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Antioxidant glutathione dependent system response to *in vivo* exposure to cadmium and copper in *Perna perna* of the Gulf of Annaba (Algeria)

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Abstract

In order to use the antioxidant glutathione-dependent system as a biomarker of oxidative stress, the effect of metals on the metabolism of glutathione has been studied at the gills of the African mussel *Perna perna* living the easternmost Gulf of Annaba (Algeria). The response of the glutathione-S- transferase activity (GST) and the rates of Glutathione (GSH) was evaluated from these bivalves, after *in vivo* exposure to three concentrations of Cadmium (50, 100 and 200 µg/l) and copper (10, 15 and 25 µg/l) during 7 days. The analyses showed a significant decrease of GSH levels depending on the concentration of cadmium in the medium compared to controls. For the GSH levels, it significantly decreased in exposed bivalves to different concentrations of copper, while the GST activity was strongly inhibited at 25 µg/l of Cu. In fact, Cadmium seems to increase GST activity using glutathione as a substrate which caused a decrease of GSH rates in the exposed mussels, while at the highest dose tested the GST was not required so other enzymes probably metallothioneins (metals detoxification proteins) support the function of antioxidant defense. In the other hand, bioaccumulation of the two metals (Cd) in exposed mussels seems to be not correlated with added concentrations. This situation can be related to the hypothesis that the antioxidant GSH dependent system is most likely involved in this phenomenon. The tested system in *Perna perna*, reported in this study may be a good biomarker to assess contamination of the marine environment particularly by metals.

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Introduction

The contamination of the marine environment, including heavy metals such as cadmium and copper, is one of the major problems in environmental toxicology. Cadmium is a toxic heavy metal but not oxide reducer element. It unlike transitional metals such as copper, iron, cadmium did not fall into a cycle of oxidation reducer (Fenton type) and does not generate the production of ROS (reactive oxygen species), it should not cause lipid peroxydation (Vlahogianni and Vlavanidis, 2007). However, cadmium increases the production of the other ROS (superoxyde anions) under some conditions, such as in cells cultured macrophages of rats treated with 0,4 and 0,6 µM cadmium (Valco *et al.,* 2005) induced membrane lipid peroxydation. The complexity of chemical pollutants metabolism and detoxification mechanisms among marine organisms encouraged to consider the interface chemistry/biology. Indeed, the accumulation of pollutants in some model organisms and their biological response to these pollutants particularly by inducing biomarkers is an essential way of biomonitoring of pollution (Sanchez and Porcher, 2009).

The oxidative stress is one of the known effects of pollution, especially in metals. It is due to the exaggeration of a phenomenon physiologically very controlled; causing the production of radical oxygen species (Bensa,2000) and to which organisms responded by developing an intracellular defence system, involving an array of enzymes to neutralize free radicals compounds to harmless, more hydrosoluble and more excretal components (Florent, 2003). Among marine organisms particularly Mytilidae, gills plays a major role in the metabolism and bioaccumulation of metals (Roméo *et al.,* 2005) so metals such as Cadmium, Cooper and Zinc could penetrate *via* this organ by diffusion (Geret *et al.,* 2002) this fact motivated choice of this model as Biological target organ.

This paper proposes the use of the response of antioxidant defense systems to evaluate the effect of *in vivo* exposure to heavy metals with the follow of the glutathione-S-transferase activities associated with the measurement of reduced glutathione rates as well as the metals were conducted in gills of the African mussel *Perna pernain vivo* exposed to cadmium and Copper.

Materials and methods

Sample preparation and protocol of contamination

Specimens of *Perna perna* with uniform size (60 mm length) were taken in the extreme Eastern Gulf of Annaba and transferred into trays filled with natural seawater (psu S = 37, T = 16 ± 2°C; 12h:12h) for an acclimatizing period of 1 day.

The renewal every 2 days of sea water of controls and exposed animals is their only source of food. During the experiment, the mussels are exposed for 7 days to different concentrations of metals (50,100 and 200 µg/l of Cd and 10, 15 and 25 µg/l of Cu). At the end of the experiment, two groups of bivalves are constituted: the first group (n = 5 per condition) used for biochemical analyses, in which each individually removed gill were used for the determination of the GST activities and for the evaluation of GSH rates. The determination of the concentrations of Cd, Cu and Zn was conducted in the dried tissues of whole mussels of the second group of animals (n = 5 per condition) by atomic absorption spectrometry (AAS).

Determination of GSH levels

Samples of gills are homogenized in EDTA 0,02 M and then déproteinised by the sulfo-salycilic acid 0,25%. After centrifugation at 1000 g for 10 min, supernatants were used for the spectrophotometric dosage at 412 nm with DTNB reagent 0, 01 M according to the method of (Weckbecker and Cory, 1988). The concentrations of GSH were expressed in nmoles/mg of proteins.

Determination of the GST activities

The GST activity was determined according to the method of (Habig and Jacoby, 1974). The S9 Samples were prepared by homogenizing gills in phosphate

buffer 100Mm; pH 6,5 and centrifuging at 9000 g for 30 min. The dosage consist to react GSTs on a CDNB (20 mM)-GSH (100mM) mixture and the change in optical density at 340nm due to the appearance of the CDNB-GSH compound is measured every 15 seconds during 2 minutes.

The determination of total proteins is carried out according to the method of (Bradford, 1976).

Trace metals analysis

The soft mass of 5 whole bivalves is dried at 70°C until a constant weight. Aliquots of 500 mg of dried samples were digested by nitric acid (HNO_3) at last for one night. Cu, Zn and Cd concentrations were analysed by an atomic absorption spectrophotometer (GBC) with flame for copper and with equipped a graphite furnace for cadmium. Quality assurance relies on the values relative to certified standard reference material (TORT-2: hepato-pancreas of lobster) provided by the National Research Council of Canada simultaneously analyzed. Our results (in µg of metal per g dry weight) are in good agreement with the certified values (Table 1).

Statistics

All results are given as mean values ± SD. To assess the significance of differences observed in metal accumulation or in biomarker responses, we used a one-way analysis of variance analysis (ANOVA).

When ANOVA was significant; post hoc pairwise comparisons between conditions were performed using Student's test to determine which values differed significantly. The statistical analyses were carried out using Minitab16 Software (Version 1.1.0).

Results

Effect of Cadmium on GST activity and GSH rates

The levels of reduced glutathion (Fig. 1) decreased significantly (p <0,001) depending on the concentration of cadmium, until reach least levels with value of 93,66 ± 10 nmoles/mg protein at 200µg Cd/l against 250,74 ± 07 nmoles/mg protein in controls. GST activity was significantly induced in gills of *P. perna* (Fig. 2) with levels of 200µg Cd /l (239, 19 ± 09 nmoles/min/mg protein) compared to controls (182, 22 ± 08 nmoles/min/mg protein).

Table 1. Analysis of the reference material Lobster Hepatopancreas TORT-2 (National Research Council Canada).

Metals	Certified values µg/g dry wt	n	Recorded values
Cd	26,7 ± 0.6	5	26, 4 ± 1. 4
Cu	106 ± 1.0	5	101 ± 1.0
Zn	180 ± 6.0	5	174 ± 8.0

Effect of copper on the GST activity and GSH rates

The biomarker responses in exposed animals to various concentrations of copper showed a dissimilar profile from that obtained with cadmium. Indeed, the levels of GSH decreased significantly all along the experiment, particularly at 25µg Cu/l which caused a highly significant decrease of GSH (71,92 ± 04 nmoles /mg protein) in comparison with control (200,14 ± 03 nmoles/mg protein) (Fig.3). For the GST activity (Fig.4), a significant response was only observed with the highest dose of Copper which manifested by a notable decrease (P<0,001).

Copper and cadmium bioaccumulation

The results of trace metals analyses in the whole body of bivalves after one week of exposure are represented in Table 2.

The Concentrations of Cadmium (µg/g dry weight) in exposed animals are significantly increased following administered concentrations in the medium with highest levels to 100µg/l of Cd which revealed about 35 times more of Cd than in controls. However, this rate is about 8 times in exposed molluscs to 200µg Cd/l; level even lower than that observed for the

lowest concentration of exposure (12 times more of Cd in exposed animals to 50 µg/l). As for Cu which showed a small accumulation during the exposure, this accumulation tends to increase only from the highest concentration of exposure (Cu 25µg/l) for which the rate of bioaccumulation doubled compared to control.

Table 2. Concentration of metals in tissues (expressed dry weigth) in *P. perna* after 7 days exposure to Cd and Cu.

Cd (n=5)				Cu (n=5)			
Control	Exposed (ug/g DW)			Control	Exposed (ug/g DW)		
	50 ug/l	100 ug/l	200 ug/l		10 ug/l	15 ug/l	25 ug/l
0,72±0.2	8,89± 3.0	26,51±1.0	5,87 ±1.0	2,08±0.6	2,03±1.0	3,79±2.0	4,44±1.0

Discussion

Common features of the *Perna perna* Cadmium exposure are decreased levels of glutathione in the gills manifested by a likely inhibition of glutathione synthesis as well as stimulation of GST activity and inhibition. Such a reduction of glutathione in the presence of metals was found in the *Mytilus galloprovincialis* mussels exposed to heavy metals (Canesi*et al.,* 1999). Amiard *et al.* (2008) report that in the freshwater mollusc *Unio tumidus*, exposed to pollutants (polycyclic aromatic hydrocarbons and metals), the decrease in glutathione is associated with that of the enzymatic activities of glutathione peroxidase depending on selenium and glutathione reductase, evidence of an anti-oxidant defense deficiency.

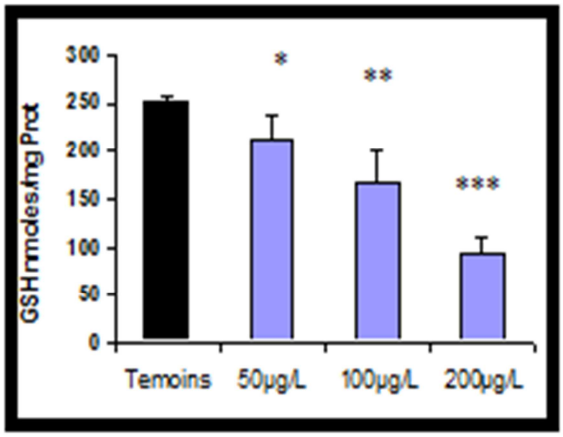

Fig. 1. Variation of GSH level (nmol/mg prot) in gills in *P. perna* after 7 days exposure to Cd *$P< 0,05$; **$P<0,01$; *** $P<0,001$. (n = 5) vs control

These authors suggest that these parameters are considered biomarkers of damage. Hoare *et al.* (1995) shows that glutathione is also involved in changes in mitochondrial metabolism, membrane permeability, and inhibition of oxidative phosphorylation and protein synthesis. The work of (Silvestre *et al.,* 2005) on Chinese crab asserts that the toxicity of Cd to its anterior gills is related to the induction of oxidative stress and to the oxidation of Cd with disulfide bridges of proteins affecting their three-dimensional conformation.

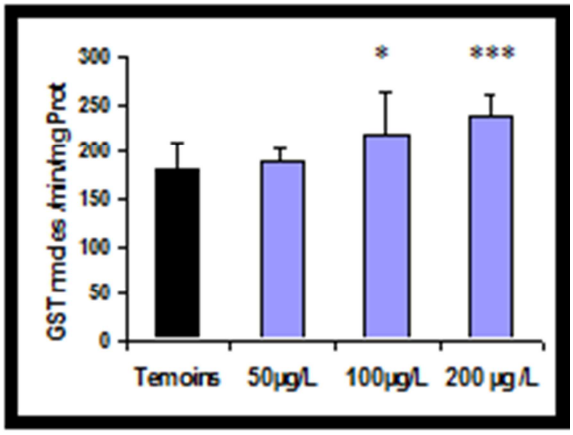

Fig. 2. Variation of GST activity (nmol/min/mg prot) in gills in *P. perna* after 7 days exposure to Cd ;*$P <0,05$;*** $P <0,001$. (n =5) vs control.

The GST involved in anti-oxydants mechanisms and its some isoforms have a fairly significant role in the detoxification of oxygen radicals and lipid peroxydation (Tsangaris *et al.,* 2015). The exposure of *Perna perna* to cadmium and copper led to a decrease of GSH. In our case, unlike Cu only the Cd induced

significantly (P <0001) GST. Several studies showed that the Cd exerts its toxicity at the cellular level, in part, by inducing reactive oxygen species production (ROS) which may cause lipid peroxydation, DNA damages and other damages like oxydation of proteins (Risso-de Faverney *et al.,* 2001 ; Moustaid *et al.,* 2005). Whereas, other studies have observed a correlation between increased resistance and the exposure to metals such as cadmium, copper, mercury and zinc (Hoare*et al.,* 1995). The biological responses observed confirmed toxic effect of cadmium.

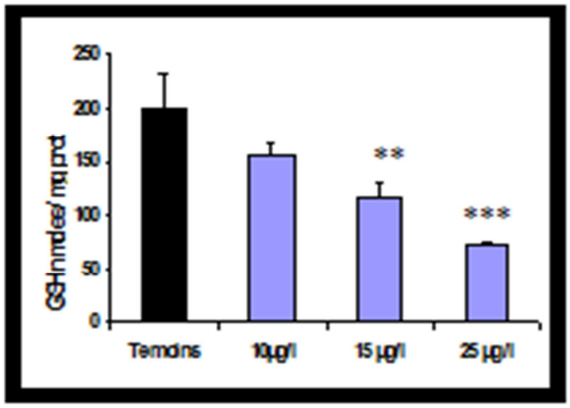

Fig. 3. Variation of GSH level (nmol/mg prot) in gills in *P. perna* after 7days exposure to Cu ; ** *P*<0,01 ; ***P*<0,001 ; n=5 vs control.

The exposure of mussels to Cu during 7 days caused a significant reduction of GSH levels (P = 0,001) similar results were recorded by (Almeida *et al.,* 2004) on mussels exposed to 40 µg/l of Cu for 24h and 72h. These authors suggested that this decline in the GSH rates was due to the increased activity of the phospholipid hydroperoxide glutathione peroxydase enzyme (PHGPx) during which the GSH play the role as electron donor or his important role intracellular sequestration Copper (Bi *et al.,* 2007). Similar results have been advanced by (Geret *et al.,* 2003) after exposing the freshwater bivalve *Unio tumidus* to 30µg Cu/ l for 72 hours who observed a decrease of GSH levels in the 72 hours exposed mussel *M. galloprovincialis* to 40µg Cu/l. In our study, the Copper appears to be increasing GST activity using glutathione as a substrate, while at the highest dose tested, the GST seems not to be required and other enzymes probably metallotioneins were involved in the processes of antioxidant defence (Khati *et al.,* 2012). The results showed that respectively at concentration of 10, 15 and 25 µ g Cu/l molluscs started its detoxification to fight against the toxicity of metal. The highest concentration of copper, has significantly inhibited the GST, this low response may reflect a lesser use of GSH in the reactions of conjugation.

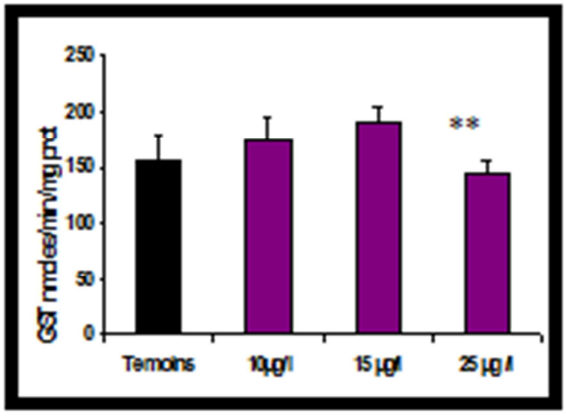

Fig. 4. Variation of GST activity (nmol/min/mg prot) in gills in *P. perna* after 7 days exposure to Cu. ** *P* <0,01. (n =5) vs control.

Some pollutants may act on the levels of GSH and reduce the activities of GST that can no longer detoxify (Roméo et *al.,* 2005). This could explain the significant reduction of the GST with the highest concentrations of copper (25 µ gCu / l). Our results are similar to the work of (Almeida *et al.,* 2004), which found no change in the activities of GST on mussels *Perna perna* exposed to 40µg Cu/ l of copper during 7 days Experimentation and this is probably related to the concentration of copper tested in this experiment. These different responses according to the authors make it difficult interpretation of results, suggesting according (Vlahogianni and Vlavanidis, 2007) that in general changes in systems defenses anti-oxidants are linked to increased ROS (Torres *et al.,* 2002), or the lower rate of MDA (Geret *et al.,* 2002).Lenartova *et al.,* (1996) reported that in vivo exposure of Ruditapes decussatus during 48 hours as phenobarbital and Benzo (a) Pyrene not resulting in any increase in GST, and that whatever the products used, no induction activities GST-CDNB was found in

the hepatopancreas. Knowing that there is a variety of mechanisms used by organisms to reduce the toxicity of heavy metals (GSH, and combined),several of them can explain this acclimatization to a metal post (Gayffon *et al.,* 2009), the exclusion of the pollutant through the mucus secreted by fish, diminishing the entry of pollutants, increased excretion of pollutant, detoxification and / or storage of the pollutant, compensatory mechanisms (protection and / or repair of structures / functions affected,...) may be the cause.

The *in vivo* determination of metals (Cd and Cu) revealed a low bioaccumulation of to all concentrations tested in relation to controls. In our study, concentrations of copper tend to increase only from 15µg Cu/l to reach concentrations (4.44 ± 1µg/g dry wt.) at 25µg Cu/l. This corresponds to a rate of approximately 37% of GSH absorbed respectively to 200µg Cd/l and 25µg Cu/l. The accumulation of Cd increased according administered concentrations in this experience, and then suddenly decreased in the highest dose (200µg Cd /l). The same phenomenon is found in the work of (Damiens *et al.,* 2006) this author gets a drop in the concentration of copper at the higher dose of 1µg Cu/l on oysters that have registered increases the concentration of previous experience, respectively 0.25 and 0.5 µg Cu/ l. These authors have suggested that the elimination of metal is probably due to the synthesis of metallothioneins (MT) which will form the copper with an insoluble complex and this will play a determining role in the detoxification (Taylor and Bill, 2013). This could have happened in the case of cadmium, which also induces MT, which has a very strong affinity for these metals. Indeed the accumulation of metals is a physiological phenomenon which has limits, and in our case the animal is contained on itself and survival.

Hence, there is many other mechanisms of acclimatization that those who would have the effect of restricting the accumulation of metal. The comparable conclusions had been drawn from other organisms and other trace metals which cannot be generalized to all species (Sahar *et al.,* 2014).

Conclusion

Any living organism can enter a state of stress when their environment is subject to change. The metabolism of glutathione seems to be involved in this experience contributing at least in part to the detoxification of metals tested. This response to stress metal could be a means of monitoring pollution, especially by metals, which can be used for evaluating the health of the natural environment in situ. However, it would be interesting to complete this study by the metallothioneins chelators of metals.

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