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PROOF

Impact study of M'zar submarine emissary (Agadir Bay, Morocco): Trace metals accumulation (Cd, Pb and Hg) and biochemical response of marine Mollusk*Donax trunculus*(Linnaeus, 1758).

M. Nadir[1], M. Agnaou[1], Z. Idardare[2], A. Chahid[3], T. Bouzid[3], A. Moukrim[1(*)]
[1]*Laboratory Aquatic Systems: Marine and continental ecosystems; Faculty of Sciences, Ibn Zohr University, Agadir (Morocco)*
[2] *High Institute of Maritime Fisheries of Agadir (ISPM)(Morocco)*
[3]*Regional Laboratory of Analysis and Research/ the National Office of Food Safety of Agadir (LRARA/ONSSA), Agadir, (Morocco)*

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**Corresponding Author. E-mail:moukrim@uiz.ac.ma;Tél :(00212.28220957)*

Abstract
The environmental quality of M'zar beach, subjected to effluent from a wastewater treatment plant and the proximity of Souss Oued estuary providing agricultural and fertilized compounds, was assessed in two different sites (S1, situated in the south of Oued souss estuary and S2 in the south of M'zar plant submarine emissary outfall) between 2009 and 2011, focusing on metal contamination (Cd, Hg, Pb) in *Donax trunculus* (Linnaeus, 1758) soft tissues. In addition, associated toxicological effects were assessed using a multibiomarker approach (catalase, acetylcholinestrerase, glutathione S-transferase, Malondialdehyd). *D. trunculus* from both sites shows different seasonal and spatial biochemical responses and heavy metals bioaccumulation pattern. The clams responded differently to metal contamination with different biomarker responses showing a clear site trend, suggesting different sources and/or magnitudes of contamination. Mercury was a source of oxidative stress in clams of both sites with a significant influence on neurotoxicity and antioxidant defense and enhancement of lipid peroxidation.

Keywords: *Donax trunculus*, multibiomarkers, Heavy metals, M'zar plant, submarine emissary, M'zar beach.

Introduction
Socio-economic life of Agadir region (South Morocco) is based on fishing, tourism and agriculture. Taking into account the arid climatic conditions and socio economic activities developed in this Region, the scientific researches and environmental management of ecosystems, particularly aquatic and natural resources, constitute a national priority to ensure sustainable development.
Different researches realized in this context, proved that wastewater represent a major source of pollution to coastal aquatic ecosystems [1,2,3,4,5,6,7,8,9]. Otherwise, some other studies, carried on the treated wastewater, present a significant potential which can be mobilized to fill a part of the water deficit, to reuse in agriculture or green spaces irrigation [10,11,12,13,14].
For those raisons (preserving coastline quality and reusing wastewater treated), local authorities has been put up a wastewater treatment plant with percolation infiltration as a processing system. But actually, the treated water is still directly discharged in marine coastal. If previous studies have been conducted to evaluate the performance of this station [11,13,14], to our knowledge, no study was engaged to estimate the impact of treated wastewater to the marine environment. Despite the fact that some results revealed that the process adopting in the plant (infiltration percolation) has shown a good effectiveness considering microbiological parameters and organic matters; whereas, it is not the case concerning chemical contaminants: heavy metals, PCB, HAP, pesticides [15,16].

The aim of this work is to evaluate the metal concentration in marine environment receiving the treated wastewater effluents, through their bioaccumulation in a filter feeding mollusk, *Donaxtrunculus*, living buried in the sand. In parallel with, the biochemical responses of this species has been also used, to contribute to the ecosystem health assessment. The choice of this species was conducted by the fact that it was utilized as a sentinel species in many other researches [4,7,17,18,19,20].
Also, the use of biochemical parameters to assess the state of the ecosystem's health has been established and applied by several environmental monitoring programs such as MEDPOL and Joint Monitoring Program of the OSPAR convention [21,22]. These parameters having multiple advantages as a monitoring tool (inexpensive, early warning to xenobiotics, a simple setting up, allow for a realistic assessment of the environment health), providing a wide application range, which constitute a substantial originality in this investigation.

2. Materials and methods

2.1. Sampling sites

To perform our investigation on the impact of the wastewater plant outfall rejection, two sampling sites are chosen: the first one is located on the north of the outfall (30°21'08,66"N; 9°36'32,23"E), the second one at the south (30°18'48,10"N; 9°37'05,80"E) (Figure 1). The choice of these two sampling sites, related to their position to the effluent, has been dictated by the fact that the marine currents in Agadir Bay have a north-south direction [23,24]. Therefore, it was important to verify if the southern site, according to the currents, presented more impact on the marine environment.

Figure 1: Map of the sampling area in Agadir Bay (Morocco). S1: The first site located in the north of the emissary, S2: The second southern site.

2.2. Specimen collection

Specimens of *D. trunculus* of standardized shell size (length between 24 and 32 mm) were collected seasonally (Spring 2009 to Summer 2011), from the two selected sites in the M'zar beach. After collection, *D. trunculus* were transported alive to the laboratory. Forty individual specimens were placed in a filtered seawater for at least 48 hours to empty the contents of their digestive glands, stored in -20°C, serving to analyze metals trace, Lead, Cadmium and Mercury (at the Regional Laboratory of Analysis and Research of the National Office of Food Safety of Agadir).Twenty individual specimens are frozen immediately at -80 °C until biochemical analysis.

2.3. Heavy metals analyzes

The tissues of samples were ground and homogenized. For analyzing Cd and Pb, 0.5 g was taken from each sample and digested with 5 ml of nitric acid (HNO3) in Teflon vessels in microwave oven. After that, 2 ml of hydrogen peroxide (30%) was added to each recipient. Concerning Hg, 4 ml of HNO3 (100%) with 2 ml of H2SO4 (100%) and 1 ml of HCL (100%) were added to 2.5g of sample and let digest for 1 hour. After these two steps, the residues were transferred to 50 ml volumetric flasks and diluted with deionized water, then were filtered using a membrane filter (0.45 µm). All procedures are followed according to the Center of expertise in environmental analysis of Quebec method [25]. Sample blanks were prepared in a similar way of the other samples.

Parallel to this, Standard solutions of cadmium, lead and mercury were prepared from concentrated solutions of 1000 mg/l and diluted to the corresponding metal solution. 1 M of nitric acid HNO3 was used for diluting mercury solution and 10 % for lead and cadmium.

The apparatus used to analyze lead and cadmium is a Shimadzu AA-6300 model atomic absorption spectrometer, having a deuterium background corrector, by a graphite furnace atomic absorption spectrophotometer using Argon. Concentration mercury was determined by an Automated Mercury Analyze AULA-254 (Gold-trap KARLSELD, GERMANY), using Argon as a gaz. All quality control results are shown in table 1. Metal concentrations were determined on wet weight (µg/g).

Table 1: Recovery of Cd, Pb and Hg added to samples fisheries

Metals	Concentration added (µg/g)	Concentration found (µg/g)	Recovery concentration (µg/g)
Cd	0	0.077	-
	0.08	0.152	0.075 ± 0.002
	0.11	0.181	0.104 ± 0.003
	0.20	0.281	0.204 ± 0.014
Pb	0	0.207	-
	0.2	0.410	0.203 ± 0.019
	0.3	0.514	0.307 ± 0.028
	0.5	0.711	0.504 ± 0.002
Hg	0	0.107	-
	0.16	0.262	0.155 ± 0.016
	0.50	0.624	0.517 ± 0.014
	0.70	0.822	0.715 ± 0.012

2.4. Biomarkers

The preparation of the post mitochondrial fraction (S9) was conducted using the animal entire soft tissue. Two specimens were pooled (n=6) and homogenized in 1/3 w/v (weight/volume) on a Tris buffer (0,1 M pH 7,5) with Ultraturax homogenizer. The homogenate was centrifuged at 9000 g for 30 minutes at 4°C. The supernatant was collected in eppendorf tube. All biochemical parameters are measured in this fraction.

Determinations of AChE activity were performed using a method described by Ellman *et al.* [26] 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.

CAT activities were assayed as described by Aebi [27], the decrease in absorbance at 240 nm caused by the consumption of hydrogen peroxide H2O2. The reaction takes place in phosphate buffer (0.1 M; pH 7.4). CAT activity was expressed as µmol/min/mg protein.

GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in a final reaction mixture containing 1 mM CDNB and 5 mM reduced glutathione [28]. The activity rate was measured as change in optical density (OD/min) at 340 nm. GST activity was expressed as nmol/min/mg protein.

Malondialdehyde (MDA) contents was measured by the thiobarbituric acid, according to Sunderman *et al.* [29] method. The absorbency was measured at 532 nm. MDA contents was expressed on nmol/mg of proteins.The protein assay is determined in S9, using method of Lowry *et al.*[30]. A standard range was prepared from a solution of BSA (bovine serum albumin) for the determination of amounts of protein in samples. The optical density (OD) was measured at 650 nm.

2.5. Statistical analysis

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

3. Results and discussion

3.1. Metal concentrations

The spatial and seasonal concentrations of heavy metals (Cd, Pb and Hg) determined in the whole tissues of *D. trunculus* from the two sampling sites, are illustrated in figure 2. Generally, the figure shows an annual variation with a decreasing tendency in Cd and Pb concentration from 2009 to 2011. This fact may be related to the operating system effectiveness of treating wastewater by M'zar plant, which should reduce pollutant load. Also, the Installations of a submarine emissary outfall allows better distribution of wastewater treated compared to the state before 2005, where wastewater were directly rejected at the M'zar coastline.

Results of a 2-way ANOVA testing the effects of site, season and interaction between the two factors on heavy metals concentrations in clams were summarized in table II. The comparison between the metal concentration in Mollusks sampled from the two sites shows no different in Cd and Pb levels ($p>0.05$, Table 2). However, Hg exhibited high levels in S2 compared to S1 with a significant variation ($p<0.05$, Table 2). This difference could be due to wastewater rich on this element, discharged directly inthe area (site 2),neighboring the Oued Souss estuary, adding to this, the effluents of wastewater treated discharged from M'zar plant. Gutiérrez-Galindo and Flores-Muñoz, [31] suggest that the spatial distribution of Hg, in *Mytilus californianus* could be influenced anthropogenically. Furthermore, our results corroborate with those revealed by Petrilli *et al.*,[32]; Balestri *et al.*,[33] concerning the effluents of Rosignano plant in Livorno (Italy) which contains high levels suspended particulate matter charged by that element. This proved that the process adopted by M'zar plant, despite its performance concerning microbiological parameters [11,13], cannot eliminate chemical contaminants.

Table 2: Results of a 2-way ANOVA testing the effects of "site", "season" and interaction between the two factors on heavy metals concentrations in clams

		SS	DF	MS	F	p
Hg	**Season**	0.757550	9	0.084172	4.9731	<0.001
	Site	0.070500	1	0.070500	4.1653	<0.05
	Season*site	0.264794	9	0.029422	1.7383	>0.05
	Error	0.541617	32	0.016926		
Cd	**Season**	0.003689	9	0.000410	5.5964	<0.001
	Site	0.000005	1	0.000005	0.0708	>0.05
	Season*site	0.000486	9	0.000054	0.7381	>0.05
	Error	0.002197	30	0.000073		
Pb	**Season**	0.104205	9	0.011578	10.6502	<0.001
	Site	0.005371	1	0.005371	4.9408	>0.05
	Season*site	0.020075	9	0.002231	2.0518	>0.05
	Error	0.023917	22	0.001087		

Concerning the seasonal concentration of the three metals, significant variations in tissue concentrations of *D.trunculus* sampled in M'zar beach, were observed. Generally, the highest values occurred in spring-summer period (0.034 and 0.049 $\mu g.g^{-1}$ wet weight for Cd in 2009 and 2010; 0.25 $\mu g.g^{-1}$ wet weight for Pb in 2009 and 1.03 $\mu g.g^{-1}$ wet weight for Hg in 2011). These results are in accordance with those reported by Idardare *et al.*,[34] at the same beach during a study period of 2004 to 2005 in *D. trunculus*. These changes could be due to biological variables appropriate to the species, particularly those linked to reproductive activity [35,36,37]. Several authors reported that reproductive cycle could influence the metal retention during the gonad development. In fact, the *D. trunculus* gametogenesis cycle began at autumn and the spawning period happened in spring [38] So, the lowest metal levels coincide with the spawning period and the highest occurred in gametogenesis one [39].Coimbra and Carraça [40] suggested the use of immature bivalves in environmental monitoring programs for heavy metals since the youngest Mollusks showed reduced gonadal growth. According to Latouche and Mix [41] the size of the biota can affect the concentration of trace element in Mollusks, Phillips [42] have highlighted the importance of a relationship between metal concentrations and body weight of mussels. Some authors reported that heavy metals distribution in bivalve organisms have been explained by the variation of the environmental factors interaction (upwelling, runoff, currents, and mixing waters from different origin) which can generate seasonal fluctuations in metal concentration levels then accumulated by organisms [35].

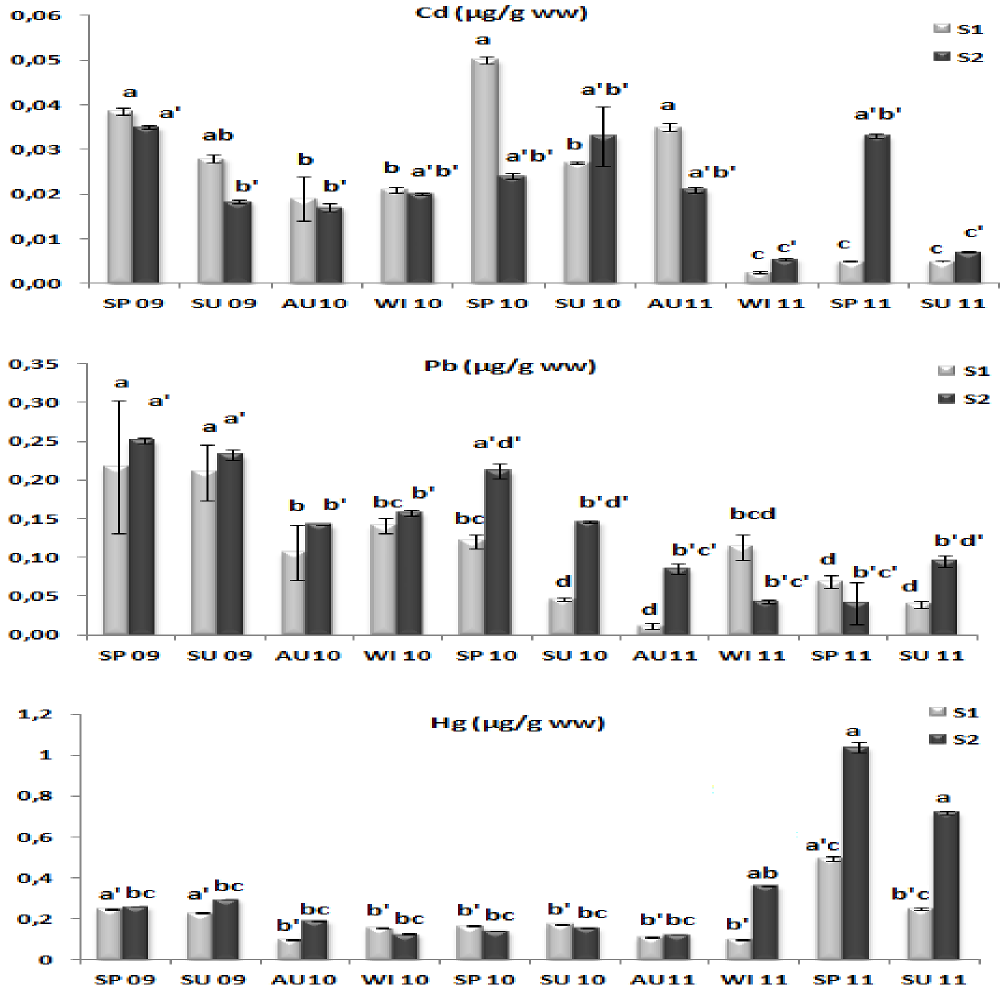

Figure 2: Seasonal variations of heavy metals in *D. trunculus* from M'zar beach at S1 and S2, sharing a common letter for the post Hoc test that is not significant (p< 0.05)

3.2. Biomarkers

The results of biomarkers showed important annual, seasonal and spatial fluctuations for different parameters studied (Figure 3). Statistical analysis illustrated in table III present a significant spatial differences for the responses of AChE ($p<0.001$), CAT ($p<0.01$) and GST ($p<0.001$). The comparison between the values in Mollusks sampled in the two sites (in North and South of the emissary) shows that the levels of different biological parameters are more important at S2 bivalves compared to S1. However, no significant differences were registered between sites for MDA contents ($p>0.05$ table 3).

Seasonal variations of studied biomarkers (AChE, CAT, GST and MDA) were exhibited in Figure 3. The two-way ANOVA registered significant effect of seasonin AChE and CAT responses ($p< 0.001$, table 3). AChE activity fluctuated to a great extent with the highest value recorded in Summer (81.6 ± 2.33 nmol/mn/mgP, in 2011) and the lowest one occurred in Spring (15.10 ± 0.78 nmol/mn/mgP, in 2011). As observed in CAT activity, high peaks are registered in Autumn and Spring (respectively, 68.25 ± 6, in 2010 and 131.42 ± 4.68 µmol/min/mg of Proteins, in 2011). For GST activity and MDA, in spite of their important seasonal variations

(Figure 3), no significant differences were noted (table 3). Concerning the effect of interaction between site and season on biomarker responses, the two-way ANOVA showed no significant interaction detected, except for CAT activity (p<0.01).

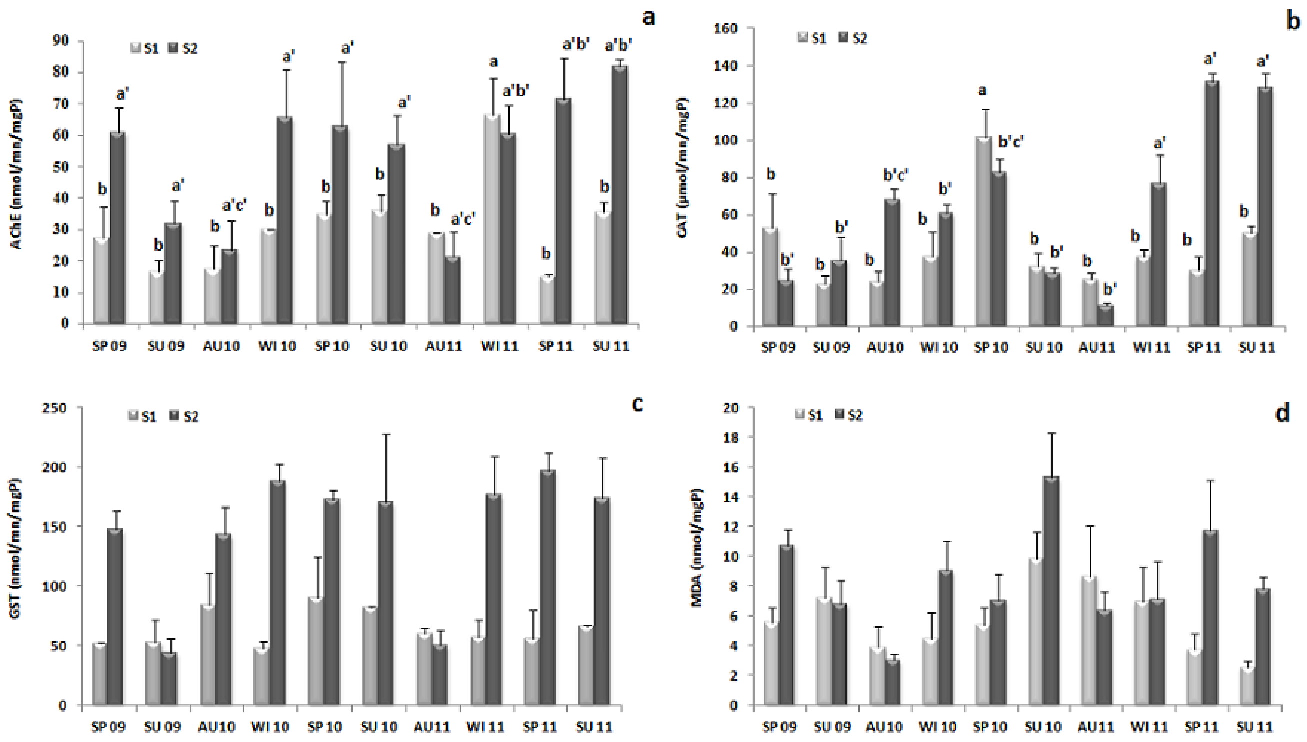

Figure 3: Seasonal variations of Acetylcholinesterase AChE (a), Catalase CAT (b), Gluthathion S-Transferase GST (c) activities and Malondealdehyde MDA content (d) (mean ± SD) (n=6) in *Donax trunculus* collected from Site 1 and Site 2 (M'zar Beach in Agadir Bay), sharing a common letter for the post Hoc test that is not significant (p< 0.05), (SP: Spring, SU: Summer, AU: Autumn and WI: Winter)

Table 3: Results of a 2-way ANOVA testing the effects of ''site'', ''season'' and interaction between the two factors on biomarkers responses in clams

		SS	DF	MS	F	p
AChE	**Season**	10999.66	9	1222.18	4.0934	<0.01
	Site	6025.60	1	6025.60	20.1811	<0.001
	Season*site	3453.50	9	383.72	1.2852	>0.05
	Error	10151.59	34	298.58		
CAT	**Season**	39782.6	9	4420.3	5.9449	<0.001
	Site	5895.4	1	5895.4	7.9288	<0.01
	Season*site	21103.5	9	2344.8	3.1536	<0.01
	Error	25280.7	34	743.5		
GST	**Season**	67380.9	9	7486.8	1.3367	>0.05
	Site	117370.6	1	117370.6	20.9550	<0.001
	Season*site	46605.7	9	5178.4	0.9245	>0.05
	Error	190436.5	34	5601.1		
MDA	**Season**	175.234	9	19.470	1.4363	>0.05
	Site	62.655	1	62.655	4.6218	>0.05
	Season*site	114.695	9	12.744	0.9401	>0.05
	Error	460.914	34	13.556		

The results of the seasonal variation of biological parameters, obtained in the present study, are agree with those reported by Moukrim et *al*., [14] using the same Mollusk as a sentinel species in polluted site of Agadir bay (polluted one, Anza beach ; and reference one, Aghroud). According to these authors, the AChE activity

exhibited high value in Summer and low one in Spring. Some authors reported seasonal variation for AChE activity in aquatic organisms related to the reproductive cycle [2,3], however the maximum of AChE activity coincided with spawning period, and the minimal coincided with sexual rest. Radenac *et al.*,[43]; Wu *et al*., [44] pointed out that environmental parameters influence the AChE activity levels. Our results corroboratewith the finding of Tsangaris *et al.*,[45] in the Greek coastline using caged mussels, and the work of Lagbouri,[4] in Anza beach (Agadir bay) for *D. trunculus*. Also, Moukrim *et al.*,[14] had recorded reduced AChE activity at the polluted site compared to pristine one. In another hand, the lowest AChE activity in soft mass of organisms from S1, could be due to the influence by its nearby to Oued Souss estuary, which drains large areas of Souss region, most of them are agricultural lands and occupied by agricultural building. So, important amounts of fertilizers and pesticides were spread in these areas and arrived to M'zar beach as endpoint according to the marine currents direction (from North to South), and then affected organisms. Agnaou *et al.*,[46] observed high concentrations residues of OCPs at Oued Souss estuary in *Scrobicularia plana* and sediment. These last authors explained their results by the excessive agricultural use of pesticides in this region. Mora [47] had documented that heavy metals could cause the inhibition of cholinesterase, however no significant correlation were identified between acetylcholinesterase and heavy metals in *D. trunculus* from the study area.
Regarding the differences of biomarker responses levels between the two sites (S1 in North and S2 in South of the emissary), our results indicate a significant elevation of lipid peroxidation (MDA, CAT, GST) in specimens from Site 2. This difference could be explained by the direction of marine currents, as mentioned previously, in Agadir Bay. In fact, spatial distribution indicated a well-defined north-south gradient of marine currents [23,24].The apparent increase in lipid peroxidation may be attributed to the accumulation of other heavy metals and pollutants inducing oxidative stress in animal soft tissues, Lesser and Kruse [48] pointed out that oxidative stress is a high seasonal phenomenon in Bivalve Mollusks, as our data indicate no significant correlations between MDA contents and metal concentrations. However, metals catalyzed the formation of ROS capable of damaging tissues such as DNA, proteins and lipids are well documented [49,50]. Our results related to MDA seem to have the same values as those reported by Tlili *et al.*, [20] in *D. trunculus* from Tunis Golf. Also, some kinds of pollutants such as heavy metals, HAPs, PCBs, and phenols can participate to provoke oxidative stress [51,52]. Furthermore, GST activities were particularly elevated at S2. The significant increase may be a response to oxidative stress caused by the presence of other metals and/or contaminants not analyzed in this study, or it could be due to leaking wastewater discharged into the marine environment during the submarine emissary reconditioning. Indeed, the correlations between GST and heavy metals measured in *D. trunculus* from M'zar beach were very weak. The increase in CAT activity is usually observed in the face of environmental pollutants since SOD-CAT system represents the first line of defense against oxidative stress [49]. However in the present study, the activity of CAT showed no site discrimination which suggests probably the presence of the same oxidative stress factors. Opposite pattern has been previously reported in *D. trunculus* from Anza and Aghroud beaches (Agadir bay) [18]. Almeida *et al.,* [53] reported that various environmental pollutants are known to induce CAT activity in aquatic organisms [54]. In our case positive correlation between CAT activity and Hg concentrations were found. Many authors documented high CAT levels especially from impacted sites [4,9,55].

3.3. Relationship between metals and biomarkers

Multiple correlations were conducted to assess the relationships between metals in *D. trunculus* whole tissues and biochemical responses, the results of correlation are depicted at table 4. No relationship was registered in biochemical parameters and the three heavy metals analyzed in the two study area. Mercury was the only metal that showed a significant relationship with biomarker levels. Hg is directly related to CAT activity ($r=0.67$, $p<0.001$). Considering the importance and potential complexity of aquatic pollution in this ecosystem, it is recommended to apply a multibiomarker approach in order to obtain a more comprehensive and integrated view of the biological responses, as chemical analysis of environmental samples alone, does not provide evidence of contaminant impacts in biota [55-57].

Table 4: Pearson correlation coefficients between trace elements concentration and biomarkers in the two study areas

	[Metal] vs AChE		[Metal] vs GST		[Metal] vs CAT		[Metal] vs MDA	
	p	**r**	**p**	**r**	**p**	**r**	**p**	**r**

Pb	0.983	-0.005	0.991	-0.0027	0.488	-0.1645	0.782	0.066
Hg	0.071	0.4127	0.071	0.4126	*0.001	*0.6758	0.37	0.2118
Cd	0.509	-0.1570	0.983	-0.0052	0.989	0.0034	0.097	0.3812

*significant correlation $p<0.05$

Conclusion

The combination between chemical approach (trace elements) and biological approach (multi-markers) seems to be more advantageous to explain distribution of pollution. In this study, generally, we did not observe a relationship between metal trace accumulation and biomarkers response. Probably, the biochemical responses observed at the sampling area are caused by other chemical contaminants than trace elements. In fact, our laboratory has conducted researches on pesticides at Oued Souss estuary (nearby zone to our study area) and explained the influence of OCPs as a principal source of pollution [46]. The activities AChE, less important in the site S2 near the river Souss estuary, is in favor of this explanation. This fact illustrated the inhibition of the enzyme activities probably under the influence of pesticides from Oued Souss, crossing agricultural farmland treated with pollutants.

In another hand, in this investigation, the levels of both biomarkers and trace elements in the bivalves associated with treated wastewater outfall from M'zar plant are remarkably lower than previous studies concerning the same sampling site and before the implantation of M'zar WWTP plant in Agadir Bay. This result reflects the performance of the treatment plant and shows its contribution to improving the environmental quality of the coast of the Bay of Agadir.

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