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Oxidative damage effects in the copepod *Tigriopus japonicus* Mori experimentally exposed to nickel

Minghua Wang · Guizhong Wang

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Abstract *Tigriopus japonicus* Mori has been recognized as a good model for toxicological testing of marine pollutants. Recently, a large number of genes have been identified from this copepod, and their mRNA expression has been studied independently against exposure to marine pollutants; however, biochemical-response information is relatively scarce. The response of *T. japonicus* to nickel (Ni) additions was examined under laboratory-controlled conditions in 12 days exposure. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), acetylcholinesterase (AchE), reduced glutathione (GSH), the ratio of reduced to oxidized glutathione (GSH/GSSG) and metallothionein (MT) were analyzed for Ni treatments (0, 0.125, 0.25, 0.75 and 3.0 mg/L) after 1, 4, 7 and 12 days. The thiobarbituric reactive species assay was used to evaluate lipid peroxidation (LPO) level in copepods after exposure. The results showed that Ni remarkably affected the biochemical parameters (SOD, GPx, GST, GSH, and GSH/GSSG) after certain exposure durations. However, the copepod's LPO level was significantly decreased under metal treatments after exposure, hinting that the factors involved in LPO might not significantly depend on the operations and functions in the antioxidant system. Ni exhibited the neurotoxicity to copepods, because its use obviously elevated AchE activity. During exposure, Ni initially displayed an inhibition effect but induced MT synthesis in *T. japonicus* by day 12, probably being responsible for metal detoxification. Thus, Ni had intervened in the detoxification process and antioxidant system of this copepod, and it could be used as a suitable bioindicator of Ni exposure via measuring SOD, GPx, GST, and MT as biomarkers.

Keywords Biomarker · Copepod · Metallothionein · Nickel · *Tigriopus japonicus*

M. Wang · G. Wang (✉)
College of Oceanography and Environmental Science, State Key Laboratory of Marine Environmental Science, Xiamen University, 361005 Xiamen, People's Republic of China
e-mail: gzwang@xmu.edu.cn

Introduction

Nickel (Ni) is ubiquitous in the biosphere, which is widely used in commerce and is also distributed throughout the environment. Ni levels in estuary and streams generally tend to range between 1 and 75 μg/L (Eisler 1998), but they are up to 500 and 2,000 μg/L (natural waters near industrial sites) with a maximum of 183,000 μg/L near a Ni refinery in Sudbury, Ontario (Chau and Kulikovsky-Cordeiro 1995). Humans are exposed to Ni via food, water and air, since it is produced from sources such as mining, extraction, refining, electroplating, food processing and Ni waste disposal (Nielsen et al. 2001). Small amounts of Ni are essential for normal growth and reproduction in some animal species, meaning that Ni can also be regarded as a trace element (Woo et al. 2009). However, Ni is toxic at elevated concentrations, with its compounds being nephrotoxic, hepatotoxic, immunotoxic, and teratogenic (Mas et al. 1985; Misra et al. 1991; Vyskocil et al. 1994; Pari and Prasath 2008; Vijayavel et al. 2009), with respect that Ni has known multisystem impacts on human health following its exposure, and major target organs include liver, kidney, brain, lung, and testes (Pari and Prasath 2008). Actually, Ni has been classified as a human carcinogen on the basis of epidemiological studies showing a higher incidence of nasal and lung cancers in occupationally exposed workers (Coogan et al. 1989; Haber et al. 2000).

Ni can generate reactive oxygen species (ROS) (Prophete et al. 2006; Gopal et al. 2009; Vijayavel et al. 2009), leading to an increase in lipid peroxidation (LPO) (Ptashynski et al. 2001, 2002), a loss of membrane integrity (Ptashynski et al. 2002), and alterations of the cellular antioxidant system (Gopal et al. 2009). Depletion in reduced glutathione (GSH) (Sidhu et al. 2004) and changes of antioxidant enzymes [e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-*S*-transferase (GST)] activities are also observed after exposure to Ni (Misra et al. 1990; Chakrabarti and Bai 1999; Hfaiedh et al. 2008). Although great attention has been paid to discussing the effects of Ni in mammalian systems via ROS formation (Mas et al. 1985; Misra et al. 1990; Vyskocil et al. 1994; Chakrabarti and Bai 1999; Kasprzak et al. 2003; Kodipura et al. 2004; Hfaiedh et al. 2008; Pari and Prasath 2008), there is a paucity of information regarding the oxidative toxicity of Ni in aquatic animals.

Metals that accumulate in organisms can be classified into two components—metabolically soluble and available metals and stored detoxified metals. All metals have the potential to be deleterious to cellular mechanisms, even though some of them are essential to normal metabolic processes, and their toxic effects are related to the metabolically soluble available form (Rainbow 2002, 2006). The complexation of metals by metallothionein (MT), a non-enzymatic metalloprotein, is one mechanism used by the invertebrate cell to prevent the activation of toxic metals in the cytoplasm (Viarengo et al. 1987). Thus, MT is generally regarded to play a role in the homeostatic control of essential metals (Cu, Zn), as well as being involved in the detoxification of excess amounts of both essential and non-essential trace metals (e.g., Cd, Ag, and Hg) (Amiard et al. 2006). Additionally, MT also seems to have a more general antioxidant defense in fighting against ROS attack (Correia et al. 2002a, b; Paris-Palacios et al. 2003; Falfushynska and Stolyar 2009). Nevertheless, for Ni, few data regarding the type of MT information mentioned earlier are available for aquatic invertebrates.

Acetylcholine is a primary neurotransmitter between sensory and neuromuscular systems in most species. The dysregulation of acetylcholinesterase (AchE) results in a build up or cut down of acetylcholine, interfering with the normal communication of the nerve/muscle fibers. Several studies indicate that this AchE activity could be prominently inhibited by contaminants including metals (Forget et al. 1999, 2003; Galgani and Bocquene 1990; Ozmen et al. 1998; Elumalai et al. 2002). So, measurement of AchE activity in aquatic organisms could be a potential biomarker for the effects of environmental contaminants.

The potential of using copepods as model organisms in ecotoxicology and environmental genomics has been recognized for some time (Raisuddin et al. 2007). For instance, the harpacticoid copepod *Tigriopus* is widely distributed and is an ecologically important organism (Wells 1984) that plays an important role in the transportation of energy (including aquatic contaminants) across food chains, due to its prominent position in marine food chains (Ruppert et al. 2003). In recent years, the sequences and functional characteristics of several toxicologically relevant genes (e.g., *GST*, *GR*, and *hsp20*) have been well studied using the intertidal copepod, *Trigriopus japonicus* Mori (Lee et al. 2007a; Raisuddin et al. 2007; Seo et al. 2006a, b, c). Thus, this harpacticoid copepod has been recognized as a good model for toxicological testing of marine pollutants (Ara et al. 2002; Marcial et al. 2003; Kwok and Leung 2005; Lee et al. 2007a; Raisuddin et al. 2007). Here, using the copepod *T. japonicus* as a model species, we examined its biochemical response to Ni treatments via measurement of various biochemical parameters (SOD, GPx, GST, AchE, GSH, and GSH/GSSG), the LPO level, and the MT content. To our knowledge, this is the first attempt to investigate Ni oxidative effects in the copepod, so as to explore the response mode of the copepod to Ni stress and sieve out a potential biomarker to Ni pollution in this copepod.

Materials and methods

Sampling

T. japonicus was collected in rocky intertidal zone pools in Xiamen Bay, People's Republic of China (Fig. 1). Reference seawater used in the experiments was obtained 20 km offshore in Xiamen Bay. The identity of this species was confirmed by morphological characteristics and the sequence similarity of mitochondrial genome sequences (Jung et al. 2006). All seawater used was filtered through a 0.45 μm acetate fiber membrane, with the background concentration of total Ni being 3.16 μg/L. Seawater characteristics were described as dissolved oxygen level 7.0–7.5 mg/L, salinity 29–30 psu, and pH 7.8–7.9. Prior to metal exposure, the copepods were acclimated in seawater at 22°C for 3 days and fed with *Tetraselmis suecica* at a density of 0.5×10^9 cells/L.

Experimental design

Adult copepods were exposed to Ni with 2 L testing solutions in glass cylinders. The experiments were carried out as semi-static tests, with daily renewal for half the exposure solution, at a temperature of 22°C with a 12:12 h light/dark cycle. Acid-washed vessels were pre-contaminated for 24 h before experiments, so as to keep the testing solution to the nominal level of Ni. Copepods were divided

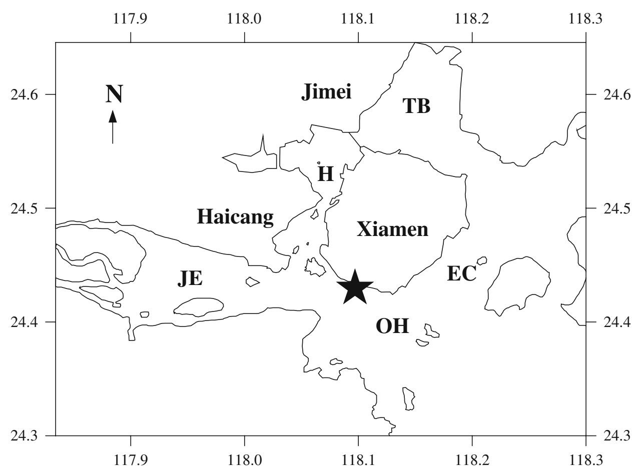

Fig. 1 *Map* of Xiamen coastal waters, Fujian Province, People's Republic of China. The *pentagram* represents the copepod sampling site. *H* Harbor, *OH* Outer Harbor, *TB* Tong'an Bay, *JE* Jiulong River Estuary, and *EC* East Channel

into five treatment groups: a seawater control and four levels of Ni^{2+} ($NiCl_2 \cdot 6H_2O$ was added to the solutions to achieve final Ni^{2+} concentrations of 0.125, 0.25, 0.75, and 3.00 mg/L). Copepods were subjected to these different Ni treatments for 1, 4, 7, and 12 days. At the end of each time exposure, ~750 copepods of mixed gender were collected from each treatment and immediately stored at −80°C. During the 12 days exposure, the copepods were fed with *T. suecica* at a density of 0.5×10^9 cells/L after the daily renewal of the testing solutions.

Biochemical assays

Metallothionein assay

For MT estimation, samples were homogenized for 5 min using a glass–glass Elvehjem-Potter homogenizer, with a solution of 0.25 M sucrose. The homogenate was centrifuged at 20,000*g* for 20 min at 4°C. Aliquots of 600 μL supernatant were analyzed for MT content using the silver-saturation method (Scheuhammer and Cherian 1991; as modified by Leung and Furness 1999). Briefly, samples were incubated with 1.0 mL glycine buffer (0.5 M, pH 8.5) and 1.0 mL of 20 mg/L silver solution for 20 min to saturate the MT metal-binding sites. Excess metal was removed by adding 0.2 mL human red blood cell hemolysate to the assay tubes followed by heat treatment in a 100°C water bath for 10 min. The heat treatment caused precipitation of Ag-bound hemoglobin and other proteins, except for heat-stable MT. The denatured proteins were removed by centrifugation at 4,000*g* for 10 min. The hemolysate addition, heat treatment, and centrifugation were repeated three times for each sample. Finally, the solution was centrifuged at 20,000*g* for 20 min. The content of Ag in the supernatant, which was proportional to the MT level, was estimated with atomic absorption spectrophotometry using a Philips PU2000 AAS with deuterium background correction. In the same way, calibration was carried out using concentrations of 2–20 μg of purified horse kidney MT (Sigma) as a standard. The protein content in samples was determined using the Bradford (1976) method with bovine serum albumin as a standard. The results were expressed as micrograms MT per milligram of protein.

Biochemical parameter determination

For biochemical assays, samples were homogenized for 5 min with 20 mM Tris-buffer (pH 7.6, containing 1 mM EDTA, 0.25 M sucrose, 0.15 mM NaCl, and 1 mM dithiothreitol) at 4°C, using a glass–glass Elvehjem-Potter homogenizer. The homogenate was centrifuged at 15,000*g* for 20 min at 4°C, and the supernatant was used for biochemical parameter determination.

The SOD activity was determined based on its ability to inhibit the reduction in cytochrome *c* by O_2^- generated in the xanthine oxidase/hypoxanthine system (McCord and Fridovich 1969). One unit of SOD was defined as the amount of sample causing 50% inhibition of cytochrome *c* reduction.

The GPx activity was measured according to the modified method of Xia and Zhu (1987). One unit of GPx activity was described as 1 nmol of GSH consumption per minute per milligram of protein at 37°C.

The GST activity was determined using 1-chloro-2,4-dinitrobenzene as the substrate, as previously described by Habig et al. (1974). One unit of GST activity was the amount of enzyme that catalyzed the formation of 1 nmol of product per min at 37°C and pH 6.5.

The measurement of AchE activity was based on the colorimetric method of Ellman et al. (1961) with acetylthiocholine iodide (AcSCh) as substrate and dithiobisnitrobenzoate as reagent at a temperature of 37°C. One unit of AchE activity was expressed as micromoles of AcSCh hydrolyzed per minute per milligram of protein.

The content of GSH and oxidized glutathione (GSSG) was determined based on spectrofluorometric assay, as previously described by Hissin and Hilf (1976) with some modifications. For GSH estimation, the assay tube contained 0.1 mL supernatant, 2.8 mL of 1 M phosphate buffer (pH 8.0), and 0.1 mL of 1 mg/mL ortho-phthalaldehyde. Then, the mixture was kept at room temperature for 20 min followed by fluorescence measurement. Calibration was carried out in the same process using 2–10 μg concentrations of GSH (Sigma) as a standard. For the GSSG assay, 0.5 mL supernatant was completely complexed with 0.2 mL of 0.04 M *N*-ethylmaleimide, and the admixture was retained at room temperature for 30 min.

Then, 0.1 mL of the mixture was thoroughly merged with 2.8 mL of 0.1 M NaOH and 0.1 mL of 1 mg/mL ortho-phthalaldehyde, followed by resting at room temperature for 20 min before the fluorescence measurement was undertaken. The standard calibration was made in the same assay using GSSG (Sigma) of concentrations 2–10 μg. The content of GSH and GSSG was expressed as micrograms per milligram of protein.

The LPO level was assessed using the thiobarbituric reactive species (TBARS) assay, which measures the production of LPO products that react with thiobarbituric acid (Ohkawa et al. 1979). Measurement of TBARS was carried out following the method of Barata et al. (2005a). TBARS concentrations were derived from an external standard curve of 1,1,3,3-tetramethoxypropane (also referred to as malonaldehyde; MDA) and the values expressed in nanomoles of MDA equivalents per milligram of protein.

Statistical tests

All measurements were replicated at least four times, and the data were expressed as mean values ± standard deviation (SD). Statistical analysis was carried out by one-way ANOVA using the Fisher's least significant difference (LSD) test to evaluate whether the means were significantly different among metal treatments at particular exposure duration. Significant difference was indicated at $P < 0.05$. Prior to any analysis, data were log-transformed to meet ANOVA assumptions of normality and variance homoscedasticity.

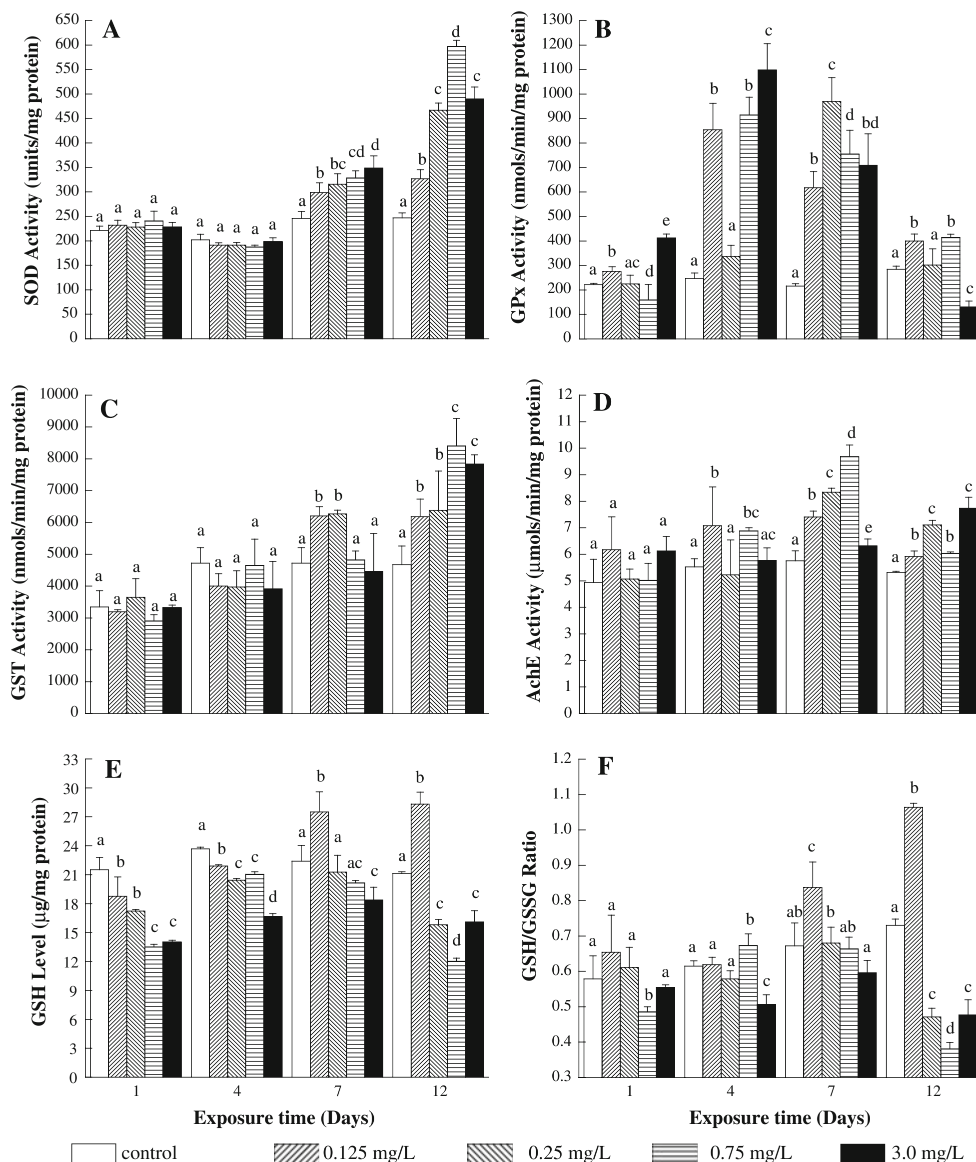

Fig. 2 Influence of Ni on biochemical parameters in *Tigriopus japonicus*: **a** superoxide dismutase (SOD), **b** glutathione peroxidase (GPx), **c** glutathione-*S*-transferase (GST), **d** acetylcholinesterase (AchE), **e** reduced glutathione (GSH), and **f** the ratio of reduced to oxidized glutathione (GSH/GSSG). Data are means ± SD ($n = 5$). Different letters indicate a significant difference among different metal treatments at $P < 0.05$

A two-way factorial ANOVA was used to determine the statistical significance of the individual biochemical variables among metal treatments and exposure durations (significant at $P < 0.5$). Correlations between variables were computed using the Pearson's test on the raw data if they passed the test of normality, or otherwise the data were transformed to fit this prerequisite. Log-transformation was used to correct the problem. The data were also subjected to a principal component analysis (PCA—significant at a factorial weight > 0.7). The PCA is generally used to reduce a set of data with a relatively high number of correlated variables (components) that keep most of the information contained in the original data. Each component consists of a number of elements (loads), which represent the correlation of the variables with the component.

Results

Effect of Ni on various biochemical parameters in the copepod *T. japonicus*

For SOD activity, Ni initiated a significantly inducible effect on the copepod's activity by day 7 (Fig. 2a, $P < 0.05$), and this stimulation attained its peak at day 12 ($P < 0.05$). Moreover, all Ni treatments exerted a significantly stimulating effect on SOD activity in a dose-dependent manner at day 7. At day 12, the stimulation attained its peak at the 0.75 mg/L metal treatment but decreased under the 3.0 mg/L Ni concentration.

Figure 2b shows the response of the copepod's GPx activity to Ni exposure. At day 1, GPx activity in the 0.25 mg/L Ni treatment showed no difference from the control, but the 0.75 mg/L metal treatment significantly depressed GPx activity, and the 0.125 and 3.0 mg/L treatments had a notable induction effect ($P < 0.05$). At day 4, GPx activity was noticeably stimulated by the 0.125, 0.75, and 3.0 mg/L metal treatments ($P < 0.05$), but the 0.25 mg/L treatment displayed little effect. At day 7, all Ni treatments exerted a significantly stimulating effect on the treated copepod's GPx activity ($P < 0.05$). When the exposure time occurred at day 12, GPx activity was significantly improved by the 0.125 and 0.75 mg/L treatments; however, the 3.0 mg/L treatment exerted a noticeably inhibitory effect on this activity, which was independent of the 0.25 mg/L treatment.

Ni treatments insignificantly affected the copepod's GST activity at day 1 and 4 (Fig. 2c). When the exposure time occurred at day 7, the 0.125 and 0.25 mg/L metal treatments significantly enhanced GST activity ($P < 0.05$), which was hardly influenced by the 0.75 and 3.0 mg/L treatments. At day 12, all Ni additions distinctly enhanced GST activity, which was linearly correlated with Ni concentrations ($P < 0.05$).

In the case of AchE activity (Fig. 2d), during the early exposure time (e.g., day 1 and 4), Ni additions had an insignificant effect, except that the 0.125 and 0.75 mg/L metal treatments displayed a remarkable induction effect at day 4 ($P < 0.05$). Subsequently, Ni treatments obviously stimulated the copepod's AchE activity at day 7 and 12.

Figure 2e shows that Ni treatments significantly depressed GSH level in the treated copepods during the whole exposure period ($P < 0.05$), except that the 0.125 mg/L metal treatment exhibited a striking stimulation effect at day 7 and 12 ($P < 0.05$). Moreover, the copepod's GSH level at day 1 and 4 linearly decreased with an increase in Ni concentration.

Figure 2f shows that the 0.125, 0.25, and 3.00 metal treatments showed no impact on the GSH/GSSG ratio at day 1, but it was noticeably prohibited by the 0.75 mg/L treatment. At day 4, the 0.125 and 0.25 mg/L treatments negligibly affected the GSH/GSSG ratio, but the 0.75 mg/L treatment displayed a significantly stimulating effect, and the 3.00 mg/L treatment exerted a noticeably prohibitive influence. At day 7, all Ni treatments exerted no impact on the GSH/GSSG ratio, except that the 0.125 mg/L metal treatment displayed an inducible effect. When the exposure duration was day 12, the 0.125 mg/L Ni concentration significantly increased the GSH/GSSG ratio ($P < 0.05$), but this was notably depressed by the 0.25, 0.75, and 3.0 mg/L metal treatments ($P < 0.05$).

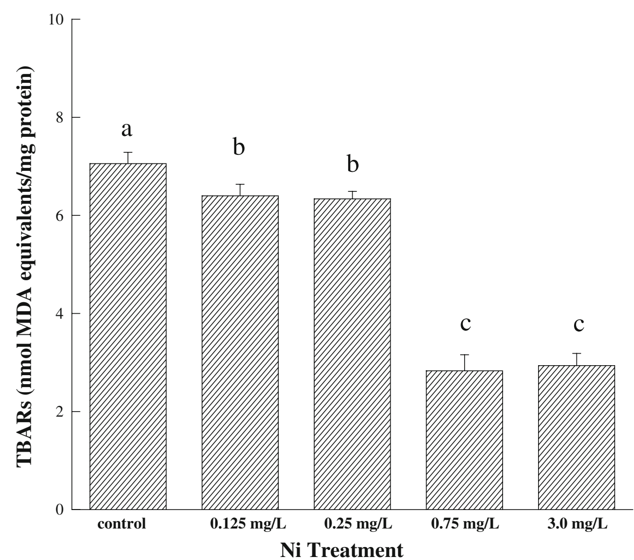

Fig. 3 Influence of Ni on LPO level in *Tigriopus japonicus*. Data are means ± SD ($n = 5$). Different letters indicate a significant difference among different metal treatments at $P < 0.05$

Effect of Ni on LPO level in the copepod *T. japonicus*

Figure 3 shows that LPO level in the copepods was markedly correlated with different Ni concentrations after 12 days of exposure ($P < 0.05$) and Ni treatments strikingly prohibited the LPO level, which tended to decrease with an increase in Ni concentration, i.e., LPO level decreased from 7.054 to 2.938 μmols/mg protein with an increase in Ni concentration from 0 to 3.0 mg/L.

Effect of Ni on MT level in the copepod *T. japonicus*

Figure 4 shows the response of MT in the copepods to Ni exposure. At day 1, Ni exerted little impact on MT level, except that the highest Ni concentration (3.0 mg/L) exhibited an inducible effect ($P < 0.05$). At day 4, 0.25 and 0.75 mg/L Ni remarkably depressed the MT level, but 3.0 mg/L Ni notably induced the synthesis of MT, and 0.125 mg/L Ni displayed little effect. When the exposure time appeared at day 7, all Ni treatments evidently depressed MT synthesis in the copepods, but at day 12, all showed obvious stimulation ($P < 0.05$).

Effect of exposure time and metal treatment on biochemical responses in the copepod *T. japonicus*

The results of two-way ANOVA (Table 1) confirmed the strong influence of concentration on the parameter variables (i.e., SOD, GPx, GST, AchE, GSH, GSH/GSSG, and MT) in the copepod ($P < 0.001$). The striking effects of exposure time were also revealed for the above biomarkers in *T. japonicus* ($P < 0.001$). Furthermore, ANOVA indicated a significant interaction between the effects of exposure time and the metal treatment on the copepod biomarkers ($P < 0.001$).

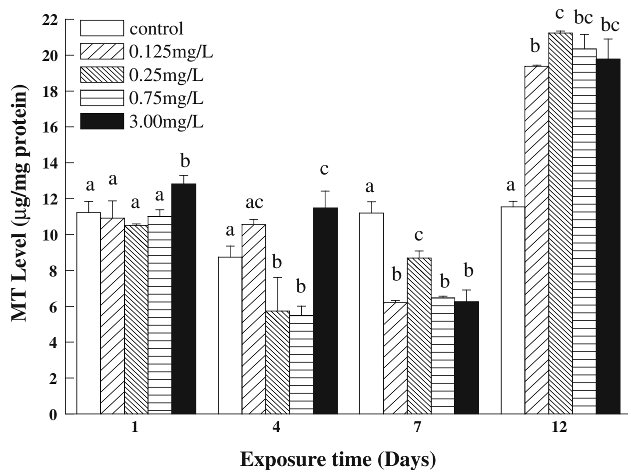

Fig. 4 Influence of Ni on metallothionein (MT) level in *Tigriopus japonicus*. Data are means ± SD ($n = 4$). Different letters indicate a significant difference among different metal treatments at $P < 0.05$

Multivariate analysis of biochemical responses in the copepod *T. japonicus*

To explore overall relationships between the different variables, we ran a PCA from matrix data at day 12 that included SOD, GPx, GST, AchE, GSH, GSH/GSSG, LPO, and MT (Table 2). The results showed that 80.63% of overall variance was explained by the first two principal components. The first component (PC1, 62.51% of variance) was built by SOD, GST, GSH, GSH/GSSG, LPO, and MT. The PC2 (18.12% of overall variance) was formed by GPx and AchE. We also made a vector plot based on the PCA result, which indicated that there was a relationship between some of the parameter variables (e.g., SOD, GST, and MT, or GSH and GSH/GSSG) (Fig. 5). In addition, various correlations between the variables were suggested by Pearson's correlation test (Table 3). The results indicated that SOD activity was positively correlated with GST activity ($P < 0.001$) and MT content ($P < 0.01$). Both GPx and GST activities showed a positive relationship with AchE activity ($P < 0.05$), and GST activity positively correlated with MT level ($P < 0.05$). Furthermore, GSH level showed a significantly positive correlation with the GSH/GSSG ratio ($P < 0.001$). It should be noted that LPO showed a negative relationship with SOD, GST, GSH, and GSH/GSSG, but this was definitely insignificant ($P > 0.05$).

Discussion

A variety of environmental contaminants can display toxicity via the induction of oxidative stress. Despite the large number of studies that have investigated the relationship between the metal exposure and the antioxidant defense system in several species of animals, according to our best knowledge, few of these studies were performed using the copepod as a model sentinel species. This might be the first attempt to examine the effects of Ni exposure on the detoxification process and its disturbance of the antioxidant system in the harpacticoid copepod *T. japonicus*.

Recent studies have shown that metals could over-produce ROS with consequent depletion of sulfhydryls groups, LPO, and DNA damage (Stohs and Bagchi 1995; Kasprzak et al. 2003; Kodipura et al. 2004). However, the antioxidant enzymes (e.g., SOD and GPx) could be induced to scavenge ROS, and so diminish oxidative damage, which was ultimately consistent with this study, i.e., SOD and GPx activities in the copepods were significantly stimulated by Ni treatments during the 12 d exposure in this present study. Therefore, Ni might compel the copepods to suffer

Table 1 Synthesis of the two-way factorial ANOVA displaying the effect of metal treatment and exposure time on biochemical parameters in *Tigriopus japonicus*

Biological parameter	Effect of metal treatment			Effect of exposure time			Treatment × time	
	F	*df*	*P*	*F*	*df*	*P*	*F*	*P*
SOD	92.58	4	0.000	637.74	3	0.000	53.35	0.000
GPx	96.42	4	0.000	312.67	3	0.000	65.78	0.000
GST	6.46	4	0.000	173.52	3	0.000	14.92	0.000
AchE	15.91	4	0.000	43.54	3	0.000	12.69	0.000
GSH	147.76	4	0.000	73.95	3	0.000	22.70	0.000
GSH/GSSG	76.30	4	0.000	20.42	3	0.000	27.68	0.000
MT	16.16	4	0.000	821.18	3	0.000	59.42	0.000

The biochemical parameters are the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), acetylcholinesterase (AchE), glutathione (GSH) content, the ratio of reduced to oxidized glutathione (GSH/GSSG), as well as metallothionein (MT) level

Table 2 Component matrix of all parameter variables at day 12 in *Tigriopus japonicus* via a principal component analysis

	Correlation coefficients	
	Component 1	Component 2
SOD	**0.993**	−0.100
GPx	0.114	**0.975**
GST	**0.954**	−0.081
AchE	0.482	**−0.712**
GSH	**−0.744**	0.391
GSH/GSSG	**−0.705**	0.491
LPO	**−0.841**	0.259
MT	**0.784**	0.053
% of variance	62.51	18.12

The variables utilized are the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), acetylcholinesterase (AchE), glutathione (GSH) content, the ratio of reduced to oxidized glutathione (GSH/GSSG), as well as levels of lipid peroxidation (LPO), and metallothionein (MT). The bold type represents biomarkers having significant factorial weights > 0.7 ($P < 0.05$)

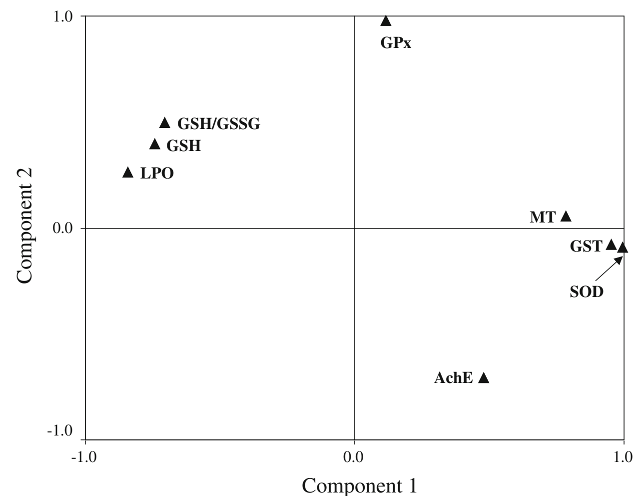

Fig. 5 Principal component analysis of all biochemical parameters at day 12 in the copepod *Tigriopus japonicus*. The parameters utilized are the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), acetylcholinesterase (AchE), glutathione (GSH) content, the ratio of reduced to oxidized glutathione (GSH/GSSG), as well as levels of lipid peroxidation (LPO), and metallothionein (MT)

from oxidative stress via ROS overformation, and the animals might correspondingly initiate their antioxidant system to counteract this stress. Hfaiedh et al. (2008) found that nickel chloride induces oxidative stress as evidenced by an increase in LPO and changes in antioxidant enzymes activities, i.e., SOD activity is found to be increased, whereas GPx and catalase activities are decreased. In fact, the most possible mechanism that may be operative in Ni toxicity is the generation of ROS, which initiates LPO, thereby causing damage to critical macromolecules such as protein or DNA as well as cell damage and death (Huang et al. 1994). For instance, Stinson et al. (1992) reported that nickel chloride could produce HO via the Fenton reaction, with consequent LPO and DNA damage, which is also exemplified by a previous study that attributes a decreasing cell viability to a significant increase in ROS, LPO, and HO levels in human lymphocyte during an acute exposure to Ni (Chen et al. 2003). In addition, our study demonstrated that SOD and GPx activities showed different responses to metal exposure, i.e., the copepod's SOD activity was significantly induced at day 7 and peaked at day 12, but its GPx activity was basically induced at day 4 or 7 and was recovered at day 12. The possible reason is that GPx might be firstly induced to eliminate the increasing H_2O_2 concentration due to Ni attack during the 12 d exposure but then, later in the exposure time, catalase initiated to play its

precious role in fighting against oxidative stress. Alternatively, the strikingly inducible GST activity in the late exposure period might be partly responsible for the recovery of GPx activity in the copepods at day 12.

GST activity showed no relationship with Ni treatments in the early exposure time but was stimulated during the late exposure time (i.e., day 7 and 12). Thus, even though SOD and GPx activities might have been induced to eliminate ROS and its metabolites caused by Ni, the copepods might have to stimulate GST activity for scavenging superfluous oxidative-products. Recently, great attention has been paid to investigate sequences and functional characteristics of several toxicologically relevant genes (e.g., *GST*) in the intertidal copepod, *T. japonicus* (Lee et al. 2007a, b; Raisuddin et al. 2007; Seo et al. 2006a, b, c), as exemplified by a preliminary study that GST gene expression in *T. japonicus* shows upregulation in response to exposure to Cu and Mn, which was in agreement with our study. Thus, we assumed that Ni accumulation might have occurred in the treated copepods in this study due to the induction of GST activity. Additionally, the previous studies also show the change of GST activity in other aquatic invertebrates exposed to metals with oxidative stress-inducing potential (Barata et al. 2005b; Jemec et al. 2007). For example, an increase in GST activity in *Daphnia magna* exposed to 5 μg/L of Cd^{2+} is observed by Barata et al. (2005b), but inhibition is found in crayfish *Procambarus clarkii* that are acutely exposed to 100 μg/L of Cd^{2+} for 96 h. However, Jemec et al. (2007) found no impact of Cd^{2+} on GST activity in *D. magna* to 40 μg/L, and similarly Cr^{6+} up to 280 μg/L shows no remarkable effect. Taking all this into account, GSTs might be recognized as biomarkers of exposure to oxidative stress-inducing chemical contaminants including metals (Moreira and Guilhermino 2005; Durou et al. 2007). Nevertheless, the GST biomarker response might be dependent on the species and the type of toxicant, and the exact mechanism of impact of Ni on this enzyme is still unknown and needs more study.

The present study showed that GSH was always maintained in the inhibitory status during the metal exposure except for the 0.125 mg/L group of day 7 and 12. Furthermore, its response was more prompt than the antioxidant and detoxificatory enzymes (e.g., SOD, GPx, and GST). Therefore, GSH could take on the first defense to metal attack, by its direct complexation with metal or participation in the detoxification process of GPx or GST (Sies 1999). In the late exposure period, the exposed GSH/GSSG ratio was significantly different from the control, which might highlight that Ni exerts toxicity on the treated copepods via alteration of the cellular redox state. Similarly, some authors attribute the toxic effects of Ni to a decrease in cellular GSH and a concomitant increase in GSSG, altering the redox state of the cells (De Luca et al. 2007). Thus, GSH/GSSG could be a suitable biomarker for oxidative stress or injury in biological organisms (Hwang et al. 1992). Additionally, the strongly positive correlation of GSH level with the GSH/GSSG ratio (Table 3) suggested that a remarkable depletion of GSH in this study might reflect an oxidative condition of the copepods induced by Ni attack via significantly interfering with the cellular redox state.

Our study showed that the LPO level in the treated copepods was markedly lower than the control value, and this was quite unexpected. Taking into account the response patterns of the antioxidant system (e.g., SOD, GPx, GST, and GSH) to Ni additions, it is quite probable that the copepods suffered from the Ni-induced oxidative stress or even oxidative damage. Several studies show that the factors involved in LPO do not seriously depend on the operations and functions of the antioxidant enzymes, i.e., the LPO level is not always correlated with ROS content,

Table 3 Pearson's correlation matrix on all variables in *Tigriopus japonicus*

	SOD	GPx	GST	AchE	GSH	GSH/GSSG	LPO	MT
SOD	1.000							
GPx	−0.141	1.000						
GST	0.855***	0.040	1.000					
AchE	0.358	0.500*	0.446*	1.000				
GSH	−0.377	0.097	−0.015	0.084	1.000			
GSH/GSSG	−0.298	0.116	−0.010	0.094	0.846***	1.000		
LPO	−0.656	−0.338	−0.629	−0.054	0.619	−0.847	1.000	
MT	0.686**	−0.394	0.555*	−0.069	−0.326	−0.198	−0.327	1.000

The variables are the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), acetylcholinesterase (AchE), glutathione (GSH) content, the ratio of reduced to oxidized glutathione (GSH/GSSG), as well as levels of lipid peroxidation (LPO), and metallothionein (MT)

Indicates a significant difference at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

which might also be controlled by other factors (Hussain et al. 1987; Strubelt et al. 1996; Nigam et al. 1999). This was basically in accordance with our results which showed that the copepod's LPO level showed insignificant correlation with all the antioxidant biomarkers used in this experiment (Table 3). Gajewska and Sklodowska (2007) also found that there is no remarkable difference between the exposed group and the control one in terms of the LPO level, although Ni addition significantly elevates the ROS level (e.g., O_2^- and H_2O_2) and remarkably influences the antioxidant enzymes. Clearly, the exact cause concerning the response of LPO to Ni exposure in the present study remains to be established in future.

We found that Ni significantly stimulated AchE activities in the late exposure time (day 7 and 12), consequently displaying neurotoxicity to the copepods, which was inconsistent with the previous studies which show that metals exert toxicity via the restraint of AchE activity (Amiard-Triquet et al. 1998; Elumalai et al. 2002; Najimi et al. 1997). However, no change is found on AchE activity in the copepod *Tigriopus brevicornis* after a 96 h exposure to LC_{50} concentrations of Cd^{2+}, but similar exposure to As or Cu exerts an inhibitory effect (Forget et al. 1999). Likewise, Jemec et al. (2007) found that concentrations of Cr^{6+} up to 280 µg/L have no effect on AchE activity in *D. magna*, which is, however, increased by about 50% at concentrations of 20–25 µg/L of Cd^{2+}. Thus, AchE biomarker response might vary with species and type of toxicant, and a further challenge would be to explore the precise mechanism with regard to Ni effect on AchE activity in this copepod. However, the strikingly positive correlation of AchE and some antioxidant biomarkers (e.g., GPx and GST) highlighted the fact that oxidative damage might be partly involved in the neurotoxicity (via AchE interference) to the treated copepods caused by Ni attack.

Generally speaking, Ni additions depressed the copepod's MT content in the initial exposure time, but markedly stimulated MT production in the late exposure time. Thus, in the initial exposure time, Ni toxicity might significantly restrain MT synthesis in the copepods, i.e., the detoxification function of MT might be destroyed or depressed by a "sudden" attack from Ni. The inhibitory effect of Ni additions on MT synthesis in the initial exposure time might be attributable to the "spill-over" hypothesis which deems that when the cellular store of MT is insufficient to capture heavy metals, the excess will "spill-over" and combine with other cellular ligands, hence engendering metal toxicity (Brown and Parsons 1978). Several laboratory studies with experimental organisms exposed to a series of metal concentrations demonstrate that high metal treatment could suppress MT level, the possible result of which being that metal attack might destroy the detoxification function of MT (George et al. 1992; Mouneyrac et al. 2002). For example, Mouneyrac et al. (2002) found that Cu and Zn exposures show little or even restraining impact on MT production in *Orchestia gammarellus*. However, due to the cooperation and protection of the antioxidant system (e.g., SOD, GPx, GST, and GSH) during Ni exposure (Table 3), the copepods might recover from Ni-induced oxidative stress and subsequently induce the synthesis of MT in the late exposure time, with the concomitant excitation of its detoxification function. Barka et al. (2001) also found that 1–40 µg/L Ni can remarkably elevate the metallothionein-like protein level in the copepod *T. brevicornis*, which is kept in balance during the 14 days of exposure. Thus, in response to Ni exposure, the copepod could induce MT synthesis to act on the detoxification function and thereafter counteract metal attack. It should be noted that the MT level measured in organisms is actually a result of the interaction between the synthesis and degradation process (Couillard et al. 1995). It could be assumed that the copepod *T. japonicus* might promptly initiate the detoxification process involving MT as a response to Ni exposure, and the increase in MT activity might result in more turnover of this protein, but not in an enhanced content, which is also demonstrated by Barka et al. (2001). Therefore, in the present study, Ni treatment exerting a prohibitive effect on the MT level in the early exposure time might be a consequence of the increasing MT activity in the copepods, which was manifested in its quicker turnover.

In this study, the PCA divided the biochemical variables into two principal components. PC1 was built by SOD, GST, GSH, GSH/GSSG, LPO, and MT, and they were involved in the antioxidant and detoxification response, which allowed us to correlate this component with the copepod's antioxidant and detoxification system. PC2 highly correlated with AchE and GPx, and therefore could be designated as an "intoxicated" condition induced by Ni attack, considering that AchE has been considered as a biomarker of exposure to neurotoxic compounds (Forget et al. 2003; Matozzo et al. 2005). Thus, the copepod first initiated the antioxidant and detoxification response to counteract the Ni attack, but when the antioxidant and detoxification system were broken down, this metal might cause neurotoxicity to the copepods. Moreover, the strongly positive correlation within the antioxidant and detoxification variables (e.g., SOD, GST, and MT) (Fig. 5 and Table 3) highlighted a highly efficient cooperation in these systems during the fight against Ni toxicity. Additionally, the significant correlation of MT with some antioxidant biomarkers (e.g., SOD and GST) suggested that this variable might have a ROS scavenger characteristic, and this is in agreement with previous studies (Correia et al. 2002a, b; Paris-Palacios et al. 2003; Falfushynska and Stolyar 2009).

In conclusion, different Ni treatments had a significant effect on the variant biochemical indexes (SOD, GPx, GST, AchE, GSH, and GSH/GSSG), and some of these parameters might be used as biomarkers of exposure to Ni. However, it is necessary to consider the influence of exposure time on biomarker response bearing in mind that the duration of exposure strongly affected the various biochemical responses in the copepod use in the present study (Table 1). Ni exposure significantly decreased LPO level in the exposed copepods in contrast to the control, which implied that the factors involved in LPO might not significantly depend on the operations and functions of the antioxidant enzymes. During the 12 d exposure, Ni originally caused an inhibition effect but then, later in the exposure time, induced MT synthesis in the copepod *T. japonicus*, and this was probably responsible for Ni detoxification. Thus, Ni exposure had intervened in the detoxification process and the antioxidant system of this copepod. PCA seems to be a useful tool when some markers vary in ways that may initially be difficult to interpret, and the PCA results indicated that the most sensitive biomarkers of Ni pollution were SOD, GPx, GST, and MT in this copepod. It is noteworthy to mention that *T. japonicus* has remarkable euryhaline and eurythermal characteristics, which could minimize the impacts of environmental change. This present study leads us to believe that *T. japonicus* is a marine species deserving of greater attention in future. Meanwhile, this copepod could be regarded as a suitable bioindicator of Ni exposure by measuring SOD, GPx, GST, and MT as biomarkers.

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