

Cytochrome *c* oxydase subunit I gene is up-regulated by cadmium in freshwater and marine bivalves

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Abstract

Inhibition of the mitochondrial electron transfer chain and induction of reactive oxygen species (ROS) production are one of the roots of cadmium (Cd) toxicity. To appreciate the impact of Cd on mitochondria, we focused on the expression of *CoxI* gene which encodes the subunit I of the Cytochrome *c* oxidase (complex IV of the respiratory chain). *CoxI* gene expression was studied by real-time quantitative PCR in three species: two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) and one marine bivalve (diploid or triploid *Crassostrea gigas*). Bivalves were exposed for 10 or 14 days to 0.13 μM Cd^{2+} and 15.3 μM Zn^{2+} in controlled laboratory conditions. We demonstrate that in the three mollusk species *CoxI* gene was up-regulated by Cd. Zinc (Zn), which is known to have antioxidant properties, had no effect on *CoxI* gene expression. In the presence of Cd and Zn, *CoxI* gene inducibility was lower than after a single Cd exposure, in each species; result that could not be fully explained by a decreased Cd accumulation. *CoxI* gene induction by Cd was 4.8-fold higher in triploid oysters than in diploid ones, indicating a possible influence of triploidy on animal responses to Cd contamination.

Introduction

Heavy metals, depending on their oxidation states, can be highly reactive and hence toxic to most organisms. Metals such as copper (Cu) and zinc (Zn) are essential for life, whereas others, such as mercury (Hg) and cadmium (Cd), are not known to perform any useful biochemical function. Cd in particular is very toxic and is considered a serious occupational and environmental health threat since it is a metal with many industrial uses which is continuously introduced into the atmosphere, into aquatic and terrestrial environments. Toxicological responses of Cd exposure include kidney damage, respiratory diseases, neurological disorders and several kinds of cancer (Waalkes *et al.* 1992).

To counter Cd toxicity, living organisms use several metal resistance mechanisms. Cells respond by increasing the expression of genes encoding a variety of proteins (Carginali *et al.* 2002): toxic ions can be segregated by thiol-containing molecules like metallothioneins (Roesijadi *et al.* 1996) or pumped out of the cell via a membrane protein (Li *et al.* 1997; Achard-Joris *et al.* 2005). A variety of mechanisms have been attributed to Cd toxicity. It interferes with the intracellular signaling network and gene regulation at multiple levels (Beyersmann & Hechtenberg 1997) and may be associated with the production of reactive oxygen species (ROS, molecules and free radicals such as superoxide anion and hydrogen peroxide derived from molecular oxygen) (Szuster-Ciesielska *et al.* 2000). Under normal conditions the production

and destruction of ROS is well regulated in cell metabolism, but under environmental oxidative stress the balance between prooxidative and anti-oxidative reactions is shifted in favor of the former.

As mitochondria are a major source of superoxide and hydrogen peroxide production in cells (Turrens 1997) several studies have focused on the effect of Cd on mitochondrial metabolism: Cd has been shown to inhibit the mitochondrial electron transfer chain and to induce ROS production (Wang *et al.* 2004). Moreover, Cd-promoted oxidative stress leads to DNA strand damage – especially mitochondrial ones (Yakes & Van Houten 1997) – and to apoptotic cell death (Bagchi *et al.* 2000). This suggests that enhanced ROS production may be at the root of Cd toxicity. Two more ways in which cells might avoid Cd toxicity can then be proposed: hypertrophied antioxidant defenses or increased consumption of molecular oxygen. As Cd can induce alterations in the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase after only a few hours exposure (Company *et al.* 2004; Ikediobi *et al.* 2004), we focused on the second way and especially on Complex IV (Cytochrome *c* oxidase (COX)) of the mitochondrial respiratory chain. This transfers electrons from reduced Cytochrome *c* to molecular oxygen, and contributes to the production of an electrochemical proton gradient across the mitochondrial inner membrane that drives the synthesis of ATP (Capaldi *et al.* 1983; Capaldi 1990). Complex IV is particularly interesting because it has been shown that oxygen-tolerant strain of HeLa cells had twice the Cytochrome *c* oxidase activity of the wild-type ones, and inhibition of Cytochrome *c* oxidase enhanced ROS production (Campian *et al.* 2004). Assuming that cell damage does arise from exaggerated mitochondrial ROS production, the site of electron leak from the electron transport chain becomes an important question.

To study the impact of Cd toxicity on mitochondria we looked at *Cox* subunit I mitochondrial gene (*CoxI*) expression. An advantage of using COX to study the impact of Cd on mitochondrial metabolism is that *CoxI* gene sequence is highly conserved between lower and higher eukaryotes (Capaldi 1990). Thus a similar *CoxI* gene regulation between species can be expected.

Many aquatic organisms are of potential interest as ecologically sensitive targets of metallic pollution. Among them, bivalve mollusks are regularly used as bioindicators of water metal pollution because of their high bioaccumulation capacities for heavy metals (Inza *et al.* 1997) and because they are well represented in aquatic ecosystems. In this study, three aquatic species were chosen: two freshwater bivalves (the Asiatic clam *Corbicula fluminea* and the zebra mussel *Dreissena polymorpha*) and one seawater bivalve (the Pacific oyster *Crassostrea gigas*). Triploid *C. gigas* oysters developed to enhance the production and selling period of commercial bivalves, were also used to investigate whether a third set of chromosomes could influence *CoxI* gene expression after metal contamination. *CoxI* gene expression level was studied in gill tissues because gills are the organ at the interface with the polluted environment and are thus the main tissues from which the metal loading in the organism will proceed (Marigomez *et al.* 2002). Cd concentration was set to 0.13 μM which is close to that found in a Cd contaminated river used for field contaminations (Achard *et al.* 2004). Since Zn has antioxidant properties (Bray & Bettger 1990), bivalves were also contaminated with Zn and a mix of Cd and Zn.

Materials and methods

Animals

C. fluminea (2 ± 0.2 cm shell length) were collected from a reference site in Cazaux-Sanguinet freshwater lake (Aquitaine region, south-west France). *D. polymorpha* (2.7 ± 0.2 cm shell length) were collected in the Garonne river, close to Agen (Aquitaine region, France).

Diploid and triploid *C. gigas* oysters (12 months old, 5.7 ± 0.1 cm shell length) were obtained from an oyster farmer in Arcachon Bay (Aquitaine region, France).

Metal contaminations

For freshwater bivalves, laboratory experiments were carried out using experimental units (EUs) based on glass tanks lined with plastic bags containing pure sand and dechlorinated tap water

permanently aerated by a diffuser connected to an air pump (Achard *et al.* 2004). Groups of 20 *C. fluminea* and 20 *D. polymorpha* were exposed for 10 days to ZnCl_2 ($15.3 \mu\text{M Zn}^{2+}$), CdCl_2 ($0.13 \mu\text{M Cd}^{2+}$) and $\text{ZnCl}_2 + \text{CdCl}_2$ ($15.3 \mu\text{M Zn}^{2+}$ and $0.13 \mu\text{M Cd}^{2+}$). Metal concentrations were maintained constant during the whole experiment by daily additions of aqueous Cd and Zn solutions, adjusted according to the decrease in metal concentration in each EU (determined by atomic absorption spectrometry – VARIAN AA 400, Victoria, Australia). The bivalves were fed every two days with 3×10^5 green algae (*Scenedesmus subspicatus*) per ml culture.

Experiments on oysters were performed using EUs based on polyethylene tanks containing aerated ($281 \mu\text{M}$ of dissolved O_2) artificial seawater prepared by mixing Instant Ocean synthetic sea salt (Mentor, OH, USA; $25 \pm 0.5\%$) with dechlorinated tap water. Groups of 36 *C. gigas* diploid and 36 *C. gigas* triploid were exposed for 14 days to ZnCl_2 ($15.3 \mu\text{M Zn}^{2+}$), CdCl_2 ($0.13 \mu\text{M Cd}^{2+}$) and $\text{ZnCl}_2 + \text{CdCl}_2$ ($15.3 \mu\text{M Zn}^{2+}$ and $0.13 \mu\text{M Cd}^{2+}$). The contaminated water was changed daily to maintain Zn and/ or Cd concentrations constant. Oysters were contaminated 4 days longer than freshwater bivalves in order to obtain an equivalent Cd concentration in tissues. Indeed, for a given metal concentration, levels of free metal ions are much less in seawater than in tap water due to the presence of metal chloride species which are less available (Campbell 1995). Oysters were fed every two days with 0.05 g green algae (*Isochrysis galbana*) per oyster.

All the experiments were performed at $20 \pm 0.3^\circ\text{C}$, $\text{pH } 8.5 \pm 0.3$ and with 12 h light per day according to a protocol already described (Achard *et al.* 2004). Cadmium chloride (CdCl_2) and zinc chloride (ZnCl_2) were purchased from Merck.

Cadmium and zinc determinations

The whole soft body of one bivalve was taken from each of the three replicates for each condition, then dissected, dried on absorbent paper and weighed (fresh weight, f.w.). Tissues and water samples were then analyzed for metal bioaccumulation by atomic absorption spectrophotometry as previously described (Achard *et al.* 2004).

Inter-individual variability for each experimental condition was defined by mean $\pm$ standard error (SE). Significant differences between metal concentrations in the whole soft body were determined using the nonparametric Mann–Whitney *U*-test ($P < 0.05$).

cDNA fragment production and SYBR green real-time PCR

Total RNA were extracted from gills of bivalves (two animals per reaction) using the Absolutely RNA RT-PCR Miniprep kit (Stratagene) according to the manufacturer's instructions. Three microgram of RNAs from laboratory bivalves were retrotranscribed in $50 \mu\text{l}$ reaction using the ProStar First-Strand RT-PCR kit (Stratagene).

Primers COXI5 5'-GGAATACCACGACG GTACTCT-3' and COXI3 5'-AGGGCAGCC GTGTAAT-3' were used on cDNA prepared from *D. polymorpha* and *C. gigas* RNA and primers CFCOXI5 5'-GATGGGTGGTTTTGGAAATT GAC-3' and CFCOXI3 5'-ACTGACGGGCCA GAATG-3' on cDNA prepared from *C. fluminea* RNA. *CoxI* gene sequence from these three species were available in GenBank (*D. polymorpha*: AF120663; *C. gigas*: AF280608; *C. fluminea*: AF120666) and primers were designed according to the LightCycler probe design software (version 1.0, Roche). Normalization was carried out detecting cDNA 28S with the following primers: 5'-CCA AGG AGT CTA ACA TGT GC-3' and 5'-GAT GGT TCG ATT AGT CTT TC-3'. Quantitative real-time PCR assay was carried out in $20 \mu\text{l}$ PCR mixture volume consisting of $1 \mu\text{l}$ LightCycler FastStart reaction mix SYBR Green I (containing FastStart Taq DNA polymerase, $0.3 \mu\text{M}$ of each oligonucleotide; Roche), 5 mM MgCl_2 , $0.3 \mu\text{M}$ of each primer and $3 \mu\text{l}$ of cDNA. Gene amplification was carried out as follows: initial activation of HotStart Taq DNA Polymerase at 95°C for 10 min; 50 cycles in three steps: 95°C for 5 s, 60°C for 5 s, 72°C for 20 s. At the end of amplification cycles, melting temperature analysis was carried out by slow increase in temperature ($0.05^\circ\text{C s}^{-1}$) from 65°C up to 95°C . Amplification, data acquisition and analysis were carried out with a LightCycler instrument (Roche, Mannheim, Germany) using LightCycler 5.3.2 software (Roche). Three replicates were performed, meaning three

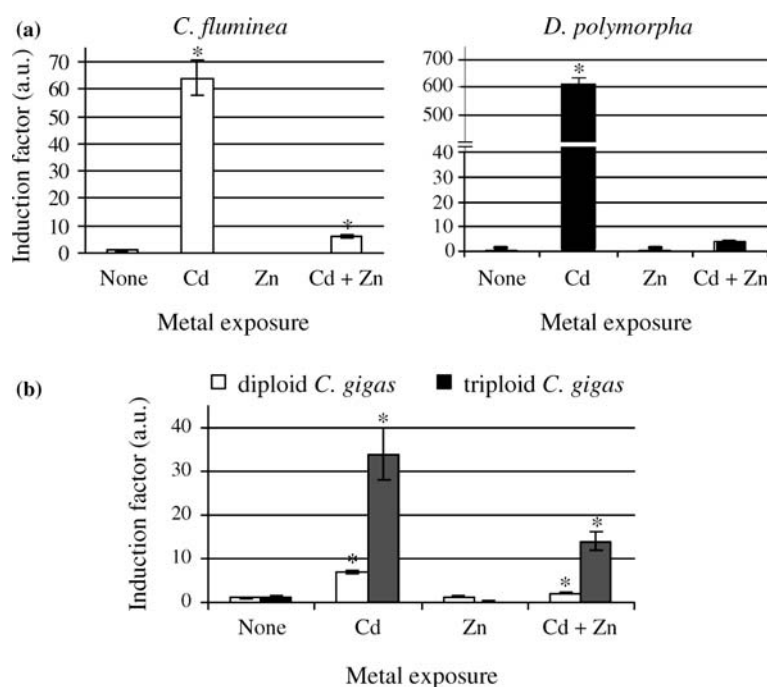


Figure 1. (a) Expression levels ($\pm$ SE) after normalization for *CoxI* transcript in gills of *C. fluminea* and *D. polymorpha* exposed for 10 days to cadmium ($0.13 \mu\text{M Cd}^{2+}$), zinc ($15.3 \mu\text{M Zn}^{2+}$), or cadmium and zinc ($0.13 \mu\text{M Cd}^{2+}$ and $15.3 \mu\text{M Zn}^{2+}$). (b) Expression levels ($\pm$ SE) after normalization for *CoxI* transcript in gills of diploid and triploid *C. gigas* exposed for 14 days to cadmium, zinc, or both metals at the same concentrations as in a. Expression ratio between *CoxI* and 28S genes in control bivalves was set to one. a.u. stands for arbitrary units. Measurements were done in triplicate. *Statistically different compared to control ($P < 0.05$).

independent RNA extractions and retrotranscriptions for each contamination condition. Relative quantifications of *CoxI* gene expression levels were normalized according to the 28S rRNA gene expression. Expression ratio between *CoxI* and 28S genes in control bivalves was set to one. Inter-individual variability for each experimental condition was defined by mean $\pm$ SE ($n = 3$). Significant differences between *CoxI* gene expression levels in the gills were determined using the nonparametric Kruskal–Wallis method ($P < 0.05$) for more than two samples comparisons. If the test was rejected, we performed the Mann–Whitney *U*-test ($P < 0.05$).

Results

Bivalves were exposed to Cd, Zn, or both metals in controlled conditions. After Cd exposure, no mortality was observed and bivalves accumulated Cd well. The Cd accumulation factor (defined as Cd concentration in Cd-contaminated bivalve versus Cd concentration in control bivalve) was 79

in *C. fluminea*, 56 in *D. polymorpha*, 15 in diploid *C. gigas*, and 16 in triploid *C. gigas*. Cd concentration in the whole soft body of bivalves was similar in each species (around $48 \text{ nmol Cd g}^{-1} \text{ fw.}$) (Table 1). After Cd and Zn exposure, a difference in Cd accumulation was observed only in *D. polymorpha*: Cd concentration was 2.4 times lower in mussels exposed to Cd and Zn than in the one exposed to Cd (Table 1); the 2.2 times lower Cd concentration observed in *C. fluminea* was not statistically significant.

Zn concentration in the whole soft body of control bivalves was different for each species. Indeed, in *C. gigas*, it was 13 and 53 times higher than in *C. fluminea* and *D. polymorpha*, respectively (Table 2). Although Zn basal levels were different for each species, similar Zn accumulation factors (defined as Zn concentration in Zn-contaminated bivalve versus Zn concentration in control bivalve) were observed between diploid *C. gigas*, triploid *C. gigas*, and *C. fluminea* after Zn exposure. Zn accumulation factor in *D. polymorpha* was calculated to be 18 and was six times higher than in the other bivalves (Table 2).

Table 1. Cadmium concentrations (measured in nanomol per gram fresh weight $\pm$ SE) in the whole soft body of bivalves after exposure to Cd, Zn, or Cd and Zn.

Species	Metal exposure, nmol Cd g ⁻¹ (fw)			
	None	Cd	Zn	Cd + Zn
<i>C. fluminea</i> ^a	0.6 $\pm$ 0.1	47.3 $\pm$ 23.1 ^c	0.9 $\pm$ 0.04	20.9 $\pm$ 10.4 ^c
<i>D. polymorpha</i> ^a	1 $\pm$ 0.2	55.5 $\pm$ 7.4 ^c	1.1 $\pm$ 0.2	23.6 $\pm$ 5.2 ^c
<i>C. gigas</i> diploid ^b	3.4 $\pm$ 0.2	52.4 $\pm$ 9 ^c	3.2 $\pm$ 0.4	40.7 $\pm$ 3.8 ^c
<i>C. gigas</i> triploid ^b	2.6 $\pm$ 0.3	41.4 $\pm$ 7.5 ^c	2.4 $\pm$ 0.1	61.2 $\pm$ 5.2 ^c

^a*C. fluminea* and *D. polymorpha* were contaminated for 10 days. ^bDiploid or triploid *C. gigas* were contaminated for 14 days.

^cSignificantly different to control ($P < 0.05$). Cadmium determination was done in triplicate for each condition.

Table 2. Zinc concentrations (measured in nanomol per gram fresh weight $\pm$ SE) in the whole soft body of bivalves after exposure to Cd, Zn, or Cd and Zn.

Species	Metal exposure, nmol Zn g ⁻¹ (fw)			
	None	Cd	Zn	Cd + Zn
<i>C. fluminea</i> ^a	309 $\pm$ 34	323 $\pm$ 16	1067 $\pm$ 158 ^c	696 $\pm$ 195 ^c
<i>D. polymorpha</i> ^a	72 $\pm$ 20	98 $\pm$ 69	1326 $\pm$ 595 ^c	1154 $\pm$ 249 ^c
<i>C. gigas</i> diploid ^b	4165 $\pm$ 310	3871 $\pm$ 368	13499 $\pm$ 171 ^c	15266 $\pm$ 955 ^c
<i>C. gigas</i> triploid ^b	3424 $\pm$ 1042	4015 $\pm$ 797	9266 $\pm$ 847 ^c	11006 $\pm$ 1540 ^c

^a*C. fluminea* and *D. polymorpha* were contaminated for 10 days. ^bDiploid or triploid *C. gigas* were contaminated for 14 days.

^cSignificantly different to control ($P < 0.05$). Zinc determination was done in triplicate for each condition.

In *C. fluminea*, *D. polymorpha*, and diploid *C. gigas*, there was a slight decrease in Zn accumulation factor after Cd and Zn exposure compared to that obtained after a single Zn exposure.

CoxI gene expression profiles of control and metal contaminated bivalves were then compared by quantitative real-time RT-PCR.

Effects of cadmium on *CoxI* gene expression

As shown in Figure 1, our experiments demonstrated for the first time that *CoxI* gene expression was up-regulated by Cd in the three different species. However, the induction factors were different between each mollusk: although freshwater bivalves accumulated the same amount of Cd (Table 1), *CoxI* induction was 9.5-fold higher in Cd-exposed *D. polymorpha* than in Cd-exposed *C. fluminea* (Figure 1a). *CoxI* expression level also differed between diploid and triploid *C. gigas*, whereas the Cd concentration in tissues was similar: a 4.8 ratio of triploid versus diploid *CoxI* gene expression was observed (Figure 1b).

Effects of cadmium on *CoxI* gene expression in presence of zinc

In each species, Zn alone did not significantly affect *CoxI* gene expression level; indeed induction factors in *C. fluminea*, *D. polymorpha*, diploid *C. gigas*, and triploid *C. gigas* were 0.1 $\pm$ 0.1, 1 $\pm$ 0.2, 1.3 $\pm$ 0.3, and 0.1 $\pm$ 0.1, respectively. When Cd and Zn were present in the water column, *CoxI* gene expression level was increased and induction factors in *C. fluminea*, *D. polymorpha*, diploid *C. gigas*, and triploid *C. gigas* were equal to 6 $\pm$ 0.6, 4 $\pm$ 0.2, 2 $\pm$ 0.2, and 14 $\pm$ 2, respectively (Figure 1). Nevertheless, gene induction was significantly lower than after a single Cd contamination; it had decreased by 10- and 200-fold in *C. fluminea* and *D. polymorpha*, respectively (Figure 1a). *CoxI* gene expression was still higher in triploid oysters than in diploid (sevenfold) but *CoxI* gene induction level underwent a 3.5- and 2.4-fold decrease in diploid and triploid *C. gigas* (respectively) as compared with single Cd contamination (Figure 1b).

Discussion

We designed this study to establish a possible relationship between Cd contamination and expression of the mitochondrial gene *CoxI*. COXI is the subunit one of the Cytochrome *c* oxydase which provides a critical function during respiration by transferring electrons from Cytochrome *c* to oxygen and contributing to ATP generation (Malatesta *et al.* 1995). Indeed it has been demonstrated that a reduced level of COX activity causes functional reduction in $\text{Na}^+ - \text{K}^+$ -ATPase capacity, an important factor responsible for neuronal death in the mammalian brain (Greenamyre *et al.* 1999), and that complex IV inhibitors cause a rapid and severe depletion of cellular ATP content, resulting in acute cell death (Zhang *et al.* 2001).

In the present study, the first result of our experiments showed that *CoxI* gene expression was up-regulated in different aquatic organisms after Cd exposure. Overexpression of *CoxI* has been demonstrated associated with a pyrethroid insecticide resistant strain of *Blatella germanica* (German cockroach) (Pridgeon & Liu 2003) and in *Danio rerio* (zebrafish) fed with diets contaminated by methyl mercury (Gonzalez *et al.* 2005), but to our knowledge, the direct relation between *CoxI* expression and Cd contamination has never been reported before. Although *CoxI* gene induction by Cd is clear, no direct link was established between Cd concentration in tissues and *CoxI* gene expression level. Indeed, *CoxI* gene expression level was 10 times higher in *D. polymorpha* than in *C. fluminea*, whereas the Cd accumulation factor was lower in *D. polymorpha* than in *C. fluminea*. The finding of 4.8-times higher up-regulation of *CoxI* gene expression in triploid than in diploid *C. gigas* cannot be explained in a simple way by the presence of an extra structural gene copy, as a quantitative effect alone would be expected to be in the order of 1.5, or even less if the background expression is higher in the triploid due to the extra gene copy. The expression level of a single gene in a polyploid organism as compared to the diploid one is hardly predictable. For instance, in cotton (*Gossypium hirsutum*) the alcohol dehydrogenase A (*adhA*) gene expression level in tetraploid leaves is two times higher than in diploid ones whereas it is two times lower in tetraploid roots than in diploid ones. And the *adhD* gene is 10 times more

expressed in the diploid leaves and roots than in tetraploid ones (Adams *et al.* 2003). This highlights the non-Mendelian mechanisms than often occurs for gene expressions in polyploid organisms.

As COX is considered as the rate-limiting step for mitochondrial respiration (Villani & Attardi 2000), *CoxI* gene overexpression could be a compensating mechanism to restore the decrease in mitochondrial activity and to efficiently consume O_2 , thus limiting Cd induced damage in the cell. Some of this damage is a consequence of increased ROS production. Indeed, Cd stimulates ROS formation in mitochondria because it blocks electron transport, especially electron transfer through the complex III which is considered as the principal site for ROS generation (Chen *et al.* 2003; Wang *et al.* 2004). Prevention of the slowing electron flow can thus be considered as a mechanism to avoid ROS production. For instance, an increased activity of complex IV, causing the depletion of electron rich intermediates, could prevent Cd-promoted oxidative stress. Moreover, Cytochrome *c* oxydase activity has been shown to be higher in oxygen-tolerant HeLa cells than in the control cells (Campian *et al.* 2004).

Another result of this study showed that *CoxI* gene inducibility was lowered when bivalves were contaminated with Cd and Zn compared with single Cd contamination. Even for *D. polymorpha*, this reduction of *CoxI* gene expression level could not be explained only by a decrease in intracellular Cd accumulation. Indeed, the ratio of *CoxI* gene induction levels after Cd exposure versus after Zn + Cd exposure was 200 in *D. polymorpha* and 10 in *C. fluminea*, whereas the decrease in Cd accumulation was similar for the two species.

Whereas Cd induced ROS production, Zn was shown to have antioxidant properties. For instance, it protected cultured skin cells against the cytotoxic effects of UV irradiation and decreased the production of ROS (Parat *et al.* 1997). Moreover, Zn added to a cell culture medium inhibited Cd-induced ROS production (Szuster-Ciesielska *et al.* 2000). These findings suggest that at least one of the mechanisms involved in the protective role of Zn against Cd-induced toxicity is connected with inhibition of Cd-induced ROS formation in cells. In our study, the decreased *CoxI* gene expression level in bivalves contaminated with Cd and Zn compared to those contaminated only with

Cd could be explained by a lower toxic effect of Cd. In any case, *CoxI* gene expression level was reduced in the presence of Zn but remained at least two times higher than in control animals (Figure 1). Thus, *CoxI* gene expression level might constitute a new tool, in addition to metallothioneins and heat shock proteins levels, for detecting Cd contamination of both freshwater and marine sentinel organisms or for detecting Cd pollution from the river to the sea.

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