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Transcriptional response of stress-regulated genes to industrial effluent exposure in the cockle *Cerastoderma glaucum*

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Abstract This study assessed the responses of molecular biomarkers and heavy metal levels in *Cerastoderma glaucum* exposed for 1 week to two industrial effluents (1 %) discharged into the Tunisian coastal area, F_1 and F_2, produced by different units of production of a phosphate treatment plant. A significant uptake of metals (Cd, Cu, Zn, and Ni) was observed in exposed cockles compared to controls, with an uptake higher for F_1 than for F_2. A decrease in LT50 (stress on stress test) was also observed after an exposure to the effluent F_1. Treatments resulted in different patterns of messenger RNA (mRNA) expression of the different genes tested in this report. Gene transcription monitoring performed on seven genes potentially involved in the tolerance to metal exposure showed that for both exposures, mechanisms are rapidly and synchronically settled down to prevent damage to cellular components, by (1) handling and exporting out metal ions through the up-regulation of ATP-binding cassette xenobiotic transporter (ABCB1) and metallothionein (MT), (2) increasing the mRNA expression of antioxidant enzymes (catalase (CAT), superoxide dismutases, CuZnSOD and MnSOD), (3) protecting and/or repairing proteins through the expression of heat shock protein 70 (HSP70) mRNAs, and (4) increasing ATP production (through the up-regulation of cytochrome c oxidase 1 (CO1)) to provide energy for cells to tolerate stress exposure. The tools developed may be useful both for future control strategies and for the use of the cockle *C. glaucum* as a sentinel species.

Keywords *Cerastoderma glaucum* · Industrial effluent · Gene expression · qPCR · Heavy metals · Phosphate treatment plant

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Introduction

With the rapid development of modern industry, various contaminants have been discharged into marine environments and have caused instability and disorder of ecosystems (Cong et al. 2012; Ladhar-Chaabouni et al. 2012). Among these contaminants, effluents are continuously discharged from municipal and industrial treatment plants and have progressively affected more and more ecosystems (Jadeja and Tewari 2007). Treated or not, these effluents can release many

pollutants such as polycyclic aromatic hydrocarbons, pharmaceuticals, and heavy metals (Sim et al. 2013). The latter are deemed to be serious pollutants because of their toxicity, persistence, and non-degradability in the environment (Gargouri et al. 2011). In general, exposure to effluents is known to cause a variety of stress-related changes in aquatic organism health such as immunotoxicity (Gagne et al. 2008; Salo et al. 2007), neurotoxicity and oxidative stress (Kamel et al. 2012), and physiological alterations (Douxfils et al. 2007). The impact of effluents on marine environments is often assessed by the measurement of pollutant body burdens in marine organisms (Haynes et al. 1997). This assessment can be done by passive biomonitoring (sampling organisms living in the investigated area) or by active biomonitoring (transplantation of organisms or laboratory experiments). However, laboratory experiences remain an alternative approach to evaluate the potential toxicity effects of a mixture of pollutants (effluents) to aquatic organisms. Using this methodology, it is possible to quantify the effects of contaminant in controlled conditions with precision. In Tunisia, the gulf of Gabès (localized on the southeastern coast) is influenced by an important industrial activity, especially a phosphate treatment plant (SIAPE: Société des Industries d'Acide Phosphoriques et d'Engrais), exposing coastal waters and marine organisms to increasing contamination (Ladhar-Chaabouni et al. 2009a). The main activity of this industry is the transformation of the crude phosphate to fertilizers. This activity generates wastewaters (effluent) rich in sulfate, fluoride, phosphorus, and heavy metals that will be discharged in the marine environment. Consequently, marine ecosystems around the crude phosphate treatment plant have been completely destroyed, and high levels of toxic metals were observed in the sediment and in bivalve species (Javied et al. 2009; Ketata et al. 2007; Mar and Okazaki 2012; Tayibi et al. 2009). *Cerastoderma glaucum* is one of the bivalves widely distributed in the gulf of Gabès, and this species has been validated as a bioindicator organism reflecting the pollution state of the Tunisian coast (Machreki-Ajmi and Hamza-Chaffai 2006; Szefer et al. 1999). This sedentary and filter-feeding organism lives in the superficial sediment and is known to be suitable for toxicology and risk assessment studies in the field (Ladhar-Chaabouni et al. 2009a; Machreki-Ajmi and Hamza-Chaffai 2006, 2008) or in laboratory experiments (Ladhar-Chaabouni et al. 2009b, c). Cockles are major preys for diverse animal groups such as crustaceans, fishes, and wading birds (Paul-Pont et al. 2010), 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. 2010). Studies carried out on this species typically focused on biochemical parameters (metal bioaccumulation and biomarker measures). In addition, experiments performed were usually conducted using a single contaminant (e.g., cadmium) (Ladhar-Chaabouni et al. 2009b, c). The originality of the present study consists of using molecular biomarkers to assess the response of cockles to a natural mixture of contaminants (effluent) of the gulf of Gabès. Given that the initial interaction between contaminant and aquatic organisms occurs at the cellular level, the molecular level is suitable for the early and sensitive detection of contaminant exposure and may allow the prediction of biological effects at higher biological organization levels. Moreover, changes in gene expression are likely to play critical roles in acclimation and/or adaptation of organisms (Schulte 2004).

The purpose of this study was therefore to investigate the responses of cockles' *C. glaucum* exposed to two different effluents originated from the SIAPE of the gulf of Gabès, through the study of (1) metal bioaccumulation in cockles' tissues, (2) the global physiological state of organisms, (3) the responses at the transcriptional level of several key genes, and (4) the relationships between gene expression and metal contents. The messenger RNA (mRNA) transcripts used 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 70, HSP70), and mitochondrial alterations (cytochrome c oxidase 1, CO1). All transcriptional responses were measured in gills because this organ constitutes a key interface for the uptake, storage, and excretion of metals and has a high capacity to synthesize proteins (Al Kaddissi et al. 2012; Marigomez et al. 2002; Navarro et al. 2011; Paul-Pont et al. 2010). In contrast to most mRNA analyses that are performed on sample pools (Ciacci et al. 2012; Navarro et al. 2009), the present report analyzes gene expression at the individual scale to highlight all the variability between cockles experiencing the same exposition.

Materials and methods

Animals and treatments

Cockles (*C. glaucum*) (28–32 mm) were collected in October from the "Luza" site (located 45 km in the north of Sfax, Tunisia; Fig. 1), which can be considered as a reference site with a low pollution level (Barhoumi et al. 2009; Kessabi et al. 2010). After a period of acclimation of 24 h, cockles were distributed in three polyethylene tanks (30×30×20 cm), each one containing a layer of autoclaved sediment and 6 l of aerated seawater. The first tank contained seawater from the reference site Luza (controls), the second tank contained seawater with the F_1 effluent, and the third tank contained seawater with the effluent F_2 (F_1 and F_2 effluents were collected at the exit of the SIAPE). Both F_1 and F_2 effluents were diluted to a final concentration of 1 % (sub-lethal concentration

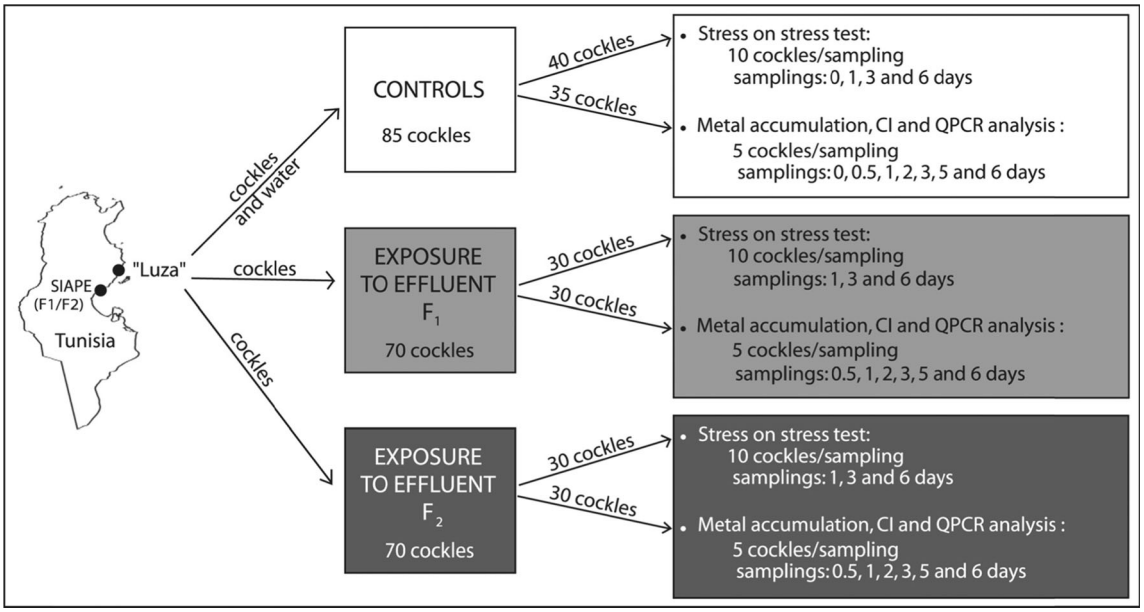

Fig. 1 Sampling and experimental exposure of cockles

determined following a preliminary set of toxicity experiments, pers. comm.). Seventy to eighty-five cockles were held in each tank (experimental design schematized in Fig. 1), and exposure time varied between 12 h and 6 days. During the experiment, effluents were renewed twice a week.

Stress on stress test

Ten animals of each of the three conditions of the experiment (controls, F_1 and F_2 groups) and at different times of exposure (after 0, 1, 3, and 6 days of exposure) were sampled and submitted to anoxia by air exposure at 15 °C (Fig. 1). For each sample, 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 and the results expressed in days for each condition and at the four different times of exposure (0, 1, 3, and 6 days).

Condition index of cockles

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

Determination of metal contents

Heavy metal analyses (Cd, Cu, Ni, and Zn) were carried out (1) on effluent waters directly from the effluent sampling site and (2) individually on five cockles from the laboratory experiment at each sampling time (Fig. 1) on the remaining animal tissues (including digestive gland, muscle, mantle, and foot). The determination of trace element concentration in effluent waters was performed using an inductively coupled plasma optical emission spectrometer (ICP-AOS, Thermo Scientific iCAP 6300 DUO), and measurement of metal concentrations in cockles was carried out as previously described (Dedourge-Geffard et al. 2009; Geffard et al. 2010) by flameless (Cd, Cu, and Ni) or flame (Zn) atomic absorption spectrophotometry with Zeeman correction, using a graphite furnace (SpectrAA Zeeman 220).

RNA extraction and cDNA synthesis

Gene transcription analyses were carried out individually on the same five individuals for which metal analyses and CI were measured and at each sampling time (Fig. 1). The 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) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm, and RNA quality was checked using the Experion system (Bio-Rad). Three micrograms of total RNA were added to dTRace primer (5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTT TTTTT-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 then the mixture was kept on ice. First strand complementary DNAs (cDNAs) were then synthesized using dTRace, dNTPs, MMLV reverse transcriptase (Promega), RNAsin (Promega), and nuclease-free water 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 Step One plus apparatus (Applied Biosystems) with Fast SYBR Green Master Mix (Applied Biosystems) and specific primers designed (Table S1 in Supplementary Data) 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 a 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 metal concentration and exposure time on ability to survive under anoxia was analyzed using the SURVREG function (survival package, R). To test for significant differences between mRNA expression values, Kolmogorov-Smirnov tests were used ($p<0.05$). After normalization of the data by $\log(x+1)$ transformation and stabilization of their variances by square root transformation, influences of effluent treatment and exposure time on mRNA expression, metal bioaccumulation, and CI were assessed by independent ANOVA significance tests. A principal component analysis (PCA) was performed for each treatment on mRNA expression, metal bioaccumulation, and CI, and correlations between all these variables were tested statistically using Pearson's test.

Results

Effects of effluents on the global physiological state

During the experiment, no mortality was recorded in the three groups of cockles. Survival under anoxia (i.e., stress on stress assay) is significantly affected by treatment ($p<0.05$), with exposure causing a decrease in survival time in air relative to controls (significant for F_1 with $p<0.05$ but not significant for F_2; Fig. 2). No difference in CI is observed with treatment (controls 11.7±2.6; F_1 12.1±1.9; F_2 13.3±2.2) nor with time of exposure (data not shown).

Metal concentrations in F_1 and F_2 effluents and metal accumulation in cockles' tissues

As indicated in Table 1, heavy metal concentrations are higher in F_1 effluent than in F_2: Cu (63-fold), Cd (29-fold), Cr (24-fold), Mn (13-fold), Ni (10-fold), Zn (6-fold), and Pb (5-fold).

The concentration of Cd, Cu, Zn, and Ni in cockles' soft tissues was also investigated during the time of the experiment (Fig. 3). Cd, Cu, Zn, and Ni concentrations (expressed with respect to dry weight) remain low and relatively constant in tissues of control cockles. Accumulation varied among metals; relative to their respective concentrations in the effluents, Cd was much less accumulated than the other metals (up to 100-fold). Their accumulation in treated cockles is dependent of the effluent treatment ($p<0.05$), i.e., cockles exposed to F_1 accumulate more Cd, Cu, Ni, and Zn than those exposed to F_2 but only about 1.5-fold, except for Cd (about 24-fold). For Cu, Ni, and Zn, a similar bioaccumulation pattern is observed between cockles exposed to F_1 and F_2. The bioaccumulation of Ni and Zn is not time dependent. On the contrary, Cu accumulation is time dependent for both effluent experiments, i.e., treated cockles accumulate Cu between 1 and 3 days (bioaccumulation higher in F_1 compared to F_2). A similar pattern is also observed for Cd (for F_1 only), with low variation during the two first days, followed by an increase in Cd concentration. This increase is quite moderate for F_2, reaching 0.224±0.106 μg/g dry weight (DW) after 5 days of exposure, while strong for F_1, with a maximum value of 6.181±1.276 μg/g DW after 6 days of exposure.

Effect of F_1 and F_2 effluent on relative gene expression

Relative expression of the seven stress-regulated genes with respect to the calibrator (controls at time 0) is represented as box plots in Figs. 4 and 5. In cockles exposed either to F_1 or to F_2, mRNA expression was globally higher than in controls for all the studied genes. The variability (represented by quartiles in boxplots) is also significantly higher in exposed cockles than controls ($p<0.001$, Fligner-Killeen test of homogeneity of variances; Figs. 4 and 5).

For MT, CO1, and HSP70, expression increased with exposure time quite linearly in cockles exposed to F_1 (Fig. 4; Table 2). In cockles exposed to F_2, exposure time had no significant influence on mRNA expression for these genes. Despite fluctuations, the expression of ABCB1 is higher in treated cockles compared to controls, but exposure time has no significant influence on its expression whatever the effluent (Table 2). Concerning the enzymes involved in the response to oxidative stress (Fig. 5), mRNA expression increased with exposure time for MnSOD and CAT in cockles exposed to F_1. In particular, the expression of these genes remained close to that of controls during the first two days of exposure and then expression increased strongly on the third day (+9- and +43-fold, the mean expression level of control samples for MnSOD and CAT, respectively) and remained high until the sixth day (Fig. 5). Exposure time has no significant influence on CuZnSOD mRNA expression (Fig. 5; Table 2). In cockles exposed to F_2, on the contrary, the expression of the genes involved in response to oxidative

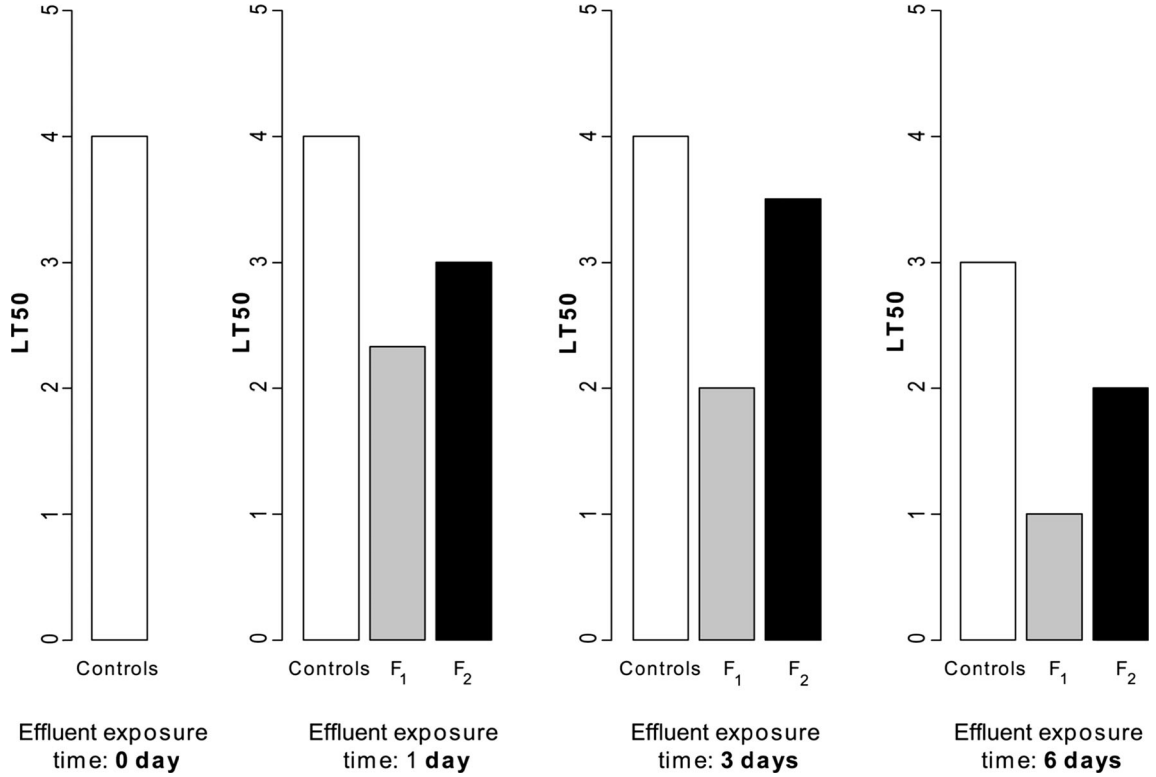

Fig. 2 Results from the stress on stress test. Some cockles were exposed to anoxia after 1, 3, or 6 days of exposure to effluents F_1 and F_2. Survival under anoxia was followed, and the median lethal time (LT50) was recorded (in days) for each combination of treatment and exposure time

stress (CAT, MnSOD, CuZnSOD) globally decreased with exposure time (Table 2). In details, the dynamics was the same for the three genes: expression increased during the first 24 h (+87-, +71-, and +58-fold, the mean expression level of control samples for CAT, MnSOD, and CuZnSOD, respectively) then decreased until the fifth day (−10-, −2-, and −3-fold for CAT, MnSOD, and CuZnSOD, respectively) (Fig. 5).

Principal component analyses (PCAs)

To highlight relationships between mRNA levels ($\Delta Ct = Ct_{sample} - C_{HKG}$), CI, and bioaccumulation of metals (Cd, Cu, Zn, and Ni), two different PCAs were carried out, one considering controls and cockles exposed to the effluent F_1 and the other one considering controls and cockles exposed to the effluent F_2 (Fig. 6).

Table 1 Trace metal concentrations in F_1 and F_2 effluent collected at the exit of the SIAPE phosphate treatment plant situated in the gulf of Gabès (Sfax, Tunisia)

	Cd	Cr	Cu	Mn	Zn	Ni	Pb
F_1 effluent	0.953	0.994	0.381	0.445	2.757	0.469	0.174
F_2 effluent	0.033	0.043	0.006	0.034	0.486	0.045	0.033
Tunisian norms	0.005	2	1.5	1	10	2	0.5
French norms	0.2	0.5	0.5	1	2	0.5	0.5

Tunisian and French norms are indicated. Results are expressed as milligrams/liter of effluent

In the F_1 PCA analysis (Fig. 6a), 59.3 % of the total variance is explained by the two main axes. All parameters appear on the same side relative to the first axis (i.e., on the left of the factorial map). Two groups of correlated parameters ($p<0.05$, Pearson's test) appear according to their location relative to the second axis (in dotted circle on the map, Fig. 6a). The first one, appearing on the top of the factorial map, is composed of Zn bioaccumulation, MT, MnSOD, CuZnSOD, CAT, and ABCB1 mRNA levels. The second one, appearing on the bottom of the factorial map, contains Ni, Cu, and Cd bioaccumulation, CO1 and HSP70 mRNA levels, and CI (Fig. 6a). The two groups are in fact overlapping with significant correlations found between some parameters of each group (Table S2 in Supplementary Data). The projection of the samples on the two main axes of the PCA (Fig. 7a) indicates that all the control samples are found quite clustered on the right of the figure with the lowest metal bioaccumulations (Ni, Zn, Cu, and Cd) and the lowest mRNA levels of the seven genes studied. On the contrary, treated cockles are found mostly on the left of the figure with the highest metal bioaccumulations and the highest mRNA expression of the biomarkers cited above (Fig. 7a). A few exposed cockles are however found clustered with the control group. The projection of the samples according to the time of exposure (Fig. 7c) indicates that the samples corresponding to the three first times of exposure

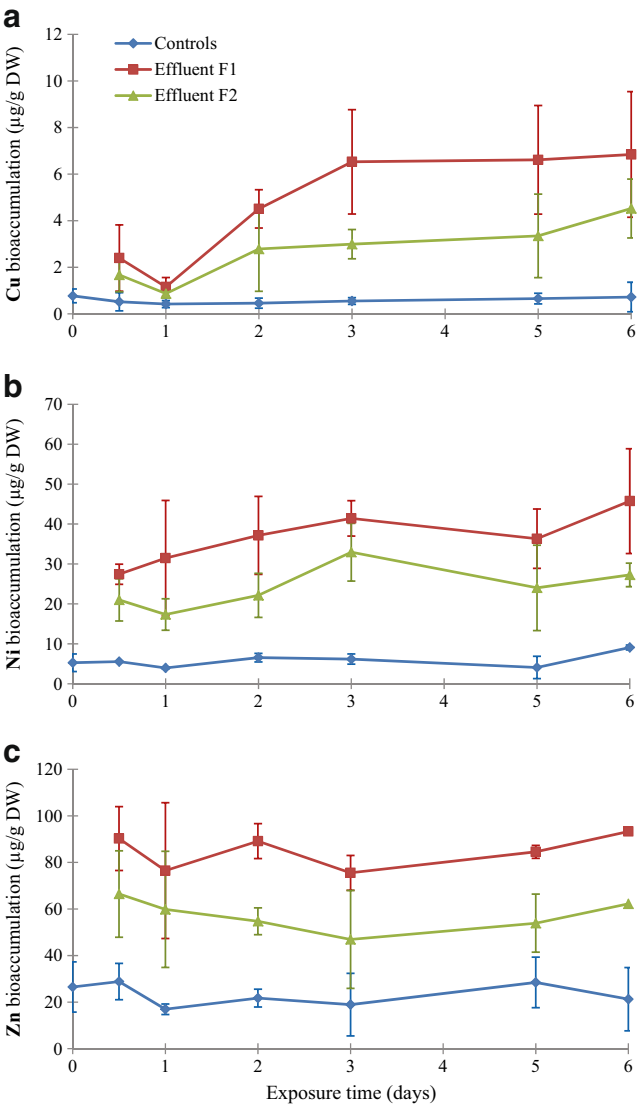

Fig. 3 Bioaccumulation of **a** Ni, **b** Zn, **c** Cu, and (**d**) Cd [µg/g (DW)] in cockles unexposed (controls) and exposed to F_1 and F_2 effluents. Data are expressed as means±SD (n=4); DW: dry weight

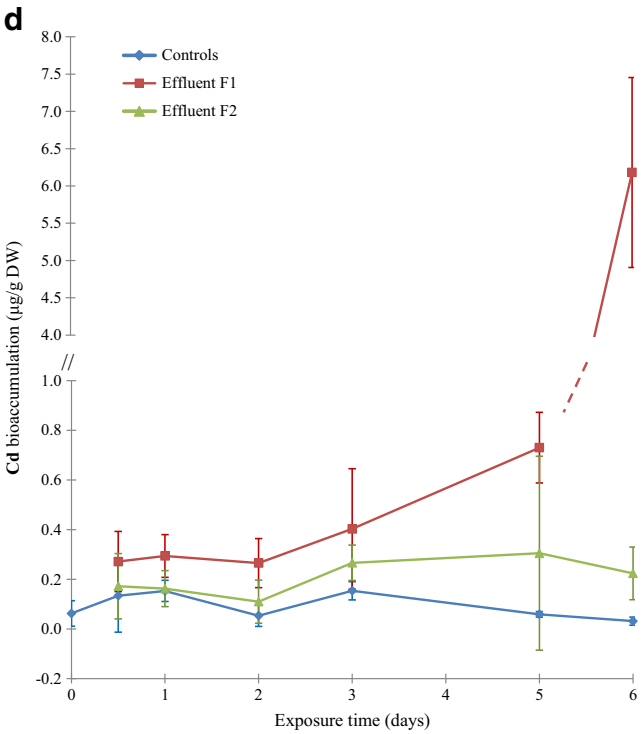

Fig. 3 (continued)

(12 hours, 1 day, and 2 days) are located on the right near the control group with low mRNA expression of all genes tested and low bioaccumulation of heavy metals compared to the other samples. Samples subjected to 3, 5, and 6 days of F_1 effluent are projected on the left of the figure with the highest mRNA expression for the different biomarkers and the highest metal bioaccumulations. It can also be noted that cockles at 3 days of exposure are located at the top of Fig. 7c with the highest expression of oxidative markers (MnSOD, CuZnSOD, CAT), MT, and ABCB1, whereas cockles at 6 days of exposure are located in the lower part of the figure with the highest expression of HSP70 and CO1.

In the F_2 PCA analysis (Fig. 6b), 62.8 % of the total variance is explained by the two main axes. A first group of correlated parameters (p<0.05, Pearson's test, Table S3 in Supplementary Data) appears on the top of the factorial map (in dotted circle) and is composed of CAT, MnSOD, CuZnSOD, and MT mRNA expression (Pearson's correlation coefficients >0.517, p<0.05). The second one appears on the bottom of the factorial map (in dotted circle) and contains bioaccumulation of metals (Cd, Cu, Zn, and Ni) and HSP70 and ABCB1 mRNA expression (Pearson's correlation coefficients >0.341, p<0.05, Table S3). The mRNA expression of CO1, appearing along the first axis, is highly correlated to the two groups (Table S3). The projection of the samples on the two main axes of the PCA (Fig. 7b) indicates that all the control samples are found quite clustered on the right of the figure with the lowest metal bioaccumulations, the lowest mRNA levels of the seven genes, and the lowest CI. On the contrary, treated cockles are again found mostly on the left of the figure with the highest metal bioaccumulations and the highest mRNA expression of the biomarkers cited above (Fig. 7b). The projection of the samples according to the time of exposure (Fig. 7d) indicates that most of the samples corresponding to the three first times of exposure (12 h, 1 day, and 2 days) are located on the top left of the factorial map (in dotted circle) with high levels of mRNA coding for antioxidant enzymes (CAT, MnSOD, CuZnSOD) and MT. In contrast, most of the samples subjected to 3, 5, and 6 days of F_2 effluent are projected on the bottom left of the factorial map (in dotted circle), i.e., with high accumulation of metals (Cd, Cu, Zn, and Ni) and high mRNA expression of HSP70 and ABCB1 genes (Fig. 7d).

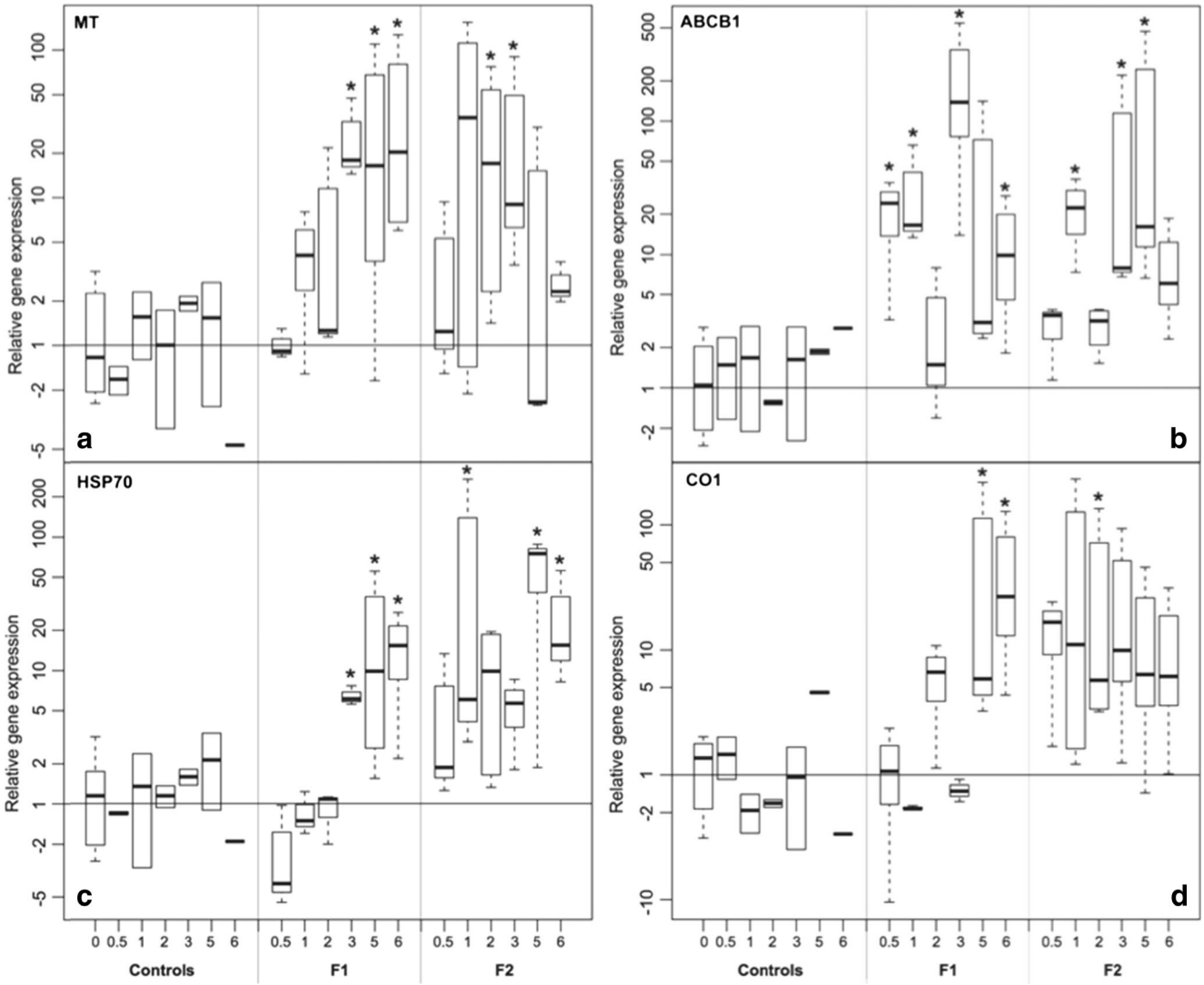

Fig. 4 Relative gene expression profiles for **a** MT, **b** ABCB1, **c** HSP70, and **d** CO1. Fold differences (log scale) are expressed relatively to controls at time 0. Significant differences from controls are indicated by an *asterisk* ($p<0.05$, Kolmogorov-Smirnov test)

Discussion

Metal concentrations in effluents

The effluents (F_1 and F_2) used in this study were discharged by a phosphate treatment plant localized near Skhira in the gulf of Gabès. F_1 was a global effluent produced by the three different units of production (sulfuric acid, phosphoric acid, and fertilizers) of the SIAPE factory, while F_2 was a product of a single unit (phosphoric acid). Therefore, it was not surprising to find higher metal concentration in effluent F_1 compared to F_2. F_1 and F_2 effluents appear in the high concentration range of industrial effluents (Hajem et al. 2007; Petala et al. 2009; Sancey et al. 2011; Santos Yabe and de Oliveira 2003), especially concerning Cd which concentration exceeds Tunisian and French norms (T.N. 106.002 and French Ministerial Decree of 22 February 1998, respectively). At 1 % of dilution (sub-lethal concentration used for the experimentation), the Cd concentration of F_1 still exceeds the Tunisian norm.

Metal accumulation in cockles' tissues

Cockles exposed continuously for 1 week to effluent F_1 or F_2 at a concentration of 1 % accumulated higher concentrations of all studied heavy metals (Cd, Cu, Zn, and Ni) than control cockles. The accumulation of metals has often been highlighted in different bivalve species (see Zuykov et al. 2013 for a review). Accumulation was higher in the cockles exposed to F_1 than in the ones exposed to F_2, in accordance with the metal concentration of the two effluents. However, accumulation did not increase proportionally with concentration, which is probably due to a saturation of the influx rate at very high concentrations (Luoma and Rainbow 2005). Cd was much less

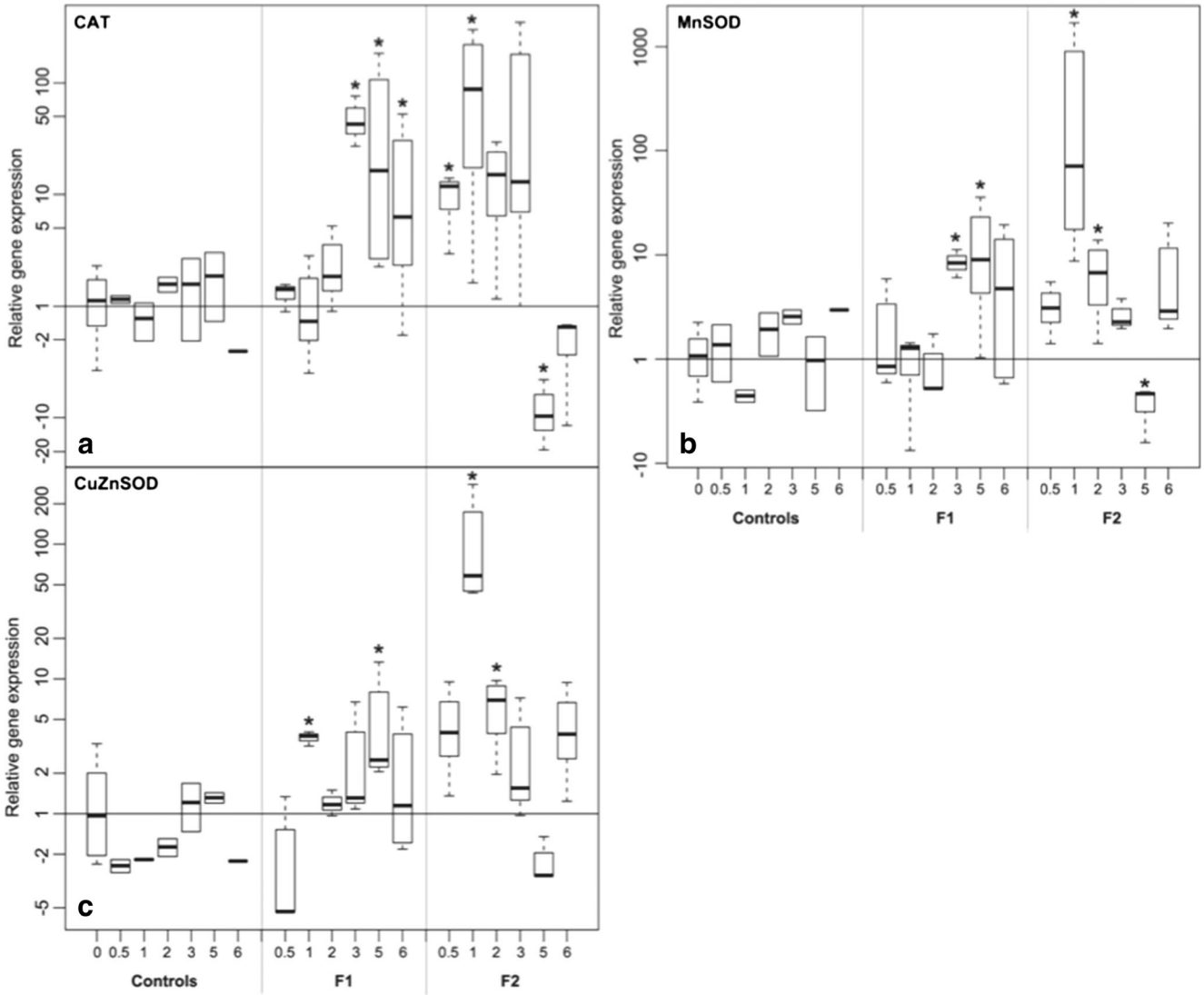

Fig. 5 Relative gene expression profiles for **a** CAT, **b** MnSOD, and **c** CuZnSOD. Fold differences (log scale) are expressed relatively to controls at time 0. Significant differences from controls are indicated by an *asterisk* ($p<0.05$, Kolmogorov-Smirnov test)

accumulated than the other metals, relative to their respective concentrations in the effluents, suggesting a lower uptake rate of this metal (Luoma and Rainbow 2005). For Ni and Zn, accumulation in cockles has been relatively constant during the length of the experiment (6 days). For Cu and Cd, accumulation increased with time. This increase was slight, except for Cd in cockles exposed to F_1 where it was exponential. According to Luoma and Rainbow (2005), low variation with exposure time may be due to a rapid rate of metal excretion, while a rapid increase in body metal concentration would

Table 2 Influence of treatment and of exposure time on relative gene expressions for each treatment versus control samples: sense of the effect and significance level

		ABCB1	CuZnSOD	MnSOD	CO1	HSP70	CAT	MT
F_1 effluent	Influence of treatment	+***	+*	+*	+**	+**	+***	+***
	Influence of exposure time	/	/	+*	+***	+***	+**	+**
F_2 effluent	Influence of treatment	+***	+***	+**	+***	+***	+**	+**
	Influence of exposure time	/	−***	−**	/	/	−***	/

Global effect: +, positive effect (increased expression); −, negative effect (decreased expression)

Statistical significance: *$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$; /, no significant effect ($p>0.05$)

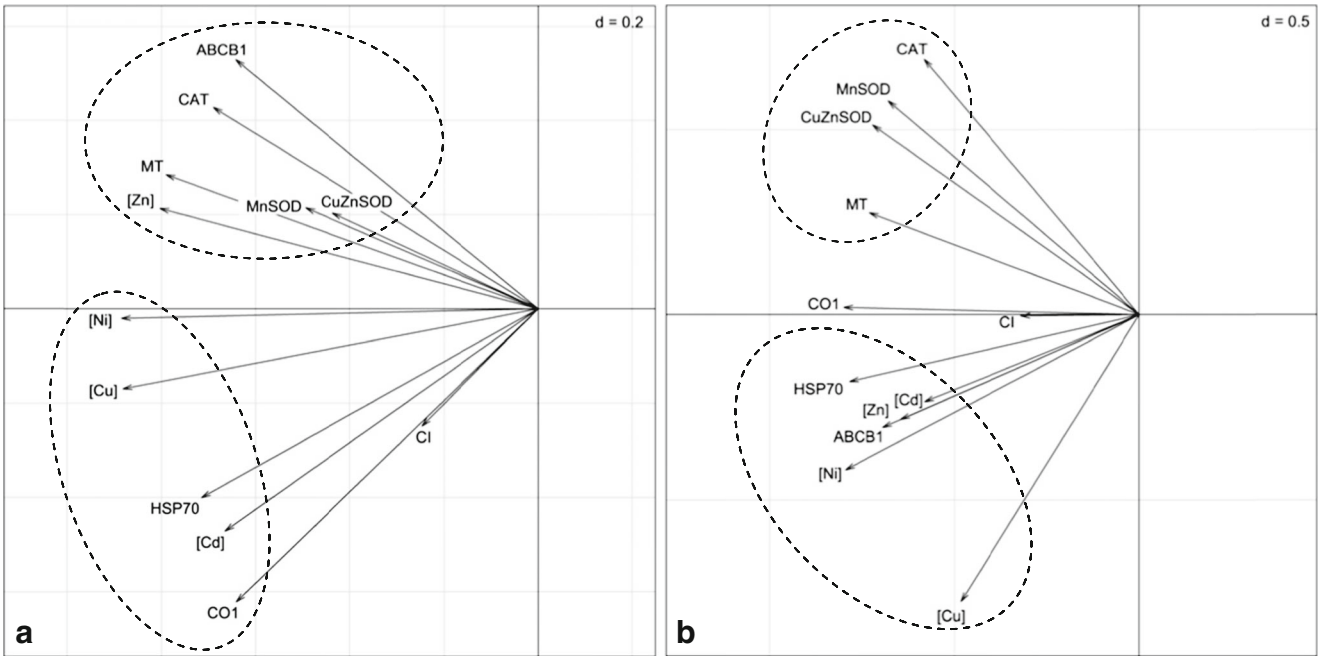

Fig. 6 Principal component analysis (PCA) on gene expressions, metal bioaccumulation (Ni, Zn, Cu, and Cd), and CI. **a** PCA performed on controls and cockles exposed to effluent F_1; **b** PCA performed on controls and cockles exposed to effluent F_2; *CI* condition index; [Ni], [Zn], [Cu], [Cd], and metal bioaccumulation in cockles

be due to the slow rate of excretion relative to the ingestion rate. Our results therefore suggest that excretion is more effective for Ni and Zn than for Cu and especially Cd in *C. glaucum*.

The global physiological state of cockles

Our data clearly demonstrate that exposure to effluent F_1 significantly affects LT50 data, reflecting metabolic perturbations and a general lower health status of cockles following effluent exposure. This result may suggest that protection and/or detoxification mechanisms as possible responses to toxicants could imply energetic costs and therefore could reduce the capacity to resist to an additional change, i.e., air exposure in our study (Viarengo et al. 1995). Such trade-offs have already been described in the literature (Forbes 2000; Handy et al. 1999; Van Straalen and Hoffman 2000). For effluent F_2, the same trend is detected though not significant. This difference may be explained by the highest concentrations in metals found in effluent F_1 compared to effluent F_2.

Measures of bivalve's condition based on weight are thought to be reliable indicators of the energetic health status and energy reserves in mollusks. 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 6 days of exposition might not be enough to produce perceptible variations between exposed cockles and control ones. This result is corroborated by the results of the two PCA analyses, which indicate that CI is a weak structuring parameter (small arrows).

Effects of effluent exposure on mRNA expression

In cockles exposed either to F_1 or F_2, mRNA expression is significantly higher than in controls for all the studied genes (Table 2; Figs. 4 and 5). Moreover, the much higher variability observed in exposed individuals (large box plots) suggests the existence of different types of responses among cockles experiencing the same exposition. Elements confirming this hypothesis may be found with the projections of individuals into the PCAs (Figs. 6 and 7). Indeed, for both effluents, control individuals are found quite grouped while exposed individuals are more scattered (Figs. 6 and 7).

The ATP-binding cassette xenobiotic transporter ABCB1 gene transcription seems to follow a wave pattern in the two effluent exposures. ABCB1 shows a rapid increase in expression (days 0.5 and 1) followed by a decrease and an additional peak at day 3 (for F_1) or 5 (for F_2). This marker is significantly correlated to Cd, Zn, Ni, and Cu accumulation in tissues following exposure to F_2 effluent. The ABCB1 transmembrane protein acts by actively exporting toxic compounds out of the cell (Pain and Parant 2003; Minier et al. 2006), and our results show that it is an actor of the response to metal exposure, its mRNA expression being modulated by the level of metals. Previous studies already highlighted a strong ABCB1 mRNA increase in marine organisms following exposure to

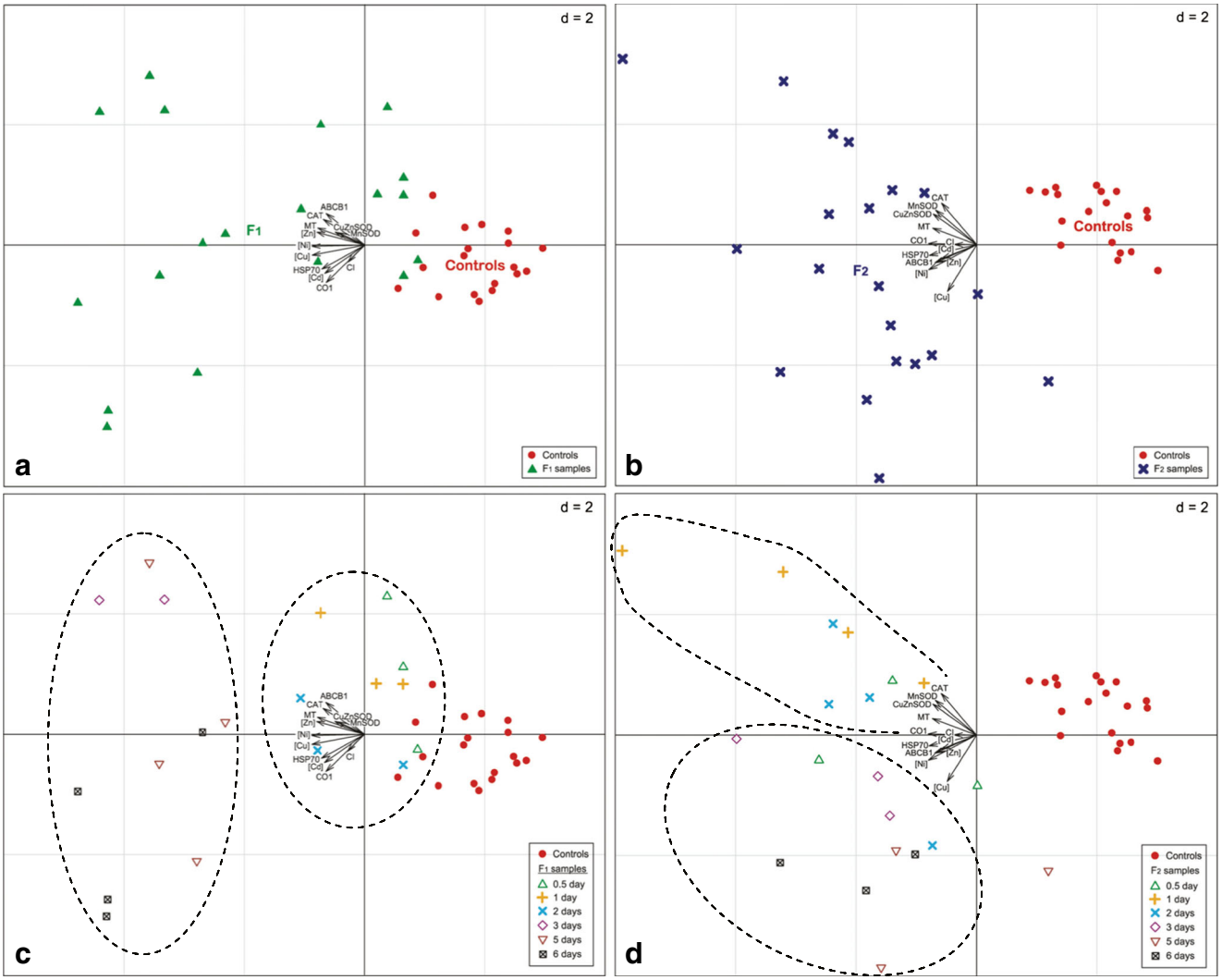

Fig. 7 **a** Projections of samples according to the different quantitative data presented in Fig. 5a (F_1 exposure); b projections of samples according to the different quantitative data presented in Fig. 5b (F_2 exposure); **c** projections of samples according to the time of exposure to F_1; **d** projections of samples according to the time of exposure to F_2

Cd and other metal exposure (Della Torre et al. 2014; Zucchi et al. 2010).

Considering both effluent exposures, antioxidative defenses are triggered through the mRNA expression of gene coding for CAT, CuZnSOD, and MnSOD. SODs (CuZnSOD and MnSOD being respectively the cytosolic and the mitochondrial form) are known to catalyze the dismutation of superoxide anions into hydrogen peroxides, which are in turn reduced by CAT into water and molecular oxygen. The increase of these antioxidant enzymes in response to effluent exposure seems to constitute an adaptation in gills to prevent and/or repair metal-induced damage in cellular component (Fernandez et al. 2010). Several studies have shown that metals can promote oxidative stress in cells (Sabatini et al. 2011; Trevisan et al. 2014; Wang et al. 2010), and in particular, Kamel et al. (2012) found a correlation between CAT, MnSOD, and metal accumulations in clams treated with municipal effluents.

MT was also induced in response to the uptake of metals in both exposures, with an inverted U shape and a significant higher expression of +35-fold at day 2 for the F_2 exposure while quite linear for the F_1 exposure, reaching a significant expression of +20 at day 6. The up-regulation of this gene is in accordance with previous experiments performed with different invertebrates following metal exposure (Al Kaddissi et al. 2012; Dondero et al. 2005; Lecoeur et al. 2004; Navarro et al. 2011; Zorita et al. 2007). It may reflect the role of MTs in the uptake and handling of metals, as well as their role as antioxidants reacting with free radicals and reactive oxygen species (ROS), reducing significantly the toxicity of ROS and preventing interactions with critical cellular components such as enzymes, structural proteins, DNA, and membrane lipids

(Amiard et al. 2006; Andrews 2000; Coyle et al. 2002; Ivanina et al. 2009; Kamel et al. 2012; Tanguy et al. 2001).

The biomarkers coding for CO1 and HSP70 showed a remarkably higher mRNA expression in cockles exposed to F_2, with in addition a high correlation to metal concentrations in cockles exposed to both effluents. For F_1 exposed cockles, this up-regulation is more observed in the second part of the experiment (3 to 6 days). The CO1 (cytochrome oxidase 1) transfers electrons from reduced cytochrome c to molecular oxygen in the mitochondrial respiratory chain and contributes to the production of an electrochemical proton gradient across the mitochondrial inner membrane that drives the synthesis of ATP (Capaldi 1990; Capaldi et al. 1983). Its increase in mRNA expression following effluent exposure indicates a higher metabolic activity in exposed cockles. This could be a cellular strategy (1) to compensate for the decrease in the number of functional mitochondria (through inhibition phenomenon or membrane potential decrease) by increasing ATP production, so as to provide enough energy for cellular needs, especially to tolerate metals (Al Kaddissi et al. 2012), and/or (2) to efficiently consume O_2 to limit metal-induced oxidative damage in cells (Chen 2003; Wang et al. 2004). It has to be noted that the gene transcription level of CO1 correlated well with those of antioxidant enzymes (CuZnSOD, MnSOD, and CAT) in cockles exposed to F_2 effluent. Previous studies have highlighted an up-regulation of the CO1 gene in invertebrates following Cd and other metals exposure (Achard-Joris et al. 2006; Al Kaddissi et al. 2012; Navarro et al. 2011). The response of HSP70 is quite similar to that of CO1 after exposure to F_1 and F_2. 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). Studies have already reported an increase in HSP70 expression in bivalves such as *Dreissena polymorpha* and *Crassostrea virginica* (Gmelin) upon the presence of metals like Cu, Cd, Pd, Pt, and Rt (Ivanina et al. 2008, 2009; Navarro et al. 2011). Even if HSP70 are known to be synthesized intensively in response to a variety of harmful stimuli (heat, metals, organic contaminants, injuries, disease, …), the strong up-regulation of its gene (reaching +15- and +75-fold, the mean expression level of controls for F_1 and F_2, respectively) reflects a mechanism highly settled to protect cells against stress-induced damage.

Finally, it has to be noted that exposure concentration (F_1 versus F_2) seems to have an impact on the global gene transcription profile over time. Indeed, mRNA up-regulations for the different genes studied (excepted ABCB1) are faster and higher in F_2 compared to F_1, though F_1 effluent contains higher concentrations of the different metals than F_2 and leads to higher metal accumulation in tissues. Similarly, recent studies on gene expression in daphnia, zebrafish, and fathead minnows following exposure to several metals over a range of concentrations highlighted that the transcription of several genes occurred at the lower metal concentrations (even though the accumulation was greater at higher concentrations). A high stress level (which is the case for F_1) may cause several transcriptional inhibitions and/or mRNA degradations that may impede or delay defense mechanisms (Klaper et al. 2008; Lerebours et al. 2010; Poynton et al. 2008). Moreover, at the end of the F_1 experiment, RNA copy number seems to be in an increasing phase for most of the seven genes studied.

Conclusion

Gene transcription monitoring performed on seven genes potentially involved in the tolerance to metal exposure showed that for both exposures (though different in concentrations), mechanisms are rapidly and synchronically settled down to prevent damage to cellular components, by (1) handling and exporting out metal ions through the up-regulation of ABCB1 and MT, (2) reducing the toxicity of ROS through the expression of antioxidant enzymes (CAT, CuZnSOD, and MnSOD), (3) protecting and/or repairing proteins through the expression of HSP70 mRNAs, and (4) increasing ATP production (through the up-regulation of CO1) to provide energy for cells to tolerate stress exposure. These molecular responses imply energetic costs and therefore might explain the reduced tolerance of cockles to anoxia observed with the stress on stress test. The tools developed may be useful both for future control strategies and for the use of the cockle *C. glaucum* as a sentinel species. However, future investigations should be performed to validate these biomarkers in field conditions. Moreover, parallel biochemical, physiological, immunological, and/or morphological/pathological data should complement this study to better assess metal toxicities and their mechanism of action.

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