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Effect of Lead Graded Doses in *Mactra Corallina* Gills: Antioxidants Status, Cholinergic Function and Histopathological Studies

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Abstract: Lead is non-essential toxic metal used in the industrial process causes severe risk to aquatic organisms. This study aimed (aims) to evaluate the effect of Pb on oxidative stress in gills of *Mactra corallina*. During the experiment, bivalves were randomly divided into four groups, control served as control and D1, D2 and D3 groups were exposed to Pb graded doses (1mg/L, 2.5mg/L and 5mg/L) during 5 days, respectively. Pb accumulation was significantly increased in all treated gills with doses dependent manner. The exposure of M. corallina to $PbCl_2$ promoted oxidative stress in gills with an increase in malondialdehyde (MDA) and in metallothionein (MTs) levels. Moreover, a decline in glutathione (GSH), non-protein–SH (NPSH) and ascorbic acid (Vit C) levels were detected in all treated groups. Thus, alterations of enzymatic antioxidants systems were confirmed by a significant increase of catalase (CAT) and decreases of glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities in doses dependent manner. The cholinergic function was confirmed by a significant decrease of acetylcholinesterase (AChE) activity in the highest exposure dose. The impairment of the gill function was confirmed by the histological study.

Keywords: Gills, Histopathological Studies, Lead Exposure, *Mactra Corallina*, Oxidative Stress

1. Introduction

Aquatic systems are contaminated by different metals through inputs from human activities [1]. Lead (Pb) is non-essential heavy metal, that can be provided from natural and industrials effluents including lead ore mining and smelting, refining, alkyl-lead petroleum combustion, batteries and cement manufacture [2]. In addition, Pb is among the most of inputs metals in water and sediment that could be accumulated by most aquatic taxa especially the faunal bivalves [3-4].

These taxa, such as *Mactra corallina* (*M.corallina*), are widely reported as bioindicators species in the monitoring applications, because of their filter feeders' mode, sedentary living and their capacity to accumulate trace elements [5-7]. The clam *M.corallina*, which generally distributed along Mediterranean and Atlantic coasts and estuaries, considered as an important sea food in Manches coast. In Tunisia, it has a large repartition from the Northern to Southern sandy beaches, occupying the lower infra-littoral zone (3to100 m depth).

Nevertheless, there is a lack of information regarding the tolerance of gill *M. corallina* to Pb potential effects. The reason for appointing gills is because this tissue forms an active site for metal uptake and oxy-radical generation in addition to enzyme biotransformation process. One of the most important established mechanisms of Pb toxicity is this capacity to boosts the ROS production, which in turn results in cell membrane damage, protein oxidation and DNA alteration [8-12].

In this study gill of *M. corallina* was examined to evaluate the effect of $PbCl_2$ graded doses exposure using biochemical parameters: lipid peroxidation, oxidative stress and histopathological changes.

2. Materials and Methods

2.1. Chemicals

Reduced glutathione (GSH), 5,5-dithio-bis-(2-nitrobenzoicacid) (DTNB), 2-thiobarbituric acid) (TBA) were purchased from Sigma chemical Co (Saint Louis, MO63103, USA). All other chemicals were purchased from standard commercials suppliers.

2.2. Experimental Exposure of Mactra Corallina

M. corallina (average shell length: 3.5±0.63 cm and weight 8.03±0.47g) were sampled from Bizerte lagoon in depths greater than 1m with scuba divers. Clams allowed acclimated for one week in twenty-liter aquaria renewed daily with fresh sea water. Control conditions systems were maintained: Temperature (18°C), Salinity (30psu), pH (7.4±0.2) and photo period (12/12). However, no type of food was attributed to *M. corallina* during the acclimatization and the experiment period. After acclimation, groups of individuals were transferred in 8L plastic aquaria and control was enclosed, therefore, exposed to unmixed $PbCl_2$ metal (Lead chloride; $PbCl_2$; Sigma-Aldrich; powder 98%) which was dissolved in pure water. The experiment was maintained for a period of 5 days under graduated $PbCl_2$ concentrations as following: CT: control; D1: 1mg/L; D2: 2.5mg/L and D3: 5mg/L with controlled conditions as mentioned above. Each treatment was performed with three replicates exposure (25 bivalves per condition). The selected Pb concentration in our experiment was based on preliminary trials focused on other bivalves [13, 14]. No mortality was recorded during the experimental period.

2.3. Samples Preparation

In this study, gills of fifty clams were pooled and 9 replicates were used for each experimental group. Samples were homogenized in Tris-HCl buffer (20 mM; pH=7.4) at cold, then, centrifuged at 10.000×g for 20 min (4°C). Gills supernatants were stored in eppendorf tube sat-80°C for oxidative stress analysis. Other portions were fixed in ethanol (70°) and embalmed in paraffin until histological analysis.

2.4. Determination of Pb Content in Gills of M.corallina

Gills of M.corallina were processed for Pb estimation according to the method described by Cheung and Wong [15]. Samples mineralization were obtained after addition of nitric acid (NHO_3) and hydrogen peroxide (H_2O_2; 37%) at a hot temperature. The mineralized solution was gauged with distillate water at 50ml until analysis. Metal content was determined by inductively coupled plasma mass spectrometry (ICP-MS) equipped with a graphite furnace. Blank samples and reference standard materials were processed to assure quality control.

2.5. Biochemical Analyses

2.5.1. Protein Quantification

Protein content was estimated according to Lowry et al., [16] method; using Folin Reagent and Bovine serum albumin (BSA) as a standard range.

2.5.2. Malondialdehyde (MDA) Levels

MDA level was determined according to Draper and Hadley (1990) [17] by spectrophotometer method at 532 nm. An aliquot of 0.5ml was incubated in heated water (37°C) for 1hour, then, mixed with 0.5ml of trichloroacetic acid (TCA 30%). After centrifugation at 3500×g for 10 min in cold 4°C; 0.5ml of thiobarbituric acid (TBA 0.67%) was added to 0.5ml of supernatant. There action was activated under heated incubation during 10min. Results were expressed as nmol/mg protein.

2.5.3. Glutathione (GSH) Levels

GSH level was measured according to Ellman [18] at 412 nm after addition of 5-dithio-bis (2-nitrobenzoicacid) (DTNB). The concentration of GSH was calculated through a standard concentration and expressed as µg/mg protein.

2.5.4. Non protein–SH (NPSH) Levels

NPSH levels were determined by the method of Ellman [18]. An aliquot of 100µl was mixed with trichloroacetic acid (10%). After centrifugation for 10min, SH groups were determined in a pure supernatant under addition of potassium phosphate buffer (pH=7.4; 1M) and DTNB (10mM). The absorbance of colorimetric reaction was measured at 412nm and NPSH level was expressed as µmol/mg protein.

2.5.5. Ascorbic Acid (Vit C) Levels

Vit C level was measured in gills and tissues according to Jaques Silva et al [19]. Protein was precipitated in cold trichloracetic acid solution and centrifuged during 10 minutes. Then, the supernatant was incubating in hot temperature (85°C) during 30 minutes with dinitro-phénylhydrazine (DNPH) and copper sulfate (CuSo4). There action product was determined after addition of sulfuric acid (65%). Dates' were expressed as µg of ascorbic acid per mg of protein.

2.5.6. Superoxide Dismutase (SOD) Activity

SOD activity was analyzed according to the method described by Beauchamp and Fridovich [20]. There action mixture contained: 50µl of tissue homogenates in 20mM Tris-HCl buffer (pH=7.4), 0.1mM EDTA, 13mM l-

methionine, 2mM riboflavin and 75mM Nitro blue Tetrazolium (NBT). The developed blue color was measured at 560nm after incubation under light. Data were expressed as µmol/mg protein.

2.5.7. Glutathione Peroxidase (GPx) Activity

Using Flohea nd Gunzler [21] procedure, glutathione peroxidase activity (GPx) was measured spectrophotometrically at 340. GPx was expressed as nmol of GSH oxidized/min/mg protein.

2.5.8. Catalase (CAT) Activity

CAT activity was determined by the method of Aebi [22] using H_2O_2 (0.5M) as a substrate. The concentration of H_2O_2 was determined every 15 seconds after initiation of the reaction by the addition of samples. One unit of CAT was defined as µmole H_2O_2 consumed/min/mg of protein.

2.5.9. Metallothionein (MTs) Levels

MTs were determined according to the method developed by Viarengo et al., [23] modified by Petrovic et al., [24]. An aliquot of supernatant (500µl) was added to ethanol/chloroform solution (95%; 1%) and centrifuged during 10 min in cold for 6000g. The obtained pellets were suspended in NaCl (0.25M) and EDTA (1mM). MTs reaction was detected under DTNB at 412nm. Results were expressed as nmol GSH/mg protein.

2.5.10. Acetylcholinesterase (AChE) Activity

AChE activity was measured using the colorimetric method of Ellman et al., [25]. Acetylthiocholine iodide was used as a substrate, in a concentration of 8.25mM. The kinetics of AChE was measured spectrophotometrically at 412 nm and determined during 5 min each 60 seconds. AChE activity was expressed as nmol of substrate/min/mg protein

2.6. Histological Analysis

Histology of gills was examined using a technique of Martoja and Martoja-Pierson [26]. Sections of the gills were fixed for 48h in buffered formalin (10%), then transferred into 70% ethanol. Sections of 6-mm thickness were cut mounted on glass slides and stained with a solution of hematoxylin and eosin according to routine histological techniques. Each histological section was examined in detail under microscopic analysis coupled with CCD camera.

2.7. Statistical Analysis

Results are expressed as means ± SE (standard error) for each analysis. The level of significance was as curtained at 0.05. Differences in antioxidants biomarkers between control and the exposure concentrations were assessed by one-way ANOVA. The mean variance in the data set was detected using principal component analysis (PCA). A Pearson correlation matrix between biochemical parameters and Pb contents in gills of *M.corallina* was established.

3. Results

3.1. Pb content in M. Corallina Gills

Significant increases of Pb contents were observed in all treated *M.corallina* gills ($p<0.001$) as compared to the control. Pb content varied between 0.22±0.010mg/kg DW and 1.45±0.09mg/kg DW in control and D3 (5mg/LPbCl$_2$) groups (Figure1). After exposure to graded dose (5mg/LPbCl$_2$), Pb was accumulated in gills more than the authorized limit for human consumption (Figure1).

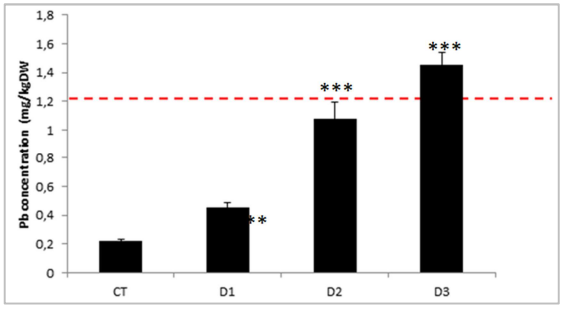

Figure 1. *Concentration of Pb in control and treated M.corallina gills with $PbCl_2$ graded doses (D1, D2, D3) after 5 days of treatment.*

The standard limit was presented by line obtained from SRM 2976 (muscle tissue, National institute of standards and technology). Values are expressed as means ± SD (n=6) D1:1mg/LPbCl$_2$; D2:2.5mg/LPbCl$_2$; D3:5mg/LPbCl$_2$ PbCl$_2$ groups VS controls: ***P<0.001

3.2. Malondialdehyde (MDA) Levels in M. Corallina Gills

Our results revealed significant increases of MDA levels (+48%, +32%, +81% respectively) in the gills of all treated groups when compared to controls (Table1). This peroxidation was confirmed by a positive correlation with MDA levels and Pb contents in all exposed gills (Table 2)

3.3. Non Enzymatic Antioxidants Levels in M Corallina Gills

Results showed significant decreases in GSH (-15 and-19%), NPSH (-26 and -34%) and Vit C (-35 and-63%) levels in treated gills *M. corallina* with 1 and 2.5mg/L of PbCl$_2$ respectively when compared to the control (Table 1).

These non enzymatic antioxidants activities seem to be correlated ($p<0.05$) positively with lipid peroxidation index (Table 2).

3.4. Enzymatic Antioxidants Activities in M.Corallina Gills

The enzymatic antioxidants activities (CAT, GPx and SOD) in control and treated bivalves are illustrated in Table1. Treated gills at different Pb concentrations showed a significant decrease in their SOD (-24, -30, -16%) and GPx activities (-33,-42 and-21%) at 1, 2.5 and 5mg/LPbCL$_2$ respectively. However, CAT activity demonstrated an increase (+95, +186 and +98%, respectively) in the treated gills with graded doses 1, 2.5 and 5mg/LPbCl$_2$ (Table 1).

The activity of CAT in gills was negatively correlated with SOD and GPx activities whereas it presents a positive correlation with AChE activity (Table 2).

Table 1. *MDA, non-enzymatic levels (GSH, NPSH and Vit C levels) and enzymatic activities (CAT, GPx, SOD) in control and treated M.corallina gills with $PbCl_2$ graded doses (D1, D2, D3) after 5 days of treatment.*

Parameters and treatments	CT	D1	D2	D3
MDA[a]	1.51±0.27	2.23±0.08***	2±0.09***	2.74±0.14***
GSH[b]	3.50±0.52	2.67±0.25**	2.19±0.33**	2.98±0.4*
NPSH[c]	0.16±0.02	0.12±0.01***	0.11±0.01***	0.13±0.01***
VitC[a]	18.91±4.23	12.23±1.30***	7.35±1.92***	15.05±2.54***
CAT[d]	5.50±1.85	10.75±1.34***	15.75±2.68***	10.89±1.55***
GPx[e]	11.52±1.26	7.63±0.88***	6.66±0.61***	9.02±0.72***
SOD[f]	19.69±2.04	14.75±0.19***	13.63±1.40***	16.49±1.60***

Values are expressed as means ± SD (n=9).
D1:1mg/L$PbCl_2$; D2:2.5mg/L$PbCl_2$; D3:5mg/L$PbCl_2$
a: nmol/mg protein
b: µg/mg protein.
c: µmol/mg protein.
d: µmol H_2O_2 consumed/min/mg protein.
e: nmolesGSH/min/mg protein.
f: U/mg protein.
$PbCl_2$ groups VS controls: **P<0.01; ***P<0.001.

3.5. Metallothionein Levels (MTs) in M.Corallina Gills

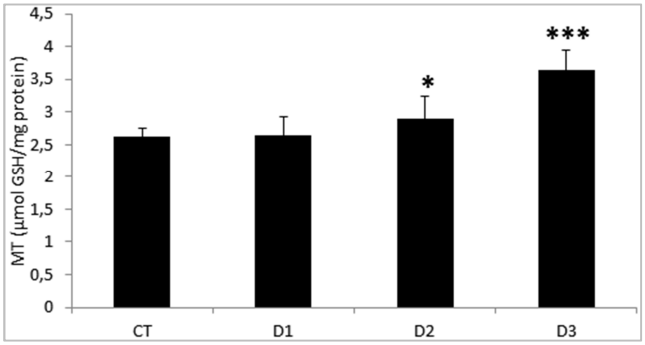

Figure 2. *MTs levels in control and treated M.corallina gills with $PbCl_2$ graded doses (D1, D2, D3) after 5 days of treatment. Values are expressed as means ± SD (n=9).*

D1:1mg/L$PbCl_2$; D2:2.5mg/L$PbCl_2$; D3:5mg/L$PbCl_2$.
$PbCl_2$ groups VS controls: *P<0.05, ***P<0.001.

A significant increase of MTs level was observed in treated *M.corallina* with 2.5 and 5mg/L of $PbCl_2$ by +55 and +88%. While, no significant change was noted in the first treatment (1mg/L of $PbCl_2$) than the controls (Figure 2). An important positive correlation between MTs and Pb contents and MDA levels was observed in our study. However, any significant correlation is recorded between metallothionein and antioxidants activities such as enzymatic and nonenzymatic (Table 2).

3.6. Acetylcholinesterase Activity

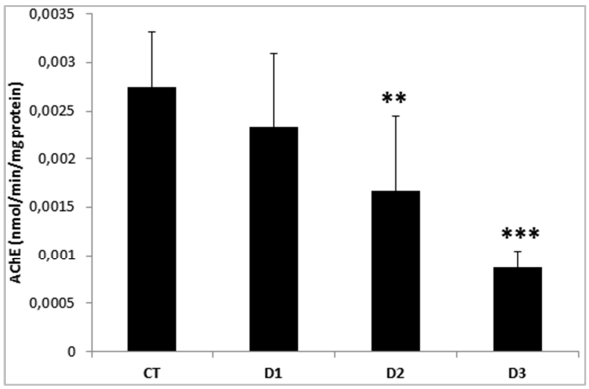

Figure 3. *AChE activity in control and treated M. coralline gills with $PbCl_2$ graded doses (D1, D2, D3) after 5days of treatment.*

Values are expressed as means ± SD (n=9).
D1:1mg/L$PbCl_2$; D2:2.5mg/L$PbCl_2$; D3:5mg/L$PbCl_2$.
$PbCl_2$ groups VS controls: **P<0.01, ***P<0.001.

Our results revealed a significant decrease of AChE activity in the gills of all treated group at graded doses (-15, -40 and -70%, respectively) when compared to those of controls (Figure 3).

A great negative correlationis observed between the AChE activity and nonenzymatic antioxidant systems (Table 2).

Table 2. *Correlation analysis (Pearson correlation) between Pb contents and biochemical parameters (MDA, GSH, NPSH, VitC, CAT, GPx, SOD, MT and AChE) in control and treated M. coralline gills with $PbCl_2$ graded doses (D1, D2, D3) after 5 days of treatment.*

Parameters	Pb contents	Protein	MDA	MT	NPSH	GSH	VitC	CAT	SOD	GPx
Protein	0.46									
MDA	0.60*	0.09								
MT	0.78*	-0.08	0.51							
NPSH	-0.50	-0.96*	0.66*	-0.03						
GSH	-0.38	-0.90*	0.61*	0.04	0.92*					
VitC	-0.32	-0.81*	0.84*	0.22	0.76*	0.71*				
CAT	0.60*	0.70*	-0.50	0.24	-0.74*	-0.66*	-0.66*			
SOD	-0.36	-0.96*	0.65*	0.15	0.93*	0.90*	0.79*	-0.65*		

Parameters	Pb contents	Protein	MDA	MT	NPSH	GSH	VitC	CAT	SOD	GPx
GPx	-0.51	-0.89*	0.55	0.02	0.87*	0.83*	0.72*	-0.71*	0.87*	
AChE	0.46	1.00*	-0.73*	-0.08	-0.96*	-0.90*	-0.81*	0.70*	-0.96*	-0.89*

*Correlation coefficients statistically significant ($p<0.05$).

3.7. Histological Analysis

Control and treated gill structures are shown in Figure 4. Gills of all experimental conditions were characterized by frontal, intermediate, abfrontal zones with ciliary discs, haemolymphatic sinus and connective tissues (Figure 4C).

Exposure to $PbCl_2$ induced degenerative changes in the gill organ. $PbCl_2$ exposure causes dilatation of haemolymphatic sinus and cilia degradation (Figure 4 D1).

The vacuolization, lipofuscion granules degradation and disorganization of the intermediate and frontal zones were also observed (Figure 4 D2. D3). The most effects were more evident at the sharpest exposure dose (5mg/L).

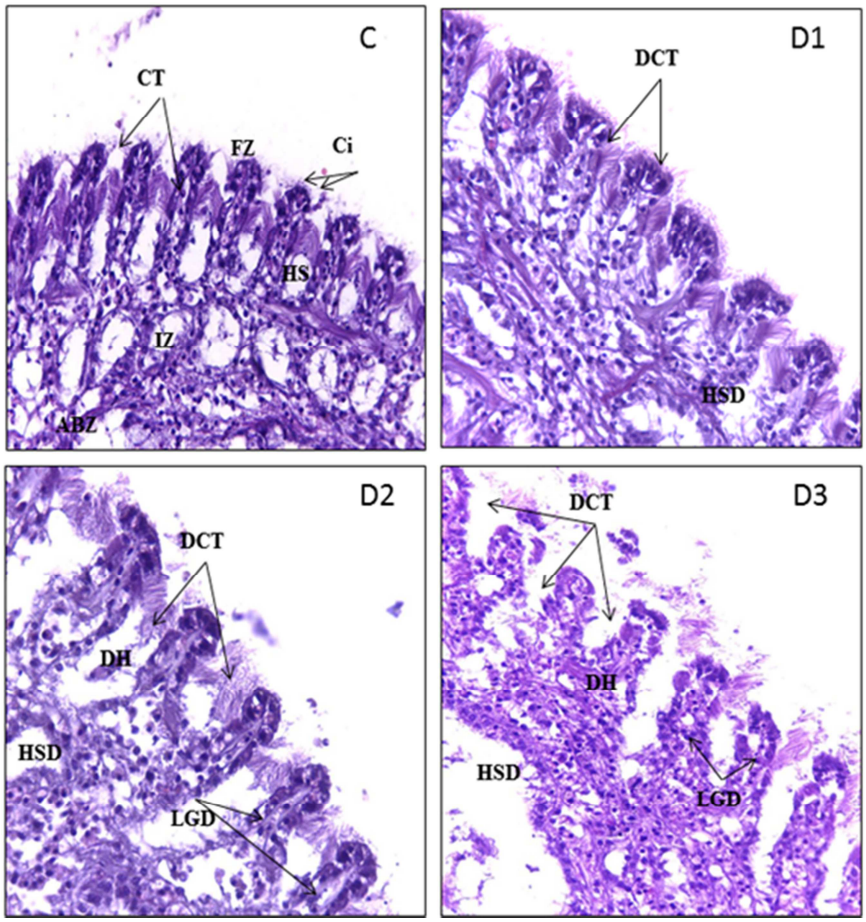

Figure 4. *Histological structure of treated (D1, D2 and D3) and control (C) gills exposed to PbCl2 graded doses during 5 days. CT: connective tissue, FZ: frontal zone, IZ: Intermediate zone, ABZ: abfrontal zone, HS: hemolymphatic sinus, Ci: cillia, DCi: deterioration of cilia, DH: dilatation of hemolymphatic sinus, DCT: degradation of the connective tissue, HSD: haemolymphatic sinus degradation, LGD: lipofuscion granules degradation.*

3.8. Principal Component Analysis (PCA)

The principal component analysis (PCA) was performed to understand the response of biomarkers after lead exposure in *Mactra corallina* gills. Results were shown in Fig (5 A, B), allowed us to retain the first two factorial axes that explain 87.94% of the total variance. Factor1displayed 69.10% of the total variance, defined by NPSH, GSH, Vit C levels and SOD, GPx and AChE activities (Figure 4A). Whereas, Factor 2 (18.83%) was characterized by a higher concentration of Pb and MTs levels. Only CAT activity and MDA levels were considered as intermediates compounds for the two axes.

PCA results showed that there was a significant separation between control and the other groups (Fig.5B). Control gills were projected in the positive side of first and negative second factorials axes, explaining by the high levels of antioxidants systems which were decreased in all treated groups. The second group was constituted by clams from D1 and D2 which were projected in the negative side of two factorials axis; showing an intermediate and closer defense state. The third one including M. coralline from D3 correlated by the most tested parameters such as Pb concentration, MTs levels. Clearly, the biomarkers response involved in oxidative stress were significantly increased in elevated $PbCl_2$ treatment groups as compared to control and D1 ones.

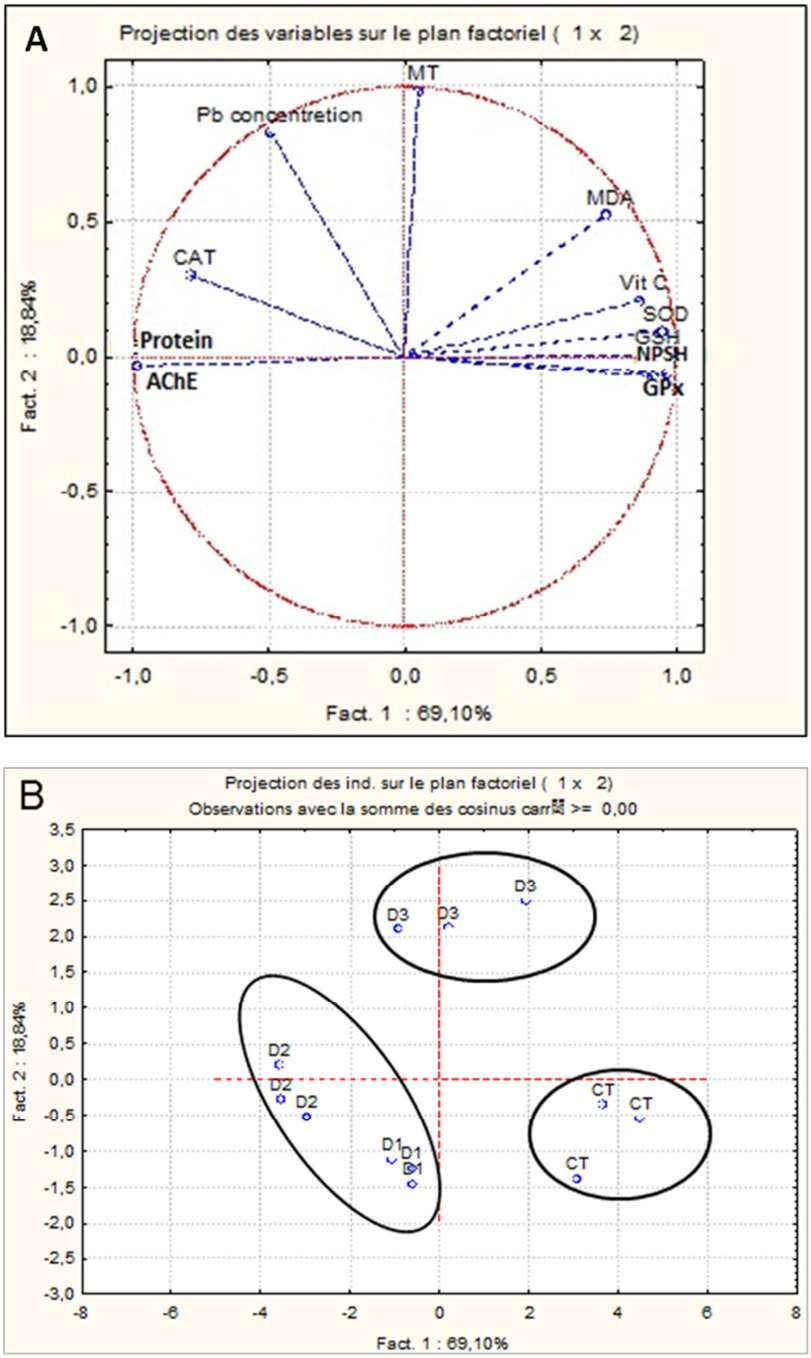

Figure 5. *Principal analysis component (PCA) represented by two factors F1 and F2 and produced by biochemical variables in control and treated M. corallina gills with $PbCl_2$ graded doses (D1, D2, D3) after 5 days of treatment: (A) Projection of the variables on the factor-plane (1×2), (B) Projection of the cases on the factor-plane (1×2).*

D1,1mg/L$PbCl_2$; D2,2.5mg/L$PbCl_2$; D3,5mg/L$PbCl_2$.

4. Discussion

Exposure to $PbCl_2$ for 5 days was sufficient to induce changes in gills metabolism. Our experimental study showed a significant accumulation of Pb levels with dose dependent manner. Important accumulation of Pb in gills was probably an indication of the ability of bivalves to lead accumulation. Thus, the change in accumulation pattern observed could associate to Pb graded doses exposure, since gills are considered as the first contact with the metal exposure that has a large surface area of the thin epithelium [27].

Previous research has demonstrated that exposure to Pb can induce free radical generation in the cell [28]. Due to the disturbance of pro-oxidants and oxidants systems, several injuries could be observed in macromolecules structures. The overproduction of ROS may cause lipid damage via reacting

with the double bonds of membrane lipids (such as PUFA), ultimately resulting in the appearance of toxic substances [29]. MDA is considered as a marker of lipid oxidation widely used in the in vivo and in vitro assessment [30-31].

In the current study, MDA was prominently increases in gills of all exposed groups compared to control. A significant and positive correlation was determined between MDA and Pb content in the gill tissue. This can lead to a rise in ROS production in lipid membrane which reacts with the double bonds of PUFA causing their peroxidation. Our results were in accordance with the study of Shenai-Tirodkar et al., [4], reporting the rise of gills MDA levels after exposure of oysters to Pb.

It's known that cellular damages induced by metals are the result of ROS overproduction and the compensatory response from endogenous and antioxidants such as enzymatic and nonenzymatic systems [8]. Among them, superoxide dismutase (SOD) is a catalyzer compound that converts superoxide radicals to hydrogen peroxide which was transformed in to H_2O via glutathione peroxidase (GPx) and catalase (CAT) [32]. In all $PbCl_2$ treated gills, the decrease of GPx and SOD activities were observed as compared to the control. This depletion indicates the probable dysfunction of those antioxidants by ROS or it' sever utilization to overcome with Pb toxicity. Nonetheless, CAT activity was increased in all groups demonstrated the protective action of this enzyme against ROS to reduce Pb toxicity. Thus, the activation of this enzyme was probably linked to Pb accumulation that shows an important correlation between its contents and enzymes responses in gills. In fact, this induction may be a compensatory adaptive mechanism to prevent the generation of highly toxic $OH^{\cdot}$ radicals via neutralization of H_2O_2 overproduction [33]. Several experimental investigations have demonstrated that Pb disturbs antioxidants enzymes activities [34].

Likewise, change in membrane permeability including MDA induction was related to the alterations of non-enzymatic antioxidants status by allowing faster ROS intake [35]. In our study, significant increases in GSH, NPSH and Vit C levels in all treated bivalves were positively correlated with lipid peroxidation (MDA levels). Such increases demonstrate the neutralize function of these enzymes against Pb toxicity, inducing an excessive generation of radicals as reported previously by Coelho et al., [36] after exposure of *Scrobicularia plana* to mercury.

As a protein of metal-binding MTs are involved in the detoxification and the accumulation of several pollutants such as metals [37] and protect the cell against the cytotoxic effects of ROS production [38]. Hence, it is widely suggested as a bioindicator in the bio-monitoring programs to asses' metals contaminations. Our results demonstrated an increase with dose dependent manner in MTs levels after exposure to gradual $PbCl_2$ doses. This increase was affirmed by a significant and positive correlation with Pb contents in gills of M. coralline that could possibly be explained by an adaptive response against its accumulation. These results were similar to the findings of Chalkiadaki et al,. [13] where the authors found an effective increase in the MTs levels in gills of *Mytilus galloprovincialis* after 20 days of exposure to Pb.

Acetylcholinesterase (AChE) activity was comm. only used as a biomarker of neurotoxicity (...). In our current study, the ROS production and the successive oxidative stress induced by $PbCl_2$ resolved the changes in the gills of $PbCl_2$-treated M. coralline established by a significant inhibition of AChE activity. This decrease could be associated with the deactivation of ChEs by metals that binding to their anionic site [39]. In other hands, the decline of AChE activity was highly correlated with the nonenzymatic antioxidant systems in our experiment. We can suggest that the inhibition of AChE activity under the effect to lead is might be caused by binding of metals to the functional groups of proteins like imidazole, sulfhydryl and carboxyl [40]. Similar to our result, Dafre et al. [41] have shown that exposure to Pb causes a marked reduction in AChE activity in *Perna perna*.

According to the histological study, the gills were extensively damaged with cilia degradation, dilatation of haemolymphatic sinus, lipofuscion granules degradation and disorganization of the intermediate and frontal zones. The vacuolization was also observed which was related to the rise of lipid peroxidation which caused membrane damages in the gills.

5. Conclusion

Based on the results of the investigated biochemical markers and histological study, we may conclude that oxidative stress generated in gills of M. coralline especially dependent on the concentration of Pb exposure. A decrease in SOD, GPx activities, and GSH, NPSH, Vit C levels which associated with an increase in MDA level in treated groups especially under sharpest dose. This biochemical modification corresponded to histological.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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