Studii şi Cercetări Ştiinţifice 2012, **13** (4), pp. 377 – 386
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

MULTIPLE BIOMARKER RESPONSE IN THE MUSSEL, *PERNA PERNA* TO ASSESS THE MARINE QUALITY IN THE BIG CASABLANCA AREA

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Received: November, 25, 2012
Accepted: December, 19, 2012

Abstract: The aim of this study is to assess the marine environment quality in the Big Casablanca area. A number of biochemical markers were measured in the brown mussel, *Perna perna*, sampled from four sampling sites characterized by a different degree of contamination and human impacts. As biochemical indices; Catalase (CAT), Glutathione S-transferase (GST), Acetylcholinesterase (AChE), as well as Malondialdehyde (MDA) and Metallothioneine (MT) were evaluated in whole soft tissues of mussels collected from the selected sites. The biomarkers showed statistically significant differences at the polluted sites when compared to the control ones. Our data indicated that CAT and GST activity, MDA and MT concentration in whole mussel bodies, are a higher and significant ($p < 0.05$) in mussels collected at polluted sites when compared to specimen sampled from control ones. In contrary the response of AChE activity was significantly ($p<0.05$) inhibited in mussels from polluted sites when compared to control values. The multiple biomarker responses obtained for October 2010 and 2011, clearly demonstrate the potential presence of different contaminants in Site1 and Site2 reflecting the intensity of pollution in these areas.

Keywords: *acetylcholinesterase, big casablanca area, biomarker responses, catalase, glutathione s-transferase, malondialdehyde, metallothioneine, mussels, Perna perna, pollution*

INTRODUCTION

Bivalve molluscs, particularly marine mussels, have been used as indicator organisms in many studies to monitor environmental pollution in coastal waters [1, 2] due to their wide distribution, sedentary lifestyle, tolerance to a large range of environmental conditions and because they are filter feeders with very low metabolism which allows the bioaccumulation of many chemicals in their tissues [3]. The mussel *Perna perna*, which is abundant along the Moroccan coast, has all the desirable characteristics of a potential biomonitor, and it has been choosing as the sentinel species for this study.
The biological responses to environmental pollution can be measured at various levels of biological organization (from molecular to community structure). Among these biological responses is Catalase (CAT), a well-known antioxidant enzyme, which converts H_2O_2 into water. The biological importance of CAT is more evident from various studies due to the fact that H_2O_2 is the main cellular precursor of the hydroxyl radical (HO^{-}) which is a highly reactive and toxic form of Reactive Oxygen Species (ROS) leading to oxidative damage to basic biological molecules. Toxicity biomarkers, such as Malondialdehyde (MDA), well-known lipid peroxidation products, have been also proposed to reflect the oxidative status of exposed species [4]. MDA is used as marker of oxidation of membrane phospholipids through lipid peroxidation [5]. Glutathione S-transferase (GST) which is a phase II enzyme involved in the metabolism of lipophilic organic contaminants. GST catalyzes the conjugation of various electrophilic compounds (e.g. epoxides of PAHs) with the tripeptide glutathione, the resulting conjugates being water soluble and thus more easily extractable. Acetylcholinesterase (AChE) is an enzyme essential to the correct transmission of nerve impulses. Its inhibition is directly linked with the mechanisms of toxic action of anticholinesterase compounds [6 – 8]. Metallothioneine (MT) are useful metal-pollution biomarkers [9], it constitutes a family of low molecular weight, cysteine-rich, and metal binding proteins that occurs throughout the animal kingdom. Biological functions of MT include homeostasis and sequestration of both essential and nonessential metals, detoxification of metals and scavenging of free radicals [9, 10].
This work aims to study the responses of a battery of biochemical biomarkers in the brown mussel *Perna perna* to assess the marine environment quality in four sites along the Moroccan Atlantic coast (Big Casablanca).

MATERIALS AND METHODS

Reagents

Hydrogen peroxide (H_2O_2), Thiobarbituric Acid (TBA), Acetylthiocholine (AtChl) and Tetramethoxypropane (TMP) were obtained from Sigma (Saint Quentin Fallavier, France). 1-chloro-2,4-dinitrobenzene (CDNB), 5,5' dithio-bis 2 nitrobenzoic acid (DTNB), Reduced Glutathione (GSH), and Bovine serum albumin (BSA) were purchased from Genome Biotechnologies (Casablanca, Morocco).

Studied areas

For this study, four stations are selected attending to various degree of human impact. Site 1 (S1) is located in Aïn Sebaâ beach. The Site 2 (S2) is located approximately 7 km in South of Mohammedia beach. Due to intense human activities, the S1 and S2 are constantly subjected to contamination. In addition, S1 and S2 have low biodiversity of intertidal organisms, indicating high levels of pollution pressure. On the contrary the Site 3 (S3), which was selected due to the absence of contamination sources, is located in the north of Mansoria beach. The Site 4 (S4) constitutes the site furthest away from the polluting industrial activities established on the coastal fringe Casablanca-Mohammedia. S4 is located in beach of the south, Skhirat which is characterised by a total prohibition of human activities. S3 and S4 are characterized by an important density of mussels and the high faunistic and floristic richness of the site are well marked.

Sampling

Ten mussels from each site were sampled during October 2010 and 2011. Following collection, the adult mussels were placed in thermally insulated boxes previously filled with water from the sampling site and immediately transported to the laboratory and stored at -80 °C until analysis.

Biochemical analyses

Whole soft tissues from each specimen (n=5 for each station) were dissected out and immediately homogenized (1:3) in phosphate buffer 100 mM, *p*H 7.4. Homogenates were then centrifuged at 9000×g at 4 °C for 30 min. After centrifugation, supernatants were collected and immediately used for the determination of enzymatic activity, MDA and MT concentration. CAT activity was measured following the decrease of absorbance at 240 nm due to H_2O_2 consumption [11]. The reaction takes place in 100 mM phosphate buffer, *p*H 7.4 containing 500 mM H_2O_2. GST activity was assayed by the method described by Habig *et al.* [12] using the CDNB as substrate, and 1 mM GSH, in 100 mM sodium phosphate buffer, pH 7.4. GST activity was determined by kinetic measurement at 340 nm. AChE activity was determined according to the method described by Ellman *et al.* [13] using 8 mM DTNB, and 45 mM AtChl as substrate in 100 mM sodium phosphate buffer, *p*H 7.4. AChE activity was determined by kinetic measurement at 412 nm. MDA was estimated according to the method described by Sunderman [14] with use of TMP as a standard. The reaction was determined at 532 nm, using TBA as reagent. MT content was evaluated in whole soft tissues according to a spectrophotometric method described by Viarengo *et al.* [15]. Tissues (n = 5 for each station) were homogenised (1:3) in Tris Buffer (Tris 20 mM, 0.5 M sucrose, pH 8.6) containing 0.5 mM phenylmethylsulphonyl fluoride and 0.01% β-mercaptoethanol. The soluble fractions containing MT were obtained by centrifuging the homogenate at 10000 g for 30 min. The supernatant was then treated with cold absolute ethanol and chloroform. Finally, MT content was spectrophotometrically determined at 412 nm using Ellman's reagent (DTNB) and GSH as standard. Protein concentrations were measured according to the Bradford [16] method, at 595 nm using BSA as standard.

Statistical analyses

The results for biomarker measurements were investigated by the use of a parametric one-way analysis of variance (ANOVA) and level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Results

The marine quality in Big Casablanca is checked in October 2010 and 2011 at four sites by analyzing a battery of biochemical biomarkers of pollution in whole mussel bodies. The biomarker responses obtained for each site studied are presented in Figure 1.

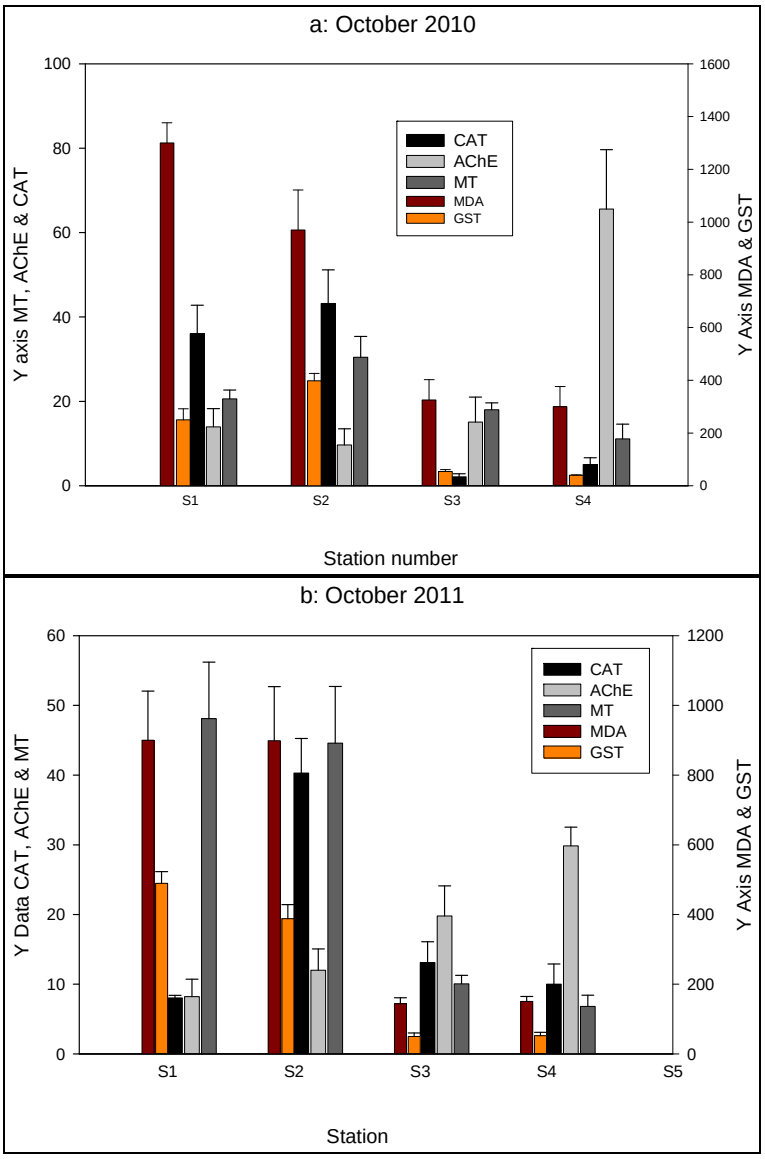

Figure 1. *Activities of CAT, GST, AChE, and the levels of MDA and MT in Perna perna collected from the studied areas*

The results relative to the biomarkers responses are reported in Figure 1. Measurements from sites S1 and S2 showed significant increased ($p<0.05$) CAT activity in October 2010 with value up to 36.06 and 43.2 μmol·min^{-1}·mg^{-1} protein, in S1 and S2 respectively, compared to mussels from S3 and S4 (2.14 and 5.01 μmol·min^{-1}·mg^{-1} protein, respectively). Our results for CAT activity in October 2011 showed a higher level in Site 2 ($p < 0.05$) with 40.3 μmol·min^{-1}·mg^{-1} protein, whereas the mussels from the Site 1 had much lower levels of CAT activity (8.01 μmol·min^{-1}·mg^{-1} protein**)**.
As shown in Figure 1, a higher and significant ($p<0.05$) accumulation of MDA in October 2010 and 2011was registered in *Perna perna* collected at S1 and S2 when compared to specimen sampled from S3 and S4 (MDA content was expressed as nmol·mg^{-1} proteins).
The results indicate that AChE activity in October 2010 was significantly ($p<0.05$) inhibited in mussels from S1, S2 and S3 with a value reaching to 13.98, 9.67 and 15.1 nmol·min^{-1}·mg^{-1} protein respectively when compared to control value (65.56 nmol·min^{-1}·mg^{-1} protein). Our results for AChE activity in October 2011 showed a trend for higher levels in Site 4 (29.86 nmol·min^{-1}·mg^{-1} protein), whereas smaller increases were detected in Site 3 (19.77 nmol·min^{-1}·mg^{-1} protein). By comparison the mussels from the S1 and S2 had much lower levels of AChE activity (8.22 and 11.99 nmol·min^{-1}·mg^{-1} protein respectively).
A higher and significant ($p<0.05$) values of GST activity in October 2010 and 2011 was registered in *Perna perna* collected at S1 and S2 when compared to specimen sampled from S3 and S4 (GST activity were expressed as nmol·min^{-1}·mg^{-1} proteins).
Our data indicated that the levels of MT in October 2010 showed a higher levels ($p<0.05$) in Site 2 (30.45 μg·mg^{-1} proteins), whereas the mussels from the Site 4 had much lower levels of MT (11.1 μg·mg^{-1} proteins). Measurements from S1 and S2 showed significant increased ($p<0.05$) MT in October 2011 (48.11 and 44.59 μg·mg^{-1} proteins respectively) compared to mussels from S3 and S4 (10.04 and 6.79 μg·mg^{-1} proteins respectively).

Discussions

Antioxidants represent the cellular defense mechanisms which counteract toxicity of reactive oxygen species (ROS), these mechanisms have been extensively investigated in sentinel organisms such as marine mussels [17 – 22]. CAT, as the first line of antioxidant defense, is very responsive to increasing levels of contaminant stimulated ROS production. The present study illustrated that mussels collected at S1 and S2 in October 2010 presented significantly higher levels of CAT activity in the whole soft body than those collected at S3 and S4. Previous studies showed that antioxidant enzymes [21, 23 – 25] also demonstrated higher activity values in response to contaminants. Considering that the induction of antioxidant enzymes represents a protective response to eliminate ROS resulting from contamination exposure, it has been hypothesized that such increase may be related to adaptations to contaminant induced stress [24, 26]. The presence of organic and metal contaminants is a possible source of oxidative stress and could induce variations in antioxidant enzyme activities [27]. Higher variability of different antioxidant enzyme in populations of aquatic organism from polluted and unpolluted areas has been observed [26, 28]. However, the induction of antioxidant enzyme activity due to the presence of high levels of

contaminants in the environment should not be considered as being a general rule, since a considerable variation of responses has been found among different species, following exposure to single or complex mixture of contaminants [26]. For example, under laboratory conditions, some authors have reported a decrease in antioxidant enzyme activities following a short-term exposure of mussels to several pollutants [29 – 30]. Others suggested that if mussels were under intense pollution degree, antioxidants appear to be overwhelmed and the antioxidant enzyme activities show a progressive decrease up to a severe depletion [28, 31] Therefore the inhibition of the CAT activity in October 2011 observed in S1 compared with reference confirms the presence of an oxidative stress that may affect mussels. Other studies have demonstrated that when invertebrates are subjected to oxidative stress, a series of defense mechanisms start to protect the organism [28, 32]. Nevertheless, when this stress increases, an inhibition of enzyme activity, such as CAT, has been found [28]. This inhibition/decrease in antioxidant defenses indicates the difficulty that the organisms have in defending against oxidative stress.

The toxicity effect of ROS can produce various damages to the cell, such as DNA damage, lipid peroxidation and lysosomal alteration [21, 33 – 36]. Lipid peroxidation is a well-known mechanism of cellular injury in vertebrates and invertebrates, and is an indicator of an oxidative damage in cells and tissues. Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation [37]. An increase in MDA concentration is found in S1 and S2. Several studies have evidenced that lipid peroxidation increases in tissues of different species of aquatic organisms, as result of being exposed to environmental pollutants [32].

Significantly higher MDA levels were found in mussels collected at S1, and S2, despite the high activity levels of CAT. Nevertheless the anti-oxidant enzyme was unable to prevent the deleterious effects on the lipid membranes (as reported in some cases by Pellerin-Massicotte) [38]. This may be due to the fact that low levels of contaminant-stimulated ROS can have a significant toxic effect, particularly upon the cell membrane and DNA, even when antioxidant enzymatic defenses are responding [30]. According to Regoli [35], when the antioxidant defenses are overwhelmed by the generation of ROS, oxidative damage (lipid peroxidation, protein degradation, enzyme inactivation) and oxidative stress occur.

The main physiological function of AChE is splitting of acetylcholine, a mediator of cholinergic synapses, during transduction of nerve impulses [39]. The inhibition of AChE activity has been widely used to diagnose exposure to anticholinesterase compounds [6 – 8]. In our study, we demonstrate that mussels in S1 and S2 present a higher inhibition of AChE activities compared to the site 3 and 4. The observed inhibition of AChE activities may be attributed to the presence of contaminants in the environment. Many studies indicate that cholinesterase activities are inhibited in the presence of some pesticides [40, 41]. In fact, in addition to anticholinesterase pesticides, a few other contaminants such as heavy metals, detergents, some pyrethroids compounds like cypermethrin and deltamethrin and complex mixtures of pollutants can also affect the AChE activity [42, 43].

The glutathione-*S*-transferases are a superfamily of dimeric, multifunctional enzymes [44]. Apart from their essential functions in intracellular transport (hem, bilirubin, and bile acids) and the biosynthesis of leukotrienes and prostaglandins, a critical role for GSTs is obviously the conjugation of various electrophilic compounds (e.g. epoxides of

PAHs) with the tripeptide glutathione, the resulting conjugates being water soluble and thus more easily extractable. The toxicity of many exogenous compounds can be modulated by induction of GST. Indeed GST activities were found to be modulated by metals or organic contaminants under both field conditions [45, 46] and laboratory exposure [47]. In this work, a significant increase of GST activity was observed in S1 and S2. The induction of GST activity can be regarded as an adaptive response to an altered environment. Several other field studies have demonstrated a similar relationship between environmental contamination and GST activity in mussels [24, 48].
Metallothioneins are low molecular weight cytosolic proteins rich in SH groups, with a high affinity for IB and IIB group metal ions (such as Zn, Cu, Cd, and Hg), known to be involved in heavy metal homeostasis and overexpressed in organisms experiencing high metal concentrations in their environment [49 – 51]. Their expression in tissues is therefore regarded as an indicator of metal contamination and widely used as a tool for biomonitoring programs Viarengo *et al.* (1999) [50]. Indeed the induction of MTs as a measure of response to metal exposure in aquatic organisms has been widely investigated in laboratory and field conditions [52, 53]. In this work, we have found that MT accumulation level is higher in mussels from S1 and S2 in comparison to S3 and S4. The elevated MT level at the site S1 and S2 may be considered as the result of higher overall level of metals pollution.

CONCLUSIONS

The aim of this work was to study the responses of a battery of biochemical biomarkers in *Perna perna* collected from four stations along the Moroccan Atlantic coast (Big Casablanca area). In this study the biomarker responses obtained for October 2010 and 2011, clearly demonstrate the potential presence of different contaminants in S1 and S2 reflecting the intensity of pollution in these areas.

ACKNOWLEDGMENTS

Authors thank the CNRST for financial support available to students.

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