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Effect of cadmium on antioxidant enzyme activities and lipid peroxidation in the gills of the clam *Ruditapes decussatus*

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Metals are known to influence the oxidative status of marine organisms, and antioxidant enzymes have been often proposed as biomarkers of effect. The clam *Ruditapes decussatus* is a well-known metal bioindicator. In this species cadmium (Cd) induces metallothionein (MT) synthesis only after 7 days of exposure. Before MT synthesis is induced, the other mechanisms capable of handling the excess of Cd are unknown. In order to identify some of these mechanisms, variations in antioxidant systems (superoxide dismutase, catalase, selenium-dependent glutathione peroxidase and non-selenium-dependent glutathione peroxidase), malondialdehyde (MDA) and MT were studied in the gills of *R. decussatus* exposed to different Cd concentrations (4, 40 and 100 $g l^{-1}$) for 28 days. These parameters, together with total proteins and Cd concentrations, were measured in the gills of the clams over different periods of exposure. Results indicate that Cd accumulation increased linearly in the gills of *R. decussatus* with the increase in Cd concentration. This increase induces an imbalance in the oxygen metabolism during the first days of Cd exposure. An increase in cytosolic superoxide dismutase (SOD) activity and a decrease in mitochondrial SOD activity was observed at the same time as or after a decrease in cytosolic and mitochondrial catalase activity and of selenium-dependent and non-selenium-dependent glutathione peroxidase activity. After 14 days of exposure, Cd no longer affect these enzymes but there was elevation of other cellular activities, such as MDA and MT production. MT bound excess Cd present in the cell. These variations in these parameters suggest their potential use as biomarkers of effects such as oxidative stress resulting from Cd contamination in molluscs.

Keywords: antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, metallothioneins, lipid peroxidation, clam, *Ruditapes decussatus*, cadmium.

Introduction

Cadmium (Cd) is a metal that is toxic to living organisms and is widely distributed in the marine environment. It enters this environment mainly from industrial processes, phosphate fertilizers and in associated with zinc extraction (Ramade 1992). Cd concentrations in suspension-feeder invertebrates, and especially in clams, increase with the time of exposure (Bebianno and Serafim 1998). Cd is involved in changes in mitochondrial metabolism (Gould *et al.* 1985), membrane permeability and inhibition of oxidative phosphorylation and protein synthesis (Viarengo 1989). Cd also binds to nucleic acid bases and phosphate groups, affecting their structures (Hughes 1981, Traore *et al.* 2000).

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Like MT, glutathione plays an important role in metal metabolism and, particularly, in detoxification mechanisms, by acting as a metal-chelating agent for the excess of Cd in the cells. Glutathione is the primary intracellular antioxidant and conjugating agent that binds Cd (Quig 1998). Metallothioneins (MTs), which are low molecular weight, cysteine-rich proteins, are induced following Cd exposure in several molluscs such as oysters (Roesijadi and Klerks 1989), mussels (Bebianno and Langston 1991) and clams (Bebianno and Serafim 1998). However, the induction of MT synthesis in the gills of the oyster *Crassostrea virginica* occurred only after 4 days of Cd exposure ($200\,\mu g\,l^{-1}$) (Roesijadi and Klerks 1989). Bebianno and Serafim (1998) reported a small increase in MT levels in the gills of the clam *Ruditapes decussatus* at the beginning of the treatment with $100\,\mu g\,l^{-1}$ of Cd.

Moreover, although it is a non-redox metal and therefore is unlikely to participate in Fenton-type reactions, Cd is known to enhance the intracellular formation of reactive oxygen species (ROS) and to promote cellular oxidative stress (Stohs *et al.* 2000). ROS alter the structure of the cell membranes by stimulating the lipid peroxidation process through oxidation of polyunsaturated fatty acids (Harris 1992, Stohs *et al.* 2000). The induction of antioxidant systems reflects an adaptation or compensatory reaction to ROS formation. In contrast, a deficiency in these mechanisms indicates a toxic effect of ROS and the organisms become more sensitive to oxidative stress (Livingstone *et al.* 1990). The cellular defence systems directed against the toxicity of oxyradicals include the activity of certain enzymes, particularly superoxide dismutase (SOD), catalase and glutathione peroxidases (total and selenium-dependent).

SODs are metalloenzymes that catalyse the dismutation of superoxide anion into hydrogen peroxide (H_2O_2). Cu/Zn-SOD is found mainly in the cytosol and nucleus, but Mn-SOD is mainly present in the mitochondria. Catalase reduces H_2O_2 to water by the reaction $2H_2O_2 \rightarrow 2H_2O + O_2$. The function of glutathione peroxidases is to detoxify peroxides (hydroperoxides or organoperoxides) in the cells. They catalyse the reduction of H_2O_2 to water (Se-dependent glutathione peroxidase) and of organic peroxides (ROOH) to stable alcohols (ROH), using reduced glutathione (GSH) as a source of reducing equivalents and electron donor to regenerate the reduced form of the selenocysteine, producing oxidized glutathione (GSSG) in the process ($ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$). Because peroxides can decompose to form highly reactive radicals, glutathione peroxidases play an important role in protecting the cells from free radical damage, particularly lipid peroxidation. Antioxidant enzymes and glutathione seem to act as the first defence mechanism against an excess of Cd in the cells, while MTs intervene later.

Bivalves, especially clams, known to accumulate metals in their soft tissues are good bioindicators of metal contamination in the marine environment (Bebianno *et al.* 1993, Romeo and Gnassia-Barelli 1995). The aim of present study was to investigate the effect of Cd exposure on the activities of protective antioxidant enzymes, lipid peroxidation, induction of MT synthesis and protein content in the gills of the marine clam *R. decussatus* in order to explain the cytotoxic and tissue-damaging effects of Cd exposure. The objective of this work was to study how these antioxidant parameters were influenced by Cd exposure and to evaluate their potential as biomarkers of the effects of Cd in molluscs.

Materials and methods

R. decussatus (shell length 34.6 ± 1.9 mm) were collected in the Ria Formosa lagoon on the southern coast of Portugal and acclimated in aerated seawater for 2 weeks prior to Cd exposure. Subsequently, groups of 35 animals were held in 5 l aquaria and exposed to three different Cd concentrations (4, 40 or 100 µg l^{-1}) for 28 days. Two aquaria were used for each Cd concentration. Control groups were maintained in clean seawater. During the experiment the water in each aquarium was changed every 2 days and the Cd concentration re-established. During the experiment only one clam died in the tank contaminated with 4 µg l^{-1} Cd and another one in the control tank.

Ten clams were removed from each treatment after 1, 3, 7, 14, 21 and 28 days. They were measured and the gills dissected, weighed, frozen in liquid nitrogen and stored at −80°C until analyses of antioxidant enzyme activities, lipid peroxidation, and MT and protein content.

Enzymatic activities

Gills were homogenized in 20 mM Tris buffer (pH 7.6) containing 1 mM of ethylene diamine tetra-acetic acid (EDTA), 0.5 M of saccharose, 0.15 M of KCl and 1 mM of dithiothreitol. The homogenates were centrifuged at 500 *g* for 15 min at 4°C to precipitate large particles. The supernatants were re-centrifuged at 12 000 *g* for 45 min at 4°C to precipitate the mitochondrial fraction, and purified on a Sephadex G-25 gel column to remove the low molecular weight proteins. SOD and catalase activities were measured in the mitochondrial and cytosolic fractions. Selenium-dependent and total glutathione peroxidase activities were measured in the cytosolic fraction.

SOD (EC 1.15.1.1). SOD activity was determined by measuring the reduction of cytochrome c by the xanthine oxidase/hypoxanthine system at 550 nm (MacCord and Fridovich 1969). One unit of SOD is defined as the amount of enzyme that inhibits the reduction of cytochrome c by 50%. The SOD activity in the gills of the clams was expressed as U mg^{-1} total protein.

Catalase (EC 1.11.1.6). Catalase activity was measured according to Greenwald (1985) by the decrease in absorbance at 240 nm due to H_2O_2 consumption. The difference in the absorbance per unit time was taken as a measure of catalase activity ($\varepsilon = -0.04\ mM^{-1}\ cm^{-1}$). The catalase activity in the gills of the clams was expressed as mmol min^{-1} mg^{-1} total protein.

Glutathione peroxidases (EC 1.11.1.9 and EC 2.5.1.18). Glutathione peroxidase activities were measured following the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm in the presence of excess glutathione reductase, reduced glutathione and corresponding peroxide (Lawrence and Burk 1976). The selenium-dependent and total glutathione peroxidase activities were measured by using H_2O_2 and cumene hydroperoxide, respectively, as substrates. The rate of the blank reaction was subtracted from the total rate. The difference in the absorbance per unit time was taken as a measure of glutathione peroxidase activity ($\varepsilon = -6.22\ mM^{-1}\ cm^{-1}$). The glutathione peroxidase activities in the gills of the clams were expressed as (mol min^{-1} mg^{-1} total protein.

Lipid peroxidation

Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) on decomposition. The method used to assess lipid peroxidation was designed to measure MDA and 4-HNE concentrations (Erdelmeier *et al.* 1998), and was based on the reaction of the chromogenic reagent *N*-methyl-2-phenylindole (R1), where two moles of R1 react with one mole of either MDA or 4-HNE at 45°C for 60 min to yield a stable chromophore with maximal absorbance at 586 nm.

For this, 10 µl of 0.5 M butylated hydroxytoluene, 650 µl of diluted R1 (6 ml of methanol with 18 ml of 10.3 mM *N*-methyl-2-phenylindole) and 150 µl of 15.4 M methanesulphonic acid were added to 200 µl of the first cytosolic fraction. This mixture was incubated at 45°C for 60 min. MDA + 4-HNE levels were estimated at 586 nm using MDA bis (tetrametoxypropan, Sigma) as standard. The concentration of lipid peroxidation compounds in the gills of the clams was expressed as nmol MDA g^{-1} total protein.

Total protein concentrations

The gills were homogenized in 20 mM Tris buffer (pH 8.6) containing 150 mM of NaCl. The homogenates were centrifuged for 30 min at 30 000 *g* at 4°C. Total protein concentrations were measured in the supernatants by the Lowry method (Lowry *et al.* 1951) using bovine serum albumin (BSA) as reference standard material. Protein concentrations were expressed as mg g^{-1} wet weight tissue.

MT concentrations

The supernatant (prepared in the same way as for measurement of total protein concentrations) was heat-treated at 80°C for 10 min to precipitate the high molecular weight proteins, and re-centrifuged at 30 000*g* for 15 min at 4°C. Aliquots of the heat-treated cytosol were used for the determination of MT concentrations by differential pulse polarography, according to the method developed by Thompson and Cosson (1984) and modified by Bebianno and Langston (1989). In the absence of a clam MT standard, quantification of MT concentrations in the cytosol of the gills of *R. decussatus* was based on rabbit liver MT (MT-I). Levels of MT were expressed as mg g^{-1} wet weight tissue.

Cd concentrations

Cd analysis was performed on dried HNO_3^- digested subsamples of the gill homogenate using atomic absorption spectrophotometry. All metal concentrations were expressed as nmol g^{-1} dry weight tissue. The quality of the data was ensured by performing Cd analyses on TORT I lobster hepatopancreas (National Research Council, Canada): the levels obtained were 26.98 ± 0.01 µg g^{-1} (mean ± SD), compared with the certified values of 26.3 ± 2.1 µg g^{-1}.

Statistical analysis

Analysis of variance (ANOVA), t-test and the Mann–Whitney test when normality was not respected were used. The level of significance was set at 0.05.

Results

Cd accumulation

The mean levels of total Cd accumulated in the gills of *R. decussatus* exposed to 4, 40 or 100 µg l^{-1} Cd for 28 days are presented in figure 1. Cd levels in the gills of controls did not change during the course of the experiments and were 0.62 ± 0.17 µg g^{-1} dry weight. In Cd-exposed clams, Cd levels in the gills ([Cd], in µg g^{-1} dry weight) increased linearly with the increase in Cd concentrations and time of exposure (t, in days): $[Cd] = 0.5t + 1.8 (r = 0.981)$, $[Cd] = 2.5t - 5.1 (r = 0.991)$ and $[Cd] = 4.2t - 6.2 (r = 0.966)$ for Cd concentrations of 4, 40 and 100 µg l^{-1}, respectively). Cd accumulation was significantly different among the different Cd treatments ($p < 0.05$).

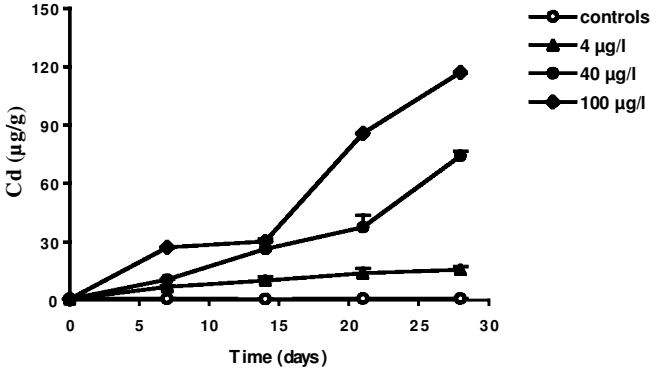

Figure 1. Mean total Cd concentrations (µg g^{-1} dry weight) in gills of control and Cd-exposed clams (4, 40 or 100 µg l^{-1} Cd) for 28 days.

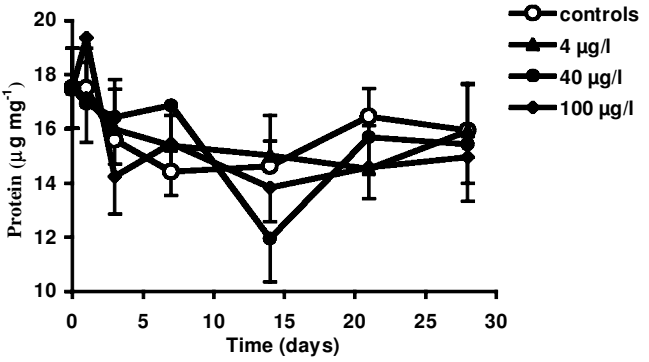

Figure 2. Total protein concentrations (mean ± SD) (µg mg^{-1} wet weight) in the cytosolic fraction of gills of control and Cd-exposed clams (4, 40 or 100 µg l^{-1} Cd) for 28 days ($n = 5$).

Total protein concentrations

Protein concentrations in the gills of controls and Cd-exposed clams (4, 40 and 100 µg l^{-1} Cd) for 28 days are presented in figure 2.

No significant change in total protein concentrations was observed for the gills of controls (16.01 ± 1.24 mg g^{-1} wet weight) or for those exposed to 4 µg l^{-1} Cd (16.98 ± 1.15 mg g^{-1} wet weight) during the whole experiment. Total protein levels in the gills of the clams exposed to 40 µg l^{-1} Cd were significantly higher than controls after 7 days of Cd exposure ($p < 0.05$). They decreased significantly the week after and remained unchanged until the end of the experiment ($p < 0.05$).

However, total protein concentrations in the gills of the clams exposed to 100 µg l^{-1} Cd significantly decreased between the first and the third day of Cd exposure ($p < 0.05$). After this period, levels remained unchanged and were not significantly different from controls.

SOD

The percentage SOD and catalase activities in the cytosol of the gills of uncontaminated *R. decussatus* and those exposed to Cd (4, 40 or 100 µg l^{-1}) are presented in table 1. SOD activities in the gills of the clams exposed to Cd (4, 40 or

Table 1. SOD and catalase cytosolic activity (% of total SOD or catalase activity) in the gills of unexposed and Cd-exposed (4, 40 and 100 µg l^{-1}) *R. decussatus* clams for 28 days (mean% ± SD) ($n = 5$).

			Cd exposure					
	Controls		4 µg l^{-1}		40 µg l^{-1}		100 µg l^{-1}	
Time (days)	SOD	Catalase	SOD	Catalase	SOD	Catalase	SOD	Catalase
0	78 ± 7	91 ± 5	78 ± 7	91 ± 5	78 ± 7	91 ± 5	78 ± 7	91 ± 5
1	78 ± 7	91 ± 5	59 ± 5	79 ± 5	81 ± 7	88 ± 1	65 ± 5	81 ± 3
3	64 ± 1	82 ± 2	89 ± 6	90 ± 3	80 ± 5	91 ± 1	75 ± 7	90 ± 3
7	72 ± 2	77 ± 10	79 ± 4	81 ± 7	80 ± 4	83 ± 3	72 ± 2	87 ± 1
14	73 ± 5	83 ± 2	77 ± 5	86 ± 3	77 ± 3	81 ± 5	73 ± 2	85 ± 3
21	76 ± 4	86 ± 5	69 ± 6	85 ± 4	74 ± 4	88 ± 2	76 ± 5	84 ± 4
28	84 ± 4	87 ± 2	69 ± 5	87 ± 2	75 ± 6	85 ± 1	81 ± 6	88 ± 1

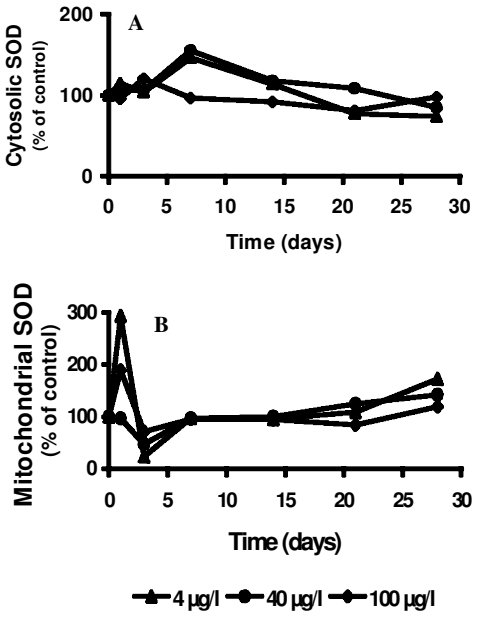

Figure 3. Cytosolic (A) and mitochondrial (B) SOD activity in gills of *R. decussatus* exposed to Cd (4, 40 or 100 µg l^{-1}) for 28 days, expressed as a percentage of the activity in unexposed, control gills ($n = 5$).

100 µg l^{-1}) for 28 days, expressed as percentage of SOD activity in the gills of controls, are presented in figure 3.

SOD was predominantly present in the cytosolic fraction (>60%) of unexposed and Cd-contaminated clam gills. Throughout the experiment, no significant difference was observed in the percentage SOD activity in the cytosol of the gills of unexposed clams (75 ± 6%). The percentage SOD activity in the cytosol of the gills from clams contaminated with 4 and 100 µg l^{-1} Cd decreased significantly during the first day of exposure when compared with non-contaminated gills ($p < 0.05$). However, after 3 days of Cd exposure (4, 40 or 100 µg l^{-1}) this percentage was significantly higher than in control gills ($p < 0.005$). For the clams exposed to 40 µg l^{-1} Cd, the percentage of cytosolic SOD in the gills continue to increase after 7 days of Cd exposure ($p < 0.05$). However, after 2 weeks, the percentage SOD activities in the cytosol of Cd-exposed gills were not significantly different from controls ($p > 0.05$).

Cytosolic SOD activity in the gills of controls increased significantly between the first (26.41 ± 5.23 U mg^{-1} protein) and the final day (49.77 ± 6.46 U mg^{-1} protein) of the experiment ($p < 0.05$). The cytosolic SOD activity in the cells of the gills of the clams exposed to 4 or 40 µg l^{-1} Cd increased significantly relative to controls ($p < 0.05$) (figure 3A). After this period, SOD activity decreased significantly ($p < 0.05$) until the end of the experiment in gills exposed to 4 or 40 µg l^{-1} Cd. Cytosolic SOD activity in gills exposed to 100 µg l^{-1} Cd increased significantly between the first and the third day ($p < 0.05$) of cadmium exposure. After this period the SOD levels remained unchanged until the end of the experiment ($p > 0.05$).

Mitochondrial SOD activity in the gills of controls ($13.55 \pm 5.31\,U\,mg^{-1}$ protein) was significantly lower than cytosolic SOD activity ($39.79 \pm 10.14\,U\,mg^{-1}$ protein) and no significant variation was observed during the course of the experiment. After the first day of Cd exposure, a significant induction of mitochondrial SOD activity in the gills exposed to 4 and $100\,\mu g\,l^{-1}$ Cd was observed ($p < 0.05$) (figure 3B). Between the first and the third day, a significant inhibition of mitochondrial SOD activity of gills exposed to the three Cd concentrations ($p < 0.05$) occurred and was more significant for the gills exposed to $4\,\mu g\,l^{-1}$ Cd ($p < 0.005$). After this period, although there was a slight increase in mitochondrial SOD activity in gills exposed to 4 and $40\,\mu g\,l^{-1}$ Cd, it was not significant for any of the three Cd treatments ($p > 0.05$).

Catalase

The catalase activity in the cytosolic and mitochondrial fractions of the gills of *R. decussatus* exposed to 4, 40 or $100\,\mu g\,l^{-1}$ Cd for 28 days, expressed as a percentage of control values, are presented in figure 4. The percentage of catalase activity in the cytosol of the gills of unexposed and Cd-exposed clams is presented in table 1.

Catalase was predominantly found in the cytosol of controls and Cd-contaminated clam gills and the percentage of catalase in the cytosol was higher than cytosolic SOD (table 1). The percentage cytosolic catalase activity of the controls did not change during the course of the experiment ($85 \pm 5\%$) (table 1). On the first day of Cd exposure, the percentages of cytosolic catalase activity in the gills of Cd-contaminated clams exposed to 4 or $100\,\mu g\,l^{-1}$ were significantly lower than in controls (79 and 81%, respectively, compared with 91%; $p < 0.05$). However, after

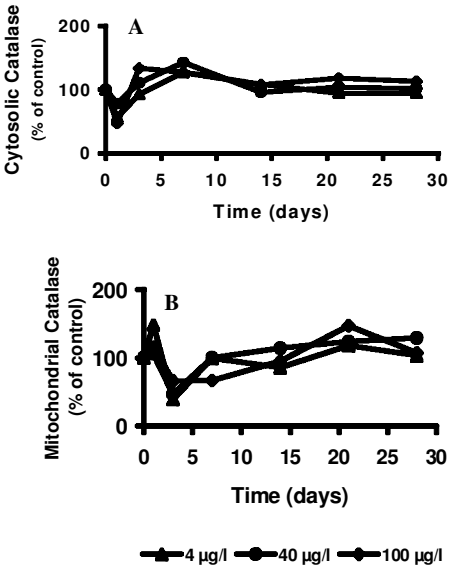

Figure 4. Cytosolic (A) and mitochondrial (B) catalase activity in gills of *R. decussatus* exposed to Cd (4, 40 or $100\,\mu g\,l^{-1}$) for 28 days, expressed as a percentage of the activity in unexposed, control gills ($n = 5$).

3 days of exposure, they were higher than in controls ($p < 0.05$) and remained unchanged, although they were not significantly different from controls ($p > 0.05$).

The cytosolic catalase activity in the gills of controls remained unchanged throughout the experiment (0.49 ± 0.04 mmol min^{-1} mg^{-1} protein).

After 1 day of Cd exposure a significant inhibition of the cytosolic catalase activity ($p < 0.05$) in the gills of Cd-exposed clams (4, 40 or 100 µg l^{-1}) was observed (figure 4A). Between the first and the third day of Cd exposure, the catalase activity in the gills of clams exposed to the three Cd concentrations increased significantly and was Cd dependent ($p < 0.05$). After this period, catalase activity remained unchanged ($p > 0.05$).

The mitochondrial catalase activity in the gills of the controls, although having some variability, did not significantly change over the course of the experiment (0.089 ± 0.019 mmol min^{-1} mg^{-1} protein). Between the first and the third day of Cd exposure, mitochondrial catalase activity significantly decreased in the gills of clams exposed to 4, 40 or 100 µg l^{-1} ($p < 0.05$) (figure 4B). Between the third and seventh day of Cd exposure, catalase activity in cells exposed to 4 and 40 µg l^{-1} significantly increased ($p < 0.05$). After this period, the catalase activity in the mitochondria was not significantly different from controls in gills exposed to 4 µg l^{-1} Cd ($p > 0.05$). However, for clams exposed to 40 µg l^{-1} Cd, mitochondrial catalase activity significantly increased until the fourteenth day of cadmium exposure. After this period, no significant variation was observed. In gills exposed to 100 µg l^{-1} Cd, mitochondrial catalase activity significantly increased between the seventh and the twenty-first day ($p < 0.05$). After this period, the mitochondrial catalase activity was not significantly different from that in the other Cd treatments.

Total glutathione peroxidase

Total glutathione peroxidase activity is presented in figure 5A. No significant variation was observed in the total glutathione peroxidase activity in the gills of controls (0.0099 ± 0.0016 µmol min^{-1} mg^{-1} protein). On the first day of exposure, a significant inhibition of total glutathione peroxidase activity was observed in the gills of clams exposed to 40 µg l^{-1} Cd ($p < 0.05$) (figure 5A). Between the first and the third day of Cd exposure, total glutathione peroxidase activity decreased in the gills of clams exposed to 4 or 100 µg l^{-1} ($p < 0.05$). Between the third and the seventh day, total glutathione peroxidase activity in gills exposed to 100 µg l^{-1} Cd increased significantly ($p < 0.05$). Between the third and the twenty-first day, total glutathione peroxidase in gills of clams exposed to 40 µg l^{-1} Cd increased significantly ($p < 0.05$). At 7 days, a significant inhibition of the total glutathione peroxidase activity in gills of clams contaminated with 4 or 40 µg l^{-1} Cd was observed ($p < 0.05$). After 14 days of Cd exposure, the total glutathione peroxidase activity remained unchanged for all three Cd concentrations until the end of the experiment ($p > 0.05$).

Selenium-dependent glutathione peroxidase

Selenium-dependent glutathione peroxidase activity in the cytosolic fraction of the gills of *R. decussatus* is presented in figure 5B.

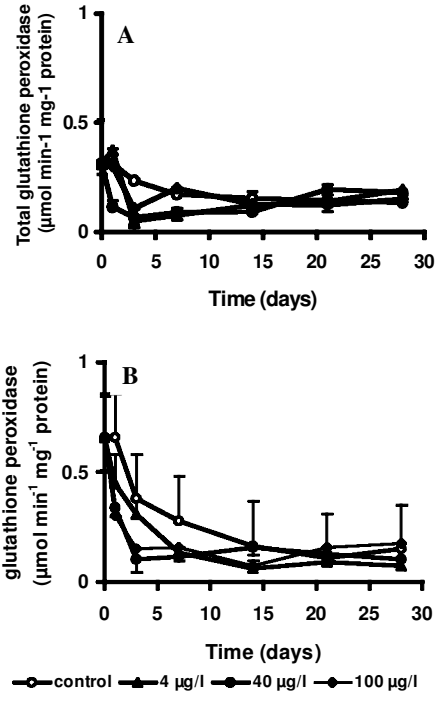

Figure 5. Total (A) and selenium-dependent (B) glutathione peroxidase activity (mean ± SD) ($\mu mol\,min^{-1}\,mg^{-1}$ protein) in the cytosolic fraction of gills of *R. decussatus* exposed to Cd (4, 40 or $100\,\mu g\,l^{-1}$) for 28 days ($n = 5$).

Selenium-dependent glutathione peroxidase activity in the gills of controls decreased exponentially ($p < 0.05$) during the experiment ($y = 0.41e^{-0.05x}$). No significant variation in selenium-dependent glutathione peroxidase activity was observed for gills exposed to $4\,\mu g\,l^{-1}$ Cd over the course of the experiment. In gills exposed to 40 or $100\,\mu g\,l^{-1}$ Cd, selenium-dependent glutathione peroxidase activity decreased significantly between the start of the experiment and the first day of Cd exposure ($p < 0.05$). By the third day of exposure (40 and $100\,\mu g\,l^{-1}$ Cd), glutathione peroxidase activity was significantly inhibited and remained unchanged until the end of the experiment ($p > 0.05$).

Lipid peroxidation

No significant variation in MDA concentration ($p > 0.05$) was observed in the gills of controls ($373.8 \pm 45.2\,nmol\,g^{-1}$ protein) and those exposed to $4\,\mu g\,l^{-1}$ Cd over the course of the experiment. However, after 21 days of Cd exposure, a significant increase in MDA concentrations was observed in the gills of clams exposed to 40 or $100\,\mu g\,l^{-1}$ ($p < 0.05$). In Cd-exposed clams, MDA levels in the gills ([MDA] in $nmol\,g^{-1}$ protein) increased linearly with the increase in Cd concentration ([Cd]) and with the time of exposure (data not shown): $[MDA] = 1.7 \times [Cd] + 376$, $r^2 = 0.32$ (figure 6).

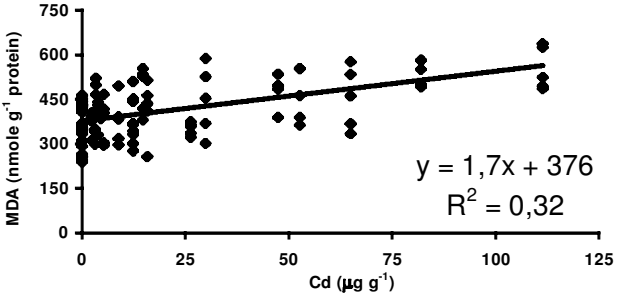

Figure 6. Relationship between MDA (nmol g^{-1} protein) and Cd concentrations (µg g^{-1} dry weight) in gills of *R. decussatus* exposed to Cd (4, 40 or 100 µg l^{-1}) for 28 days.

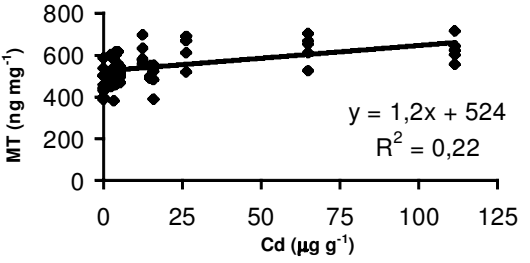

Figure 7. Relationship between MT (mg g^{-1} wet weight) and Cd concentrations (µg g^{-1} dry weight) in gills of *R. decussatus* exposed to Cd (4, 40 or 100 µg l^{-1}) for 28 days.

MT concentrations

No significant variation in MT concentrations was observed between the gills of clams exposed to 4 µg l^{-1} Cd (512 ± 59.8 ng mg^{-1} wet weight) and unexposed gills (504 ± 60.2 ng mg^{-1} wet weight) over the course of the experiment. However, after 7 days of Cd exposure, a significant induction of MT synthesis in the gills of clams exposed to the other two concentrations (40 and 100 µg l^{-1}) was observed until the end of the experiment ($p < 0.05$). In Cd-exposed clams, MT levels in the gills ([MT] in ng mg^{-1} wet weight) increased linearly with the increase in Cd concentration ([Cd]) and with the time of exposure (data not shown): $[MT] = 1.2 \times [Cd] + 524$, $r^2 = 0.22$ (figure 7).

Discussion

Laboratory studies are a useful tool to evaluate the impact of xenobiotic compounds on antioxidant enzyme systems and to explain the intervention and relationship of the different antioxidant enzyme mechanisms as a function of metal exposure. Our work aimed to study how the response of antioxidant parameters could be affected by Cd exposure (4, 40 and 100 µg l^{-1}) in the gills of the clam *R. decussatus,* to explain the tissue damage and cytotoxic effects of Cd, and to evaluate the potential use of antioxidant parameters as biomarkers of response to Cd.

Cd accumulated in the gills of the clams and the accumulation increased with the time of exposure and concentration in the water (figure 1). The gills of the clam *R. decussatus* are in direct contact with the water, and are very sensitive to oxidative

stress, at least during the first 7 days of Cd exposure (figures 1–5). Several mechanisms are able to remove ROS, including SOD, catalase and glutathione peroxidases. Changes in the antioxidant enzyme activity of SOD, catalase and glutathione peroxidases were observed in our study during this period. Similarly, changes in antioxidant enzyme activities in molluscs in response to Cd (Regoli and Principato 1995; Doyotte *et al.* 1997) or organic contaminations with polycyclic aromatic hydrocarbons (PAHs) (Livingstone *et al.* 1990; Akcha *et al.* 2000) or polychlorinated biphenyls (PCBs) (Ribera *et al.* 1991; Krishnakumar *et al.* 1997) have been highlighted in other laboratory and field studies. Antioxidant enzyme activities (glutathione reductase, glyoxalases, glutathione transferases, glutathione peroxidases, catalase, SOD) analysed in the gills and digestive gland of the mussel *Mytilus galloprovincialis* from a clean site and a site contaminated with several metals (As, Se, Mn, Fe, Pb) were altered by metal contamination (Regoli and Principato 1995). Similarly, variations were also observed in antioxidant enzyme activities (glutathione peroxidases, glutathione reductase, catalase, SOD) in the gills and digestive gland of the freshwater bivalve *Unio tumidus* collected from different contaminated sites (close to a cokery) (Cossu *et al.* 1997, 2000). However, the authors were unable to relate the depletion of the antioxidant systems to any of the metals (As, Cr, Cu, Cd, Pb, Hg, Ni, Zn, Fe) or organochlorinated compounds (PAHs, organochlorine pesticides, PCBs) present in the environment (Cossu *et al.* 2000).

The subcellular distribution of SOD observed in the present study supports the fact that most of SOD is present in the form of Cu/Zn-SOD. Cytochemical studies have shown that Cu/Zn-SOD is mainly a cytosolic protein, although it was also detected in additional subcellular sites (peroxisomes, nuclei), whereas Mn-SOD was restricted to the mitochondria (Orbea *et al.* 2000). Similar results were obtained for *M. edulis* (Livingstone *et al.* 1992).

Like SOD, catalase was also localized in the cytosolic fraction. The proportion of cytosolic catalase observed for gills from the clam *R. decussatus* is higher than those for the mussel *M. edulis* (Livingstone *et al.* 1992) and for *U. tumidus* (Cossu *et al.* 1997). Generally, catalase is localized in peroxisomes (insoluble fraction), removing H_2O_2 generated by SOD (Livingstone *et al.* 1992, Orbea *et al.* 2000). The use of frozen rather than fresh tissue resulted in an increase in cytosolic activity to 72.3% of the total in the digestive gland of the mussel *M. edulis* (Livingstone *et al.*, 1992).

The results of the present study showed an increase in cytosolic SOD activity from the first to the seventh day of Cd exposure, along with a decrease in cytosolic and mitochondrial catalase activity between the beginning of the experiment (cytosolic catalase) and the third day (mitochondrial catalase). Cd has a similar ionic radius to that of calcium, and the interference of Cd with calcium homeostasis is well documented and may play an important role in Cd toxicity (Yang *et al.* 2000). The interaction of Cd with calcium is especially important with regard to antioxidant enzyme activities. Cd can replace calcium in the conversion of xanthine dehydrogenase to xanthine oxidase by calcain, a calcium-dependent protease (Stark *et al.* 1989). Xanthine oxidase then catalyses the oxidation of xanthine, producing $O_2^{\cdot -}$. The increase in Cd accumulated in the tissues stimulates this reaction, and the increase in $O_2^{\cdot -}$ along with an induction of SOD from the first day results in the transformation of the superoxide anion radical to hydrogen peroxide. The production of superoxide anion activates SOD, which

produces H_2O_2, which then activates catalase. The stimulation of catalase activity begins on the first day of Cd exposure for cytosolic catalase and on the third day for mitochondrial catalase. During the first days of Cd exposure, catalase activity (cytosolic and mitochondrial) decreased due to the presence of anion superoxide peroxide in the cells of the clam gills. Kono and Fridovich (1982) previously reported catalase inhibition by $O_2^{\cdot -}$ generated by the aerobic xanthine oxidase reaction in *Lactobacillus*. A decrease in catalase activity was also observed in the cytosolic fraction of the kidney of the sea bass *Dicentrarchus labrax* exposed to increasing Cd concentrations (Romeo *et al*. 2000). One of the most striking effects induced by Cd in the gills of *R. decussatus* was the decrease in glutathione peroxidase activity in the first few days of Cd exposure, with the levelling off of these enzymes after that period. Reactions involving glutathione peroxidase use reduced glutathione as a cofactor. However, Cd has a strong affinity for reduced glutathione. The reactions of Cd with glutathione result in the formation of Cd complexes (Regoli and Principato 1995). Metals have been shown to cause glutathione depletion in the gills and digestive gland of the mussel *M. galloprovincialis* from a multi-metal (Mn, Pb, As, Zn) polluted site (Regoli 1998). Reduced glutathione is used by glutathione peroxidase to reduce both hydrogen peroxide and organic hydroperoxides. Thus the depletion of cellular glutathione leads to a decrease in glutathione peroxidase activity. On the other hand, after 7 days of Cd exposure, MT synthesis was induced in the cytosol of the gills of clams exposed to the two higher Cd concentrations (40 and 100 $\mu g\,l^{-1}$). Although the function of MT in metal metabolism is not fully understood, it has been suggested that this protein acts as a detoxification mechanism for an excess of metals, particularly Cd. Cd has been shown to induce MT synthesis in *R. decussatus* (Bebianno and Serafim 1998) as well as in several other molluscs such as mussels (Bebianno and Langston 1991) and oysters (Roesijadi and Klerks 1989, Geret *et al*. 2002). However, in the gills of the oyster *Crassostrea virginica*, the induction of MT synthesis only started after 4 days of Cd exposure (200 $\mu g\,l^{-1}$) (Roesijadi and Klerk 1989). Moreover, MT acts as a component of the antioxidant system in protecting cells against the cytotoxic effects of ROS (Viarengo *et al*. 2000). The activation of MT gene expression by oxidative stress is mediated, in part, by an increase in free zinc ion in the cell (Andrews 2000). The free zinc ion acts as a second messenger to activate the DNA-binding activity of metal-responsive transcription factor 1. In mammals, the presence of H_2O_2 rapidly induced MT-I mRNA in a dose-dependent manner in the cells of the mouse (Andrews 2000). In the gills of *R. decussatus* we observed the setting up of antioxidant elements in cascade. After some days of exposure, the antioxidant system is functional and MTs capture free Cd ions present in the cells.

The MDA results demonstrated that exposure to sublethal Cd concentrations (40 and 100 $\mu g\,l^{-1}$) stimulates lipid peroxidation in the gills of *R. decussatus* after 21 days of exposure. An increase in lipid peroxidation was also observed in *in vitro* studies with the giant fresh water prawn *Macrobrachium rasenbergii* after exposure of the crude homogenate to 500 μM $CdCl_2$ for 30 min (Dandapat *et al*. 1999). An increase in basal peroxidation was also observed when the supernatant of gills of the clam *R. decussatus* was incubated *in vitro* with 500 $\mu g\,l^{-1}$ Cd for 20 min (Romeo and Gnassia-Barelli 1997). However, no modification of the levels of lipid peroxidation in the mussels *M. galloprovincialis* and *Perna viridis* was observed

after short-term Cd exposure (1–7 days with 40 $\mu g\,l^{-1}$ and 500 $\mu g\,l^{-1}$ Cd, respectively) (Viarengo *et al.* 1990, Arasu and Reddy 1995).

Cd does not induce the production of ROS through a Fenton-like redox cycling mechanism. However, it does increase the production of other ROS (superoxide anions and nitric oxide) in cultured macrophage cells of rats treated with 0.4 or 0.6 µM of Cd (Stohs *et al.* 2000) and nitric oxide is known to induce membrane lipid peroxidation (Radi *et al.* 1991). However, nitric oxide is produced by nitric oxyde synthase, the two genes for which (*nNOS* and *eNOS*) are constitutively expressed and their activity is mainly regulated by changes in free intracellular Ca^{2+} concentrations leading to a low-output production of nitric oxide (Marletta 1993). Cd can replace calcium and stimulate the production of nitric oxide, increasing the lipid peroxidation of cell membranes.

The work presented here demonstrates the kinetic effect of Cd concentrations on antioxidant enzyme activities, lipid peroxidation and MT concentrations, and the relationship between different elements of the antioxidant system and calcium, which is a major constituent of the cell. Antioxidant enzymes such as the glutathione peroxidases may be a promising biomarker, especially as the response of these enzymes is aspecific and can vary with several contaminants in aquatic ecosystems (Doyotte *et al.* 1997). Therefore, glutathione peroxidases together with MDA and MT concentrations might be used as biomarkers of the effects of Cd in the gills of *R. decussatus*.

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