Biochemical Markers Of Oxidative Stress In *Donax Trunculus* Exposed To Mercury Contamination In Aghroud Beach (Moroccan South)

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Abstract— **The aim of the present work was to investigate bioaccumulation and biological responses of *Donax trunculus* collected along the sampling area of Aghroud beach (Moroccan south). Samples were collected over a 27-month period in 2009–2011. The study focused on mercury (Hg) as a trace element known to be potential reactive oxygen species inducers. Enzymatic activities related to cellular defense systems including antioxidant enzymes as catalase (CAT) and Acetylcholinesterase (AChE) were measured in soft tissue. Concentrations of mercury were significantly higher in *Donax trunculus* in three months during sampling period. The values are respectively of 0.51 ± 0.02 µg/g wet weight at May 2009; 0.73 ± 0.008 µg /g wwt at September 2009 and 1.43 ± 0.009 µg/g wwt at August 2010. These values exceeded the environmental quality standards (0.5 mg kg−1 wwt) established by the European Union commission. Concerning this study, no significant correlation was observed between mercury and biochemical parameters measured in *D. trunculus*.**

Keywords—mercury; bioaccumulation; pollution; biomarkers; acetylcholineterase; catalase; Donax trunculus; Aghroud beach; Agadir Bay; Moroccan coastline

I. INTRODUCTION

Coastal areas are among the most threatened ecosystems and mainly affected by wastes of urban development and industrialization, intensive agricultural practices. Consequently, complex mixtures of contaminants are continuously released into these systems, degrading water quality and generating severe damage on organisms that may causes a decrease of natural resources [1]. The use of biota to get information on the quality of aquatic ecosystems has been suggested, and has now become an accepted methodology for assessing contaminant bioavailability [2].

Agadir Bay is considered one of the most important coastlines of Morocco (southern). It is among the richest areas in fisheries resources. However, despite this importance, the Agadir region uses the marine environment as a discharge of domestic and industrial wastewater. This fact causes disturbances in fauna and flora as well as water physicochemical quality. So, it seems necessary to promote researches and studies related to the assessment of the state of the bay health. Recent papers have focused on biota, water and sediment sampled from areas hot spots in Agadir Bay. It concerns especially pesticides [3]. The findings indicated that the presence of pesticides in this area may represent a potential health concern for the whole ecosystem and the species living in. Trace elements have been of great concern in marine and coastal ecosystems, since they cause several biological alterations from molecular to tissue level depending to their concentrations.

Bivalves are filter feeders they absorb trace elements not only from water or sediment, but even from inorganic particulate materials ingested [4]. Indeed, they are well established as bioindicators for monitoring of trace elements bioconcentration in several exposed areas [5]. Previous reports highlighted the usefulness of *Donax trunculus* as a bioindicator organism of trace elements contamination, both in field studies [6,7] and laboratory experiments [8], and from bioaccumulation [9,10,11] to biomarker responses [7], suggesting *D. trunculus* to be an adequate species to monitor mercury contamination and toxicity in sandy beaches.

Among the internationally recognized sandy beaches is Aghroud Beach. This coastline, which receives tourists from all over the world, is subjected to profound disturbances, especially in terms of ecotoxicology and the disruption of the physicochemical quality of its waters. Indeed, a decade ago, this site was adopted as a reference site for investigations [8,12,13,14] conducted by our laboratory which selected *Donax trunculus* as a specific bioindicator species for sandy beaches. The coastline of Aghroud is now a construction site for new subdivisions and entertainment and tourism projects. For these reasons it was necessary to conduct investigations to assess the state of health of this ecosystem so considered a pristine area through two complementary approaches biological and chemical.

Chemical analyses of trace metal (mercury) in *D.Trunculus* soft tissues were expected to reflect changes of their bioaccumulation during different periods in collecting area. These results were integrated with a biomarker approach, measuring a large range of biological responses. Such biomarkers are the first warning signals of chemical disturbance, being very sensitive and/or specific to particular category of pollutants, and reflecting different levels of cellular disturb and toxicity [15]. Catalase activity and acetylcholinesterase activity were chosen as specific responses toward exposure to mercury. Mercury has been recognized as severe environmental threats to the safeness of aquatic species principally due to its accumulation and persistence in sediments [16]. Therefore, mercury toxicity and its accumulation in bivalve tissues should be considered as a primordial concern because fauna is in close contact with contaminated sediments. These specimens are exposed to mercury from sediments, water, and suspended particulates from which they feed [17]. Acethylcholinesterase inhibition in sentinel species has been generally used as a marker of exposure to organophosphate and carbamate in biomonitoring programs [18] and alteration of the activity have been also attributed to other types of contaminants. Antioxidant enzymes, which help to protect cells against oxyradical damage resulting from exposure to some pollutants, were also involved [19]. The antioxidant enzymes catalase (CAT) along with auxiliary enzymes and conjugating enzymes, help to protect organisms from oxidative stress induced by exposure to contaminants in the environment [15].

Despite the considerable arrangement of data presented on the levels of contaminants in mollusks around the world, there is little information about contaminants and/or toxic chemical in *Donax trunculus* at Agadir coastline. Such as mercury Hg that has become extremely studied in current years, due to the fact that the element and its products are persistent, bioaccumulate, and toxic which could cause human and ecosystem risks.

In this paper, levels of mercury accumulation and biomarkers responses measured in *D. trunculus* soft tissues at the period 2009-2011 at Aghroud beach in Agadir Bay are presented and discussed together as a contribution to the evaluation of the impact of pollution on ecosystem health of the study area.

II. MATERIALS AND METHODS

A. *Site and Experimental Design*

Latitude and longitude respectively of 30 ° 36 'North and 9 ° 47' West, the site is located 35 km north of the Agadir city "*Fig.1*". This sandy beach has some fishing and tourist activities. *Donax trunculus* is abundant throughout the year. In this beach, the most dominant sands have a size between 0.125 and 0.2 mm with a median particle size of 0.180 mm [20], which makes Donacidae living buried in. *D. trunculus* is considered to be a substrate-sensitive organism because of its sensitivity to sediment grain size variations during its life cycle, in particular during its early growth stages [21].

This sampling area was far from any industrial or agricultural activities. The different studies carried out in our laboratory [8,22,23,24] considered Aghroud beach as a pristine area. But in five years after, this site has been under human-caused stressors until those days (housing estates constructions, new projects and tourist complexes).

Specimens of *D. trunculus* of standardized shell size (length between 24 and 32 mm) were collected monthly (May 2009 to July 2011) from selected area of Aghroud beach. Animals were transported alive to laboratory. Forty individual organisms were placed in filtered seawater for at least 48 hours to empty the contents of their digestive glands and then stored in -20°C in order to analyze mercury concentration. In parallel with, twenty individual specimens are frozen immediately at -80 °C until biochemical analysis.

Fig.1. Sampling area (Aghroud Beach, Morocco)

B. *Biomarkers*

- *Acetylcholinesterase activity*

AChE activity were performed using a method described by [25] with the use of acetylthiocoline (ASCh) as substrate. The activity rate was measured as change in OD/min at 412 nm. Activity was expressed as nmol/min/mg protein.

- *Catalase activity*

CAT activity was determined according to [26] by following the decrease in absorbance at 240 nm due to H_2O_2 consumption. Results were expressed as units per milligram of proteins.

Total protein contents were determined according to the Biuret method [27], using bovine serum albumin as a standard.

C. *Mercury Analyzes*

The method consists on digesting samples in the presence of acids and potassium permanganate (KMnO4). This step allows decomposing the organic matter and transforming the mercury into Hg^{2+} form. Hydrochloric acid promotes the rapid decomposition of cinnabar (HgS), resistant to the attack of nitric acid and sulfuric acid. Potassium permanganate ensures complete oxidation of organic compounds refractory to acid decomposition. During this step, the manganese is reduced from Mn^{7+} form to Mn^{4+} (MnO_2) form. After digestion, a solution of hydroxylamine chloride (NH_2OH-HCl) reduces MnO_2 and the excess of $KMnO_4$ without reducing Hg^{2+}.

Secondly, mercuric ions reduced to elemental mercury by stannous chloride (SnCl2) and transformed to gaseous form in a cell by bubbling air. The mercury contained at the cell is assayed by atomic absorption spectrophotometry with vapor formation at 253.7 nm [28].

Sample concentration is determined by comparing the respective absorbances with those of a range of standard solutions.

The procedure requires to take 4 ml of HNO_3 (100%) with 2 ml of H_2SO_4 (100%) and 1 ml of HCL (100%) which are added to 2.5g of sample and let to digest (1 hour). After, residues were transferred to 50 ml volumetric flasks and diluted with deionized water, then filtered using a membrane filter (0.45 µm). Concentrations were determined on wet weight basis as $mg.kg^{-1}$. The samples were analyzed in triplicate. The accuracy and precision of the analytical methodology were assessed by replicate analysis of certified reference materials. Precision of the measurements based on replicate digestions was between 92% and 109%.

D. *Statistical Analysis*

Data are expressed as mean standard deviation (SD) of independent experiment. Two-way ANOVA was used to compare biomarker concentrations in species between months in order to determine if there were differences in biomarker concentrations and if the patterns of biomarker response at sampling area were similar over cycles. Where a significant main effect was detected over, Fisher LSD multiple range test was used to locate differences between levels of the significant main effect. The same analysis was performed to compare mercury concentrations between months in the Aghroud beach clams. Simple positive/negative correlation was used to establish significant relationships between the biological responses and mercury concentrations. The analyses were carried out using the STATISTICA with 5% as the level of significance.

III. Results

A. *Biomaker Response*

Cholinesterase and catalase activities were carried out during two annual cycles in *D.trunculus* soft tissues from the study area. The results are shown in "*Fig.2*".

- *Acetylcholinesterase activity AChE*

Over the first annual cycle, high levels of AChE activity were recorded at the beginning of summer and autumn 2009 (101.05 ± 11.27 nmol/min/mg P in July and 89.21 ± 15.97 nmol/min/mg P in October 2009). A minimum activity is established during the winter season, with the lowest value recorded in January 2010 (41.89 ± 15.31 nmol/min/mg P). During the second annual cycle, significant fluctuations were observed with marked high activity in winter and spring 2011. Indeed, two noticeable peaks are observed at the end of summer and the start of spring (100.05 ± 10.92 and 187.99 ± 30.6 nmol/min/mg P) respectively in September 2010 and April 2011). However, the minimum is observed on the ending of spring (39.05 ± 7.78 nmol/min/mg P in June 2010).

- *Catalase activity CAT*

During the first cycle at Aghroud beach, catalase activity was very important from spring to autumn starting with two distinct peaks (227.26 ± 71.80 µmol/mn/mg P in June 2009 and 292.06 ± 126.40 µmol/mn/mg P in October 2009). The activity decreased with a minimum recorded in winter ending (127.85 ± 15.78 µmol/min/mg P in March 2010). This seasonal pattern is less pronounced in the second cycle, with a fall which begins in summer and was persisting until the start of winter (84.37 ± 1.28 µmol/min/mg P in January 2011). The activity increased giving the highest point in spring (319.68 ± 12.26 µmol/min/mg P in April 2011).

B. *Metal Concentrations*

"Fig.3" shows significant monthly fluctuations (from May 2009 to July 2011) in mercury levels of *Donax trunculus* soft tissues at Aghroud beach. Mercury concentrations in the Bivalve recorded two distinct peaks during the first annual cycle. Indeed, the first one was noted in the spring (0.51 ± 0.02 µg/g wwt at May 2009). A second peak was detected in the end of summer 2009 (0.73 ± 0.008 µg /g wwt at September 2009), followed by a decrease of mercury concentration, which stabilized during the following seasons. The same trend was observed in the second annual cycle in the Bivalve *D. trunculus*, two peaks are noticeable in the spring and summer of 2010. The values are respectively 0.36 ± 0.001 µg/g wwt at April 2010 and 1.43 ± 0.009 µg/g wwt at August 2010. This peak is followed by a significant decrease of the trace element concentration which remains low down during the cycle.

C. *Relationship Between Metals and Biomarkers*

Multiple correlations were conducted to assess the relationships between mercury in *D. trunculus* whole tissues and biochemical responses (Acetylcholinesterase and catalase), the results of correlation are depicted at table 1. No relationship was registered in biochemical parameters and the mercury analyzed in specimens from the study area.

Table 1: Pearson correlation coefficients between trace element concentration and biomarkers in the study area (p:Probability, r: Regression)

	AChE		CAT	
Hg	p	r	p	r
	0,775	-0,0576	0,595	-0,107

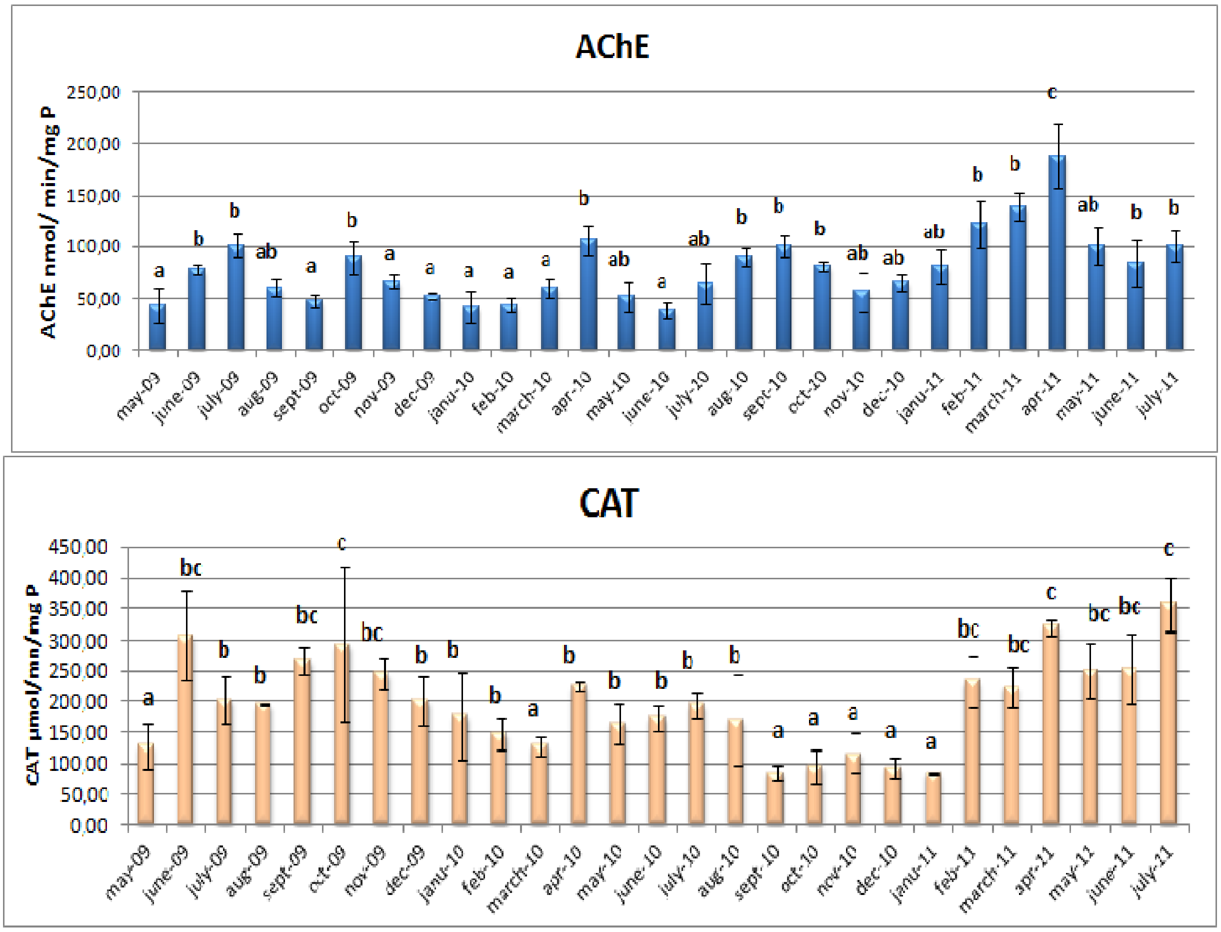

Fig.2. Variations of Acetylcholinesterase (AChE) and Catalase (CAT) activities (mean ± SD) (n = 6) in *Donax trunculus* collected from Aghroud Beach in Agadir Bay between May 2009 and July 2011 (sharing a common letter for the Hoc Post test that is not significant (p <0.05))

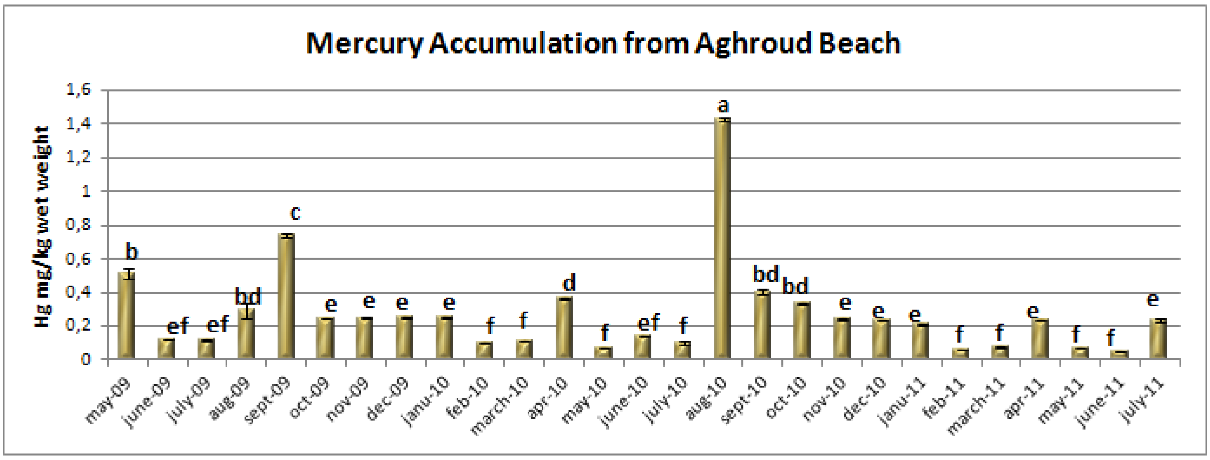

Fig.3. Monthly changes in mercury concentration in the soft mass of *D. trunculus* taken from samplig study site in Agadir Bay. The shared common letters for each month did not show any significant differences (p <0.05)

I. Discussion

A. *Biochemical Responding*

Various studies have shown that Donax is one of the most representative species of sandy ecosystems and can be considered as a bioindicator species to assess the health of the ecosystem [7,10,12,29,30].

Following the results of the two biomarkers response, the values show seasonal variations which are more or less important. Several studies have revealed similar variations in marine invertebrates [31]. These fluctuations are related to the influence of various biotic and abiotic factors in the environment, such as temperature that seems to influence the response of catalase activity [32] in some marine and estuarine molluscs [12,18]. In the same way, the temperature is considered as a factor which intervenes in the enzymatic metabolisms. Nevertheless, nutrient availability, pH and salinity [33] have a significant effect too. For other authors the variations would be correlated with intrinsic factors such as the reproductive cycle or animal growth.

- *AChE activity*

In biomonitoring studies AChE is particularly used as a biomarker for exposure to organophosphorus pesticides and carbamates [9-15]. However, other studies have shown that trace elements and polycyclic aromatic hydrocarbons could modulate and inhibit cholinesterase activity [34] as well as pesticides. In addition, some authors [35] point out that, specifically in marine bivalves, AChE inhibition is correlated to high concentrations of neurotoxic substances.

The variations observed in this study recorded maxima in winter and spring, while inhibitions occurred during the summer period. Several authors have reported seasonal variations in cholinesterase activity in marine invertebrates. Reference [12] showed that highest values are recorded in summer and lowest in spring for the same specimen, while reference [9] observed decreases in spring cholinesterase activity in Aghroud beach. Other studies concerned this Mollusc from Gulf of Tunis coasts showed similar results to ours [30]. Reference [36] noted seasonal variations in AChE activity in polychaete *Nereis diversicolor* at the Oued Souss estuary. Reference [37] observed lower levels of cholinesterase activity at *Perna perna* gills in Brazilian sites, the activity of the cholinesterases were manifested in the digestive glands of the species. While [38] noted clear inhibitions of AChE in *Mytilus galloprovincialis* transplanted in areas impacted by different effluents from anthropogenic activities along the Greek coasts.

Some authors maintained that AChE activity is inhibited by the presence of trace elements, indicating a kind of metallic pollution. In the literature, it is found that AChE activity is stimulated by exposure to contaminants such as lindane, aluminum, toluene, vinyl chloride, also ethyl parathion in rats [39] and metals in fish [40]. However, other authors confirmed the influence of environmental parameters. Indeed, temperature is one of the environmental parameters that modulate cholinesterase activity, as water temperature increases the ability of bivalves to accumulate pesticides in the environment. Thus, [41] showed that in crustacean *Crangon crangon*, AChE increases with temperature.

The present study showed high levels of AChE activity in Aghroud beach organisms, particularly during the second cycle. In the Bay of Agadir, the location of the sampling site explains the spatial variation of the AChE response. For example, relatively inhibited cholinesterase activities were observed during the same study period in animals from sites adjacent to the Oued Souss estuary, where this site continuously receives treated wastewater from the M'Zar purification (STEP) [7]. Releases from this STEP enriched the region with all kinds of pollutants, including organophosphates involved in the composition of various household products as well as trace elements. Indeed, positive correlations were noted between trace elements and AChE [7,24]. Reference [30] have reported at the Golf of Tunis (site receiving sewage discharges) inhibitions of AChE and attributed this to the consequences caused by intense use of insecticides nearby the Golf. Concerning Aghroud beach, it seems that the area is enriched with aromatic hydrocarbons via the transit of fishing boats, taking a count the effect of parceling project which contributes to the enrichment of this site in various types of domestic and urban wastes. Keeping in mind that studied site is also threatened by untreated urban water coming from neighboring agglomerations without a wastewater treatment plant. On the other hand, the results obtained suggest that Aghroud beach is not contaminated by cholinesterase inhibitors (organophosphates). Moreover, no significant correlation was observed between AChE and the trace element measured (Hg) in *D. trunculus.*

- *Catalase activity*

Catalase is used as a biomarker of oxidative stress that can be induced by a wide range of contaminants, including organic xenobiotics, PCBs, PAHs, phenols [42] and trace elements [43]. The abiotic parameters of the environment act also on the response of the catalase activity [30]. This activity is not only used as a marker involved in defense against oxidative stress, it is at the same time a seasonal phenomenon in molluscs [32].

The present study revealed significant seasonal variations of catalase at Aghroud beach. Similar results were observed at the same species *D. trunculus* from Sidi Jehmi coast, a touristic beach in the Tunis Golf [15]. This seasonality seems to be related to the fluctuations of the abiotic parameters of the environment. Several authors have shown that seasonal variations in catalase activity in marine invertebrates are closely related to these factors, including temperature, salinity and oxygen. The biota temperature is an essential element considering its direct intervention on the metabolism and the enzymatic activity. The effect of high temperature on catalase activity has already been observed in marine molluscs [32]. These authors observed that the response of catalase activity depends on water temperature (positive correlation) in *M. galloprovinvialis* from the Bizerte lagoon in Tunisia. Some authors have reported that the increase in salinity and the duration of photoperiod induce catalase activity in *M. galloprovincialis*. Other authors have explained that the elevation of enzymatic activities is related to endogenous factors such as the reproductive cycle and fluctuations in the physiological state of organisms. However, [44] attributed the increase in catalase to oxidative stress generated by trace elements [45] and the reproductive cycle of *S. plana* and *C. edule* [46]. Indeed, [47] recorded a positive correlation between cadmium and catalase in *Solea senegalensis* at the estuary of Huelva in Spain.

Maintaining catalase activity at high levels during most of the sampling period at Aghroud beach showed that animals are continuously subjected to an important oxidative stress. However, the non-specificity of a biomarker such as catalase prohibits to identify the real cause of the observed stress. It could be the result of a purely anthropogenic disturbance caused by domestic discharges from the surrounding area, as well as the influence of the development of the new tourist subdivision in this region, also the planned landing point neighboring the sampling area which may considered a source of HAP contamination. Indeed, [15] reported that the elevation of catalase activity exhibited an increase in peroxisomal proliferation induced by organic xenobiotics such as HAP. Similarly, [37] recorded a significant activity of catalase at mussel gills from polluted site. These authors explained that the high levels of catalase activities were due to the increase in antioxidant products in order to eliminate the H_2O_2 induced, directly or indirectly, by contaminants such as PAHs in bivalves.

The investigations carried out on oxidative stress biomarker as catalase explained that the aspecific character of the response is an advantage indicating a mixed pollution state of any ecosystem [48].

B. *Mercury Accumulation*

The monthly monitoring of Hg bioaccumulation at *D. trunculus* soft tissues revealed seasonal variations. Comparable variations have been reported in previous works on this species and in different other filtering Molluscs. The same trend was observed for the species in the Red Sea coast [4]. Similar results have been reported by [10] in *D. trunculus* for mercury and in *M. galloprovincialis* [49].

Several hypotheses have been proposed to explain the seasonal variation of trace element bioaccumulation in bivalves. Indeed, physiological processes related to metabolism and reproductive activity played a key role in determining seasonal variations in metallic contents in the tissues of aquatic organisms. Seasonal variations may be due to seasonal changes in soft tissue weight rather than to variability in the metal content of the body. Reference [50] have shown that the highest levels of Hg in *S. plana* are generally noted at spawning period. On the other side, some authors [2] indicate that significant differences in mercury concentrations were observed between small and larger sized individuals in the most contaminated sampling sites in several Portuguese estuaries systems, suggesting progressive mercury accumulation with size.

Other factors than the reproductive cycle could influence seasonal fluctuations in trace element concentrations such as temperature, precipitation, suspended matter load, and the ability to regulate trace elements in the body. Indeed, [51] noted that the increase in temperature has an effect on the filtration rate of *M. galloprovincialis* and the accumulation of the bioavailable metallic element. Food availability may also be responsible for seasonal fluctuations in metal concentrations. Thus, the increase in phytoplankton food during a certain period of the year (during the summer period) in the Agadir Bay is associated with an increase in primary production, which can lead to an increase in the concentration of metabolites in seawater. Salinity was also a significant factor affecting the community, being responsible for seasonal variations of mercury accumulation in the macrobenthic assemblages of Ria de Aveiro in Portugal [52].

The difference in metal content in species also seems to imply longevity as a parameter affecting the rate of bioaccumulation of a trace element in a species. Indeed, reference [53] found that *S. plana* registers higher levels of total Hg than polychaetes. They explained this difference in part by the fact that *S. plana* is a long-lived species with a lifetime of 5 years [54], while for polychaetes their longevity varies between 12 and 15 months. The species *D. trunculus* has a lifetime of 3 to 5 years. Therefore, a possible explanation for the measured element levels in *D. trunculus* may be related to the fact that the Bivalve accumulates a metal level relative to its lifetime.

Moreover, reference [55] conducted investigations into the effect of certain neighboring urban effluents of a chemical industrial complex (Haifa Bay) on detoxification system settings in the species *D. trunculus* taken upstream and downstream of these discharges. The results showed a disruption of antioxidant mechanisms and an intensification of energy metabolism in specimens chronically exposed to industrial discharges. These authors also present the ability of *D. trunculus* to survive in highly polluted environments and believe that is a genetic adaptation when chemical stress continues to increase in the bay.

II. CONCLUSION

Biological responses of specimens exposed to an environmental stress are often hardly interpretable because of the high complexity of the pollutant mixture which can generate different and sometimes conflicting responses, and also because chronic exposure to low levels of contaminants can guide to physiological mechanisms of adaptation and consequently may reduce animal sensitivity to contaminants.

High mercury levels were determined in the species studied, which shows that Aghroud beach is an ecosystem contaminated. The probable origin of pollutant may be attributed to artisanal fisheries wastes. Note that Aghroud has always been considered as a reference site for previous ecotoxicological investigations. To confirm this hypothesis, an analysis of the trace elements is recommended for the sediment and the water column in order to have more informations.

This research work enabled an insight into the current status of Aghroud beach regarding mercury contamination, highlighting contamination that may be of ecological concern. While regarding mercury concentration in *D.trunculus* soft tissue, some of exceeded the environmental quality standards (0.5 mg kg^{-1} wwt) established by the EU commission directive 2008/105/EC.

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