

Note

Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve mollusks: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*)

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Abstract

The impact of metals (silver, cadmium, copper, mercury and zinc) on metallothionein (MT) and malondialdehyde (MDA) levels of the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*) was studied after 4 or 21 days of metal exposure. Moreover, total protein levels were determined. After 4 days of metal exposure, although *C. gigas* and *M. edulis* accumulated cadmium and mercury concentrations in the gills and digestive gland, no significant variation of total protein level was occurred. After 21 days of exposure, metals were bioaccumulated in the gills and the digestive gland of both mollusks. A decrease of total protein concentrations in the gills of oysters and the digestive gland of mussels and an increase on metallothionein concentrations in the gills of both mollusks were observed. An increase of MDA levels was noticed for the gills and the digestive gland of mussels exposed for 21 days to either cadmium, silver or mercury whereas a decrease of MDA levels was observed in the gills of the oysters exposed for the same time to the same metals. The levels of proteins, MDA and MT were metal, species or organ dependent.   2002 Ifremer/CNRS/Inra/Cemagref/ ditions scientifiques et m dicales Elsevier SAS. All rights reserved.

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1. Introduction

Many marine ecosystems are contaminated by different pollutants, and especially metals, as a result of human activities. Bivalve mollusks such as mussels and oysters can accumulate high concentrations of heavy metals and serve as bioindicators of metal contamination in the marine environment (Langston et al., 1998). Negative effects of toxic metals, and particularly cadmium and mercury, on the general metabolic activity was also demonstrated by Viarengo et al. (1980). The hypothesis that inorganic cations can stimulate lipid peroxidation processus via the oxidation of poly-unsaturated fatty acids is often proposed. Viarengo et al. (1990) showed the relationship between copper exposure

and changes in lipid peroxidation in the mussel *Mytilus galloprovincialis*. Lipid peroxidation and resulting damages are modulated by antioxidant systems (superoxide dismutase, catalase, glutathione peroxidases, glutathione) (Quig, 1998) and metallothioneins (MT) (Cherian and Chan, 1993). MT, play an important role in metal metabolism and particularly in the detoxification mechanisms by acting as a metal-chelating agent for the excess of metals in the cells. MT that are low-molecular weight, cysteine-rich proteins are induced following metal exposure in several mollusks (Langston et al., 1998). Moreover, MT could protect cells from oxidative stress not only by acting as an oxyradical scavenger, but also through metal binding/release dynamics (Viarengo et al., 2000). The present study was designed to investigate the biomarker response of MT and MDA and to examine the links between the MT status and symptoms of cellular toxicity (MDA) in *Mytilus edulis* and *Crassostrea gigas* to different metal exposure.

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Table 1
Mortality (%) of mussels and oysters in controls and after exposure to metals (silver, cadmium, copper, mercury and zinc) in solution for 21 days.

	Controls	Ag	Cd	Cu	Hg	Zn
Mussels	6	4	6	17	2	2
Oysters	0	4	17	13	0	13

2. Materials and methods

2.1. Experimental exposure of bivalves

Oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) were collected in November 1997 from the Bourgneuf Bay (Loire Atlantique, France). They were acclimated in aerated natural sea water (salinity: 31, 15 °C) for one week prior to the experiments. Groups of 20 individuals were transferred to 4 l aquaria. Mollusks were exposed to mono-metallic solutions (Ag, Cd, Cu, Hg and Zn respectively 20; 200; 40; 20 and 1000 $\mu\text{g.l}^{-1}$) in sea water for a period of 4 or 21 days. These concentrations have been used in several other laboratory experiences with bivalves and can be found in contaminated environments (Roesijadi and Fellinham, 1987; Bebianno and Langston, 1991; Berthet et al., 1992). During the 4 day contamination experiment, the sea water was changed every day and oysters were not fed. During the 21 day contamination experiment, control and exposed oysters were fed with a mixture of nutritive substance and vitamins (Organ Planer SEE) every other day before was renewed the water (contaminated or not). No mortality was recorded during the 4 day experiment. During the 21 day experiment, the mortality of oysters and mussels are presented in Table 1.

2.2. Preparation of the samples for biochemical analyses

The gills and digestive glands were separated from the soft part of the mollusks, weighed and homogenized in 5 volumes of buffer (20 mM Tris, 150 mM NaCl, pH 8.6, 10 mM β mercaptoethanol and 3 protease inhibitors: 20 μM leupeptin, 2 μM aprotinin and 100 μM benzamidine). Homogenates were centrifuged for 30 minutes at 30 000 g at 4 °C. Pellets (P1) and aliquots of supernatants (S1) of 5 samples were digested with nitric acid at 60 °C for metal analysis. All the remaining supernatants were submitted to heat-denaturation (75 °C, 15 min) and centrifuged (10 000 g, 15 min). Newly obtained supernatants were frozen at -80 °C until MT and MDA determination.

2.3. Metal analysis

Metal quantification (Ag, Cd, Cu, Zn) was performed with a polarized Zeeman atomic absorption spectrophotometer (Amiard et al., 1987). Mercury was analyzed by cold vapor atomic spectrophotometry after addition of potassium permanganate to oxidise all mercury to a steady state

(Thibaud, 1983; Cosson et al., 1988). Metal concentrations were expressed as $\mu\text{g.g}^{-1}$ wet weight tissue.

2.4. Metallothionein analysis

The levels of metallothionein (MT) in heat-denatured supernatants were estimated by differential pulse polarography (DPP) (Olafson and Sim, 1979, improved by Thompson and Cosson, 1984).

The quantification of MT in the supernatants was done by the standard addition method, using rabbit liver metallothionein (SIGMA, M-7641) as reference material.

Olafson and Olsson (1991) have confirmed, by quantitative analyses on chromatographic fractions, that the method is specific for MT after removal of contaminating proteins in tissue homogenate by heat denaturation. Mercaptoethanol and low-molecular-weight sulfhydryl-containing species (such as glutathione and free cysteins) do not interfere with the polarographic determination of MT (Olafson and Olsson, 1991; pers. obs.). MT concentrations were expressed as $\mu\text{g.mg}^{-1}$ of protein.

2.5. Protein analysis

Total protein concentrations in the supernatant S1 were measured by the Lowry method (Lowry et al., 1951). The protein levels were calculated using BSA (bovine serum albumin) as reference material and expressed as mg of protein (BSA equivalent) per g of tissue wet weight (mg.g^{-1}).

2.6. Malondialdehyde analysis

Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) upon decomposition. The method used was designed to measure (thiobarbituric acid reactives) (TBARs) including malondialdehyde (MDA) (Quintanilha et al., 1982).

2 ml of reactional mixture (thiobarbituric acid 0.375%, trichloroacetic acid 15% and chloridric acid 0.25 N) were added to 1 ml of the heat denatured supernatant. TBARs levels were estimated at 535 nm using malonaldehyde bis (tetrametoxipropan, 10,838-3, SIGMA) as standard. The concentration of lipid peroxidation compounds in the gills and digestive gland of the mollusks is expressed as nmoles of MDA per gram of wet weight tissue.

2.7. Statistical analysis

The data were analysed by analysis of variance (ANOVA), *t*-test or Mann-Whitney test when the normality was not respected with the Sigma Stat 2.0 program. The level of significance was set at 0.05.

Table 2
Total mean metal concentrations \pm standard deviation ($\mu\text{g}\cdot\text{g}^{-1}$) in the gills (G) and the digestive gland (D.G) of unexposed (Control) and exposed oysters and mussels (Ag, Cd, Cu, Hg, and Zn) for 4 days and 21 days.

Oyster	Control	Ag	Control	Cd	Control	Cu	Control	Hg	Control	Zn	
4 days	G.	1.86 \pm 0.02	5.19 \pm 0.20 ^a	0.27 \pm 0.01	19.3 \pm 0.99 ^b	48.1 \pm 5.16	76.70 \pm 9.20 ^c	0.12 \pm 0.03	8.06 \pm 1.50 ^d	379.6 \pm 60.6	374.8 \pm 61.6
	D.G.	3.50 \pm 0.44	7.97 \pm 0.64 ^a	0.12 \pm 0.01	16.60 \pm 1.2 ^b	97.77 \pm 22.24	96.03 \pm 7.62	0.10 \pm 0.03	5.86 \pm 2.49 ^d	667.4 \pm 123.4	400.1 \pm 102.0
21 days	G.	4.15 \pm 0.54	4.41 \pm 0.40	0.17 \pm 0.01	45.50 \pm 10.46 ^b	38.09 \pm 6.25	115.25 \pm 6.47 ^c	0.13 \pm 0.03	74.81 \pm 14.02 ^d	327.0 \pm 36.5	557.4 \pm 38.6 ^e
	D.G.	9.86 \pm 0.54	13.50 \pm 2.13	0.39 \pm 0.02	78.16 \pm 9.44 ^b	61.40 \pm 4.78	95.52 \pm 16.96 ^c	0.16 \pm 0.02	22.63 \pm 3.86 ^d	488.7 \pm 34.6	830.4 \pm 55.2 ^e
Mussel											
4 days	G.	< DL	0.94 \pm 0.12 ^a	0.10 \pm 0.01	11.61 \pm 1.23 ^b	0.69 \pm 0.07	7.17 \pm 0.59 ^c	0.08 \pm 0.02	51.98 \pm 4.64 ^d	20.77 \pm 3.11	17.80 \pm 2.18
	D.G.	< DL	0.38 \pm 0.04 ^a	0.05 \pm 0.01	9.20 \pm 0.97 ^b	2.04 \pm 0.16	3.07 \pm 0.29 ^c	0.25 \pm 0.05	5.89 \pm 0.81 ^d	24.20 \pm 1.78	47.93 \pm 3.92 ^e
21 days	G.	< DL	1.30 \pm 0.10 ^a	0.03 \pm 0.01	48.90 \pm 3.00 ^b	0.97 \pm 0.04	16.04 \pm 1.50 ^c	0.41 \pm 0.14	131.10 \pm 12.19 ^d	17.79 \pm 3.24	27.23 \pm 2.89 ^e
	D.G.	< DL	1.33 \pm 0.10 ^a	0.06 \pm 0.07	56.78 \pm 4.75 ^b	2.20 \pm 0.16	5.32 \pm 0.27 ^c	0.34 \pm 0.04	62.95 \pm 7.32 ^d	22.85 \pm 2.43	39.83 \pm 2.24 ^e

a,b,c,d indicate significant differences between control and Ag, Cd, Cu, Hg or Zn exposure in oysters and mussels respectively.

3. Results

3.1. Metal accumulation

Metal concentrations in the oysters were significantly higher ($P < 0.05$) than in the mussels (Table 2). In these mollusks, total metal concentrations in the gills were significantly lower ($P < 0.05$) than in the digestive gland (except for exposed oyster for 21 days). After 4 days of exposure, toxic metals were bioaccumulated in both gills and digestive gland of mussels and oysters ($P < 0.05$). Cu was significantly bioaccumulated only in the gills of the mussels while Zn was significantly bioaccumulated in the digestive gland.

After 21 days of metal exposure, Cd and Hg were bioaccumulated in both mollusk organs but the accumulation of Cd was higher in the digestive gland than in gills while the accumulation of Hg was higher in the gills. Ag was significantly bioaccumulated in mussels. Cu was accumulated in the gills and digestive gland of mollusks (except in the digestive gland of oysters after 4 days of Cu exposure). The accumulation factor was higher in the gills than in the digestive gland while Zn was not significantly accumulated ($P > 0.05$). After 21 days of metal exposure, the percentage of mortality of Cu exposed bivalves was high and showed the strong toxicity of this element when present in excess.

3.2. Protein concentrations

Between the fourth and the twenty-first day of metal exposure, no significant variation of total protein concentrations was observed in the digestive gland of control oysters (Fig. 1). On the other hand, these concentrations decreased in the gills of control oysters. After 21 days of exposure, total protein concentrations in the gills of oysters exposed to Ag, Cd, Cu and Hg significantly decreased ($P < 0.05$). No significant modification in total protein concentrations was observed in the gills of mussels

($P > 0.05$) while an increase of the metals was observed in this organ. However, total protein concentrations in the digestive gland of mussels exposed to Ag, Cd, Hg and Zn significantly decreased while no significant variation was observed in the digestive gland of oysters ($P > 0.05$).

3.3. MDA concentrations

In both mussels and oysters, MDA concentrations were higher in digestive gland than in gills (Fig. 2). A significant decrease of MDA concentrations was observed in the gills of oysters exposed to Cd, Cu and Hg and mussels exposed to Cu during 21 days, while a significant increase was observed in gills of mussels exposed to Ag, Cd and Hg. A significant increase of MDA concentrations was observed in digestive gland of mussels exposed to Cd and Hg while no variation was occurred in digestive gland of oysters.

3.4. Metallothionein concentrations

Metallothionein concentrations in mussels were higher than in oysters (Fig. 2). After 4 days of metal exposure, no variation of MT concentrations was observed between control and exposed mollusks (results not shown). After 21 days of metal exposure, a significant induction of MT synthesis in the gills of metal exposed bivalves (except for the gills of Cu exposed mussels) and in the digestive gland of oysters exposed to Cd and Hg was observed. In both mussels and oysters.

4. Discussion

The results presented in this work demonstrate that the exposure to sublethal concentrations of metals caused modification of protein, MT and MDA levels in mussels and oysters.

The accumulation of essential metals was low, specially for Zn. Similar results were observed in *Macoma balthica*

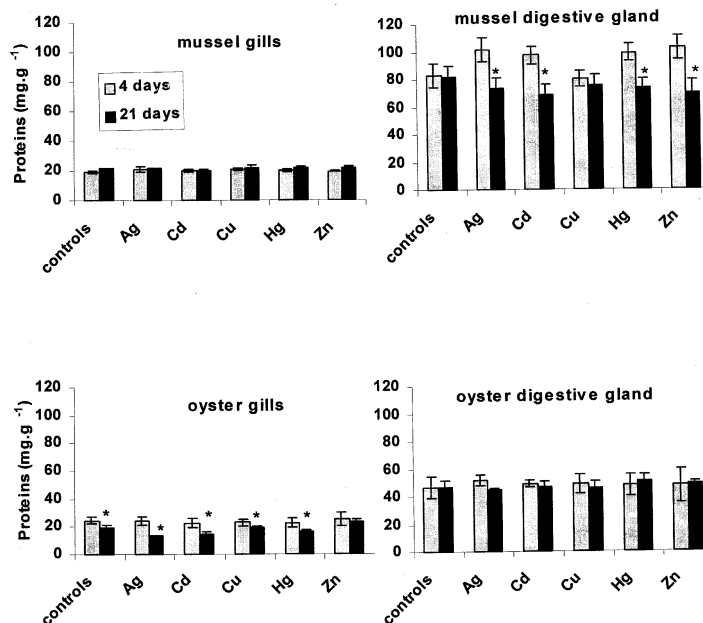


Fig. 1. Mean concentrations (+ confidence limits) of total proteins in the gills (a) and digestive gland (b) of oysters and mussels: unexposed and exposed to silver, cadmium, copper, mercury and zinc during 4 or 21 days.

*: significant difference between total protein concentrations in the digestive gland of mollusks exposed for 4 days and for 21 days respectively ($P = 0.05$).

(Bordin et al., 1994). Most of the species seem to regulate the levels of this metal in their tissues (White and Rainbow, 1982; Amiard-Triquet, 1989). Toxic metals were bioaccumulated, particularly Cd and Hg. The values of Cd accumulation obtained in the gills and digestive gland of mussels are in agreement with those described by Serra et al. (1995) in the bivalve *Scapharca inaequalvis* after 7 or 21 days of exposure. After the same time of exposure, toxic metals modified the general metabolism in the gills of the oysters

and in the digestive gland of the mussels expressed by a decrease of total protein levels. This decrease amplified a phenomenon already observed in controls between 4 and 21 days of experiment. Baudrimont et al. (1997) showed also a decrease of total protein levels in *Corbicula fluminea* explained that by a decrease of general metabolic activity. On the other hand, after 21 days of metal exposure, MT synthesis was induced in the cytosol of the gills of both mollusks (except in the gills of Cu exposed mussels).

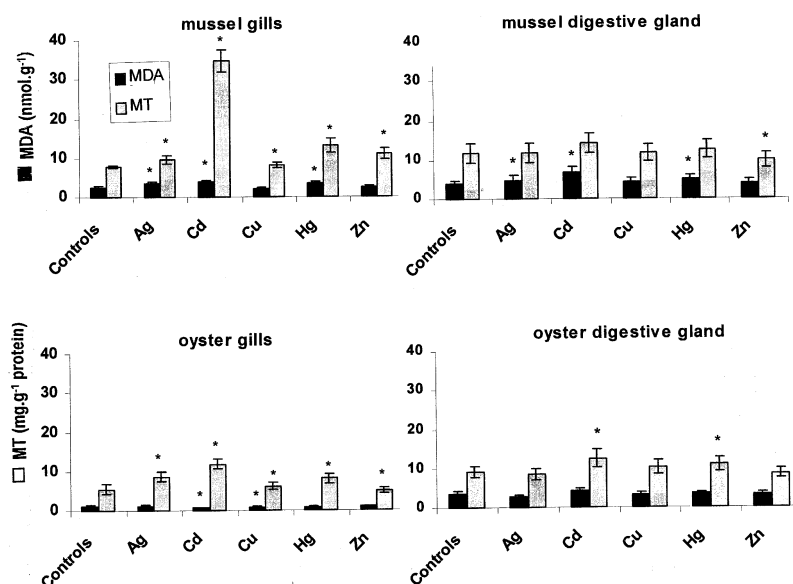


Fig. 2. Mean concentrations (+ confidence limits) of malondialdehyde (MDA) and metallothioneins (MTs) in the gills (a) and the digestive gland (b) of oysters and mussels: unexposed and exposed to silver, cadmium, copper, mercury and zinc for 21 days.

*: significant difference between MDA or MTs concentrations of unexposed and exposed mollusks ($P = 0.05$).

Although the function of MT in metal metabolism is not fully understood, there is a particular concensus that this protein acts as a detoxification mechanism of the excess of toxic metals in mollusks (Langston et al., 1998). MT levels after Cd exposure were higher in the mussels than in the oysters (factor 4 against factor 2). MT levels are in agreement with the results presented by Bebianno and Serafim (1998) for the mussels *Mytilus galloprovincialis* exposed to 100 $\mu\text{g Cd l}^{-1}$ during 40 days.

Moreover, metals are known to produce reactive oxygen species (ROS) (Viarengo et al., 1990; Stohs et al., 2000). ROS can act on membrane lipids leading to lipid peroxidation and to the formation of MDA. However, MT could protect cells against toxic effects of ROS (Viarengo et al., 2000; Andrews, 2000). The activation of MT-I gene expression by oxidative stress is mediated, in part, by an increase in free Zn ions in the cell (Andrews, 2000). So, it is interesting to associate the study of cellular toxicity indicators (MDA) and antioxidant molecules as MT.

These results demonstrate that metal exposure modify the production of MDA in mollusks. An increase in lipid peroxidation was observed in the gills and digestive gland of mussels exposed to Cd and Hg during 21 days. These results are similar to those obtained in Cd exposed mussels, *Perna viridis* (Prakash and Jagannatha Rao, 1995) or clams, *R. decussatus* (Romeo and Gnassia-Barelli, 1997; G eret et al., unpublished data). Cd and Hg have high affinities for glutathione (GSH) which is the primary intracellular antioxidant agent and can bind and cause the irreversible excretion of GSH leading to the depletion of GSH and the increase of MDA (Quig, 1998). However, in the gills of oysters exposed to Cd, Cu or Hg, a decrease in MDA levels paired up an increase in MT levels. This decrease could result in an intensification of antioxidant systems including MT, limiting the MDA formation. On the other hand, in the gills of mussels exposed to toxic metals, the increase of MDA levels was associated with an increase in MT levels. A relationship between MT and MDA enhancement was shown in the freshwater bivalve, *Pyganodon grandis*, in response to Cd and Zn exposure (Couillard et al., 1995). This increase of MDA could result in a saturation of antioxidant systems.

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