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Biomarkers of Oxidative Stress in the Polychaete *Eurythoe complanata* (Amphinomidae) Under Short Term Copper Exposure

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Metal exposure may induce cellular injury due to membrane breakdown, which would affect cellular structures and functions. Metal toxicity is usually related to a redox cycling phenomenon which promotes the formation of free radicals, identified as responsible for oxidative stress. The free radical-mediated effects include DNA damage, enzyme inactivation, and cellular or subcellular membrane peroxidation resulting in the generation of lipid hydroperoxides and complexes of carbonyl compounds including malondialdehyde (MDA) (Di Giulio *et al.* 1989; Viarengo *et al.* 1990; Winston *et al.* 1991). Cellular damage is usually preceded by the impairment of antioxidant biochemical mechanisms that quench radicals before they initiate their molecular effects. Among oxidative defenses, the antioxidant enzymes catalase (CAT, EC. 1.11.1.6), gluthatione peroxidase (GPX, EC. 1.11.1.9), gluthatione reductase (GR, EC. 1.6.4.2.), and superoxide dismutase (SOD, EC. 1.15.1.1) appear to be the most sensitive to radical proliferation (Wenning and Di Giulio 1988; Livingstone *et al.* 1999; Porte *et al.* 1991; Solé *et al.* 1994; Regoli and Principato 1995; Labrot *et al.* 1996; Regoli *et al.* 1998).

The modulation of antioxidant enzymes by heavy metals, linked to oxidative stress, has been observed in marine invertebrates, principally in molluscs. Although, these enzymes are potential biomarkers for environmental stress, they have seldom been used for this purpose in different taxonomic groups for hypothesis testing. In general, the evaluation of the responses of antioxidant enzymes in marine invertebrates related to heavy metal exposure and the understanding of these processes involved is scanty. This paper examines the effects of a short-term, sublethal copper exposure on membrane peroxidation (MDA) and antioxidant enzyme biomarkers in the polychaete *Eurythoe complanata* (fire worm; Amphinomidae). This cosmopolitan tropical organism has proved suitable as a bioindicator for testing benthic pollution (Marcano *et al.* 1997; Nusetti *et al.* 1998).

MATERIALS AND METHODS

Adult specimens of *Eurythoe complanata* (fire worms) were collected from the Gulf of Cariaco, in Eastern Venezuela, and kept in a well aerated aquarium containing seawater (36‰, pH 7.8), sand and gravel from the sampling site to provide food and refuge for 2 weeks prior to the

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experiments. Temperature was maintained at 24 ± 1°C. The experimental work was conducted during 7 and 15 days to determine microsomal membrane peroxidation and enzyme activities, respectively. Worms weighing between 1.2 and 1.4 g were exposed to nominal sublethal concentrations of 0.39 mg L^{-1} Cu^{2+} (30% LC50, from $CuSO_4.5H_20$) in 6 L glass vessels containing aerated seawater and a 2 cm layer of sediment (300 g). Control and 7-d copper exposed worms were periodically sampled for the determination of microsomal malondialdehyde (MDA) concentrations and copper levels in the carcass tissue. Tissue copper concentrations were recorded by flame atomic spectrophotometry (Nusetti *et al.* 1998), using a group of 5 individuals for each sampling period. Parallel to these measurements, 3 groups of 5 individuals were sampled to estimate the microsomal peroxidation by the formation of thiobarbituric acid reactive substances (TBARS), colorimetrically quantified as MDA equivalents mg^{-1} protein (Livingstone *et al.* 1990). Microsomal proteins were dissolved in 10% sodium deoxycholate and measured following Lowry *et al.* 1951. Antioxidant enzymes were assayed in the carcass tissue following 15 d copper exposure when maximal copper uptake by the polychaete occurs, as has been previously described in similar experimental conditions (Marcano *et al.* 1996; Nusetti *et al.* 1998).

Crude enzymatic and microsomal fractions were prepared as follows. The polychaete carcass tissue was homogenized in ice cold 20mM Tris-HCl buffer pH 7.4 containing 1mM EDTA, 1mM dithiothreitol, 0.5 M sucrose, 0.15 M KCl, and 0.2 mM phenylmethyl-sulphonic acid (PMSF). The homogenate was centrifuged at 5,000 and 12,000 g at 4°C for 20 min to obtain the supernatant samples for the antioxidant enzyme assays. Microsomes were obtained after centrifugation at 105,000 g for 90 min at 4°C, and were used for measuring membrane peroxidation. Prior to the biochemical tests, the cellular preparations were stored at -70°C for 2 weeks.

Antioxidant enzymes were measured by spectrophotometry at 25°C basically as described in Livingstone *et al.* (1990), with modifications of final substrate concentrations, as follows: GPX activity was recorded at 340 nm following the oxidation of NADPH (0.06 mM, ext. coeff. 6.2 mM^{-1} cm^{-1}) during the formation of reduced glutathione by commercial glutathione reductase (1U.mL^{-1}) using 10mM H_2O_2 as a substrate and 2mM sodium azide in 100mM phosphate buffer, pH 7.5. GR activity was measured at 340 nm following the oxidation of NADPH (0.06 mM) by oxidized glutathione (GSSG) (12 mM) in 100 mM phosphate buffer, pH 7.5. CAT activity was determined by recording the decrease in absorbance at 240 nm using 75mM H_2O_2 (ext. coeff. 40 M^{-1} cm^{-1}) in 50 mM phosphate buffer, pH 7.0. The total enzyme activity was expressed in terms of units (μmoles substrate converted to product min^{-1}) g^{-1} of wet tissue.

The effects of copper exposure on microsomal MDA concentration were examined using a one-way ANOVA. Significant differences among MDA mean values were tested by the Duncan multiple range test. The student t-test was used

to detect significant differences in enzyme activities between control and copper exposed worms.

RESULTS AND DISCUSSION

The figure 1 indicates the profiles of copper and MDA concentrations in the carcass tissue during the 7d-experiment. In general copper uptake markedly increased accompanied with significant changes in microsomal membrane peroxidation. The microsomal content of MDA increased significantly after 6 hours of exposure to copper, compared to the control (2.354 ± 0.210 and 0.958 ± 0.076 nmol MDA/mg protein, respectively. $\overline{X}$ ± SD, N=3). Concentration values of 4.00 ± 0.30 - 8.00 ± 0.30 nmol MDA mg^{-1}protein (N=12) were recorded within 24 and 120 h exposure. At the end of the exposure time (168 h), concentrations of MDA increased about ten fold (9.773 ± 0.722 nmol MDA/mg protein, N=3).

Table 1 shows a significant inhibition of glutathione reductase in copper exposed worms. This is indicative of a lowered capacity to synthesize reduced glutathione, a free radical scavenger. The activities of glutathione peroxidase and catalase were not significantly affected by the Cu-experimental treatment. Thus, the polychaete *E. complanata* manifested changes in markers of oxidative stress in response to short-term copper treatments, as demonstrated by both the enhancement of microsomal malondialdehyde (MDA) and the inhibition of glutathione reductase (GR).

Table 1. Antioxidant enzyme activities in carcass tissue from the polychaete *E.complanata* exposed for 7 days sublethal copper concentration.

Enzymes	$\overline{X}$ ±SD (U/g wet mass)		Student t
	Controls	Experimentals	
Glutathione Peroxidase	0.0127 ± 0.0007 (6)	0.0137 ± 0.0019 (5)	1.19 NS
Glutathione Reductase	0.0044 ± 0.0001 (4)	0.0019 ± 0.0004 (4)	12.6 *
Catalase	3.7600 ± 0.7700 (6)	3.1300 ± 1.1700 (5)	1.03 NS

*p< 0.001; NS: p>0.5; (n): sample size.

The increased levels of microsomal MDA associated with the higher concentrations of tissue copper suggest that the metal burden on the polychaete is responsible for membrane lipid peroxidation. This process is thought to involve the activation of free radical chain reactions leading to the formation of toxic substances, including lipid hydroperoxides, endoperoxides, and MDA which is an end product of the oxidation of unsaturated fatty acids (Halliwell and Chirico1993). Increased membrane peroxidation has also been observed in marine bivalves under short-term copper exposure (Viarengo *et al.* 1990). This biochemical alteration results in structural and physiological deterioration (Randal 1995).

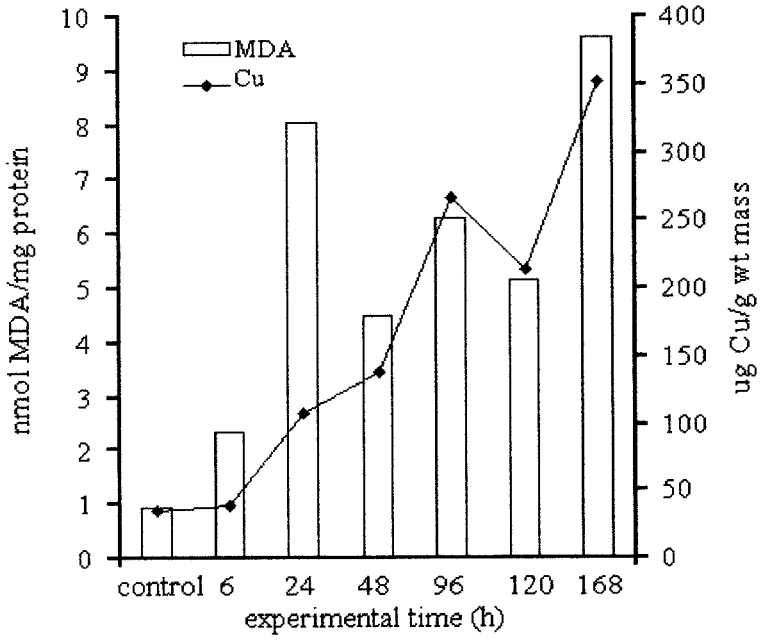

Figure 1. Patterns of copper uptake and malondialdehide formation by the carcass tissue from the worms during 168 h (7d)-Cu^{+2} exposure.

The inhibition of GR in the copper-exposed polychaetes is indicative of potential toxic effects on the normal pathways by which the free radicals and their derived metabolites are removed; this metabolic change may bring pathological consequences. The enzyme catalyzes the intracellular production of reduced glutathione, a nucleophilic molecule, which plays a predominant role in contaminant detoxification, such as quenching radicals directly and supplying reducing equivalents for the reduction of peroxides by GPX. The limited production of reduced glutathione could also affect glutathione-S-transferase, which conjugates a variety of electrophylic metabolites with reduced glutathione transforming them into water soluble and easily excretable products (Rand 1995). *E. complanata* possesses a fairly high activity of this enzyme (unpublished). Thus, the inhibition of glutathione reductase by sublethal copper exposure may indirectly cause profound metabolic alterations in the organism, raising the possibility of developing oxidative stress and compromising its survival. Interestingly, it appears that short-term exposure of mussels to sublethal copper concentrations causes tissue peroxidation linked to a decreased production of reduced glutathione, possibly related to the inhibition of GR (Viarengo *et al.* 1990; Regoli and Principato 1995).

Based in our data, the polychaete *E. complanata* appears suitable as an experimental organism for the development of biochemical tests for revealing the

occurrence of oxidative stress during environmental pollution studies. Measurement of microsomal MDA and the enzymes regulating glutathione levels could provide an early warning of toxic effects of subtle chemical contamination in the benthic habitat.

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