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Integrated Effect of Metal Accumulation, Oxidative Stress Responses and DNA Damage in *Venerupis Decussata* Gills Collected From Two Coast Tunisian Lagoons

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Abstract: The bivalve *Venerupis decussata* has been proposed as a sentinel species for assessment of lagoon water. Our study aimed to evaluate the spatial and temporal variation of oxidative stress biomarkers, metal content and DNA damage in *Venerupis decussata* digestive gland collected seasonally from contaminated (BOUGHRARA "L2") and comparatively cleaner (GHAR EL MELH "L1") lagoons. Trace metal contents (Cu, Pb and Cd) in *Venerupis decussata* collected at polluted site were 1-2 folds higher compared to the control site and showed maximum variation especially during summer and spring seasons. The current findings indicate a seasonal increase of malondialdehyde (MDA), protein carbonyl (PCO), glutathione (GSH) and metallothioneins (MT) levels in relation to trace element accumulation in *Venerupis decussata* digestive gland from BOUGHRARA sampling lagoon compared to those from GHAR EL MELH. We found an increase in glutathione-S-transfers (GST) and glutathione peroxides (GPx) activities in clams collected from BOUGHRARA lagoon. A random DNA degradation was observed mostly in digestive gland from the polluted site. The principal component examination of the physiological parameters showed a clear separation between *Venerupis decussata* collected from the polluted lagoon (L2) and those from the clean one (L1). Our study delivers basic information on the toxicological effects of environmental pollutants in clam through the combination of metabolic and physiological methodologies.

Keywords: Antioxidants Defense, Bioaccumulation, Calms, DNA Damage, Gills, Lagoons

1. Introduction

Marine ecosystems close to industrial and urban activities are contaminated by a large variety of xenobiotics that may be altered into novel harmful compounds. These anthropogenic imputes may have serious impacts on the marine environment and particularly in coastal areas such as lagoons [1]. In fact, that body water, which is the most vulnerable to pressures located at the interface between coastal, terrestrial and marine ecosystems, are valuable natural habitat for many life forms and offer socioeconomic advantages due to their highly productive system [2]. However, due to the often restricted water exchange with the sea these shallow ecosystems are particularly vulnerable to anthropogenic pressure and eutrophication processes [3].

Marine pollution along the Tunisian coast is at present limited to a few specific areas such as Gabes gulf, where, most part of the industrial activities are concentrated there [1]. Boughrara lagoon, located in the southern side of the gulf

of Gabes is subjected for decades to increased pollution [4]. While, Ghar el Melh lagoon is considered as the most unpolluted ecosystem used as a reference site in several monitoring programs [5].

Mollusk bivalves considered as the major targets of heavy metal contamination, are in direct contact with metal contaminants in the aquatic environment through water filtration including the food chain [6]. Bivalves such as *Venerupis decussata* (*V. decussata*) are mainly used for the assessment of aquatic environment pollution and considered as bio-indicators of environmental pollution [1, 7]. Gills tissues of bivalves possess a various essential functions (such as osmoregulation, respiration and nitrogenous waste excretion) have been widely used in the monitoring programs of aquatic pollutants, since it come in contact with environmental pollutants which absorbed toxic chemicals lead to a rapid toxic response in the gills [8].

Metals accumulation in calms gills catalyzes redox reactions that produce reactive oxygen species (ROS) which may induce oxidative stress causing morphological, physiological and biochemical alterations [9]. In order to prevent ROS generation, organisms have established antioxidant defense system to limit and eliminate ROS damaging [10].

Defensive mechanisms, including various antioxidant defense enzymes become activated to counteract ROS production [11]. Among the various antioxidant mechanisms, glutathione peroxidase (GPx) is an important intracellular enzyme that failure the hydrogen peroxides (H_2O_2) to water; and lipid peroxides to their corresponding alcohols mainly in the mitochondria and sometimes in the cytosol [12]. Glutathione -S-transferase (GST) is an antioxidant and phase II detoxification enzymes belongs to multigene family, involved in xenobiotic biotransformation and are important in the assessment of oxidative stress [13]. Glutathione (GSH) protects cells beside ROS generation due to exposure to xenobiotic and environmental stressor as it is the most abundant intracellular thiol-based antioxidant that functions as a cofactor for antioxidant enzymes [14].

This imbalance between antioxidants and pro-oxidants causing macromolecules damages such as DNA, lipids and proteins [15] which had been widely reported in numerous studies and considered as the seriousness effect of the oxidation [16]. Also, as a metal-binding protein that is induced by metal injuriousness, metallothionein (MT) is able to bind metals for detoxification and can be a sensitive and reliable biomarker of metal contamination in aquatic ecosystems [17].

The aim of our study was therefore to quantify the accumulation of metals and DNA deterioration in clams' gills and apply a battery of antioxidants defense and macromolecules injuries as protective systems to assess environmental metal accumulation of two coastal lagoons impacted with different degree of pollution.

2. Materials and *Methods*

2.1. Sampling and Tissue Preparations

Clams of Venerupis decussata (39.5±1.5 mm length and 13.5±2.0 weight) were collected from Ghar el Melh (L1) and Boughrara (L2) lagoon seasonally (Figure 1) and transferred directly in ice box to the laboratory where they were immediately dissected. Gills (30 individual calms per season) were carefully removed and some portions of the tissue were homogenized in 10% of Tris-HCl buffer (20mM, pH: 7.4) then, centrifuged at 10000g for 25min at 4°C. The obtained homogenates were then stored at -80°C for subsequent biochemical analysis. Other gills portions were cleaned and stored (-80°C) to be used either for DNA extraction or for trace elements analysis.

2.2. Trace Elements (TE) Analysis

Metals accumulation was carried out according to Carvalho et al. [18] method. The lyophilized gill tissues were mineralized with nitric acid (NHO3) and hydrogen peroxide (H2O2). Metals concentrations were analyzed using ICP-MS (model: E2M18 with Agilent ASX-500 ICP-MS Autosampler) and expressed as mg/ kg of dray weight.

2.3. Metallothionein (MT) Level

MT level was determined in V. decussata gills following the method of Viarengo et al. [19] modified by Petrovic et al. [20]. MT levels were expressed as nmol GSH. mg^{-1} protein.

2.4. Malondialdehyde (MDA) Level

MDA level was determined spectrophotometrically at 532nm by the thiobarbituric reactive species (TBARS) assay based on Draper and Hardely [21] method. Briefly, 500 µl of gills extract was mixed with 1 ml of trichloroacetic acid (10%) and centrifuged at cold for 10 min. Then, 1ml of the previous mixter was added to 1ml of thiobarbituric acid (0.67%) and incubated for 15 min at 100°C. Results were expressed as nomoles of thiobarbituric acid reactive substances, using 1,1,3,3-tetra-ethoxypropane as standard (TEP) and expressed as µmoles. mg^{-1} of protein.

2.5. Protein Carbonyl (PCO) Level

PCO level was evaluated according to Reznick and Packer [22]. 100 µl of gills extract was added to500 µl of 2,4-dinitrophenylhydrazine (10 mM) dissolved in HCl (2 N). Samples were incubated for 1 h at 37°C and vortexed each 10 min. After that, 500 µl of trichloroacetic acid (20%) was added and the mixtures undergo centrifugation for 10 min at 3500×g. The pellet contained protein was washed several times with ethanol–ethyl acetate (v/v) and dissolved in 600 µl of guanidine hydrochloride (6 M). The absorbance was determined at 370 nm. Carbonyl content was calculated using the molar extinction coefficient ε02.2×104cm−1M−1 and expressed as nomoles. mg^{-1} protein.

2.6. Glutathione (GSH) Level

GSH level was determined in gills supernatants by measuring the intensity of the absorbance under DTNB (10mm) addition using the spectrophotometrical method of

Ellman [23] at 412nm. Results were expressed as µg.mg^{-1} protein.

2.7. Glutathione-S-Transfers (GST) Level

GST activity was quantified following the protocol of Habig et al. [24]. Briefly, 100µl of diluted sample was added to 200µl of reactive solution contained phosphate buffer (pH: 6.5; 100mm), GSH (10mm) and CDNB (60mm). The reaction was measured by calculating the rate of connection between 1-Choloro-2, 4 -dinitrobenzene (CDNB) with reduced L-Glutathion (GSH). This reaction in the range of ultraviolet ray was measured at 340nm. Results were expressed as µmol. mg^{-1} protein.

2.8. Glutathione Peroxides (GPx) Level

GPx activity was measured by Flohe and Gunzler [25] method. Absorbance at 340 nm was detected after addition of supernatant. Data were expressed as nmol of GSH oxidized. min-1. mg-1 protein.

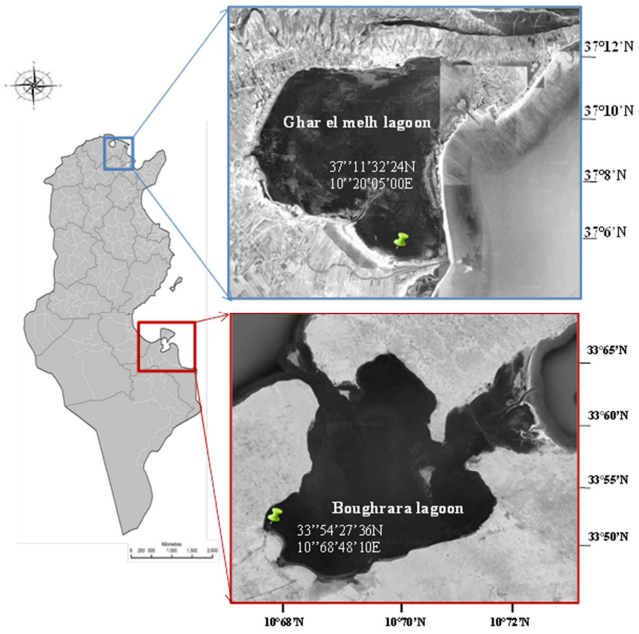

Figure 1. *Sampled sites along the Tunisian coastal.*

2.9. DNA Damage

DNA extraction was performed using Clarke et al. [26] procedure based on cetyltrimethyl ammonium bromide as reactive buffer (CTAB; 2%). The purified DNA has undergone migration on agarose gel (1%) and deterioration was observed and photographed in a dark room under an ultraviolet lamp.

2.10. Statistical Analysis

Signification was applied at 0.05. Normality was carried out according to Statistica version 8. Data were analyzed through non parametric and ANOVA tests between seasons. Correlation and principal component analysis (PCA) were performed to integrate all dates' and evaluate the relation between them.

3. Results

3.1. Traces Elements (TE) Accumulation

TE concentrations (Pb, Cu and Cd) in gills of *V. decussata* from Ghar el Melh and Boughrara lagoons are presented in Table 1. The most accumulate element in both population was cooper (Cu) followed by lead (Pb) and cadmium (Cd). However, results indicate that samples from L2 contain higher TE concentration as compared to L1 over studied seasons (<0.05). Furthermore, the important significant change was observed during spring and summer for Cd (33% and 29% respectively), Pb (38% and 23% respectively) and Cu (23% and 30% respectively). Only cd exhibited significant increases in clams' gills from L2 during autumn (27%) and winter (27%) than those from L1. While, Pb and Cu concentrations did not shows any differences between the studied lagoons during the cold seasons.

3.2. Metallothioneins Levels (MTs)

Our results revealed (Table 1) an increase of MTs levels in gills tissues of *V. decussata* collected from L2 during spring (34%), summer (41%) and autumn (30%) as compared to L1. No significant differences were recorded between sampling sites during winter season.

Table 1. *Seasonal variation of metallothioneins and Traces elements levels in clams' gills from two coastal lagoons.*

	MTs	Cd	Pb	Cu
Spring				
L1	0.41 ± 0.05	0.66 ± 0.04	1.17 ± 0.04	4.50 ± 0.40
L2	0.55 ± 0.07**	0.88 ± 0.07**	1.62 ± 0.25 *	5.55 ± 0.46 *
Summer				
L1	0.87± 0.05	1.11 ± 0.08	1.77 ± 0.28	5.20 ± 0.40
L2	1.23 ± 0.21**	1.57 ± 0.18 **	2.19 ± 0.07 *	6.76± 0.41 *
Autumn				
L1	0.49 ± 0.08	0.70± 0.08	1.44 ± 0.12	3.89 ± 0.78
L2	0.64 ± 0.08**	0.89 ± 0.64 *	1.61 ± 0.13	3.98 ± 0.78
Winter				
L1	0.42 ± 0.09	0.63 ± 0.08	0.94 ± 0.07	2.47 ± 0.21
L2	0.44 ± 0.08	0.87 ± 0.08 *	1.06 ± 0.20	2.56 ± 0.21

Values are means ± SD for 6 clams (per season and per site). Significant difference between L1 and L2 are detected at *p<0.05 and **p<0.01.

3.3. Malondialdehyde Levels (MDA)

V. decussata from L2 showed a significant increase of MDA levels during spring (19%), summer (23%) and autumn (28%) seasons than those from L1 (Table 2). However, winter season exhibited similar variation between the two studied lagoons.

3.4. Protein carbonyl Levels (PCO)

The levels of PCO were determined in *V. decussata* gills (Table 2). Results showed significant increases of PCO levels by 38%, 33% and 19% during spring, summer and autumn respectively in gills tissues of clams from L2 as compared to L1. When compared between the two lagoons, PCO levels reached similar values during winter season.

3.5. Principal Compound Analysis (PCA)

Principal component analysis (PCA) was performed on the trace elements, biomarkers responses, lipid peroxidation and protein oxidation of clams gills collected from two coastal Tunisian lagoons (Figure 2). PCA results showed a significant separation between studied lagoons in relation to seasonality, explained by 81.01% of total variance (F1=69.37%; F2=11.65%).

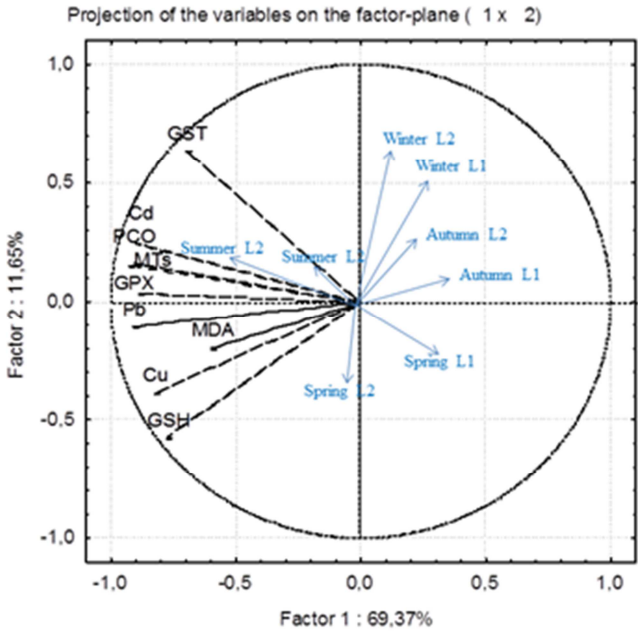

Figure 2. *Principal compound analysis (PCA) represents 81.01% of the total variance of biomarkers (MTs, GSH, GST and GPx), trace elements (Pb, Cd, Cu) and macromolecules indices (MDA and PCO).*

As shown in fig.(1) Boughrara lagoon has significantly higher levels of MDA (F1= -0.700), PCO (F1= -0.905), MTs (F1= -0.924), GSH (F1= -0.775), GPx (F2=-0.888) and GST (F1= -0.700) with an important accumulation of Cu (F1= -0.829), Cd (F1= -0.901) and Pb (F1= -0.911) in gills tissues as compared to Ghar el Melh lagoon, especially spring summer season.

Table 2. *Seasonal variation of malondialdehyde and protein carbonyl levels in clams' gills from two coastal lagoons.*

	MDA (nmol.mg^{-1}.pr)	PCO (nmol.mg^{-1}.pr)
Spring		
L1	14.88 ± 2.76	1.26 ± 0.23
L2	17.84 ± 2.69*	1.75 ± 0.51*
Summer		
L1	17.25 ± 1.38	2.40 ± 0.52
L2	21.24 ± 3.17*	3.20 ± 0.52*
Autumn		
L1	15.35 ± 2.13	1.72 ± 0.15
L2	19.70 ± 4.12*	2.05 ± 0.20*
Winter		
L1	16.31 ± 1.47	1.58 ± 0.31
L2	17.47 ± 2.46	1.65 ± 0.38

Values are means ± SD for 15 clams (per season and per site).
Significant difference between L1 and L2 are detected at *p<0.05.

3.6. Enzymatic Antioxidants Responses

Antioxidants enzyme responses of GSH, GST and GPx in *V. decussata* gills from two coastal lagoons are represented in Table 3. Our results showed that GSH levels in clams' from L2 were significantly elevated by 18% and 26% during spring and summer respectively compared to those of L1.

However, GSH levels seem stable during the cold seasons over the studied lagoons. As shown in Table 3, clams from L2 characterized by significant increases of GPx and GST activities during spring (27% and 29% respectively), summer (36% and 24% respectively), autumn (32% and 12% respectively) and winter (34% and 24% respectively) as compared to those collected from L1

Table 3. *Seasonal variation of enzymatic antioxidants responses in calms gills collected from two coastal lagoons.*

	GSH (µg.mg^{-1}.pr)	GST (nmol.min^{-1}. mg^{-1}.pr)	GPx (nmol GSH. min^{-1}. mg^{-1}. pr)
Spring			
L1	3.47 ± 0.50	0.52 ± 0.06	9.08 ± 0.61
L2	4.13 ± 0.54*	0.68 ± 0.05*	11.60 ± 1.17**
Summer			
L1	3.72 ± 0.61	0.83 ± 0.11	11.36 ± 1.16
L2	4.71 ± 0.55**	1.04 ± 0.12*	15.52 ± 1.13***
Autumn			
L1	2.92 ± 0.26	0.56 ± 0.02	8.65 ± 0.64
L2	3.16 ± 0.35	0.63 ± 0.04*	11.45 ± 0.97***
Winter			
L1	2.33 ± 0.45	0.18 ± 0.01	8.60 ± 1.00
L2	2.69 ± 0.39	0.23 ± 0.02*	11.56 ±1.52***

Values are means ± SD for 15 clams (per season and per site).
Significant difference between L1 and L2 are detected at *p<0.05, **p<0.01 and ***p<0.00

3.7. DNA Damage

The analyzed gels of *V. decussata* gills are summarized in Figure 3. Results demonstrated clear damage in DNA quality of gills tissues from L2 as compared to L1 during spring and summer. These alterations were presented by smear and fragmentations. However, during autumn and winter DNA structure between the studied lagoons were characterized with clear DNA band within deterioration.

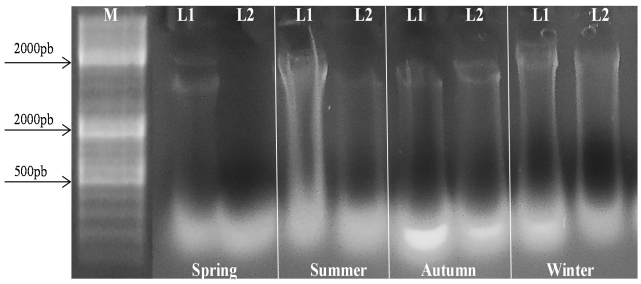

Figure 3. *DNA degradation in gills tissues of V. decussata sampled seasonally from Ghar el melh (L1) and Boughrara lagoons (L2). M: marker (3 kb DNA ladder).*

4. Discussion

Nowadays, the extensive dispersion of metals and metalloids used in many industrials activities allow as investigating their potential hazardous effects on aquatic organisms health.

In the present study, the gradient of metal uptake was detected in gills of clams that were collected from the two studied lagoons. Boughrara lagoon was found to be the ecosystem with the highest TE accumulation, in concordance with previous field studies [27]. While, Ghar el Melh lagoon considered as unpolluted ecosystem which was selected as one of the primary sites within the MELMARINA project (Monitoring and Modelling Coastal Lagoons: Making Management Tools for Aquatic Resources in North Africa) [28]. This lagoon had a lower TE accumulation than the previous one. Among season, TE accumulation varied largely with highest uptake recorded during summer, which could be explained by weather's. In this context, summer meteorological conditions joined effects of important evaporation and slight precipitation that might occur to higher TE accumulation as described in previous work [29]. During winter the accumulation of TE decreased as compared to summer in both studied lagoons. This may be due to lixiviation caused by increasing of rainfall which contributes largely to dilute TE [30].

Exposures to TE was associated with a variety of toxicological outcomes in aquatic species, including change in MTs levels, which largely act as a regulator of metal homeostasis in tissues and have an important role in the capture of harmful oxidant radical species [31]. In marine invertebrates, MTs have been employed in the detoxification, storage, transport, and exchange of essential and non-essential trace elements [32].

The rise of MTs concentration in *V. decussata* from Boughrara lagoon than those from Ghar el Melh, represents the typical response to metal toxicity. Meanwhile, the higher accumulation was noted during summer as reported by Bigot et al. [33] showed that MT levels were increased significantly during summer in *Corbicula fluminca* gills from a polluted site. Our data corroborated with previous investigations described a higher MTs level in relation with TE accumulation [1, 16].

The stability between the pro-oxidant stimulated by ROS and the organism's capacity to eliminate the xenobiotic and/or excessive TE accumulation, namely through the antioxidant defense system, is essential to assess severe cellular injury, either in macromolecules such as DNA, proteins and lipids [34]. Thus, lipid peroxidation such as MDA commonly used as a suitable index to assess lipid peroxidation of species exposure to the various stressors [35]. Our results reported that clams collected from Boughrara lagoon exhibited a significant elevation of MDA levels among seasons. It has been reported that such increases could be associated to H_2O_2 generation in gills tissues that provoke deficiency of mitochondrial respiratory chain. Furthermore, an important generation of H_2O_2 of clams' gills from Boughrara lagoon might affect the membrane structure through polyunsaturated (PUFA) attack which resulting to enhancement of MDA levels. In this context, many filed have demonstrated that gills are rich with PUFA that make them more vulnerable to lipid peroxidation as compared to other organs [36, 37]. Our results are in agreement with others research's reported that environmental metals pollution, increased MDA levels in several bivalves' tissues [38, 49]. As lipids, protein oxidation caused by hydroxyl radicals' generation form H_2O_2 via Fenton reaction, could attack directly protein backbone and amino acid chain [40]. Proteins are the biological targets for ROS generation giving rise to the carbonyl group formation into side chains [41]. In this respect, ROS, probably generated as a result of TE accumulation in clams gills from Boughrara lagoon induced an increase of PCO levels, considered as suitable biomarker for protein damage. Recent studies indicate the effect of higher environmental pollution in the enhancement of cellular (ROS) release, thereby increasing the risk of PCO [42].

The excessive generation of ROS and free radical production has been described to mediate genotoxicity and cell death [43]. Results showed that in gills of *V. decussata* collected from Boughrara lagoon, DNA structure was deteriorating gravelly presenting by smear. While, clams from Ghar el Melh lagoon presented with no DNA fragmentation and no subsequent format of DNA smear. In line with our findings, previous works reported that release of free radicals causing by environmental metals pollution was associated with DNA damage and cell death [16]. Oxidative stress refers to situation of marked imbalance between the production and the remove of reactive oxygen species. To defend against oxidative stress, bivalves are equipped with an effective and complex antioxidants system [44]. Among them, GPx and GST considered as regulators of endogenous reactive oxygens at relatively low levels to continue normal cell function and to reduce the damage caused by their high reactivity [10]. Also, these enzymes catalyze the conversion of hydrogen peroxide to water and have the ability to reduce tissue injury by removing H_2O_2 [16].

In the present study, GST and GPx activities increased significantly in clams from Boughrara lagoon which might reflect an adaptive mechanism of the anti-oxidative defense system. Similar results have been reported that GPx activities

increase in relation to the high metal accumulation and abiotic parameters [45]. Our results were in total agreement with previous works demonstrated that metal accumulation during summer stimulated GST activities in gills and digestive gland tissues of *Mytillus galloprovincialis* and *Perna viridis* respectively [45].

Non-enzymatic antioxidants such as GSH considered a second line of cellular defense against free radical generation and the major cellular redox buffer [10]. GSH is the most abundant non protein thiol involved in the elimination of ROS and the preservation of membrane protein thiols, and it serves as a substrate for GPx and GST [11]. The difference in GSH levels showed that clams from Boughrara lagoon exhibited the highest adaptation against environmental stresses. There are several reports on increasing GSH levels in bivalves in the presence of excess free radical caused by TE accumulation [46, 47].

The bioavailability of metals is an important tool to comprehend the biological effects of its bioaccumulation that can occur in exposed organisms' when metals were spilled in aquatic ecosystems. However, numerous biotic and abiotic parameters may influence metal bioaccumulation of organisms; thus, it is more appropriate to assess metals bioavailability by measuring the bioaccumulation and the biomarkers status of specific organs in exposed organisms as indicators [1]. In our study, principal component analysis (PCA) separated studied lagoons according to the physiological status of *V. decussata* among seasons. Further, PCA analysis indicated that the anthropogenic activities of Boughrara lagoon were more important as compared to Ghar el Melh lagoon.

5. Conclusion

The present study was undertaken with an objective to explore the eco-physiological interaction and the impact of lagoon pollution in the two natural populations of a *venerupis decussata*. Trace elements uptake and MTs levels were higher in specimens from Boughrara lagoon. Also, the highest adaptation against environmental pollution was observed through increases of GSH, GPx, GST, MDA and PCO responses in the same population. Our genotoxicity examination showed serious fragmentation under stressful conditions in *V. decussata* gills collected from Boughrara lagoon. Seasonal changes in physiological biomarkers must be carefully taken in toads in order to use them in the environmental assessment programs which deliver information's that could help improving the health status lagoons.

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Conflict of Interest

The authors have no conflict to declare.

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