 compared to the control (Fig. 2, $p < 0.01$). However, this was not associated with a significant increase in the percentage of Zn in other fractions (Fig. 2, $p > 0.05$). In general, the percentage of Zn in each fraction was not affected by Zn exposure ($p > 0.05$).

The clearance rate of the clams was about 6 l g^{-1} h^{-1}, and did not decrease in the metal-exposure treatments, suggesting that Ag, Cd or Zn did not have any toxic effects on the clams (Fig. 3). In the Cd and Zn

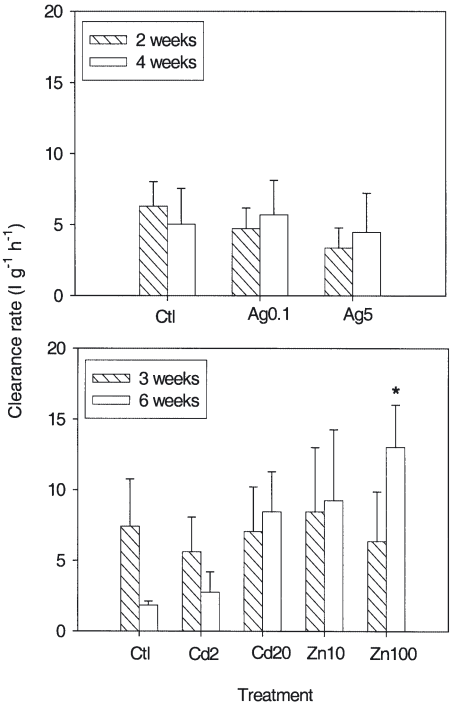

Fig. 3. *Ruditapes philippinarum*. Clearance rate after Ag, Cd or Zn exposure (mean ± SD, n = 8). Ctl, Ag0.1, Ag5, Cd2, Cd20, Zn10 and Zn100 = control, 0.1, 5, 2, 20, 10 and 100 µg l^{-1} of corresponding metals, respectively. ⋆:Significant difference at $p < 0.010$ compared to control (Tukey's HSD multiple comparison test)

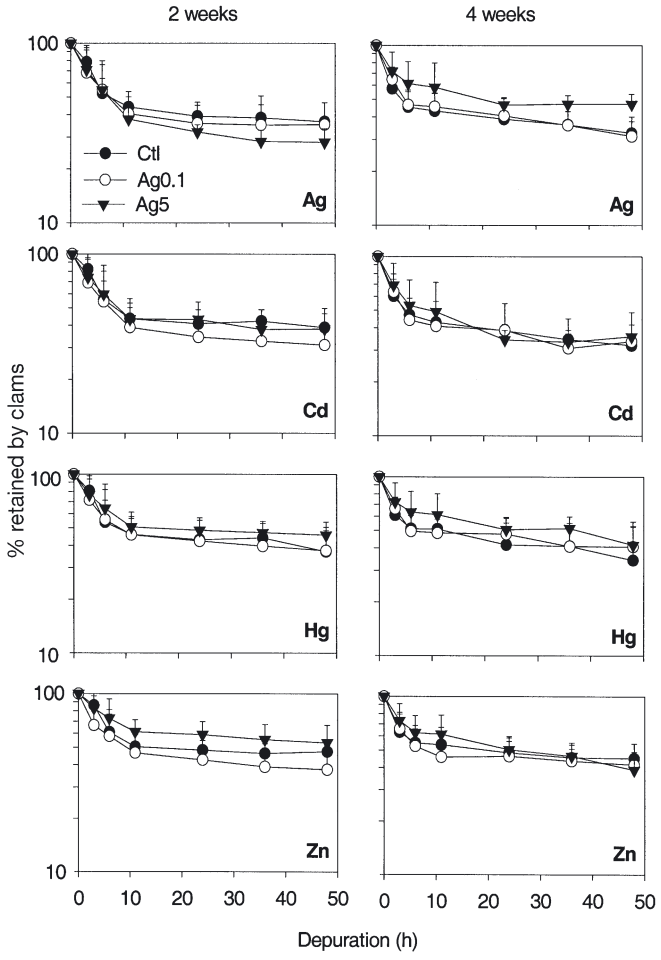

Fig. 4. *Ruditapes philippinarum*. Percentage of metals retained by the clams pre-exposed to Ag at different concentrations and for different periods (mean ± SD, n = 5). Ctl, Ag0.2 and Ag5 are the control, 0.1 and 5 µg l^{-1}, respectively

exposure experiments, the clams in the control and 2 µgCd l^{-1} treatments had a low clearance rate at Week 6 (2 l g^{-1} h^{-1}), while those that had been exposed to Cd 20 µgCd l^{-1} or Zn (all concentrations) had a higher clearance rate, especially for clams in 100 µgZn l^{-1} (12 l g^{-1} h^{-1}, Fig. 3, $p < 0.01$).

The depuration of the unassimilated metals was faster within the first 6 h of ingestion of radiolabeled diatoms, becoming slower and almost constant by 36 to 48 h (Figs. 4 & 5). The calculated AEs of each metal after Ag, Cd, or Zn pre-exposure are shown in Table 2. There was variation in the Ag and Hg depuration patterns between Weeks 3 and 6 in the Cd and Zn exposures (Fig. 5). The depuration of both Ag and Hg was almost linear (semilog scale) throughout Week 3, whereas the pattern in Week 6 was similar to that of the other metals. This resulted in higher AEs of Ag and Hg in Week 3 than in Week 6 in most treatments (e.g. for Ag, 52% at Week 3 and 30% at Week 6 in the control treatment). The AEs of Ag and other metals (Cd, Hg or Zn) by *Thalassiosira pseudonana* were not affected by Ag pre-exposure (Table 2, $p > 0.05$). The AEs of these metals was also not affected after exposure to Cd or Zn for 3 wk (Table 2). However, at Week 6, there was a 10% increase in the Zn retention of clams in 20 µgCd l^{-1} ($p < 0.05$) and 100 µgZn l^{-1} ($p < 0.05$) (Fig. 5), but the AEs were not significantly different (Table 2, $p = 0.203$).

The uptake rates of dissolved Cd and Hg were reduced by 3 to 4 times ($p < 0.01$) in the Ag exposure treatments after 2 wk (Table 3). By Week 4, only the uptake rate of Cd in 5 µgAg l^{-1} was reduced ($p < 0.01$). A reduction in uptake rate of Cd or Hg only occurred in the earlier part of the exposure period, i.e. Week 3 in the Cd and Zn exposures (Table 3). The uptake rate of Cd decreased by 2 times in the 20 µgCd l^{-1} treatment

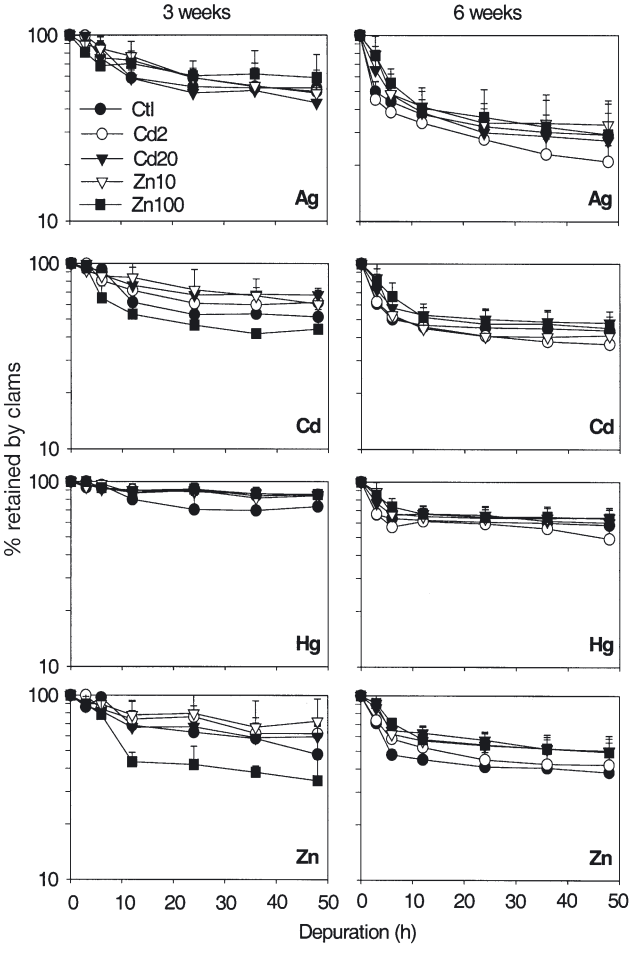

Fig. 5. *Ruditapes philippinarum*. Percentage of metals retained by the clams pre-exposed to Cd or Zn at different concentrations and for different periods (mean ± SD, n = 5). Ctl, Cd2, Cd20, Zn10 and Zn100 are the control, 2, 20, 10 and 100 µg l^{-1} of the corresponding metals, respectively

Table 2. *Ruditapes philippinarum*. Assimilation efficiency (AE) of metals in clams after pre-exposure to Ag, Cd or Zn. Further details as in Table 1

Expt	AE (%)			
	Ag	**Cd**	**Hg**	**Zn**
Ag exposure				
2 wk				
Ctl	38.3 ± 12.4	42.2 ± 6.6	43.9 ± 9.1	46.4 ± 6.1
Ag0.1	25.0 ± 10.3	32.7 ± 7.1	39.6 ± 7.3	39.0 ± 7.2
Ag5	28.4 ± 4.3	37.9 ± 11.1	47.1 ± 7.3	55.3 ± 11.9
4 wk				
Ctl	36.1 ± 6.7	34.2 ± 2.6	40.9 ± 14.2	45.4 ± 9.7
Ag0.1	36.0 ± 7.0	30.8 ± 14.2	40.8 ± 11.1	43.5 ± 7.4
Ag5	47.0 ± 5.6	33.8 ± 5.5	51.5 ± 8.3	46.3 ± 7.4
Cd or Zn exposure				
3 wk				
Ctl	51.9 ± 4.0	53.5 ± 3.2	69.7 ± 11.9	58.2 ± 1.7
Cd2	52.7 ± 13.7	59.9 ± 9.1	83.8 ± 0.8	62.0 ± 8.5
Cd20	50.4 ± 20.3	68.0 ± 6.1	86.2 ± 6.3	58.8 ± 16.8
Zn10	53.4 ± 2.4	66.9 ± 15.6	81.4 ± 3.9	67.0 ± 26.1
Zn100	61.8 ± 20.5	41.8 ± 1.5	85.9 ± 7.2	38.2 ± 3.1
6 wk				
Ctl	30.2 ± 4.8	44.8 ± 0.9	60.0 ± 8.3	40.6 ± 3.7
Cd2	23.0 ± 4.9	38.1 ± 4.6	56.0 ± 12.7	42.6 ± 5.6
Cd20	28.7 ± 16.1	48.7 ± 6.6	61.5 ± 10.6	51.5 ± 6.5
Zn10	33.64 ± 3.8	40.4 ± 1.4	63.7 ± 1.5	51.7 ± 8.0
Zn100	32.2 ± 15.7	47.4 ± 8.8	64.8 ± 8.8	51.7 ± 9.7

($p < 0.001$) and the 100 µgZn l^{-1} ($p < 0.01$), whereas the uptake rate of Hg decreased by 3 to 6 times in 2 µgCd l^{-1} ($p < 0.01$), 20 µgCd l^{-1} ($p < 0.001$), or 10 µgZn l^{-1} ($p < 0.01$) (Table 3). Despite the variation in metal uptake rates between weeks, the variation in absorption efficiency was small. Higher absorption efficiency of Ag, Cd and Hg in the control and 2 µgCd l^{-1} at Week 6 (Table 3) may be explained by the lower clearance rates in these treatments (Fig. 3). Metal absorption efficiency of the clams was in the order Hg/Ag > Zn > Cd (Table 3). Our results suggested that Ag and Cd exposure had differential effects on metal absorption. Ag absorption efficiency of the clams in 5 µgAg l^{-1} increased by 2 times after 2 wk ($p < 0.01$) and Hg absorption efficiency in 20 µgCd l^{-1} was reduced by 6× after 3 wk ($p < 0.01$). In addition, the dissolved metal uptake rates of Ag and Cd, Ag and Hg, Cd and Hg, Cd and Zn, Zn and Hg were significantly correlated (Fig. 6).

Since the clearance rate, the AE and the dissolved metal uptake rate varied from week to week, the percentage of these values relative to the control was calculated as an index and the relationship between the metal uptake and the metal concentration in the soft tissue was determined. An increase in the Cd concentration in the tissue was slightly related to an increase in the clearance rate (Fig. 7, $r^2 = 0.67$, $p < 0.05$), but there was no correlation between Ag or Zn tissue concentration and clearance rate (Fig. 7, $p > 0.05$). There was also no relationship between Ag, Cd or Zn

Table 3. *Ruditapes philippinarum*. Uptake rate and absorption efficiency of dissolved metals after pre-exposure to Ag, Cd or Zn. Mean ± SD (n = 8). Further details as in Table 1

Expt	Uptake rate (ng g^{-1} h^{-1})				Absorption efficiency (%)			
	Ag	**Cd**	**Hg**	**Zn**	**Ag**	**Cd**	**Hg**	**Zn**
Ag exposure								
2 wk								
Ctl	3.6 ± 1.1	5.2 ± 1.3	6.8 ± 2.0	128.9 ± 41.9	2.1 ± 0.8	0.04 ± 0.02	2.5 ± 1.0	0.4 ± 0.1
Ag0.1	1.6 ± 0.4	6.0 ± 2.9	2.5 ± 0.9*	85.5 ± 25.8	1.1 ± 0.3	0.04 ± 0.01	1.2 ± 0.2	0.30 ± 0.04
Ag5	2.8 ± 1.0	1.6 ± 0.6*	1.8 ± 0.7*	62.2 ± 32.3	4.2 ± 1.8*	0.03 ± 0.02	1.7 ± 1.0	0.7 ± 0.5
4 wk								
Ctl	1.1 ± 0.3	3.0 ± 0.8	3.4 ± 1.6	42.8 ± 10.3	1.1 ± 0.5	0.04 ± 0.02	1.8 ± 0.5	0.2 ± 0.1
Ag0.1	1.5 ± 0.6	2.7 ± 0.5	4.7 ± 2.0	53.3 ± 21.0	1.3 ± 0.5	0.03 ± 0.02	2.8 ± 1.3	0.2 ± 0.1
Ag5	1.4 ± 0.8	1.6 ± 0.5*	3.0 ± 2.0	34.6 ± 14.8	1.7 ± 0.9	0.02 ± 0.01	2.4 ± 1.4	0.3 ± 0.2
Cd or Zn exposure								
3 wk								
Ctl	4.0 ± 3.0	3.0 ± 0.5	6.4 ± 1.4	64.6 ± 18.7	3.3 ± 3.5	0.02 ± 0.01	2.6 ± 0.7	0.2 ± 0.2
Cd2	1.3 ± 0.3	2.2 ± 0.6	3.6 ± 1.2*	65.2 ± 11.3	1.1 ± 0.4	0.02 ± 0.01	2.1 ± 1.0	0.3 ± 0.2
Cd20	1.3 ± 0.7	1.3 ± 0.5*	1.2 ± 0.7*	44.1 ± 9.2	1.2 ± 1.1	0.01 ± 0.01	0.4 ± 0.3*	0.2 ± 0.2
Zn10	2.2 ± 0.9	1.9 ± 0.5	3.4 ± 1.3*	59.8 ± 11.7	1.2 ± 0.9	0.01 ± 0.01	1.1 ± 0.8	0.2 ± 0.1
Zn100	5.1 ± 2.2	1.3 ± 0.5*	4.6 ± 0.9	40.0 ± 19.6	2.6 ± 0.8	0.02 ± 0.01	1.4 ± 0.5	0.2 ± 0.2
6 wk								
Ctl	2.5 ± 0.0	3.4 ± 1.0	11.6 ± 7.6	58.4 ± 11.4	5.1 ± 1.2	0.1 ± 0.1	16.4 ± 17.4	0.6 ± 0.3
Cd2	1.7 ± 0.0	3.0 ± 0.0	8.2 ± 2.9	51.9 ± 1.0	2.5 ± 1.6	0.04 ± 0.01	5.9 ± 4.5	0.3 ± 0.2
Cd20	1.6 ± 0.5	2.9 ± 0.5	7.0 ± 3.0	60.4 ± 15.4	0.9 ± 0.2*	0.02 ± 0.01*	2.7 ± 0.8	0.2 ± 0.1*
Zn10	2.3 ± 0.5	4.1 ± 1.5	6.4 ± 2.8	56.0 ± 14.9	1.4 ± 0.8*	0.03 ± 0.02*	2.3 ± 0.9	0.2 ± 0.1*
Zn100	3.0 ± 1.3	2.1 ± 0.7	5.9 ± 2.5	45.9 ± 11.0	1.2 ± 0.7*	0.010 ± 0.002*	1.3 ± 0.4	0.09 ± 0.01*

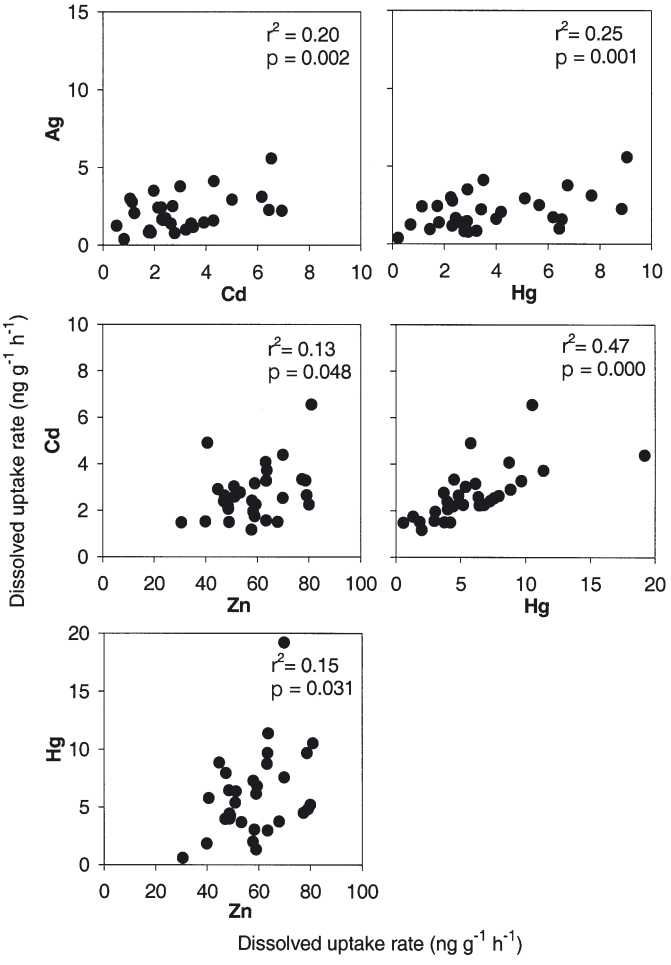

Fig. 6. *Ruditapes philippinarum*. Correlation of dissolved metal uptake rates (ng g^{-1} h^{-1}) of individual clams after Ag, Cd or Zn pre-exposure. r^2 is the proportion of data that fits the linear regression lines which describe the relationships between the parameters

concentration in the tissue and the uptake of these metals in the clams (data for relevant metals shown in Fig. 7, $p > 0.05$; data for other metals not shown, $p > 0.05$).

DISCUSSION

The increase in Ag body burden over time was correlated with higher levels of MT in *Ruditapes philippinarum*. MT is regulated in response to elevated metal levels (e.g. Bebianno & Langston 1995, Hamza-Chaffai et al. 2000) and MT may bind to Ag, resulting in an increase in the metal body burden, and preventing toxic effects to the clams. The detoxification mechanism for Ag may vary among bivalve species. Ag was mainly associated with the sulphide complex, which is concerned with detoxification in mussels (Fisher & Wang 1998, Shi et al. 2003). However, Ag was predominantly bound to the insoluble fraction (Mouneyrac et al. 2000), or associated with MTLP (Johansson et al. 1986) in the clam *Macoma balthica*. In our study, although Ag uptake was correlated with MT induction, the distribution of Ag in different tissue fractions of the clams was not examined. In addition, MT synthesis can be due to endogenous biological processes rather than the metal exposure; thus, the role of MT induction following Ag exposure remains speculative.

Cd exposure did not produce a very high body burden in the clams (the highest body burden in exposed clams was about 18 times the concentration in unexposed clams), in contrast to green mussels exposed in the laboratory (Blackmore & Wang 2002). This may have been due to the lower Cd uptake rate of clams than of mussels (4 times lower: Chong & Wang 2001). The percentages of Cd in the insoluble fraction (metal-rich granules, cellular debris and organelles) and cytosolic proteins (HSP and MTLP) in *Ruditapes philippinarum* agreed well with those reported for marine and freshwater bivalves (Baudrimont et al. 2003). Our results imply that cytosolic proteins and the insoluble fraction play a role in detoxification of Cd in *R. philippinarum*. Thus, there was no adverse physiological effect at these exposure concentrations. In the cytosolic fraction, MT (in the MTLP fraction) may be responsible for detoxifying Cd, since MT synthesis was found to correlate with an increase in Cd body burden. In fact, MT is commonly known to detoxify Cd in marine invertebrates, e.g. oysters (Geret et al. 2002), gastropods (Bebianno & Langston 1995) and mussels (Hamza-Chaffai et al. 2000). In contrast to Ag and Cd, the Zn concentration in *R. philippinarum* was stable, and mainly bound to the insoluble fraction (>80%). A high percentage of Zn has also been found in the metal-rich granules and the insoluble fraction in the mussel *Perna viridis* (Blackmore & Wang 2002), clams *R. decussatus* (Hamza-Chaffai et al. 2000, Baudrimont et al. 2003, Bebianno & Serafim 2003) and *R. philippinarum* (Shi & Wang 2004). Although the toxicological importance of the insoluble fraction in *R. philippinarum* is unknown, the inert granules or mineral concretions in the insoluble fraction may immobilize Zn for detoxification similar to the process in other mollusks (Mason & Jenkins 1995).

The subcellular distribution of Cd or Zn was unaffected by exposure. In contrast, in the mussel *Perna viridis* exposed to 20 µgCd l^{-1} or 100 µgZn l^{-1} for 21 d, a higher proportion of Cd was associated with MTLP, concomitant with an increase in Cd body burden and a higher percentage of Zn associated with the metal-rich granules, but no increase in the Zn body burden (Blackmore & Wang 2002). In our study, the relatively

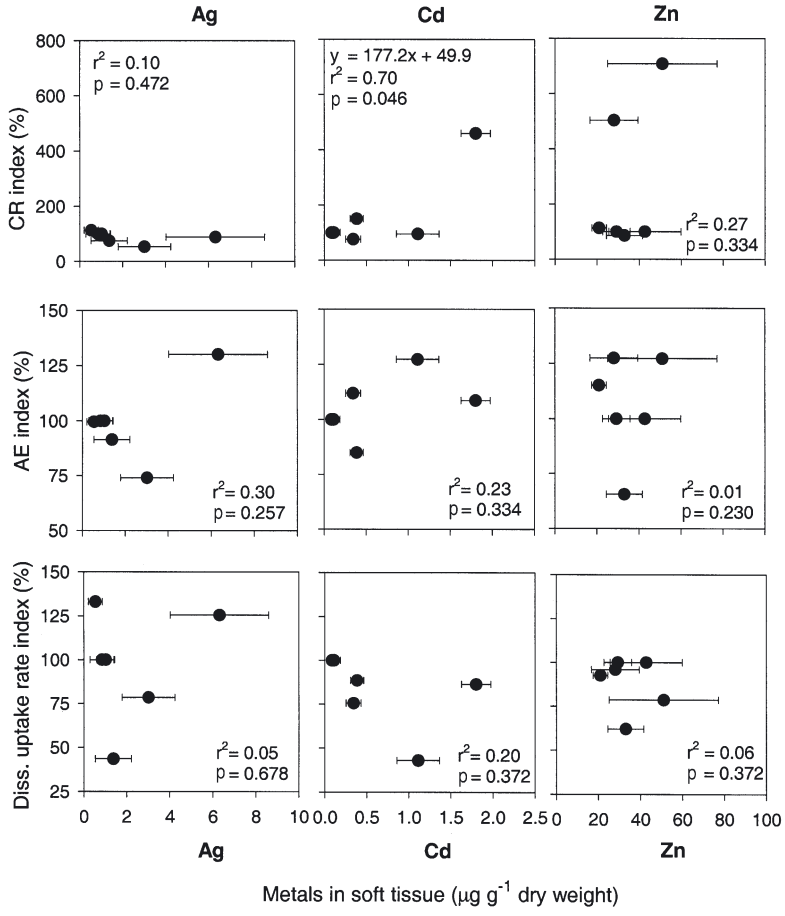

Fig. 7. *Ruditapes philippinarum*. Correlation between the clearance rate (CR), assimilation efficiency (AE) or dissolved uptake rate of the metals and their concentration in the soft tissue of the clams after Ag, Cd or Zn exposure. The index is the percentage of the values relative to the control, the equations describe the linear regression relationship between the parameters and r^2 is the proportion of data that fits the linear regression lines

constant metal distribution in the Cd treatments may be explained by 2 possible mechanisms. (1) The Cd body burden in the clams was low, and thus the metal-binding ligands were not saturated. Consequently, Cd exposure did not cause any shift in metal distribution with an equilibrium-dependent exchange of metals, as shown in a few studies (Roesijadi et al. 1984, Sanders & Jenkins 1984, Giguere et al. 2003). (2) The MTLP, HSP and the insoluble fraction were almost equal in importance for Cd detoxification, such that Cd exposure caused a similar increase of Cd in each fraction. The constant Zn distribution in each fraction may imply that Zn exposure did not cause any metabolic stress to the clams.

There was, in general, no physiological toxic effect of Ag, Cd or Zn on the clams under the experimental concentrations. Clearance or filtration rate may be an indicator of the physiological conditions of animals (Anandraj et al. 2002), and has been used to assess the toxic effects of metals on marine organisms (e.g. Naimo 1995). Metals may depress the filtration rate of bivalves: for example, Abel (1976) showed that 180 and 230 µgCu l^{-1} and 1900 µgZn l^{-1} caused a 50% reduction in the filtration rate of *Mytilus edulis*. Shi et al. (2003) found no significant toxic effects of dissolved (0.5, 5 µg l^{-1}) or dietary (30 µg l^{-1}) Ag exposure on the mussel *Perna viridis*, after exposure for 5 wk. Our data suggest that the clearance rate of *Ruditapes philippinarum* was not affected by exposure to 5 µgAg l^{-1} for 4 wk. Caution should be exercised in interpreting the results on the clam's clearance rate in the Cd or Zn exposure experiments: the clearance rates of the clams in the control and 2 µgCd l^{-1} treatments at Week 6 were very low compared to measurements in the other treatments, and the reasons for this low clearance rate remain unexplained. There was no significant increase in the clearance rate of the clams in 100 µgZn l^{-1} compared to the typical clearance rate of the clams (e.g. the control at Week 2).

In this study, the assimilation of metals by the clams varied among individuals and experiments, resulting in different AE patterns for the same metal. AE is typically variable because it is affected by physiological differences in bivalves and the condition of their algal food (Wang et al. 1995, Wang & Fisher 1996, Wang et al. 1996, Chong & Wang 2000). The AEs of Ag, Cd, Hg and Zn quantified for in this study (Ag: 30 to 52%; Cd: 34 to 54%; Hg: 41 to 70%; Zn: 41 to 58%) on *Ruditapes philippinarum* were comparable to those in previous studies on the same species (Chong & Wang 2000) or other clam species; e.g. *Macoma balthica* (Reinfelder et al. 1997, Griscom et al. 2002), *Potamocorbula amurensis* (Lee & Luoma 1998) and *Mercenaria mercenaria* (Reinfelder et al. 1997). Shi et al. (2003) recently found a 2 to 3 times greater Ag AE in mussel *Perna viridis* that had been pre-exposed to dissolved Ag at 5 µg l^{-1} for 2 wk and dietary Ag for 5 wk, presumably caused by the induction of a sulphide complex that can bind with Ag after Ag exposure. In our study, the percentage of Ag retained after pulse-chase feeding was 10% higher in clams that had been exposed to 5 µgAg l^{-1} for 4 wk, concomitant with induction of MT, although this increase was not statistically significant. Thus, Ag uptake from food in the

clams was less affected by Ag pre-exposure than uptake in the green mussel.

Our study contributes to the growing body of literature on mechanisms affecting interspecific differences in metal bioaccumulation. The increase in Cd AEs in green mussels following Cd exposure was mainly attributed to the induction of MT synthesis that sequestered Cd and increased the cytosolic Cd concentration (Blackmore & Wang 2002). Similarly, the higher Cd AE observed in polluted populations of the clams *Ruditapes philippinarum* and *Mactra veneriformis* was related to the higher fraction of Cd associated with the MTLP (Shi & Wang 2004). However, in our present study, the Cd AE of clams after Cd pre-exposure remained unchanged, irrespective of the level of MT synthesis. There is evidence that clams shorten their digestion times of Cr-contaminated food and consequently reduce the Cr AEs after prolonged metal exposure (Decho & Luoma 1996). It thus appears that the effects of metal exposure on AE may arise through a change in gut physiology.

Metal exposure had little effect on the dissolved metal uptake rates of the clams. A reduction in dissolved metal uptake following metal exposure has been demonstrated in a few studies (Boisson et al. 1998, Blackmore & Wang 2002). Our exposure did not produce any effects, possibly because the concentrations were not sufficiently high or the exposure period was not sufficiently long to result in a significant change in metal uptake rate. Nevertheless, the uptake of other dissolved metals (Cd and Hg) was reduced after Ag, Cd or Zn pre-exposure. These effects were generally more obvious during the earlier period (3 wk) of exposure, presumably reflecting a short-term protection mechanism of the clams. Following a longer period of exposure, the clams may have adopted a better detoxification mechanism (e.g. induction of MT synthesis or mobilization of more metal-rich granules) to prevent the metals from damaging the cells or interfering with the metabolism. It is assumed that, following metal exposure, the uptake of the relevant dissolved metal may be reduced due to physiological regulation of uptake. When the uptake pathways of 2 metals are similar, regulation of one metal may also occur for the other. The coupling relationship of dissolved metal uptake between one metal and another (Ag and Cd, Ag and Hg, Cd and Hg, Cd and Zn, Zn and Hg) may be related to the effects of exposure to one metal on the uptake of the dissolved form of other metals. Interestingly, it was also recently found that pre-exposure of green mussels to metals (Ag, Cu, Cd, and Zn) reduced the uptake of dissolved Hg (D. Shi & W.-X. Wang unpubl. data).

The metal absorption efficiency of the clams in this study was comparable to that of previous studies (Lee et al. 1998, Wang & Fisher 1999, Chong & Wang 2001). Although metal exposure reduced the uptake rate of some dissolved metals, it did not affect their relative absorption by the clams, except for Hg absorption following Cd exposure.

Our study has demonstrated that dietary metal uptake by the clams is not related to body burden of the relevant metal. Previous studies have shown an indirect relationship between metal body burden and the uptake of dietary metals in *Perna viridis* (Blackmore & Wang 2002, Shi et al. 2003) and gastropods (G. Blackmore & W.-X. Wang unpubl. data). Metal pre-exposure may induce some biochemical changes (e.g. MT synthesis), resulting in more metals being bound to the metal-binding ligands and possibly an increase in metal uptake. In our study, MT was also induced by Ag and Cd exposure at different levels, but it did not affect the AE of metals extensively. It is likely that the induced MT level was still not high enough to affect dietary uptake. The highest MT level in this study was 20.3 ± 9.7 µg g^{-1} compared to 46.2 ± 5.7 µg g^{-1} observed for the same species concomitant with an increase in Cd AE (Shi & Wang 2004). However, it has also been suggested that metal AE is not related to MT levels, since repartitioning of metals into MTLP requires a long period of time e.g. longer than 21 d (Evtushenko et al. 1986). Therefore, the involvement of MT in dietary metal uptake is still unclear.

Dissolved metal uptake was similarly not related to metal body burden in the clams. In general, dissolved metal uptake is largely a physiochemical process and little affected by intracellular physiological changes such as MT induction or metal body burden. For example, the uptake of dissolved metals by different populations of mussel species (*Perna viridis*, *Mytilus* spp.) in different locations (England, China and USA) was in general similar, despite differing soft-tissue metal concentrations (Blackmore & Wang 2003). In addition, MT related to the metal body burden, has been shown to have no clear effect on the dissolved uptake of Cd and Zn in bivalves (Blackmore & Wang 2002) or gastropods (Blackmore & Wang unpubl. data), or of Ag in bivalves (Shi et al. 2003).

To summarize, the clam *Ruditapes philippinarum* accumulates Ag and Cd, but not Zn. MT is a possible detoxification pathway for Ag and Cd, but Cd and Zn may also both use the insoluble fraction for detoxification. The induction of MT in response to metal exposure with subsequent metal binding had little influence on metal uptake by the clams. In addition, metal uptake from food and water was in general independent of the clams' metal body burden. However, there was an effect of pre-exposure of one metal on the uptake of other dissolved metals. Any change in metal uptake as a result of physiological and biochemical

metal modification may have important implications for the interpretation of biomonitoring data. The influence of exposure to one metal on the bioaccumulation of other metals clearly needs further clarification. In addition, our data imply strong differences between clams and mussels in their responses to metal pre-exposure, largely as a result of differences in their metal sequestration and storage.

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