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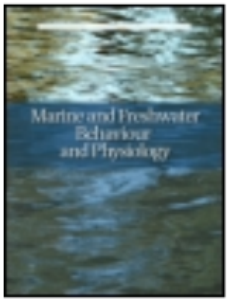

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Effect of copper on survival, osmoregulation, and gill structures of freshwater prawn (*Macrobrachium rosenbergii,* de Man) at different development stages

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This study evaluated the acute toxicity of copper at different life stages of freshwater prawn, *Macrobrachium rosenbergii*, to determine the effect of sub-lethal copper concentration on osmoregulatory capacity (OC), to measure the copper level in gills, and to investigate the effect of copper on the histological change of the gills of adult prawns. The 24-, 48-, and 96-h medium lethal concentration (LC_{50}) of copper on *M. rosenbergii* increased progressively along with the increasing life stage, from postlarvae, juvenile to adult. The 24-, 48-, and 96-h LC_{50} values for copper were higher at 0 ppt than those at 12 ppt for both juvenile and adult. After 7 d exposure to 0.75 mg Cu L^{-1} at 0 and 12 ppt, the OC values of exposed prawns were reduced by 12 and 47%, respectively, compared with control animals. However, the OC value of prawns exposed to 0.5 mg Cu L^{-1} was not significantly different from the OC value of control prawns both at 0 and at 12 ppt. The copper concentrations on gill tissues increased significantly in prawns exposed to copper both at 0 and at 12 ppt. After the copper exposure, swelling of lamellae, multiple hyperplasia and necrosis were observed in gill lamellae, resulting in abnormal gill tips. An obvious relation between the impairment of osmoregulation and the structural damage of gills are reported in this study.

Keywords: prawn; *Macrobrachium rosenbergii*; copper; toxicity; life-stages; survival; osmoregulation; gills

Introduction

Copper (Cu) is a normal constituent of aquatic environments (Spicer & Weber 1992). Due to anthropogenic input (i.e. industries, agriculture, mining and harbors), the concentration of copper in aquatic environments has considerably increased (Stauber et al. 2005; Dan'azumi & Bichi 2010). Copper is an essential element required by all living organisms because it plays indispensable roles in a variety of physiological and biochemical processes (Martins et al. 2011). In crustaceans, copper is also required for the synthesis of respiratory pigment hemocyanin, participating in oxygen transport (Rainer & Brouwer 1993; Mendez et al. 2001). Although copper is an essential element, it can be potentially toxic to crustaceans if an excess of copper is used (Brouwer et al. 2002; Rainbow 2002).

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Crustacean tissues are able to accumulate copper in accordance with the external copper concentrations (Rainbow 2002). Copper uptake occurs throughout epithelial surfaces related to absorption and excretion of ion such as the gill membranes (Scott-Fordsmand & Depledge 1997; Soegianto et al. 1999). In crustaceans, gills are important organs of respiration as well as of osmoregulation (Mantel & Farmer 1983; Romano & Zeng 2012). Osmoregulatory organs, particularly gills, can be damaged by copper as demonstrated in some crustacean species after exposure to different levels of copper (Lawson et al. 1995; Soegianto et al. 1999; Li et al. 2007; Frias-Espericueta et al. 2008). Osmoregulation of crustaceans could therefore potentially be altered by copper. The cause of death in crustaceans exposed to lethal Cu concentrations is reported to result from disruption of gill function leading to perturbation of osmoregulatory capacity (OC) (Bambang et al. 1995; Usman et al. 2013).

The toxic effects of Cu exposure have been determined in a limited number of freshwater decapods species (Taylor et al. 1995; Osunde et al. 2004; Li et al. 2005; Reddy et al. 2006), and these studies focused mainly on a single stage (postlarvae or juvenile), whereas few data are available regarding the effects of Cu on freshwater prawn (particularly *Macrobrachium rosenbergii*) at different life stages (Osunde et al. 2004; Li et al. 2005; Reddy et al. 2006).

The giant freshwater prawn, *M. rosenbergii*, is a common inhabitant of rivers and estuaries throughout the Indo-Pacific region (Cheng et al. 2003). This species is one of the most commercially important crustaceans because of its fast growth in subtropical and tropical countries including Indonesia (New 2002). In nature, *M. rosenbergii* inhabits a wide range of environmental salinities (0–18 ppt) during its life cycle. During the reproductive season, adults migrate from freshwater habitats to estuarine regions, where eggs hatch and larval development occurs (Nelson et al. 1977). The life cycle of *M. rosenbergii* consists of several developmental stages, including eleven postlarval stages, followed by juvenile and adult phases (New 2002).

Prawn farms are often located near the water resources (i.e. rivers and coasts) as the water is directly used to rear prawn. However, today many water resources are contaminated by many kinds of pollutants including heavy metals due to human activities (Amado et al. 2006). The level of copper in unpolluted waters is about $0.05\,\mu g\,L^{-1}$, while near industrial effluent discharge, it may be much higher reaching $1.4–2\,mg\,L^{-1}$ (Wong 1993; Saager et al. 1997; Stauber et al. 2005; Dan'azumi & Bichi 2010). The impacts of copper on prawn culture should therefore be better understood. This study was undertaken to evaluate the acute toxicity of copper at different life stages of freshwater prawn *M. rosenbergii*, to determine the effect of sublethal copper concentration on OC, to measure the copper level in gills, and to investigate the effect of copper on the histological change of the gills of adult prawns.

Materials and methods

Experimental organisms and media

Postlarvae (PL1, PL4, PL7, and PL11), juvenile and adult stages of *M. rosenbergii* were used in the experiments. They were obtained from a commercial prawn farm located in Trenggalek, East Java, Indonesia. The values of salinity, temperature, dissolved oxygen, and pH at the prawn farm were 12–15 ppt, 28–29 °C, $5.6–6.8\,mg\,L^{-1}$, and 7.6–8.2, respectively. After transportation to the laboratory, the postlarvae were acclimated to laboratory conditions for a few hours before being used for toxicity tests. The juvenile mean body weight was 8.2 ± 1.1 g, and the adult mean body weight was 16.0 ± 1.8 g.

The juveniles and adults were kept in acclimation tanks for 4 d in aerated and filtered recirculating brackish water (12 ppt salinity) and freshwater (0 ppt salinity), respectively, before the experiments. Seawater was obtained from the Surabaya coast adjacent to the university, and the freshwater was obtained from dechlorinated municipal tap water. Both seawater and freshwater were filtered through gravel, sand and sponge filter system by airlift. Animals were fed (*ad libitum*) twice daily with commercial fish food (30% protein, 3% fat, and 4% fiber) during the acclimation period. Uneaten food was removed from the tank every day to avoid the diminution of water quality. The values of salinity, dissolved oxygen, pH, and temperature were 0 and 12 ppt, 6.0–7.0 mg L^{-1}, 7.5–8.0, and 28–29 °C, respectively, throughout the experiments. The light cycle was 12-h light/12-h dark.

Since crustacean physiology, and particularly hemolymph osmolality, fluctuates during the moult cycle (Charmantier et al. 1994), it was a prime consideration in the selection of experimental animals. Only adults at intermolt or first premolt stages were used in the experiments. Molting stages were determined by microscopical examination of an antennal scale according to Drach and Tchernigovtzeff's method (1967).

Acute toxicity

Because of the short duration of some postlarval stages, the acute toxicity tests lasted 24 h for postlarvae prawns (PL)1 and 48 h for PL4 and PL7. For PL11, juvenile and adult prawns, the tests lasted 96 h. The toxicity tests for postlarvae prawns were conducted in a media with the salinity of 12 ppt, meanwhile the tests for juvenile and adult prawns were conducted in a media with 0 and 12 ppt salinity. The different salinities used in this experiment were based on the salinities commonly used in local prawn farms.

A stock solution (1000 mg Cu L^{-1}) was prepared from 3.080 g of Cu $(CH_3COO)_2 \cdot H_2O$ (Merck, Germany) in 1000 mL of deionized water. Selected experimental concentrations were made by adding the necessary volume of the stock solution to freshwater (dechlorinated municipal tap water) or dilute seawater. The dissolved Cu concentrations of control test media determined by flame atomic absorption spectrophotometer (Shimadzu, AA-6200) were 0.0023 ± 0.0006 mg L^{-1} for 12 ppt and <0.0015–0.0023 mg L^{-1} for 0 ppt.

A toxicity range-finding test was conducted prior to initiating a static, acute, definitive toxicity test. Definitive toxicity tests were conducted with triplicate groups of 10 individuals (30 for each Cu concentration) kept either in plastic boxes ($15 \times 10 \times 5$ cm^3) containing 500 mL of test solution for postlarvae or in plastic containers ($35 \times 30 \times 25$ cm^3) containing 20 L of test solution for juvenile and adult prawns. Test copper concentrations were 0, 0.0025, 0.0063, 0.016, 0.040, 0.1 mg Cu L^{-1} for PL1 and PL4; 0, 0.025, 0.063, 0.160, 0.4, 1 mg Cu L^{-1} for PL7 and PL11; 0, 0.25, 0.63, 1.60, 4, 10 mg Cu L^{-1} for juvenile and adult prawns. The water was aerated continuously by an air stone. Each test solution was renewed at 48-h interval. During acute toxicity tests, the prawns were not fed. Regular observations were made and dead individuals were removed 1, 3, 6, 12, 24, 48, and 96 h, after the tests started. The absence of body movement, immobility of the heart and lack of response after repeated touches with a probe were used as criteria for mortality. Median lethal concentrations (LC_{50}) and 95% confidence intervals were calculated with trimmed Spearman–Karber method (Martins et al. 2011). LC_{50}s were calculated at 24 h for PL1, 48 h for PL4 and PL7, and 96 h for PL11, juvenile and adult prawns.

Sublethal toxicity

Effect on OC

OC was determined in adult prawns. OC corresponds to the difference between the hemolymph osmolality and the osmolality of the medium at a given salinity. Two groups of 10 adult *M. rosenbergii* (20 for each Cu concentration) were exposed for 7 d to sublethal concentrations of copper: 0 (control), 0.50, and 0.75 mg Cu L^{-1}, at salinity of 0 and 12 ppt, respectively. These concentrations were chosen because no mortality was observed up to 7 d. Test media were aerated and renewed every 48 h. During the experiments, the prawns were fed with commercial fish food twice a day (*ad libitum*). To maintain the quality of the media, uneaten feed was removed every day. Hemolymph was sampled from the prawns on the last day of the experiment. Hemolymph was sampled by inserting the needle of a 1-ml hypodermic syringe into the ventrolateral sinus of the abdomen. About 20 µl of hemolymph was drawn from each of the control and exposed prawn, allowing osmolality determination. The osmolalities of the hemolymph and of the media were measured using an osmometer (Fiske® 210 Micro-Sample Osmometer, USA) and expressed in mOsm kg^{-1}.

Copper accumulation in gill tissues

At the end of the period of copper exposure, the adult prawns were dissected, and the gills were removed from each individual of each experimental group. These tissue samples were dried at 65 °C for 48 h to a constant weight then homogenized. Approximately, 1 g of homogenized tissue sample was transferred to 3 ml concentrated nitric acid and digested at 90 °C for 4 h. After the cooling, samples were filtered and diluted to 50 ml with deionized water; copper concentrations were measured on a Shimadzu type AA-6200 flame atomic absorption spectrophotometer. Copper concentrations of samples were quoted as mg kg^{-1} dry weight (dw). The detection limit of copper was 0.0015 mg kg^{-1} dw. Analytical blanks were run in the same way as the samples, and concentrations were determined using standard solutions prepared in the same acid matrix. Validity of analytical methods was checked using dogfish muscle reference materials (DORM-2) provided by the National Research Council of Canada. The recovery for Cu in the tissue standard reference material DORM-2 was 106–108% (certified value: 2.34 ± 0.16 mg kg^{-1} dw, measured value: 2.52 ± 0.03 mg kg^{-1} dw, the values obtained for the analysis of three replicates).

Effect on gills structure

On the last day of the experiment, gill samples of adult prawns were carefully dissected out and fixed in 4% buffered formalin, embedded in paraffin, sectioned at 8 µm thickness on a microtome (Microm HM 315, Germany), stained with hematoxylin and eosin, and examined with a microscope (Olympus CX41, Japan). Histological damage to the gills was observed and confirmed in at least six samples of each treatment.

Statistical analysis

All data were tested for normality using a Kolmogorov–Smirnov tests. If not normally distributed, they were transformed and subjected to parametric statistics. The comparisons of the effects of the different treatments on Cu concentration in the gills and OC were analyzed using one-way analysis of variance with the confidence level set at

$p < 0.05$. Duncan's multiple range test was used to determine which treatments resulted in significant effects on OC and concentration of Cu in the gills of adult prawns at a significance level of 0.05. The comparison of the effects of different treatment on the Cu concentration in the gills at different salinities was analyzed using a Student's *t*-test.

Results

Acute toxicity

Table 1 shows the 24-, 48- and 96-h LC_{50} values for copper at12 ppt of different life stages of the freshwater prawn *M. rosenbergii*. No death was observed among control animals at any stage in the experiments. The tolerance to copper increased with increasing life stage: the less tolerant stage was postlarvae (PL)1, and the most tolerant stage was adult. PL11 was more tolerant than PL1, 4 and 7. The 24-h LC_{50} values were low in postlarvae (0.01–0.92 mg Cu L^{-1}) and increased substantially in juveniles (1.26 mg Cu L^{-1}) and adults (2.24 mg Cu L^{-1}). The 48-h LC_{50} values in postlarvae (PL1, 4, 7, and 11) ranged from 0.01 to 0.71 mg Cu L^{-1} and increased in juveniles (1.20 mg Cu L^{-1}) and adults (1.51 mg Cu L^{-1}). The 96-h LC_{50} values in PL11, juveniles and adults were 0.59, 0.94, and 1.20 mg Cu L^{-1}, respectively.

The 24-, 48-, and 96-h LC_{50} values for copper at 0 ppt for juvenile and adult *M. rosenbergii* are presented in Table 2. The 24-, 48-, and 96-h LC_{50} values for copper were higher at 0 ppt than those at 12 ppt for both juvenile and adult prawn. It is important to note that the percentage of survival in control juvenile and adult prawns (no copper addition into the media) was 100% in both 0 and 12 ppt, confirming the euryhalinity of juvenile and adult *M. rosenbergii*.

Effect on OC

No prawns died after exposure to copper concentrations within 7 d at both 0 and 12 ppt. Alterations in OC values of adult *M. rosenbergii* exposed to sublethal concentration of copper during 7 d at 0 and 12 ppt are shown in Figures 1 and 2, respectively. The *post hoc* analysis (Duncan's test) revealed that OC values of controls and prawns exposed to 0.5 mg Cu L^{-1} at 0 ppt were not significantly different. The OC of prawns exposed to 0.75 mg Cu L^{-1} was significantly different (or reduced by 12%) from the OC of the control animals ($p < 0.01$). After exposure to 0.75 mg Cu L^{-1} at 12 ppt, the OC of exposed prawns was significantly different (or reduced by 47%) from the OC of the control prawns ($p < 0.01$). However, the OC value of prawns exposed to 0.5 mg Cu L^{-1} was not significantly different from the OC value of control prawns.

Table 1. The 24-, 48-, and 96-h LC_{50} values with 95% confidence intervals of copper (mg Cu L^{-1}) to postlarvae, juvenile and adult of *M. rosenbergii* at 12 ppt salinity.

Life stage	24-h LC_{50}	48-h LC_{50}	96-h LC_{50}
Postlarvae 1	0.01 (0.01–0.02)	–	–
Postlarvae 4	0.01 (0.01–0.02)	0.01 (0.01–0.02)	–
Postlarvae 7	0.34 (0.26–0.45)	0.21 (0.17–0.26)	–
Postlarvae 11	0.92 (0.57–1.49)	0.71 (0.42–1.19)	0.59 (0.21–1.68)
Juvenile	1.26 (1.05–1.52)	1.20 (1.00–1.45)	0.94 (0.75–1.19)
Adult	2.24 (1.83–2.73)	1.51 (1.25–1.83)	1.20 (1.00–1.45)

Note: – = no data.

Table 2. The 24-, 48-, and 96-h LC_{50} values with 95% confidence intervals of copper ($mg\,Cu\,L^{-1}$) to juvenile and adult of *M. rosenbergii* at 0 ppt salinity (freshwater).

Life stage	24-h LC_{50}	48-h LC_{50}	96-h LC_{50}
Juvenile	3.50 (2.11–5.81)	2.22 (1.44–3.40)	1.94 (1.33–2.84)
Adult	3.10 (2.31–4.17)	2.37 (1.87–2.99)	1.81 (1.40–2.34)

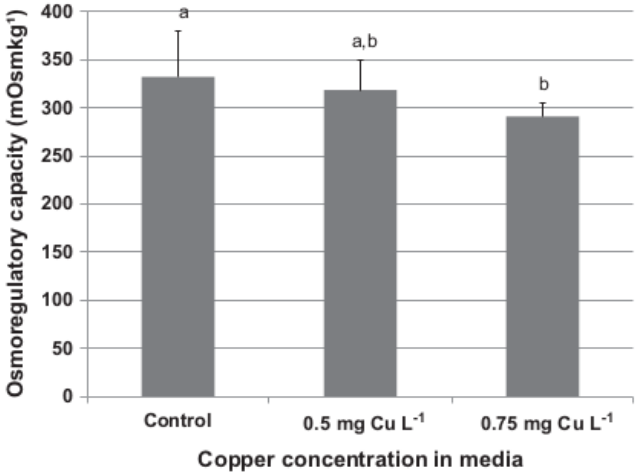

Figure 1. OC (mean ± SD) of control *M. rosenbergii*, as well as individuals exposed to 0.5 and 0.75 $mg\,Cu\,L^{-1}$ for 7 d at 0 ppt salinity. Different letters indicate significant differences ($p < 0.01$; $a > b$). Data are means of 10 determinations.

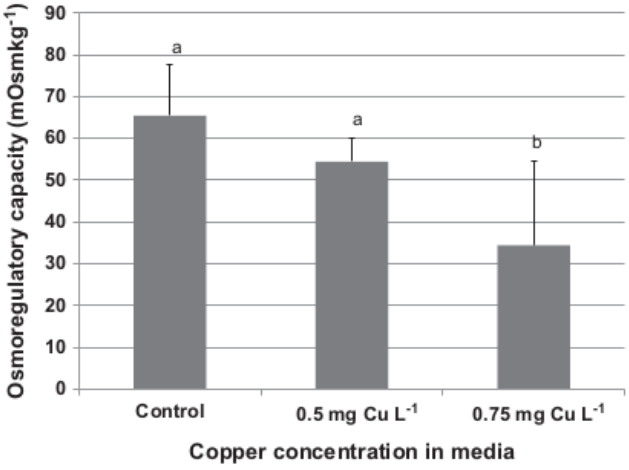

Figure 2. OC (mean ± SD) of control *M. rosenbergii*, as well as individuals exposed to 0.5 and 0.75 $mg\,Cu\,L^{-1}$ for 7 d at 12 ppt salinity. Different letters indicate significant differences ($p < 0.01$; $a > b$). Data are means of 10 determinations.

Copper accumulation in gill tissues

The concentration of copper in the gill tissue of control and exposed prawns is presented in Figures 3 and 4. In the control prawns, the concentration of copper in the gills is 0.0022 ± 0.0006 (at 0 ppt salinity) and $0.0023 \pm 0.0005\,\mathrm{mg\,kg^{-1}}$ (at 12 ppt salinity). Compared with control prawns, the copper concentrations in gill tissues increased significantly by 565 and 826% in prawns exposed to 0.5 and $0.75\,\mathrm{mg\,Cu\,L^{-1}}$ at 0 ppt ($p < 0.01$), respectively, and by 765 and 1452% in prawns exposed to 0.5 and $0.75\,\mathrm{mg\,Cu\,L^{-1}}$ at 12 ppt ($p < 0.01$). The copper concentrations in gills increased significantly with increasing copper concentrations of both media. The concentration of Cu in gills of prawns exposed to 0.5 and $0.75\,\mathrm{mg\,Cu\,L^{-1}}$ at 12 ppt was significantly higher than those exposed at 0 ppt ($p < 0.05$) (Figure 5).

Effect on gills structure

In the control gills both at 0 and at 12 ppt, each gill lamellae is limited by a thin epithelium. The tip of lamellae is widened to form a marginal canal (Figures 6, 1(A) and (B)). Exposure to $0.5\,\mathrm{mg\,Cu\,L^{-1}}$ for 7 d at both 0 and 12 ppt did not change the gills structure of exposed prawns. The histological structure of exposed gills was similar to that of the control gills (Figures 6, 2(A) and (B)). However, after 7 d of copper exposure of the sublethal concentrations of $0.75\,\mathrm{mg\,Cu\,L^{-1}}$ at both 0 and 12 ppt, swelling of lamellae to adult prawns, multiple hyperplasia and necrosis were observed in gill lamellae, resulting in abnormal gill tips (loss of marginal canal) (Figures 6, 3(A) and (B)).

Discussion

The sensitivity of *M. rosenbergii* to copper decreased from postlarval stages to adult stages. The 24-, 48-, and 96-h LC_{50} values show that postlarval stages are the most sensitive, while adult stages are the most tolerant to copper. Only limited studies on Cu toxicity to different life stages of freshwater decapod crustacean species have been

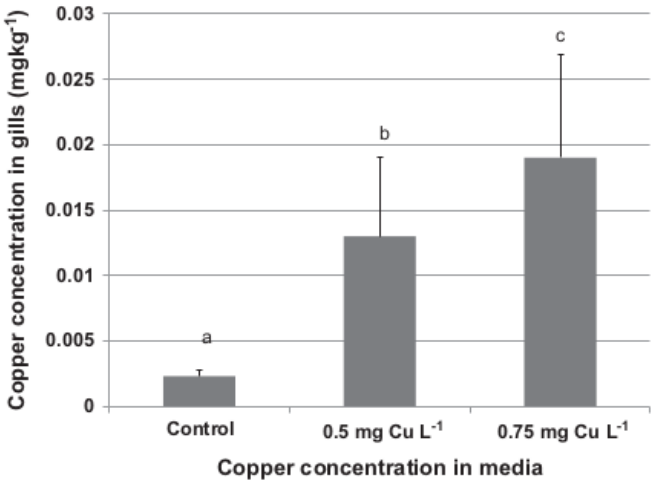

Figure 3. Copper concentrations (mean ± SD) in gill tissues of adult *M. rosenbergii* after animals were exposed to various Cu concentrations for 7 d at 0 ppt salinity. Different letters indicate significant differences ($p < 0.01$; $a < b < c$). Data are means of 10 determinations.

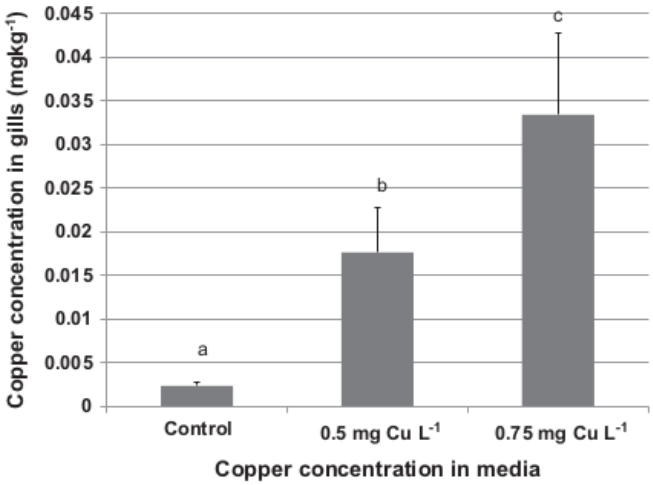

Figure 4. Copper concentrations (mean ± SD) in gill tissues of adult *M. rosenbergii* after animals were exposed to various Cu concentrations for 7 d at 12 ppt salinity. Different letters indicate significant differences ($p < 0.01$; $a < b < c$). Data are means of 10 determinations.

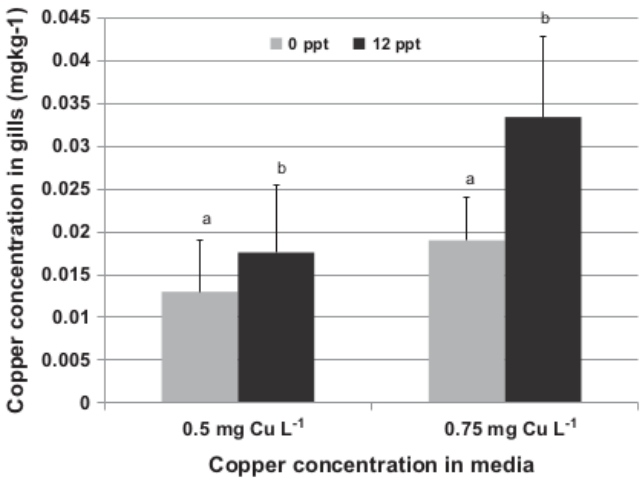

Figure 5. Copper concentrations (mean ± SD) in gill tissues of adult *M. rosenbergii* after animals were exposed to 0.5 and 0.75 mg Cu L^{-1} for 7 d at 0 and 12 ppt salinity, respectively. Different letters indicate significant differences ($p < 0.05$; $a < b$). Data are means of 10 determinations.

conducted. Almost all previous studies investigated marine crustacean species. Bambang et al. (1995) and Usman et al. (2013) found the same trend of tolerance to Cu of different life stages of *Penaeus japonicus* and *Litopenaeus vannamei*. The less tolerant stages are the larvae and postlarvae and the most tolerant stage was the juvenile.

The comparable data with this study were reported by Li et al. (2005) and Reddy et al. (2006), who used the juvenile *M. rosenbergii*. They reported that 96-h LC_{50} values were 0.452 mg Cu L^{-1} to a juvenile with a mean body weight of 3.5 g (Li et al. 2005) and 0.39 mg Cu L^{-1} to a juvenile with a mean body weight of 1.25 g (Reddy et al. 2006) in freshwater (0 ppt). Meanwhile, our results noted that the 96-h LC_{50} in juvenile prawns (mean body weight of 8.2 g) was 0.94 mg Cu L^{-1}. This difference might have

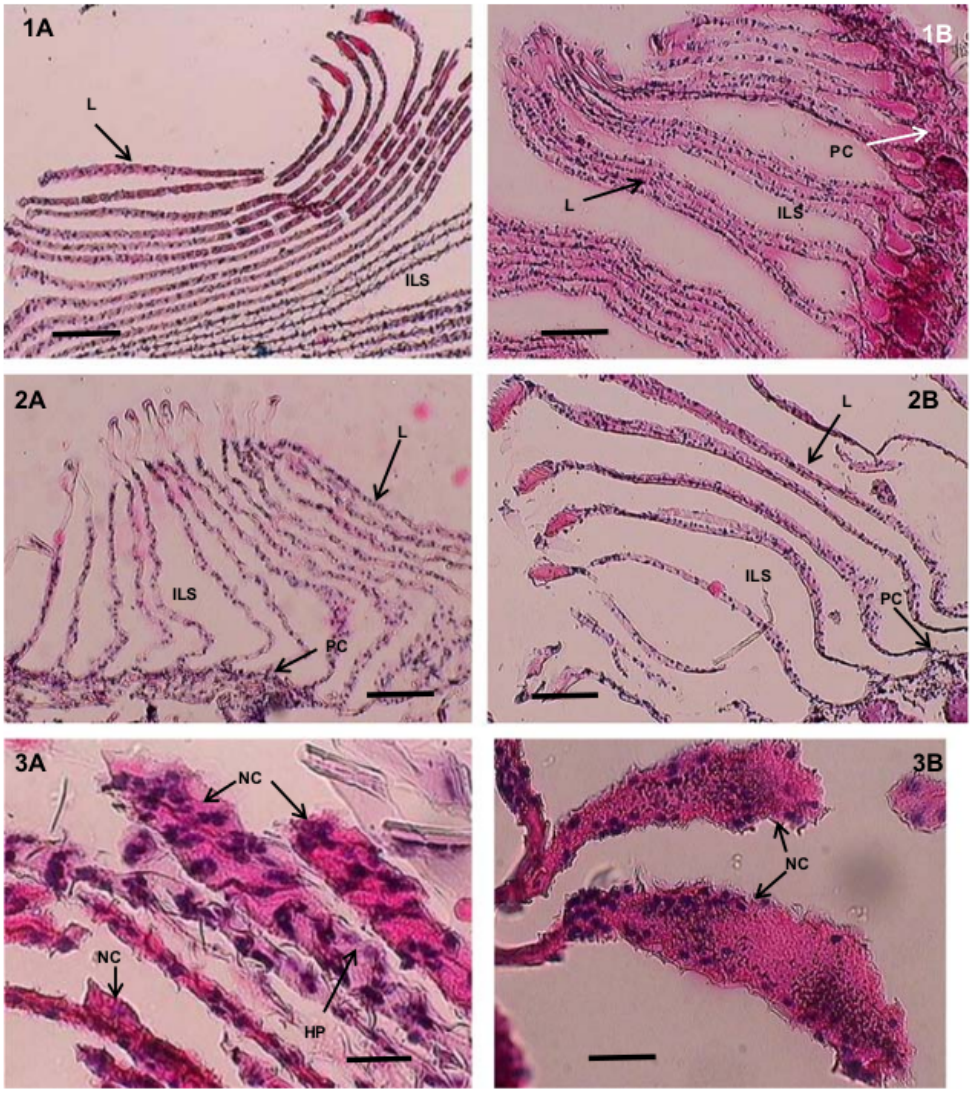

Figure 6. Histological structure of gills of *M. rosenbergii*. 1A. Control gills at 0 ppt; 1B. Control gills at 12 ppt; 2A. Gills exposed to 0.5 g Cu L^{-1} for 7 d at 0 ppt; 2B. Gills exposed to 0.5 g Cu L^{-1} for 7 d at 12 ppt; 3A. Gills exposed to 0.75 g Cu L^{-1} for 7 d at 0 ppt, hyperplasia resulting swelling of gill tips (marginal canals); 3B. Gills exposed to 0.75 g Cu L^{-1} for 7 d at 12 ppt, necrosis and swelling of gill tips; (bar size = 10 µm for 1A, 1B, 2A and 2B; bar size = 40 µm for 3A and 3B; L = lamellae, PC = pillar cells, ILS = interlamellar space, NC = necrosis, HP = hyperplasia).

been caused by the different sizes of juvenile prawns used in toxicity test. The mean body weight of the prawns used in our study was higher than that used in their experiments. This is in agreement with the results of Bambang et al. (1995) and Usman et al. (2013) and the results show that the sensitivity of crustaceans to copper decreases with increasing development stage and size.

The 24-, 48-, and 96-h LC_{50} values for copper were higher at 0 ppt than those at 12 ppt for both juvenile and adult prawns. An increase in salinity causes an increase in copper uptake or toxicity by juvenile and adult *M. rosenbergii*. The results of the

current study are different from the results of previous studies in which uptake, toxicity and bioavailability of metals increase in dilute seawater (e.g. Sunda et al. 1978; Nugegoda & Rainbow 1989; Bervoets et al. 1995; Ardiansyah et al. 2012). How then can the findings of this study be interpreted? Funge-Smith et al. (1995) reported that juvenile and adult *M. rosenbergii* is a hyperosmoregulator over a range of salinity from 0 to 15 ppt and maintains osmolalities close to the medium in the range of 15–28 ppt. In hyperosmoregulating prawns, the energetic demand of ion and osmoregulation may be reduced at increasing salinity by increasing the permeability of the exchanges surfaces (Rainbow et al. 1993; Bervoets et al. 1995). As a consequence, juvenile and adult *M. rosenbergii* exposed to higher salinities will have a higher permeability for the major cations, so that the uptake of other metals that use the same pathways is expected to increase. Thus, although Cu bioavailability decreased along with the increasing salinity, the prawns became increasingly permeable to Cu, so that the uptake and toxicity of Cu increased. Therefore, as presented in our findings, the concentration of Cu in gills of prawns exposed to 0.5 and 0.75 mg Cu L^{-1} at 12 ppt was significantly higher than those exposed at 0 ppt (Figure 5). A similar finding was reported by Chan et al (1992) in the case of zinc and cadmium in crabs *Carcinus maenas*. These crabs showed a decrease in zinc and cadmium uptake rate with a salinity decreased from 35 to 15 ppt. It appeared that any reduction in apparent water permeability (AWP) at 15 ppt is sufficient to limit Zn and Cd uptake despite greater bioavailability of Zn and Cd (Chan et al. 1992). The AWP of *C. maenas* decreased appropriately with reduced salinity (Smith 1970).

The present study shows that increasing copper concentration in media both at 0 and at 12 ppt affected significantly the OC values of adult *M. rosenbergii*, indicating that imbalance in plasma and ions occurred. Imbalance in the plasma ion concentrations has also been observed in shrimps *P. japonicus* and *L. vannamei* after they were exposed to various copper concentrations (Bambang et al. 1995; Usman et al. 2013). The hypo-OC of *P. japonicus* in seawater (37–38 ppt), and its hyper-OC in dilute seawater (16–17 ppt), decreased significantly with increasing ambient copper concentrations (Bambang et al. 1995). The juvenile *L. vannamei* also had low ability to maintain hemolymph osmoregulation affected by sublethal concentrations of copper. After the exposure to 0.675, 1.325, and 2.010 mg Cu L^{-1} at salinity 15 ppt, the OC of exposed shrimps significantly reduced by 70.3, 77.2, and 77.8%, respectively, compared with control animals (Usman et al. 2013).

Several studies have also shown that other heavy metals adversely affected the osmoregulation of crustacean species. After exposure to 3 mg Cd L^{-1} or 3 mg Zn L^{-1} for 12 h, osmotic pressures of *L. vannamei* were lower than those of control animals. Exposure for 24 h caused more severe effects on osmotic pressures of exposed shrimps (Wu and Chen 2004). Ardiansyah et al (2012) reported that exposure to sublethal concentration of Cd and Zn reduced the hyper-OC of *L. vannamei* at 5 and 15 ppt salinity and increased the hypo-OC of exposed shrimps at 27 ppt salinity.

Many factors affect the hemolymph osmolality value of crustacean such as the tissue volume regulation, the ionic permeability of the exchange surface epithelia and the ratio of surface to volume of the organism (Bouaricha et al. 1994; Lignot et al. 1998; Amado et al. 2006). The perturbation in OC observed in this study was due to direct inhibition of the osmoregulation mechanisms (e.g. the increasing water or ions and/or, inhibition of ion transport mechanisms) (Bjerregaard & Vislie 1986; Boitel & Truchot 1989; Hansen et al. 1992; Weeks et al. 1993; Amado et al. 2006). Copper is known to affect the ionic and osmotic regulatory systems of crustaceans species

negatively by reducing the hemolymph ion concentrations (Na^+, Ca^{2+}, K^+, Mg^{2+}, and Cl^-) and by inhibiting gill Na^+-K^+-ATPase activity of the crabs *C. maenas* and *Cancer irroratus* (Thurberg et al. 1973). Hemolymph osmolality and Na^+, K^+, and Cl^- concentrations in *C. maenas* were reduced after exposure to various copper concentrations (Bjerregaard & Vislie 1986). In other studies, copper exposure also reduced hemolymph ionic concentrations and gill Na^+-K^+-ATPase activity in *C. maenas* (Boitel & Truchot 1989; Hansen et al. 1992; Weeks et al. 1993). We thus supposed that the diminution of OC of *M. rosenbergii* after being exposed to copper originated in a direct disruption effect of copper on hemolymph ionic concentrations and the alteration on gill Na^+-K^+-ATPase activity.

This study shows that copper significantly accumulated in gills of adult *M. rosenbergii* after sublethal exposures for 7 d at both 0 and 12 ppt. Their toxic actions substantially altered the structure of gills. Gill hyperplasia, necrosis, swelling of lamellae, and abnormality of gill tips were observed in this study. The histological damage increased along with the increasing copper concentration in gills. Li et al. (2007) reported that exposure to copper concentrations ranging from 0.01 to 0.4 mg Cu L^{-1} for 7 d resulted in profound structural changes including the accumulation of hemocytes in the hemocoelic space, swelling and fusion of the lamellae, abnormal gill tips, hyperplastic, necrotic, and clavate–globate lamellae in the gills of *M. rosenbergii*. Similar histological damages had been observed in other crustaceans exposed to various heavy metals (Lawson et al. 1995; Soegianto et al. 1999; Frias-Espericueta et al. 2008; Wu et al. 2009). They demonstrated that a blackened appearance of the gill, an increased number of nephrocytes in gill filaments, hyperplasia of the gill, swelling of gill filaments, necrosis of gill cells resulting in narrowed or obstructed hemolymphatic lacuna at gill tips, abnormal dilation of the lacuna of the filaments, loss of regular structure of the epithelium, the appearance of a space between the cuticle and the epithelial cells, disorganization of gill organelles and even fragmentation of nuclei within gill cells could be observed when crustaceans were exposed to different levels of metals. Since copper concentrations resulted in serious damage to the gill of *M. rosenbergii*, the copper might consequently inhibit the physiological function of this organ. The adverse effects of copper on gill structures of the exposed prawns might be one of the main factors responsible for reduction of OC. These findings are supported by the present works that show that the OC of juvenile *M. rosenbergii* was significantly affected by 0.5 and 0.75 mg Cu L^{-1}. An obvious relationship can therefore be established between the impairment of osmoregulation and the structural damages to gills reported in this study.

Implications for aquaculture

Prawn farms (both hatchery and culture pond) are often located close to water resources (i.e. river and coast) due to their potential as a main source to cultivate prawns. However, today many water resources are contaminated by heavy metals from anthropogenic activities (Stauber et al. 2005; Amado et al. 2006; Dan'azumi & Bichi 2010). The level of copper in unpolluted water is about 0.05 µg L^{-1}, while it registers as high as 2 mg L^{-1} in polluted coastal waters (Wong 1993; Saager et al. 1997; Stauber et al. 2005; Dan'azumi & Bichi 2010). The potential negative impacts of copper on prawn hatcheries and culture should therefore be anticipated when we consider utilizing these water sources. The experiment of this study demonstrated that all life stages of *M. rosenbergii* are sensitive to copper and their survival, osmoregulation and gill structure may be negatively impacted. Treating source waters that may be contaminated

with copper before entering prawn's hatchery tanks and culture ponds is a practical recommendation that could be implemented.

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