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Differential metallothionein, reduced glutathione and metal levels in *Perna perna* mussels in two environmentally impacted tropical bays in southeastern Brazil

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ABSTRACT

Mussel farming is an important economic activity in Brazil, and these organisms are consumed by the majority of the population in most coastal zones in the country. However, despite the increasing pollution of aquatic ecosystems in Brazil, little is known about the biochemical activity in mussels in response to metal exposure. In this context, the aim of the present study was to investigate metal and metalloid exposure effects in *Perna perna* mussels, by determining metal levels, the induction of metallothionein (MT) synthesis, and oxidative stress, in the form of reduced glutathione (GSH) in 3 contaminated areas from the Guanabara Bay in comparison to a reference site, Ilha Grande Bay, both in summer and winter. Metal and metalloid concentrations were also compared to Brazilian and international guidelines, to verify potential health risks to human consumers. Mussels from all sampling sites were shown to be improper for human consumption due to metal contamination, including Ilha Grande Bay, which has previously been considered a reference site. Several statistically significant correlations and seasonal differences were observed between MT, GSH and metals and metalloids in both analyzed tissues. A Discriminant Canonical Analysis indicated that the digestive gland is a better bioindicator for environmental contamination by metals and metalloids in this species and offers further proof that MT variations observed are due to metal exposure and not oxidative stress, since GSH influence for both muscle tissue and the digestive glands was non-significant in this analysis. These results show that *P. perna* mussels are an adequate sentinel species for metal contamination with significant effects on oxidative stress and metal exposure biomarkers. To the best of our knowledge, this is the first study to report metals, metalloids, MT and GSH levels in the muscle tissue of this species.

1. Introduction

The contamination of the aquatic environment by metals and metalloid species has become a global problem in the last decades. Among environmental pollutants, these contaminants are of particular concern due to their potential toxic effects and their ability to bioaccumulate in aquatic ecosystems (MacFarlane and Burchett, 2000; Miller et al., 2002). Often, the criterion used to classify a substance as harmful or not is the death (or not) of an organism. This, however, is a very extreme option, where no preventive action can be taken. Therefore, it is more accurate to measure the sublethal effects of pollutants in the biota, which arise long before death. This is conducted through the use of biomarkers (Moore et al., 2004; Van Der Oost et al., 2003; Viarengo et al., 2007).

Certain biomarkers are useful in indicating oxidative stress, which takes place in response to exposure to metal and metalloid species. Oxidative stress occurs when cellular defense mechanisms are unable to act on free radicals or on their deleterious effects. The glutathione system is one of the first lines of defense against oxidative stress, which includes reduced glutathione (GSH), in addition to antioxidant enzymes such as catalase and superoxide dismutase, being of vital importance in aquatic ecotoxicology analyses to understand and unravel the mechanisms of action of environmental contaminants. The second line of defense against

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oxidative stress is composed of metallothioneins (MT). These metalloproteins, due to their abundant cysteine residues, are also known for their protective free radical scavenging activity, playing an active role in the capture of harmful oxidant radical species (Kumari et al., 1998). This, though, has been mostly reported for mammals regarding MT in the central nervous system, while studies in aquatic organisms such as fish as fish and mussels are still scarce. Some studies in this regard with mussels have demonstrated that MT is induced in response to factors promoting oxidative stress (Viarengo et al., 1999). However, the main role investigated regarding MT is the fact that they have a high affinity for metal ions and function as both mediators of physiological metal homeostasis and the detoxification of essential and non-essential metals. The induction of this metalloprotein is, in fact, one of the main biomarkers established in biomonitoring programs in the European community (Garrigues et al., 2002; Ramos et al., 2000; Viarengo et al., 1997).

Despite the increasing pollution of aquatic ecosystems in Brazil, little is known about the biochemical response of certain organisms, such as mussels, in response to metal exposure. The Guanabara Bay is considered heavily contaminated by these pollutants (Almeida, 2003), while Ilha Grande Bay is a reasonably preserved area, with no history of environmental contamination by metals and metalloid species, and is, therefore, regularly used as a reference area for environmental monitoring studies (Cardoso et al., 2001; Junior et al., 2002; Lavradas et al., 2014).

In this context, the aim of the present study was to investigate metal and metalloid exposure in *Perna perna* mussels, by determining metal levels and the induction of metallothionein synthesis and oxidative stress, in the form of GSH, comparing these biochemical responses throughout the different sampling sites in two seasons, summer and winter. Among the different species of mussel, *P. perna* is the most cultivated along the Brazilian coast, since it reaches larger sizes, grows relatively fast, has a high production rate, is nutritious and easily collected (Baraj et al., 2003a). In addition, metal and metalloid concentrations were compared to Brazilian and international guidelines, in order to verify if the consumption of this species can bring potential health risks to human consumers due to metal bioaccumulation.

2. Methodology

2.1. Study area

The Guanabara Bay, located in the state of Rio de Janeiro, latitude 22°24′S–22°57′S and longitude 43°33′W–43°19′W (Kjerfve et al., 1997), is one of the most important coastal bays in Brazil (Amador, 1997). Several sources of pollution are present in this area, such as surrounding industries (over 6000) of which 52 are responsible for 80% of the industrial pollution of the bay, including the Duque de Caxias refinery (REDUC), marine oil terminals (16); commercial ports (2); advanced fuel stations (2000) and shipyards (32). In addition, domestic sewage, waste leakage and the occupation of public land bordering the rivers and hillsides increase the daily pollutant loads discharged into the bay (Almeida, 2003).

Ilha Grande Bay, located on the southern coast of the state of Rio de Janeiro, latitude 22°50′S–20′S and 23°45′W longitude 44°–44°00′W (Creed et al., 2007), is a very rugged bay, composed of several small bays inside, and dotted with many islands that decrease the hydrodynamics of the area. It is an important tourist region for Rio de Janeiro and is considered one of the most important areas of this state in terms of fishing productivity (Bizerril and Costa, 2001; Kehrig et al., 1998). This area has been repeatedly considered a reference area due to low environmental metal levels (Cardoso et al., 2001; Lacerda et al., 1989; Lavradas et al., 2014).

2.2. Mussel sampling

P. perna mussels were sampled from different beaches belonging to the Guanabara Bay (GB, Diabo Beach, Urca Beach and Vermelha Beach – DB, UB and VB) and from Ilha Grande Bay (IG) during the summer and winter seasons of the same year. Individuals were measured (shell length, width and height) and grouped according to size range, totalling four composite samples, each containing 10 individuals. The digestive glands and adductor muscle tissue were removed and freeze-dried for 48 h (Liotop 101, Liobrás, São Paulo, Brazil).

2.3. Metal and metalloid determinations

The freeze-dried samples were decomposed in an acid medium with subdistilled nitric acid at 100 °C for 5 h. After cooling, the volumes were adjusted with ultra pure water (resistivity $> 18\ M\Omega\ cm$) for subsequent analysis by inductively coupled plasma mass spectrometry (ICP-MS). The metals and metalloids were determined on an Elan DRC II (Perkin-Elmer Sciex, Norwalk, CT, USA) spectrometer without the use of a reaction cell. Sample introduction was conducted using a Meinhard nebulizer with cyclonic chamber and twister. The following isotopes were monitored: ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{114}Cd, ^{202}Hg, ^{208}Pb. Quality control was performed by a strict blank control, the analyses of replicates and certified reference materials. Accuracy was assessed through the analysis of certified material DORM-4 (National Research Council, Canada), composed of dog-fish muscle impregnated with known amounts of metals and trace-elements (Ni: $1.36 \pm 0.22\ \mu g\ g^{-1}$; Cu: $15.9 \pm 0.9\ \mu g\ g^{-1}$; Zn: $52.2 \pm 3.2\ \mu g\ g^{-1}$; As: $6.80 \pm 0.64\ \mu g\ g^{-1}$; Se: $3.56 \pm 0.34\ \mu g\ g^{-1}$; Cd: $0.306 \pm 0.015\ \mu g\ g^{-1}$; Pb: $0.416 \pm 0.053\ \mu g\ g^{-1}$). No statistically significant difference was observed between certified DORM-4 and observed values (Ni: $1.28 \pm 0.15\ \mu g\ g^{-1}$; Cu: $15.6 \pm 0.5\ \mu g\ g^{-1}$; Zn: $51.5 \pm 1.7\ \mu g\ g^{-1}$; As: $7.03 \pm 0.20\ \mu g\ g^{-1}$; Se: $3.63 \pm 0.25\ \mu g\ g^{-1}$; Cd: $0.310 \pm 0.012\ \mu g\ g^{-1}$; Pb: $0.410 \pm 0.05\ \mu g\ g^{-1}$). Recoveries ranged from 94% to 103% and were considered appropriate for the present study.

2.4. GSH extraction and determination

GSH extraction was performed according to Beutler (1975), with modifications introduced by Wilhelm-Filho et al. (2005). Briefly, approximately 25 mg of each sample was homogenized in 350 μL of 0.1 mol L^{-1} pH 7.0 sodium phosphate buffer containing sucrose 0.25 mol L^{-1} in an inert atmosphere (nitrogen 99.9%). The samples were then centrifuged at $11.000 \times g$ in a Mikro 220R centrifuge (Hettich, Alemanha) for 30 min at 4 °C. The supernatants were removed, transferred to sterile microtubes and subsequently treated with 0.1 mol L^{-1} DTNB in pH 8.0 sodium phosphate buffer at a 1:1 ratio. After a 15 min incubation in the dark the sample absorbances were determined at 412 nm on a UV–vis spectrophotometer (Lambda 35, Perkin Elmer). GSH concentrations were estimated using an analytical curve plotted with GSH as the external standard (Monteiro, 2006).

2.5. MT extraction and determination

MT was extracted according to the thermal procedure described by Erk and collaborators, using tris-2-carboxyethyl-phosphine (TCEP) as the reducing agent (Sigma-Aldrich, São Paulo, Brazil) (Erk et al., 2002; Getz et al., 1999; Tenório-Daussat et al., 2014). Briefly, the samples were homogenized in sterile polypropylene microtubes at a 1:20 ration in a buffer solution containing Tris–HCl, 20 mmol L^{-1} pH 8.6, phenyl methyl sulfonyl fluoride 0.5 mmol L^{-1} and 0.01% TCEP. The samples were then