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# Transcriptional response of stress-regulated genes to cadmium exposure in the cockle *Cerastoderma glaucum* from the gulf of Gabès area (Tunisia)

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**Abstract** This study investigates cadmium effects on key messenger RNA (mRNA) expression (MT, MnSOD, CuZnSOD, CAT, ABCB1, HSP70, and CO1) by qPCR in the cockle *Cerastoderma glaucum* after chronic exposure to two high but environmentally relevant concentrations of $CdCl_2$ (50 μg/L and 5 mg/L) for 12 h to 18 days. Cd accumulation measured in cockles' tissues is significantly higher in both treatment conditions compared to controls and in a dose-dependent manner. Stress on stress tests performed at different times of the experiment clearly demonstrated that exposure to both concentrations of Cd significantly affects cockle survival time in air. Important changes in gene transcription were also highlighted. In particular, MT, HSP70, CAT, and CuZnSOD seem to be relevant biomarkers of Cd exposure because (1) their mRNA levels increase upon exposure and (2) they are highly correlated to Cd accumulation in tissues. Results may be useful for control strategies and for the use of cockles as sentinel organisms.

**Keywords** Cockle · Cadmium · Stress response · Gene transcription · qPCR · Detoxication · Oxidative stress

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## Introduction

Cadmium is released in the aquatic environment from both anthropogenic and natural sources (Choi et al. 2007). It is one of the most toxic metals principally obtained as a by-product in zinc refining (Pinot et al. 2000) and found in phosphate fertilizers (Cupit et al. 2002). Cadmium can enter the aquatic food chain through direct consumption of water or biota and has the potential to accumulate at each level of the food chain so that human may amass large quantity of Cd through diet (Zhu et al. 2012). Therefore, Cd is considered as a serious environmental health threat. Major programs of freshwater safeguard have studied the deleterious impacts of Cd at the ecosystem, community, or population levels of organization (Brooks et al. 2004; Al Kaddissi et al. 2012).

Many aquatic organisms are of potential interest as ecologically sensitive targets of metallic pollution. Among them, bivalves 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. Responses to Cd exposure have been largely investigated in different bivalve species such as cockles (Ladhar-Chaabouni et al. 2008; Paul-Pont et al. 2012), zebra mussels (Navarro et al. 2011), oysters (Ivanina et al. 2008a, b; Sanni et al. 2008), bay scallops (Wang

et al. 2009), clams (Wang et al. 2010), and freshwater bivalves (Bigot et al. 2009). To highlight the response to Cd exposure, authors used different biomarkers at various biological organization levels, i.e., biochemical, transcriptional, proteomic, and histochemical. These studies showed that Cd exposure could inhibit the activity of some mitochondrial enzymes and usually induces expression of detoxification and other defense genes, but their regulation remains complex. In Tunisia, the Gulf of Gabès was shown to be contaminated by trace metals, especially Cd, which originated from the crude phosphate treatment from plants (Hamza-Chaffai et al. 2003; Smaoui-Damak et al. 2003). Cadmium concentrations in bivalves collected from some localities along this area exceeded the consumption safety level currently authorized by the World Health Organization (Smaoui-Damak et al. 2003; Machreki-Ajmi and Hamza-Chaffai 2006).

The cockle *Cerastoderma glaucum* is widely distributed from the Baltic to the Mediterranean Sea in a wide range of ecological conditions (Derbali et al. 2012) and has been validated as a bioindicator organism reflecting the pollution state of the coast. This sedentary and filter-feeding species lives in the superficial sediment and is known to be suitable for toxicology and risk assessment studies since it tends to accumulate large amounts of pollutants and especially metals such as Cd (Szefer et al. 1999; Cheggour et al. 2001; Machreki-Ajmi et al. 2008). Cockles are major preys for diverse animal groups such as crustaceans, fishes, and wading birds (Paul-Pont et al. 2010a), and they may also contribute to reduce the particulate organic load (Derbali et al. 2012). Beyond these ecological roles, they are also commercially important resources (Paul-Pont et al. 2010a). Some investigators have recently focused on the biology of *Cerastoderma glaucum* and the responses to different biochemical, immunotoxicological, and endocrine disruptors were studied using biomarkers that have been employed for the analysis of pollution impacts in estuaries and toxicity risk assessment of specific contaminants (Matozzo et al. 2007; Machreki-Ajmi and Hamza-Chaffai 2008; Marin et al. 2008). The qRT-PCR analysis of gene transcription has become one of the most robust tools of molecular biology. Changes in gene expression are likely to play a critical role in both acclimation and adaptation to environmental changes (Schulte 2004). Key changes in gene expression are also thought to precede the manifestation of strong functional alterations, giving expression profiling a great potential for early detection of alterations (Steiner and Anderson 2000; Schulte 2001; Mirbahai and Chipman 2014).

The present work aims to unravel the modifications of key messenger RNA (mRNA) expression underlying physiological responses of the cockle *Cerastoderma glaucum* to Cd exposure. The mRNA transcripts used for this analysis encode proteins involved in diverse pathways such as metal and xenobiotic detoxification (metallothionein, MT; ATP-binding cassette xenobiotic transporter, ABCB1), protection against oxidative stress (superoxide dismutases, MnSOD and CuZnSOD; catalase, CAT), general stress (heat shock protein, HSP70), and mitochondrial alterations (cytochrome c oxidase 1, CO1). In this study, gills were chosen for gene transcription monitoring. This organ constitutes a key interface for the uptake of dissolved metal ions from water and therefore was considered as the main target organ in metal exposure studies (Marigomez et al. 2002; Paul-Pont et al. 2010b).

Two concentrations of cadmium were used during an experimentation of 18 days: the lowest $CdCl_2$ dose employed in this study (50 μg/L) is a high environmentally relevant concentration since it has been found in several contaminated sites (Blackmore 1998; Serafim and Bebianno 2007; Zhang et al. 2011; Zhao et al. 2014). The highest $CdCl_2$ dose employed (5 mg/L) is less encountered, but it might be found in very highly polluted sites and especially during accidental pollution (Henry et al. 1984; Belabed et al. 1994). Survival rates of *Cerastoderma glaucum* and stress on stress tests (survival to air exposure) were assessed. Cd bioaccumulation levels were examined in the same individuals, and relationships between gene transcription and Cd levels were further examined in an attempt to highlight an mRNA expression pattern in cockles exposed in vivo to Cd.

## Materials and methods

### Animals and treatments

Cockles (*Cerastoderma glaucum*) (28–32 mm) were collected from the "Luza" site (located 45 km in the north of Sfax, Tunisia; Fig. 1), which is considered as a reference site with a low pollution level (Barhoumi et al. 2009; Kessabi et al. 2010). After an acclimation period of 24 h, cockles were assigned to three polyethylene tanks (30×30×20 cm), each one containing a layer of autoclaved sediment collected from the Luza site and 6 L of aerated seawater. The first tank contained seawater without $CdCl_2$ (control), the second tank contained seawater with 50 μg/L of $CdCl_2$ (low concentration), and the third tank contained seawater with 5 mg/L of $CdCl_2$ (high concentration). Eighty to 90 cockles were held in each tank (experimental design schematized in Fig. 1), and exposure time varied between 12 h and 18 days (Fig. 1). During the experiment, seawater and $CdCl_2$ were renewed twice a week.

### Verification of metal content in the sediment used for the experimentation

A lyophilized sediment (0.5 g) from the Luza site was placed in glass tubes previously washed with hydrochloric acid (10 %) and then mineralized using 5 mL of nitric acid

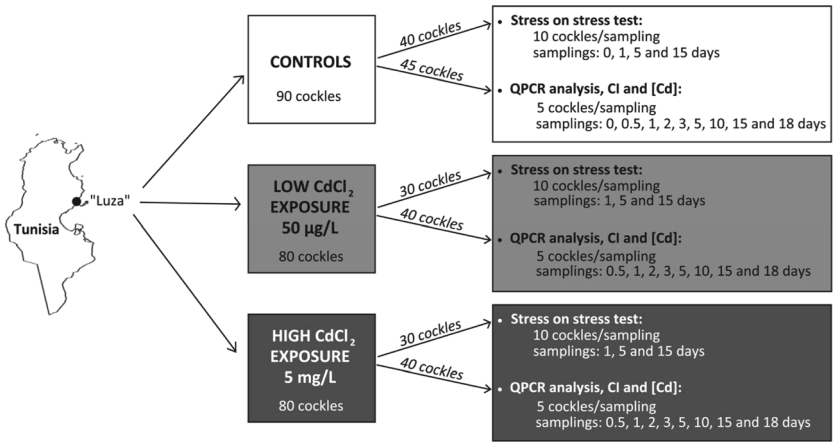


**Fig. 1** Sampling and experimental exposure of cockles

($HNO_3$) and 3 mL of hydrochloric acid (HCl). Ten milliliters of hydrochloric acid is then added to the dry residue. Determination of trace concentrations of Cd, Cu, Zn, Ni, Cr, Mn, and Pb was performed using inductively coupled plasma atomic emission spectrophotometry (ICP AES, Thermo Scientific iCAP 6000 series) (Geffard et al. 2001).

### Stress on stress test

Ten animals of each condition (controls and $CdCl_2$ contaminations) were sampled after 0, 1, 5, and 15 days of exposure and submitted to anoxia by air exposure at 15 °C (Fig. 1). Survival was assessed daily according to the method of Viarengo et al. (1995). Death symptoms were considered to be open valves and absence of muscular activity. Lethal time corresponding to 50 % of dead animals ($LT_{50}$) was measured.

### Condition index of cockles

Condition index (CI) was calculated individually on five cockles at each sampling time (Fig. 1). Before dissection, all cockles were weighted (total and soft weight), and the CI was expressed as a percentage of the ratio of fresh weight of soft tissues to total weight (Lobel et al. 1991).

### Determination of cadmium content in cockles

Measurement of Cd concentration in cockles was carried out as previously described (Geffard et al. 2010) by electrothermic atomic absorption spectrophotometry with Zeeman correction, using a graphite furnace (SpectrAA Zeeman 220). The measure was done individually on five cockles at each sampling time (Fig. 1) on the remaining animal tissues including digestive gland, muscle, mantle, and foot.

### RNA extraction and cDNA synthesis

Gene transcription analyses were carried out individually on the same five individuals for which Cd concentration and CI were measured and at each sampling time (Fig. 1). Gills of each sample were dissected, conserved in RNA later (Sigma-Aldrich), and stored at −20 °C. Total RNA was isolated from 100 mg of gills using Tri-Reagent (Invitrogen). RNA concentration and quality were determined using the Experion system (Bio-Rad). Three micrograms of total RNA was added to dTRace primer (5′-GACCACGCGTATCGATGTCGACTT TTTTTTTTTTTTTTT-3′) and nuclease free water in a final volume of 12.25 µL. A denaturation at 70 °C was carried out for 5 min and the mixture kept on ice. First-strand cDNAs were then synthetized using dTRace, RNAsin (Promega), and MMLV reverse transcriptase (Promega) in a final volume of 25 µL for 90 min at 42 °C.

### Real-time quantitative PCR

Real-time PCR reactions were performed using a StepOnePlus apparatus (Applied Biosystems) with Fast SYBR Green Master Mix (Applied Biosystems) and specific primers (Table S1 and S2 in Supplementary data) designed on the basis of previously characterized sequences as described in details in Supplementary Data. The best housekeeping gene was selected among four genes tested using the Bestkeeper Software (Pfaffl et al. 2004), and the relative expression was determined by the comparative Ct method (Livak and Schmittgen 2001) using control cockles at time 0 ($T_0$) as calibrator (cf Supplementary Data).

### Statistical analyses

All statistical analyses were performed using the R statistical Software version 2.14.1. In the stress on stress experiment, influence of Cd concentration and exposure time on ability to

survive under anoxia was analyzed using the SURVREG function (survival package, R; Lumley and Therneau 2003). To test for significant differences, *t* tests were used for bioaccumulation values and Kolmogorov-Smirnov tests for gene transcription values. After normalization of the data by $\log(x+1)$ transformation and stabilization of their variances by square-root transformation, influences of Cd treatment and exposure time on gene transcription, Cd bioaccumulation, and CI were assessed by independent ANOVA significance tests. Principal component analyses (PCAs) were performed and correlations were tested statistically using Pearson's method to highlight correlations between gene transcription, Cd bioaccumulation, and CI.

## Results

Heavy metal content analyzed from the sediment used for the experimentation showed that all the concentrations (Cd 0.109, Cu 0.941, Zn 7.082, Ni 0.727, Cr 2.717, Mn 6.301, Pb 1.654 μg/g of dry weight) were considerably below the maximum levels authorized by the Tunisian and French norms (T.N 106.002 and French Ministerial Decree of 22 February 1998).

### Survival, stress on stress response, and CI

Survival rates of *Cerastoderma glaucum* were assessed for each exposure time, and the Cd-induced mortality remain below 10 % for each concentration and time point. Survival under anoxia (i.e., stress on stress assay) is significantly affected by Cd exposure (Fig. 2). Indeed, a significant decrease of lethal time ($LT_{50}$) values is observed with the increase of Cd concentration ($p<0.001$; SURVREG function, R) and exposure time ($p<0.001$; Fig. 2). $LT_{50}$ of controls reaches 8 days while decreasing from 8 days (after 1 day of exposure) to 2 days (after 15 days of exposure) for the concentration of 50 μg/L and from 6 days (at day 1) to 1 day (at day 15) for the concentration of 5 mg/L.

Neither low nor high exposure to $CdCl_2$ affected the condition index (CI) of *Cerastoderma glaucum* after 18 days. No significant difference was observed comparing the mean CI for control (9.14±2.42) and exposed groups (50 μg/L, 7.39±1.75; 5 mg/L, 8.39±1.98).

### Cd bioaccumulation in cockles' tissues

Cadmium concentration remains low and relatively constant in tissues of control cockles (Fig. 3). Following exposure to 50 μg/L of $CdCl_2$, the tissue cadmium concentration was significantly increased to about 0.5 μg/g and displayed no significant variation in time ($p>0.05$). On the contrary, the treatment with 5 mg/L of $CdCl_2$ resulted in a dramatic accumulation of Cd that increased with exposure time ($p<0.001$) up to 35.9±10.850 μg/g at day 18 (Fig. 3).

### Effect of Cd on relative gene transcription

Relative expression of the seven stress-regulated genes is represented as box plots with respect to the calibrator (controls at time 0) in Figs. 4 and 5. The global comparison of the gene transcriptions indicates a lower variability within sampling points in controls compared to exposed cockles (either 50 μg/L and 5 mg/L) for all the target genes studied (Figs. 4 and 5). However, exposition to $CdCl_2$ globally led to an increase in gene transcription for CuZnSOD, CAT, MT, and HSP70 (up to +243, +49, +44, and +379 fold the mean expression level of control samples respectively; Fig. 4a–d, Table 1). Gene transcription was dependent of exposure time in some cases. In particular, gene transcription globally increased with exposure time for CuZnSOD, HSP70, and MT in cockles exposed to 50 μg/L of $CdCl_2$ and for CAT and MT in cockles exposed to 5 mg/L of $CdCl_2$ (Fig. 4a–d, Table 1). However, variation was rarely linear: Gene transcriptions of HSP70 and MT at Cd concentration of 50 μg/L and MT at Cd concentration of 5 mg/L first decreased to a minimum (−3, −19, and −47 fold the mean expression level of control samples respectively, after 1 to 3 days of exposure) and then increased (up to +379, +16, and +44 fold the mean expression level of control samples respectively; Fig. 4c, d). Concerning CO1, MnSOD, and ABCB1, expression was not influenced by the $CdCl_2$ treatment in itself, but some of them were influenced by exposure time. Gene transcription globally decreased with time for MnSOD in cockles exposed to 50 μg/L of $CdCl_2$ and for CO1 in cockles exposed to 5 mg/L of $CdCl_2$ (Table 1). As previously, this decrease was nonlinear; expression first increased to a maximum, decreased abruptly, and slightly increased again (Fig. 5a, b).

### PCAs

To highlight relationships between mRNA levels ($\Delta Ct = Ct_{sample} - C_{HKG}$), CI, and Cd bioaccumulation, two different PCAs were carried out, one considering controls and cockles exposed to 50 μg/L and the other one considering controls and cockles exposed to 5 mg/L (Fig. 6). All pair-wise correlations were tested statistically (Pearson's correlation test), and the results are presented in Supplementary data (Tables S2 and S3).

In the 50 μg/L analyses (Fig. 6a, c), 58.3 % of the total variance is explained by the two main axes. Positive

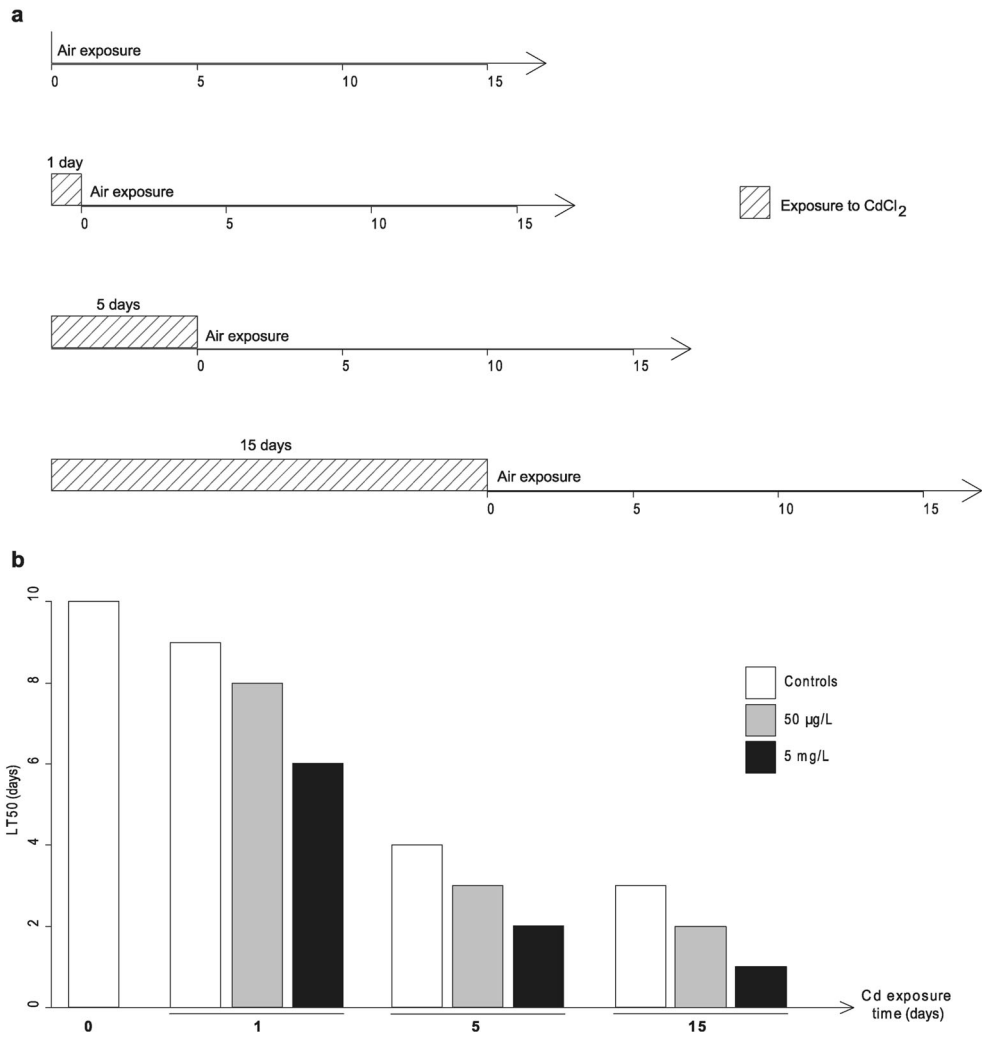


**Fig. 2** Protocol and results of the stress on stress test. Some cockles were exposed to anoxia after 0, 1, 5, or 15 days of exposure to either 0, 50 µg/L, or 5 mg/L of $CdCl_2$. Survival under anoxia was followed, and the median lethal time (LT50) was recorded for each combination of treatment and exposure time. **a** Experimental conditions: the *length of the hatched bar* indicates the time of cadmium exposure. The *arrow* figures the following of survival under anoxia after cadmium exposure. **b** Results: median lethal time (in days) of cockles submitted to anoxia after cadmium exposure for each treatment and exposure time

correlations are observed between Cd bioaccumulation and MT, HSP70, CuZnSOD, CO1, CAT, and ABCB1 mRNA levels (Fig. 6a, Table S3). No correlation is found between this cluster and MnSOD and CI (Fig. 6a and Table S3). The projection of the samples on the two main axes (Fig. 6c) indicates that all the control samples are found quite grouped on the right of the figure with the lowest Cd bioaccumulation and the lowest mRNA levels of HSP70, MT, CuZnSOD, CO1, CAT, and ABCB1. On the contrary, the cockles exposed to 50 µg/L of $CdCl_2$ are found mostly on the left of the figure with the highest Cd bioaccumulation and the highest mRNA expression of the biomarkers cited above (Fig. 6c).

In the 5 mg/L analysis (Fig. 6b, d), 61.4 % of the total variance is explained by the two main axes represented in the figure. Strong positive correlations ($p<0.05$, Table S4) are observed between Cd bioaccumulation, CAT, HSP70, MT, and CuZnSOD mRNA levels (Fig. 6b). This cluster is negatively correlated to MnSOD and CI while not correlated to CO1 and ABCB1 (Fig. 6b, Table S4). CO1 and ABCB1 are however clearly correlated with each other (Fig. 6b, Table S4, $p<0.05$). The projection of the samples on the two main axes (Fig. 6d) indicates that all the control samples are found quite grouped on the right of the figure with the lowest Cd bioaccumulation and the lowest mRNA levels of HSP70, MT, CuZnSOD, and CAT and the highest CI and mRNA levels of MnSOD. On the contrary, the cockles exposed to 5 mg/L of $CdCl_2$ are found mostly on the left of the figure with the highest Cd bioaccumulation and the highest mRNA expression of the biomarkers cited above (Fig. 6d). Two of the exposed samples are closely related to the CO1/ABCB1 cluster.

## Discussion

The global physiological state of cockles was assessed along experiments by performing stress on stress tests and

measuring condition index. According to Bayne (1986), stress results in a reduced capacity of individuals to adapt to changes in their environment. This concept was applied in this study through the surimposition of exposure to air (a natural stressor) over the effect of Cd stress (stress on stress tests) (Viarengo et al. 1995). The LT50 data demonstrates that exposure to both concentrations of $CdCl_2$ significantly affects cockle survival time in air. Such trend probably reflects metabolic perturbations following $CdCl_2$ exposure and a decrease of anoxia tolerance in accordance with previous data obtained on bivalves (Viarengo et al. 1995; Hamza-Chaffai et al. 1998; Ladhar-Chaabouni et al. 2008). This may suggest a decreased general health status among cockles exposed to $CdCl_2$ and imply potential "trade-offs", e.g., physiological allocations between detoxification and other processes linked to the survival of the organism, as previously described (Handy et al. 1999; Forbes 2000; Van Straalen and Hoffman 2000).

Measures of bivalve's condition based on weight are thought to be reliable indicators of the energetic health status and energy reserves in mollusks. Lannig et al. (2006) found significant variations in condition index in oysters (*Crassotrea virginica*) after 20 days of exposure to both $CdCl_2$ (50 μg/L) and temperature (28 °C). The results obtained in our study do not show any difference in the condition index between controls and exposed cockles. This finding probably highlights that this parameter is too much integrative and that only 18 days of exposition might not be enough to perceive or to produce variations between exposed cockles and control ones.

Because bivalves are filter-feeding organisms able to accumulate high concentrations of metals (Paul-Pont et al. 2012), a dose-dependent Cd accumulation in treated cockles was an expected result. Moreover, our study indicates that at a high level of $CdCl_2$ exposure (5 mg/l), Cd accumulation is time-dependent. Ladhar-Chaabouni et al. (2008) also demonstrated in *Cerastoderma glaucum* that Cd accumulation was time- and dose-dependent for concentrations ranging from 50 to 150 μg/L, giving this species a great potential for long-term ecotoxicological studies. This bioaccumulation capacity of bivalves exposed to metallic contaminations was already highlighted in several species such as *Cerastoderma edule* (Paul-Pont et al. 2012), *Ruditapes decussatus* (Bebianno and Langston 1993; Smaoui-Damak et al. 2003, 2004), *Corbicula fluminea* (Legeay et al. 2005), and *Dreissena polymorpha* (Marie et al. 2006a, b). The present study adds the fact that *Cerastoderma glaucum* are particularly tolerant to higher cadmium concentration (5 mg/L) without exhibiting dramatic effects, indicating a strong resistance and the establishment of effective acclimation strategies.

The protection and/or detoxification processes probably settled by cockles may be studied at different levels and especially at the transcriptional level. Indeed, changes in gene expression are likely to play critical roles in acclimation of organisms (Schulte 2004). The aim of our study was to

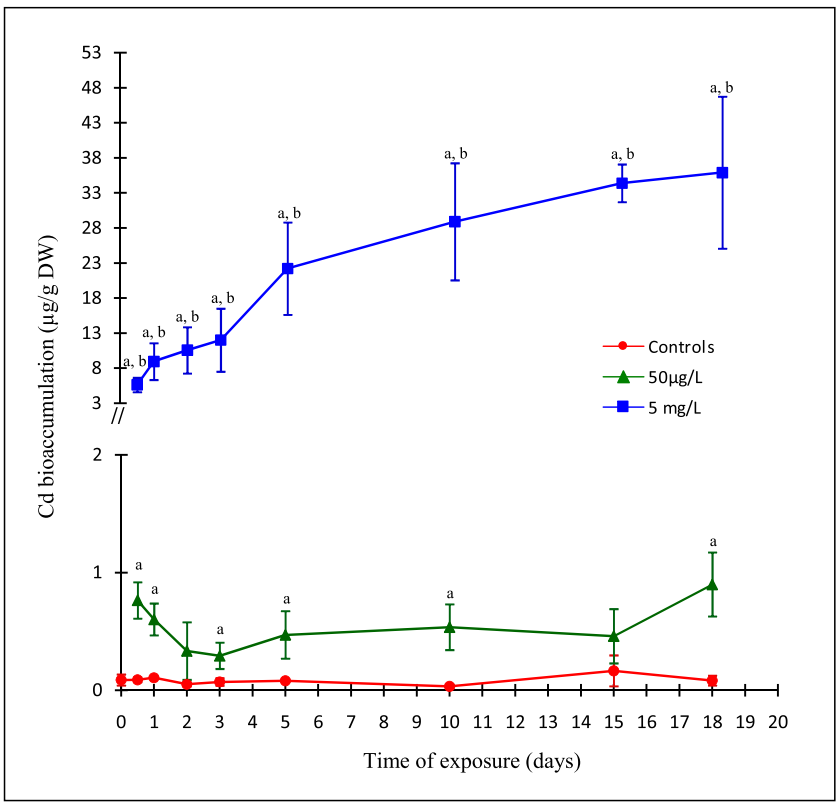


**Fig. 3** Cd bioaccumulation [μg/g (DW)] in cockles unexposed (controls) and exposed to 50 μg/L or to 5 mg/L. Data are expressed as means±SD ($n$=4). *DW* dry weight. Significant differences ($p$<0.05) are indicated with *letters* (**a**, **b**); statistical comparisons per time point between treated cockles and controls (*a*), and statistical comparisons per time point between cockles exposed to 50 μg/L and cockles exposed to 5 mg/L (*b*)

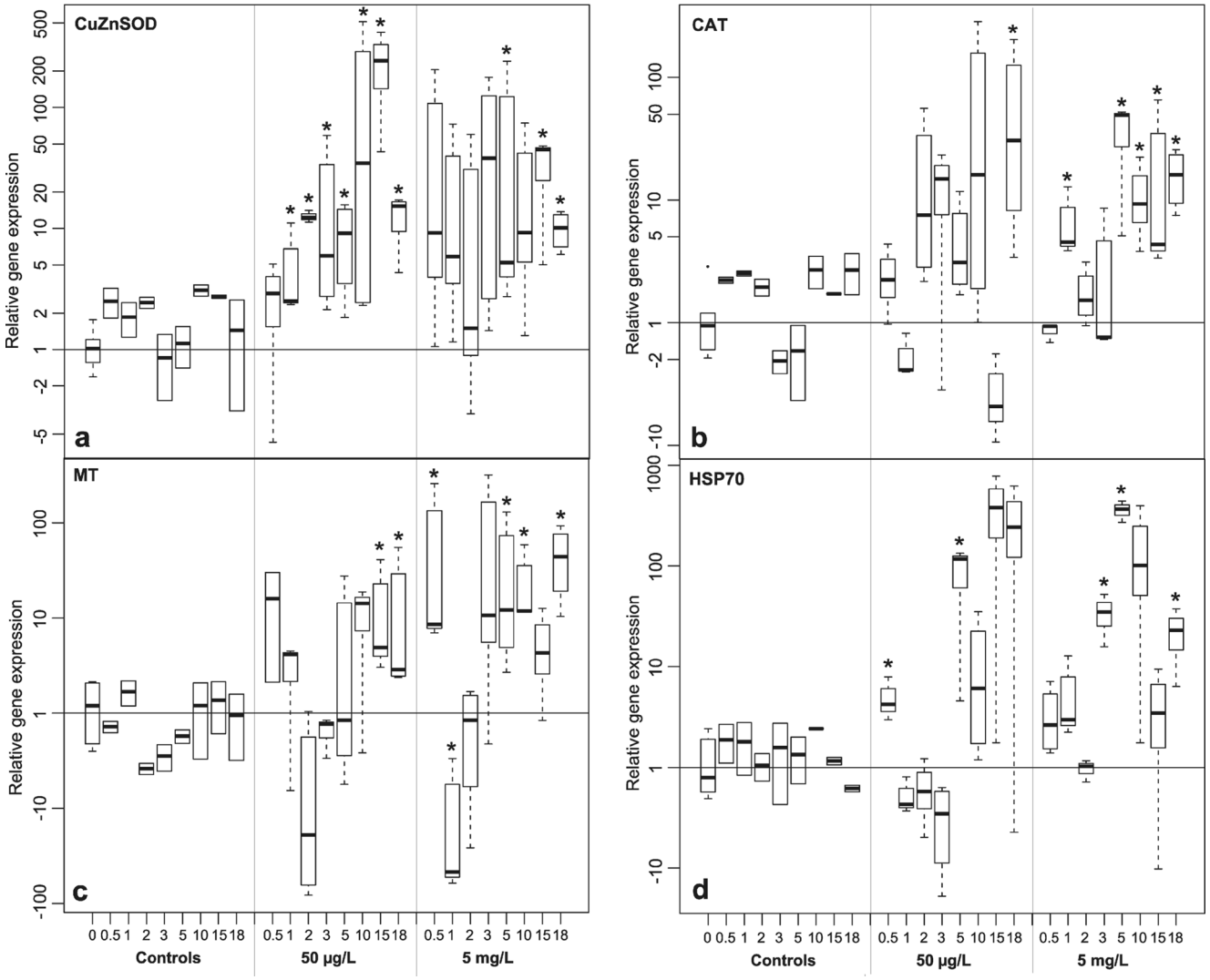


**Fig. 4** Relative gene expression profiles for **a** CuZnSOD, **b** CAT, **c** MT, and **d** HSP70. Fold differences (log scale) are expressed relatively to controls at time 0. Significant differences from controls are indicated by an *asterisk* ($p<0.05$, Kolmogorov-Smirnov test)

highlight modifications in mRNA expression of several genes involved in the tolerance to Cd exposure. Defense proteins involved in xenobiotic detoxification (MT, ABCB1), protection against oxidative stress (MnSOD, CuZnSOD, CAT), general stress (HSP70), and mitochondrial alterations (CO1) were chosen because their functions make them primary candidates for cellular protection against stress and because modifications of their gene expression had already been shown in invertebrates (Achard-Joris et al. 2006; Ivanina et al. 2008a, b; Navarro et al. 2011; Al Kaddissi et al. 2012).

A relatively low variation is observed in gene transcription along the experiment in control cockles indicating that mRNA expression is not affected by the experimental procedure. A high variability of gene transcription is observed for all target genes in exposed cockle biological variability that reflects the natural genotypic and phenotypic variation among individuals (Bustin 2010), but the much higher variability observed in exposed individuals suggests the existence of different types of response among individuals exposed to the same contaminant. Elements confirming this hypothesis may be found with the projections of individuals into the PCAs (Fig. 6c, d). For both concentrations of $CdCl_2$, control individuals are found quite grouped, while exposed individuals are more scattered, some even found close to the controls (Fig. 6c, d).

Exposure to 50 µg/L and 5 mg/L resulted in important variations in the mRNA expression of four genes (MT, HSP70, CAT, and CuZnSOD). A time-dependent increase of MT mRNA abundance is observed after exposure to the two levels of Cd toxicity. MT gene transcription is highly correlated to Cd accumulation in the cockles' tissues, meaning that individuals that accumulate the most Cd are also the ones with the highest MT mRNA levels. The MT isoform studied here is the MT1 that has been reported to be specific to Cd contamination (Gonzalez et al. 2006). The upregulation of this gene is

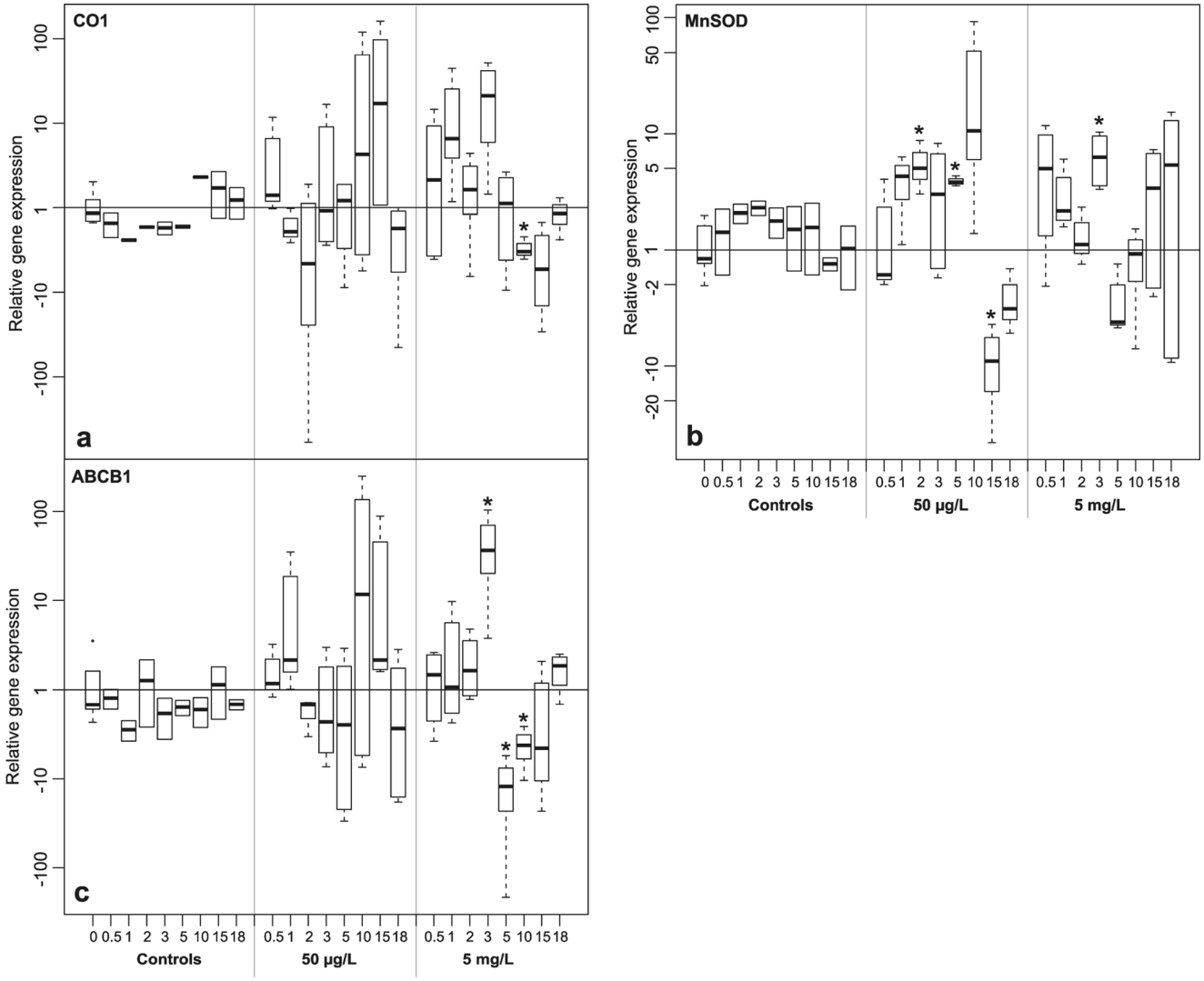


**Fig. 5** Relative gene expression profiles for **a** CO1, **b** MnSOD, and **c** ABCB1. Fold differences (log scale) are expressed relatively to controls at time 0. Significant differences from controls are indicated by an *asterisk* ($p<0.05$, Kolmogorov-Smirnov test)

in accordance with previous experiments performed with different invertebrates following metal exposure (Lecoeur et al. 2004; Dondero et al. 2005; Zorita et al. 2007; Navarro et al. 2011; Al Kaddissi et al. 2012). It may reflect the role of MTs in uptake and handling of metals, as well as antioxidants reacting with free radicals and reactive oxygen species (ROS), preventing interactions with critical cellular components such as enzymes, structural proteins, DNA, and membrane lipids (Andrews 2000; Amiard et al. 2006). The response of HSP70 is quite similar to that of MT after $CdCl_2$ exposure and also

**Table 1** Influence of treatment and of exposure time on relative gene expression for each Cd treatment versus control samples: sense of the effect and significance level

| | | CuZnSOD | CAT | MT | HSP70 | CO1 | MnSOD | ABCB1 |
|---|---|---|---|---|---|---|---|---|
| 50 µg/L Cd | Influence of treatment | +[a]*** | +* | +* | +* | / | / | / |
| | Influence of exposure time | +** | / | +** | +*** | / | −*** | / |
| 5 mg/L Cd | Influence of treatment | +*** | +*** | +*** | +*** | / | / | / |
| | Influence of exposure time | / | +*** | +** | / | −*** | / | / |

[a] global effect, + positive effect (increased expression), − negative effect (decreased expression), / no significant effect ($p>0.05$)

*$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$

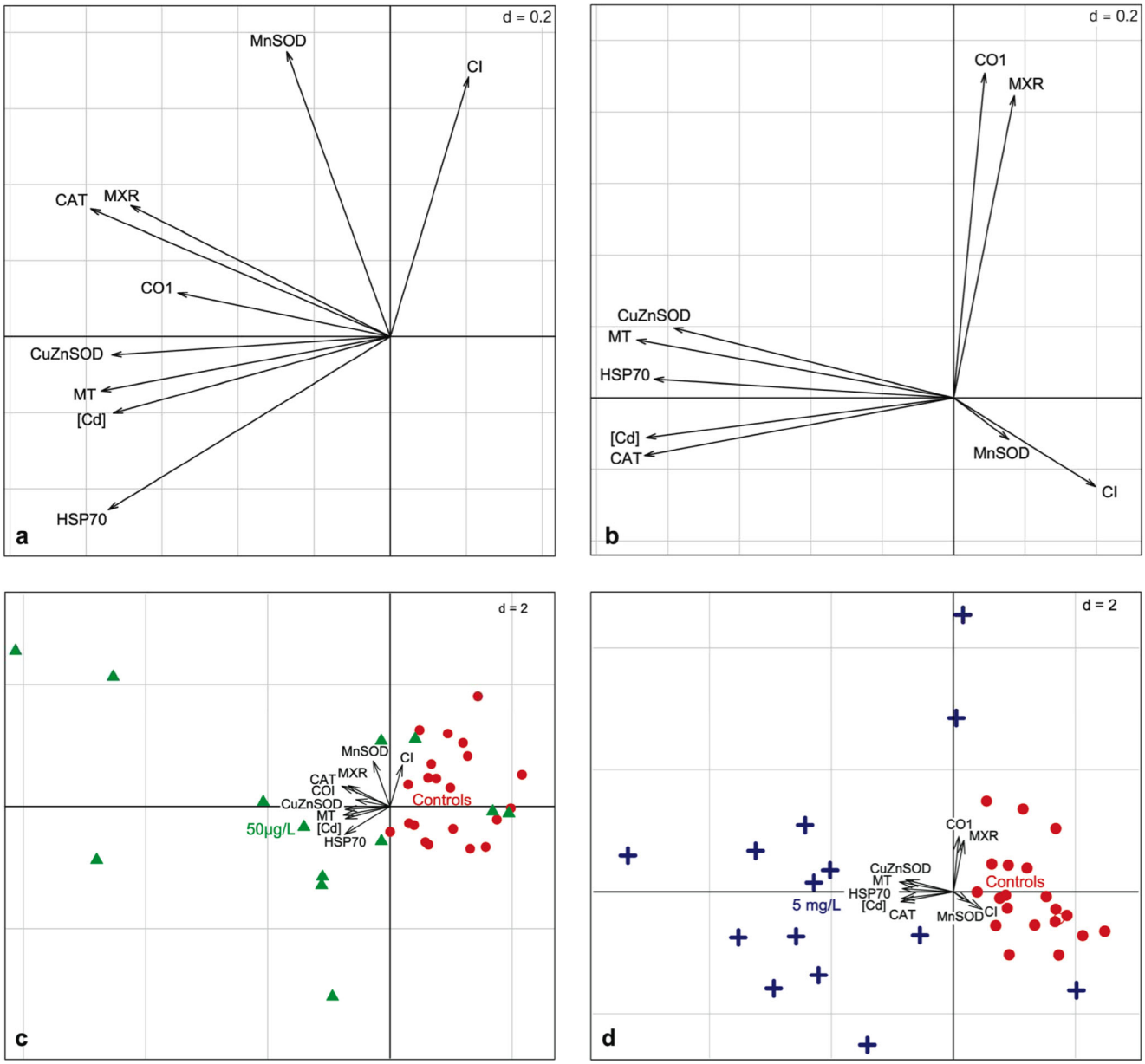


**Fig. 6** Principal component analysis (PCA) on gene expressions, Cd bioaccumulation, and CI. **a** PCA performed on controls and cockles exposed to 50 μg/L of Cd. **b** PCA performed on controls and cockles exposed to 5 mg/L of Cd. **c** Projections of samples according to the different quantitative data presented in **a.** **d** Projections of samples according to the different quantitative data presented in **b**. CI condition index, [Cd] Cd bioaccumulation in cockles. *Red circles* indicate control samples, *green triangles* indicate 50 μg/L, and *blue cross* indicate 5 mg/L

highly correlated to Cd accumulation in tissues. Some studies have shown that HSP70s are synthesized intensively in the cell in response to a variety of harmful stimuli, including heat, heavy metals ($Pb^{2+}$, $Cd^{2+}$), organic contaminants, injuries, diseases, and other stressors (Singer et al. 2005). Navarro et al. (2011) and Ivanina et al. (2008a) also showed a strong increase in the response of this gene after Cd exposure in *D. polymorpha* and in *Crassostrea virginica*, respectively. HSP70 are involved in folding/refolding of newly synthesized and damaged proteins as well as in sequestering and degradation of proteins that are damaged beyond repair (Mayer and Bukau 2005). A strong upregulation of its gene (reaching +379 and +366 fold the mean expression level of controls for 50 μg/L and 5 mg/L of $CdCl_2$, respectively) reflects a mechanism highly settled to protect cells against stress-induced damage. However, variations in expression of genes coding for MT and HSP70 were not completely linear (with a decrease in gene transcription following the first increase), reflecting a potential time-lag between gene and protein regulation. Analyses

of protein concentrations would be particularly useful to give complementary data and to disentangle more precisely the regulation.

Several investigations have shown that Cd can indirectly promote oxidative stress in cells. This metal can bind to antioxidants or to their substrates. Consequently, impairment of the defense mechanism against ROS leads to an increase in ROS concentration (Wang et al. 2004; Cao et al. 2010). This fact could explain the increase in gene transcription of the antioxidant enzymes CAT and CuZnSOD following high and low $CdCl_2$ exposure and their correlation to Cd accumulation in tissues. CuZnSOD (the cytosolic form of SODs) is known to catalyze the dismutation of superoxide anion into hydrogen peroxide, which is in turn reduced by CAT into water and molecular oxygen. The increase of these antioxidant enzymes in response to Cd exposure constitutes an adaptation in gills to prevent and repair metal-induced damage in cellular component (Fernandez et al. 2010). Similar results were reported for the clam larvae *Meretrix meretrix*; the SOD and CAT activities increased after exposure to 25 μg/L of Cd (Wang et al. 2010). Jo et al. (2008) also found that SOD and CAT mRNA expression levels increased significantly after 24 h of exposure to 0.1 ppm of Cd in the oyster *Crassostrea gigas*. In our study, the expression of MnSOD (the mitochondrial form of SODs) is not influenced by the $CdCl_2$ treatment whatever its concentration and reflects natural physiological differences between cockles rather than a response to Cd. It can also be explained by the presence of sufficient basal concentration of antioxidant enzymes to protect cockles against oxidative stress.

Earlier studies have shown that energy misbalance is implicated in Cd-induced stress and toxicity in aquatic organisms, especially mitochondrial dysfunctions that lead to impaired ATP production, reduced aerobic capacity, and elevated oxidative stress (Sokolova et al. 2005; Cherkasov et al. 2007). As part of the mitochondrial respiratory chain, CO1 expression may be an interesting biomarker of the metabolic activity. Results showed that the CO1 mRNA expression is correlated to Cd accumulation at the concentration of 50 μg/L of $CdCl_2$ with however no clear pattern along the 18 days of the experiment (an increase in expression might be observed but not significant). At the concentration of 5 mg/L of $CdCl_2$, no correlation between CO1 expression and Cd accumulation was observed, and a downregulation of this gene along the experiment is even observed. The expression of CO1 thus appears to be modulated by the level of Cd concentration. Lerebours et al. (2010) previously stated that the intensity of gene response may not correlate positively with toxicant concentrations and that different gene expression patterns are expected for different concentrations of exposure. This is in concordance with our findings and tends to indicate that a modification in the metabolism might occur. Low concentration of Cd exposure tends to increase the level of CO1 mRNA and thus the mitochondrial metabolism. This could be a cellular strategy (1) to compensate for the decrease in the number of functional mitochondria by increasing ATP production, so as to provide enough energy for cellular needs, especially to tolerate Cd (Al Kaddissi et al. 2012), and (2) to efficiently consume $O_2$ to limit Cd-induced oxidative damage in cells (Wang et al. 2004). It has to be noted that the transcription level of CO1 correlated well with those of CuZnSOD and CAT. Achard-Joris et al. (2006) found the same upregulation of the CO1 gene in three mollusk species following high Cd exposure. In contrast, in our study, exposure to the high concentration of $CdCl_2$ (5 mg/L) led to a progressive decrease of the CO1 mRNA content, reflecting a potential decrease in the metabolic activity. This result tends to indicate a strong disturbance of cellular energy balance, with individuals no more able to compensate for the probable inhibition of the mitochondria function. Negative impacts on physiological performances and survival of organisms are expected (Sokolova and Lanning 2008). This is partially observed with the mortality rate higher in the 5 mg/L tank compared to the others and the $LT_{50}$ (stress on stress) lower in this condition. Cases of repression/decrease of CO1 mRNA/activity were also recorded in the crayfish *Procambarus clarkii* and in *Crassostrea gigas* after acute and medium-term Cd exposure (Ivanina et al. 2008b; Al Kaddissi et al. 2012).

The ATP-binding cassette xenobiotic transporter ABCB1 gene transcription is significantly correlated to Cd accumulation at the concentration of 50 μg/L of $CdCl_2$ but not at the concentration of 5 mg/L. However, it does not exhibit a clear pattern along the experiments. This protein, which acts by transporting toxic compounds out of the cell (Minier et al. 2006), seems to be an actor of the response to the medium exposure to $CdCl_2$ (50 μg/L), but a strong variability in expression between individuals reduces the power of this biomarker.

In conclusion, the physiological trend detected in *Cerastoderma glaucum* exposed to low and high concentration of $CdCl_2$ suggests a general decreased health status along the experiment. Gene transcription monitoring performed on seven genes potentially involved in the tolerance to Cd exposure showed that MT, HSP70, CuZnSOD, and CAT are relevant biomarkers of Cd exposure because of (1) their relative gene transcription higher in exposed individuals compared to controls and (2) their significant correlation to Cd accumulation. The upregulation of these genes indicates that Cd detoxification is promoted as well as protection against free radicals and intracellular oxidative damage. Following the mRNA expression of CO1 and ABCB1 gives complementary information on the status of exposed individuals, in particular their metabolic activity, but these biomarkers do not seem to be relevant with a chronic exposure. The molecular mechanisms underlined in this study imply energetic costs and therefore might explain the reduced tolerance of cockles to anoxia

(stress on stress). The influence of contaminants other than metals of the environment from which the cockles were harvested as well as the influence of the experiment itself should however not be discarded. The tools developed may be useful both for future control strategies and for the use of the cockle *Cerastoderma glaucum* as a sentinel species. However, future investigations should be performed to validate these biomarkers in field conditions. Moreover, parallel biochemical, physiological, immunological, and morphological/pathological data should complement this study to better assess Cd toxicity and its mechanism of action.

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