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Research Paper

Metabolism and Oxidative Stress Biomarkers in Clam *Ruditapes decussatus* to Assess Pollution in Industrial Area of Mediterranean Lagoon

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Abstract: *Coastal lagoons are complex and dynamic ecosystems, characterized by a constant fluctuation of environmental conditions. Due to urban development, industrialisation and tourism, lagoons are mainly considered as extensively modified and threatened ecosystem. The purpose of this study is to assess the efficiency of a multibiomarker approach in clam Ruditapes decussatus sampled seasonally from industrial area of Tunis lagoon. Control samples were collected at the reference site "Louza". Benzo[a]pyrene hydroxylase (BPH) and Glutathione-S-transferase (GST) activities were used as a phase I and phase II detoxification enzyme. Catalase (CAT) activity and lipid peroxidation were analyzed as oxidative stress markers. Lipid peroxidation was assessed using malondialdehyde (MDA) accumulation. Results showed a significant increase of all biomarker levels compared with controls. Moreover, the responses of four biomarkers fluctuated over time. BPH and GST activities showed a progressive increase from winter to autumn. CAT activity reached a maximum value in spring; while MDA concentration recorded a maximum value in summer. Seasonal variation of biomarkers arises from a complex interaction between exogenous and endogenous factors. These results confirm the efficiency of the multibiomarker approach used in this study for the detection of biologic responses in Ruditapes decussatus to environmental pollution and to confirm the presence of anthropogenic contaminants in the area of study, which essentially due to the industrial rejections.*

Keywords: Multibiomarker approach; environmental risk assessment; *Ruditapes decussatus*; Tunis lagoon, seasonal variation.

Introduction

Littoral ecosystems are constantly threatened by pollution due to their proximity to human settlements with their associated agricultural and industrial activities. The rapid increase of anthropogenic activities has lead to a continual influx of both organic and inorganic xenobiotics into estuaries and coastal waters, including polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), organophosphorus compounds and heavy metals. Aquatic organisms are able to accumulate in their tissues a wide range of both organic and inorganic compounds, and this feature has been used to assess the concentration of selected chemicals present in the environment. However, even though chemical analyses of tissues concentrations are able to detect a wide range of contaminants, this approach does not provide any indication of biological significance and deleterious effects on the biota[1].

Moreover, the complex mixture of contaminants present in the marine environment contains substances for which chemical analyses are not yet available or are extremely expensive. Thus, there is a need to develop strategies allowing assessment of whether a given environment is under stress or not. Therefore, techniques for measuring biological effects are critical for any pollution monitoring program[2].

The presence of toxic compounds can be detected more quickly and specifically by measuring an organism's response to a pollutant at the biochemical or physiological levels, allowing earlier identification of change, before deleterious effects reach higher organization levels[3]. The application of biomarkers under field conditions has been proposed by many authors in order to assess chronic responses in aquatic populations exposed under environmental realistic conditions[4,5].

The potential use of multibiomarker approach for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during the recent years[4,6,7]. Several field studies have proved to be successful using a multibiomarker approach in invertebrates[8-10] and nowadays this is a recommended tool for pollution monitoring studies within many governmental institutions such as MAFF, IFREMER, UNEP, OECD, EU projects (e.g. BEEP), and it has been adopted by international conventions OSPAR, ICES[11].

The mechanism of detoxification of organic pollutants involves a suite of enzymes mainly enclosed in phase I (or functional reactions) and phase II (or conjugative reactions) of biotransformation. The first stage in metabolism of many organic compounds is mediated by the cytochrome P450 dependent mixed-function oxygenase (MFO) system which is involved in the metabolism of many xenobiotics and the induction of the activities of some related enzymes as benzo(a)pyrene hydroxylase (BPH) and NADPH cythocrome c (P450) reductase could be indicative of the presence of these chemicals[12,13]. Several field studies have shown direct relationships between the induction of MFO components and environmental PAHs and PCBs concentrations[14].

Glutathione S-transferases (GST) are enzymes from a family of multi-functional proteins involved in the phase II detoxification processes that play a fundamental role in protection against endogenous and exogenous toxic chemicals[15]. In *Ruditapes decussatus* at least 7 GST isoforms have been described that catalyse the conjugation of the substrate 1,chloro-2,4 dinitrobenzene (CDNB) with GSH; this in vitro co-substrate is currently used for the evaluation of the level of whole GST isoforms as biochemical biomarker[16]. This enzyme is used as biomarkers of several groups of pollutants including organochlorine pesticides, PCBs and petrochemical products in invertebrates[17-19].

The cytochrome P450 system is often associated with redox cycling mechanisms that generate oxyradicals[20,21]. These reactive oxygen species (ROS) are detoxified and controlled by antioxidant defenses which include several antioxidant enzymes such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPx). When antioxidant defenses are unable to cope with the generation of oxyradicals there is an imbalance between the production and removal of oxidants, a situation known as oxidative stress[20,21]. Oxidative stress includes oxidative damage, enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (LPO)[22]. Lipid peroxidation is considered a major mechanism, by which oxyradicals can cause tissue damage, leading to impaired cellular function and alteration in physicochemical properties of cell membranes, which in turn disrupt vital functions[23].

Over the years, some marine bivalves, particularly mussels and clams, have been widely and successfully used as bioindicators for pollution monitoring in many coastal environments[24-26]. Filter-feeding invertebrates like mussels and clams live abundantly in the intertidal zone and tolerate a wide range of environmental conditions[27]. Many studies have demonstrated that several bivalve species have a great ability to accumulate organic pollutants and heavy metals and, thus, can be used as sensitive in situ indicators for pollution assessment[28-30].

To our knowledge there are no recent works interested in biomarkers responses in marine organisms from Tunis lagoon. In addition, this study is the first to focus on seasonal monitoring of pollution in Tunis lagoon through a multibiomarker approach. The present work aims to assess the marine environment quality in the industrial area of Tunis lagoon using a battery of biomarkers (metabolism and oxidative stress biomarkers) during four seasons.

Material and Methods

Sampling

Located in northern Tunisia, between Tunis and La Goulette, Tunis lagoon consists of a north lagoon, a south lagoon, and a navigation canal. An industrial zone is located near the navigation canal (Figure 1). Clams *R. decussatus* of similar size (n = 30) were seasonally collected at the industrial area of Tunis lagoon (36°48'02.2"N 10°16'55.5"E), during one year and transported to the laboratory, where their digestive glands were isolated and stored at –80 °C. Control clams were collected from Louza site (35°01'11.1"N 11°00'24.6"E), which has been considered as a reference site in monitoring programs along the Tunisian coasts[31].

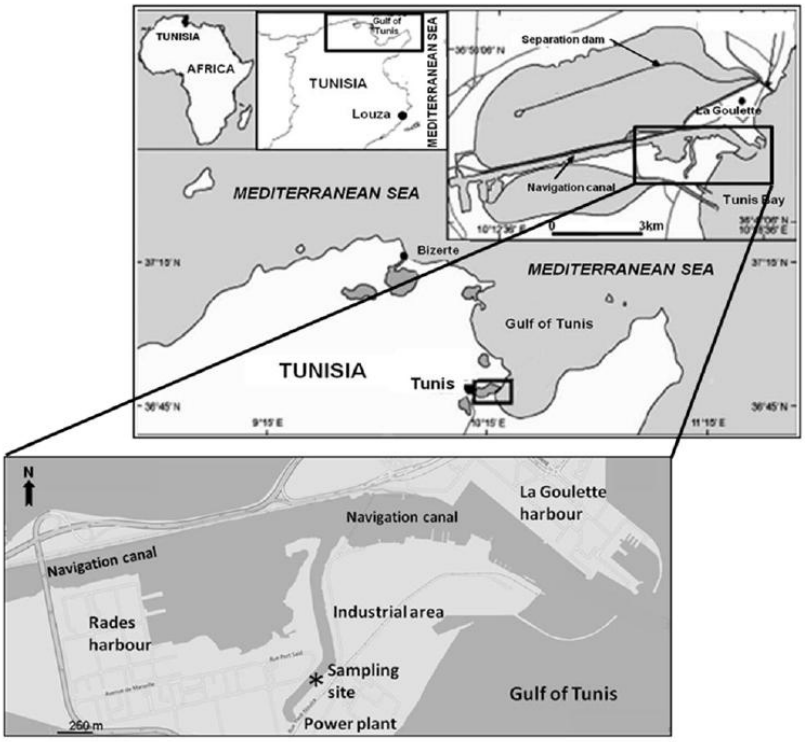

Figure 1: Map of the study area and location of sampling sites in Tunis lagoon and Louza

Sample procedure for biochemical determinations

Prior to biochemical analysis, digestive glands were ground in phosphate buffer (0.1 M, pH 7.5). The homogenate obtained was centrifuged at 100 000 g for microsomal fraction (S100) or at 9000 g for cytosolic fractions (S9).

Benzo[a]pyrene hydroxylase measurement

BPH activity was assessed in digestive gland microsomes (S100 fraction) by the adapted fluorometric method of Michel et al.[32] to a microplate reader. Following incubation with B[a]P, there action was stopped by the addition of TritonX-100. Fluorescence of the sample was obtained by difference in fluorescence between the respective emission/excitation wavelengths of 492/430 and 510/430 nm. Results were expressed in pmoles per min per mg protein.

Glutathione S-transferase measurement

GST activity was measured in digestive gland cytosol (fraction S9) according to Habig et al.[33] using 10 µg of cytosolic proteins, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich, St Louis, MO, USA) as substrate, and 4 mM reduced glutathione (GSH), in 100 mM sodium phosphate buffer, pH 7.5. GST activity was determined by kinetic measurement at 25 °C using a spectro UV-VIS double beam PC scanning spectrophotometer UVD-2960 (λ = 340 nm). Results were expressed as nmoles GS-CDNB produced per min and per mg proteins.

Catalase measurement

CAT activity was determined in S9 fraction according to Clairborne's method[34]. Reaction mixture (final volume of 1 mL) contained 0.78 mL 0.1 M phosphate buffer (pH 7.5) and 0.2 mL 0.5 mM H_2O_2. After 30 s preincubation, the reaction was started by adding 0.02 mL of S9 fraction. CAT activity was evaluated by kinetic measurement at 25 °C using a spectro UV-VIS double beam PC scanning spectrophotometer UVD-2960 (λ = 240 nm). Results were expressed as µmoles hydrogen peroxide transformed per min and per mg proteins.

Malondialdehyde determination

Lipid peroxidation (LPO) was estimated in S9 fraction by measuring the formation of thiobarbituric acid reactive substances (TBARS) and quantified as MDA equivalents. The 1,1,3,3- tetramethoxypropane was used as standard. The reaction was assessed at 532 nm using thiobarbituric acid (TBA) reagent as described by Buege and Aust[35]. MDA content was expressed as nanomoles MDA per milligram proteins.

Total protein determination

The quantities of proteins were determined according to the Bradford[36] method using Coomassie

blue reagent (BioRad) and bovine serum albumin as standard protein.

Statistical analysis

The experimental data was initially tested for normality and homogeneity of variance, in order to meet statistical demands. Data was expressed as mean ± standard deviation (SD). Data statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan's test for multiple range comparison, $p < 0.05$ was considered as significant. Statistical analysis was carried out with SigmaStat software 3.5 (Systat Software, Inc.).

Results and Discussion

Metabolism biomarkers

Our aim was to study the seasonal variation of oxidative stress and metabolism biomarkers in the clam *R. decussatus* sampled in field, focusing on the determination of usual biomarkers that respond to the generation of oxidative stress and to the organic pollution.

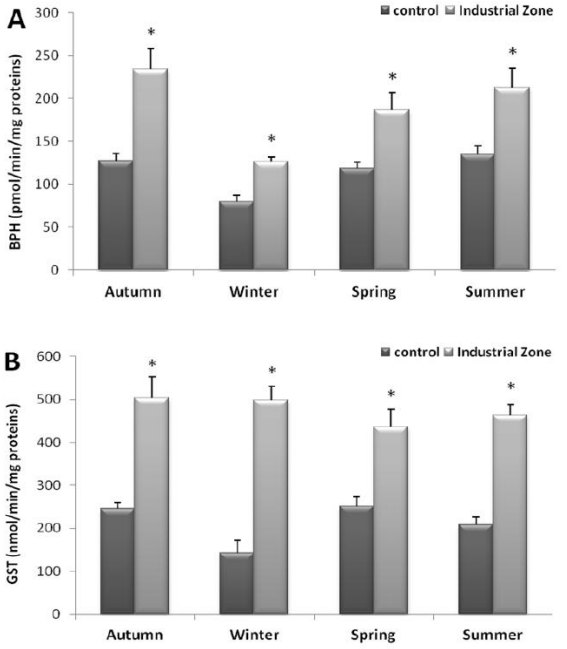

Figure 2: Seasonal variation of benzo(a)pyrene hydroxylase (A) and glutathione S-transferase (B) activities in digestive gland of clam *R. decussatus* sampled from industrial area of Tunis lagoon and control. Results are expressed as mean ± SD (n = 10). * indicates $p < 0.05$ compared with control (significantly different by ANOVA, multiple comparison, and Duncan's test across sites)

Figure 2 reports the levels of phases I and II enzymes (A: BPH activity and B: GST activity) detected in digestive gland of *R. decussates* from industrial area of Tunis lagoon and control animals. The results show a significant increase in the enzymatic activity of both metabolism biomarkers BPH and GST in animals sampled during four seasons from industrial area. This increase is very pronounced during spring, summer and autumn. The maximum values recorded (in autumn) for BPH and GST activities are equal to 234.16 pmole/mine/mg proteins and 503.01 nmole/min/mg proteins, respectively.

BPH and GSTs, which are involved in the metabolism of xenobiotic organic compounds, have often been assayed to indicate metabolisation of planar hydrophobic contaminants. BPH activity indicates CYP1A (a terminal component of the MFO system) activity in fish and mollusks[37]. GSTs are a group of multifunctional enzymes catalysing the conjugation of a broad range of electrophilic substrates, generally produced during phase I of xenobiotics metabolism, to glutathione[38]. BPH and GSTs are involved in the cellular detoxification and excretion of many physiological and xenobiotic substances such as PAHs, PCBs and DDE[18,39].

Increased BPH activity was observed in mussels *Mytilus galloprovincialis* exposed to benzo[a]pyrene (50 mg/kg dry weight)[40] and in clams *Mercenaria mercenaria* transplanted to PAHs contaminated environments in Tampa Bay[41]. The increased activity of this enzyme in *R. decussatus* from the industrial area of Tunis lagoon compared to control values (especially in summer and autumn) indicates that these animals were exposed, *in situ*, to organic contaminants, particularly PAH. Bebianno and Barreira[42] reported that no BPH activity was detected in the gills of *R. decussatus* transplanted in contaminated site (Ria Formosa lagoon, Portugal), while in the digestive gland, BPH significantly increased. Therefore, the digestive gland is often used in bivalve toxicology because it is the main organ of metabolism of organic compounds and the main site of biotransformation activities[43].

GST activity is part of the phase II of the metabolism process. These enzymes have a relatively complex response towards to different classes of pollutants, certain metals such as copper may inhibit them[44], while pesticides and polychlorinated biphenyls (PCB) induce them[45]. Our data showed a significant increase of GST activity in area study compared with control values (especially in spring, summer and autumn). Moreover, we signed a correlation between the two metabolism biomarkers BPH and GST activities. This activity increase confirms pollution of investigated site by organic contaminants. Banni et al.[46] showed an increase of GST activity in digestive gland of mussels *M. galloprovincialis* after exposure to 75 nM benzo[a]pyrene (B[a]P) for 48 and 72 h. Akcha et al.[40]

reported after 4 week exposure to B[a]P (via food supply to a daily theoretical dose of 50 mg B[a]P kg 1 dry weight mussel) a relative decrease of the phase II enzyme in mussel gills when it was unchanged in digestive gland tissues, thus suggesting distinct response of mussels to B[a]P depending on the way and the exposure period. Hoarau et al.[18] determined an increase in GST activity in clams *Ruditapes decussatus* exposed to B[a]P concentrations (1.5 µg/l and 3 µg/l). Attig et al.[47] reported an increase of GST activity in digestive gland of mussels *M. galloprovincialis* exposed to sublethal nickel concentrations (2.5 and 13 µM) for 96 h and 8 days. Levels of GSTs can be modified by a large range of xenobiotics and also by abiotic factors. GST activity in bivalve tissues is known to increase with the temperature of the water, while it decreases with the increase in salinity[48].

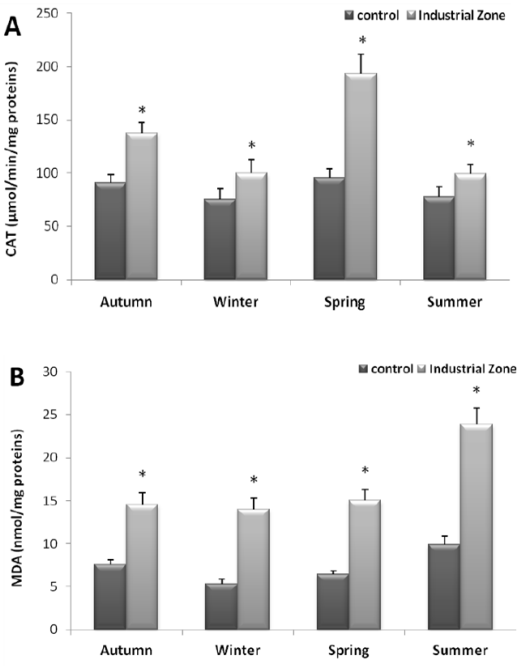

Figure 3: Seasonal variation of catalase activity (A) and malondialdehyde concentration (B) in digestive gland of clam *R. decussatus* sampled from industrial area of Tunis lagoon and control. Results are expressed as mean ± SD (*n* = 10). * indicates *p* <0.05 compared with control (significantly different by ANOVA, multiple comparison, and Duncan's test across sites

Oxidative stress biomarkers

Lipid peroxidation in biological membranes depends on two systems; the availability of oxidative substances-polyunsaturated fatty acids and the antioxidant-defense system. The levels of oxidative stress in clam *R. decussatus* caused by environmental pollution have been studied in terms of antioxidant defense systems (CAT activity) and lipid peroxidation levels (malondialdehyde accumulation). Figure 3 shows the levels of oxidative stress biomarkers (CAT activity and MDA accumulation) detected in digestive glands of *R. decussates* from industrial area of Tunis lagoon. The clams from the site of study presented significant increased levels of CAT in spring and autumn compared to control values. The maximum value (193.42 µmole/min/mg proteins) reached at spring. The increased activity of this enzyme in *R. decussatus* from the investigated site compared to control values indicates that these animals are facing an oxidative challenge, probably associated with the presence of contaminants in the environment. The CAT activity found in clams was in accordance to the levels previously reported for this species[49,50].

Increase in CAT activity signifies oxidative stress, often connected to excessive oxyradical formation during the catabolism of various organic compounds[34,51]. Moreover, it has been known that metals are involved in ROS generation through Fenton reactions, the inactivation of antioxidant defenses, or by inhibiting electron transport chain, allowing the transference of electrons directly to oxygen[52,53]. Thus, the exposure of organisms to metals and organic compounds can cause oxidative stress by increasing the rate of ROS production. The increases in CAT activity seen in *R. decussatus* from industrial area of Tunis lagoon compared to control values would be a response to an increase in ROS generation due to organic contaminants and metal exposure. Antioxidant enzyme levels are also linked to seasonal variability in the metabolic status of individuals, shown to follow changes in temperature, food availability and reproductive state[54,55].

MDA is a metabolite derived from lipid peroxidation which has been widely used as an indicator of oxidative damages to membranes and, therefore, of oxidative stress[31]. Figure 3B shows a significant MDA accumulation in clams from site of study during all seasons. The higher MDA accumulation (23.85 nmole/mg proteins) was registered during summer. This fact could be partially linked to the eutrophication phenomena[31]. Heavy metals as nickel increase the MDA accumulation in bivalves[47]. Bebianno and Barreira[42] reported that *R. decussatus* exposed, *in situ*, to PAH presented a lipid peroxydation increase. Banni et al.[31] registered a higher and significant MDA accumulation in *R. decussatus* from several sites in Tunisian coastal during summer and autumn. The seasonal nature of metabolism in marine invertebrates is well established[56,57]. Developmental and seasonal metabolic cycles reflect the complex interaction between exogenous factors such as food availability,

temperature, growth and possible contaminants and endogenous factors such as reproductive activities.

Conclusion

In the present study, the use of a battery of exposure biomarkers has been validated for *in situ* determination of material toxicity. The battery of biomarkers proposed has addressed a potential link between metal and organic contamination and bioavailability of contaminants. Seasonal variation arises from a complex interaction between exogenous and endogenous factors. It is difficult therefore to draw unambiguous conclusion from such variation. However, it appear from data discussed in this work, that significant biochemical changes occur in digestive gland tissues of clams during the period of spring, summer and autumn. The clam *Ruditapes decussatus* has been found to be potential bioindicators of marine contaminant exposure.

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