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Biochemical response of the mussel *Mytilus coruscus* (Mytiloida: Mytilidae) exposed to in vivo sub-lethal copper concentrations*

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Abstract Many aquatic organisms are negatively affected by exposure to high copper concentrations. We investigated the biochemical response of the mussel *Mytilus coruscus* (Mytiloida: Mytilidae) to copper exposure. In vivo bioassays using *M. coruscus* and different copper concentrations were conducted. The activity of six biomarkers, namely superoxide dismutase (SOD), catalase (CAT), acid phosphatase (ACP), alkaline phosphatase (AKP), glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were measured. Survival rates decreased with increased copper concentrations and exposure times. The LC_{50} values at 48, 72, and 96 h exposure were 0.48, 0.37, and 0.32 mg/L, respectively. Within digestive glands, CAT activity increased with increasing Cu concentrations. The activity of AKP showed no significant change, while the remaining four enzymes showed decreasing activity with increasing Cu concentrations. Within the gills, AKP activity increased when the Cu concentration was 0.05 mg/L, but showed no significant changes at higher concentrations. Activity of CAT and ACP within gills tended to decrease with increasing Cu concentration. The activity of SOD and GPT decreased at an exposure concentration of 0.2 mg/L. GOT activity within gills decreased at 0.1 mg/L and increased at an exposure concentration of 0.2 mg/L. Within the adductor muscle, AKP activity increased at 0.05 mg/L but did not change at higher exposure concentrations. ACP activity within adductor muscle tissue showed no change, while activities of CAT, GOT and GPT decreased with increasing Cu concentrations. SOD activity within the adductor muscle tissue significantly decreased at the 0.02, 0.05 and 0.2 mg/L exposure concentrations. Our results show tissue specific differences for the six biomarkers in for *M. coruscus*. Our findings provide the basis for the establishment of reference activity levels against which biomarker changes can be estimated, and are essential preliminary steps in development of in vivo bioassays.

Keyword: *Mytilus coruscus*; copper; biomarker; antioxidant enzymes; metabolic enzyme

1 INTRODUCTION

Over the last few decades, concerns have grown within many countries and regions in regards to metal pollution within the environment (Chandran et al., 2005; Jing et al., 2006; Li et al., 2009). According to the report of the Bulletin of the East China Sea (East China Sea Branch of State Oceanic Administration, 2009), metal contamination and especially copper (Cu), have caused environmental poisoning within the coastal areas of the East China Sea. The main source of Cu within the East China Sea is derived from terrigenous discharge, especially from rivers which account for 88%, with the highest contribution of the fluxes derived from the Changjiang (Yangtze) River (Wang et al., 2010).

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Copper is known to be essential for enabling the metabolism of marine invertebrates. However, excessive copper can be toxic (White and Rainbow, 1985; Viarengo, 1989; Arnold et al., 2010). With the phasing out of triorganotin-based formulations, copper has become a commonly used biocide within antifouling paints (Voulvoulis et al., 1999; Lorenzo et al., 2002; Omae, 2003; Yebra et al., 2004; Xie et al., 2005; Parks et al., 2010). Within the natural marine environment, concentration of copper is usually <5 μg/L, but within polluted areas, it could be as high as 800–1 000 μg/L (Soegianto et al., 1999).

Copper can bioaccumulate within the soft tissues of mollusks, and induce corresponding enzymatic responses (Viarengo and Canesi, 1991; Arun and Subramanian, 1998; Mazorra et al., 2002; Jing et al., 2006). It has been reported that copper can interfere with the antioxidant enzymatic defense system in many species of mollusks such as *Onchidium struma* (Li et al., 2009), *Crassostrea rivularis* (Jiang and Niu, 2006), *Pinctada fucata* (Jing et al., 2006), and *Adamussium colbecki* (Regoli et al., 1998). In addition, it has been demonstrated that copper affected the systems of metabolic enzymes within mollusks, such as *Ruditapes philippinarum* (Blasco et al., 1993), *Scrobicularia plana* (Mazorra et al., 2002), *Pinctada fucata* (Jing et al., 2006) and *O. struma* (Li et al., 2009).

Using tests in the laboratory, Regoli and Principato (1995) investigated the effects of exposure to copper on the Mediterranean mussel *M. galloprovincialis*, and found that distinct antioxidant responses were dependent on tissue type. The presence of antioxidant enzymes within the digestive gland of mussels, and the evaluation of the effects of copper exposure on the antioxidant defenses of the mussel *Perna perna*, was investigated by de Almeida et al. (2004). Company et al. (2008) studied the influence of copper exposure on the antioxidant response of the mussel *Bathymodiolus azoricus*, and demonstrated that the activity of antioxidant enzymes within gills was higher than that found in the mantle after Cu exposure. Although Cu exposure effects on these enzymatic systems have been reported for some species of mussels, information on the enzymatic response to Cu exposure within the mussel *M. coruscus* was identified as a gap in research.

The mussel *M. coruscus* (Gould) is a common species inhabiting the temperate zone along the coastal water of East Asia (Chang, 2007). Within China, this species is one of the most heavily commercially exploited marine bivalves, particularly within Zhejiang Province (Zhang and Zhao, 2003; Chang and Wu, 2007). In the present study, the authors investigated the biochemical response of three tissue types (digestive gland, adductor muscle and gills) of the adult mussels to exposure to copper. The response was assessed through the measurement of six selected biomarkers, namely the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and the metabolic enzymes acid phosphatase (ACP), alkaline phosphatase (AKP), glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT).

2 MATERIAL AND METHOD

2.1 Animal culture

Adult *M. coruscus* were collected in September and October 2010 from Gouqi Island, Zhoushan, China (30°43'N, 122°46'E). Adult mussels were maintained in 120 L plastic containers containing sea water of 30±1 and acclimated for one week within laboratory conditions prior to exposure to copper. During the period of acclimation, the collected *M. coruscus* individuals were fed daily with a mixed diet of *Chaetoceros gracilis*, *Chlorella* spp. and *Dicrateria inornata*. The culture water was changed daily and the temperature was maintained at 20±1°C. The wet weight (ww), shell length (SL) and shell height (SH) of *M. coruscus* used within the bioassays were 49.52±9.53 g, 8.70±0.64 cm and 4.65±0.05 cm, respectively.

2.2 Copper solution

Copper chloride ($CuCl_2$) used in the experiments was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). A 1 g/L stock solution was prepared by dissolving $CuCl_2$ in autoclaved filtered sea water (AFSW) on the day of the assays. Test solutions were prepared immediately prior to the assays by diluting the stock solution in AFSW to the desired concentrations. The background total copper (Cu) content within the assays was measured using an atomic absorption spectrophotometer and was found to be 7.40±0.23 μg/L.

2.3 Acute and sublethal toxicity tests

To perform the acute toxicity test, adults were placed in a container (20 L) containing 10 L of the test solution. The treatments were 0 (control), 0.1, 0.2, 0.3, 0.4, and 0.5 mg/L of Cu. Each treatment consisted of three replicates and each replicate contained 20 individuals. Test solutions were renewed daily but no

additional food was added. Dissolved oxygen (DO) and temperature of each container was monitored every 12 h during the assay. DO ranged from 8.82–9.47 mg/L and water temperature was maintained at 20±1°C. A photoperiod of 12 h light and 12 h darkness was used. Within the assay, mussel survival was examined at 24, 48, 72 and 96 h. The criterion for death was no response toward repeated touches with a glass rod (Winner and Farrel, 1976; APHA et al., 1985). Dead mussels were removed.

Based on results of the acute toxicity test, effects of sub-lethal concentrations of copper on antioxidant and metabolic enzymes were investigated. Adult mussels were exposed to Cu concentrations of 0, 0.01, 0.02, 0.05, 0.1 and 0.2 mg/L for a period of 96 h. Tissue samples of the digestive gland, gills and adductor muscle of 10–16 *M. coruscus* individuals from each treatment container were dissected on ice, and immediately stored in liquid nitrogen at -80°C until the bioassays were carried out.

2.4 Biochemical assays

Approximately 0.5 g samples of gill, digestive gland and adductor muscle tissue were individually homogenized at 15 000 r/min for 1 min within 4.5 mL ice-cold mussel physiologic saline (Lowe et al., 1995) using an automatic homogenizer (Ika T 10 b, Germany). Mussel physiological saline was prepared with Millipore ultra-pure water to avoid adding exogenous toxic metal ions. Homogenized samples were centrifuged at 1 431×*g* for 20 min at 4°C, and the resulting supernatants were used to determine enzyme activity. Activities of antioxidant and metabolic enzymes (detailed below) were determined using substrates obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to protocols provided by the manufacturer. Optical density values were measured with a spectrophotometer (UV-2600, Unico, China) and used to calculate enzyme activities.

2.4.1 Total superoxide dismutase (T-SOD; EC 1.15.1.1) activity

T-SOD activity was measured through the inhibition of nitro blue tetrazolium (NBT) reduction by O_2^- generated by the xanthine/xanthine oxidase system (Beauchamp and Fridovich, 1971). One unit (U) of enzyme activity was defined as the amount of enzyme that caused a 50% inhibition in a 1-mL reaction solution/mg protein. Data were expressed as U/mg protein.

2.4.2 Catalase (CAT; EC 1.11.1.6) activity

CAT activity was detected using the ammonium molybdate method (Shen et al., 2007). Briefly, a H_2O_2 degradation reaction catalyzed by CAT was terminated by adding ammonium molybdate, and the intensity of a yellow complex formed by molybdate and H_2O_2 at 405 nm was measured. One U of enzyme activity was defined as the degradation of 1 μmol H_2O_2 per second per mg tissue protein and the enzyme activity was expressed as U/mg protein.

2.4.3 Acid phosphatase (ACP; EC 3.1.3.2) and alkaline phosphatase (AKP; EC 3.1.3.1) activities

ACP and AKP activities were assessed by the method of King (1965) using disodium phenylphosphate as the substrate. The enzyme unit definitions of ACP (U/g protein) and AKP (U/g protein) were expressed as the degradation of 1 mg phenol/mg protein at 37°C within 30 min and 15 min, respectively.

2.4.4 Glutamic-oxaloacetic transaminase (GOT; EC 2.6.1.1) and glutamic-pyruvic transaminase (GPT; EC 2.6.1.2) activities

GOT and GPT activities were detected following the method of Reitman and Frankel (1957). For determining the activity of GPT, 0.1 mL of enzyme source was taken and 0.5 mL of GPT substrate. Similarly, for determination of GOT, 0.1 mL of enzyme source was taken and 0.5 mL of GOT substrate was added to it. After 30 min of incubation at 37°C, 0.5 mL of 2, 4-dinitrophenyl hydrazine solution was added and contents were incubated for 20 min at 37°C. Subsequently, 5 mL of 0.4 mol/L NaOH was added, rigorously mixed and allowed to stand at room temperature for 5 minutes. The optical density was read at 505 nm after setting the blank. One unit of either GOT or GPT enzymatic activity was defined as tissue activity (Reitman-Frankel unit) per mg protein.

2.4.5 Protein assays

The concentration of total protein in the supernatants was determined using Coomassie Brilliant Blue G250 (Li and Jiao, 1980) according to the provided protocol.

2.5 Statistical analysis

Prior to one-way Analysis of variance, the normality and homogeneity of the analyzed data were

both assessed. When normality and homogeneity of variance assumptions were not satisfied, the equivalent non-parametric Kruskal-Wallis test was applied. The LC_{50} values of adult mussels were calculated by the Probit analysis method (Finney, 1971). All statistical computations were performed using JMP™ software (JMP 6, SAS Institute, Cary, NC, USA). Differences were considered significant at $P<0.05$.

3 RESULT

3.1 Effect of copper on the survival of adult mussels

The survival rates of adult mussel after exposure to Cu for 24, 48, 72 and 96 h are shown in Fig.1. Within the control and Cu treated samples up to 0.2 mg/L, no dead animals were observed, even after 96 h. Within the 0.3 and 0.4 mg/L treatments, the percentage of survival decreased significantly after 48 h (Kruskal-Wallis Tests, $P<0.000\,1$). Within the 0.5 mg/L treatment, no surviving adults were found after 96 h. Based on the results shown in Fig.1, the concentrations that caused 50% mortality (LC_{50}) in the Cu treated specimens were calculated. The LC_{50} decreased with increasing exposure time and the LC_{50}s at 48, 72 and 96 h were calculated to be 0.48, 0.37 and 0.32 mg/L, respectively.

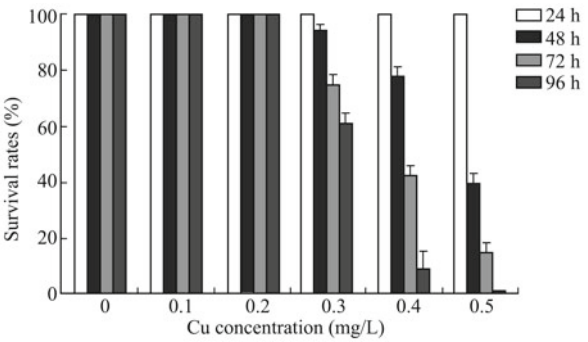

Fig.1 Survival rates of adult mussels following exposure to different Cu concentrations for 24, 48, 72, and 96 h

Data are means (± SD) of the nine replicates.

3.2 Biochemical response

3.2.1 Effect of copper on the activities of antioxidant enzymes

The effect of a 96 h Cu exposure on the activities of the antioxidant enzymes, SOD and CAT, in digestive gland, gill and adductor muscle tissue of adult *M. coruscus* are presented in Fig.2. In general, the SOD activity tended to decrease in digestive gland tissue with increasing Cu concentrations ($P<0.05$). When compared with the control, the activity of SOD in gill tissue decreased only at the 0.2 mg/L concentration ($P<0.05$) and the SOD activity in adductor muscle tissue significantly decreased at 0.02, 0.05 and 0.2 mg/L exposure concentrations ($P<0.05$). SOD activities were higher in gill tissue than in the other investigated tissue types ($P<0.05$). The CAT activity was higher in digestive gland tissue compared to the other two tissue types ($P<0.05$) and showed an increasing trend with increasing copper concentration ($P<0.05$), while the opposite effects were observed within gill and adductor muscle tissue.

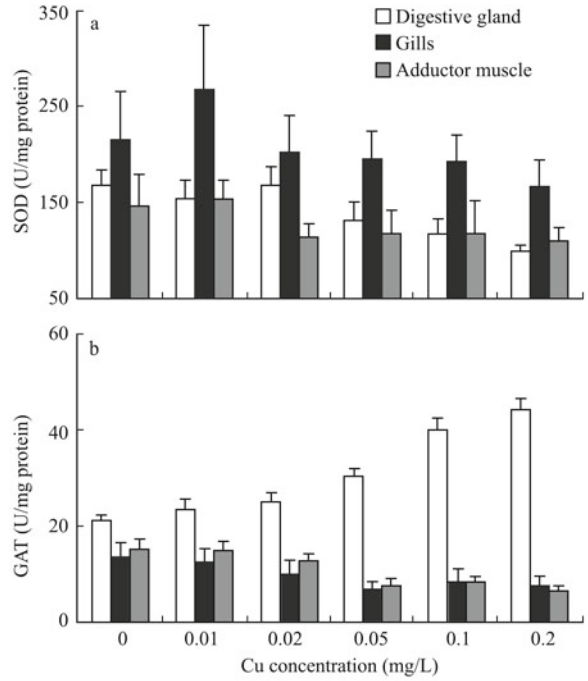

Fig.2 Effect of 96 h exposure to Cu concentrations (mg/L) on the activities (U/mg protein) of SOD (a) and CAT (b) in digestive gland, gills and adductor muscle of adult *M. coruscus*

Data are the means (± SD) of nine replicates.

3.2.2 Effect of copper on the activities of metabolic enzymes

The effect of a 96 h Cu exposure on the activities of the metabolic enzymes, ACP, AKP, GOT and GPT, within digestive gland, gill and adductor muscle tissue of adult *M. coruscus* are shown in Fig.3. The ACP and AKP activities were significantly higher in the digestive gland tissue than that in gill or adductor muscle tissue (Fig.3a, b; $P<0.05$). The ACP activity decreased in digestive gland and gill tissue whereas no change in adductor muscle tissue occurred with increasing copper concentration (Fig.3a; $P<0.05$). The GOT and GPT activities within digestive gland

tissue were significantly lower compared to those in gill and adductor muscle tissue (Fig.3c, d; $P<0.05$). The activity of GOT within gill and adductor muscle tissue decreased significantly in the 0.1 mg/L treatment ($P<0.05$) and increased significantly within the 0.2 mg/L treatment ($P<0.05$).

4 DISCUSSION

In the present study, results showed that the survival of *M. coruscus* decreased with increasing copper concentrations and exposure times. The LC_{50} for 96 h Cu exposure was 0.32 mg/L, which was lower than

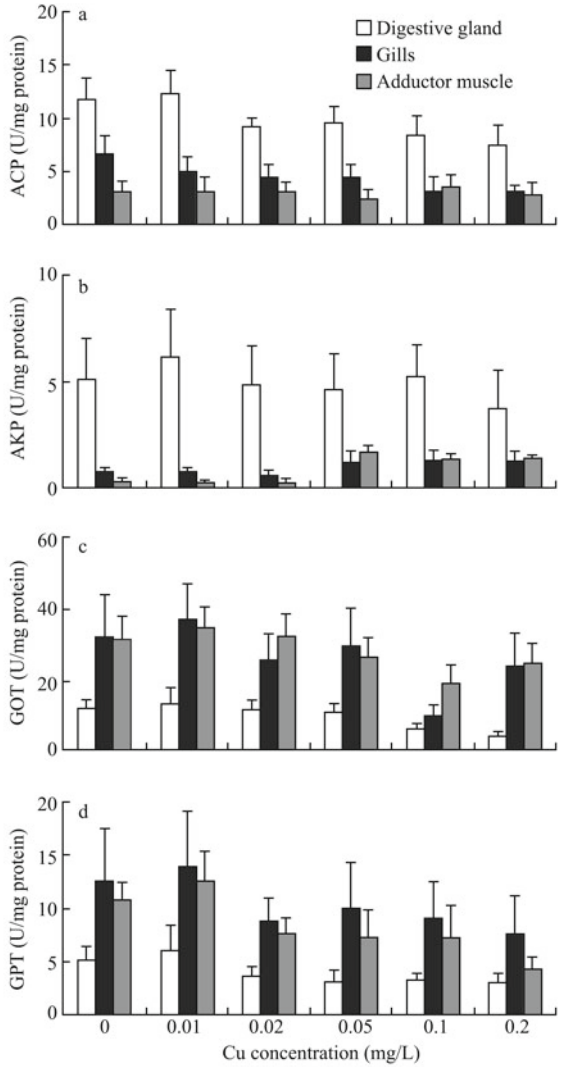

Fig.3 Effect of 96 h exposure of various concentrations of Cu (mg/L) on the activities (U/mg protein) of ACP (a), AKP (b), GOT (c) and GPT (d) in digestive gland, gills and adductor muscle of adult *M. coruscus*

Data are the means (± SD) of nine replicates.

that for many other aquatic organisms, such as the molluscs *Mytilus edulis* (Amiard-Triquet et al., 1986; LC_{50}, 0.48 mg/L), *Perna viridis* (Chan, 1988; LC_{50}, 0.62 mg/L), and *O. struma* (Li et al., 2009; LC_{50}, 7.48 mg/L), and the crustaceans, *Callianassa australiensis* (Ahsanullah et al., 1981; LC_{50}, 1.03 mg/L) and *Crangon crangon* (Portmann and wilson, 1971; LC_{50}, 1.9 mg/L). Contrastingly, the LC_{50}s for 96 h Cu exposure in some marine invertebrates were lower than those for the species presented in this study (Bryan, 1976; Heslinga, 1976; Reish et al., 1976; Pesch and Morgan, 1978; Bielmyer et al., 2005; Wang et al., 2007). Values of LC_{50} in these marine invertebrates ranged from 0.025 mg/L to 0.3 mg/L. Although the reasons for these differences in copper sensitivity are unknown, it may be related to the differences in age, size and condition of the test organisms, and the environmental conditions (Chan, 1988). The different sensitivity to Cu may be also correlated with the species of test animal.

Marine invertebrates, especially mollusks, have been known to bioaccumulate copper to varying degrees, and body Cu concentration may reach high levels (Bryan, 1979; Chan 1988). Cu accumulation has been known to be important for understand the varying response of different tissues to Cu concentrations. In the present study, no data on the actual Cu accumulation was provided, and this needs to be investigated in future research.

To cope with stress induced by environmental pollution, organisms have evolved antioxidant systems to scavenge reactive oxygen species (ROS) and to control the oxidative damage they induce (Cheeseman and Slater, 1993). Enzymatic antioxidant systems are known to include SOD and CAT (Fridovich, 1978), and these are regarded as the two main antioxidant enzymes to protect organisms against damages induced by oxygen radical production (Borković et al., 2008). An important feature of these enzymes is their inducibility under conditions of oxidative stress, and such an induction may be an important adaptation to pollutant-induced stress (Liu et al., 2006). The effect of Metal exposure has been known as one of the different ROS-generating mechanisms, and consequently metal exposure causes oxidative stress within biological systems (Stohs and Bagchi, 1995). Cu exposure may activate redox cycling, which is a typical sign of damage due to oxidative stress (Roméo et al., 2000; Liu et al., 2006; Company et al., 2008).

This study showed that SOD activities within three

tissue types showed an inhibitory trend and decreased at the highest (0.2 mg/L) concentrations of copper. SOD activities within gill tissue were higher than those in the other two tissue types. The finding that tissue type specific variation of SOD activity exists, is consistent with previous reports of SOD activity in other organisms (Company et al., 2008; Li et al., 2009). For example, Company et al. (2008) demonstrated that the activity of SOD in gill tissue was significantly higher than that in the mantle tissue of the mussel *Bathymodiolus azoricus* after copper exposure. Li et al. (2009) also suggested that in SOD activity in hepatopancreas tissue was significantly higher than that in muscle tissue of *O. struma*. Although the reason is unknown, it may be due to the different physiological function and responses to Cu (Company et al., 2008).

The antioxidant enzyme CAT is a major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to H_2O (Borković et al., 2008). In contrast to SOD activity, CAT activities in the present study were significantly induced within digestive gland tissue with increasing Cu concentrations. Activities of CAT were inhibited within gill and adductor muscle tissue, suggesting that different tissues have a qualitatively different response pattern (Li et al., 2009) or different physiological functions (Company et al., 2006). Among the three tissue types, the results presented here demonstrate that digestive gland tissue is very sensitive to Cu stress, and is therefore a suitable tissue type for further study.

ACP acts as a marker enzyme for the detection of lysosomes within cell fractions, and can be utilized as a reliable tool for the biological assessment of metal pollution (Blasco et al., 1993; Mazorra et al., 2002; Rajalakshmi and Mohandas, 2005). AKP are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells, and are sensitive to metals (Blasco et al., 1993; Mazorra et al., 2002; Jing et al., 2006).

In the present study, significantly higher activities of ACP and AKP were observed in digestive gland tissue compared to the other two tissues types. Previous studies on ACP and AKP activities within different tissues of aquatic organisms also suggested that the enzymatic activity and response to metal pollution is tissue type specific (Mazorra et al., 2002; Rajalakshmi and Mohandas, 2005; Li et al., 2008; Li et al., 2009; Aanand et al., 2010). In addition, variation of ACP and AKP activities due to Cu exposure in the same tissue type were different within different species. For example, the results presented in this study suggested that the ACP activity in digestive gland tissue decreased when Cu concentrations were >0.01 mg/L, while AKP activity remained unchanged. In *Eriocheir sinensis*, activities of ACP and AKP in digestive gland tissue increased initially at low Cu concentrations but decreased with increasing Cu concentrations (Yang et al., 2006).

The transaminases GOT and GPT have been known to be involved in cellular transfer systems, protein and nucleic acid synthetic processes and energy producing mechanisms (Bradfield, 1950; Rao and Panda, 1981). A recent study on the green mussel (*P. viridis*) found that exposure to copper increased the activity of GOT and GPT in adductor muscle tissue (Aanand et al., 2010). In the present study, activities of GOT and GPT in adductor muscle tissue initially increased after exposure to Cu in the 0.01 mg/L treatment, which was followed by decrease of activity at higher Cu concentrations. Activities of GOT and GPT within digestive gland tissue were inhibited by increasing Cu concentrations, indicating that copper produced serious deformation of the membrane of mitochondria in cells, and hence reduced the ability of mitochondria to produce sufficient adenosine triphosphate (ATP) as energy for living organisms (Bubel, 1976). The differences in activities of GOT and GPT may be due to the variation among species.

5 CONCLUSION

The results presented in this study provide valuable information for determining the Cu tolerance levels for the important bioindicator species *M. coruscus*, which could be applied in the assessment of the levels of coastal Cu pollution. In the current study, tissue specific differences for the six selected biomarkers SOD, CAT, ACP, AKP, GOT and GPT do exist within *M. coruscus*. In general, the activity of CAT within digestive gland tissue showed an increasing tendency with increasing Cu concentrations, while the activity of other enzymes tended to decrease as a consequence of increasing Cu concentrations. The digestive gland can be viewed as potential target tissue for further study, and activities of CAT are suitable to indicate Cu contamination and may be used as a potentially useful biomarker due to sensitivity to Cu exposure. Field studies to develop a comprehensive profile of the biochemical response within *M. coruscus* to

copper are needed. Overall, *M. coruscus* could be used as a potential bioindicator species for Cu monitoring.

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