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Sub-lethal effects of cadmium on the antioxidant defence system of the hydrothermal vent mussel *Bathymodiolus azoricus*

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ABSTRACT

The mussel *Bathymodiolus azoricus* is one of the most abundant species in the Mid-Atlantic Ridge hydrothermal vents and is continually exposed to the high-temperature venting fluids containing high metal concentrations and enriched in sulphides and methane, which constitute a potential toxic environment for marine species. The aim of this study was to assess the effects of a sub-lethal Cd concentration on the antioxidant defence system of this mussel. *B. azoricus* were collected at Menez Gwen vent site (37°51'N, 32°31'W) and exposed to Cd (50 $\mu g\,l^{-1}$) during 24 days, followed by a depuration period of six days. A battery of stress related biomarkers including antioxidant enzymes (superoxide dismutase–SOD, catalase–CAT; glutathione peroxidases–GPx), metallothioneins (MT), lipid peroxidation (LPO) and total oxyradical scavenging capacity (TOSC) were measured in the gills and mantle of *B. azoricus*.

Cd was accumulated linearly during the exposure period in both tissues and no significant elimination occurred after the 6 days of depuration. Antioxidant enzymes activities were significantly higher in the gills. Cyt-SOD, T-GPx and Se-GPx were induced during the experiment but this was also observed in control organisms. Mit-SOD and CAT activities remained relatively unchanged. MT levels increased linearly in the gills of exposed mussels in the first 18 days of exposure. No significant differences were observed between LPO levels of control and exposed mussels. TOSC levels remained unchanged in control and exposed mussels. This suggests that although Cd is being accumulated in the tissues of exposed mussels, MT defence system is enough to detoxify the effect of Cd accumulated in the tissues. Furthermore, other factors besides the presence of Cd are influencing the antioxidant defence system in *B. azoricus*.

1. Introduction

The hydrothermal vent mussel *Bathymodiolus azoricus* are exposed to an extreme physico-chemical and potentially life threatening environment during its entire life cycle that most coastal mussels never experience, even in heavily polluted sites. Nevertheless *B. azoricus* is the most abundant and representative specie of the hydrothermal vents from North Mid-Atlantic Ridge, such as Menez Gwen, Lucky Strike and Rainbow (Colaço et al., 1998). The capacity to accumulate high amounts of metals by hydrothermal organisms, especially bivalves, has been studied almost since the discovery of these metal enriched environments back in 1977 (Cosson-Mannevy, et al., 1988; Ruelas-Inzunza et al., 2003). *B. azoricus* and other bathymodiolus mussels are no exception and they are frequently proposed as a hydrothermal model to study the effects of metals in a naturally polluted system (Cosson et al., 2008), as their coastal counterparts are used for marine and estuarine areas (e.g. Mytilus genus).

Recently, special attention has been devoted to study the effects of metals in the antioxidant defence system of *B. azoricus*. Like any other marine bivalve, *B. azoricus* was found to possess the classical antioxidant arsenal to deal with the formation of reactive oxygen species (ROS) within its tissues, including the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) (total and Se-dependant) and the

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specific activity of these enzymes are tissue and site specific following the accumulation of metals (Bebianno et al., 2005). Along with the antioxidant enzymes, it is also frequent to assess the Total Oxyradical Scavenging Capacity (TOSC), to measure the balance between antioxidant parameters and prooxidants factors. The TOSC assay measures the capability of a tissue to neutralise ROS in quantifiable terms (Regoli, 2000).

Moreover, the exposure of *B. azoricus* to metals in laboratory experiments can disturb the activity of antioxidant enzymes and enhance lipid peroxidation, a membrane level damage caused by increasing ROS inside the cells (Company et al., 2006, 2008). Cadmium (Cd) is a very toxic metal to marine organisms in general, and even though it is unable to undergo Fenton-type reactions and therefore is considered a non-redox metal, it has been associated with the enhancement of ROS (Kumar et al., 1996; Stohs et al., 2001; Watanabe et al., 2003). In a previous work, where *B. azoricus* were exposed to 100 $\mu g\,l^{-1}$ Cd in a pressurised tank for 6 days, there was a linear increase in LPO products after 24, 48 and 144 h, and Cd seems to inhibit the antioxidant enzymatic capacity of SOD and CAT (Company et al., 2006). Nevertheless, in their natural environment these organisms are constantly exposed to high metal concentrations and therefore vent species can have a higher tolerance to Cd and less susceptible to Cd toxicity. In this context there was the need to extend the exposure to this metal for a longer period to better understand the effects of Cd in the antioxidant system of *B. azoricus*. Therefore, the purpose of this study was to evaluate a sub-lethal 24 days Cd exposure in the vent mussel *B. azoricus*, specifically in regard to metal accumulation, responses of antioxidant enzymes and TOSC and evaluation of MT and LPO content.

2. Materials and methods

2.1. Sample collection

Hydrothermal mussels *B. azoricus* from Menez Gwen hydrothermal vent site (37°51'N, 32°31'W, 840 m) located in the Mid-Atlantic Ridge (MAR) south-west of the Azores archipelago, were transferred from the vent field to acoustically retrievable cages during the ATOS cruise in Summer 2001 (Sarradin et al., 2001) using the Remote Operated Vehicle (ROV) Victor6000 (IFREMER). The acoustically retrievable cages were specially developed for the collection of hydrothermal vent organisms of the VENTOX consortium at Southampton Oceanography Centre (Dixon et al., 2001) and subsequent studies have shown that mussels brought to surface in these cages experience a lower degree of stress compared to those collected using the ROV (Dixon et al., 2004). After collection, mussels were transported to the Azorean island of Faial, a journey, which took approximately 14 h, mussels were kept in chilled seawater at approximately 9 °C, their natural environmental temperature.

2.2. Sub-lethal Cd exposure experiment

Mussels acclimatized during 48 h in filtered seawater collected from the Azores coastal zone was established prior to the experiments. A total of 120 mussels (7.73 ± 0.82 cm shell length) were used in this experiment; sixty *B. azoricus* were exposed to a nominal concentration of 50 $\mu g\,l^{-1}$ Cd ($CdCl_2 \cdot H_2O$, Merck) during 24 days, followed by a 6 days period of depuration, on a land-based laboratory "LabHorta" (University of Azores). Another groups of 60 mussels were maintained in the same conditions with clean seawater (control). The exposed and control mussels were maintained at 9 ± 1 °C at atmospheric pressure. During the experiment the water was changed every two days and Cd concentration re-established. After 24 days of experiment, the Cd treated mussels were placed in uncontaminated seawater during 6 days for depuration. Ten mussels were sampled from control and Cd-exposed aquaria after 0, 6, 12, 18, 24 and 30 days. During both the acclimatizing and experimental periods the water was enriched with methane to maintain the methanotrophic symbiotic bacteria in the gills of *B. azoricus*. Ultra-pure methane (N-45 grade, Air Liquide) was supplied from pressurised gas cylinders across a 40 cm length porous rubber tube secured to the bottom of aquaria using lead-filled glass vials. Dissolved methane in the water column was measured once a day using a portable CH_4 sensor (GMI Gasurveyor 500) following the extraction of the gas. Similarly, the addition of sulphur in the tanks would help in the maintenance of thioautotrophic bacteria in the *B. Azoricus* tissues, however this element was not added to avoid metal precipitation in the water solution.

2.3. Metal analysis

Cadmium was analysed in both gills and mantle of *B. azoricus* in the soluble and insoluble fractions obtained by centrifugation (30,000 g, 30 min, 4 °C) during tissue processing for MT quantification. The total levels of Cd (total homogenate) were calculated adding soluble and corresponding insoluble levels. Metal levels were determined by Flame atomic absorption spectrometry (Z-5000 polarised Zeeman AAS Hitachi). The accuracy of the analytical procedure was checked using certified reference material that covered the range of metal levels that could be expected for *B. azoricus*. Therefore, three reference materials were used, namely defatted lobster hepatopancreas (*Homarus americanus*) (TORT-2), dogfish (*Squalus acanthias*) muscle (DORM-2) and liver (DOLT-2) from the National Research Council, Canada. Results were in good agreement with certified values. Cd concentrations are expressed as $\mu g\,g^{-1}$ dry weight tissue.

2.4. Biochemical determinations

Antioxidant enzymes were determined spectrophotometrically in the gills and mantle of mussels. Superoxide dismutase activity (SOD; EC 1.15.1.1) was measured at 550 nm (McCord and Fridovich, 1969), catalase (CAT, EC 1.11.1.6) at 240 nm (Greenwald, 1985) and Se-GPx (EC 1.11.1.9) and total GPx at 340 nm (Lawrence and Burk, 1976). Detailed techniques are described elsewhere (Bebianno et al., 2005). Since symbiotic bacteria were not separated from the gills, enzymatic activities in this tissue reflect the contributions of both host and symbionts.

Total oxyradical scavenging capacity (TOSC) was only assessed in the gills since this tissue exhibits significantly higher levels, compared to the mantle (Bebianno et al., 2005) and for this reason could indicate more easily the changes in the oxidative stress susceptibility in *B. azoricus*. TOSC was determined by the method based on Winston et al. (1998) and Regoli and Winston (1999), modified by adjusting the buffers used for marine bivalves (Regoli et al., 2000) and the technique is described in detail in Bebianno et al. 2005. Data are expressed as TOSC unit mg^{-1} protein.

Metallothioneins (MTs) were determined in both the gills and mantle according to the method described by Olafson and Sim (1979) modified by Thompson and Cosson (1984) using differential pulse polarography. The levels of MTs were expressed as $mg\,g^{-1}$ wet weight.

For the determination of total proteins concentrations, the tissues were homogenised in 20 mM Tris buffer, pH 8.6, containing 150 mM of NaCl. The homogenates were centrifuged for 30 min at 30,000*g* at 4 °C. Total protein concentrations were measured on supernatants by the Lowry method (Lowry et al., 1951) using BSA (Bovin Serum Albumin) as reference standard material. Protein concentrations are expressed as $mg\,g^{-1}$ wet weight tissue.

Lipid peroxidation was determined in the supernatant used for total proteins quantification according to the method described by Erdelmeier et al. (1998) that measures the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) produced during decomposition of polyunsaturated fatty acid peroxides of membrane lipids. The procedure is fully described in Bebianno et al. (2005). The concentration of lipid peroxidation compounds in the gills and mantle of *B. azoricus* are expressed as nmoles of MDA g^{-1} total protein concentrations.

2.5. Statistical analysis

Values are expressed as means ± standard deviation (SD). The data was previously tested for normality and homogeneity. Analysis of variance (ANOVA) was used to determine significant statistical differences between treatments regarding antioxidant enzymatic activity (SOD, CAT and GPx), TOSC levels, MTs and LPO concentrations. The level of significance was set at 0.05.

3. Results

3.1. Metal concentrations

Fig. 1 shows Cd concentrations in total homogenate, soluble and insoluble subcellular fractions from the gills and mantle of *B. azoricus* exposed to this metal for 24 days, followed by 6 days of depuration. The levels of total Cd in control organisms did not vary significantly throughout the experiment ($1.87 \pm 0.25\ \mu g\,g^{-1}$ in the gills and $0.26 \pm 0.06\ \mu g\,g^{-1}$ in the mantle) (data not showed in Fig. 1). In exposed mussels, Cd concentrations (in total