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Short-term metal accumulation and MTLP induction in the digestive glands of *Perna virdis* exposed to Zn and Cd

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Abstract

Time-dependent Zn and Cd accumulation and metallothionein like protein (MTLP) induction in the digestive glands of mussels, *Perna virdis*, were measured under different exposure conditions. The initial uptake rate at start of chase (ρ_0) and mean residence time (τ) were calculated to determine the physiological response of organisms and their potential detoxification mechanisms. It was found that in digestive glands, Zn had obviously higher ρ_0 and shorter mean residence time than Cd, indicating that these two metals had different accumulation dynamics even though they were very close in the periodic element table. MTLP levels in digestive glands varied from 0.51 to 1.05 μg/g ww (wet weight). The MTLP level increased continuously when mussels were exposed to low and middle levels of Zn and Cd media, and reached maximal levels at day 4, then decreased when they were exposed to high level Zn and Cd solutions. With regard to the fraction of Zn and Cd accumulated in the digestive glands, the ratios of soluble metal to total metal decreased continuously after exposure in low and middle levels of Zn and Cd media, and decreased continuously in the first 4 days and then to level off when mussels were exposed to media with high concentration of Zn and Cd. Results suggested that both MTLP induction and metal insolubilization were detoxification processes in digestive glands of mussels.

Key words: Zn; Cd; *Perna viridis*; metallothionein like protein; insolubilization
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Introduction

Marine animals can uptake and accumulate heavy metals from both dissolved phase and dietary source. And in the process of uptake or bioaccumulation, animals themselves will adjust to a variety of physiological mechanisms of tolerance to resist the toxic effects of heavy metals, including releasing organic compounds to form extracellular complexes with metal ions, increasing the synthesis of metallothionein (MT) for metal homeostasis and detoxification (Grill et al., 1985; Cajaraville et al., 2000; Berthet et al., 2003).

As an early warning indicator of metallic pollution, the properties of MTs in marine organisms and their relationships with metal exposure have been given considerable attention by environmental scientists (Ikemoto et al., 2004; Geffard et al., 2005). MTs are low molecular mass, cysteine-rich and thermally-stable proteins, which can be inducible, and of characteristic metal-binding properties. Circumstantial evidence revealed that MTs could play an important role in homeostasis of essential metals and detoxification of toxic metals. MTs have been found to be conserved nearly throughout the animal kingdom, and some proteins rich thiolate (–SH) but not completely identical to MTs as named with metallothionein like protein (MTLP) or metallothiopeptide (MP) (Kraak et al., 1993; George and Olsson, 1994). MTLP is generally taken as a detoxification mechanism by organisms due to its latent capacity to bind metals for cysteine contents.

Mussel is a filter-feeding bivalve species, mainly feeding on small zooplankton, phytoplankton and other suspended organic debris. Due to its sedentary lifestyle and nutritional feature, mussel plays an important role in the biogeochemical recycles of trace elements including macronutrients and heavy metals, and the species has been recognized as a good indicator of marine metallic pollution in coastal and estuarine waters. Mussels have been also proposed as appropriate biomonitors of marine pollution, especially for monitoring metallic pollution based on variations of metallothionein as biomarkers. In field studies, the potential rules among those characteristics indicators may be concealed by low signal-to-noise ratio (Cairns, 1992) for complex influence and interaction of various environmental and climatic factors. Therefore, laboratory exposure experiment is a feasible strategy for studying the effects of

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metals and latent relationships between metals and MTLP synthesis in organisms.

Zn is an important trace element for the organism as a cofactor for approximately 300 enzymes involved in nearly all aspects of metabolism (Vallee and Auld, 1990). Cd is usually thought as a toxic metal, and has similar chemical characteristics with Zn as the same IIB elements. Both Zn and Cd are primary metal pollutants in Daya Bay, which located in the northern part of the South China Sea (Qiu et al., 2005). There has been an increasing awareness of metallic toxicity and detoxification in mussels (Kraak et al., 1993; Bebianno and Machado, 1997; Geffard et al., 2005). However, only few and sporadic work has been carried out by Chinese scientists on physiological interaction between mussels and heavy metals till now even they are ubiquitous in China's coastal regions (Wang and Pan, 2004). In the present study, the coastal marine mussel, *Perna viridis*, a local dominant sedentary animal in Daya Bay (Xu, 1989), was exposed to different levels of Zn and Cd media, and the total accumulated and subcellularly distributed metals in the digestive tissues were determined. The aims of our study are: (1) to determine Cd and Zn uptake dynamics and MTLP induction by digestive glands of *P. virdis*; (2) to analysis the relationship between metal accumulation and MTLP induction; and (3) to analyse the possible detoxification mechanisms of Zn and Cd by digestive glands of mussel, *P. virdis*.

1 Materials and methods

1.1 Organisms and exposure media

About 400 individuals of marine mussels, *P. viridis* ((6.5 ± 0.5) cm shell length), a local dominant species in Daya Bay, China, were collected from a locate fishery farm, and maintained immediately under laboratory conditions for 48 hr before exposure experiments. In the process of laboratory acclimatization and exposure experiments, mussels were fed with fresh green algae (*Platymonus* spp.) supplied by Alga Culture Lab in South China Sea Institute of Oceanology. The condition index (CI) of mussels used in our experiments was determined as: CI = drained weight of soft tissues/total weight × 100% = (27.5 ± 2.8)% (n = 6). The conversion factor of wet weight and dry weight of mussels is 5.42 ± 0.37 (n = 6) in the experiments.

In the present study, the exposure concentrations of Zn and Cd were designed higher than their contents in natural seawater (Qiu et al., 2005), but lower than the reported LC_{50} value (Forget et al., 1998; Barka et al., 2001; Yap et al., 2004). Rather high metal exposure levels were designed in our experiments (Table 1) to highlight the physiological responses of mussels, which could help to understand the stress response of marine organisms when suffering from accidental severe metal pollution. Exposure media was prepared using natural seawater collected form Daya Bay to which the corresponding inorganic salts ($ZnCl_2$ and $CdCl_2$) were added and equilibrated for at least 24 hr prior to experiments. The background concentration of Zn was (13.0 ± 1.0) μg/L in natural seawater, while Cd was undetected. Each exposure treatment contained about 50 individual bivalves in a 20 L clean plastic container. The seawater was changed daily to maintain constant metal contents. The exposure media (salinity 31‰, pH 8.2) was aerated constantly at room temperature (25–31°C) and under good natural illumination conditions.

1.2 Samples preparation for MTLP, total and soluble metal contents in digestive glands test

The digestive glands of 4–6 animals were sampled at day 1, 2, 4, 7, 10 of exposure before feeding to avoid food debris, and then stored frozen at –20°C immediately until analysis. Newly sampled digestive glands (2 mussels/pool) were homogenized using a ultrasonic homogenizer in a pH 8.6 buffer solution (1:3, *W/V*) composed of 20 mmol/L Tris, 150 mmol/L NaCl, and 0.5 mmol/L PMSF (phenylmethylsulfonyfluoride), 2.5 mmol/L DTT (dithiothreitol). PMSF was added as protease inhibitor and DTT as reducing agent to avoid MT degradation by proteolytic enzymes and oxidation. Then, 1 mL homogenate sample was pipetted for total metal (T-metal) analysis. The remaining portion was centrifuged for 30 min at 5000 r/min. Aliquot supernatant was sampled and heated at 75°C for 15 min in a water bath to remove the heat-denatured high molecular mass proteins which could interfere with the subsequent MTLP quantification, and then centrifuged for 45 min at 15,000 r/min (4°C). The S_{30} aliquot was separated for the analysis of MTLP and soluble metal.

1.3 Total and soluble metal analysis

For total and soluble metal analysis, the homogenate samples and S_{30} supernatants were digested thoroughly in a mixture of super-pure concentrated HNO_3 and H_2O_2 (2:1, *V/V*) at 70°C. After neutralization pH at the range of 3–5 with NaOH and volume-fixation, total and soluble Zn and Cd contents in digestive gland sample were determined with the 797VA computrace in the DP mode, and the following specific analysis conditions were adopted: purge time 300 sec, accumulation time 60 sec, pulse amplitude 50 mV, pulse time 0.04 sec, sweep rate 0.059 V/sec, rotation rate 2000 r/min, scanning potential for Zn: –1.10 to –0.80 V, for Cd: –0.80 to –0.40 V. During the polarography determination, the sample was buffered with KCl-NaAc solution. Three replica measurements were conducted for each sample and the results were expressed as μg/g ww

Table 1 Zn and Cd concentrations in different exposure experiments

Treatment	Control	Low		Middle		High	
		C_W	C_N	C_W	C_N	C_W	C_N
Zn (μg/L)	13 ± 1	45 ± 2	40	95 ± 5	100	826 ± 15	1000
Cd (μg/L)	n.d.	6.3 ± 0.2	6	34 ± 5	30	293 ± 12	300

C_W: measured metal concentration; C_N: nominal metal concentration; n.d.: not determined.

(wet weight). The detection limit for both the Zn and Cd analysis is 50 ng/L using this method.

1.4 MTLP determination

MTLP induction in mussels digestive glands in our exposure experiments were determined by Brdicka procedure with a 797VA computrace (Metrohm, Switzerland) using differential pulse mode, and the standard MT isolated from rabbit liver was used as the reference material in the experiment. Brdicka procedure is a polarographic method to determine the proteins containing SH-groups in ammonia buffered Co(III) solution (Brdicka, 1993; Raspor et al., 2001), and has been well used in evaluating MTs levels of environmental organisms (Thompson and Cosson, 1984; Erk et al., 2002; Raspor et al., 2004). Briefly, a three-electrode system was used including working electrode HMDE (surface area 0.4 mm^2), reference electrode (Ag/AgCl, 3 mol/L KCl), and auxiliary electrode (Pt wire). The analysis was performed on the 10 mL supporting electrolyte: 0.6 mmol/L $Co(NH_3)_6Cl_3$ and 1 mol/L ammonia buffer (NH_4OH + NH_4Cl). The measurement conditions were as following: scanning potential, –0.90 to –1.70 V; purging time, 420 sec; accumulation time, 90 sec; accumulation potential, –1.4 V; pulse amplitude, 50 mV; pulse time, 0.04 sec; sweep rate, 0.011 V/sec; rotation rate, 2000 r/min. All measurements were performed at constant temperature 15–16°C and each sample was scanned for three times. A calibration curve was set up by the standard addition method, and MT isolated from rabbit liver (M 7641, Sigma, USA) was used as the calibration material. Levels of MTLP are given relative to the wet mass of tissue (μg/g wt).

1.5 Quality control and statistical analysis

All chemical reagents used in our experiments were purchased from Sigma-Aldrich Cooperation, USA. All glasswares used in the experiments were acid washed (5% HNO_3) and rinsed thoroughly with Milli-Q water (Arium 613UV, Sartorius, Germany). All solutions used in the experiments were made with Milli-Q water. Commercially available Zn and Cd standard solutions (Fluka, Switzerland) were used for metal polarograph calibration. Random checks for animal sample digestion and tests with standard reference material (oyster tissue 1566a, US Department of Commerce, Technology Administration, National Institute of Standards and Technology, USA) were made. Agreement was considered good, i.e., within 10%.

Data were analyzed using the software statistica version 5.1 (1997). Student's t-tests (or t'-tests when variances were not homogenous) were used to metal contents in digestive glands and MTLP induction in different exposures.

The replicates used for all parameters test in our experiments were 4–6, and the results were expressed as mean value ± standard error.

2 Results

2.1 Zn and Cd accumulation by digestive glands

The total Zn concentrations in the control mussels digestive glands were determined as (18.88 ± 2.27) μg/g ww, and Cd was not detected for the technique reason. As shown in Fig. 1, a 10-day exposure resulted in a prominent increase of Zn and Cd contents in digestive glands of mussels. The metal uptake of high-level Zn exposure group achieved the saturated capacity almost on

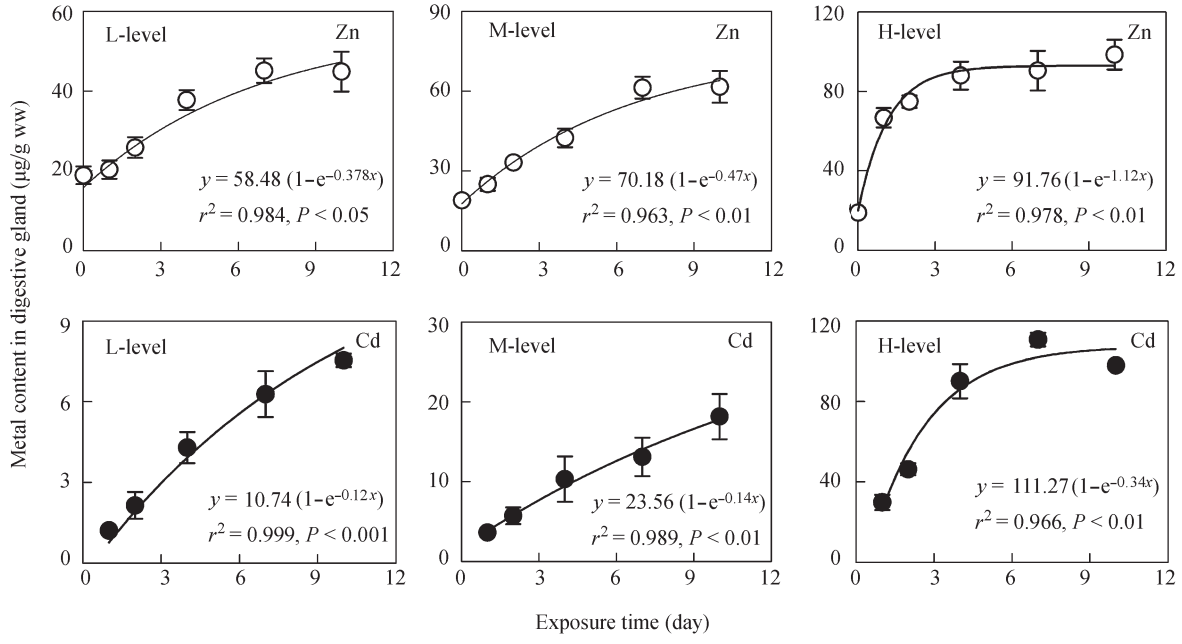

Fig. 1 Total metals concentration in the digestive glands of mussels exposed to low (L-level), middle (M-level), and high (H-level) levels Zn, Cd for 1, 2, 4, 7, 10 days and total metal uptake modes from the two compartment models. It is marked with the equations and statistically significant differences are accepted at $P < 0.05$. Data are expressed as mean ± standard error.

day 4 of exposure. The most uptake of the high-level Cd exposure group occurred on day 7. All other groups were still in the rising period of metal accumulation in the digestive glands at the end of exposure. In general, both Zn and Cd contents in glands increased obviously with the metals exposure concentrations. For instance, on day 4 of exposure, Cd contents in glands were 90.19 ± 8.50 (high level), 10.29 ± 2.85 (middle level), and 4.29 ± 0.58 (low level) μg/g ww, and total Cd contents in digestive glands of mussels in middle and high Cd exposure concentration treatments were respectively 2.39 and 21.02 folds higher than their counterparts in low Cd exposure treatment.

Metals accumulated in digestive glands of mussels are directly transported into target organism after they are uptake into animal bodies from exposure media. Thus, the time course of metal internalization into digestive glands can be described with pulse-chase kinetics model (Hudson and Morel, 1990). For transient uptake, model parameters are evaluated from the following equation:

$$C_A = \rho_0 \tau (1 - e^{-t/\tau})$$

where, C_A (μg/g) is the total metal contents in digestive glands; ρ_0 (μg/(g·day)) is the initial uptake rate at start of chase, t (day) is exposure time, and τ (day) is mean residence time of metal in digestive glands.

Table 2 Simulated parameters of kinetic modes of Zn and Cd in different treatments

Parameter	Zn			Cd		
	Low	Middle	High	Low	Middle	High
ρ_0 (μg/(g·day))	21.66	33.26	102.43	1.32	3.19	38.01
τ (day)	2.71	2.11	0.89	8.13	7.39	2.93

ρ_0: uptake rate at start of chase; τ: mean residence time of metal in digestive glands.

All kinetic parameters calculated for Zn and Cd uptake by mussel digestive glands are listed in Table 2. It was found that the initial uptake rates for Zn and Cd increased significantly with increasing their exposure levels and the mean residence time for both metals had the contrary trends. For example, the calculated initial uptake rates for Zn by digestive glands were 102.43 (high level), 33.26 (middle level), and 21.66 (low level) μg/(g·day) respectively, while the mean residence time for their corresponding treatments were 0.89, 2.11, and 2.71 days respectively.

2.2 Metal fraction in digestive glands of mussels

The metals accumulated in glands of mussels were operationally fractionalized into soluble and insoluble metals in our experiments, which was helpful to learn the distributed state of metals and possible metal assimilation mechanisms in the mussel bodies. There was no significant difference of ratios of soluble to total metal contents in digestive glands among all exposure treatments, and Zn and Cd had similar such ratios in digestive glands (Fig. 2). In general, for both Zn and Cd, in middle and low concentration treatments, the ratios of soluble to total metals in glands decreased linearly on the whole exposure course, while in the high concentration treatments, the ratios decreased obviously in first 4 days and leveled off after then on. For instance, when mussels were exposed to 30 μg/L Cd solution (nominal concentration, middle treatment), the ratio of soluble Cd to total Cd in glands decreased from 0.62 ± 0.15 to 0.31± 0.08 continually during the whole course, and in the 300 μg/L Cd treatment (nominal concentration, high level), the ratio decreased rapidly from 0.64 ± 0.09 on day 1 to 0.44 ± 0.11 on day 4, and after that, the ratios leveled off basically, the values were 0.40 ± 0.08 and 0.40 ± 0.10 on day 7 and day 10 day of exposure.

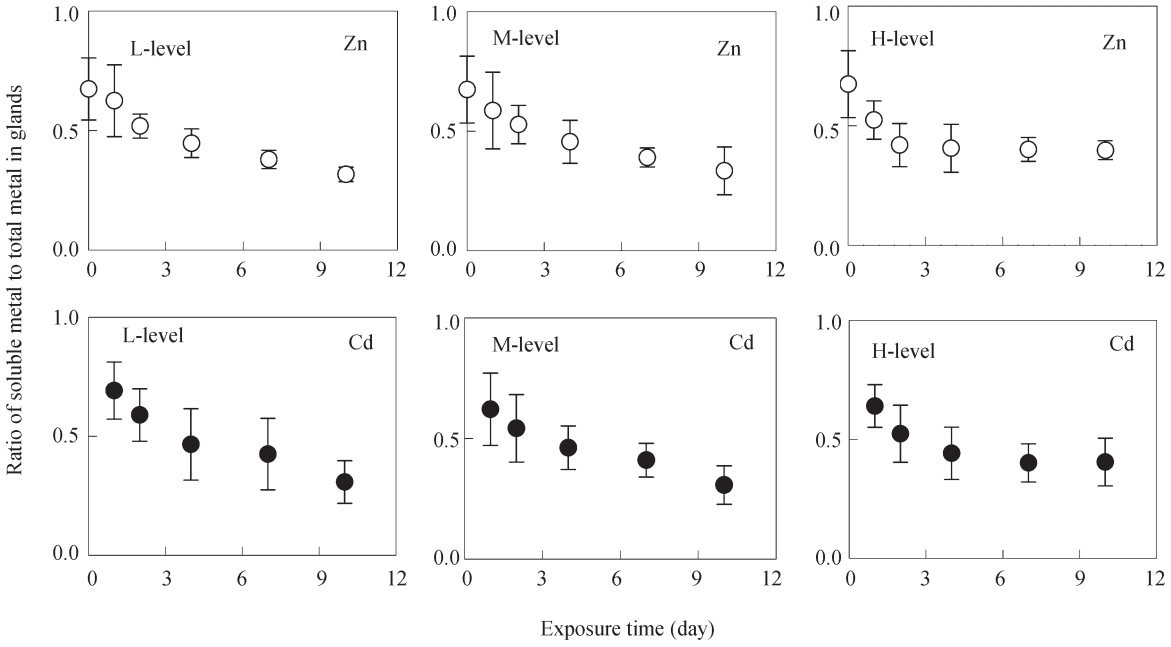

Fig. 2 Changes of the ratios of soluble metal to total metal in the digestive glands of mussels in different metal treated groups during the exposure time. Data are expressed as mean ± standard error.

2.3 MTLP induction by digestive glands

The heights of hydrogen reduction wave (Cat) in scanning current-potential curves of MTLP samples by Brdicka reaction was used to evaluate the MTLP content. Figure 3 depicts time-dependent MTLP induction by digestive glands of mussels exposed to different concentrations of Zn and Cd. The mean MTLP content of control mussels was (0.551 ± 0.037) μg/g ww (n = 8) and did not vary significantly (p > 0.05). MTLP synthesis by digestive glands of mussels was observed in almost all treatments in our exposure experiments and increased with the exposure metal levels. In general, MTLP contents in digestive glands were observed to increase continuously when the organisms were exposed to low and middle levels of Zn and Cd media, while amounted to maximal levels on day 4 and decreased after then when they were exposed to high level Zn and Cd solutions. For instance, in Zn exposure treatments, digestive glands MTLP contents in low level and middle level groups increased 30% and 51% at the end of experiment, while in high level group, those mussels had responded quickly at first and attained to the maximal MTLP level (1.05 μg/g ww) on day 4, and then decreased obviously.

2.4 Relationship of MTLP and metal contents in digestive glands

As depicted in Figs. 1 and 3, for both Zn and Cd exposure, metal and MTLP contents in the mussel digestive glands had similar variation behaviors. When mussels were exposed to low and middle levels of Zn and Cd, metal and MTLP contents in digestive glands increased in the whole exposure course. While in the high Zn and Cd treatments, metal contents in glands increased up to maximum on day 4 and level off then on, the MTLP levels also increased continuously in the first 4 days but decreased after that. The relationships between the accumulated metal concentrations and MTLP levels in the digestive glands of mussels have been examined and shown in Fig. 4. The results indicated that MTLP significantly correlated with Zn and Cd accumulated in digestive glands. For instance, when mussels were exposed to low level of Zn and Cd, MTLP and metal contents in digestive glands had positive linear relationship (r^2 = 0.963 and 0.965 for Zn and Cd respectively). However, such relationship did not exhibit in high level treatment.

3 Discussion

The observed metal contents in digestive glands of mussel, *P. viridis*, were generally comparable to previous measurements (Dragun et al., 2004; Shi and Wang, 2004). MTLP measured by Brdicka protocol in our experiments was obviously higher than other reported values obtained by Ag-saturation method (Lecoeur et al., 2004; Shi and Wang, 2004), but was in a good agreement with the results reported by Dragun et al. (2004), Geffard et al. (2005) and Raspor et al. (2004) with the electrochemical method. Calculated uptake rate at start of chase, ρ_0, for Zn (21.66–102.43 μg/(g·day)) were obviously higher than Cd ρ_0 (1.32–38.01 μg/(g·day)), while calculated mean residence time of metal in digestive glands, τ, for Zn (0.89–2.71 days) were much lower than their counterparts for Cd (2.93–8.13 days). Zn and Cd had a similar metal fraction in digestive glands of mussels in our experiments, the ratios of soluble to total metal contents for Zn were 0.32–0.67, for Cd were 0.31–0.69.

Metal Zn and Cd contents in digestive glands of mussels, *P. viridis*, increased with increasing ambient metal concentrations and duration of metal exposure in general.

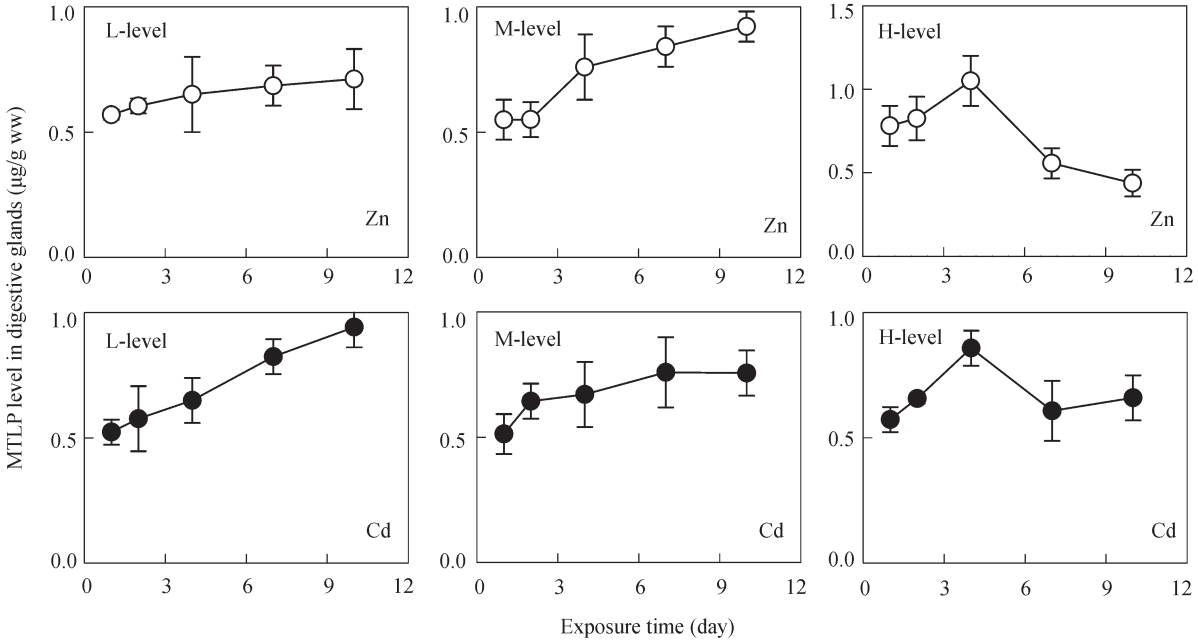

Fig. 3 MTLP concentrations in the digestive glands of mussels exposed to different levels Zn, Cd for 1, 2, 4, 7, 10 days. Data were expressed as mean values ± standard error.

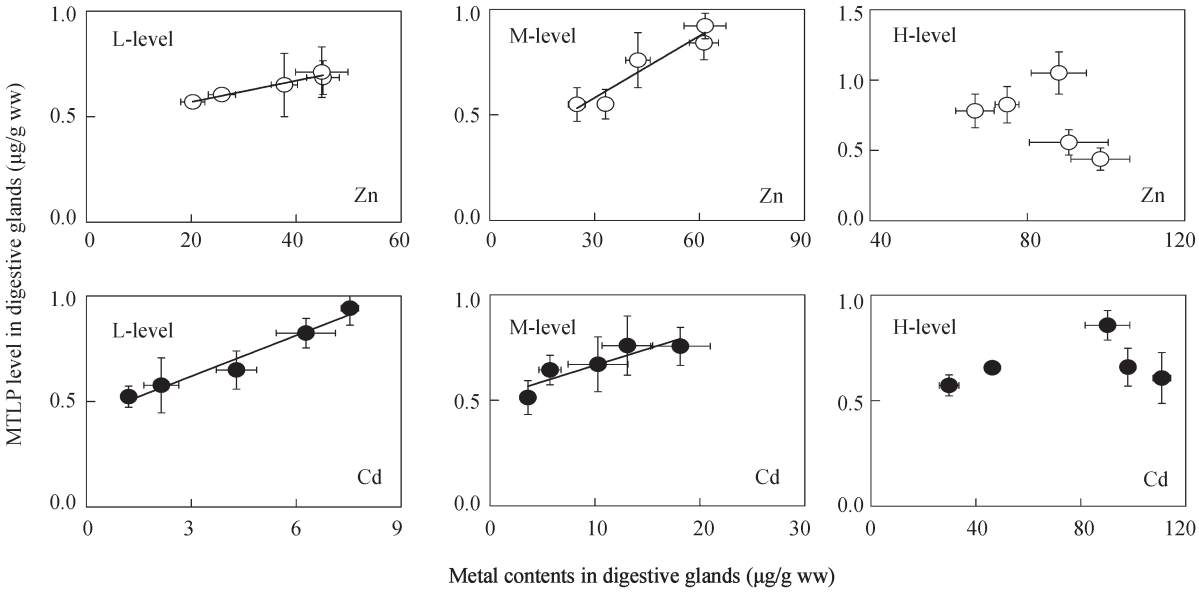

Fig. 4 Relationships between MTLP and metals (Zn, Cd) accumulated in the digestive glands of mussels (the linear regression equations and Pearson's correlation coefficients are exhibited and statistically significant differences are accepted at $P < 0.05$).

Calculated uptake rate at start of chase, ρ_0, increased for both Zn and Cd with increasing exposure concentrations. The findings in our experiments revealed that mussels accumulate heavy metals at high uptake rates when exposed to high level dissolved metals in the seawater. The obviously higher uptake rate at start of chase and shorter mean residence time of Zn than Cd in digestive glands of mussels indicated that metal Zn and Cd had significant different accumulation behaviors even though they had a close location in element table. Digestive glands accumulated Zn at a faster velocity than Cd, and maintained Zn for a shorter time than Cd. The results in our study suggested that the uptake dynamics of metals were metal-specific, even the metals have a similar location in element table.

Our results also revealed that the ratio of soluble Zn and Cd to total contents of them in the mussel digestive glands decreased in the whole process of exposure experiments in the low and middle levels treatments, and decreased first and leveled off then in high concentration exposure experiments. The results suggested that more and more Zn and Cd accumulated by mussel digestive glands were stabilized during the exposure process (in low and middle level treatments and the first stage of high level exposure) until the metal uptake was saturated (at the last stage of high concentrations treatments). We could conclude that the insolubilization was an important mechanism of metal detoxification by mussel digestive glands.

Heavy metal poisoning is usually thought to involve the inhibition of enzymes function through the formation of metal mercaptides with enzyme sulphydryl groups, also alter the antioxidant balance of bodies and directly compete with nutrient trace elements for binding sites on some proteins (Van and Clijsters, 1990; Lou and Shen, 2001). The organisms could adjust a variety of response and tolerance mechanisms to resist the poisonous effects. It had been reported (Brown, 1982; Viarengo et al., 1988; Marigomez et al., 2002) that metal detoxification in vertebrates was mainly through binding these ions to MTs. In our experiments, MTLP contents in mussel digestive glands were observed to have such an expected trend: in low and middle concentrations of Zn and Cd exposure treatments, MTLP contents in digestive glands increased continuously while increased firstly and decreased after 4 days of exposure. Similar relationships of MTLP and metals (Zn and Cd) contents in digestive glands in different treatments in our study verified the important physiological function of MTLP in metal accumulation and detoxification in mussels. These findings implied that MT anabolic system in mussels digestive glands is very sensitive to the sudden metallic stress.

Metals accumulated in the organisms are likely to be present in the two phases (Barka et al., 2001; Narriri et al., 2000): dissolved metals acted mainly as chelate complexes with metal-binding proteins such as MTLP, and incorporated into metal-rich granules. Based on the findings of our experiments on MTLP induction and ratios of soluble metal to total metal contents in mussels digestive glands, we could conclude that these two metal detoxification mechanisms work simultaneously. When exposed to severe metal stress, the organism would regulate all possible strategies to resist. However, we did not determine which detoxification process dominated in digestive glands of mussels, *P. virdis*.

4 Conclusions

The MTLP level and metal (Zn, Cd) accumulation in the digestive glands of mussels were investigated in this study. The results indicated that the organisms could adjust some physiological mechanisms to resist the metal poisonous effect. Accumulated metals would combine with metal-binding proteins, MTLP, and the superfluous metals

accumulated in the organisms were mainly separated by the insolubilization process when mussels were exposed to severe metal conditions. Generally, the MTLP levels in the tissues increased with metal concentration in organisms and seawater, and attained the maximum state about for 4–7 days of exposure, then the synthesis could be inhibited for long duration of metal exposure and elevated metal uptake in the bodies. Compared to combination of MTLP and metals accumulated by digestive glands of mussels, insolubilization process was a stable mechanism for metal detoxification.

Acknowledgments

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