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Biomarker approach to the assessment of the health status of Moroccan marine ecosystems: Preliminary study in Sidi Ifni coast (South of Morocco)

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Abstract
The present study, which has not yet been done, constitutes a preliminary contribution to the assessment of the marine ecosystem health in the coast of Sidi Ifni (South of Morocco) in which some issues still receives wastewater. Measurements of biochemical indicators: Acetylcholinesterase, Glutation S-transeferase, Catalase and Malondialehyde (AChE, GST, CAT and MDA) in the Mediterranean mussel (*Mytilus galloprovincialis)* collected from the two sites on the coast of Sidi Ifni, showed that these biomarkers are measurable and inducible by pollution existing in the study sites. Seasonal variations of the measured biochemical parameters show that it is imperative to take into account such variations in the development and validation of these biomarkers.

Keywords: Biomarker, Marine ecosystem, Morocco, *Mytilus galloprovincialis*, Sidi Ifni

1. Introduction
Morocco's Mediterranean and Atlantic coastlines, extending for nearly 3500 km, play an important socio-economic role in the country, which is often accompanied by a demographic and urban significant growth. However, due to diverse human pressures, many coastal areas are already experiencing acute environmental problems, such as pollution. Indeed several types of pollutants are discharged into the seawater without any treatment in number of areas along these ecosystems. In Sidi Ifni city, the marine environment is considered as a receptacle of wastewater loaded with contaminants from agricultural urban and fishing port activities which are therefore likely to cause serious disruption of the physical and chemical quality of water, which can act directly and / or indirectly to the wealth and quality of coastline ecosystem.
As part of the efforts of our country for the implementation of a strategy of continuous monitoring and vigilance on the quality and health of the marine environment and its resources, our laboratory contributes by developing biochemical parameters called biomarkers and considered as indicators of pollution and water quality [1-2]. Such diagnostic and prognostic early-warming parameters, complementary to chemical analysis, offer the potential of specificity, sensitivity and application to a wide range of organisms and for discrimination water contamination over broad geographic regions. Many biomarkers, like Acetylcholinesterase (AChE), Glutathione S-tranferases (GSTs), Catalase (CAT) and Malondialdehyde (MDA), are actually subject of several international research programs of pollution monitoring [3-4].
The enzyme AChE hydrolyzes the neurotransmitter acetylcholine to acetate and choline at the cholinergic synapses, terminating nerve impulse transmission. It is known that AChE is strongly inhibited by organophosphate and carbamate pesticides, and also by metals [5-6-7]. For this reason, the evaluation of AChE inhibition in marine organisms has been widely used as an indicator of marine contamination by these compounds [8].
CAT is a major antioxidant enzyme of the defense system protecting organisms against oxidative stress. This enzyme removes hydrogen peroxide from cells during basal aerobic metabolism or exposure of animal species to some environmental parameters and chemicals contaminants, like metals [9-10]. CAT activity was used as biomarker of pollution in many studies. The MDA is considered as a product of lipid peroxidation which

reflected membrane degradation in a variety of pathological conditions and after exposure of organisms to chemical pollution [11-12]. It has been widely used to assess the effects of many pollutants [13-14-15].
GSTs are a ubiquitous family of phase II detoxification enzymes [16] and induced by exposure to various foreign compound like PAH, PC, Phenobarbital and some pesticides [17]. It was proposed as biomarker of pollution exposure and/or effect by several authors [18-19-20].
In previous studies, in which assays of AChE, GST, CAT and MDA were developed and used as biomarkers of pollution in Agadir bay, we have demonstrated that marine organisms (like *Mytilus galloprovincialis*, *Perna perna*, *Donax trunculus*, *Nereis deversicolors*) living at the sites receiving wastewater were significantly affected [20-21-22-23]. The study of the biology of these organisms showed also many perturbations in the reproductive cycle and growth [24-25].
The aim of this work is to initiate a program of research about the study of the health state of the coastline of Sidi Ifni (South of Morocco), not yet studied, and to test as biomarkers of pollution a battery of biochemical parameters already validated in large scale marine ecosystems. About that we have established a research program focused on the study of the four biomarkers, mentioned above, in the Mediterranean mussel (*Mytilus galloprovincialis)* collected from the two sites on the coast of Sidi Ifni region (South of Morocco).

2. Materials and methods

2.1 Studied Areas and sampling

Our study was conducted on samples of standardized size (30 to 50 mm) of *Mytilus galloprovincialis* collected in two sites representative of Sidi Ifni's coastline (Figure 1): *i)* Mirleft (MIR), which is considered as reference site, located at 30 Km to the north of Sidi Ifni, far from any source of pollution, and *ii)* Cheikh Sidi Ali Ifni (CHK) located at the entrance of Sidi Ifni and receives untreated waste waters of the city. For this preliminary study, the sampling period was from June to November 2013.

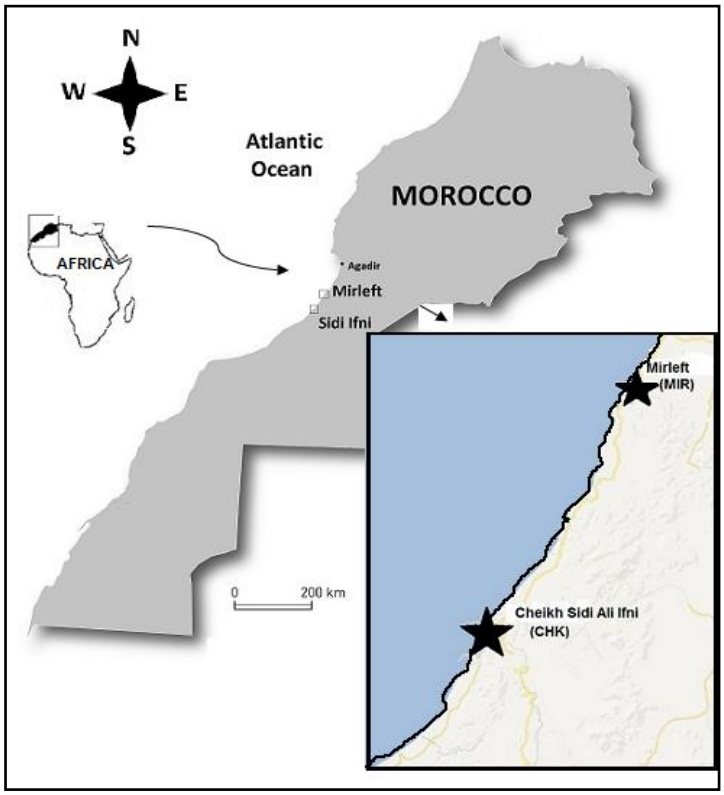

Figure 1: Map of the sites sampled in coastline of Sidi Ifni [CHK: Cheikh Sidi Ali Ifni (Polluted site), MIR: Mirleft (Reference site)]

2.2 Biochemical Analyses

2.2.1 Preparation of homogenate fractions

Mussels (30 individuals for each station) collected monthly were washed in fresh seawater and rapidly transported to laboratory and frozen at -30°C until analysis.

All the preparation procedures were conducted at 4°C. Soft tissue (whole animal) were collected, thawed, washed with cold 100 mM Tris buffer (pH 7.4), weighed and homogenized in three volumes (w/v) of the same buffer with an Ultra Turax homogenizer. Homogenates were centrifuged at 9000g for 30 minutes and the resulting supernatant (post-mitochondrial fraction or S9) frozen (- 30°C) until use.

2.2.2 Acetylcholinesterase activity (AChE)
The AChE activity was determined by the method described by Ellman et al. [26] using acetylthiocholine iodide as substrate. Spectrophotometric measurement was performed at 412 nm every 15 seconds for 2 minutes at 25°C. The reaction mixture contain 1.05 ml of 100 mM Tris buffer (pH 7.4), 50 µl of dithio-bis (2-nitrobenzoat) (80 mM in the assay), 50 µl of acetylthiochoilne (45 mM in the assay) and 50 µl of S9. Enzyme activity was expressed as nanomoles of acetylthiocholine/min/mg of S9 protein using the molar coefficient of extinction 13.6×10^3 $M^{-1}\times$ cm^{-1}.

2.2.3 Glutathion S-transferase activity (GST)
GST activities were measured according to Habig et al. [27] using 1-chloro-2-4-dinitrobenzene as a substrate. This latter is often preferred choice when total GST is being measured and is recommended for determination of GST activities. Assay was carried out in a reaction mixture containing 1 ml of 100 mM Tris buffer (pH 7.4), 50 µl of 1-Chloro-2 ,4-Dinitrobenzen (CDNB) (1 mM in the assay), 50 µl of reduced glutathione (1 mM in the assay) and 50 µl of S9. Enzyme activity was determined by monitoring changes in absorbency at 340 nm for 2 minutes at 25°C. GST activities are expressed as nmoles of dinitrophenyl glutathione (produced by conjugation of CDNB and GSH) per minute per milligram of S9 protein using the molar coefficient of extinction 9.6 $mM^{-1}\times$ cm^{-1}.

2.2.4 Catalase activity (CAT)
Assay of CAT activity was accomplished according the method of Aebi [28] which quantify the loss of hydrogen peroxide (H_2O_2) per minute at 240 nm in a reaction mixture containing 1 ml of potassium phosphate buffer (pH 7.4), 1 ml of H_2O_2 and 50 µl of S9. The activity of CAT was expressed in nmoles of H_2O_2 transformed per minute per milligram of S9 protein using the molar coefficient of extinction 40 $M^{-1}\times$ cm^{-1}.

2.2.5 Malondialdehyde rate
The determination of the MDA was estimated in terms of thiobarbituric acid reactive species, with the use of 1, 1, 3, 3-treaethyloxypropane as standard. The reaction was assessed at 532 nm using TBA reagent as described by Sunderman [29]. MDA content was expressed as mg of MDA/mg S9 protein.

2.2.6 Protein assay
The protein content in samples was evaluated according the method of Lowry et al. [30] using BSA as standard.

2.3 Statistcal analyses
Data were expressed as mean ± standard error (SD). The statistical significance of the differences between samples was determined by the "t" test using the Statistical software. A p value of less 0.05 was considered as statistically significant.

3. Results and discussion
During the sampling period, ACHE activity measured in *Mytilus galloprovincialis* (Figure 2) showed that this biomarker of neurotoxicity was inhibited in mussels populations living in polluted site (CHK) versus those sampled in Mirleft considered as reference site. The amplitude of the inhibition is more significantly marked in september and november (with a percent inhibition of 13,85% and 31,69% in october and November respectively). Such changes would certainly be related to the state of pollution of the CHK site that receives domestic wastewater and agricultural discharge without any prior treatment of contaminants that would be responsible for the inhibition of AChE.
Indeed, many studies have demonstrated that ACHE activities were inhibited in the presence of some contaminants like pesticides linked to agricultural activities. Its inhibition is considered a typical effect of

organophosphate and carbamate pesticides [31]. Similar effects may be caused by other factors which are known to modulate this enzymatic activity, including trace metals [32-33].
The observed inhibition of AChE activities may be attributed to the presence of contaminants in the environment. The same inhibitions were observed in *Mytilus galloprovincialis* and *Perna perna* mussels [8] living in stations receiving wastewater and contaminated by PAHs [34], metals such as Fe, Zn, Cd, and Cu [35] and pesticides [36] in Agadir Bay.

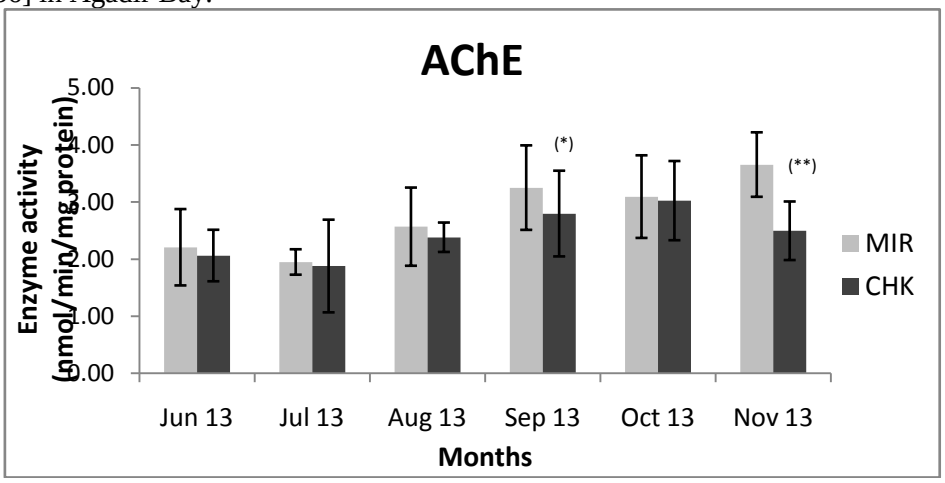

Figure 2: Acethylcolinesterase activity (ACHE) in *Mytilus galloprovincialis* collected the coastline of Sidi Ifni [CHK: Cheikh Sidi Ali Ifni (Polluted site), MIR: Mirleft (Reference site)]. *Data were expressed as mean ± standard error (SD). N=6, Values indicate significant difference from reference site: (*): $p < 0.05$ and (**): $p < 0.01$*

The ACHE activity seems to follow a seasonal profile. Higher levels of ACHE activity were observed in mussels collected in the two sites during the summer months than such collected at the autumn. Previous studies in our laboratory have shown seasonal variation for this enzyme activity in *Mytilus galloprovincialis* and *Perna perna*, which could be related to different levels of cholinergic system activation during the reproductive cycle of mussels [8].
Concerning GST, the present study shows a markedly higher enzyme activity in mussels living in CHK site versus Mirleft site (Figure 3). The amplitude of the induction is more significantly marked in summer months (with a percent induction of 208,02%, 138,07% and 221,52% in june, july and august respectively). Results like ours have already obtained the *Mytilus galloprovincialis* and *Perna perna* in Moroccan coasts (Agadir bay) [20]. Several other studies have described a similar relationship between environmental pollution and GST activity in mussels and other organisms in many marine ecosystems [37-38-39-40-41].

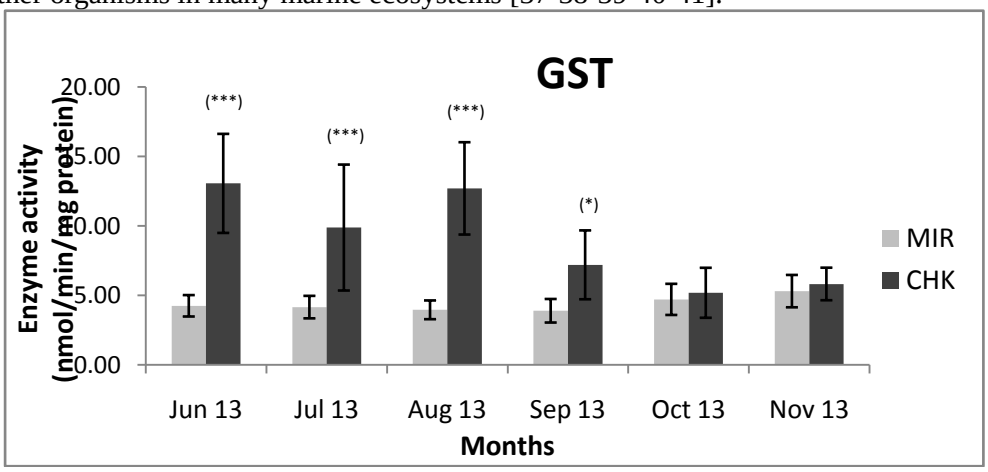

Figure 3: Glutathione-Stransferase activity (GST) in *Mytilus galloprovincialis* collected the coastline of Sidi Ifni [CHK: Cheikh Sidi Ali Ifni (Polluted site), MIR: Mirleft (Reference site)]. *Data were expressed as mean ± standard error (SD). N=6, Values indicate significant difference from reference site: (*): $p < 0.05$, and (***): $p < 0.001$*

Indeed, it is known that GST activity is a phase II enzyme involved in the detoxification metabolism of lipophilic organic contaminants. This enzyme catalyzes the conjugation of various organic electrophilic

compounds making it easily extractable. Its induction will seem to be, in our opinion, an adaptive response to altered environment by wastewater in CHK site.

Otherwise and like ACHE, GST activity seems present a seasonal evolution and variation in presence of pollution. Higher enzyme activity was obtained in summer. Seasonal variations of this activity were already described in *Mytilus galloprovincialis* and *Perna perna* in Moroccan coasts (Agadir bay) [8] and others organisms in different regions [42-43].

Figure 4 show that changes in catalase activity during the six months of study recorded high values in CHK site versus Mirleft. The induction of this enzyme activity in mussels living in polluted site was significantly marked in the beginning of summer (with a percent induction of 50%, and 48,632% in june and july respectively) and the end of autumn (with a percent induction of 57,73% in november). Results obtained in our study are in general agreement with induction linked to pollution described X in Bizerte lagoon (Tunisia) [44] and in the coast of Casablanca [45], using the mussels *Mytilus galloprovincialis* and *Perna perna* as sentinel species, respectively. Such variations are due to various environmental stimuli that may induce prooxidative processes in organisms which are often associated with biochemical and histological alterations in mollusks [46-47].

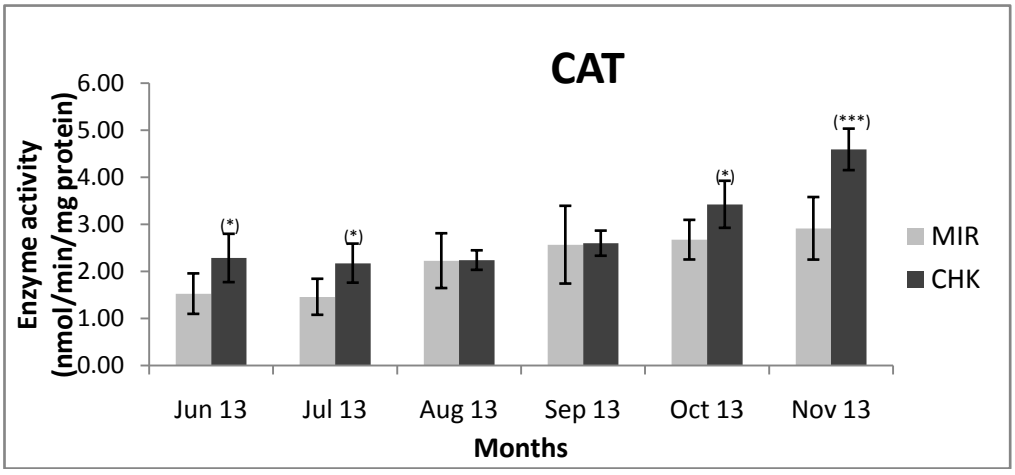

Figure 4: Catalase activity (CAT) in *Mytilus galloprovincialis* collected the coastline of Sidi Ifni [CHK: Cheikh Sidi Ali Ifni (Polluted site), MIR: Mirleft (Reference site)]. *Data were expressed as mean ± standard error (SD). N=6, Values indicate significant difference from reference site: (*): $p < 0.05$ and (***): $p < 0.001$*

Indeed, CAT activity, considered as primary enzyme (or is the earliest enzyme) in the antioxidant defense system of organisms, is induced [48] against higher H_2O_2 directly or indirectly generated by contaminants present the CHK site which received domestic wastewater and agricultural discharge without any treatment. The higher H_2O_2 produced in mussels of CHK site, which receive domestic wastewater and agricultural discharge without any treatment, may stimulate this antioxidant enzyme.

The seasonal trend of CAT activity obtained in our study confirmed the results described in *Mytilus galloprovincialis* living in Basque Estuaries (Bay of Biscay) [49] and in the Saronikos Gulf of Greece [50] and in *Ruditapes decussatus* in Tunisian coastal areas [51-52].

The monthly monitoring of MDA levels shows high values in *Mytilus galloprovincialis* collected in the polluted site (CHK) during the period of our study (Figure 5). The percentage of induction is comparable during the period of study and including between 13 and 30%. Such variation linked to pollution has also been reported by El Jourmi et al. [53] in *Perna perna* along the moroccan atlantic coast (big Casablanca) and by Kamel at al. [54] in *Mytilus galloprovincialis* sampled in Bizerte lagoon (Tunisia). Indeed, measurement of MDA is widely used as an indicator of lipid peroxidation [55]. Several other studies have described that MDA levels may be positively correlated with the level of certain pollutants. Indeed, the capacity for heavy metals and organic compounds to induce lipo-peroxidation was reported by Livingstone et al. [56], Viarengo et al. [57], Narbonne et al. [58] and Labrot et al. [59].

An apparent and seasonal evolution of MDA level was obtained in our study. This kind of results was described in *Perna viridis* living in sea beach of Bambolim (India) [60] and translate the relationship between reproductive cycle and oxidative stress described by Filho et al. [61].

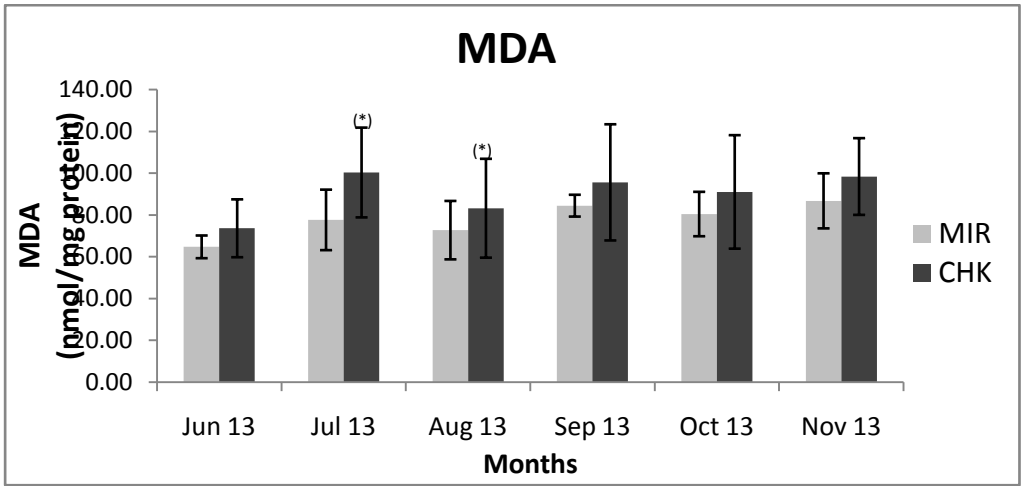

Figure 5: Malondialdehyde level (MDA) in *Mytilus galloprovincialis* collected the coastline of Sidi Ifni [CHK: Cheikh Sidi Ali Ifni (Polluted site), MIR: Mirleft (Reference site)]. *Data were expressed as mean ± standard error (SD). N=6, Values indicate significant difference from reference site: (*): p < 0.05*

Conclusion

The aim of this work is to initiate a research program for the health status of Sidi Ifni's coastline (South of Morocco), not yet studied, and to test a battery of biochemical parameters considered as biomarkers of pollution already validated in large scale marine ecosystems models.

The result obtained in our preliminary work, despite its limitations in time and space, indicates a significant influence of pollution on all parameters tested (ACHE, GST, CAT and MDA) in *Mytilus galloprovincialis*.

The biomarkers responses obtained during the study period (June - November 2013) shown clearly the presence of different contaminants in CHK site which receives untreated waste waters and validated the large application of biomarkers in Moroccan marine ecosystems using *Mytilus galloprovincialis* as sentinel organisms

Our result indicates also a significant influence of season on all biomarkers tested. This variation must be correlated to the complex interactions between biomarkers and exogenous and endogenous factors. They oblige us to take these variations into account in the development and validation of biomarkers. This biological approach constitutes a useful tool for monitoring Moroccan marine ecosystems which must be integrated in environmental surveillance programs. They show also a real disturbance of the marine ecosystem of Sidi Ifni and led us, in the present conditions, to strengthen coastal surveillance of the city and neighboring sites and to develop effective prevention against pollution. In addition, think long term, to treat all wastewater from the city.

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