

RESEARCH ARTICLE

Cadmium Accumulation and Metallothionein Biosynthesis in Cadmium-Treated Freshwater Mussel *Anodonta woodiana*

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Abstract

This study investigated the distribution of cadmium (Cd) and the protein level of metallothionein (MT) and examined the relationship of Cd accumulation and the MT concentration in different tissues of freshwater mussel *Anodonta woodiana* following Cd treatment. The mussels were exposed to Cd (4.21, 8.43, 16.86, 33.72 and 67.45 mg L⁻¹) for 24, 48, 72 and 96 h, respectively. After Cd treatment, the gills, mantle, foot, visceral mass and digestive gland tissues were collected for analysis. We found that, in the controls, Cd distributed in all tissues in the concentration order of gills>mantle>foot>visceral mass>digestive gland. Upon Cd treatment, Cd concentration significantly increased in all tissues. The highest Cd accumulation was found in the digestive gland, which was 0.142 mg g⁻¹ ($P<0.05$). MT levels in the gills and mantle of the mussels increased significantly ($P<0.05$), which were in positive correlation with Cd accumulation in the tissues ($P<0.05$). In conclusion, our results demonstrated a correlation between Cd accumulation and MT up-regulation in gills and mantle of the mussels after Cd treatment. It is suggested that the protein level of MT in gills and mantle of *Anodonta woodiana* is a good biomarker for Cd contamination.

Introduction

Cadmium (Cd) is a biotoxic element and one of the major metals that are ubiquitously distributed in aquatic systems [1–3]. It is also a widely used heavy metal in the industry. In recent years, serious Cd pollution in rivers, estuaries, and near-shore waters has become a serious problem [4]. Anthropogenic input is considered to be the main source of Cd contamination in aquatic environments [5]. Aquatic organisms that encounter waterborne Cd over a long period of time can get adapted and show Cd tolerance [6].

Metallothioneins (MTs) are low molecular weight non-enzymatic proteins that are rich in cysteine, are free of aromatic amino acids and are heat stable. The thiol groups of cysteine residues enable MTs to bind essential and non-essential metals with high affinity [7]. MTs are ubiquitous among mollusc species and play a role in the homeostatic control of essential metals (Cu, Zn) to fulfill enzymatic and metabolic demands [8]. They also play an important role in the detoxification of non-essential trace metals such as Ag, Cd and Hg, which protects organisms against

oxidative stress by scavenging intracellular free radicals [9–12]. MT has been used as a biomarker of metal contamination in various species for pollution biomonitoring [7, 13, 14]. This assists in the protections of potamic, lacustrine, estuarine and coastal structure and function.

Recent field studies have demonstrated that Cd can accumulate in aquatic invertebrates, which can cause an elevation of the intracellular level of MT [7, 15, 16]. In freshwater crab *Sinopotamon henanense*, Cd increases the MT levels in the gills and hepatopancreas in time- and dose-dependent, as well as tissue-specific manner [12]. But, there is no relationship between the total Cd, cytosolic Cd and MT level in the digestive gland of most cephalopods from the Bay of Biscay (France) and the Faroe Islands, suggesting the involvement of other Cd-binding ligands [17]. In *Pyganodon grandis*, limnological factors and Cd field contamination can influence the variations in the concentrations of Cd and MT. The pH, calcium (Ca) and Cd at the sediment–water interface explain 74% of the variability in Cd concentrations in the gills. Ca and Cd together explain 62% of the variation in MT concentrations in the gills [18]. Cd detoxification appears to be reasonably effective in the gills and digestive gland of *Pyganodon grandis*, as judged from the protection of the heat-denaturable protein fraction. However, Cd concentration dose increase in potentially metal-sensitive organelles (i.e. mitochondria) in both organs [19]. Moreover, MT protein levels are clearly related to accumulated Cd concentrations in the soft body of *Pyganodon grandis* [20], which is also noted in the whole soft body of two other freshwater bivalves, *Dreissena polymorpha* and *Corbicula fluminea* [21]. The levels of Cu and Cd thioneins increase during metal exposure (3–4 weeks) in the gills and digestive gland of *Mytilus galloprovincialis*, confirming that MTs play a fundamental role in the accumulation of these metals [22]. In aquatic environments, mussels have been widely used for assessing contamination levels, particularly those caused by heavy metals, because of their high bioconcentrating capacities and sensitivity to contaminants [23–26]. The freshwater mussel *Anodonta woodiana* is a common, inexpensive and economically valuable species. This species is widely distributed in rivers and lakes in China, and it plays an important functional role in fresh water ecosystems [27]. Some studies investigated the metabolism and the activities of different enzymes, attempting to use them as potential biomarkers for contamination [28–30]. However, no study has focused on *in vivo* Cd accumulation and MT expression as well as their relationship in the mussel *Anodonta woodiana*.

The present study investigated Cd accumulation and MT protein levels in various tissues of *Anodonta woodiana*, which include the gills, mantle, foot, digestive gland and visceral mass. The relationship between Cd accumulation and MT levels was also examined.

Materials and Methods

Ethics Statement

Our study was permitted by Taiyuan government, Shanxi Province, China. We also confirm that the current study did not involve endangered or protected species.

Chemicals

Cadmium chloride (CdCl_2), nitric acid and perchloric acid were obtained from Beijing Chemical Reagent Co., Ltd. (Beijing, China). Bovine hemoglobin was purchased from Sigma Co., (St. Louis, MO).

Animals and experimental design

Anodonta woodiana were collected from the Fen River (37°55' N, 112°14' E) of Shanxi Province, China. They were acclimated for 2 weeks in glass aquaria filled with dechlorinated and UV-treated tap water (pH 7.5±0.3, dissolved oxygen 8.0±1 mg L⁻¹, temperature 20±2°C) under a regime of 12 h light/12 h dark before the experiments. Water was changed every three days. The mussels were fed with commercial feeds every other day.

Experimental treatment and sample preparation

After acclimation, adult mussels (48.0 ± 4.0 g weight, 6.8 ± 0.3 cm in length) were randomly divided into six groups, with 20 mussels for each group in glass aquaria kept at the same conditions as in the acclimation period, one of which was used as control group. The mussels in other groups were treated with five sub-lethal concentrations of Cd (1/32, 1/16, 1/8, 1/4 and 1/2 of the 96 h LC_{50} ; the 96 h LC_{50} of acute Cd poisoning is 134.9 mg L^{-1}) [30], which were equivalent to 4.21, 8.43, 16.86, 33.72 and 67.45 mg L^{-1} of Cd, respectively. During the experiments, mussels were not fed. Mortality in the treated groups was ($10 \pm 5\%$), which was similar to that of the controls. Mussels were treated by Cd under controlled laboratory conditions.

Mussels from the control and treatment groups were collected after 24, 48, 72 and 96 h, respectively. They were depurated in aquarium water for three times and sacrificed on ice. The gills, mantle, foot, digestive gland and visceral mass tissues were dissected, frozen immediately in liquid nitrogen and stored at -80°C until use. Each tissue sample was divided into two parts that were used for Cd and MT analyses, respectively.

Determination of Cd and MT concentration

To estimate the amount of Cd, the gills, mantle, foot, digestive gland and visceral mass (including gonad and intestine) tissues were digested in 20 ml nitric acid for 12 h at 20°C , then in nitric acid (10 ml) plus perchloric acid (2 ml) for 3 h at 105°C . The concentration of Cd was determined using atomic absorption spectrophotometry (Varian AA240, America) at 228.8 nm according to the method described by Amiard [31], which was expressed as mg g^{-1} wet weight.

The level of MT was determined using the method described by Onosaka [32]. Tissue samples were weighed and homogenized with an ultraturax homogenizer in Tris-HCl buffer (0.01 M, pH 8.6) at a ratio of 1:9 w/v on ice. The homogenates were then centrifuged at 15000 g for 20 min at 4°C , and aliquots of the supernatant (400 μl) were incubated with 400 μl of CdCl_2 solution (20 mg L^{-1}) at room temperature for 10 min to saturate the metal binding sites of MT. Following an addition of bovine hemoglobin (2%, 200 μl), samples were incubated on ice for 5 min at 4°C and then heat-treated in a water bath for 2 min at 100°C . MT was heat stable and other denatured proteins were removed by centrifugation at 12000 g for 10 min at 4°C . Bovine hemoglobin addition, heat treatment and centrifugation were repeated three times. Cd concentrations were measured in the supernatant by atomic absorption spectrophotometry (Varian AA240, America). Data were expressed as $\mu\text{g g}^{-1}$ wet weight.

Statistical analysis

Data were presented as mean \pm S.D. Multi-group comparison was performed with one-way analysis of variance (ANOVA) using the computer software package SPSS 15.0. Differences among individual groups were determined using the least significant difference test. Pearson correlation coefficient and linear regression analysis were calculated between Cd concentrations and MT levels. $P < 0.05$ was considered as statistical significant.

Results

Cd accumulation in the tissues of the mussels

As shown in Figs. 1–5, Cd accumulation was measured in different tissues of the mussels. In the controls, Cd was detected in different tissues in the following concentration order: gills > mantle > foot > visceral mass > digestive gland.

In the experimental groups, Cd concentration increased markedly in the gills of the mussels treated with Cd at 8.43, 16.86, 33.72 and 67.45 mg L^{-1} Cd for 24 and 72 h ($P < 0.05$). Cd

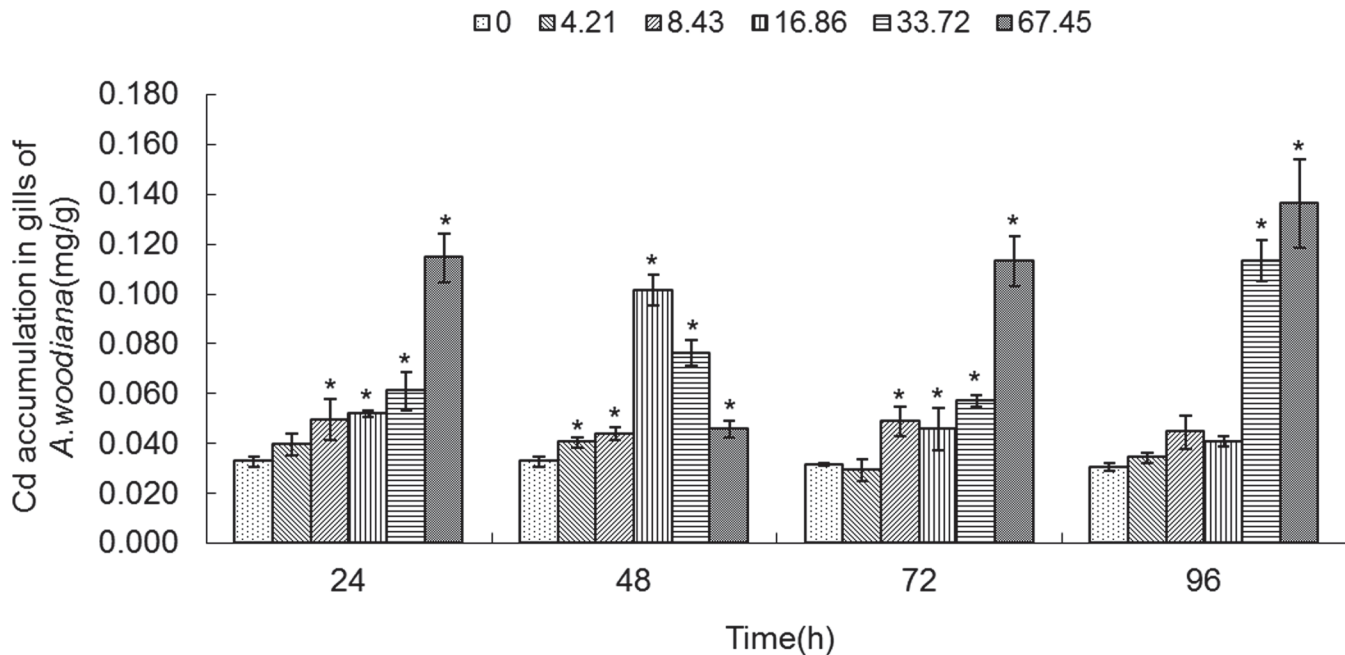


Fig 1. Cd accumulation in gills of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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concentration increased in all experimental groups treated with Cd for 48 h ($P < 0.05$). At 96 h, it increased to the highest level in the gills treated with Cd (67.45 mg L^{-1}) ($P < 0.05$, Fig. 1). The Cd concentration in mantle of the mussels increased significantly ($P < 0.05$) in the following four groups: a. 8.43, 16.86, 33.72 and 67.45 mg L^{-1} Cd for 24 h; b. 67.45 mg L^{-1} Cd for 48 h; c. 16.86, 33.72 and 67.45 mg L^{-1} Cd for 72 h; and d. 33.72 and 67.45 mg L^{-1} Cd for 96 h, which peaked at 67.45 mg L^{-1} Cd for 96 h ($P < 0.05$, Fig. 2). As shown in Fig. 3, when the mussels were exposed to Cd for 24 h, the Cd concentration in foot increased significantly at all of the experimental groups, which peaked in the group of 67.45 mg L^{-1} Cd (all at $P < 0.05$). At 48 h, Cd concentration increased notably in the groups treated with Cd at 8.43, 16.86, 33.72 and 67.45 mg L^{-1} ($P < 0.05$). At 72 h, it increased notably in the 16.86 and 67.45 mg L^{-1} Cd groups ($P < 0.05$). At 96 h, Cd concentration increased in the 33.72 and 67.45 mg L^{-1} Cd groups ($P < 0.05$, Fig. 3). Fig. 4 summarized the Cd concentrations in the visceral mass in all experimental groups. At 24 h, the Cd concentration remained unchanged in all experimental groups, except that it increased in the 67.45 mg L^{-1} Cd group ($P < 0.05$). Cd concentration increased significantly (all at $P < 0.05$) in all other experimental groups of different treatment concentrations and different exposure time points, except the 4.21 mg L^{-1} Cd 72 h group (Fig. 4). In the digestive gland, the Cd concentration increased significantly in all of the experimental groups ($P < 0.05$), except the three 48 h groups (4.21, 8.43, 16.86 mg L^{-1} , respectively), and it reached the highest level in the 67.45 mg L^{-1} Cd 48 h group (Fig. 5).

MT concentration in tissues of mussels treated with Cd

MT levels in different tissues of the mussels exposed to Cd were shown in Figs. 6–10. In the controls, the order of MT content in different tissues was digestive gland > foot > mantle > visceral mass > gills.

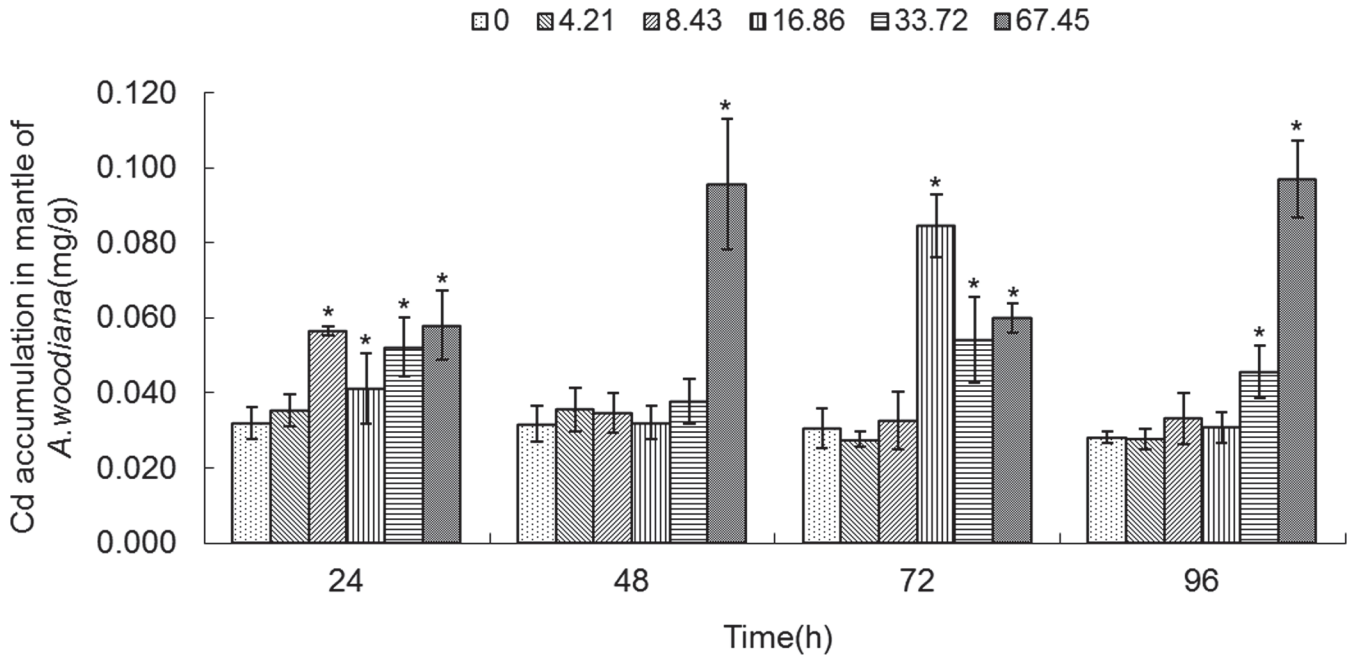


Fig 2. Cd accumulation in mantle of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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As shown in Fig. 6, the MT level increased in gills in all of the experimental groups ($P < 0.05$), except the 4.21 mg L⁻¹ Cd 96 h group. MT level reached a maximum in the 16.86 mg L⁻¹ Cd 24 h group (Fig. 6). As shown in Fig. 7, the MT level in mantle tissue increased only in

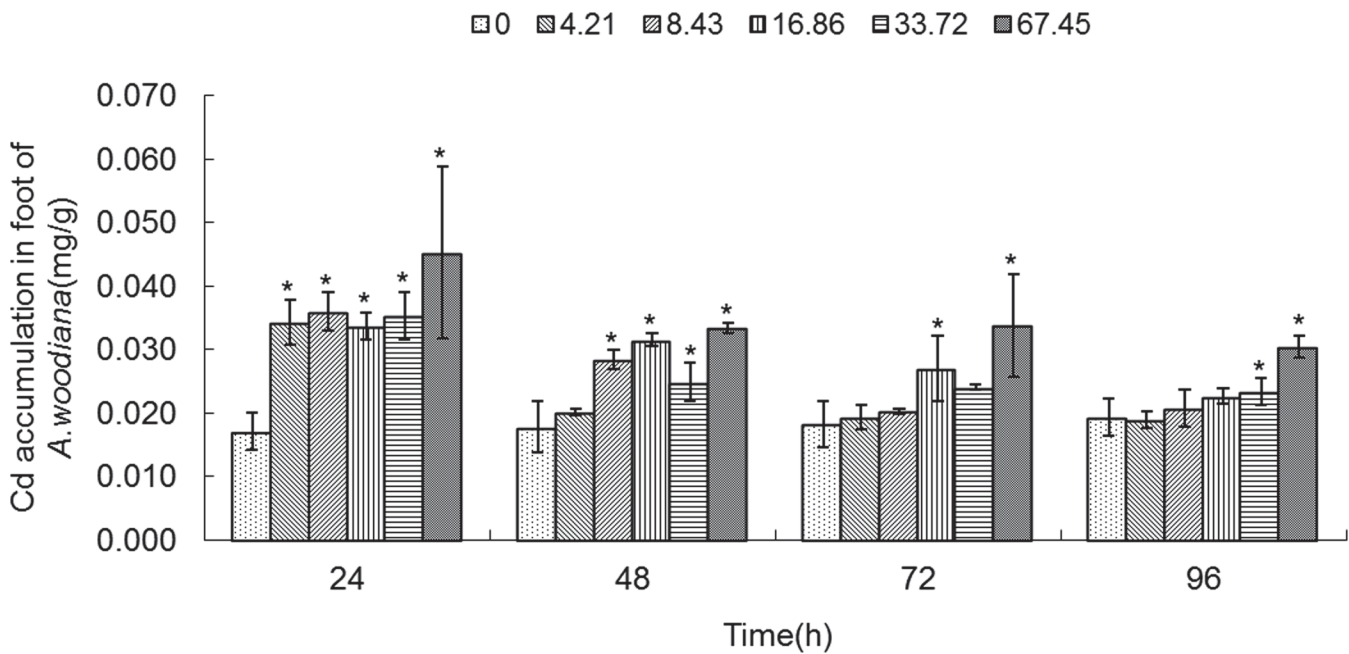


Fig 3. Cd accumulation in foot of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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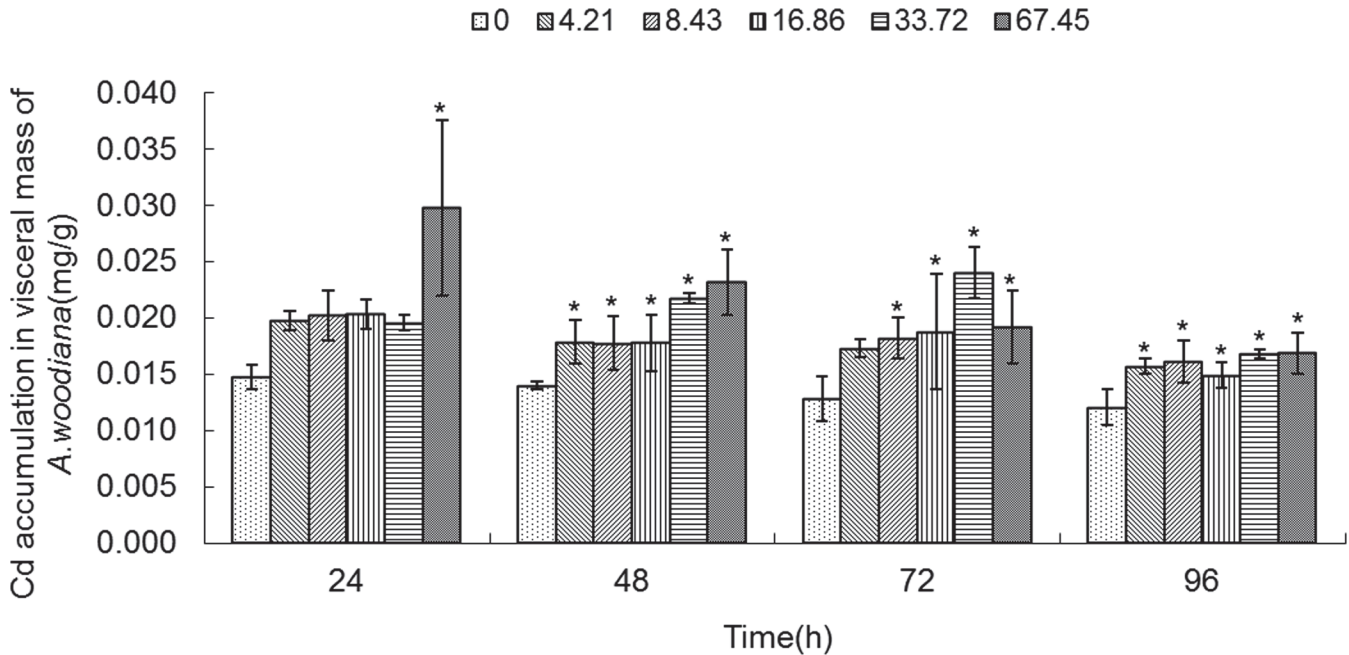


Fig 4. Cd accumulation in visceral mass of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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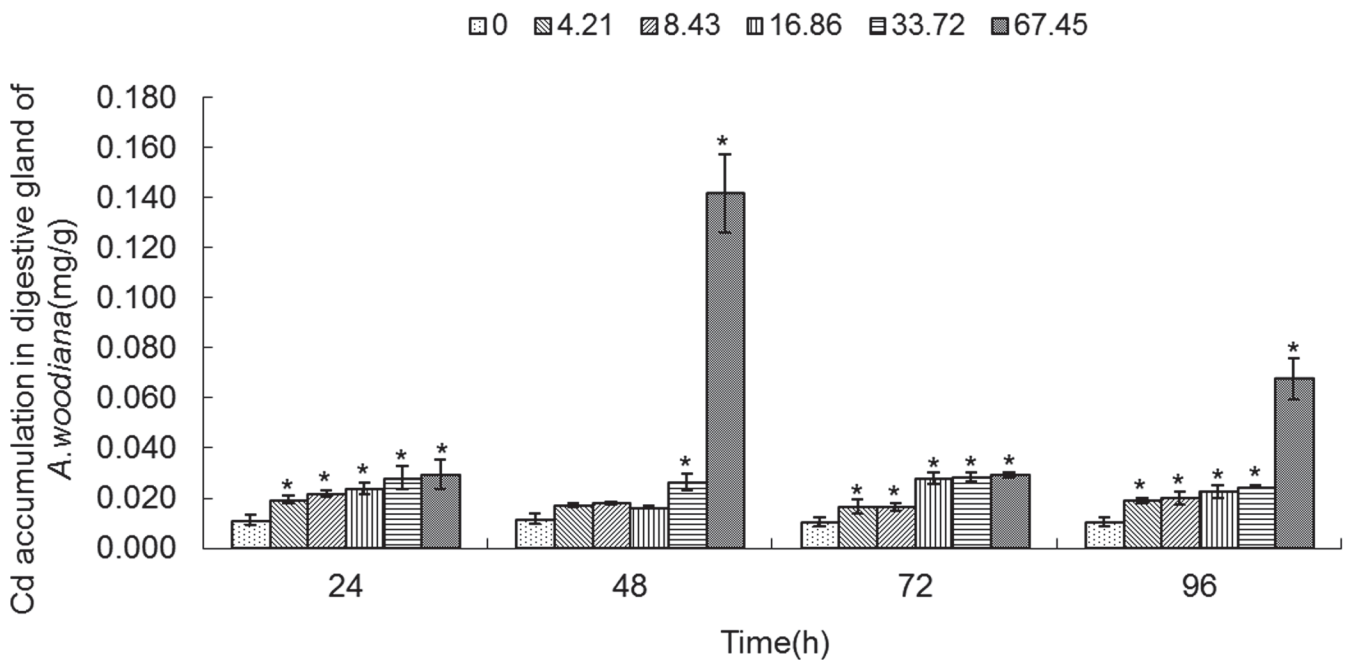


Fig 5. Cd accumulation in digestive gland of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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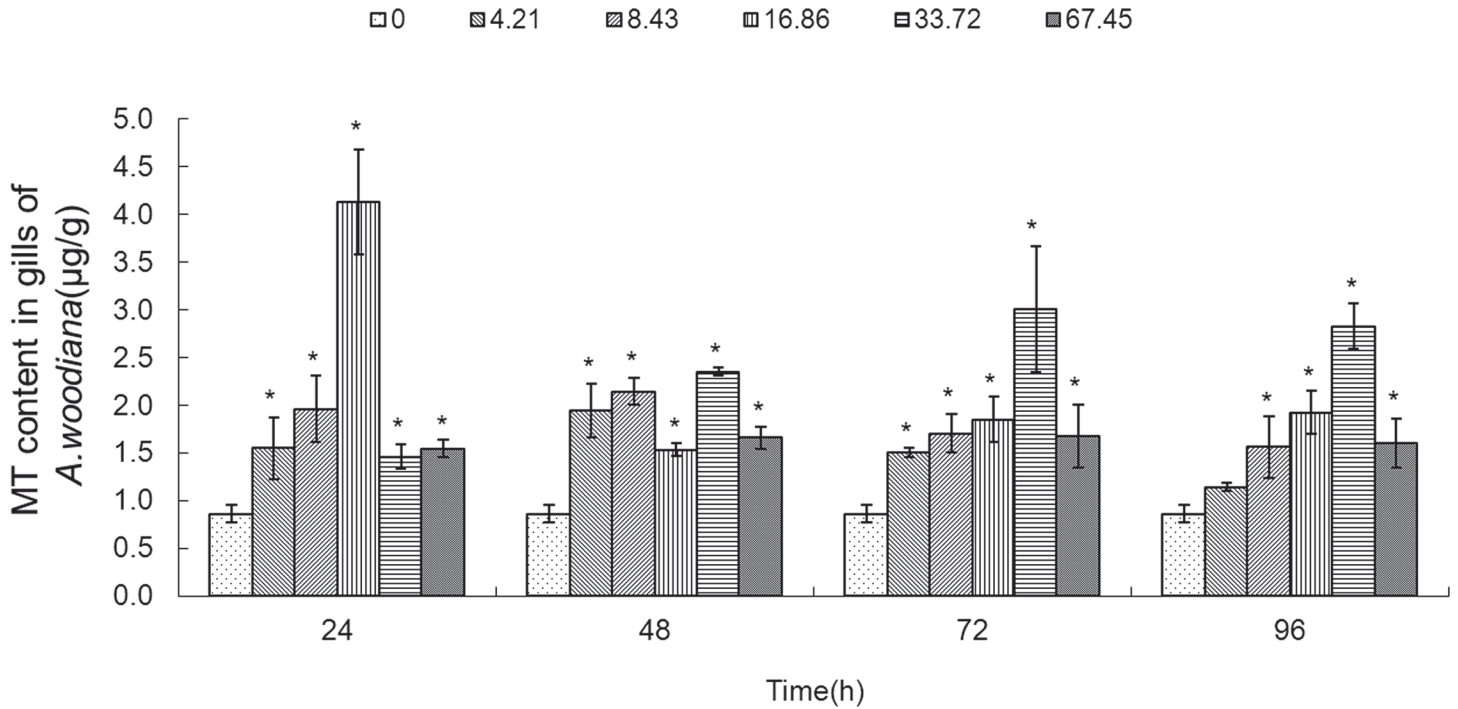


Fig 6. MT content in gills of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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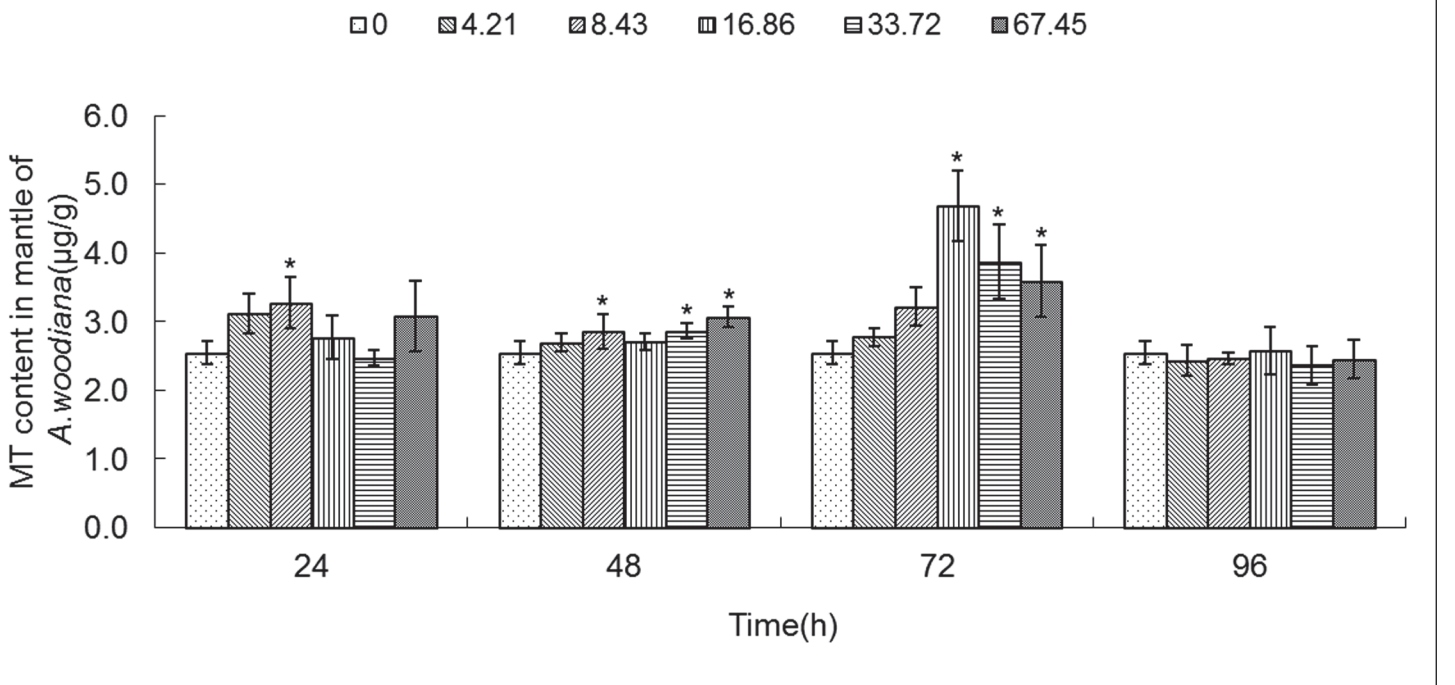


Fig 7. MT content in mantle of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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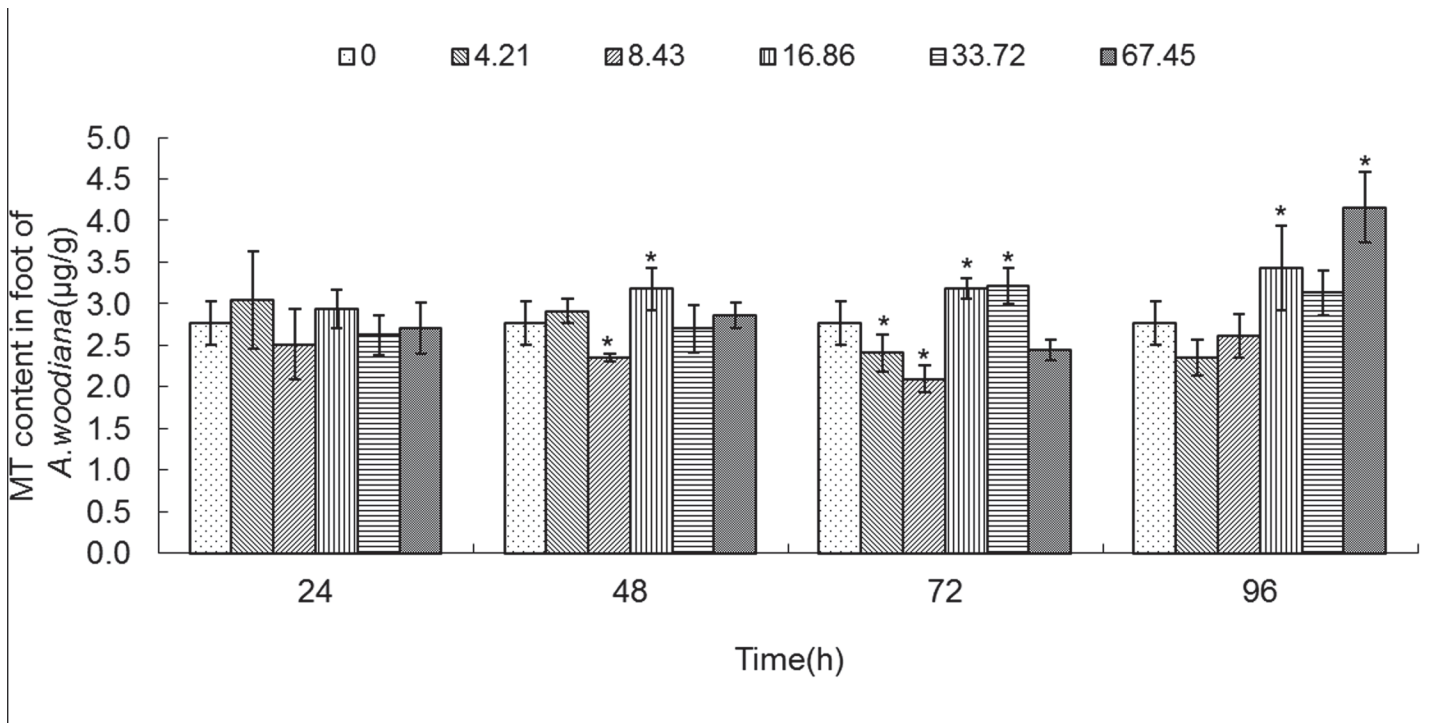


Fig 8. MT content in foot of *Anodonta woodiana*. Data are expressed as mean \pm standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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the 8.43 mg L⁻¹ Cd group for 24 h. At 48 h, the MT level increased in the 8.43, 33.72 and 67.45 mg L⁻¹ Cd groups. At 72 h, the MT level increased in the 16.86, 33.72 and 67.45 mg L⁻¹ Cd groups, which reached the highest level in the 16.86 mg L⁻¹ Cd group. It remained unchanged in all the experimental groups for 96 h (Fig. 7). In the foot (Fig. 8), the MT level decreased in the 8.43 mg L⁻¹ Cd group, but increased in the 16.86 mg L⁻¹ Cd group for 48 h ($P < 0.05$). At 72 h, the MT protein concentration decreased significantly in the groups treated with lower Cd concentrations (4.21 and 8.43 mg L⁻¹), but increased in the groups treated with higher Cd concentrations (16.86 and 33.72 mg L⁻¹, $P < 0.05$). The MT protein concentration peaked in the 67.45 mg L⁻¹ Cd 96 h group ($P < 0.05$, Fig. 8). In the visceral mass of the mussels exposed to Cd (4.21, 8.43, 16.86 and 67.45 mg L⁻¹) for 24 h, the MT level decreased significantly ($P < 0.05$) and it remained unchanged for 48 and 72 h. In contrast, it increased notably in the 67.45 mg L⁻¹ 96 h group (Fig. 9). As shown in Fig. 10, the MT level in the digestive gland decreased at 24 h in all of the treatment groups ($P < 0.05$). A decrease in MT level was also noted in some other experimental groups which included 4.21 and 8.43 mg L⁻¹ groups (72 h) as well as 16.86 and 67.45 mg L⁻¹ groups (96 h). The MT level remained unchanged in the experimental groups treated for 48 h (Fig. 10).

Correlations between Cd accumulation and MT concentration

In order to examine the relationship between Cd accumulation and the protein level of MT, we performed statistical analyses using the Pearson correlation coefficient and linear regression analysis. As shown in Table 1, in the gills, Cd treatment markedly increased the MT levels in a concentration-dependent manner (96 h, $P < 0.05$), and the tissue Cd accumulation correlated positively with the protein level of MT ($P < 0.05$). Similarly, a positive correlation between Cd

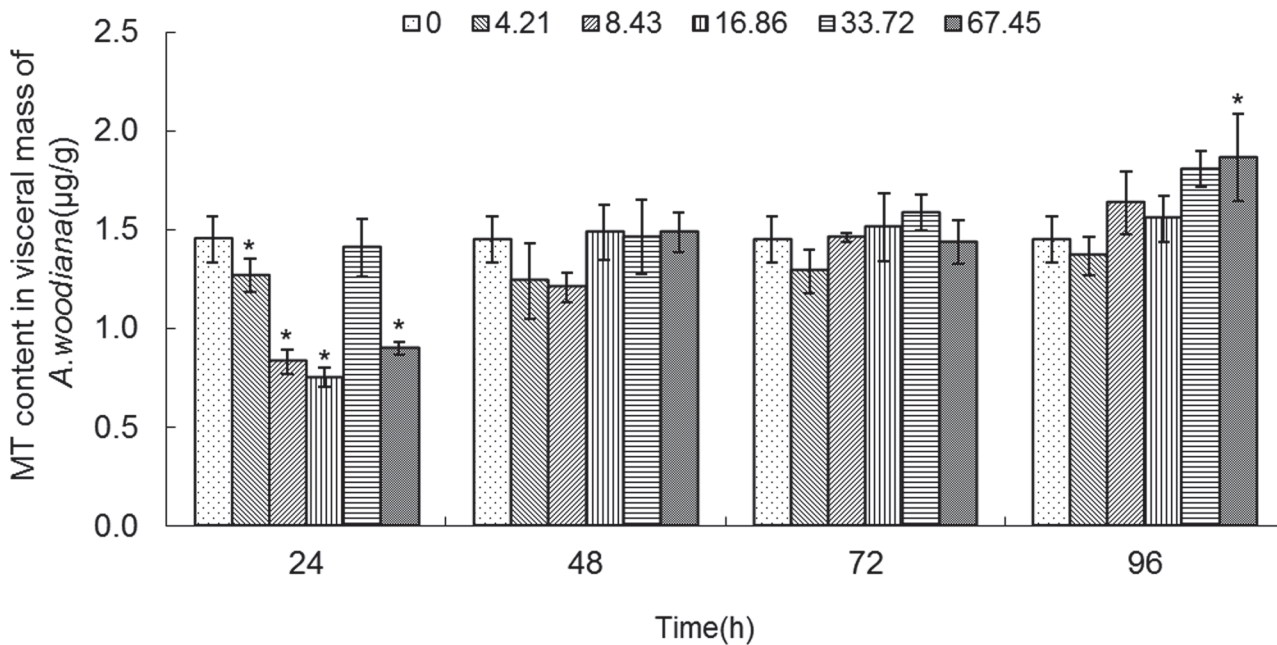


Fig 9. MT content in visceral mass of *Anodonta woodiana*. Data are expressed as mean ± standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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concentration and the protein level of MT was found in the mantle (48h and 72h, $P < 0.05$). Surprisingly, the MT protein concentration in the digestive gland showed a negative correlation with Cd accumulation (24 h, $P < 0.05$).

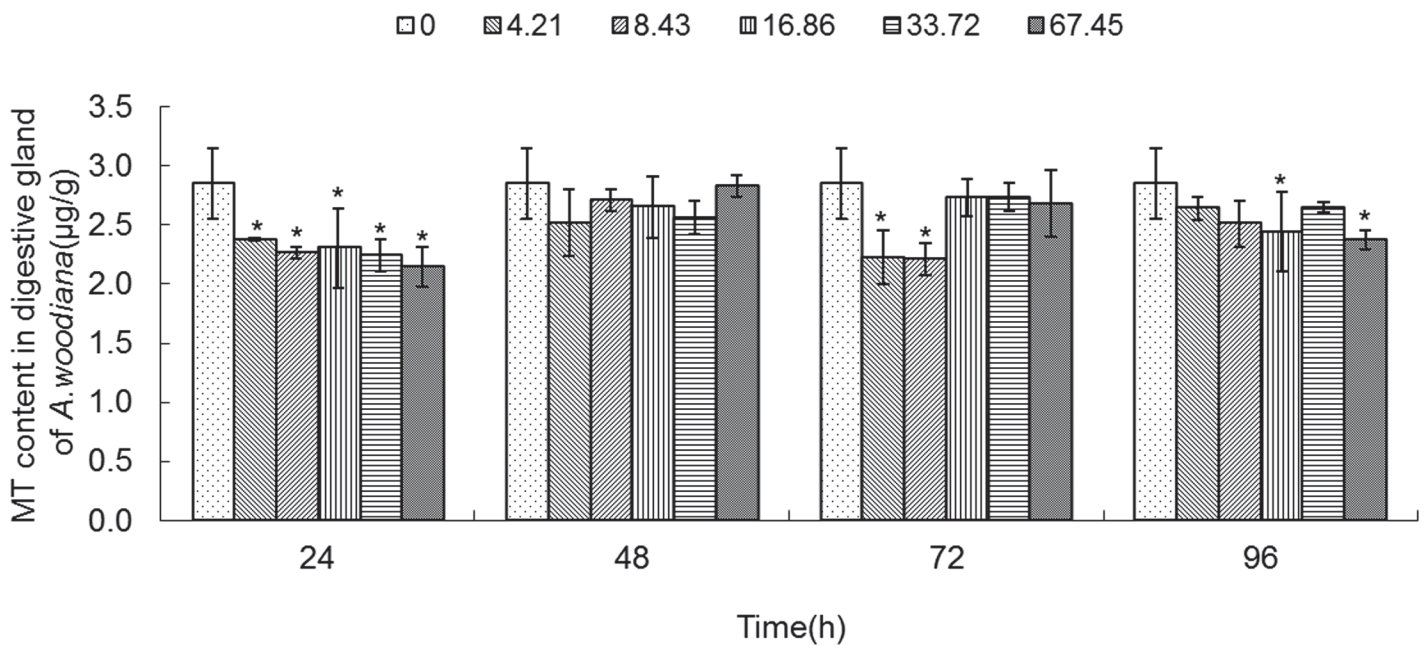


Fig 10. MT content in digestive gland of *Anodonta woodiana*. Data are expressed as mean ± standard deviation (n = 3). Comparison between the control and treatment groups is notified as * $p < 0.05$.

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Table 1. Correlations between Cd accumulation and MT levels.

Tissue	Time (h)	Correlation coefficients	Regression equation	P value. (2-tailed)
Gills	96	0.568	$y = 8.767x + 1.066$	0.014
Mantle	48	0.628	$y = 5.598x + 2.542$	0.005
	72	0.815	$y = 30.504x + 1.971$	0.000
Digestive gland	24	-0.665	$y = -28.077x + 2.991$	0.003

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Discussion

Assessing the biological effects of pollutants during biomonitoring attempts show that bio-markers are of high demand for the biomonitoring in aquatic ecosystems [33]. Ecotoxicological programs recommend the use of even a set of biomarkers for ecosystems health and environmental risk assessments [34].

MTs are generally considered as useful biomarkers for heavy metal pollution, because their protein concentration often increases upon exposure to heavy metals in different organs [35]. When bivalves *Mytilus edulis* and *Mytilus galloprovincialis* are exposed to Cd, a linear relationship between Cd and MT concentrations is observed in the whole soft tissues [36, 37]. In the oysters *Crassostrea gigas*, the MT levels in the digestive gland are only occasionally correlated with accumulated metal concentrations [38].

Although metal induction of MT synthesis is often observed, some studies do not show a positive correlation between heavy metal accumulation and MT biosynthesis in invertebrates. In the clam *Ruditapes decussatus* exposed to Cd ($400 \mu\text{g L}^{-1}$) for 30 days, MT has no significant increase in the soft tissues [39]. On the other hand, a significant increase in MT10 appears only after 9 days of exposure to Cd in the digestive gland of *Mytilus galloprovincialis*. In contrast, the protein concentration of MT10 is significantly reduced after 2 days exposure to Cu [40], suggesting that MT expression in the digestive gland is different in response to different heavy metals. Some studies even show the negative relationships between Cd and MT concentrations. In the copepods *Tigriopus brevicornis*, Cd can decrease the concentrations of MTs [41]. Thus, the relationship between heavy metal contamination and the increase in MT concentration is controversial. At sites where metals are present at high concentrations, some species do not show increased MT concentrations, at least in some organs [38, 42–44]. Further studies are required to clarify the relationship between metal contamination and MT level and to validate the use of MTs as biomarkers [7].

In the present work, we found that Cd accumulation provoked a significant increase of MT protein concentration in the gills and mantle of *Anodonta woodiana* (Fig. 1; Fig. 2; Fig. 6; Fig. 7; Table 1). In contrast, there was no correlation between Cd accumulation and MT protein level in the foot and visceral mass. This supports the view of a positive correlation between Cd and MT in specific tissues [12, 40, 45]. Mussels filter large volumes of water through their gills and mantle, and thus concentrate contaminants in these tissues. Gills and mantle are the main interface between the organism and its environment, and hence these tissues are frequent targets of environmental pollutants. Any adverse change in the ambient environment, such as oxidative stress, is easily reflected in these tissues [46, 47]. In the digestive gland, we even found a negative correlation between Cd accumulation and MT protein level (Fig. 5; Fig. 10; Table 1), suggesting that MT did not play a main role in the detoxification or homeostasis of Cd in the tissue. Cadmium detoxification can occur with other metalloproteins rather than MTs. The digestive gland consists of numerous blind ending tubules that are composed of digestive and basophilic cells. The digestive gland is involved in digestive and absorptive

functions, and its epithelium is composed of cells rich in lysosomes which contain several hydrolytic enzymes [17]. In invertebrates, similar to bivalves, lysosomes are known to accumulate both essential and toxic metals from the cytosol of the digestive gland cells, and play an important role in Cd detoxification [17, 48]. After the freshwater mussel *Dreissena polymorpha* are treated with $200 \mu\text{g L}^{-1}$ of Cd for 7 days, digestive lysosomal system is activated, as evidenced by an increase in both the number and the size of lysosomes [34]. So, it is not surprising that metals are found in lysosomal residual bodies [49]. Lysosomal system has several typical structures, i.e. heterolysosomes and heterophagosomes, “boules”, residual bodies and brown bodies. Such lysosomal structures are probably involved in the compartmentalisation of metals in the digestive gland cells [17, 50]. It appears that the effect of Cd on MTs protein level is tissue-specific. Our data suggest that Cd and MT levels in the gills and mantle of *Anodonta woodiana* provide good indicators for Cd pollution, although, MT levels in the digestive gland have been widely used as biomarkers for pollution by metals including Cd [13, 51–54].

The mechanisms of MT gene expression are poorly characterized as yet, particularly during oxidative stress. The Zn-sensitive inhibitor regulates MT gene expression, which provides a conceptual model for interpreting the significance of MT induction in response to toxic metals [55]. In this model, Cd has greater ligand affinity than Zn and it can displace Zn from other Zn binding sites via metal-metal exchange reaction. The displaced Zn is then available for binding the metal transcription inhibitor (MTI), releasing the metal transcriptional factor (MTF) from inhibition, and initiating MT expression [8]. The presence of metal regulatory elements (MREs) in the upstream sequences of MT gene is indicative of the specificity of this induction. Studies indicate that MREs play a positive role in initiating MT gene expression [56]. In mice, MT gene expression is regulated by Zn-mediated release of an inhibitor. The six zinc-finger metal-responsive transcription factor MTF-1 plays a central role in the transcriptional activation of the MT-I gene in response to zinc. MTF-1 is induced to bind to the metal response elements in the proximal MT promoter in cells during oxidative stress [10]. The basic helix-loop-helix-leucine zipper protein, upstream stimulatory factor family (USF), also plays a role in regulating the transcription of the mouse MT-I gene in response to Cd or H_2O_2 [57–59]. Studies also demonstrate that cis-acting elements mediate the induction of MT gene expression by metals and oxidative stress, which is present in the chicken MT promoter. This suggests a role for increased binding of the transcription factor MTF-1 to the metal response elements [60]. Whether MTF-1- or USF-like protein functions in the freshwater mussel *Anodonta woodiana* in response to metals has not yet been investigated. Our results showed that Cd increased the MT biosynthesis in the freshwater mussel *Anodonta woodiana*, which may be mediated by the transcription factor of MTF-1- or USF-like protein.

We have evidence that H_2O_2 accumulated in various tissues of freshwater mussel *Anodonta woodiana* after Cd treatment (data not shown), indicating that Cd triggers ROS production. ROS can induce protein oxidation, especially targeting the proteins rich in cysteines. MTs are low molecular weight proteins rich in cysteines. Within a total of 61–62 amino acids, depending on their source, there are 32 cysteines [61]. In our experiment, we found an increase in MT in gills and mantle, suggesting that the increase in MT maybe one of the responses to Cd, which is to reduce the Cd-induced ROS. From this point of view, a higher level of MT is beneficial, which can reduce oxidative damages induced by Cd.

MTs play important roles in the detoxification of heavy metals including Cd and Hg by binding with heavy metals through the thiol group of cysteine residues [62, 63]. Therefore, an increase in MT mRNA/protein levels may reflect an elevated demand to detoxify Cd. This is the reason why MTs have been widely used as significant biomarkers for metal pollution. However, it is worth noting that no increase in MT proteins was observed in organs of fish exposed to metal, despite that the MT gene transcription was up-regulated. This suggests that the

physiological role of MT gene transcription can be increased in response to MT degradation by metal, attempting by cells to maintain MT levels; increase in transcription alone does not increase metal sequestration capacities of MT [64].

Conclusions

In conclusion, our results showed that Cd accumulated in all tissues to different levels. Concomitantly, there was an increase in protein levels of MT only in gills and mantle. Positive correlations between Cd and MT levels were found in these two tissues (Table 1). Our results indicate that positive correlation between Cd and MT is tissue-specific, and suggest that protein level of MT in gills and mantle of *Anodonta woodiana* is a useful biomarker for Cd pollution.

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Author Contributions

Conceived and designed the experiments: YL LW HY. Performed the experiments: YL HY. Analyzed the data: YL HY LW. Contributed reagents/materials/analysis tools: YL LW HY QW. Wrote the paper: YL NL JL LW.

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