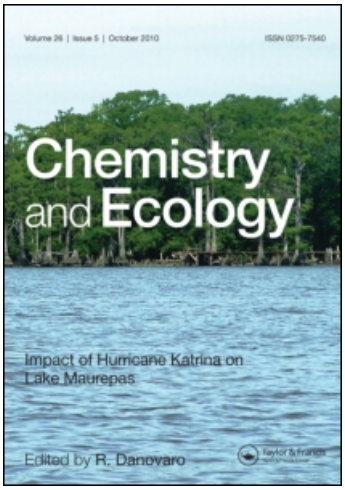


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# Changes in Lipid Peroxidation in the Gill and Muscle of the Marine Bivalve (*Perna Viridis*) During Exposure to Cadmium and Copper

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# CHANGES IN LIPID PEROXIDATION IN THE GILL AND MUSCLE OF THE MARINE BIVALVE (*PERNA VIRIDIS*) DURING EXPOSURE TO CADMIUM AND COPPER

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The marine bivalve, *Perna viridis* was exposed to sublethal concentration (one third of 24 h $LC_{50}$) of either cadmium or copper for 1, 3 and 7 days and alterations in lipid peroxidation in the gill and muscle were investigated. Significant increase of the level of malondialdehyde, which is indicative of the peroxidative process, and a decrease of activity levels of glutathione peroxidase and glutathione transferase were observed in both the tissues of copper exposed mussels. The exposure of mussels to cadmium did not elicit any of the above changes.

KEY WORDS: Malondialdehyde, glutathione peroxidase, glutathione transferase, lipid peroxidation, copper and cadmium, gill and muscle.



## INTRODUCTION

Indiscriminate discharge of raw and partially treated industrial effluents into aquatic systems leads to the deterioration of the environment. Various metals are present in industrial effluents in significant quantities. Since these metals are immutable, bioaccumulative and non-biodegradable, aquatic organisms, some of them important members of food chains such as fish and shellfish, are adversely affected. The tragic incidences of 'Minimata' and 'Itai-Itai' diseases in man have been attributed to the long-term consumption of sea food exposed to mercury and cadmium respectively (Tsuchiya, 1969; Ui, 1972). In view of this, an elaborate programme to evaluate the impact of heavy metals on the physiology and biochemistry of some marine edible organisms has been undertaken.

The research presented in this report is an initial phase of a project designed to determine the influence of heavy metals on lipid peroxidation in edible marine organisms. Cadmium and copper are the model elements and the green mussel (*Perna viridis*) is the test species. The reason for this choice is that mussels are filter-feeding organisms, able to accumulate within their tissues many of the contaminants (metals, pesticides, hydrocarbons etc.,) present in sea water and are probably the best sentinel organisms for use in monitoring programmes and for the evaluation of the biological effects of pollutants (Viarengo and Canesi, 1991). These mussels are abundant locally and are usually considered as "poor man's protein".

## MATERIALS AND METHODS

Indian green mussels, *Perna viridis*, weighing $50 \pm 3$ g, were collected from the Pondicherry coast. Prior to use, they were maintained in the laboratory in large aquaria for 7 days at $25 \pm 2$ °C with a normal photoperiod of 12 h darkness. Cadmium chloride and copper sulphate, obtained from S.D Fine Chemicals (India) were used in the present study. $LC_{50}$ values for 24 h exposure were determined from percentage and probit mortality vs log concentration (Finney, 1971) and were found to be 1.5 mg $l^{-1}$ for cadmium and 0.75 mg $l^{-1}$ for copper. One third of the 24 h $LC_{50}$ value was selected as a sublethal concentration for the present study. The experimental mussels could tolerate this concentration for more than a month without mortality.

### *Exposure conditions*

During the experiments, animals were exposed for different periods of time to either 0.5 mg $l^{-1}$ of cadmium or 0.25 mg $l^{-1}$ of copper. Metals were added daily to the sea water in the form of standard solutions of $CdCl_2$ and $CuSO_4$. The sea water (5 l/animal) was changed daily. Mussels were removed from the exposure tanks after 1, 3 and 7 days of exposure. Control animals were maintained in the same conditions without the addition of any metal. No deaths occurred in either the exposed or control groups.



### *Sample preparation and biochemical assays*

On each sampling occasion, gill and muscle tissues were dissected out, damp-dried and used immediately for analysis. Eight samples were taken on each occasion. In these organs from both control and metal-exposed mussels, levels of malondialdehyde, activity levels of glutathione S-transferase and glutathione peroxidase, and the concentration of metals were estimated.

### *Analysis for metal content*

The metal concentrations in the gill and muscle tissues were determined by atomic absorption spectrophotometry (Perkin-Elmer Model 2380) as described by Reddy and Bhagyalakshmi (1994). Standard calibration curves were prepared using standard cadmium and copper solutions and used for analysis of samples.

### *Evaluation of the malondialdehyde (MDA) content*

The tissues were homogenised (10% W/V) in 30 mM Tris-HCl buffer, pH 7.4. Aliquots of the homogenates were added with an equal volume of acetonitril and subsequently centrifuged at 5000 g for 15 min at 0 °C. The supernatants were utilized for the evaluation of MDA concentrations by high performance liquid chromatography (HPLC), using a Waters carbohydrate analysis column with 30 mM Tris-HCl:acetonitril (9:1) as elution buffer. A standard solution of MDA was prepared as described by Viarengo *et al.* (1990) and used for calibration.

### *Enzyme assays*

The tissues were homogenized in 5 volumes of 50 mM Tris HCl, pH 7.4, containing 1 mM EDTA and lmM diethyldithiocarbamate. The homogenates were centrifuged

at 150,000 g for 60 minutes in a Hitachi ultracentrifuge. The clear supernatant fraction (cytosol) was used as an enzyme source.

*Glutathione peroxidase (GPX)*

GPX activity was assayed with a glutathione reduction coupled to reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidation by glutathione reductase according to the method described by Schisler and Singh (1988). Activity of GPX was monitored at 340 nm by the disappearance of NADPH using $H_2O_2$ as the substrate. The reaction mixture in a volume of 1.0 ml contained: 100 mM potassium phosphate buffer, pH 7.5, 0.003 M EDTA, 0.003 M sodium azide, 0.03 M glutathione, 0.006 M NADPH, 3 units of glutathione reductase, 2 mM $H_2O_2$, and an appropriate amount of enzyme protein. The reaction was initiated by the addition of $H_2O_2$ and the activity was expressed as μmole of $H_2O_2$ reduced/mg protein/min.

*Glutathione-S-transferase (GST)*

GST activity was evaluated as decribed by Habig *et al.* (1974), utilizing 1-chloro-2, 4, dinitro benzene (CDNB) as substrate. The reaction mixture in a final volume of 3.0 ml contained: 150 mM potassium phosphate buffer, pH 7.5, 1 mM CDNB, 5 mM glutathione, and an appropriate amount of enzyme protein. Thio-ether concentration was determined from the slopes of initial reaction rates. A molar extinction coefficient of $9.6 \times 10^3$ $cm^{-1}$ was used in the calculation. The activity was expressed as μmoles of thio-ether formed/mg protein/min.

*Analysis for protein content*

The tissue protein content was determined using bovine serum albumin as a standard, following the method of Lowry *et al.* (1951).

*Statistical analysis*

The statistical significance of the difference between the means of control and experimental groups was analyzed by Student's t-test (Pillai and Sinha, 1968).

## RESULTS AND DISCUSSION

In Table I, data on the heavy metal content in the gill and muscle of mussels exposed for 1, 3 and 7 days to cadmium and copper are reported. A significant and progressive increase of metal concentration in both tissues was noticed. Table II shows the GST activity levels in the gill and muscle of *Perna viridis* exposed to cadmium and copper. The results indicate that the activity levels of this enzyme increased significantly in both the tissues following exposure to copper. In contrast, the activity level of GST did not show significant variation in the tissues of cadmium exposed mussels. The results in Table III show that GPX activity increased significantly in both the tissues of copper exposed mussels. The exposure to cadmium did not significantly affect the tissue GPX activity levels. The exposure to copper significantly increased tissue MDA levels, whereas exposure to cadmium did not affect the MDA content in either of the tissues studied (Table IV).

**Table I** Concentration of metals in the gill and muscle tissues of *Perna viridis* during exposure to either copper or cadmium.

| | | *Exposure Time (days)* | | |
|---|---|---|---|---|
| *Metal* | *Control* | *1* | *3* | *7* |
| | | Gill | | |
| Copper | 4.64 ±0.19 | 6.96 ±0.31 (50) P<0.001 | 11.04 ±0.46 (138) P<0.001 | 14.61 ±0.63 (215) P<0.001 |
| Cadmium | 0.82 ±0.039 | 1.47 ±0.06 (79) P<0.001 | 2.68 ±0.09 (227) P<0.001 | 3.73 ±0.12 (355) P<0.001 |
| | | Muscle | | |
| Copper | 1.02 ±0.03 | 2.11 ±0.05 (107) P<0.001 | 2.99 ±0.11 (193) P<0.001 | 3.08 ±0.13 (202) P<0.001 |
| Cadmium | 0.64 ±0.03 | 1.21 ±0.05 (89) P<0.001 | 2.02 ±0.12 (216) P<0.001 | 2.98 ±0.13 (366) P<0.001 |

Each value (μg/g dry tissue) represents mean ±S.D of eight individual observations. Values in parentheses represent percent increase from control. 'P' is calculated between control and experimental values.



**Table II** Changes in glutathione-S-transferase activity levels in the gill and muscle tissues of *Perna viridis* during exposure to either copper or cadmium exposed to a sublethal (0.25 ppm) concentration of copper sulphate and cadmium chloride.

| | | *Exposure Time (days)* | | |
|---|---|---|---|---|
| *Metal* | *Control* | *1* | *3* | *7* |
| | | Gill | | |
| Copper | 2.05 ±0.21 | 2.81 ±0.24 (37) P<0.001 | 3.04 ±0.19 (48) P<0.001 | 3.97 ±0.31 (94) P<0.001 |
| Cadmium | 2.05 ±0.21 | 2.04 ±0.19 (−0.48) NS | 2.19 ±0.23 (6.8) NS | 2.83 ±0.32 (38) NS |
| | | Muscle | | |
| Copper | 3.16 ±0.21 | 3.91 ±0.23 (6.8) NS | 5.43 ±0.27 (48) P<0.001 | 6.09 ±0.38 (66) P<0.001 |
| Cadmium | 3.66 ±0.21 | 3.71 ±0.31 (1.4) NS | 3.60 ±0.14 (−1.6) NS | 3.79 ±0.29 (3.5) NS |

Each value (μmoles of thio-ether formed/mg protein/min) represents mean ±S.D of eight individual observations. Values in parentheses represent percent change from control. 'P' is calculated between control and experimental values.
NS: Not significant.

**Table III** Changes in glutathione peroxidase activity in the gill and muscle tissues of *Perna viridis* during exposure to either copper or cadmium.

| | | *Exposure Time (days)* | | |
|---|---|---|---|---|
| *Metal* | *Control* | *1* | *3* | *7* |
| | | Gill | | |
| Copper | 0.28 ±0.03 | 0.36 ±0.04 (29) P<0.001 | 0.40 ±0.03 (43) P<0.001 | 0.94 ±0.05 (75) P<0.001 |
| Cadmium | 0.28 ±0.03 | 0.26 ±0.03 (−7.1) NS | 0.31 ±0.04 (11) NS | 0.29 ±0.02 (3.6) NS |
| | | Muscle | | |
| Copper | 0.23 ±0.02 | 0.23 ±0.02 (21) P<0.01 | 0.29 ±0.03 (53) P<0.001 | 0.31 ±0.03 (63) P<0.001 |
| Cadmium | 0.19 ±0.02 | 0.19 ±0.03 (0.00) NS | 0.21 ±0.02 (11) NS | 0.23 ±0.03 (21) NS |

Each value (μmoles of $H_2O_2$ reduced/mg protein/min) represents mean ±S.D of eight individual observations. Values in parentheses represent percent change from control. 'P' is calculated between control and experimental values.
NS: Not significant.



**Table IV** Changes in malondialdehyde levels in the gill and muscle tissues of *Perna viridis* during exposure to either copper or cadmium.

| | | *Exposure Time (days)* | | |
|---|---|---|---|---|
| *Tissue* | *Control* | *1* | *3* | *7* |
| | | Gill | | |
| Copper | 22.91 ±4.61 | 27.31 ±4.09 (19) P<0.001 | 36.44 ±4.53 (59) P<0.001 | 38.47 ±3.97 (68) P<0.001 |
| Cadmium | 22.91 ±4.61 | 24.04 ±3.42 (4.9) NS | 25.09 ±3.21 (9.5) NS | 24.61 ±3.05 (7.4) NS |
| | | Muscle | | |
| Copper | 18.09 ±3.42 | 24.64 ±3.81 (36) P<0.001 | 29.88 ±3.47 (65) P<0.001 | 29.17 ±2.91 (61)) P<0.001 |
| Cadmium | 18.09 ±3.42 | 20.01 ±3.07 (11) NS | 19.87 ±2.88 (9.8) NS | 20.43 ±3.24 (13) NS |

Each value (nmoles/g wet tissue) represents mean ±S.D of eight individual observations. Values in parentheses represent percent increase from control. 'P' is calculated between control and experimental values.
NS: Not significant.

The results presented in this work demonstrate that exposure to sublethal concentrations of copper stimulate lipid peroxidation in the mussel tissues, as shown by the increased MDA concentration in the gill and muscle of the copper exposed mussels. On the contrary, exposure of mussels to cadmium did not significantly affect lipid peroxidation.

It has been reported that in mammalian cells, transition metal ions, such as iron and copper, may stimulate the peroxidation of polyunsaturated fatty acids by acting as catalysts in the generation of oxygen radicals (Hunt *et al.*, 1988; Auroma *et al.*, 1989). In particular, cuprous ions react with hydrogen peroxide with a much greater rate constant than do ferrous ions, giving rise to the highly reactive hydroxyl radical in the Fenton reaction (Halliwell and Gutteridge, 1984):

$$Cu^{+}+H_2O_2 \rightarrow Cu^{2+}+OH^{\cdot}+OH^{-}.$$

Homologous reaction is thought to occur with organic hydroperoxides (ROOH), leading to the formation of the more reactive peroxy ($ROO^{\cdot}$), and extremely reactive alkoxy ($RO^{\cdot}$), radicals:

$$ROOH+Cu^{2+} \rightarrow ROO^{\cdot}+Cu^{+}+H^{+}$$

$$ROOH+Cu^{+} \rightarrow RO^{\cdot}+Cu^{2+}+OH^{-}.$$



Although data on free radical generation in metal-exposed animals are not available, recent studies demonstrated that exposure to sublethal concentrations of copper stimulate lipid peroxidation in the gill and digestive gland of *Mytilus* (Viarengo *et al.*, 1990). The data suggest that the copper ions may participate both in initiation and propagation of lipid peroxidation there by generating free radicals. These free radicals are toxic and should be removed from the cell environment by the organisms (Livingstone *et al.*, 1990, 1992; Winston and Giulio, 1991; Keherer, 1993).

The increased activity levels of GST and GPX in the tissues during copper exposure may be viewed as a protective mechanism to eliminate the highly reactive free radicals. Glutathione- dependent enzymes are involved in scavenging the free radicals in the tissues thereby blocking the propagation of lipid peroxidation (Benson *et al.*, 1978; Hassan *et al.*, 1985; Viarengo *et al.*, 1988,1990, 1991)). Such a possibility could also explain that cadmium, which does not undergo redox cycling, was unable to stimulate the peroxidation process in the tissues of mussel.

Besides lipid peroxidation, these metals are also involved in inducing synthesis of metallothioneins. These soluble, SH-rich, heat stable, low molecular weight (7400 daltons) proteins are able to bind the metal in a non-toxic form. It has been demonstrated recently that copper-thioneins accumulate in the lysosomes in insoluble form due to formation of intramolecular disulphide bridges after binding to copper (Viarengo *et al.*, 1989). It is tempting to speculate that copper induced lipid peroxidation, as shown in the present study, may contribute to the accumulation of polymerized, insoluble copper-thioneins in lysosomes.

The copper is bound both to lipid peroxidation products (lipofuscin) and oxidized thioneins and is subsequently eliminated from the cell by exocytosis. These routes of

metal excretion do not seem to be active in the case of cadmium. The differential influence on lipid peroxidation might be the reason for the differential biological half-life of these metals in *Perna*, which is longer for cadmium (6 months) and shorter for copper (20 days) (author's unpublished data).

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