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Biochemical Response of the Cyclopoida Copepod *Apocyclops borneoensis* Exposed to Nickel

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Abstract

The use of biomarkers to evaluate the biological effects of pollutants in marine organisms represents a recent tool in biomontoring programs. The cyclopoida copepod *Apocyclops borneoensis* was exposed to different Ni treatments [0 (control), 10, 100, 1000 μg l^{-1}Ni] for 1, 2, 4, 7, 14 days. At each exposure time, acetylchlinesterase (AChE), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione (GSH) were analyzed. Additionally, lipid peroxidation (LPO) level was measured after a 14-day exposure. The results show that Ni treatment significantly stimulated copepod's antioxidants SOD, GST and GSH at environmentally relevant concentrations after a certain exposure time. On the other hand, the exposure time significantly affected SOD and GSH. In contrast, Ni exposure significantly decreased LPO level, implying that the factor involved in LPO might not significantly depend on the operations and functions in the antioxidant defense system. In addition, Ni might also be a neurotoxic agent to copepods via changing AChE activity.

Keywords: Nickel; *Apocyclops borneoensis*; Biochemical Response; Oxidative Stress; Neurotoxiciy.

1. Introduction

Nickel (Ni) is a metallic element that is ubiquitously present in the environment. Ni is released into the marine environment from the discharge of metal industries, mining, refining, power plants, waste incinerators, and direct leaching from rocks and soil (Fishbein, 1981; Denkhaus and Salnikow, 2002). Meanwhile, Ni also is present in crude oils and, in the event of an oil spill, is released into the marine environment (Sadiq, 1989). Ni concentration in estuaries and streams generally ranges from 1 to 75 μg L^{-1} (Eisler, 1998) and could reach as high as 500 to 2000 μg L^{-1} in natural waters near industrial sites (Chau and Kulikovsky-Cordeiro, 1995).

The metabolism of a great variety of pollutants, including metals, can enhance reactive oxygen species (ROS) production (Regoli *et al*., 2002; Limón-Pacheco and Gonsebatt, 2009). At high concentrations, ROS can be important mediators of damage to cell structures, including lipids, membranes, proteins and nucleic acids and often leading to oxidative damage in organisms (Poli *et al*., 2004; Valko *et al*., 2006). The biological defense mechanisms against ROS include enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), and glutathione peroxidases (GPx), and non-enzymatic antioxidants, such as glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene, and urate (de Zwart *et al*., 1999; Valko *et al*., 2006). Higher Ni concentrations can generate ROS, subsequently it can induce oxidative stress in organisms and alter the cellular antioxidant defense system (Denkhaus and Salnikow, 2002; Kasprzak *et al*., 2003). Measurement of antioxidants response in aquatic organisms has been used as a biomarker of heavy metals effect (Paris-Palacios *et al*., 2000; Elumalai *et al*., 2002; Brown *et al*., 2004; Cunha *et al*., 2007; Elumalai *et al*., 2007).

Acetylchlinesterase (AChE) is a key enzyme in the nervous and sensory systems in most species. AChE terminates the transmission of neural impulses by the rapid hydrolysis of acetylchline (ACh) into the inactive products of choline and acetic acid (Barnard, 1974). Previous studies demonstrated that heavy metals may be neurotoxic agents to aquatic organisms, via affecting their AChE activity (Forget *et al*., 1999; Tsangaris *et al*., 2007; Pretto *et al*., 2009).

Copepods are widely distributed and are key secondary producers in the ocean (Zhong *et al*., 1989). They play an important ecological role in aquatic ecosystems, because of their position in the trophic chain (i.e., essential link between the phytoplankton and the higher trophic levels), their rapid turnover (Runge, 1988), and their role in ocean biogeochemical cycles (Wang and Fisher, 1998). As they are the essential link between the primary producer, phytoplankton, and other organisms of higher trophic

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levels, they also play significant roles in transportation of aquatic pollutants across the food chain (Raisuddin *et al*., 2007). Although several studies have highlighted the effect of heavy metals on survival, growth, development, reproductive performance and the community structure of copepods (Hook and Fisher, 2001; Lee and Correa, 2005; Kwok *et al*., 2008; Lee *et al*., 2008; Mohammed *et al*., 2010), few studies have been carried out on the biochemical response of copepods to heavy metals exposure (Barka *et al*., 2001; Wang and Wang, 2009, 2010). Using the cyclopoida copepod *Apocyclops borneoensis*, the present study primarily aims at assessing the biochemical response to sublethal Ni treatments in a long exposure time, via measurement of AChE, SOD, GST; GSH, and LPO as biomarkers.

2. Materials and Methods

2.1. Copepod Collection and Maintenance

In most cases, the species belong to the genus *Apocyclops* are dominant in coastal brackish waters (Støttrup, 2006). *Apocyclops borneoensis* was collected using 64 μm mesh size plankton net from Xiamen bay, PR. China. Since collection, copepods had been maintained at 28 to 31° C and 18 to 22 ppt salinity in continuous stock cultures in our laboratory under static-renewal conditions and under 12D: 12L photoperiod cycle. The culture water was 0.45 μm Millipore filtered seawater (cellulose filter paper), with 7 to 7.9 mg L^{-1} dissolve oxygen and a pH ranging from 7.90 to 8.25. Copepods were fed a mixed algal diet of *Isochrysis galbana* and *Platymonas subcordiformis*. The algae were cultured in filtered seawater contain f/2 enriched media at 20°C.

2.2. Test Solutions

Nickel was provided as a chloride salt ($NiCl_2 .6H_2O$) from Guang Fu Chemical Institute, China (≥ 98.0% pure). Stock solutions were prepared in double distilled water. The stock solutions were subsequently diluted in different volumes of 0.45 μm filtered seawater to create various Ni nominal test concentrations (0, 10, 100, 1000 μg L^{-1}).

2.3. Exposure

Adult copepods were exposed to Ni test concentrations for 1, 2, 4, 7, 14 days. The experimental salinity and temperature were 20 ppt and 30°C, respectively. The experiments were carried out as semi-static (renewal) tests, with daily renewal of half of the exposure solution, each treatment with 2-L exposure solution. After the daily renewal of the test solutions, copepods were fed with *Isochrysis galbana* at a density of 5×10^5 cell mL^{-1} during 14 days exposure. At the end of each exposure time, about 1000 adult copepods of mixed gender were collected from each treatment and immediately stored at -80°C.

2.4. Biochemical Analysis

In order to determine biochemical parameters, samples were homogenized by digital sonifier cell disrupter (model 450, Branson, USA) for 2 min with 20 mmol/L Tris-buffer (pH 7.6, containing 1 mmol/L EDTA, 0.25 mol/L sucrose, 0.15 mmol/L NaCl and 1 mmol/L dithiothreitol) at 4°C. The homogenate was centrifuged at 15,000g for 20 min at 4°C, and the supernatant was used for the biochemical analysis. Protein determination was performed using the method of Bradford (1976) with bovine serum albumim as a standard.

The activity of GST, SOD, and AChE, and the level of GSH and LPO (as MDA) were measured spectrophotometrically with the test kits supplied by Jian-Cheng Bio-engineering Institute of China. The test kits were made of reagents from Sigma-Aldrich Co.

The content of GSH was determined based on spectrophotometric method of Rahman *et al*. (2006). DTNB (5,5-dithiobis-2-nitrobenzoic acid) was developed for the detection of thiol compounds. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid (TNB) and glutathione disulfide (GSSG). The rate of formation of TNB is proportional to the concentration of GSH in the sample. The content of GSH was expressed as micrograms per milligram of protein.

The GST activity was determined according to Habig *et al*. (1974), by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH. The rate of GSH content decrease is directly proportional to the GST activity in the sample. One unit of GST is defined as the amount of enzyme that will reduce 1μmol/L GSH in the reaction system at 37° C per minute in 1 mg protein.

The SOD activity was determined based on its ability to inhibit the reduction of cytochrome-*c* by the O_2^- generated in the xanthine oxidase/hypoxanthine system (McCord and Fridovich, 1969). One unit of SOD will inhibit the rate of oxidation of hydroxylamine by 50% in a coupled system, using xanthine and xanthine oxidase at 37°C in 1.0 mg/ml protein concentration of tissue homogenate.

The lipid peroxidation (LPO) level was assessed by the thiobarbituric acid (TBA) assay. The measurement of thiobarbituric reactive species (TBARS) quantified as MDA equivalents was based on the method previously described by Ohkawa *et al*. (1979). MDA is formed as an end product of lipid peroxidation which reacts with TBA to generate a colored product that measured spectrophotometrically. The amount of MDA formed was expressed as nmol MDA per mg of protein.

The AChE activity was spectrophotometrically determined according to Ellman method (Ellman *et al*., 1961). Acetycholine is hydrolyzed by acetylchlinesterase producing acetic acid and thiocholine. Thiocholine reacts with the Ellman reagent DNTB (5,5-dithiobis-2-nitrobenzoic acid) to produce the anion of 5-thio-2-nitrobenzoic acid (TNB) and the increase of its absorption indicates AChE enzyme activity. One unit of AChE catalytic activity is defined as the amount of enzyme that will cause decompose of 1 μmol acetycholine per 6 minutes at 37° C in 1.0 mg protein of tissue homogenate.

2.5. Statistical Analysis

Two statistical programs (Microsoft excel 2003 package; SPSS 17.0, Chicago, IL, USA) were used to analyze the data. All biochemical measurements were replicated at least three times. The data were expressed as mean values ± standard deviation (S.D). Prior to any statistical analysis, data were log-transformed to meet ANOVA assumption of normality and variance homoscedasticity. The statistical analysis was carried out using one-way ANOVA and Fisher's least significant

difference (LSD) test to evaluate whether the means were significantly different among Ni treatments at particular exposure time. Significant differences were indicated at $p< 0.05$.

A two-way factorial ANOVA was used to evaluate whether Ni treatments and exposure time used had significant effects on the biochemical parameters tested (significant at $p< 0.05$).

3. Results

3.1. Effect Of Ni Exposure on Various Biochemical Parameters in The Copepod A. Borneoensis

The GSH level was significantly (one-way ANOVA, $p< 0.05$) stimulated by day 1 under the highest Ni treatment (1000 µg L^{-1}), and this stimulation reached its peak at day 2, and then was significantly decreased with the increase of exposure time (figure 1A, $p< 0.05$). For 10 and 100 µg L^{-1} Ni treatments, the level of GSH was significantly stimulated by day 7 with a significantly higher level under100 µg L^{-1} than 10 µg L^{-1} and then was significantly decreased at day 14 for both treatments (fig. 1A, $p< 0.05$).

For all Ni treatments, the GST activity was not significantly different from control at 1, 2, 4 days; however, it increased by day 7 with the increase of Ni concentration, except the highest concentration, and was significantly stimulated at 100 µg L^{-1} Ni (fig. 1B, one-way ANOVA, $p< 0.05$). The highest Ni treatment (1000 µg L^{-1}) had a noticeably inhibition effect on GST activity at day 14, but not other treatments ($p< 0.05$).

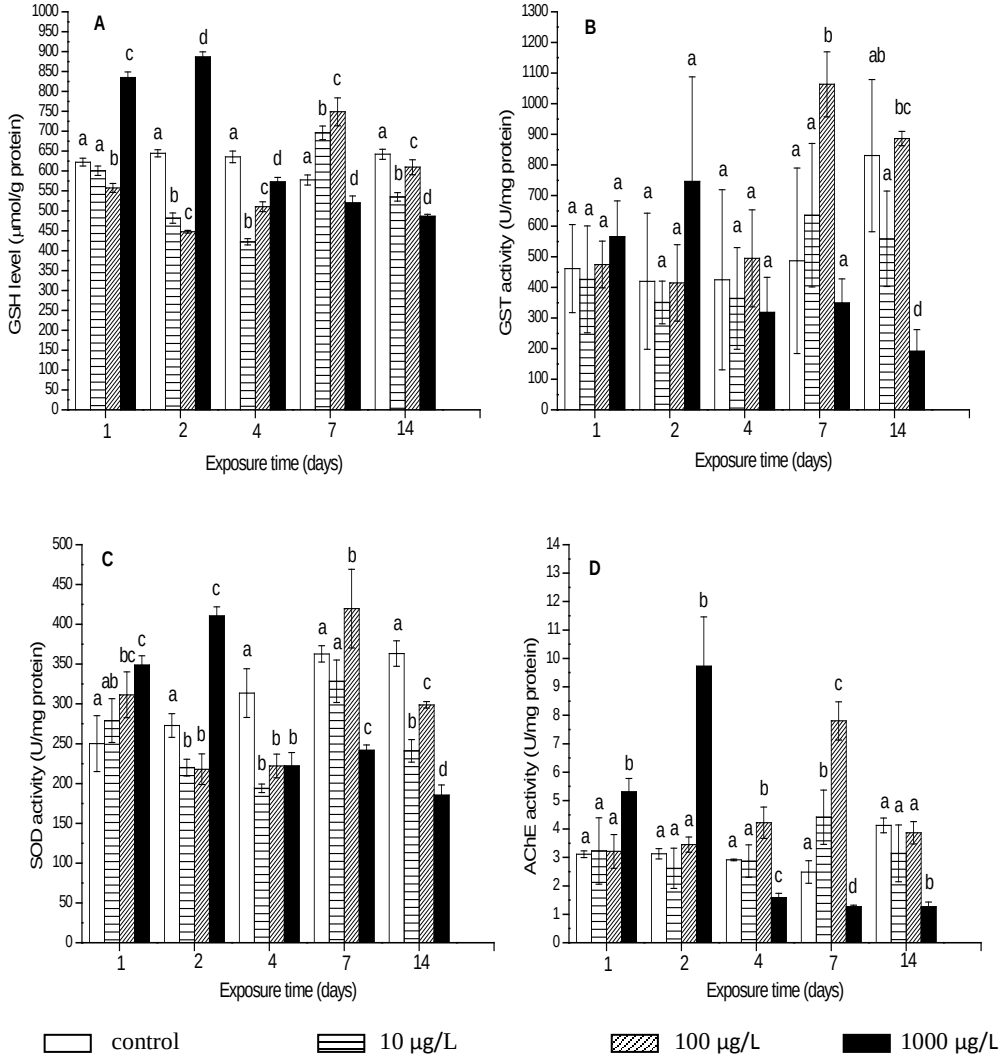

Figure 1. Effect of Nickel on various biochemical parameters (**A**) GSH level (**B**) GST activity (**C**) SOD activity (**D**) AChE activity in *Apocyclops borneoensis*. Data are described as mean ± standard deviation. Different letters indicate a significant difference among different Ni treatments at $p < 0.05$.

Figure 1C shows the response of copepod's SOD activity to different Ni treatments. By day 1, both 100 µg L^{-1} and 1000 µg L^{-1} Ni treatments were significantly stimulated copepod's SOD activity, but not the lowest Ni treatment which showed a little effect (Figure 1C, $p< 0.05$). The highest Ni treatment 1000 µg L^{-1} reached its peak stimulation of SOD activity by day 2, and then was significantly decreased at 4, 7, 14 days ($p< 0.05$). Both 10 µg L^{-1} and 100 µg L^{-1} Ni treatments significantly decreased the SOD activity at days 2 and 4; however, by day 7, 100 µg L^{-1} Ni treatment significantly stimulated its activity but not 10 µg L^{-1} ($p< 0.05$). The inhibition effect of all Ni treatments on copepod's SOD activity was shown by day 14.

In the case of copepod's AChE activity, the highest Ni treatment (1000 µg L^{-1}) displayed a significant stimulated effect at day 1 and attained its peak by day 2, and then was significantly decreased with the increase of exposure time (Figure 1D, $p< 0.05$). The other Ni treatments had insignificant effect on AChE activity at 1, 2, 4 days; however, by day 7 they significantly stimulated its activity (Figure 1D, $p< 0.05$). The highest Ni treatment (1000 µg L^{-1}) had a noticeably inhibition effect on copepod's AChE activity at days 4, 7 and 14, but not other treatments ($p< 0.05$).

The influence of Ni on copepod's LPO was measured as MDA level at day 14. Figure 2 shows that the copepod's MDA level significantly decreased with the increase of Ni concentration ($p< 0.05$). There was no significant difference in MDA level between 10 µg l^{-1} and 100 µg l^{-1} Ni treatments.

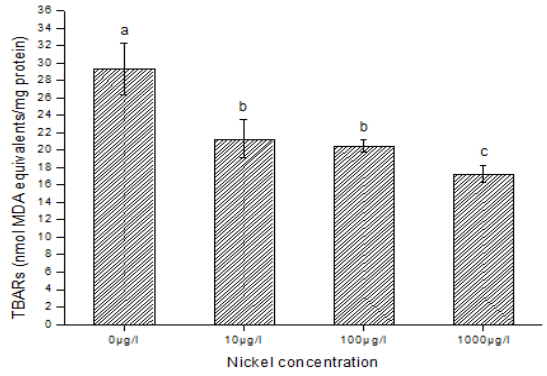

Figure 2. Effect of Nickel on LPO measured as MDA level in *Apocyclops borneoensis* exposed for 14 days. Data are described as mean ± standard deviation. Different letters indicate a significant difference among different Ni treatments at $p < 0.05$.

3.2. Effect of Ni Exposure Time and Treatment on Biochemical Responses in The Copepod A. Borneoensis

The two-way ANOVA statistical analysis of the effect of Ni treatment and exposure time (table 1) confirmed the significant influence of Ni treatment on the biochemical parameters GSH ($p<0.001$), SOD ($p<0.001$), AChE ($P<0.001$) and GST ($p< 0.05$). Also, Ni exposure time significantly ($p<0.001$) influenced GSH, SOD, and AChE; however, its influence on GST was insignificant ($p>0.05$). Moreover, the two-way ANOVA showed a significant interaction between the effect of Ni treatment and exposure time on the copepod biochemical parameters GSH ($p<0.001$), SOD ($p<0.001$), AChE ($p<0.001$) and GST ($p< 0.05$).

Table 1. Effect of Ni treatment and exposure time on biochemical parameters in *Apocyclops borneoensis*

	***P*-value**			
	GSH	GST	SOD	AchE
Effect of Ni treatment	<0.001*	0.024*	<0.001*	<0.001*
Effect of exposure time	<0.001*	0.142	<0.001*	<0.001*
Treatment × time	<0.001*	0.005*	<0.001*	<0.001*

Note: * indicates a significant effect, two-way ANOVA ($P< 0.05$)

4. Discussion

Exposure to some xenobiotics, especially toxic chemical pollutants, plays an important role in the mechanistic aspects of oxidative damage. Such a diverse array of pollutants stimulates a variety of toxicity mechanism, such as oxidative damage to membrane lipids, proteins, and DNA and changes to antioxidant enzymes activity and non-enzymatic antioxidant content (Poli *et al*., 2004; Valko *et al*., 2006; Valko *et al*., 2007). Living organisms have the ability to synthesize and control specific enzymatic systems, which can be used for repairing and removing of the damaged proteins, lipids, and DNA. Also, since oxidative stress levels may vary from time to time, organisms are able to adapt to such fluctuating stresses by inducing additional synthesis of enzymatic and non-enzymatic antioxidants to regulate oxidative damage (Valavanidis *et al*., 2006). Thus, the balance between prooxidant endogenous and exogenous factors (pollutants) and the antioxidant defenses (enzymatic and non-enzymatic) in the biological systems can be used to assess toxic effects of chemical pollutants on living organisms. With respect to other aquatic organisms, few studies have been carried out on the biochemical response of copepods to heavy metals. The major findings of the present study were that Ni treatment significantly stimulated some of the measured biochemical parameters in the cyclopoida *A. borneoensis* after certain exposure time.

GSH is the main intracellular thiol antioxidant and has a key role in the detoxification process of pollutants, not only as a substrate of antioxidant enzymes, but also as a direct reducing agent and a nucleophile able to block the toxicity of heavy metals and organic chemicals with thiol affinity by covalent binding (Vasseur and Leguille, 2004). It has an important role in scavenging of cellular ROS (such as H_2O_2, $O_2^{\bullet -}$ and $^{\bullet}OH$). A variety of environmental pollutants are known to change the GSH level in aquatic organisms, including heavy metals (Canesi *et al*, 1999; Wang *et al*., 2008). Our results show that the copepod's GSH level was significantly ($p< 0.05$), increasing by day 1 under the highest Ni treatment used (1000 µg L^{-1}), and by day 7 under the lowest Ni treatments (10 µg L^{-1} and 100 µg L^{-1}), and then significantly decreased by day 14 for all Ni treatments (figure 1A, $p< 0.05$). The induction of GSH in the tissue of the copepod *A. borneoensis* exposed to Ni was probably due to a primary defense system in which the GSH is involved to protect copepod from oxidative stress. Similarly, an increase in GSH level has been reported in different tissues of aquatic animals exposed to heavy metals (Schlenk and Rice, 1998; Zaroogian and Norwood, 2002; Atli and Canli,

2008). However, Wang and Wang (2009; 2010) demonstrated that heavy metals (Cd and Ni) cause a decrease in the GSH level of the benthic copepod *Tigriopus japonicus* in early exposure time (1 day) and an increase in its level in late exposure time. The decrease of GSH level in early exposure time is due to the GSH's ability to become the first defense line to heavy metal attack. Our results show that Ni significantly inhibited GSH content at the end of exposure time for all Ni treatments. This inhibition might be explained by Ni accumulation and the reduction of the cellular ability to eliminate ROS, so that it raises the general oxidative stress potential in the cells.

Among phase II enzymes, the expression of GST is a family of multifunctional enzymes related to cellular antioxidant defenses. GST conjugates different electrophilic toxic chemicals with GSH during phase II of biotransformation reactions, enhancing the polarity of these chemicals by neutralizing their active electrophilic sites and subsequently making the toxic chemical compound more water soluble in order to enable their excretion (Sáenz *et al*., 2010). The present study show that Ni treatments increased GST activity at day 7, and was significantly different from control under 100 μg L^{-1} Ni treatment (figure 1B, $p< 0.05$). However, the highest Ni treatment (1000 μg L^{-1}) had a noticeable inhibition effect on GST activity at day 14 ($p< 0.05$). In agreement with our results, Wang and Wang (2009; 2010) found that heavy metals (Cd and Ni) significantly increased GST activity of the benthic copepod *Tigriopus japonicus* after 7 days exposure. Additionally, previous studies showed a significant change of GST activity in other marine invertebrates exposed to heavy metals (Canesi *et al*., 1999; Cunha *et al*., 2007; Attig *et al*., 2010). Canesi *et al*. (1999) reported that Cu and Hg significantly stimulated GST activity in gill and digestive gland of mussel *Mytilus galloprovincialis*. Also Attig *et al*. (2010) observed an increase of the digestive gland GST activity of mussel *M. galloprovincialis* after being exposed to Ni. However, Cunha *et al*. (2007) found that Cu caused a significant reduction of marine gastropod *Nucella lapillus* GST activity but had no effect on *Monodonta lineate* GST, while Cd had no significant effects on the GST activity of either species. Therefore, GST biomarker response might be dependent on species, type of metal, metal concentrations and experimental time. The mechanism of impact of Ni on this enzyme is still unknown and needs more investigation.

The cellular defense system against metal toxicity includes induction of SOD, which catalyses scavenging of superoxide anion radicals ($O_2^{\bullet -}$) to hydrogen peroxide H_2O_2 (Limón-Pacheco and Gonsebatt, 2009). Our study reveals that Ni increased copepod's SOD activity by day 1; this induction was significantly different from control under 100 μg L^{-1} and 1000 μg L^{-1} Ni treatments (fig. 1C, $p< 0.05$). The inhibition effect of all Ni treatments on the copepod's SOD activity was shown by day 14. The induction of SOD by day 1 might be to eliminate ROS caused by Ni, and so counteract oxidative stress. Thus, SOD might act as the first defense line against ROS attraction in the copepod *A. borneoensis* exposed to Ni, dismutating superoxide to H_2O_2. H_2O_2 is then subsequently detoxified by several enzymes such as, CAT and GPx (Limón-Pacheco and Gonsebatt, 2009). Similarly, Wang and Wang (2009; 2010) reported an increase of SOD activity in the benthic copepod *T. japonicus* after being exposed to Cd and Ni. Inhibition of the copepod's SOD activity at the end of exposure time (14 days) might be associated with Ni accumulation, accumulated H_2O_2 and the oxidative damage potential in the copepod cells. Significant inhibition of SOD activity was reported in aquatic animals exposed to heavy metals (Chandran *et al*., 2005; Vutukuru *et al*., 2006).

Heavy metals may alter the structure of cell membranes by stimulating the lipid peroxidation process, a complex sequence of biochemical reactions, broadly defined as oxidative deterioration of unsaturated fatty acids. Lipid peroxidation results in the production of lipid radicals and in the formation of a complex mixture of lipid degradation or secondary products (malondialdehyde and other aldehydes such as formaldehyde, acetaldehyde, etc.), which are extremely toxic to cells due to their high affinity to thiol and amino groups of peptides, enzymes, and nucleic acids (Roméo and Gnassia-Barelli, 1997; Benedet and Shibamoto, 2008). Malondialdehyde (MDA), which is a major cytotoxic product of lipid peroxidation, acts as oxidative stress biomarker of heavy metal exposure in aquatic animals (Roméo and Gnassia-Barelli, 1997; Jena *et al*., 2009; Wang and Wang, 2009; Attig *et al*., 2010). Unexpectedly, *A. borneoensis* LPO measured as MDA level significantly decreased with the increase of Ni concentration at the end of exposure time (14 days) (fig. 2, $p< 0.05$). Taking into account that Ni treatment significantly inhibited GSH level, GST and SOD activity at day 14, it is quite probable that the copepod cells suffered from oxidative stress or even oxidative damage, which might lead to mutagenic and carcinogenic effects to the copepod, since MDA is a reactive electrophile reacts with protein and DNA (Marnett, 1999; Nair *et al*., 2007). Similarly, Wang and Wang (2010) found that Ni treatment strictly prohibited LOP level in the copepod *T. japonicus* after 12 days of exposure.

Previous studies demonstrated that heavy metals may be neurotoxic agents to copepods, via affecting AChE activity (Forget *et al*., 1999; Wang and Wang, 2009; 2010). *A. borneoensis* AChE activity was not significantly affected after being exposed to lowest Ni treatments (10 and 100 μg L^{-1}) for 1, 2, 4 days except for 100 μg L^{-1} Ni treatment of day 4; however, by day 7, they significantly stimulated its activity (fig. 1D, $p< 0.05$). The highest Ni treatment (1000 μg L^{-1}) significantly stimulated the copepod's AChE activity at day 1 and reach its peak by day 2, and then had a noticeable inhibition effect with the increase of exposure time. Thus, Ni might cause neurotoxicity to the copepod by stimulating AChE activity during certain exposure time. In agreement with our results, Cd and Ni significantly increased AChE activity in the copepod *T. japonicus* after a certain exposure time (Wang and Wang, 2009; 2010). However, no significant change is found on AChE activity in the copepod *T. brevicornis* after a 96 h exposure to LC_{50} concentration of Cd, but similar exposure to As or Cu exerts an inhibitory effect (Forget *et al*., 1999). Taken together, exposure of copepods to heavy metals causes different responses of AChE activity. Thus, AChE biomarker response might

vary with copepod species, type of metal and metal concentration.

5. Conclusion

Biological markers or biomarkers have been used to diagnose environmental contamination and to assess its effects on living organisms. Particularly, copepods are considered sensitive indicators of metal toxicity, being employed ecotoxicological studies both in the laboratory and in the field (Raisuddin *et al*., 2007). The present study investigated the biochemical response of the cyclopoida copepod *A. borneoensis* following exposure to sublethal Ni treatments. In conclusion, our results showed that Ni treatment had a significant effect on the copepod's AChE, SOD and GST activity and GSH level at environmentally relevant concentrations after a certain exposure time. Thus, changes in SOD, GST and GSH might be detoxificative defense of copepods against Ni addition. In addition, Ni might also be a neurotoxic agent to copepods via changing AChE activity. In contrast Ni exposure significantly decreased the LPO level of copepods compared to control, implying that the factor involved in LPO might not significantly depend on the operations and functions in the antioxidant defense system. Thus, we suggest that AChE, SOD and GST activities and GSH level be used as suitable biomarkers for Ni pollution.

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