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Accumulation, Distribution, and Toxicology of Copper Sulfate in Juvenile Giant Freshwater Prawns, *Macrobrachium rosenbergii*

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The giant freshwater prawn, *Macrobrachium rosenbergii,* is one of the most important crustaceans cultured commercially in mainland China and other Pacific Rim countries (Sung et al., 2003). In recent years, chemical treatment for maintaining optimal water quality has gained attention because farmers use antiviral and antibacterial chemicals to prevent vibriosis and several diseases (Chen and Lin , 2001).

Copper sulfate is one of the chemicals that are commonly applied to shrimp ponds to eradicate filamentous algae. The application of copper sulfate in ponds is also very effective in reducing the abundance of *Microcystis* and other blue-green algae. The application rate of copper sulfate varies from 0.025 to 2 mg/l and is directly related to total alkalinity (Boyd, 1990). However, farmers often use an excess amount of copper sulfate in shrimp pond management. As a result, the growth and survival rate of animals decrease significantly, and accordingly, the pond management cost tends to increase year by year. Therefore, the remaining copper sulfate in water and its toxicity are of primary concern.

There has been considerable work done in toxicity of copper on crustaceans. Bambang et al. (1995) and Chen and Chen (1996) investigated the toxicity of copper sulfate to *Penaeus japonicus* and *P. monodon*, respectively. However, our knowledge remains limited regarding the toxicity of metals in *M. rosenbergii*. The aim of the present study was to provide information about copper toxicity and accumulation in juvenile *M. rosenbergii*.

MATERIALS AND METHODS

M. rosenbergii juveniles were obtained from a private nursery in the Songjiang District, Shanghai. They were acclimated in 70×45×60 cm plastic containers, each with a total volume of 300 l. The containers are filled with 50 l of dechlorinated tap water for 3 days at 29±1℃ prior to experiments. Stocking densities were 20 prawns per container within 50 l water. The wet weight and average standard body length of shrimps was 3.5±0.1 g and 4.20±0.05 cm on average, respectively. The prawns were divided into three treatment groups and each treatment group had a control for comparison.

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We first dissolved 57.53 g of copper sulfate in 500 ml of distilled water to prepare 50 g/l of copper stock solution. Short-term median lethal concentration (LC50) toxicity test was carried out according to the method of equal logarithmic basis method (Zhou and Zhang, 1989). Nominal test concentrations of 0.00, 0.32, 0.42, 0.56, 0.75, 1.00, 1.35, and 1.80 mg/l copper for the acute toxicity test, and 0.00, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/l for the bioaccumulation test were prepared by adding appropriate volumes of the stock solution to dechlorinated tap water. The Cu^{2+} content in the dechlorinated tap water, 0.004±0.001 mg/l, was examined by an inductively coupled plasma atomic emission spectrometry (ICP-AES, Plasma, 2000, Derkin Elmer).

Bioassay experiments to establish tolerance limits were conducted in polyethylene tanks containing 50 l test solution. An air stone continuously aerated each tank. The test solution was renewed daily, in accordance with the semi-static renewal method for toxicity tests (Buikema et al., 1982; American Public Health Association et al., 1985). Shrimps were not fed during the acute toxicity test. Water was maintained at 29±1℃, pH 6.7 to 7.3. Observations were usually made at 24 h intervals throughout 96 h. The criteria for death were total lack of movement, immobility of the heart and no response to stimuli after repeated touches with a glass rod (APHA, 1980; Winner et al., 1976). The dead shrimps were removed whenever they are detected. The concentration-response of test shrimps was determined for calculations of LC50s for copper.

Copper accumulation was conducted essentially in a similar way of the acute toxicity test. But shrimps were fed twice daily (09:00 and 21:00 h) with the commercial diet at 5% of body weight. Dead juveniles and uneaten feed were removed daily, in the morning (8:00–9:00) when the water was renewed. Exuviae were also removed daily. After seven days of exposure tissues were prepared for Cu^{2+} analysis as follows: gill filaments were removed from the gill arch; the hepatopancreas was separated, cut longitudinally, lightly and scraped using a spatula; the exoskeleton was dissected according to Duquesne et al. (2000). All the tissue samples were stored at -70℃. The gill, hepatopancreas, and exoskeleton samples were oven dried at 135℃ for 10h in order to evaporate most water in samples and improve the efficiency of the following steps (Tilstone, 1997). Then all the samples were subjected to the carbolite at 450℃ (Peng, 1999) for 4~5h until all the samples were dried into ashes. Charred samples were treated with 500 μl additional analytical-grade nitric acid and volumes were adjusted to 20 ml with distilled water. Cu^{2+} concentration was analyzed by the ICP-AES following the instrument operation manual. Cu^{2+} concentration in tap water was analyzed in the same way as tissue samples, with 19.5 ml tap water sample plus 500μl additional analytical-grade nitric acid for test. All the data for metal concentrations in tissues are expressed as μg/g wet weight.

Statistical analysis was performed by SPSS v. 9.0 software. One-way ANOVA was used to test for differences between control and copper-treated groups. Regression analysis was used to test for concentration-dependent patterns of accumulation in tissues because of the small sample size. All data are expressed as

the mean ± S.E.

RESULTS AND DISCUSSION

No mortality was noted in control groups (0.00 mg/l copper). All the shrimps exposed to 1.80 and 1.35mg/l copper died after 24 h, while those exposed to 1.00 mg/l copper died after 48 h. No mortality occurred among the shrimps exposed to 0.32 mg/l copper after 96 h (Table 1). The LC50 values of copper and their 95% confidence limits at different time periods are presented in Table 2. The 24-, 48-, 72- and 96-h LC50 of copper for *M. rosenbergii* juveniles was 0.525, 0.453, 0.454 and 0.452 mg/l, respectively.

In southeast China, the tolerance to copper of juvenile *M. rosenbergii* in stage I to stage X increased with developmental stage (Wang et al. 2000). The 96-h LC50 of copper for juveniles was 0.32 mg/l. The present study showed an increased tolerance of copper in the juvenile prawns that developed more completely than those in Wang's experiment. The results of our present study seem to be mainly associated with some physiological mechanisms of crustaceans. It has been reported that crustaceans are more sensitive to toxic chemicals, especially heavy metals, when exuviating (Dell et al. 1999; Wright 1979). Ecdysis is the molting, permitting the formation of a new larger exoskeleton, and involves profound changes in the rate of absorption of waters and ions (including Cu^{2+}) to expand the body volume (Lockwood and Inman, 1973). *M. rosenbergii* larvae in the stage between I and X molt frequently and possess a relatively weak exoskeleton compared with the shrimps used in our research. Therefore, juveniles in stages I-X are more susceptible to water-born copper and with a lower 96-h LC50. Moreover, the mortality rate of shrimps increased following longer exposures (from 48h to 96h), which was probably partly caused by weakness of shrimp after molting.

Concentration of 0.084 mg/l copper sulfate (i.e. 0.033 mg/l copper) will reduce *Microcystis* blooms in ponds (Boyd 1990). This dose did not significantly affect the mortality of juvenile *M. rosenbergii* in the present study. However, juvenile *M. rosenbergii* experienced a decrease in both vitality and mobility after 7 days following exposure to copper even at low levels between 0.06 and 0.10 mg/l. This shows that juvenile *M. rosenbergii* to be quite sensitive to copper and the safe concentration should be around 0.045 mg/l. Safe concentration here is defined as concentration of toxins that have no deleterious effect on the test subjects (animals, typically crabs and shrimps) during the full life stage test (Zhou and Zhang, 1989). Wang (2000) recommended that the Short-term Safety Concentration of shrimp is 0.05 mg/l copper. The Short-term Safety Concentration 0.045 mg/l copper in the present study is similar to that reported previously, which would be useful in pond management of *M. rosenbergii* aquaculture.

The toxic action and the physiological consequences of copper on crab have been widely studied and are summarized by Boitel and Truchot (1989). They reported metabolic acidosis without marked changes in haemolymph ions in European green crab (*Carcinus maenas*) following 18 days of exposure to 1.0 mg/l copper.

Table 1. Acute toxicity of copper to the juvenile *Macrobrachium rosenbergii* (mean ± S.E).

Copper concentration (mg/l)	24h	48h	72h	96h
0.00	N.D.	N.D.	N.D.	N.D.
0.32	N.D.	N.D.	N.D.	N.D.
0.42	7.0±0.00/ 35.0±0.0*	9.0±0.00/ 45.0±0.0	9.0±0.00/ 45.0±0.0	9.0±0.00/ 45.0±0.0
0.56	11.5±0.14/ 60.0±7.1	15.0±2.83/ 75.0±14.2	15.0±2.83/ 75.0±14.2	15.0±2.83/ 75.0±14.1
0.75	13.5±2.12/ 67.5±10.6	17.0±1.41/ 85.0±7.1	18.5±0.71/ 92.5±3.5	19.0±0.0/ 95.0±0.0
1.00	17.0±1.41/ 85.0±7.0	A.D.	A.D.	A.D.
1.35	A.D.	A.D.	A.D.	A.D.
1.80	A.D.	A.D.	A.D.	A.D.

Note: The average wet weight of shrimps was 3.5±0.1 g.
* Denotes number of shrimp died /death rate (%).
N.D.: No death.
A.D.: All death.

Table 2. The LC_{50} values of copper (mg/l) and their 95% confidence limits at different time periods (h) to the juvenile *Macrobrachium rosenbergii* (mean±S.E).

	24h	48h	72h	96h
LC_{50}	0.53±0.04	0.45±0.05	0.45±0.04	0.45±0.04

Further research is needed to reveal osmotic and ionic regulation in *Macrobrachium* shrimps under copper stress.

The threshold concentration that produces a statistically significant deleterious effect is usually expressed as the Maximum Acceptable Toxicant Concentration (MATC) (Wickins 1976; Chen and Chen 1996). The MATC is 0.045 mg/l copper based on the weight, length, and molting frequency of *M. rosenbergii* weighing 3.5±0.1 g in the present study.

Copper accumulation in all three tissues of juvenile *M. rosenbergii* tended to increase with increased water-borne copper concentration in a positive concentration-response relationship. Average copper concentrations in exoskeleton, gills and hepatopancreas were 0.013, 0.072, 0.028 mg/g wet weight, respectively. With increasing the concentration of water-borne copper, copper level increased significantly in all the tissues studied, especially the hepatopancreas. In the group exposed to 0.50 mg/l copper level, the average copper concentrations in the exoskeleton, gill, and hepatopancreas were 0.082,

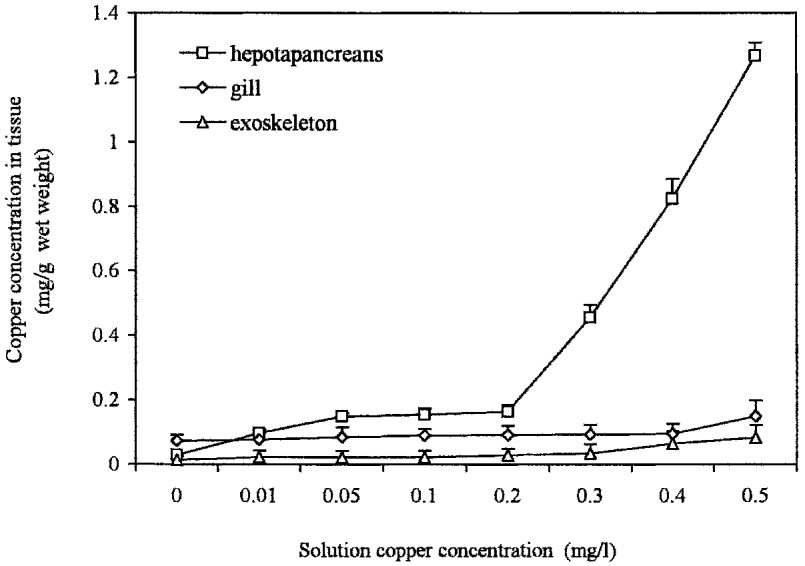

Figure 1. Copper accumulation in the hepatopancreas, gill, and exoskeleton of juvenile *Macrobrachium rosenbergii* exposure to gradient concentration of copper. Vertical bar show confidence intervals at 95%.

0.149, 1.268 mg/g wet weight, respectively (Fig. 1). In general, the evidence of copper bioaccumulation in each tissue decreased in order of the hepatopancreas > the gills > the exoskeleton. The exoskeleton generally serves as the most direct protective sheath in *M. rosenbergii*, and it is always exposed to the ambient environment. Therefore, exoskeleton has a high likelihood of accumulating copper and transports this metal to the other tissues or organs in body subsequently. In addition, the major component in the exoskeleton is calcium, which shares a similar chemical characteristic with copper, and, hence, the water-borne copper probably took the place of calcium in exoskeleton and accumulated in this tissue. However, copper may either absorb to the surface of the exoskeleton or bind to the inner exoskeleton after uptake and are transported through the haemolymph. Therefore, there is no significant copper content increase in the exoskeleton as the results in the present study. As an important aspect of crustacean physiology, molting may also influence metal concentrations and distributions between soft tissues and exoskeleton. All the information listed above may be provided an explanation for the pattern of copper accumulation in the exoskeleton of *M. rosenbergii*.

The hepatopancreas plays a significant role in the regulation of copper absorption (Wong et al. 1995). There is a plateau phrase for copper concentration in the hepatopancreas of *M. rosenbergii* from 0.01 mg/l to 0.2 mg/l, and then copper concentration further obviously increased from 0.097 mg/g to 1.268 mg/g when

exposed to higher water-borne copper level ranging between 0.2 mg/l and 0.5 mg/l. Similar phenomena cannot be observed in the gill and exoskeleton (Fig. 1). These results may indicate that the hepatopancreas is the most important site for copper accumulation in *M. rosenbergii*. Future study will be designed to investigate the biochemical response to copper in the hepatopancreas because the capacity of hepatopancreas to detoxify assimilated copper is quite limited, and can be totally out of function under a high water-borne copper level (Piyan et al. 1985). In addition, metals bound to the inner exoskeleton matrix and gills can be transported through the hemocyanin to the hepatopancreas (Hare 1992), and this may also increase copper content in hepatopancreas.

Gills play vital roles in gaseous exchange and ionregulation. In addition, the hemocyanin (an indispensable copper-oxygen binding protein) also exists in gills although it is the principal protein in the haemolymph. Therefore, it is no surprise that the copper concentration in gills of controls (0.072 mg/g) is the highest among all the three organs studied. However, as toxic substance can be transported from the gills into hemocyanin and other organs or tissues subsequently, copper bioaccumulation in the gills does not increase significantly in our exposure experiments. Even if shrimps were exposed to 0.5 mg/l copper, copper content in the gills is lower than that in the hepatopancreas.

Many studies of penaeid shrimps have already been conducted in the past. Ahsanullah and Ying (1995) concluded that the 96-h LC50 and its 95% confidence intervals for *Panaeus Merguiensis* were 0.38 and 0.21-0.68 mg/l copper, respectively. *P. monodon* exhibited no acute toxic effects to copper in sea water, at or below concentration of 2.5 mg/l copper. Meanwhile, they observed that both species accumulated copper during experiment as the order of gills > hepatopancreas > exoskeleton. Our results are slightly different when exposed to 96-h LC50 (0.452mg/l copper). The tissular copper accumulated in order of hepatopancreas > gills > exoskeleton in *M. rosenbergii*. This difference can be attributed to a series of endogenous (e.g. developmental stages, physiological conditions) and exogenous (e.g. temperature, pH) factors. The mechanism of bioaccumulation and toxicity of heavy metals in aquatic animals is generally synergistic effects of these factors mentioned above.

This study suggested that (1) juvenile *M. rosenbergii* was sensitive to the copper toxicity. The 24-, 48-, 72- and 96-h LC_{50} of copper for *M. rosenbergii* were 0.525, 0.453, 0.454, and 0.452 mg/l, respectively, and the safety concentration for copper was around 0.045 mg/l, which might be effective in the regulation of *M. rosenbergii* pond management; (2) bioaccumulation of copper in the hepatopancreas, gills, and exoskeleton varied obviously for juvenile *M. rosenbergii*: there is no significant copper level increase in the gills and the exoskeleton, in contrast, the hepatopancreas has a significant copper level increase. Therefore, the hepatopancreas is regarded as the most important copper-accumulation site, and it must play an indispensable and special role in the metabolism of copper. Further work will be conducted to determine how hepatopancreas function in heavy metal detoxification of shrimp *M. rosenbergii*.

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