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Acute toxicity of organochlorine insecticide endosulfan to the giant freshwater prawn *Macrobrochium rosenbergii**

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Abstract Endosulfan, an organochlorine pesticide, is highly toxic and effective at controlling pests in agriculture, horticulture, and public health programs. In this study, static bioassays were used to evaluate the toxicity of endosulfan to freshwater prawns (*Macrobrachium rosenbergii*) of various lengths (1.5±0.03, 4±0.08, and 7±0.06 cm). Additionally, the activities of peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase, acetylcholinesterase (AChE), and Na^+/K^+-ATPase were analyzed to reflect the effects of endosulfan exposure. The 96 h LC_{50} of endosulfan for prawns 1.5, 4, and 7 cm long were 1.86, 4.53, and 6.09 μg/L, respectively, improved tolerance to endosulfan with growth. The POD activities of test organisms exposed to low concentrations of endosulfan were inhibited, indicating the presence of oxygen damaged tissue. Moreover, a notable decrease in AChE activity was observed due to overstimulation of neurotransmission, which might result in abnormal behavior. The effect caused by endosulfan on phosphatase production in the hepatopancreas of prawns 1.5, 4, and 7 cm long was different because the ability of nonspecific immune regulation increased with growth. The 96 h LC_{50} values obtained in this study could be used in the formulation of water-quality criteria in China. Moreover, the changes in enzymes activities of *M. rosenbergii* under stress of endosulfan could be applied in the establishment of early warning indicators for bio-safety.

Keyword: hepatopancreas; acetylcholinesterase (AChE); peroxidase (POD); alkaline phosphatase (AKP); acid phosphatase (ACP); Na^+/K^+-ATPase

1 INTRODUCTION

Crustacean aquaculture constitutes an integral part of the economies of many developing countries. *Macrobrochium rosenbergii* (*M. rosenbergii*) is an important commercial freshwater species because of its large body, rapid growth and abundant nutritive value (Pillai et al., 2011). In 2010, global production of this freshwater prawn reached 996 610 t (FAO, 2010). China is the largest producer of *M. rosenbergii* because of high production from traditional aquaculture ponds (Achuthankutty et al., 1993; Suryavanshi et al., 2009).

The application of pesticides is a common and extensive approach used in agriculture (McKinlay et al., 2008). Because of their high stability and persistence in the environment, organochlorine (OC) pesticides (dieldrin, endosulfan, DDT, mirex, chlorothalonil) have attracted a great deal of attention (Sutherland et al., 2000; Saiyed et al., 2003). Such compounds result in increased environmental damage with increasing residence time in the soil, as well as adverse effects on aquatic systems they enter via runoff (Matos et al., 2007; Suryavanshi et al., 2009). Indeed, there have been massive die-offs in some shrimp hatcheries in response to inadvertent exposure to OC in the Gulf of California, Mexico (Tumburu et al., 2012), and fish were killed because of OC exposure at a breeding farm in Florida Bay (Wirth et al., 2001; Tumburu et al., 2012).

Endosulfan, a broad-spectrum OC pesticides

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belonging to the cyclodiene group, is an inexpensive and effective compound primarily used to control invertebrates (Rao, 2006). China is one of the largest endosulfan-consuming countries in Asia, using an estimated 25 700 t in agriculture production between 1994 and 2004 (Jia et al., 2009; Wang et al., 2012a). However, endosulfan has likely produced adverse effects on non-target organisms in China as a result of runoff into nearby rivers. Moreover, endosulfan has been found to be toxic to crustaceans (Wan et al., 2005; Capkin et al., 2006; Hii et al., 2007). Despite this, there is a lack of in-depth knowledge regarding endosulfan toxicity to *M. rosenbergii*. Therefore, the present study was conducted to investigate the susceptibility of *M. rosenbergii* to endosulfan.

2 MATERIAL AND METHOD

2.1 Test organism

M. rosenbergii post-larvae were obtained from Shanghai Shencao Special Fisheries Development Company, and cultured in an indoor traditional aquaculture pond. Active juvenile shrimp in lengths of 1.5±0.03, 4±0.08, and 7±0.06 cm and no remarkable indication of disease were selected from this culture pond, and immediately transferred to aquariums (experiment container, 200 L), where they were allowed to acclimate for 7 days prior to the experiments.

2.2 Chemicals

Commercial-grade endosulfan (active ingredient of 35% EC) was purchased from Jizhou Kaiming Pesticide Co. Ltd., in Hebei Province of China. Endosulfan solutions were directly dissolved in distilled water, after which a series of dilutions were conducted to obtain solutions of the required concentration for acute toxicity tests.

2.3 Tap water

The aquariums used in all experiments were cleaned and filled with tap water without chlorine. To dechlorination, fresh tap water was aerated by continuous aeration for at least 2 weeks and tested by iodine reagent before using. The water-quality parameters after dechlorination were as follows: dissolved oxygen 6.8±0.2 mg O_2/L, temperature 30±1°C, pH 7.6±0.2.

2.4 Acute toxicity

All acute toxicity bioassays were conducted under static conditions. Endosulfan concentrations of 0.01, 0.04, 0.12, 0.43, 1.51, 5.31, and 18.62 μg/L were used for toxicity tests of juvenile shrimp 1.5±0.03 cm long, while 0.01, 0.05, 0.25, 1.22, 6.08, 30.20, and 149.97 μg/L were used for shrimp 4 cm long, and 0.01, 0.05, 0.27, 1.41, 7.37, 38.37, and 199.89 μg/L were used for those 7 cm long. A control group (0.00 μg/L) was set in each toxicity test and repeated four times. Ten shrimp were distributed randomly into each aquarium. During the experiments, continuous aeration was applied to obtain a homogeneous concentration of endosulfan, and no feed was provided. Mortality was assessed at 24, 48, 72, and 96 h after exposure, and dead individuals were removed. After 96 h, the surviving shrimp were sacrificed and frozen at -40°C for biochemical assay.

2.5 Biochemical assay

Each sample (hepatopancreas wet weight about 1 g) was homogenized at ratios of 1:9 (w:v) in pre-cooling saline (0.86%). Homogenates were then centrifuged at 3 500 r/min for 30 min at 4°C. The enzyme activities of acetylcholinesterase (AChE), alkaline phostase (AKP), acid phostase (ACP), peroxidase (POD), and Na^+/K^+-ATPase were subsequently analyzed with AChE, AKP, ACP, POD and Na^+/K^+-ATPase kits (Jiancheng Ltd., Nanjing, China). ACP and AKP activity were both expressed as U/g protein, POD and AChE activity were expressed as U/mg protein, and Na^+-K^+ATPase activity was expressed as the amount of μmol pi per mrprotein in one hour (Wang et al., 2012b).

2.6 Behavior observation

Behavioral observation was conducted after 1, 4, 8, 12, 24, 48, 72, and 96 h of exposure. Abnormal behavior was defined as body twisting, lethargy, erratic swimming, and convulsions occurring throughout the observation period (60 s).

2.7 Safe concentration

The safe concentration (SC) was calculated as follows: SC=0.1×96 h LC_{50}.

2.8 Statistical analysis

Initial mortality data were used for evaluation of mean values; Probit analysis was used to assess the 24, 48, 72, and 96 h lethal concentration of endosulfan with 95% confidence limits (Das and Mukherjee, 2003). One-way ANOVA was conducted

Table 1 LC_{50} and safe concentration for *M. rosenbergii* in different lengths exposed to endosulfan

Length (cm)	Exposure time (h)	LC_{50} (μg/L) and 95% confidence interval	SC (μg/L)
1.5	24	10.44 (8.50–12.80)	0.19
	48	3.88 (3.26–4.62)	
	72	2.81 (2.33–3.39)	
	96	1.86 (1.52–2.84)	
4	24	82.21 (63.46–106.49)	0.45
	48	23.78 (19.85–28.49)	
	72	9.12 (7.75–10.75)	
	96	4.53 (3.81–5.39)	
7	24	174.27 (144.77–209.78)	0.61
	48	79.32 (54.32–115.81)	
	72	7.02 (5.92–8.32)	
	96	6.09 (5.15–7.20)	

using SPSS 17.0 identify differences between groups, and Dunnett's test was conducted to examine the variance.

3 RESULT

3.1 Acute toxicity of *M. rosenbergii* exposed to endosulfan

As shown in Table 1, endosulfan was highly toxic to the freshwater prawn, *M. rosenbergii*, and morality visibly increased with exposure to increasing endosulfan concentrations and increased exposure time. Specifically, the 96 h LC_{50} values of endosulfan toward *M. rosenbergii* in lengths of 1.5, 4, and 7 cm were 1.86, 4.53, and 6.09 μg/L, respectively.

No remarkable changes were observed in the control group. However, prawns exposed to high levels of endosulfan exhibited a series of abnormal behavioral patters. For example, individuals exposed to the highest concentration of endosulfan exhibited body twisting, erratic swimming, flashing, and restlessness.

3.2 Effect of endosulfan on ACP and AKP activities of *M. rosenbergii*

After 96 h of exposure endosulfan, there was no significant relationship observed between concentration of endosulfan and phosphatase levels in the hepatopancreas of shrimp in lengths of 1.5 cm (P>0.05) (Fig.1a). However, phosphatase levels in hepatopancreas of 4-cm lengths prawns (regardless of exposure concentration) were lower than in the control group. Moreover, there was significant decrease in AKP in the hepatopancreas of test prawns exposed to endosulfan at 0.01, 0.05, and 0.25, 149.97 μg/L (P<0.01) and 1.22 and 30.20 μg/L (P<0.05). ACP levels were also significantly reduced in response to treatment with endosulfan at 6.08, 30.20, and 149.97 μg/L (P<0.05) (Fig.2a). Furthermore, there was a reduction in AKP in the

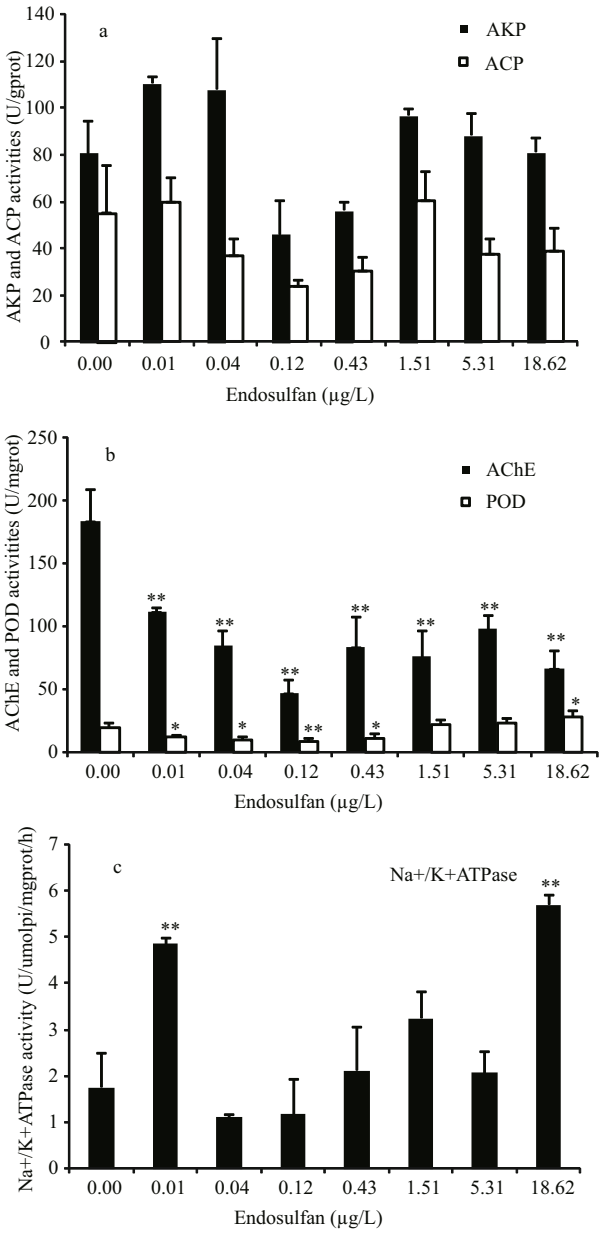

Fig.1 Effect of 96 h exposure to various endosulfan concentration (μg/L) on the activities of ACP(a), AKP (a), AChE (b), POD(b), and Na^+/K^+ATPase(c) in hepatopancreas of *M. rosenbergii* 1.5 cm long

Difference from control values *P<0.05, **P<0.01. Data expressed as Mean±SE, n=3.

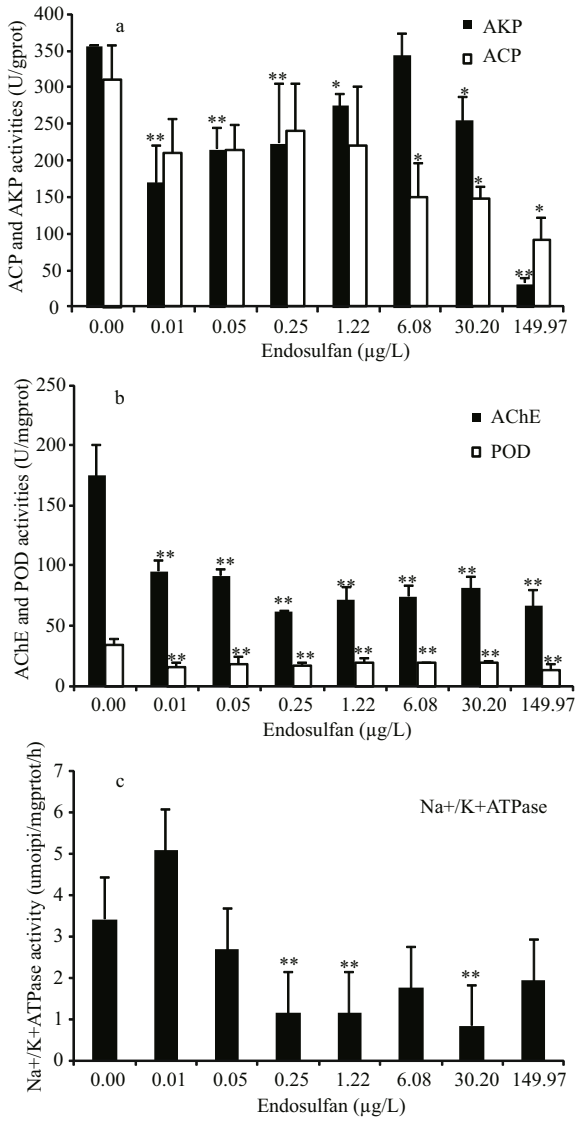

Fig.2 Effect of 96 h exposure to various endosulfan concentration (μg/L) on the activities of ACP (a), AKP (a), AChE (b), POD (b), and Na^+/K^+ATPase (c) in hepatopancreas of *M. rosenbergii* 4 cm long

Difference from control values *P<0.05, **P<0.01. Data expressed as Mean±SE, n=3.

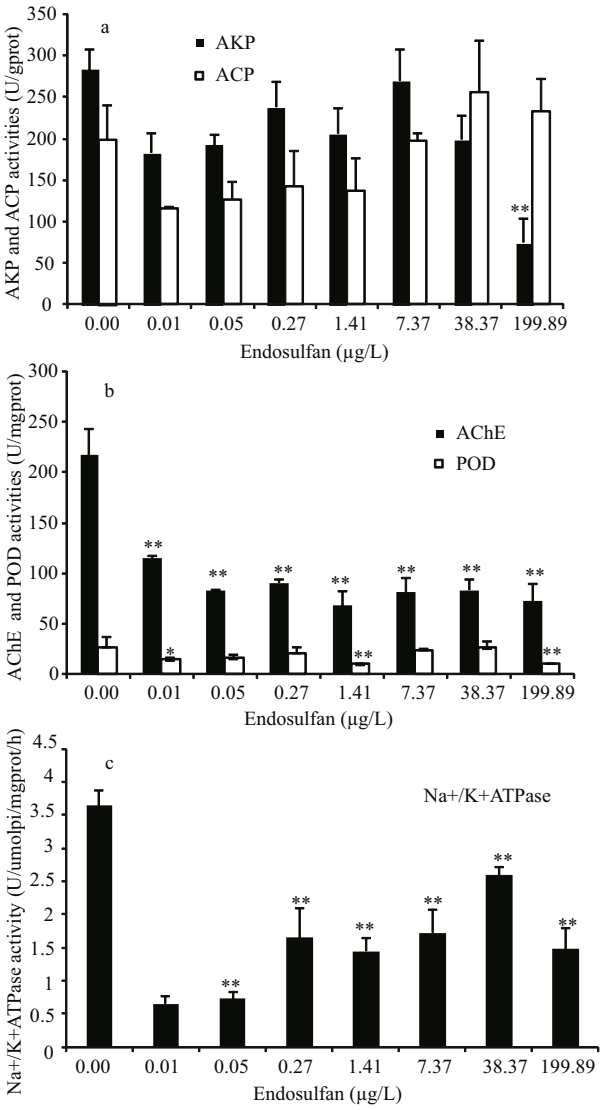

Fig.3 Effect of 96 h exposure to various endosulfan concentration (μg/L) on the activities of ACP (a), AKP (a), AChE (b), POD (b), and Na^+/K^+ATPase (c) in hepatopancreas of *M.rosenbergii* 7 cm long

Difference from control values *P<0.05, **P<0.01. Data expressed as Mean±SE, n=3.

hepatopancreas of test prawns 7 cm long (regardless of concentration), with an extreme reduction being observed in response to treatment 199.89 μg/L (P<0.01). ACP activities in the same tissues of test prawns exposed to endosulfan at 0.01–7.37 μg/L were found to be lower than those of the control group (P>0.05) (Fig.3a).

3.3 Effect of endosulfan on POD and AChE activities of *M. rosenbergii*

Exposure to endosulfan at 0.01–0.12 μg/L resulted in decreased POD activity in the hepatopancreas of 1.5 cm long test prawn. However, POD in 1.5 cm long prawns exposed to endsouflan at 0.43–18.62 μg/L increased in a dose-dependent fashion (P<0.05) (Fig.1b). Endosulfan also caused significant inhibition of POD activity in hepatopancreas of 4-cm long test prawns (P<0.01), but this inhibition was not dose dependent (Fig.2b). POD activity in the hepatopancreas of 7 cm long test prawns were found lower in all treatments than in those of control group, POD activities exposed to endosulfan at 0.01, 1.41, and 199.89 μg/L (P<0.05) were significantly inhibited unlike the control group (Fig.3b).

Toxicity of endosulfan produced a notable inhibition of AChE activity in all test organisms (Figs.1b, 2b, and 3b). After 96 h of exposure to endouslfan at 0.01, 0.04, 0.12, 0.43, 1.51, 5.31, and 18.62 μg/L, the AChE activities in hepatopancreas of 1.5 cm long test prawn were significantly lower than in the control group (P<0.01). Moreover, the AChE activities in the hepatopancreas of 1.5 cm long prawns decreased significantly in response to treatment with endosulfan at 0.01, 0.05, 0.25, 1.22, 6.08, 30.20, and 149.97 μg/L (P<0.01). Finally, extremely large differences in AChE activities were observed in the hepatopancreas of 7 cm long prawns when the control group and groups treated with endosulfan at 0.01, 0.05, 0.27, 1.41, 7.37, 38.37, and 199.89 μg/L were compared.

3.4 Effect of endosulfan on Na^+/K^+-ATPase of *M. rosenbergii*

Endosulfan at 0.01 and 18.62 μg/L caused a significant increase in Na^+/K^+-ATPase level in the hepatopancreas (P<0.01), but did not cause a concentration-related change in the tissue of 1.5 cm long prawns (Fig.1c).

The Na^+/K^+-ATPase activities were significantly inhibited in response to endosulfan at 0.25, 1.22, and 30.20 μg/L (P<0.01) (Fig.2c). Moreover, the Na^+/K^+-ATPase activities in the hepatopancreas of 7-cm long prawns were much lower than those in control prawns, regardless of exposure concentration (P<0.01) (Fig.3c). Interestingly, exposure to endosulfan at 0.01 μg/L promoted reduction of Na^+/K^+-ATPase levels for 7 cm long prawns, but not for those of other lengths.

4 DISCUSSION

Endosulfan is toxic to fish (Bacchetta et al., 2011), as indicated by 96 h LC_{50} values of endosulfan against *Oncorhynchus mykiss* and *Channa punctatus* of 1.75 μg/L (Capkin et al., 2006) and 7.75 μg/L (Pandey et al., 2006), respectively. In the present study, the 96 h LC_{50} value of endosulfan toward *M. rosenbergii* was 1.86–6.09 μg/L, indicating that endosulfan is toxic to *M. rosenbergii*.

The *SC* for *M. rosenbergii* 1.5, 4, and 7 cm was 0.19, 0.45, and 0.61 μg/L, respectively, indicating that younger prawns are less tolerant of ensoulfan. Lombardi et al. (2001) reported a 96 h LC_{50} value for *M. rosenbergii* post-larvae exposed to endosulfan under static conditions of 0.93 μg/L, which was lower than 1.86 μg/L (prawns 1.5 cm long). The results of the present study are also consistent with those of a study conducted by DeLorenzo and De Leon (2010), who reported that *Palaemonetes pugio* larvae were more sensitiveto etofenprox than adults.

Sucahyo et al. (2008) found a 96 h LC_{50} value of endousulfan to *Caridina laevis*v Heller of 1.02 μg/L, which was lower that of 1.86 μg/L observed in the present study. These findings indicate that the tolerance of various species to endsoulfan varies. Moreover, the toxicity of endosulfan might be directly correlated with environmental factors including pH, alkalinity, turbidity and temperature (Pillai et al., 2011). For example, a pH<5 would accelerate hydrolyzation of endosulfan to endosulfan sulfate, which is more toxic (Lee et al., 2003). Natarajan et al. (1992) reported that the 96 h LC_{50} of *M. rosenbergii* 4 cm long in high alkalinity media was 6.0 μg/L, which was higher than the value of 4.53 μg/L obtained in the present study. Moreover, McLeese and Metcalfe (1980) found that the 96 h LC_{50} of endosulfan toward *Crangon septemspinosa* (4–5 cm long) increased from 0.2 μg/L in seawater to 6.9 μg/L in sediment.

The lowest 96 h LC_{50} value of endosulfan toward *M. rosenbergii* observed in the present study was 1.86 μg/L. Endosulfan levels greater than 0.22 μg/L in aquatic systems have been reported to produce negative effects on the organisms (Mersie et al., 2003; Ballesteros et al., 2011b). Moreover, endosulfan could accumulate in the fatty tissues because of its lipophilicity, thereby posing a potential threat to human health (Fagotti et al., 2005; Ballesteros et al., 2009a). In this study, the toxicity toward *M. rosenbergii* was reflected in various enzyme activities in hepatopancreas tissue sample.

Similar to other environment contaminants, endosulfan may induce the generation of reactive oxygen species (ROS) during its metabolism (Almeida et al., 1997). Intracellular free radical levels exceeding the capacity of antioxidant defense result in altered cell structure and function (Bagchi et al., 1992). Peroxidase (POD) is an antioxidant enzymes that could scavenges excessive ROS (Ahmad et al., 2000; Perez and Maureira, 2003; Agrahari and Gopal, 2008). In the present study, POD activities in the hepatopancreas were inhibited because of the toxicity of endosulfan. These findings indicate that endosulfan exposure caused oxygen stress, which might have caused damage to antioxidant defense, similar to the results reported by Zhong et al. (2011). Moreover, the result of the present study showed that POD activity

in aquatic organisms was sensitive to endosulfan levels as low as 0.01 μg/L. Therefore, POD activity might be useful as an indicator of *M. rosenbergii* health following exposure to endosulfan.

Alkaline phosphate (AKP) and acid phosphatase (ACP) are polyfunctional enzymes (Bernet et al., 2001). AKP participates directly in the mineralization of skeleton tissues (Atli and Canli, 2007a); while ACP is associated with autolytic degradation of dead cells (Bhavan et al., 2001). The highest ACP and AKP values were observed in 4 cm long prawns, while the lowest observed in 1.5 cm prawns. This is likely a result of increased production of phosophatase for consumption during the growth stage, and the same is the ability of nonspecific immune mechanism of 1.5, 4, and 7 cm long prawns, resulting in different change on phosophatase production in the hepatopancreas of 1.5, 4, and 7 cm long prawns even they all exposed to endosulfan 0.01 μg/L.

In this study, concentrations of AKP in the hepatopancreas of 7 cm long prawns exposed to various endosulfan concentrations were lower than those of the control. These findings were likely a result of oxygen stress, indicating that the capacity for transphosphorylation was impaired, and resulting in degeneration alteration and hypofunction of hepatocytes (Borges et al., 2007). A similar reeducation in AKP activity also appeared when *Salmo trutta* were exposed to sewage conditions (Bernet et al., 2001). Moreover, a notable decrease in AKP in the hepatopancreas of 7 cm long prawns occurred in response to treatment with endosulfan 38.37 μg/L, indicating the exposure limit for proper nonspecific immune regulation.

Na^+/K^+-ATPase is a special class of carrying enzymes found in animal cell membranes, plays a vital role in the transport of glucose, amino acids and other macromolecules, as well as in regulation of transmembrane transportation of ions, and provides balance within the cell (Agrahari and Gopal, 2008). When an organism subjected to stress from environmental contaminants, a change would occur in ATP content, causing damage to the energy supply and ion balance, as well as alteration of ATPase. Many studies have shown that environmental contaminants such as pesticides, heavy metals, fuel and industrial waste water may inhibit Na^+/K^+-ATPase activity (Bianchini and Castilho, 1999).

The results of the present study reveal significant inhibition of Na^+/K^+-ATPase following exposure to endosulfan at 0.01 μg/L in 7 cm long prawns when compared with 1.5 cm and 4 cm long prawns. These findings may indicate that the ability to tolerate endosulfan improves as size increases. As endosulfan increased to a certain level, more Na^+/K^+-ATPase was produced to achieve the transmembrane transport of substances and defend against endosulfan toxicity (Atli and Canli, 2011b), as reflected by the Na^+/K^+-ATPase activities of 7 cm long prawns being higher in response to exposure to endosulfan at 0.27–38.37 μg/L than 0.05 μg/L. However, it likely resulted in too much endosulfan entering the prawns, leading to imbalanced intercellular ions, as well as damage to the cell structure and synthesis of protein, and causing decreases in Na^+/K^+-ATPase levels (Suvetha et al., 2010). Similarly, the decreased Na^+/K^+-ATPase activities in response to treatment with 149.97 μg/L and 199.89 μg/L endosulfan indicated that the concentrations had already exceeded the maximum tolerable range for *M. rosenbergii* 4 cm and 7 cm in length, respectively.

It is well known that inhibition of AChE (Dutta and Arends, 2003) contributes to overstimulation of neurotransmission, which results in depression, paralysis and even death (Arufea et al., 2007; Ballesteros et al., 2009a). Kumar et al. (2010) reported that AChE inhibition exceeding 20%, indicating toxicity due to environmental contamination, and that inhibition exceeding 50% would harm normal survival. In this study, the inhibition of AChE ranged from 39.3% to 72.3%, which is likely responsible for the abnormal behavior and mortality that was observed.

Swimming ability can be used as a potential indicator to physiological stress, and its strength is closely related to the ecological environment (Rosenshield et al., 1999). In this study, the incidences of abnormal swimming such as body twisting, body roll, lethargy, erratic swimming, and convulsion increased with exposure concentration. Similar behavioral responses have been observed in amphibians exposed to various concentrations of endosulfan (Brunelli et al., 2009).

Biological behavior is positively related to the nervous system, indicating that endosulfan impairs neural signal transmission. Neurotoxicity in fish is induced by endosulfan at 0.2–1 μg/L, which also results in reduced AChE activity in the brain (Giusi et al., 2005). Similar phenomena were observed in the present study. Toxicity of endosulfan to aquatic organisms is known to be caused by antagonistic action with γ-amino butyric acid (GABA), resulting

in inhibition of the aggregation of GABA receptors (Werner, 1994). Moreover, the synthesis, decomposition, release, and absorption of neurotransmitter hormones of the brain are affected by short-term exposure to endosulfan. GABA is an inhibitory neurotransmitter, and endosulfan neurotoxicity can be induced by its receptor (Kalmijima and Casida, 2000; Key et al., 2003).

Overall, the results of this study indicate that endosulfan is toxic to *M. rosenbergii* and that tolerance of *M. rosenbergii* to endosulfan was improved by individual growth. Moreover, changes of various enzymes in hepatopancreas were exhibited the status of prawn in acute energy crises, which could be applied in establishment of early warming indicator for bio-safety.

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