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Multimarker approach analysis in the brown mussel to evaluate the anthropogenic stress: A preliminary study

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Abstract
In this study, a suite of biochemical biomarkers, possible signs of stress, were applied to the mussel *Perna perna* collected from various areas of the Big Casablanca, to verify the pollution status in the referred areas. A battery of biochemical biomarkers, extensively used worldwide, was assessed in the whole body. As biochemical indices; Catalase (CAT), Glutathione S-transferase (GST), Acetylcholinesterase (AChE), as well as Malondialdehyde (MDA) and Metallothioneine (MT). All biomarkers showed statistically significant differences at the polluted sites when compared to the control ones. In fact, our data indicated that CAT and GST activity, MDA and MT concentration in whole mussel bodies, are more significant ($p < 0.05$) in the mussels collected at polluted sites than specimen samples 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 January 2010 and December 2011, clearly demonstrate the potential presence of different contaminants in Site 1 and Site 2 reflecting the intensity of pollution in these areas.

Keywords: Multimarker approach, Catalase, Malondialdehyde, Glutathione S-Transferase, Acetylcholinesterase, Metallothioneine, Mussels, Perna perna, Marine pollution, Anthropogenic stress.

Introduction
Invertebrates, particularly bivalve molluscs such as mussels are very suitable organisms for studying the biological effects of pollutants. They are sessile, filter-feeding, resistant, easy to collect, widely distributed and abundant in coastal and estuarine areas and able to accumulate several classes of pollutants, thus providing a time-integrated picture of their bioavailability. For such characteristics, these organisms are widely used in biomonitoring programs, in which chemical analyses are integrated with the use of biomarkers, to evaluate molecular, biochemical and cellular effects induced by pollutants [1]. 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. It should be noted that *Perna perna* have been used as indicator organisms in many studies to monitor environmental pollution in Moroccan coastal waters [2-5]. An important role in environmental toxicity of both metals and organic contaminants is assumed by the enhancement of intracellular reactive oxygen species (ROS). The reactivity of ROS is normally counteracted by antioxidants such as catalase (CAT) and changes the levels or activities of these defenses that have been proposed as biomarkers of contaminant-mediated prooxidant challenge [6]. An impaired capability to neutralize ROS can anticipate the appearance of oxidative damages at cellular level as widely demonstrated. Malondialdehyde (MDA) is a product of lipid peroxidation it is a result of oxidative damages of the cell membrane. The MDA level which is proportional to the extent of lipid peroxidation [7], serves as a marker for oxidative stress. Glutathione-S-transferase (GST) is a well-known Phase II detoxification enzyme catalyzing the initial step of mercapturic acid synthesis and the conjugation of glutathione with xenobiotics and their metabolites such as the alkyl transferase and epoxide transferase, detoxifying PAH epoxide produced by P450 [8]. GST has been used as a biomarker of exposure to anthropogenic organics [9]. Acetylcholinesterase (AChE) is an enzymatic biomarker of neurotoxicity, and responsible for acetylcholine degradation. AChE activity is inhibited in the presence of pesticides such as organophosphorus compounds and carbamates and some heavy metals in mussels [10]. This biomarker has been extensively used in coastal biosurveillance programs [10-15].

Metallothioneines (MT) are proteins that have been identified in various vertebrate and invertebrate tissues. They are involved in the regulation of essential metals such as Cu and Zn and the detoxification of nonessential ones (Cd, Hg, Ag) [16]. MT are useful metal-pollution biomarkers [17].
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).

2. Materials and methods

2.1. 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).

2.2. Sampling sites and mussel handling

Perna perna was sampled during January 2010 and December 2011 in four differently contaminated sites of the Big Casablanca: S1 and S2, were both impacted by pollution (the first is located in Aïn Sebaâ beach, the second is located in Mohammedia beach), and S3 and S4, were both used as references (the third is located in Mansoria beach, the fourth is located in Skhirat beach). At each site, 10 mussels were collected at low tide. The mussels were immediately placed in coolers (kept at 4°C) for transport to the laboratory. It were then shipped to the laboratory and stored at -80°C until analysis.

2.3. Preparation of homogenate fractions and 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, pH 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 activities and MDA concentrations.
CAT activity was measured following the decrease of absorbance at 240 nm due to H_2O_2 consumption [18]. The reaction takes place in 100 mM phosphate buffer, pH 7.4 containing 500 mM H_2O_2. GST activity was assayed by the method described by Habig and al. [19] using 1 mM CDNB and 1 mM GSH as substrate, 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 and al. [20] using 8 mM DTNB and 45 mM AtChl as substrate in 100 mM sodium phosphate buffer, pH 7.4. AChE activity was determined by kinetic measurement at 412 nm. MDA was estimated according to the method described by Sunderman [21] 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 and al. [22]. 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 [23] method, at 595 nm using BSA as standard.

2.4. 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 $p < 0.05$. *Perna perna* mussels collected from different stations located along costal shores of grand Casablanca were homogenized individually and biomarkers was determined as indicated. Data are expressed as average values from 5 mussels (±SD). Statistically significant differences with respect to S1 and S2 stations are indicated (ANOVA, * = $p \leq 0.05$).

3. Results and discussion

The biomarker responses obtained for each studied site are presented in Fig. 1. The spatial biomarker responses obtained in January 2010 showed generally a similar pattern to December 2011. Measurements from sites S1 and S2 showed significant increased ($p < 0.05$) CAT activity compared to mussels from S3 and S4 (CAT activity was expressed as μmol min^{-1} mg^{-1} protein).
As shown in Fig. 1, a higher and significant ($p < 0.05$) accumulation of MDA was 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 proteins). The results indicate that AChE activity was significantly ($p<0.05$) inhibited in mussels from S1 and S2 when compared to controls value (AChE activity was expressed as nmol $min^{-1}mg^{-1}$ protein).
A higher and significant ($p < 0.05$) values of GST activity 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/mg proteins).

Our data indicated that the mussels from S1 and S2 showed significant increased ($p < 0.05$) MT compared to mussels from S3 and S4 (MT content was expressed as µg mg^{-1} proteins).
Identical spatial variability of biomarkers in *perna perna* species from polluted and unpolluted areas has been observed. In this context, Kaaya [2] and Bouhaimi [3] recorded high levels of CAT and GST activities, as well as a significant higher MDA concentration in *Perna perna* mussel tissues exposed to contaminated stations (Agadir Bay, Morocco), while AChE activity showed low values compared to the reference site. These results confirmed those found in *Perna perna* mussels [4] sampled from the same study sites.

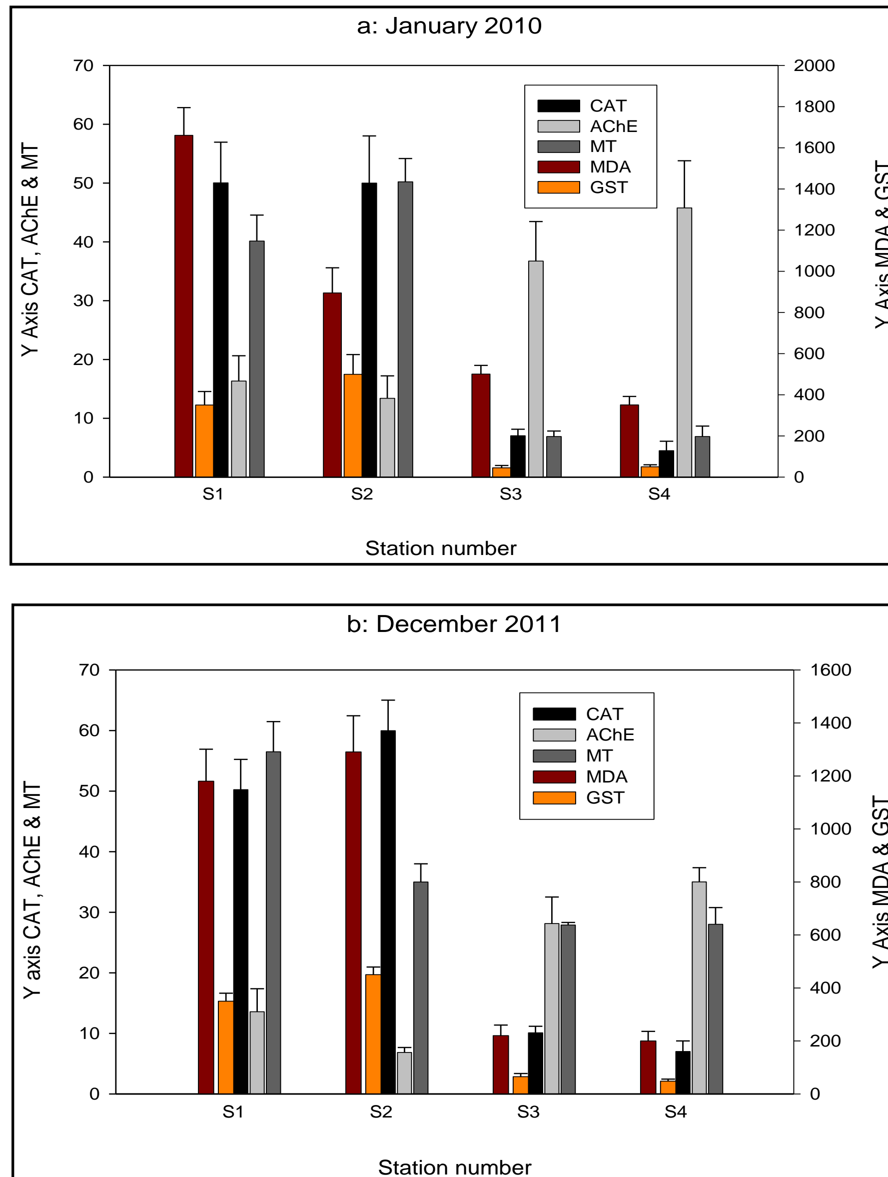

Figure 1: Activities of CAT, GST, AChE, and the levels of MDA and MT in *Perna perna* collected from the studied areas.

CAT response showed higher activities in mussels from the S1 and S2 compared to those collected at S3 and S4. Previous studies showed that CAT [24-26] had also demonstrated higher activity values in response to contaminants. It is well known that organic compounds are possible sources of oxidative stress which can bring changes into antioxidant enzyme activities. However, the antioxidant enzyme activities may also be induced by metals as it was reported by numerous studies [27]. The overall significance of such responses was reflected by an increased capability to neutralize peroxyl or hydroxyl radicals, thus indicating a more integrated unbalance of oxyradical metabolism [28,29]. Considering that the induction of antioxidant enzymes represents a protective response to eliminate ROS resulting from contamination exposure, it has been hypothesised that such increase may be related to adaptations to contaminant induced stress [30,31].

An increase in MDA concentration was found in S1 and S2. Several studies showed evidence of lipid peroxidation increases in tissues of different species of aquatic organisms, as result of being exposed to environmental pollutants [32]. It is known that lipid peroxidation in mussel can be stimulated via oxidation of polyunsaturated fatty acids, not only by various inorganic cations as Cu, Cd, Ag and Hg [33, 34] but also by PCBs and PAHs [35,36].

Higher inhibition of AChE activity at S1 and S2 than at site 3 and 4 was noted. Many studies indicate that Cholinesterase activities were inhibited in the presence of some pesticides [12,37]. In fact, AChE has a fundamental role in the nervous system of both vertebrates and invertebrates, and its inhibition is considered a typical effect of organophosphate and carbammate pesticides [38]. Similar effects may be caused by other factors which are known to modulate this enzymatic activity, including trace metals (cadmium, copper, mercury, zinc) [27,39,40]. The observed inhibition of AChE activities may be attributed to the presence of contaminants in the environment. The same inhibitions were observed in *Perna perna* mussels [3-5] originating from stations of the Agadir Bay (South-Western Morocco) contaminated by PAHs [41] and metals [42] such as Fe, Zn, Cd, and Cu.

Mussels at S1 and S2 had significantly higher GST levels than those at S3 and S4. The assessment of GST activity after exposure to certain pollutants has already been demonstrated both in fish and invertebrates [9, 30, 43]. Thus, several authors have proposed the use of these enzymes as indicators or biomarkers of exposure to pollution. 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 [44, 45] and laboratory exposure [46]. For example laboratory and field studies reported a strong GST induction in fish and mussels exposed to PCBs commercial mixtures or pure congener. The induction of GST activity can be regarded as an adaptive response to an altered environment.

An increase in the GST activity has also been observed in *Perna perna* (Agadir Bay, Morocco) tissues exposed *in situ* to organic compounds, such as PCBs and PAHs [2, 4].

Inter-site differences were well marked for MT concentrations in mussels. MT concentrations were significantly higher in mussels from S1 and S2 than in those from S3 and S4. The biological role of MTs is still a topic of discussion but it is generally admitted that they play a role in the homeostasis of essential metals and in the detoxification of toxic metals. In fact metals are the best known and the most potent inducers of MT biosynthesis [47,48]. MT induction as a response to metal exposure is well documented, and thus MTs have been proposed as biomarkers of trace metal pollution in numerous species from different zoological groups [48]. The elevated MT level at the site S1 and S2 may be considered as the result of high overall level of metals pollution.

It is obvious that Atlantic Casablanca–Mohammedia area is the most important in Morocco because of a high population and intensive industrial development. In fact, the Atlantic coastal belt contains 39% of the industrial activities of Morocco. But the most of these activities uses the sea for final disposal of untreated wastewater [49] and contributes to the contamination of the marine environment with a large and varied contaminant load. In this context, many studies [50-52] indicated high toxic metals and organic compounds concentrations in mussel tissue, seawater and around the sewage effluent of Mohammedia and Casablanca cities. Indeed, the mussels from Ain Sebaâ (Casablanca) exhibited high levels of heavy metals such as Hg [53], Pb [54] and Cr [55] as well as high values of hydrocarbons and PCBs. Similarly, physico-chemical analyzes of effluents carried out regularly by INRH (Casablanca) showed high concentrations not only by metals but also by organic compounds [56]. In the *Perna perna* mussel sampled along the Mohammedia coast, high levels of Pb [54] and Hg [53] were also revealed in stations heavily polluted by industrial and domestic discharges of the region. In Ouled Hmimoune beach (Mohammedia), the Hg levels in mussels have reached significant concentrations with a methyl mercury percentage (most toxic form) of 20% [57].

Basing on the results of this study, the ANOVAs dataset generally indicated that the temporal evolution of biomarkers was generally found to be related to season. The annual physiological cycle of the species, the period of food availability, the sea water temperature and other factors, probably explains the variability in biomarker responses of the model organisms.

As GST activity showed no seasonal variability, it was therefore slightly affected by natural variables. As consequence, GST activity could be considered the most reliable biomarker, and thus the more appropriate compared with other biochemical parameters to assess and bio monitor the coastal waters quality of the region.

Conclusion

In the present study, the levels of CAT, GST, AChE, MDA and MT in *perna perna*, clearly demonstrate the potential presence of different contaminants in S1 and S2 reflecting the intensity of pollution in these areas. Several works involving integrated studies of biomarkers as a function of natural variables and chemical mixtures in specific natural habitats, and this would appear to be the most suitable approach for monitoring of environmental stress. Future investigations should get more insights in the seasonal variability of biomarkers in mussels living under environmental constraints typical for the temperate waters, to support a different response to natural factors in coastal from those due to anthropic stress.

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