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Redox status Modulation and DNA Impairment in Gills *Cerastoderma edule*, after Kinetic Exposure to Lead Chloride

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Abstract: This study assessed the impact of $PbCl_2$ on the antioxidant and macromolecules statues in *C. edule* gills. Cockles were exposed to graded concentrations of $PbCl_2$ (1, 10 and 100 µg/ L) for 3 and 6 days. The exposure to these concentrations stimulated macromolecules alterations revealed with increases of malondialdehyde, protein carbonyl, and advanced oxidation protein product levels. Furthermore, our investigation revealed a random of DNA degradation in gills after kinetic exposure at 100µg/L. Moreover, the increase in superoxide dismutase, glutathione S transferees and glutathione peroxidase activities were observed especially at the sharpest exposure concentration. Yet, the increases of the metallothionein and reduced glutathione levels were also registered in treated gills. Our multivariate results demonstrated that the main changes in the physiological status were recorded after 6 days of exposure. This study highlighted the toxicity of $PbCl_2$ and confirmed the usefulness of *C. edule* as a sensitive bio-indicator of metals exposure.

Keywords: *Cerastoderma edule*, gills, kinetic exposure, oxidative stress, Lead chloride graded concentration, DNA alteration.

1. INTRODUCTION

Lead (Pb) is one of the most abundant elements on the Earth's crust since concentrations described in sediments, waters, estuarine, lagoons and coastal ecosystems are great, varying between 2 and 150mg/Kg [1, 2], revealing its high solubility in water [3]. Furthermost, the toxicity of Pb has been designed to be important to aquatic organisms because of these high concentrations [4].

Currently, Pb is widely used in the additives of several products (such as cement manufacture, paint color, batteries…etc.) and, certainly, it released amount into aquatic systems will increase [5]. In the aquatic environment, the interface of Pb with water components may possibly improve the availability of Pb for organisms; conversely, such interactions aren't still understood [1]. The toxicity of Pb to aquatic bivalves has been recently demonstrated in some species, such as *Mytilus galloprovincialis* [6] *and Venerupis philippinarum* [7].

Effects on metabolism, including changes in cell function capacity, are between the most frequently defined biological responses detected in bivalve species at short exposure period to metals [7, 8]. Similar to other metals with no biological roles, Pb was able to mimic biological function, using natural transporters for some essential metals [9]. Due to its reactive property, Pb can increase the intracellular generation of reactive oxygen species (ROS) and stimulate oxidative stress responses [8].

Furthermore, the interaction of ROS with cell structures can cause cellular damage to macromolecules (lipids, proteins and DNA, for example) when the antioxidant defense system fails to attenuate the increase rates in ROS production, which has already been well described in bivalve species [1, 10]. To detoxify cells from ROS, organisms can implicate non-enzymatic and enzymatic defenses mechanisms, including the reduced glutathione (GSH), metallothioneins (MTs), glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione S-transferases (GSTs) whose functions protect organisms from oxidative stress [11].

Assuming the increase of Pb content in the marine environment as a consequence of the use of this element in numerous industrial activities, it is of the highest importance to assess its toxicity towards the infaunal organisms such as *Cerastoderma edule (C. edule).* Since, these bivalves are abundant in the Tunisian coasts and considered as an excellent species for the bio-monitors programs as there is resistance to a broad range of environmental conditions, have a sedentary lifestyle, and tendency to accumulate contaminants in their tissues [12]. The effect of heavy metal exposure on bivalves has been widely studied; nevertheless, there is no information about the antioxidants responses of lead chloride ($PbCl_2$) toxicity. Thus, the present study investigated the impacts of graded $PbCl_2$ concentrations on the redox status and macromolecules performance of *C. edule* after exposure to a kinetic period of 3 and 6 days.

2. MATERIALS AND METHODS

2.1. Experimental design

Experiments sets were performed in order to assess the toxic effect of $PbCl_2$ via the responses of *C. edule* successively exposed to graded concentrations (1, 10 and 100µg/L). Cockles, with mean length (3.5 ± 0.2 cm) and weight (5.2 ± 0.6 g), were collected during March 2017 from the Bizerte lagoon (north coast of Tunisia), where there acclimated for one week in 20 L aquaria with renewable seawater, submitted to a continuous aeration, regular photoperiod (12 D/12 L), normal temperature (ca. 18 °C) and salinity (ca. 37 psu).After the acclimation period, thirty *C. edule* were put in 20 L tanks with seawater that was contained0 (C), 1 (C1), 10 (C2) and 100 (C3) µg/L of $PbCl_2$ and all conditions were treated in triplicate during a kinetic period of 3 and 6 days. During the experimental days, seawater was renewed every 2 days and the water conditions were re-established ($PbCl_2$ concentrations). After each exposure period, 15 individuals per exposure concentrations were collected and directly frozen in liquid nitrogen and conserved at ⁻80 °C.

2.2. Lead quantification

The quantification of Pb in *C. edule* gills was realized through several steps when 0.5g of powder gills tissue were weighed into a Teflon tube to which 4 mL of HNO_3 and 2 mL of H_2O_2 were added. The mineralization procedure was made using a closed programmable microwave (Ethos D, Milestone Inc.). After cooling, the mineralized gills solution were gauged with ultrapure water in polyethylene tubes to a final volume of 50 mL and stored until Pb quantification by an **inductively coupled plasma mass spectrometer (**ICP-MS, **Agilent™ 7900).** All results of Pb content in gills tissue were given in mg/Kg of a dry weight of triplicate analysis. The blank samples and the certified reference materials were also determined.

2.3. Tissues preparation

Cockles' gills tissues (15 individuals per condition) were extracted with Tris-HCL buffer (100mM, pH 7.4), centrifuged at 10.000g during 20 min at 4 °C, and the obtained supernatants were collected in aliquots and stored at ⁻80 °C until oxidative stress analysis. For DNA analysis, fractions of 100µg of gills were directly fixed in ethanol (70°) until analysis.

2.4. Indicator of lipid peroxidation

Lipid peroxidation was determined based on the quantification of malondialdehyde (MDA) according to Draper and Hadley [13]. The reaction was measured at 540 nm and results were calculated using 1,1,3,3-tetraethoxypropane (TEP), served as standard, and expressed as nmol of MDA/mg of protein.

2.5. Indicator of protein oxidation

The indicators of protein oxidation such advanced oxidation protein product (AOPP) and protein carbonyl (PCO) were determined following the methods of Kayali et al. [14] and Reznick and Packer [15], respectively. The assessment of AOPP was performed using potassium iodide (1.16M) and glacial acetic acid, absorbance was measured at 340 nm andthe results expressed in nmol /mg of proteins basing onthe extinction coefficient $261cm^{-1}$ mM^{-1}. PCO levels were evaluated based on the molar extinction coefficient of 2.4 dinitrophénylhydrozine (DNPH) (e02.2104 cm^{-1} M^{-1}), measured at 370nm and expressed as nmol/ mg of protein.

2.6. DNA assay

DNA extraction from gill tissues was performed according to the method of Clark and Melki [16]. The smear method was achieved to check the extent of DNA alteration. It consists of electrophoresis on the complete genomic DNA extracted in an agarose gel (1%) that was detected and photographed under an ultraviolet lamp.

2.7. Non-enzymatic responses

Non-enzymatic antioxidant activities such GSH and MTs were measured following Ellman [17] and Viarengo et al. [18], respectively. GSH analysis was established on the reductive cleavage of 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) by the sulfhydryl (-SH) group to produce a yellow color. GSH levels were measured as the change in the absorbance values in the presence and the absence of DTNB at 412 nm of the tested samples. Results were expressed as µmol/ mg of protein. MTs levels was quantified by evaluating the SH residue content by a spectrophotometric method, using 5,5dithiobis 2 nitrobenzoic acid reagent, at at 412 nm. The results were expressed as µmol GSH/mg of protein.

2.8. Enzymatic responses

Antioxidant enzymes activities such SOD, GSTs and GPx were measured following Beauchamp and Fridovich [19], Habig et al. [20], and Flohe and Gunzler [21], respectively. SOD activity was measured spectrophotometrically at 560 nm and was calculated using the inhibition percentage of nitro blue tetrazolium (NBT) reduction. The results were expressed as U /mg of protein for which U represents 50% reduction of NBT. GSTs activities were determined at 340 nm (ε = 9.6 mM^{-1} cm^{-1}) using 1-Chloro-2,4-Dinitrobenzene (CDNB) regents. The amount of enzyme was expressed as µmol/mg of protein. GPx activity was measured at 340 nm. Results were expressed in nmol of GSH oxidized/mg of protein.

2.9. Data analysis

Data on Pb content and oxidative stress responses were analyzed through the non-parametric multivariate analysis of variance using STATISTICA software (version 13.0 USA), due to lack of

homogeneity of variance that was tested previously by Kolmogorov–Smirnov test. In the represented results, significance levels (among control and the experimental conditions) are presented with different letters asterisk at 0.05. Principal component analysis (PCA) was performed to observe the correlation between the control cockles and the exposure ones with graded $PbCl_2$ concentrations.

3. RESULTS

3.1. Pb quantification in gills tissues

As shown in Table 1, there was a significant increase of Pb content in treated cockles with 100µg/L by 21% after 3 days of exposure. However, all exposed *C. edule* for 6 days showed a significant raise of Pb content (+30%, +40% and +116%), respectively, when compared to those of controls.

3.2. Lipid peroxidation levels in gills tissues

Lipid peroxidation, in treated bivalves with 10 and 100µg/L, was enhanced as evidence by increases of MDA levels with 19% and 25% respectively, after 3 days of exposure and by 35% and 54% respectively, after 6 days of exposure as compared to the control (Figure 1).

3.3. Indicator of protein oxidation

The effect of $PbCl_2$ on protein oxidation was determined by evaluating AOPP and PCO levels in *C. edule* gills exposed to kinetic period (Table 2). Our result revealed an increase of AOPP (+66% and +76% respectively) and PCO (+32% and +54% respectively) levels at the high exposed concentration (100µg/L) after 3 and 6 days compared to control group. However, no significant changes in AOPP and PCO levels were observed after the kinetic exposure to the 1µg/L and 10µg/L of $PbCl_2$..

3.4. DNA damage in gills tissues

As shown in Figure 2, DNA structures of control and treated gills are presented. Our results showed a smear without ladder formation of *C. edule* that were exposed to 10 and 100µg/L of $PbCl_2$ during both kinetic periods, indicating a random of DNA degradation.

3.5. Superoxide dismutase (SOD) activities in gills tissues

Our results revealed significant increases of SOD activities mostly after 6 days of exposure by +77% and 151% in C2 and C3 treated groups (Table 3). Similar results were observed in C3 treated group after 3 days of exposure (+43%) than the control group. However, similar responses for SOD activities were detected between control and C1 groups, after all the experimental period ($p>0.05$).

3.6. Glutathione S-transferases (GSTs) activities in gills tissues

Our results revealed increases of GSTs activities after 3 days (+41% and +43%) and 6 days (+52% and +75%) of exposure to $PbCl_2$ at 10 and 100µg/L, respectively, as compared to the control (Table 3).

3.7. Glutathione peroxidase (GPx) activities in gills tissues

Significant increases of GPx activities were recorded in treated *C. edule* with 10 and 100 µg/L under kinetic exposure of 3 (+26% and +68%) and 6 (+40% and +90%) days respectively, when compared to the cockles of controls (Table 3). Conversely, these enzymes weren't changed in C1 group that exposed to the lowest dose (1µg/L of $PbCl_2$) ($p>0.05$).

3.8. Metallothioneins (MTs) levels in gills tissues

Concerning MTs levels (Table 3) increases of +36%, +102% and +284% were recorded in all treated cockles after 6 days of exposure. Comparable raise were observed after 3 days of exposure with 10 (+26%) and 100µg/L (+57%) of PbCl2. However, no significant change was observed in C1 group treated with the lowest concentration as compared to the control cockles.

3.9. Reduced glutathione (GSH) levels in gills tissues

Our result showed a significant increase in GSH levels by +32% and +69% in C2 groups after 3 and 6 days of exposure than the control (Table 3). In addition, GSH levels were more pronounced in the sharpest exposed concentrations (100µg/L) under the kinetic period by +54% and 160% respectively. While no change was observed in C1 groups ($p>0.05$) (Table 3).

3.10. Principal component analysis (PCA)

Multivariate analysis was established to evaluate the correlation among the physiological responses of *C. edule* gills and the kinetic exposure to graded $PbCl_2$. The PCA (PC1 and PC2) accounted for 93.62% of the total variance as reported in the dimensional plot (Figure 3). Our results revealed that exposure to $PbCl_2$ separate treated groups according to the exposure dose and time. Closeness between control and treated-gills with 1µg/L of $PbCl_2$was observed during the over experimental period. However, progressive changes were recorded at 10 and 100µg/L concentrations during the 3th days of exposure as compared to the control. Furthermore, the enormous separation was detected after 6 days of exposure at both sharpest concentrations (10 and 100µg/L) which coincided with the higher impairments of lipids, proteins and DNA as well as the activation of antioxidants systems.

4. DISCUSSION

In this study, the metabolic changes in gills exposed to graded concentrations of $PbCl_2$during kinetic period were reported in*C. edule.* In this concern, it is known that changes in redox status of marine

organisms were associated to metals uptakes and accumulation [22]. In our study, the contents of Pb in *C. edule* are not particularly high when compared to similar investigations [7, 8] and was by far below to the tolerable limit registered by FAO [23] (1.19±0.18 mg/kg wet wt.). Our data showed that Pb was accumulated mostly in all treated gills after 6 days of exposure. In fact, according to other experimental reports, bivalves' gills are considered as a short time storage organ, due probably to its continuous exposure surroundings and the large surface area of thin epithelium. Our results are in agreement with Shenai-Tirodkar et al. [8], also determined a high accumulation of Pb in gills than other tissues of bivalves.

A compulsory importance effects of Pb accumulation in gills were ROS generations that establishing by the simultaneous release of MDA, end product of lipid peroxidation. An important and positive correlation was established between MDA and $PbCl_2$ levels in gills tissues ($p<0.001$). This could be the consequence of ROS overproduction in gills, organ involved in metabolic function, which related to Pb concentration that may directly affect its metabolic pathways. The enhanced lipid peroxidation observed under our experimental conditions might be ascribed to the increase resulting from Pb exposure. This result corroborates the findings of previous studies in bivalves after metals toxicity [24, 25].

It is widely established that lipid peroxidation products are involved in cell genotoxicity [11]. In our current study, the occurrence of MDA production was suggested to aggravate DNA destruction which considered as a target molecule of various agents (such as carcinogenic, mutagenic…etc), leading to cell modification and death [26]. Our study showed that exposure to $PbCl_2$ graded concentrations during 3 and 6 days have resulted with considerable DNA damages and smear on the agarose gel as compared to the control group which was characterized with an imperceptible smear of DNA. However, despite the obvious effect of Pb that have been previously described [7], it effect on molecular mechanisms stay mostly unclear.

Moreover, free radicals attack all macromolecules structures including proteins that could lead to its modification through the oxidation and carbonization of some amino acids [27]. In this current study, a significant increase in the level of AOPP and PCO was observed in $PbCl_2$ treated bivalves by increasing the exposure time. The high level of proteins oxidation may due to in both increases production and accumulation of ROS. The results of our present study are corroborated by the findings of previous research where authors have found increased of protein oxidation in metal toxicity in mussels [28] and in sea cucumber [29].

To neutralize the harmful effects of oxidative stress, gills tissues are set with antioxidant defense system which works simultaneously to combat oxidative stress. To demonstrate the mechanisms underlying $PbCl_2$ toxicity, the enzymatic antioxidants systems such as SOD, CSTs and GPx in gills were also tested. This buttery has an ability to avoid free radical development and to limit their harmful outcome [30]. SOD is considered as a catalyze compound that converts superoxide radicals to hydrogen peroxide which is therefore transformed to H_2O by GPx [31]. Likewise, GSTs is a major metabolic enzyme that has a key role in detoxification of several xenobiotic [32]. In the present study, gills showed initially less SOD, GPx and GSTs responses which were similar to control group. While, it increased progressively during treatment especially at the sharpest concentrations, showing the important role of gill in detoxification process in the oxidative defense systems. Similar studies have described the increase of SOD, GPx and GSTs activities in bivalves' tissues as an adaptation response to metals toxicity exposure [33].

The second cellular defense line beside the toxic effect of $PbCl_2$ is presented by the non-enzymatic antioxidants. In this regard, GSH is the major cytosolic sulfydryl compounds, acts as a principal decreasing agent and protective marker beside severe toxic substances, such as metals [34]. Our results demonstrated that exposure to graded-$PbCl_2$ concentrations increase progressively the levels of GSH especially at the severest doses. Such results could be either a result of increased production or either to their important involvement in the neutralization of ROS through the SH group. Analogue studies have demonstrated that the increase of GSH level in aquatic organisms revealed their adaptation on response to oxidative metals toxicity [35]. Our data were correlated with Aouini et al. [7] who demonstrated that Pb exposure can generate (ROS) in *Venerupis philippinarum*, which in turn might have contributed to the development of this antioxidants.

Another interesting group of non enzymatic antioxidants, metallothioneins (MTs) are powerful scavangers of hydroxyl radicals and also provide toward giving protection against oxidative injuries and other environmental stressors [36]. The level of MTs in gills exposed to $PbCl_2$ graded doses enhanced after 3 and 6 days of the treatment indicating the activation of the detoxification mechanisms in this organ. These results are in the accordance with previous studies carried by Perić et al. [37] on bivalves.

The global multivariate analysis was complements with our results and approves that physiological response in *C. edule* gills depending in concentrations (graded exposure) and durations (kinetic exposure). This indicates the alteration of macromolecules compounds and the activation of a

mechanism systems defenses against the obvious accumulation of Pb.

5. CONCLUSION

On the whole, our study shows that Pb generate an oxidative stress in gills of *C. edule* and induce progressively the activation of non enzymatic and enzymatic antioxidants systems. Moreover, based on the results of the investigated biomarkers we conclude that the oxidative stress generated in gills is specifically depending on the concentration and duration of exposure. However, in future, other studies should be assessed for chronic exposure with other biochemical parameters and molecular analysis to better get the mechanistic pathways into the toxic effect of Pb in the gills tissues.

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Figures

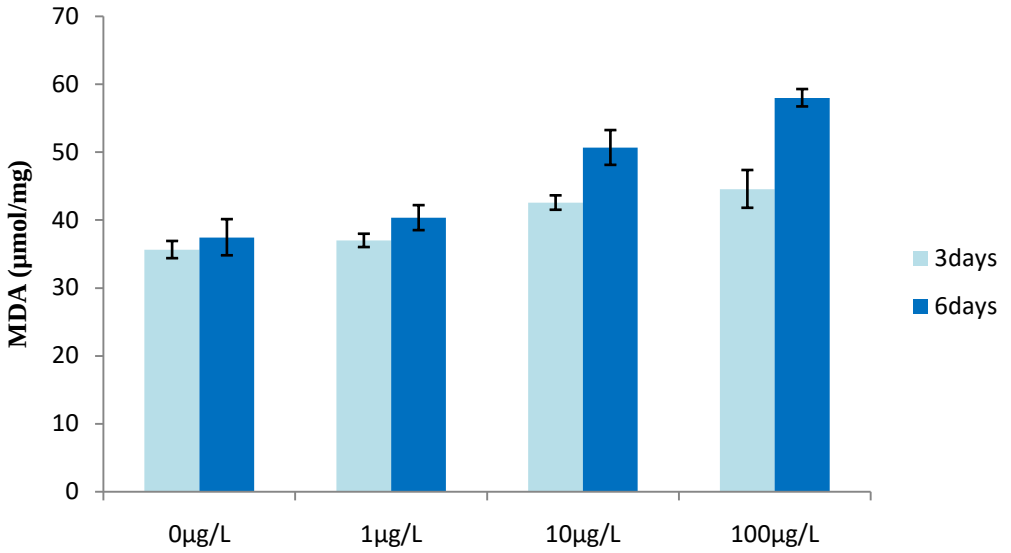

Figure 1. *Malondialdehyde (MDA) levels in C. edule gills after a kinetic exposure to graded $PbCl_2$ concentrations.*

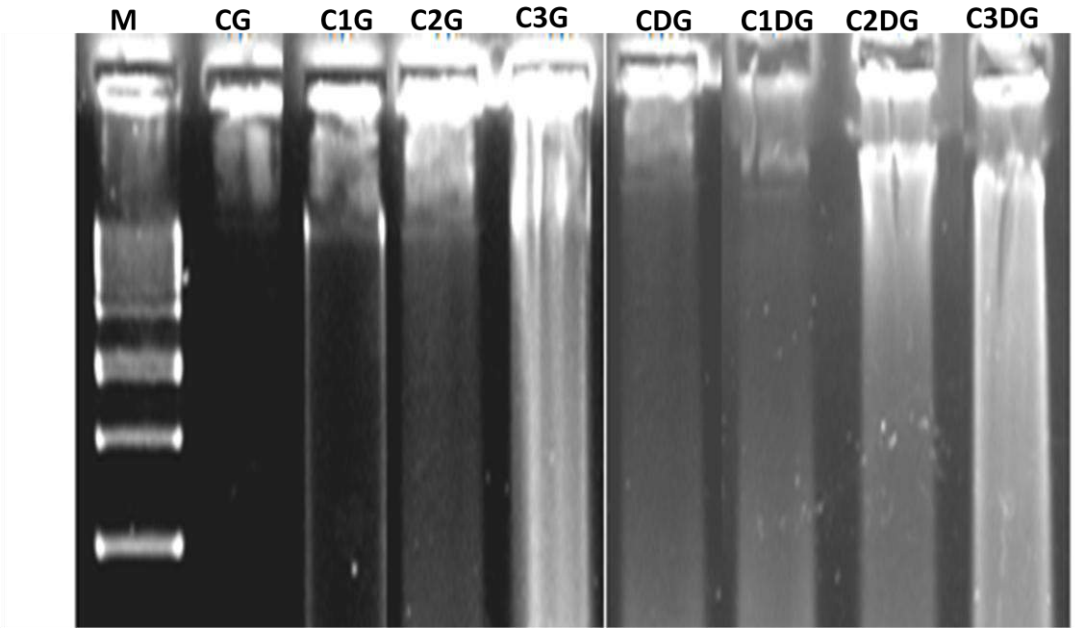

Figure 2. The electrophoresis of DNA isolated from the gills of the control and treated *C. edule.* M: marker , CG: gills of control group, C1G: gills of treated group with 1µg/L of $HgCl_2$, C2G: gills of treated group with 10µg/L of $HgCl_2$, C3G: gills of treated group with 100µg/L of $HgCl_2$, CDG: digestive gland of control group, C1DG: digestive gland of treated group with 1µg/L of $HgCl_2$, C2DG: digestive gland of treated group with 10µg/L of $HgCl_2$, C3DG: digestive gland of treated group with 100µg/L of $HgCl_2$.

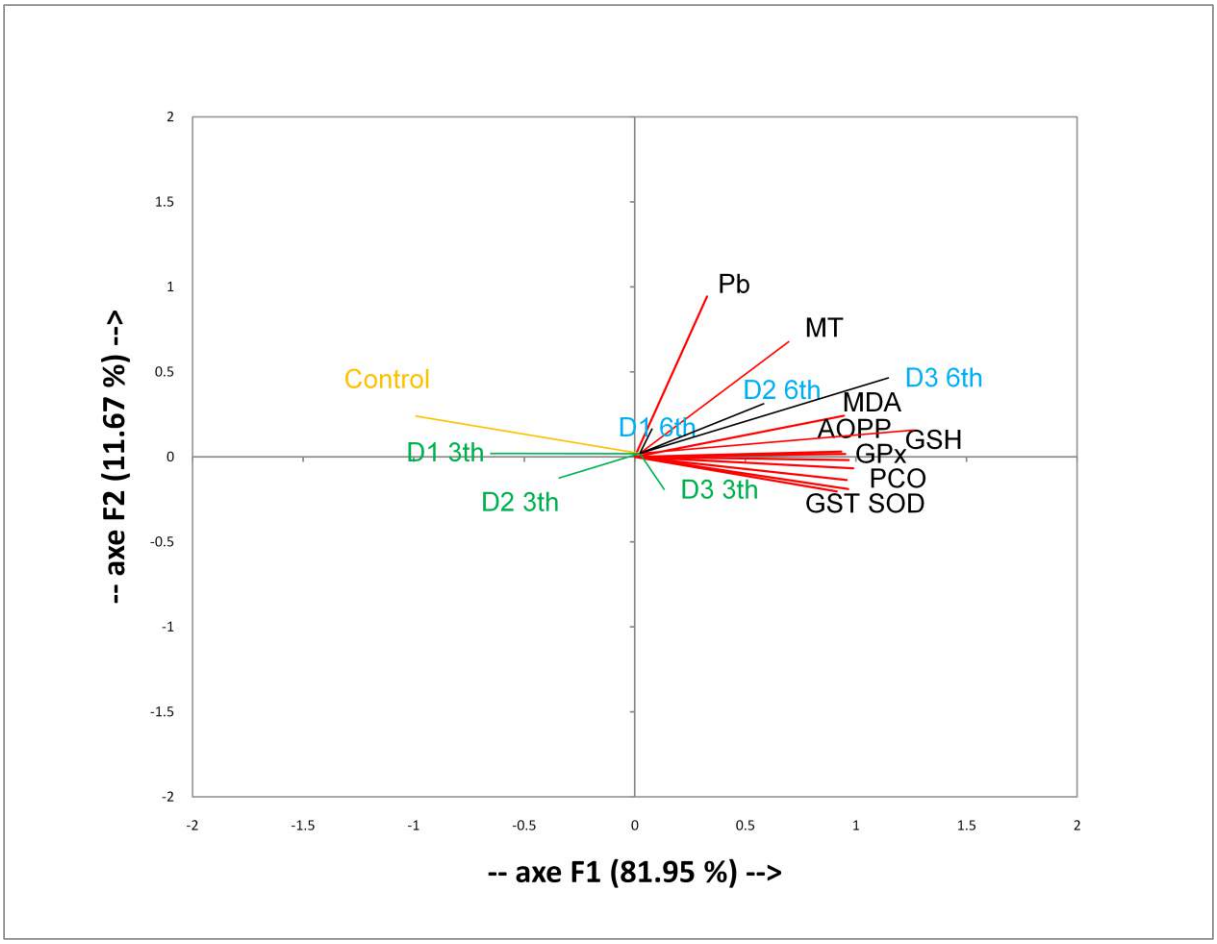

Figure 3. Principal component analysis (PCA) including all measured markers (Pb accumulation, MDA, PCO, AOPP, GSH, SOD, GST, GPx, and MT) in *C. edule* gills exposed to $HgCl_2$ graded doses [C1 (1µg/L), C2 (10µg/L) and C3 (100 µg/L)] during 7 days.

TABLES

Table 1. *Lead content in Cerastoderma edule gills after exposure to graded $PbCl_2$concentrations during a kinetic period of 6 days.*

	3th days	**6th days**
0µg/L	0.65±0.05	0.65±0.05
1µg/L	0.70±0.04	0.85±0.07***
10µg/L	0.72±0.03	0.91±0.08***
100µg/L	0.79±0.06**	1.41±0.33***

Results are given in means ± standard deviation of six replicates in each group.
Treated-gills vs Control: **p<0.01 and ***p<0.001.

Table 2. *Protein carbonyl (PCO), and advanced oxidation protein product (AOPP) levels in Cerastoderma edule gills after exposure to graded $PbCl_2$concentrations during a kinetic period of 6 days.*

		PCO (µmol/mg)	**AOPP** (nmol/mg)
3 th	**0µg/L**	6.61±0.17	1.01±0.07
	1µg/L	6.51±0.25	1.21±0.33
	10µg/L	6.95±0.31	1.18±0.41
	100µg/L	8.78±0.41***	1.68±0.41**
6 th	**0µg/L**	6.61±0.17	1.01±0.07
	1µg/L	6.68±0.14	1.33±0.09
	10µg/L	7.03±0.45	1.30±0.28
	100µg/L	10.22±0.36***	1.78±0.22***

Results are given in means ± standard deviation of six replicates in each group.
Treated-gills vs Control: ***p<0.001.

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Table 3. Redox systems including enzymatic (superoxide dismutase SOD, glutathione S transferees GSTs and glutathione peroxidase GPx) and non-enzymatic (metallothioneins MTs and reduced glutathione GSH) antioxidants responses in *Cerastoderma edule* gills after exposure to graded $PbCl_2$concentrations during a kinetic period of 6 days.

		MTs (µmol GSH/mg of pr)	**GSH** (µmol/mg of pr)	**SOD** (U/mg of pr)	**GSTs** (µmol/mg of pr)	**GPx** (nmol GSH/mg of pr)
3th	0µg/L	1.75±0.06	7.18±0.03	8.76±0.17	0.53±0.02	20.57±2.27
	1µg/L	1.71±0.07	7.23±0.18	8.64±0.07	0.55±0.05	20.64±1.75
	10µg/L	2.21±0.11*	9.51±0.05**	8.91±0.42	0.75±0.10*	26.01±2.05*
	100µg/L	2.76±0.34***	11.09±0.31***	12.54±0.39***	0.78±0.08**	34.56±1.15***
6th	0µg/L	1.75±0.06	7.18±0.03	8.76±0.17	0.53±0.02	20.57±2.27
	1µg/L	2.38±0.01*	7.55±0.23	9.01±0.31	0.60±0.04	21.97±2.38
	10µg/L	3.54±0.24***	12.17±0.9***	15.55±0.25***	0.81±0.04***	28.80±4.21**
	100µg/L	6.75±0.55***	18.73±0.08***	21.73±0.11***	0.93±0.12***	39.09±3.61***

Results are given in means ± standard deviation of six replicates in each group.

Treated-gills vs Control: *p<0.05, **p<0.01 and ***p<0.001.

