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Responses of abalone *Haliotis diversicolor* to sublethal exposure of waterborne and dietary silver and cadmium

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ABSTRACT

In this study, we examined the chronic waterborne and dietary exposure of silver (Ag) and cadmium (Cd) to the abalone *Haliotis diversicolor* using various endpoints such as growth and feeding rates, metal body burden, subcellular distribution, and metallothionein (MT) concentration over a period of 7 weeks of exposure. The growth and feeding rates of abalones were inhibited during the early stage of exposure to different extents, but then recovered to nearly the control levels. A large portion of Ag was redistributed to organelles and metal-rich granules from the cellular debris fraction, whereas cellular debris and metallothionein-like protein were the dominant pools for the storage of Cd, which remained comparable during the exposure period. The MT concentrations were significantly elevated (in a dose-dependent manner) within the first 2 weeks of exposure, after which the MT concentrations started to decrease. All these results implied that abalones respond rapidly to metal exposure, but apparently developed subsequent acclimation.

1. Introduction

Abalone is one of the most important commercial sea delicacies worldwide, and is a common marine gastropod distributed widely in coastal regions. The survival, growth, and reproduction of abalones rely greatly on the ambient water quality. Factors that tend to cause population decline include exploitation of fishing activities, deterioration of natural habitats and food availability as well as water quality (Gorski and Nugegoda, 2006). During the process of farming of abalones that is currently practiced in many countries (including China), abalones are fed macroalgae such as *Gracilaria*, *Laminaria*, *Porphyra*, and *Ulva*. Marine macroalgae are known to accumulate significant amounts of metals from the ambient environment (Pawlik-Skowronska et al., 2007; Sanchez-Rodriguez et al., 2001). The bioconcentration factors (BCF, the quotients of the concentration of a chemical in aquatic organisms divided by the concentration in the surrounding water) of Ag and Cd in different macroalgae are 5000 and 20,000, respectively (IAEA 2004). Wang and Dei (1999) measured the uptake kinetics of four metals (Cd, Cr, Se, and Zn) in two marine macroalgae (the green alga *Ulva lactuca* and the red alga *Gracilaria blodgettii*). The predicted bioconcentration factor was 30,000 for Cd, 2000 for Cr, 40–150 for Se, and 10,000–20,000 for Zn in *U. lactuca*. Given the considerable potential of bioconcentrating metals from the water, the macroalgae may act as vectors for metal transfer to abalones, which may lead to toxic effects in the abalones.

Information on metal toxicity in abalones, via water or dietary phase, will enhance the management of abalone aquaculture as well as improve the seafood safety. However, the influences of long-term metal exposure on the growth and toxicity of abalones have been rarely considered (Tsai et al., 2004). Although a few previous studies on terrestrial gastropods also focused on the dietary exposure pathway (Gomot, 1997; Swaileh and Ezzughayyar, 2000), most studies on the toxic effects of metals on marine gastropod species, including abalones, focused on the waterborne exposure pathway (Chen and Liao, 2004; Lin and Liao, 1999). Lin and Liao (1999) determined the bioconcentration factor (180) and biomagnification factor (1.06) of zinc in *Haliotis diversicolor supertexta*. Tsai et al. (2004) examined the chronic toxic effects of Zn in *Haliotis diversicolor supertexta* from the waterborne phase, and found that the shell growth was greatly inhibited with increasing metal concentration.

Recent studies have indicated that dietary uptake is a dominant pathway for metal accumulation in some aquatic animals (Meyer et al., 2005). Therefore concerns about the toxicity of dietary metals in aquatic animals have increased. For example, Cheung et al. (2006) investigated the bioaccumulation and toxicity of Cd in a marine predatory whelk, *Thais clavigera*, via the dietary exposure pathway, and found that MT was the most

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sensitive biomarker for dietary Cd exposure. Our previous work (Huang et al., 2008) found that at the median bioconcentration factor of metals in the macroalgal diet, more than 50% of Ag, Cd, and Hg in abalone came from the dietary source. Furthermore, the calculated trophic transfer factors (TTF, ratio of metal concentration in the animals to that in their macroalgal food) for Ag, Cd, and Hg were determined to be greater than one (in the order of Ag > Cd > Hg), implying a potential for biomagnification of these metals due to their high dietary assimilation. However, whether or not dietary metals have any toxic effects in the aquaculture of abalones has not been examined in the literature.

Given the importance of dietary exposure to metals in abalones, it is imperative to examine the potential dietary toxicity of metals and contrast the results with waterborne metal toxicity (Meyer et al., 2005). In this study, we conducted an investigation on the long-term sublethal effects of two metals (Ag and Cd) on abalones through waterborne and dietary exposure separately. Ag and Cd were selected in this study because our earlier study indicated that the source of both Ag and Cd in abalones is mostly (> 50%) from trophic transfer and it is possible that both metals are biomagnified during trophic transfer from macroalgae (Huang et al., 2008). The abalones were exposed to two dietary concentrations (via macroalgal food) or one waterborne concentration of either Ag or Cd for 7 weeks. The Ag and Cd concentration in the water exposure was about 10–100 times than that in the environment. Five different endpoints, including growth and feeding rate, bioaccumulation, subcellular distribution, and metallothionein (MT) concentration, were chosen to investigate the chronic metal toxicity as well as metal detoxification in abalones during the period of metal exposure. Growth and feeding are used as indicators of metal pollution stress in marine gastropods (Leung and Furness, 2001; Wo et al., 1999). MT and subcellular fractionation have also been commonly used as predictors of metal pollution and toxicity (Cheung et al., 2006; Leung and Furness, 2001; Wallace et al., 2003). To understand the differences between waterborne and dietborne metal toxicity, the abalones were exposed to different dosages of waterborne and dietborne metals, such that their tissue-accumulated concentrations were comparable, indicating similar total exposures to bioavailable metals.

2. Materials and methods

2.1. Abalones and macroalgae

Small abalones (*H. diversicolor* with a shell width of 30.9 ± 1.2 mm) and their food macroalgae *Gracilaria tenuistipitata* var. *liui* were collected from an abalone farm in Dapeng Bay in Guangdong Province, China in April 2008. The abalones were carefully cleaned of their epibionts and maintained in aerated seawater at 20 °C and salinity of 31 psu for at least 1 week prior to the exposure experiments. The epiphytes of the seaweeds were removed and they were similarly kept at the same temperature and salinity as the abalones with a 14:10 h light/dark cycle. The water was changed each day in the morning and the abalones were fed *G. tenuistipitata* var. *liui* in the evening.

2.2. Experimental treatments

There were seven experimental treatments in this study, including a control, two water exposures (waterborne Cd and Ag) and four dietary exposures (low and high Cd and low and high Ag), each with three replicated tanks (5 L). In the control treatment, the abalones were fed *G. tenuistipitata* var. *liui* that had not been exposed to Cd or Ag. In the water exposure treatments, the abalones were exposed in 0.22 μm filtered seawater (collected from Clearwater Bay, 31 psu) containing 5 μg L^{-1} Ag (nominal, in $AgNO_3$) or 50 μg L^{-1} Cd (nominal, in $CdCl_2$), for 12 h each day, followed by 12 h feeding on macroalgae that had not been exposed to Cd or Ag. In the dietary exposure treatments, the macroalgae *G. tenuistipitata* var. *liui* were first exposed to Ag (5 or 50 μg L^{-1} as the low dietary or high dietary Ag treatment, respectively) or Cd (50 or 500 μg L^{-1}, as the low dietary or high dietary Cd treatment, respectively) in 0.22 μm filtered seawater for 5 days before being fed to the abalones. The actual metal concentrations in the water were not directly measured. However, the accumulation of metals in the tissues of macroalgae and abalones was quantified, thus all the toxicity endpoints could be directly related to the tissue accumulation. Each day, the abalones were fed metal-exposed macroalgae for 12 h and then placed in clean seawater for another 12 h. In order to explain the results more accurately, the metal concentrations in *G. tenuistipitata* var. *liui* were measured after 5 days of exposure before feeding to abalones. In each treatment, 27 individual abalones were placed in each replicated tank (a total of 21 tanks) with aeration. During the exposure, the abalones in each tank were fed 15 g (either control or metal exposed) *G. tenuistipitata* var. *liui* each day at night (10 h), and the water was renewed twice per day (before feeding and after feeding). They were placed in clean seawater (except in the waterborne treatment in which the seawater contained the spiked metals) during the day. The abalones were collected at weeks 0, 2, 4 and 7 for measurements as described below. No mortality was observed during the 7 weeks of exposure in all treatments.

2.3. Growth and feeding rate

In each replicated tank, five abalones were tagged with water-proof labels using super glue in order to measure their shell length and fresh weight at weeks 0, 2, 4, and 7 without dissection. The daily growth rate of individuals was calculated by the following equation (Allen et al., 2006):

$$g = \frac{\ln(w_t/w_0)}{t} \times 100, \quad (1)$$

where w_0 and w_t are the fresh weight (blotted with paper towels) of an abalone at the beginning and at time t (days), respectively. The w_0 and w_t are replaced by respective shell lengths to calculate the growth rate of the shells.

Feeding rates were measured at weeks 4 and 7 in abalones from different treatments. Briefly, the fresh weights of *G. tenuistipitata* var. *liui* before and after (12 h) being fed to abalones in different treatments were weighed. The feeding rate (F, g per individual per h) can be calculated by

$$F = \frac{A - A_L}{N \times 12}, \quad (2)$$

where A and A_L are the weight of the food before and after 12 h of feeding, respectively, and N is the number of abalones in the feeding tank.

2.4. Bioaccumulation, subcellular distribution and metallothionein (MT) concentration

Two abalones from each replicated tank (a total of 6 abalones from each treatment) were dissected to remove their shells. The soft tissues were then dried at 80 °C until they reached a constant weight. The dried tissues (about 0.2 mg) were weighed and digested with 70% nitric acid (5 ml, Fisher Scientific®, UK) at room temperature for 12 h and at 80 °C for 2 h and then finally heated at 110 °C in an auto-regulated heating block until the tissues were digested thoroughly. At the same time, oyster tissue standard (1566a, National Institute of Standards and Technology, Gaithersburg, MD, USA) was digested as a reference material. The digests were diluted with double-distilled water to the appropriate range of concentrations before they were quantified using an HITACHI Z-8100 polarized Zeeman atomic absorption spectroscopy (AAS) system. The recovery of metals in the oyster standards was 95%. The method detection limit of the AAS was 0.01 μg L^{-1} for both Cd and Ag. The metal concentrations were expressed as μg g^{-1} dry weight.

Another two abalones were collected from each replicate and dissected. The subcelluar Ag and Cd distributions were determined using the method described by Wallace et al. (2003) with a slight modification (Huang et al., 2008). Five different fractions, including cellular debris, metal-rich granules (MRG), organelles, heat-denaturable protein (HDP), and metallothionein-like protein (MTLP), were obtained. These fractions were dried, digested with 70% nitric acid as described above and finally their metal contents were measured by AAS.

Two abalones from each replicate were weighed and placed into a homogenizing tube containing 20 mM Tris-base buffer (pH 8.0; 0.1 mM antioxidant 2-mercaptoethanol; 5 mM phenylmethanesulfonyl fluoride) in 1:5 weight to volume ratio. They were then homogenized in a tissue homogenizer (Ultra-Turrax T25 basic, IKA) at medium speed for 5 min on ice. The homogenate was centrifuged at 16,000g for 20 min at 4 °C. Aliquots of 300 μl supernatant were removed for the quantification of MT by the silver-saturation method described by Scheuhammer and Cherian (1991) with a slight modification (Cheung et al., 2006). They were incubated with 0.5 ml of 20 μg ml^{-1} of stable Ag and ^{110m}Ag (7.4 kBq ml^{-1}) in 0.5 M glycine buffer at room temperature for 10 min, to saturate the metal binding sites of MT. Afterwards, 100 μl of rabbit blood cell hemolysate was added, followed by heat treatment at 100 °C for 5 min and centrifugation at 3000 g for 5 min, to remove the excess Ag ions. The supernatants were collected and the steps of hemolysate addition, heat treatment and centrifugation were repeated another two times. Finally, the supernatants were centrifuged at 16,000g for 20 min, and the radioactivity of the final supernatant was counted by the

gamma counter. The MT concentration was calculated as 3.54-fold the Ag concentration and expressed as $\mu g\ g^{-1}$ wet weight (Cheung et al., 2006).

2.5. Statistical analysis

Differences of growth rates were analyzed using one-way ANOVA (SPSS for Windows, version 16.0) within temporal groupings (not between them). And Tukey post hoc tests were conducted to make multiple comparisons. Independent-samples *t*-test was employed to compare the differences of feeding rates, metal concentrations and MT concentrations between treatments and those of the controls or between temporal groupings. The significance level was set at $p < 0.05$.

3. Results

3.1. Growth rate and feeding rate

The shell length and fresh weight of whole abalones in each treatment at weeks 0, 2, 4, and 7 were monitored to calculate the daily growth rate (Fig. 1). The shells were not removed from the abalones when the fresh weights were measured. Within the first 2 weeks, all the metal exposure treatments exhibited some differences from the control treatment (Fig. 1). A significant decrease in the growth of the shells was found in the waterborne Ag, waterborne Cd, and high dietary Cd treatments ($p < 0.05$). However, the inhibition from metal exposure on the growth of abalones was less pronounced in the subsequent weeks of metal exposure, except in the waterborne and low dietary Ag treatments, which was probably caused by the considerable elevation of the body burden of Ag between weeks 2 and 4 (see later). There was no significant difference between any of the treatments from 4 to 7 weeks. The increase in the fresh weight followed a similar pattern as that of shell length (Fig. 1). However, only the high dietary Cd treatment had a significant influence on the increase in fresh weight within the first 2 weeks ($p < 0.05$). In addition, the fresh weight did not increase as much as the shell length from 2 to 4 weeks. The shell length growth calculated over the 7 week period (0–7 weeks) suggested that low dietary Ag, low dietary Cd and high dietary Cd significantly decreased the growth rate (%) of abalones. In terms of the fresh wet weight, waterborne Ag and high dietary Cd significantly decreased the growth of abalones.

The quantified feeding rates of abalones at weeks 4 and 7 are shown in Fig. 2. Based on the homogeneity test of the variance, one-way ANOVA was appropriate to compare the difference of feeding rates except for the Ag grouping at week 4 even after logarithmic transformation. Nonparametric tests indicated no significant difference within Ag grouping at week 4 ($p > 0.05$). In order to examine the difference between the control and the metal-exposed treatments, independent-sample *t*-test was used to compare the feeding rates in both the Ag and Cd groups. Significant difference was found between the control and the waterborne Cd treatment, and between the waterborne Cd and the low dietary Cd treatments at week 4. No significant difference was however found between the control and the waterborne Ag treatment at week 4 since the equal variances were not assumed, although the average feeding rate in waterborne Ag treatment was much lower. There was no significant difference in the feeding rate between any of the treatments in week 7. During this period, the growth of the shell length and fresh weight in the two dietary Ag treatments did not decrease significantly (Fig. 1). Correspondingly, the feeding rates of the abalones in these treatments were relatively high, which would contribute to the increase in the growth rate (particularly the fresh weight growth) at weeks 4–7. There was no significant difference in the feeding rate between any of the treatments in week 7.

3.2. Metal accumulation, subcellular distribution, and MT induction

Five days of exposure to Cd and Ag resulted in a significant increase in the metal concentrations in the macroalgae (Table 1). This increase appeared to be proportional to the ambient metal concentrations. For example, when both the Cd and Ag concentrations increased by 10-fold, their accumulation in the macroalgae increased by 12.5- and 8.2-fold, respectively. There

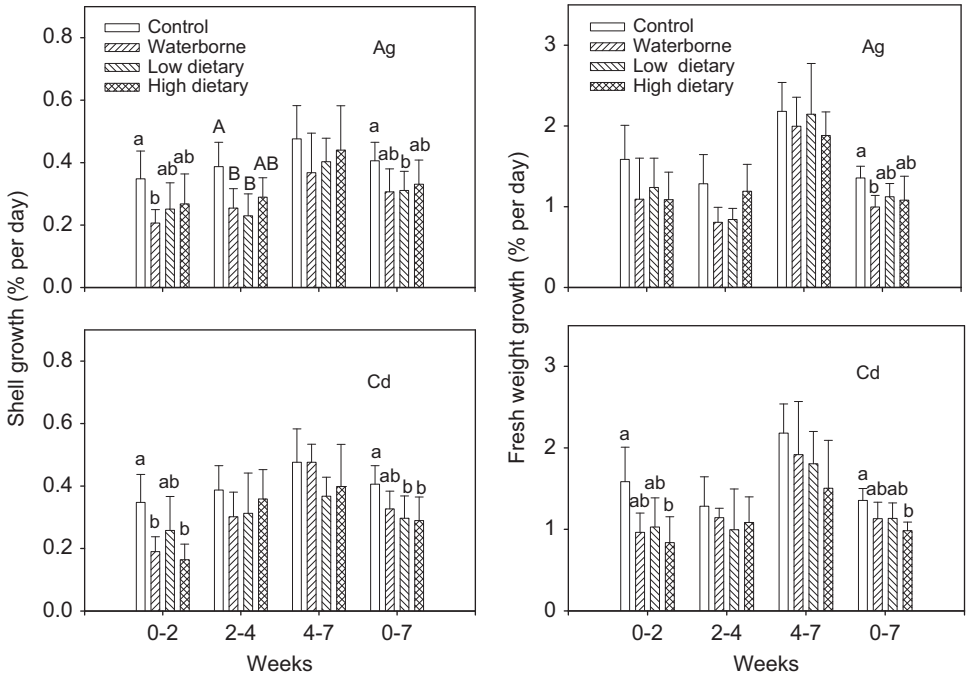

Fig. 1. The calculated growth rates of shell lengths and wet weights of abalones *H. diversicolor* during 7 weeks of exposure to waterborne and dietary metals. Dietary metal concentrations are shown in Table 1. Different letters indicate significant differences within temporal groupings not between them, analyzed by one-way ANOVA. Values are means ± SD ($n = 5 \times 3$, five abalones from each of three tanks). The significance level was set at $p < 0.05$

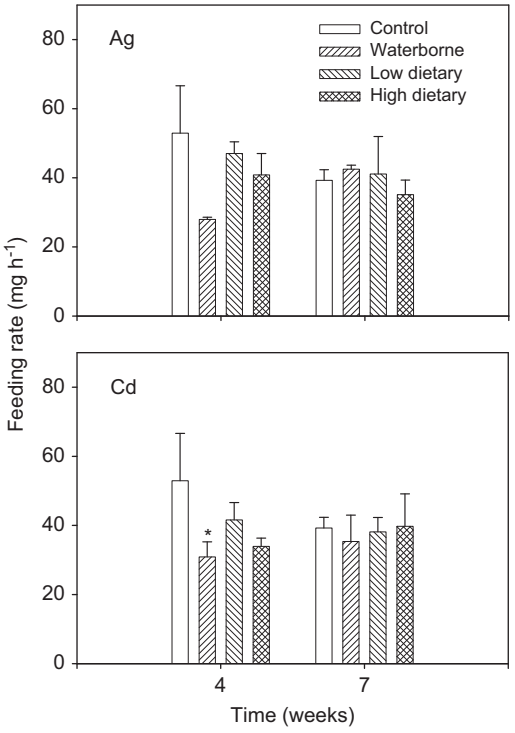

Fig. 2. Feeding rates of abalones *H. diversicolor* at week 4 and week 7 exposure to waterborne and dietary metals. Dietary metal concentrations are shown in Table 1. Values with an asterisk are significantly different from those of the controls at $p < 0.05$. Means ± SD ($n=3$).

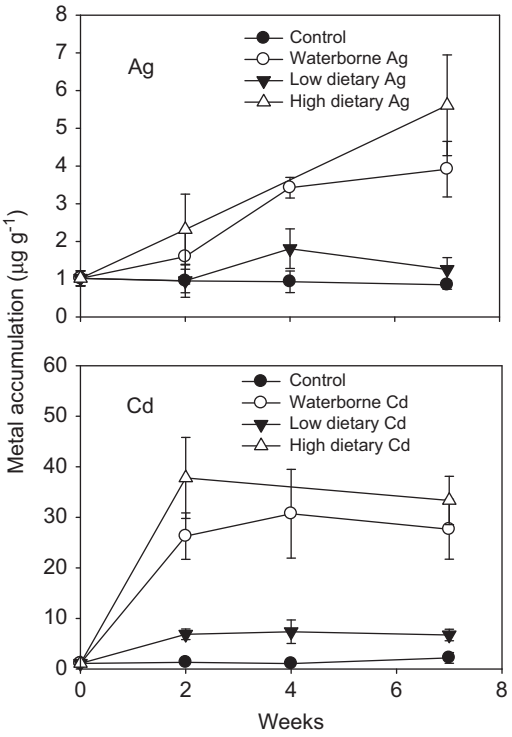

Fig. 3. The Ag and Cd concentrations in the abalone, *H. diversicolor* during 7 weeks of exposure to waterborne and dietary metals. Dietary metal concentrations are shown in Table 1. Values are mean ± SD ($n=2\times3$, two abalones from each of the three tanks).

Table 1
Cadmium and silver concentrations in macroalgae, *Gracilaria tenuistipitata* var. *liui* after 5 days of exposure to dissolved metals. Data are mean ± SD ($n=3$).

Metal	Exposure conc. ($\mu g\ L^{-1}$)	Metal conc. in macroalgae ($\mu g\ g^{-1}$ dry weight)
Cadmium	Control	0.73 ± 0.07
	50	9.56 ± 0.51
	500	120 ± 1.50
Silver	Control	0.13 ± 0.01
	5	5.52 ± 1.73
	50	45.1 ± 5.58

was no significant increase in metal concentration in the abalones in the control treatment throughout the 7-week period (Fig. 3). The Ag concentrations in the abalones increased significantly ($F=5.279$, df=7, $p < 0.05$) in the high dietary Ag treatment after 2 weeks of exposure. Ag concentrations in the abalones of waterborne treatment did not increase significantly within the first 2 weeks, but continued to increase during the remaining exposure period. By the end of the 7 weeks, the Ag concentrations in abalones in these two treatments were not significantly different from each other ($F=1.448$, df=10, $p > 0.05$) and were 4–5 times higher than that in the control treatment. In the low dietary Ag treatment, the Ag accumulation in the abalones increased slightly and was not significantly different from the control treatment ($F=4.892$, df=10, $p < 0.05$). The Cd concentrations in the abalones also increased significantly in all of the Cd-exposed treatments within the first 2 weeks of metal exposure, but they remained rather constant during the remaining 5 weeks of exposure (Fig. 3). By the end of the exposure period, Cd concentrations in the abalones were comparable ($F=0.478$, df=10, $p > 0.05$) between the waterborne and high dietary Cd treatments.

The Ag and Cd subcellular distributions are shown in Fig. 4. Generally, most Ag in the control treatment was deposited in the cellular debris and MRG fractions, whereas only a small amount of Ag was found in the HDP and MTLP fractions. During Ag exposure, the cellular debris and MRG continued to be the dominant pools for Ag to bind with, but the importance of organelles and MRG fractions increased as the period of Ag exposure increased, whereas the concentration of Ag in the MTLP fraction decreased with exposure. At the end of 7 weeks of exposure, Ag was nearly equally distributed in the MRG, cellular debris and organelles fractions in the waterborne and high dietary treatments, suggesting a significant repartitioning of metals among the different subcellular pools during Ag exposure.

The cellular debris and MTLP fractions were the dominant pools for Cd accumulation in the control treatment, and Cd concentrations in the five fractions did not change appreciably during 7 weeks of exposure. In the Cd exposure treatments, the cellular debris and MTLP fractions were also the dominant Cd storage pools throughout the exposure period, but ratio of Cd in the MTLP to cellular debris fractions appeared to increase slightly in the high dietary Cd treatment (from 0.09 in week 2 to 0.89 in week 7), which indicated that Cd was repartitioned between these two pools.

Before metal exposure, the MT concentrations in the abalones were 73–79 $\mu g\ g^{-1}$ (Fig. 5). After 2 weeks of exposure, the MT concentration increased significantly ($p < 0.05$) in all six exposure treatments. However, these concentrations then decreased during the remaining 5 weeks of exposure and returned to the control levels after 7 weeks of exposure. There was no significant relationship between the MT concentration and the accumulated body Ag or Cd concentrations in the abalones when all data were considered ($p > 0.05$).

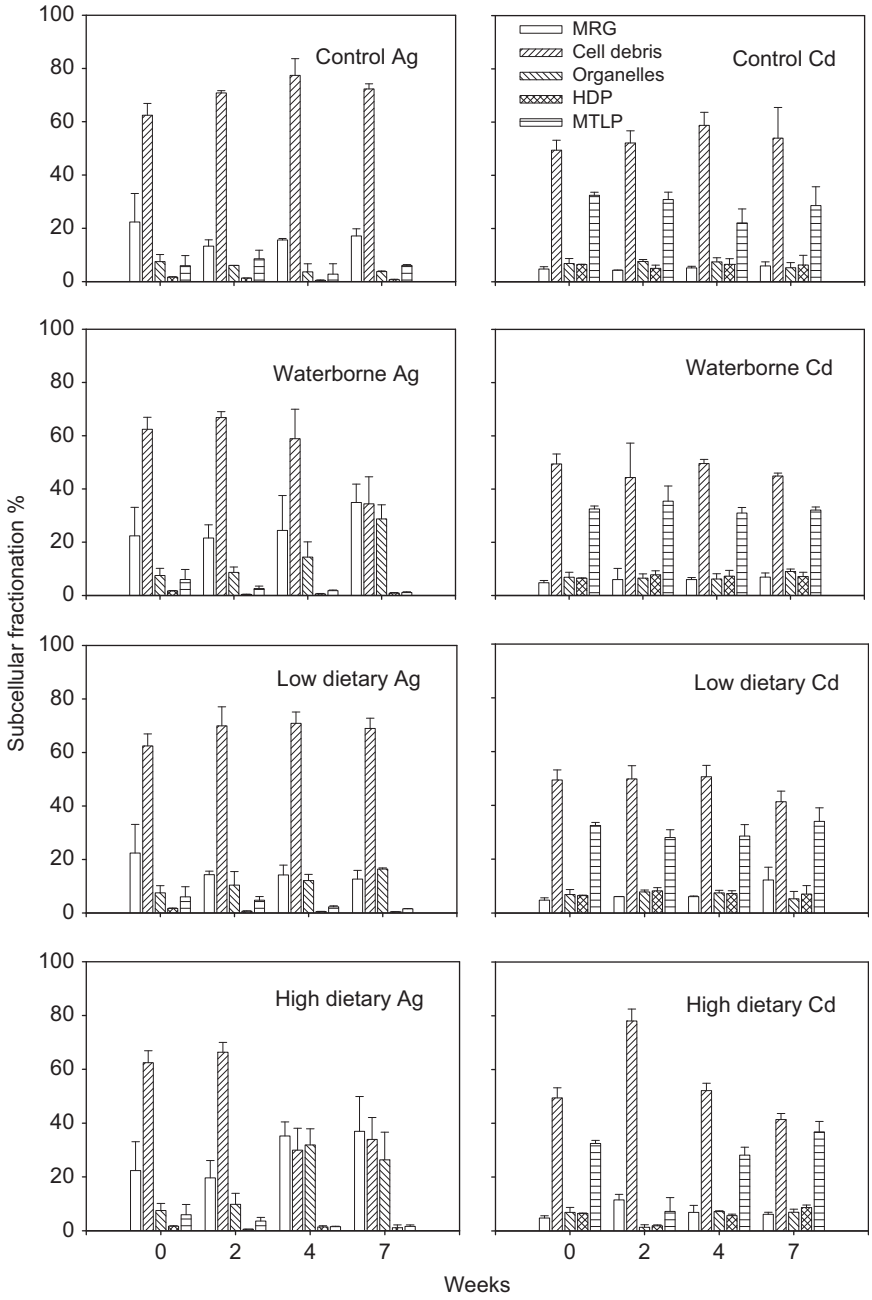

Fig. 4. The subcellular Ag and Cd distribution in abalones *H. diversicolor* during 7 weeks of exposure to waterborne and dietary metals. Dietary metal concentrations are shown in Table 1. Values are means ± SD ($n=2\times3$). MRG: metal-rich granule; HDP: heat-denatured protein; MTLP: heat-stable protein.

4. Discussion

Growth is an important fitness component of individual organisms, because it reflects the sublethal effects on an animal and appears to be sensitive to a wide range of stressors, e.g., temperature, salinity, and metals (Wo et al., 1999). Therefore, growth has been used as an indicator of metal pollution stress in marine invertebrates, e.g., intertidal gastropods (Burrows and Hughes, 1990; Forbes and Depledge, 1992; Leung and Furness, 2001; Wo et al., 1999). Waterborne Cd exposure can inhibit the growth of these gastropods, both the shell length and wet weight of *Nassarius festivus* were reduced at a Cd concentration greater than 220 μg L^{-1} (Wo et al., 1999). Forbes and Depledge (1992) found that the growth of *Hydrobia ulvae* decreased by 1.9-fold when they were exposed to 100 μg L^{-1} Cd for 3 weeks. Similarly, the individual growth rate of *Nucella lapillus* decreased with increasing Cd in the digestive gland/gonad complex (Leung et al., 2001). Likewise, the daily growth rate of abalones exposed to waterborne Cd in our study decreased significantly from 0.348% d^{-1} (control) to 0.190% d^{-1} within the first 2 weeks ($p<0.05$). Dietary effects of metal exposure on the growth of terrestrial gastropods have also been observed (Gomot, 1997; Swaileh and Ezzughayyar, 2000); three species of snails had the same lowest-observed-effect concentration (LOEC) of Cd (50 μg g^{-1} dry food). Two dietary doses of Cd were used in this study, and the high dietary concentration of Cd had a significant negative influence on the growth of abalones within the first 2 weeks ($p<0.05$). There is no available report on the growth effect of Ag in marine and terrestrial gastropods to compare with our data.

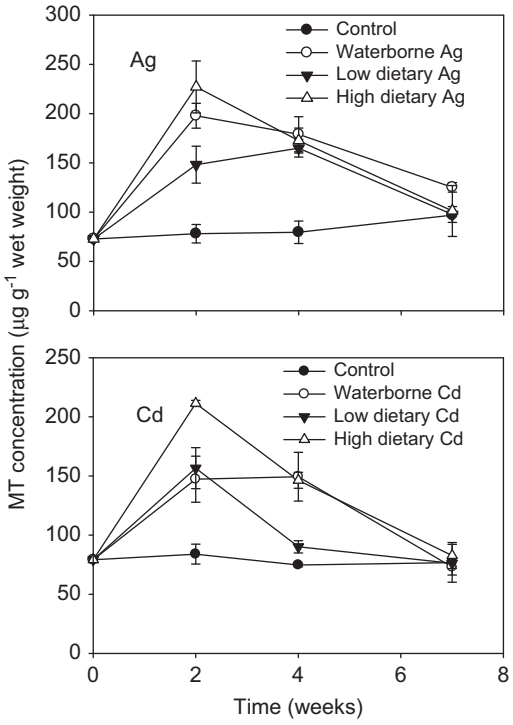

Fig. 5. Metallothionein (MT) concentration in abalones *H. diversicolor* during 7 weeks of exposure to waterborne and dietary metals. Dietary metal concentrations are shown in Table 1. Values are means ± SD ($n=2\times3$).

Change in feeding is one of the first responses to environmental perturbations and it has potential effect on other life traits such as growth, reproduction, and ultimately on the survival of organisms if impaired (Maltby, 1999; McLoughlin et al., 2000; Croteau and Luoma, 2008). For example, the number of *N. festivus* engaging in feeding was significantly reduced with increasing dissolved Cd concentrations (Cheung et al., 2002; Wo et al., 1999). In addition, the time spent on feeding was greater for individuals exposed to Cd concentrations higher than 100 μg L^{-1} (Cheung et al., 2002), which subsequently depressed the average feeding rate. The increased metal levels in the diet can decrease the food consumption of land snails (Gomot, 1997; Swaileh and Ezzughayyar, 2000). In our study, both the waterborne Cd and Ag exposure and high Cd diet significantly reduced the feeding rates of abalones at week 4. Although the reason for why food consumption decreases when marine animals are exposed to high metal concentrations in the water or in their food remains to be further clarified, reduced food consumption leads to a decrease of available energy and poses a direct impact on growth (Notten et al., 2006). Nevertheless, the feeding rate then recovered subsequently, which strongly suggested that the abalones acclimated to the metal exposure. Such acclimation is consistent with the observations about their growth, which did not show significant differences with the control during 4–7 weeks of exposure.

The dependence of metal concentration on body size has been found in a variety of animals since 1970s (Boyden, 1974; Pan and Wang, 2008). Leung et al. (2001) found that growth rate was a confounding factor in the use of dogwhelk *N. lapillus* as biomonitor of metal (Cd, Zn, Pb, and Cu) contamination because the tissue burden and MT content were both inversely related to the body size. In our study, the increase of shell length of abalones from week 0 to 7 was 15–22%, while that of wet weight (including shell) was 59–92%. Therefore, the relative constant of Cd body burden after 2 weeks of rapid accumulation could be partially ascribed to the growth dilution effect (i.e., the increase of body mass would lead to a decrease of metal body concentration while metals continued to be accumulated by the abalones) or to changes in Cd biokinetics in the abalones.

Metals can be detoxified by storage as a non-toxic form or excreted by the animals. Despite the rapid accumulation of metals in the abalones within the first 2 weeks of exposure, the subcellular distribution of the metals appeared to respond to metal exposure at a slower rate than the metals were accumulated. The subcellular distribution pattern of this study is consistent with other studies of marine molluscs that Ag is generally detoxified by binding with MRG and cellular debris while only a small fraction is associated with the protein fraction (HDP or MTLP) (Huang et al., 2008; Shi et al., 2003; Wang and Rainbow, 2008). Likewise, different from Ag, the MTLP fraction was an important pool for Cd storage, in addition to the cellular debris fraction. Previous studies categorized the MRG and MTLP fractions as the biological detoxified metal fraction (BDM), while the organelles and HDP fractions were described as the metal-sensitive fraction (MSF) (Cheung et al., 2006; Wallace et al., 2003). It is obvious that the distribution Ag in the organelles increased with increasing Ag exposure, thus leading to an increase in metal in the proposed MSF (although Ag in HDP fraction decreased). However, we found that Ag in BDM fraction also increased remarkably because of the great increase of MRG in all the experimental groups except the low dietary treatment. A large amount of metals were stored in MRG (for Ag) or MTLP (for Cd) with increasing metal body concentration, which may contribute to the zero mortality of abalones in this study.

MTs can be induced by essential metals (e.g., Cu and Zn) and non-essential metals (e.g., Cd, Ag, and Hg) following metal exposure (Amiard et al., 2006). Previous works generally demonstrated that MT induction is dose dependent. For example, Cheung et al. (2006) reported a significant correlation between the MT concentration in the leiblein gland and the accumulated Cd concentration in whelks *T. clavigera*. In a field study, Blackmore and Wang (2004) also demonstrated a significant relationship between MT concentrations in the whelks and the body concentration of Cd. The dose-dependent induction of MT was found in our study after 2 weeks of exposure. It was, however, surprising that the MT concentrations in the abalones started to decrease afterwards and returned to the control levels after 7 weeks, despite the fact that the Ag and Cd concentrations continued to increase or were maintained at a constant level. The reasons for the decrease of the MT concentrations in abalones remain unknown, but it may be due to the breakdown of MT without further significant accumulation of metals. Barka et al. (2001) proposed a similar phenomenon in the copepods *Tigriopus brevicornis*, e.g., Cd induced MT on the first day of exposure, but MT decreased on the following days (with Zn and Ag exposure, the MT concentration remained constant after the first day of exposure). In addition to metal sequestration, MT also plays a role in antioxidation (Leung and Furness, 2001). Thus, it appears that MT synthesis is an acute response to metal exposure within the first 2 weeks in abalones. With increasing metal exposure, the animals may acclimate to the metals and MT may play a less important role in metal storage and detoxification. In fact, even though MT was significantly induced by Ag exposure, most of the accumulated Ag was concentrated in the other subcellular fractions. The distribution of Cd in the MTLP fraction also remained rather constant throughout the 7 weeks of exposure.

The inhibition and recovery of growth and feeding rate within the exposure duration implied some degrees of tolerance development in the abalones. Klerks and Weis (1987) classified tolerance that aquatic animals may achieve after metal exposure into two categories: physiological acclimation and genetic

adaptation. Certain mechanisms may occur to increase metal tolerance, such as the alteration of metal biokinetics, development of defense system, or change in enzyme sensitivity (Guan and Wang, 2006) In the present study, other than growth and feeding rate, rate of metal accumulation for all treatments decreased after 2 weeks of exposure, and the MT contents also decreased simultaneously and nearly to the control levels by the end of 7 weeks of exposure. Furthermore, a great portion of Ag of waterborne and high dietary treatment was redistributed to organelles and MRG fractions at a late exposure period. These data suggested that physiological acclimation and detoxification both operated in abalones to resist the environmental stress. In recent years, metal pre-exposure studies were conducted to quantify metal biokinetics and stress resistance and to examine the development of physiological acclimation (Shi et al., 2003; Wang and Rainbow, 2005). For example, Shi et al. (2003) found that the Ag dietary assimilation increased whereas the efflux decreased in the green mussels *Perna viridis* in response to aqueous and dietary pre-exposure.

The target organs may be different between the waterborne and dietary exposure pathways. Therefore, it is difficult to compare metal toxicity on the abalones simply by the endpoints measured in this study. However, the exposure regimes were designed such that the tissue burdens of Ag and Cd between waterborne and high dietary treatments were comparable during the 7 weeks of exposure. We can thus compare the waterborne and dietary metal toxicity in abalones with comparable tissue burdens. In general, no significant difference of growth rate and feeding rate was observed between these two treatments, and tissue burden alone posed a strong effect on Ag and Cd toxicity of abalones. These data indicated that the effects of Ag and Cd in abalones were probably not dependent on the exposure route under long-term exposure. However, the high dietary Cd appeared to induce more MT than did the waterborne Cd exposure. Blackmore and Wang (2004)also found that dietary Cd exposure induced more MT in *T. clavigera* than waterborne Cd exposure under similar metal body burdens.

5. Conclusion

Although no mortality was observed during the long-term exposure, some biological and physiological responses were observed in the treatments of waterborne and dietary Ag and Cd within the first few weeks. The feeding and growth rates of abalones decreased to some extent initially, during which the abalones initiated some detoxification strategies such as MT induction and subcellular transfer. Nevertheless, these responses then disappeared, although the abalones continued to accumulate metals. Such responses strongly suggested that the abalones acclimated to metal exposure. Consequently, abalones may develop an ability to acclimate to metal stress, which can only be revealed through chronic metal exposure instead of acute exposure. Future metal toxicity study should consider metal acclimation during chronic exposure. Our study also indicated that the toxic effects of Ag and Cd were independent of the exposure pathways under long-term exposure.

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