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Response of the pearl oyster *Pinctada margaritifera* to cadmium and chromium: Identification of molecular biomarkers

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ABSTRACT

This study was designed to identify in the pearl oyster *Pinctada margaritifera*, used as a bio-accumulator, molecular biomarkers for the presence of heavy metals in the lagoon environment. Pearl oysters were exposed to 2 concentrations (1 and 10 μg L^{-1}) of cadmium (Cd) and chromium (Cr) compared to a control. Twelve target genes encoding proteins potentially involved in the response to heavy metal contamination with antioxidant, detoxification or apoptosis activities were selected. *P. margaritifera* accumulated Cd but not Cr, and mortality was related to the amount of Cd accumulated in tissues. In response to Cd-Cr contamination, metallothionein (MT) was significantly up-regulated by Cd-Cr at both concentrations, while 7 others (SOD, CAT, GPX, GSTO, GSTM, CASP, MDR) were down-regulated. Based on the development of these molecular tools, we propose that the pearl oyster, *P. margaritifera*, could be used as a sentinel species for heavy metal contamination in the lagoons of tropical ecosystems.

Heavy metal contamination in coastal and marine environments has become an increasingly serious threat to both the naturally stressed marine ecosystems and humans that rely on marine resources for food, industry and recreation. Heavy metals contribute to the degradation of marine ecosystems by reducing species diversity and abundance and through their accumulation and reminiscence in living organisms and throughout the food chains (Hosono et al., 2011). They can be introduced into coastal and marine environments via a variety of sources, including industry, wastewater and domestic effluent (Fu and Wang, 2011). The biomonitoring of the aquatic environment and the assessment of ecosystem health play essential roles in the development of effective strategies for the protection of the environment, human health and sustainable development.

The use of biological responses to contaminant exposure by sentinel species has become a useful tool in environmental quality evaluation and risk assessment. Among the numerous ecotoxicological biomarkers proposed in recent decades, those based on responses at the molecular and cellular levels usually provide the earliest signals of environmental disturbance and are commonly used for biomonitoring (Moore et al., 2004; Viarengo et al., 2007). Looking at heavy metals in the water column or in the sediment is very difficult because of the low concentrations and transient process, but many organisms have the potential to absorb heavy metals from ambient waters to extremely high levels. Because of the widely different environments that they inhabit, bivalves such as mussels, oysters and pearl oysters have been extensively utilised as bioindicators in environmental toxicology (Sidoumou et al., 2005; Viarengo et al., 2007; Maanan, 2008; Giarratano et al., 2010; Naser, 2013; Taylor et al., 2013; Izagirre et al., 2014). These bivalves are sessile and filter-feeding organisms and are appropriate sentinel organisms for pollution-monitoring studies.

The coral reefs of French Polynesia are considered to be healthy overall (Salvat et al., 2008), but increasing anthropogenic activity is leading to significant impacts in some areas, especially near urban centers. In this context, the objective of this study was to evaluate the use of the pearl oyster *Pinctada margaritifera* as a model sentinel species to characterise the health status of coral ecosystems. *P. margaritifera* pearl oysters were exposed to cadmium and chromium at different concentrations within a controlled environment in order to evaluate mortality and the transcriptional response of 12 selected putative marker genes. These genes encoded candidate biomarkers, including molecules commonly used for biomonitoring in other species (proteins with antioxidant, detoxification or apoptosis activities) (Taylor et al., 2013; Kim et al., 2014). This targeted transcriptome analysis of *P. margaritifera* identified predictive molecular biomarkers and indicators of the presence of heavy metal contamination in a coral ecosystem.

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P. margaritifera (n = 150) from Takaroa Atoll (Tuamotu Archipelago, French Polynesia) were grown in the Vairao lagoon for 2.5 years and brought to the Ifremer laboratory in Tahiti, French Polynesia.

The monitoring was carried out under controlled conditions for 1 month and involved 124individuals (n = 124) with an average height of 82 mm. After 1 week of acclimation, the contaminants cadmium (Cd) and chromium (Cr) were introduced at the same time as the feeding of oysters, at 2 concentrations for each metal (1 μg L^{-1} and 10 μg L^{-1}) (Table 1). Control conditions corresponded to a tank with no contamination. A decontamination phase followed, and the concentration levels of the 2 metals were measured in triplicate (Table 1). During the bioassay test period, the pearl oysters were fed with the microalgae *Isochrysis galbana* (T-Iso) and *Chaetoceros gracilis* at a constant concentration of 10 cell μL^{-1} throughout the experiments. The temperature was stable, around 27.9 ± 0.2 °C.

Three oysters per treatment were sampled at different times (Table 2). For low concentration tanks, samples were taken 1, 4 and 17 days after the start of contamination (D1, D4 and D17). Contamination was stopped at D17, and sampling was done at D21 and D25 during the decontamination phase. For high concentration tanks, samples were taken 1, 4 and 8 days after the start of contamination (D1, D4 and D8). Contamination was stopped at D8, and sampling was done at D11 during the decontamination phase. This sampling strategy in its entirety is shown in Table 2. For each harvested oyster, a fragment of the gill was sampled and subsequently stored at −80 °C. Dead oysters were removed daily from the tanks.

Cadmium $CdCl_2$ (cadmium chloride anhydrous, CAS 10108-64-2, Alfa Aesar) and chromium CrO_3 (chromium [VI] oxide, CAS: 1333-82-0, Sigma-Aldrich) were used for conducting the heavy metal toxicity studies. The two heavy metals, cadmium (Cd) and chromium (Cr), were quantified in sea water soft tissue through atomic absorption spectrometry with flame (Usero et al., 2005; Izagirre et al., 2014). A calibration range was prepared for each heavy metal. Metal dissolution was performed in sea water. For each metal (Cd and Cr), the salts were placed in seawater for 24 h, and the solutions were then filtered to remove undissolved residue. Previously calibrated filters were dried and weighed after filtration to obtain the mass balance of the two phases: particulate and dissolved. Each collected oyster that had previously been drained and weighed to measure fresh weight was frozen for 24 h and then freeze-dried and milled. After mineralization at 550 °C, the oyster tissues were digested in a mixture of concentrated acid (HNO_3 and 5% HCl). The heavy metal concentration was analysed following complete digestion.

The target genes were selected based on a literature study and existing molecular data for the *P. margaritifera* pearl oyster (Joubert et al., 2010; Marie et al., 2012). The selected candidate genes were as follows: Cu/Zn superoxide dismutase (*Pmarg*-SOD), metallothionein (*Pmarg*-Metal), cytochrome *c* oxidase (*Pmarg*-CytC), caspase (*Pmarg*-Casp), heat-shock proteins (*Pmarg*-HSP70, *Pmarg*-HSP90), catalase (*Pmarg*-Cat), glutathione peroxidase (*Pmarg*-GPX), multidrug resistance protein (Pmarg-MDR1), inhibitor of apoptosis (*Pmarg*-IAp) and glutathione S-transferase omega class (*Pmarg*-GSTO) and Mu-class (*Pmarg*-GSTM). The primer sequences of target genes for real-time qRT-PCR were determined using Primer3 software (Table 3). The primers were synthesised by Eurogentec (www.eurogentec.com).

Gill samples of three oysters per treatment for qPCR analyses were performed at different points in time. Total cellular RNA was extracted using TRIZOL® Reagent (Life Technologies) according to the manufacturer's recommendations. After precipitation with sodium acetate, the RNA was treated with DNase I (DNA-free Kit™ DNase Treatment and Removal Reagents, Ambion), and the RNA integrity was assessed on a 1% agarose gel. The RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Inc.). The gene expression levels were analysed with quantitative RT-PCR analysis using a set of forward and reverse primers (Table 3). A universal set of primers for the 18S rRNA gene was used as a primary reference gene; these were designed based on the 18S rRNA sequence alignment of different bivalve species (Uni1304F: TTAGTTGGTGGAGCGATTT; Uni1670R: TAGCGACGGGCGGTGTG). A second reference gene was chosen based on its ubiquitous and constitutive expression pattern in *P. margaritifera* tissue (REF1S: AGCCTAGTGTGGGGGTTGG; EF1AS: ACAGCGATGTACCCATTTCC). First-strand cDNA was synthesised from 800 ng total RNA using the Transcriptor First-Strand cDNA Synthesis Kit (Roche) and a mix of poly(dT) and random hexamer primers. The qPCR amplifications were carried out on a StratageneMX3000P, using Brilliant II SYBR® Green QPCR Master Mix (Stratagene) with 400 nM of each primer and a 1 μL cDNA template. The following protocol was used: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60°Cfor 1 min and extension at 72 °C for 30 s. Finally, the amplicon melting temperature curve was analysed using a melting curve program: 45–95 °C with a heating rate of 0.1 °C s^{-1} and continuous fluorescence measurements. All measurements were performed in duplicate. The comparative Ct (threshold cycle) method was used to analyse the expression levels of the genes of interest. All analyses were based on the Ct values of the PCR products. The relative expression ratio of each analysed cDNA was calculated based on the delta–delta method, normalised by the two reference genes for comparing the relative expression results, which is defined as follows: ratio $= 2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ calibrator]} = 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Here, the ΔCt calibrator of each gene is the ΔCt value mean of each gene.

The Cd and Cr concentrations in pearl oysters during the experiment were compared with the concentrations in pearl oysters at T0 using Dunnett's test. The expression values of the 12 candidate genes were compared using two-way ANOVAs after normalisation with the Box-Cox transformation to meet the condition for parametric ANOVAs (Shapiro-Wilk and Bartlett tests). Two-way ANOVAs were done to determine the effect of metal on gene expression compared to the control condition, the changes of gene expression during time exposure and the 'metal × time' interaction. Post hoc comparison was done using Tukey's post hoc test.

Following the exposure of *P. margaritifera* pearl oysters to high Cd and Cr heavy metal concentrations (10 μg L^{-1}), the cumulative mortalities were 42.8% at the end of the contamination step on D7 (Fig. 1).

Table 2
Sampling protocol: number of pearl oyster sampled according to the exposition level and duration. Numbers in bold indicate that the pearl oysters were in the decontamination step.

	D1	D4	D8	D11	D17	D21	D25	Total sampling
High	3	3	3	**3**				12
Low	3	3			3	**3**	**3**	15
Control	3	3	3	3	3	3	3	21

Table 1
Experimental design.

	Cadmium μg L^{-1}	Chromium μg L^{-1}	Pearl oyster number/tank	Replicate tank	Total number of pearl oyster
High	10.22 ± 0.11	11.47 ± 1.64	15	3	45
Low	1.02 ± 0.001	1.15 ± 0.13	15	3	45
Control	0	0	17	2	34

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Table 3
Primer sequences for qRT-PCR. Cu/Zn superoxide dismutase (Pmarg-SOD), metallothionein (Pmarg-Metal), cytochrome *c* oxidase (Pmarg-CytC), caspase (Pmarg-Casp), heat-shock proteins (Pmarg-HSP70, Pmarg-HSP90), catalase (Pmarg-Cat), glutathione peroxidase (Pmarg-GPX), multi-drug resistance protein (Pmarg-MDR1), inhibitor of apoptosis (Pmarg-IAp1), glutathione S-transferase omega class (Pmarg-GSTO) and mu-class (Pmarg-GSTM).

Gene name	Forward primer (5′–>3′)	Reverse primer (3′–>5′)
Pmarg-SOD	ATCTTGGAAATGTCACAGCAG	CATCAATATCAGCATGCACCA
Pmarg-Metal	CCATGTGCTTGTTCTGACTC	TACATCCTACTCCACATCCAC
Pmarg-CytC	GGATAGATGTAGACACTCGG	CAATGTCTAAAGAGGCGCTG
Pmarg-Casp	ATGGTGACAATGGAGTGGTG	TGACAGGCTTGGATAAAGAAC
Pmarg-HSP9	0 CAAGGCAGAGTTTGAAGGAC	CAGCCTGTTAGATACCACCA
Pmarg-HSP7	0 TCTGTGCCTTGTCCATCTTG	AACCCTGTCATCCAGTACAC
Pmarg-CAT	AGATTATGCCATCCGTGACC	TCCTGTTCAACACCATCCTG
Pmarg-GPX	ACTTCTACGGAATCTGACGG	GTCTCACATAGCGTAGACCA
Pmarg-MDR1	AACACATAGGCTTAGTTGGTC	CAATGTCTCGTAGCCATCTG
Pmarg-IAp1	ATTGAGGAATTGGGAGCCAG	GCTGACAAAGCAACATTGCC
Pmarg-GSTO	ACAGTATGAGGTTCTGTCCC	ATCTTTCCGTCCTTCTCCAG
Pmarg-GSTM	GACAAACAATATGAGCAGGGA	CCAGAAGTCCATGTTTAGCAG

Mortality significantly increased during the decontamination step, reaching 62%. At low concentrations (1 μg L^{-1}), cumulative mortality reached 36% on D17 at the end of the contamination phase, and mortality increased by only 6% by D25 (Fig. 1). Finally, mortality affecting control oysters reached 10% at the end of the experiment on D25. Mortality appears to be independent of the concentration of Cr. However, the concentrations of Cd accumulated in the pearl oysters reached 59 and 108 μg kg^{-1} dw at the mortality threshold of 50% for the 2 metal tested concentrations 1 and 10 μg L^{-1}, respectively (Table 4).

The concentrations of contaminant were measured using total soft tissue of pearl oysters. In the control, the Cd concentration in pearl oysters was between 3.19 and 10.85 mg kg^{-1} dry weight throughout the experiment. No significant variation in these concentrations was observed over time (Fig. 2a). In the contaminated tanks, Cd accumulated in pearl oysters. For the 1 μg L^{-1} condition of contamination, the mean concentrations ranged from 10 (control value) to 60 mg kg^{-1} dry weight after 17 days of contamination (Fig. 2a). For the 10 μg L^{-1} contamination treatment, the mean concentrations for 3 oysters were between 18 and 85 mg kg^{-1} dry weight after 7 days of contamination (Fig. 2a). The contact time was long enough to observe the accumulation of Cd in the animal tissue. The cessation of the daily addition of metals (D17 and D8 for the 1 μg L^{-1} and the 10 μg L^{-1} contamination treatments, respectively) coincided with the end of the increase in the concentration of Cd in the oyster tissues. However, no subsequent decrease in the heavy metal concentration in pearl oyster tissue was observed, showing that decontamination takes much longer than contamination.

Table 4
Metal concentration (μg kg^{-1} dw) in tissues of animals when mortality reached 50% of the population.

Metal dose		Cd
0	Mean value	7.8
1	LD 50 value	59
10	LD 50 value	108

There was no accumulation of Cr in pearl oyster tissues during the contamination (Fig. 2b). Indeed, throughout the experiment, the measured concentration of Cr was < 10 mg kg^{-1} of dry weight, and no difference between control and contaminated oysters was observed.

Data analysis consisted of the study of gene expression kinetics associated with the accumulation of heavy metals in pearl oysters. The results highlighted two categories of genes: those whose expression was independent of the contamination with heavy metals and those that significantly changed after contamination by heavy metals.

At the dose of 1 μg L^{-1}, only 3 genes, of the 12 tested, showed that their relative expression significantly changed as a result of exposure to heavy metals (Table 5, Fig. 3). These were the MT, HSP90 and CAT genes. The MT gene expression was significantly up-regulated during the contamination phase; this state persisted during the decontamination phase. The response of the gene HSP90 appeared later on, only in the decontamination phase. The CAT gene was significantly under-regulated with the exposure to heavy metals (Fig. 3). The expression of 3 genes varies significantly under the effect of the interaction between metal and the duration of exposure. Those are the SOD, GSTO and HSP70 genes. This interaction may be due to a sub-expression during the phase of contamination and a recovery of the expression at the end phase of decontamination for the SOD gene. This phenomenon

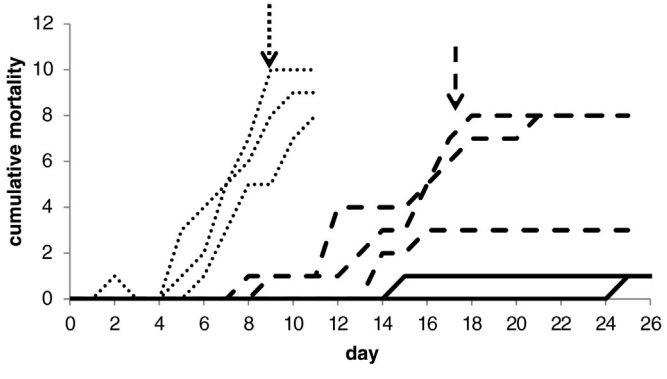

Fig. 1. Cumulative mortality of pearl oysters during the experimental contamination by cadmium (Cd) and chromium (Cr) in controlled conditions. Initially, each tank contained 15 pearl oysters. Contaminated tanks were in triplicate (dotted and dash lines for high and low metal contamination respectively), control tank were in duplicate (continuous line). Arrows represent the end of the contamination phase and the beginning of the decontamination phase (D8 and D17 for high and low metal contamination respectively).

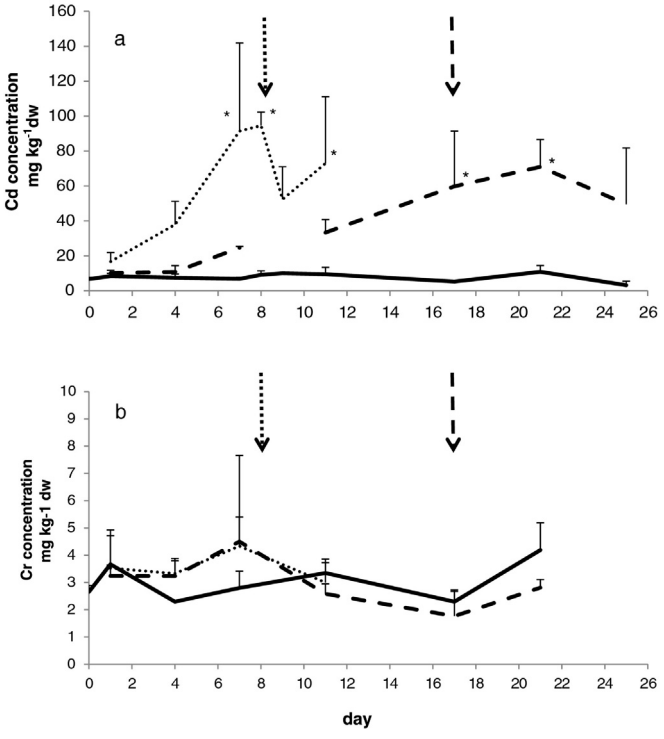

Fig. 2. (a) Cadmium (Cd) and (b) chromium (Cr) concentrations in the pearl oyster (n = 3). The error bars represent the standard error. Dotted and dash lines are for high and low metal contamination, respectively, and continuous line for control. Arrows represent the end of the contamination phase and the beginning of the decontamination phase (D8 and D17 for high and low metal contamination, respectively). Asterisks indicate the significantly different averages compare to T0 control.

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Table 5
ANOVA significance level of gene expression according to the metal dose and the kinetic of contamination and decontamination.

	Factors	SOD	Metal	GSTO	HSP90	CAT	MDR	CytC	HSP70	GPX	CASP	IAp1	GSTM
0 vs 1 µg	Dose	0.166	0.009	0.164	0.033	0.041	0.660	0.060	0.348	0.974	0.062	0.440	0.648
	Time	0.082	0.823	0.957	0.021	0.177	0.696	0.018	0.048	0.029	0.190	0.020	0.001
	Interaction	0.002	0.474	0.036	0.258	0.201	0.999	0.348	0.031	0.172	0.165	0.142	0.397
0 vs 10 µg	Dose	0.005	<0.0001	0.013	0.101	<0.0001	0.025	0.716	0.274	0.018	0.001	0.465	0.028
	Time	0.143	0.048	0.252	0.638	0.726	0.349	0.054	0.765	0.025	0.375	0.325	0.002
	Interaction	0.083	0.485	0.150	0.658	0.268	0.645	0.088	0.304	0.009	0.206	0.911	0.076

applies with greater force to the expression of the HSP70 gene. The expression of the gene GSTO transiently decreased to the D4 phase of contamination and became similar to the control at the end of the phase of contamination at D17 (Fig. 3).

At the dose of 10 µg L^{-1} (Table 5, Fig. 4), the expression of 8 genes was affected by the contamination with heavy metals. Those genes were SOD, metal, GSTO, CAT, MDR, GPX, PSAPS and GSTM. The effects translate into different patterns of response: an over-expression during the contamination phase (SOD, GPX, PSAP, GSTM), a permanent under-expression (GSTO, CAT, lAp1) and a permanent over-expression for MT. The GPX gene was significantly over-expressed only in the decontamination phase (Fig. 4).

The relationship between the Cd content in the tissue of the pearl oysters and the average relative expression of the gene coding for MT is a non-linear regression equation as follows:

$$MT = 3.03 \times \ln(Cd) - 4.844$$

The correlation between modeled and measured values of the expression of MT in relation to the Cd content in tissues is highly significant ($r = 0.890$). This non-linear regression includes a phase where the expression of MT increases with the amount of Cd accumulated in tissues between 0 and 25 mg Cd kg^{-1} dw, followed by a phase where the expression of MT reaches its maximum regardless of the Cd content accumulated in tissues (Fig. 5).

The heavy metal concentrations used in the experiment were within the dose range previously cited in the literature for laboratory metal exposure experiments (Thompson et al., 2011; Taylor et al., 2013; Kim et al., 2014) and are also comparable to those observed in some heavily polluted marine environments (Giarratano et al., 2010; Taylor et al., 2013; Chakraborty et al., 2014). In our study, mortality was positively correlated with increasing concentrations of metals as well as increasing exposure periods. Indeed, the cumulative mortality during the exposure period showed that half of the contaminated pearl oysters died at D9 and D22 at high and low Cd-Cr concentrations, respectively. Similar reports on the mortality of marine mollusks such as oysters, mussels, snails and clams were observed in relation to heavy metal toxicity (Sommanee, 1980; Mc Innes and Calabrese, 1978; Eisler and Hennekey, 1977). To a thousand times higher concentration, the lethal time 50% occurs between 1 and 3 days in *Perna viridis*, *Modiolus carvalhoi* and *Donax speculum* exposed to Cd (Mohan et al., 1984).

In the present study, the Cd concentration in the control group of pearl oysters was of a 7.8 mg kg^{-1} dry weight throughout the experiment, which was within the range of those previously observed for *Mytilus edulis* or *M. galloprovincialis* mussels and *Crassostrea gigas* oysters exposed in the natural environment (Giarratano et al., 2010; Maanan, 2008; Sidoumou et al., 2005). The Cr mean concentration in the control group of pearl oysters was of a 3.04 mg kg^{-1} dry weight in the range of 1.68–5.44 mg kg^{-1} dry weight. The concentration scale found in this investigation is within the ranges found in many other bivalves (Eisler, 1981; Nicolaidou, 1994; Conti and Cecchetti, 2003; Tiapa et al., 2010).

In the contaminated tanks, pearl oysters accumulated Cd but not Cr. The contact time was long enough to observe the accumulation of Cd in the animal tissue, which reached 59 and 108 mg kg^{-1} dw at the threshold of 50% mortality at 1 and 10 µg L^{-1}, respectively. A similar degree of Cd accumulation in *P. margaritifera* was also observed following experimental contamination of *M. galloprovincialis* mussels (Izagirre et al., 2014). The cessation of the daily addition of metals coincided with the end of the increase in the concentration of Cd in the oyster tissues. However, no subsequent decrease in the heavy metal concentration in pearl oyster tissues was observed, indicating that decontamination takes

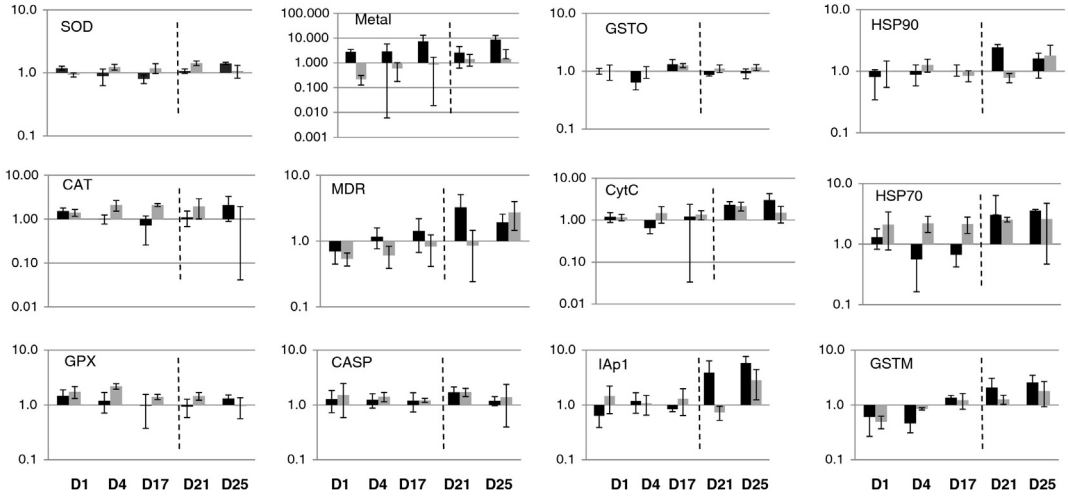

Fig. 3. Relative expression levels of genes encoding proteins potentially involved in the stress response to low cadmium (Cd) and chromium (Cr) exposure in the pearl oyster *P. margaritifera* during the kinetic of contamination and decontamination. The dotted lines represent the end of the contamination phase and the beginning of the decontamination phase (D17).

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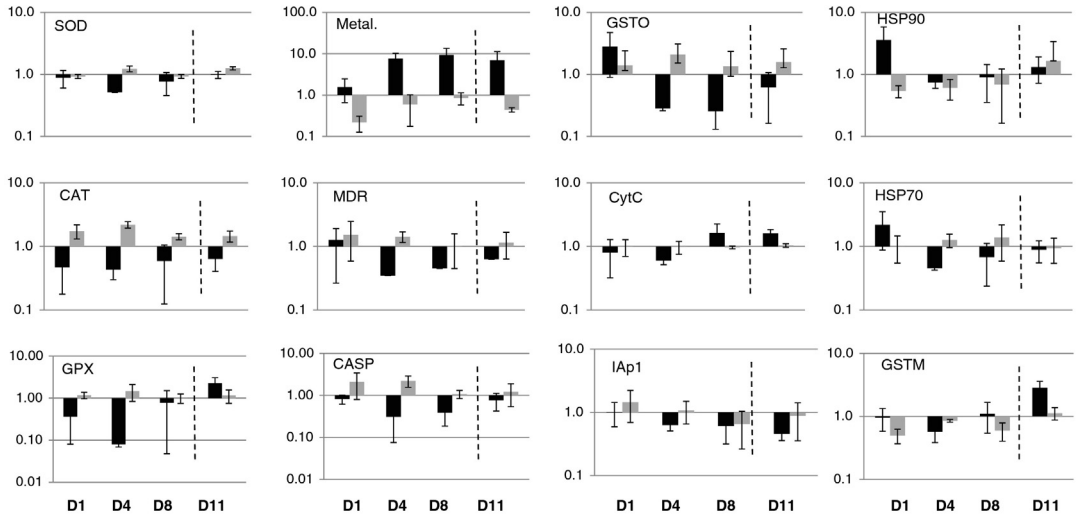

Fig. 4. Relative expression levels of genes encoding proteins potentially involved in the stress response to high cadmium (Cd) and chromium (Cr) exposure in the pearl oyster *P. margaritifera* during the kinetic of contamination and decontamination. The vertical dotted lines represent the end of the contamination phase and the beginning of the decontamination phase (D8 for the high contamination treatments).

much longer than contamination. This is strong support for the use of *P. margaritifera* as a sentinel species, since Cd contamination shows long-term retention and detectability in the gills.

There was no accumulation of Cr in the pearl oyster tissues during the contamination. These results differ from those of Medina et al. (1986), who observed a high concentration of Cr in bivalves from the Mediterranean Sea. However, apart from the measured traces of Cr in the environment, few contamination experiments with Cr have been performed with bivalves. A similar experiment showed a significant accumulation of Cr in mussel gills after 96 h treatment with 0.1 and 1 μg L^{-1} Cr (Ciacci et al., 2012). These different results might be explained by the experimental conditions or the form of Cr used, or by the limit of sensitivity of the detection method, but they also emphasise that Cr is poorly retained by pearl oysters.

Cd ions entering cells are transported to target tissues and act by a molecular and ionic mechanism, substituting the proper ions in their metabolic sites (Bridges and Zalups, 2005). Since Cd is a permanent metal ion, it is accumulated by many organisms that determine oxidative stress, DNA damage, increase in stress proteins (HSPs) and macromolecular damage (Ercal et al., 2001). In the different tissues or cells in the body, Cd selectively focuses on a number of molecules, such as MT, which ensure its detoxification. As a result, Cd is in competition with other metals (zinc, copper). Exposure to Cd may also stimulate the production of MTs; indeed, recent studies have shown that Cd can accumulate in aquatic invertebrates, causing a rise in the intracellular level of MT (Amiard et al., 2006; Ma et al., 2008, 2009). In the present study, the expression of the gene coding for MT has been strongly stimulated as a result of the Cd exposure, and as in other taxa such as fish (Espinoza et al., 2012), the response is as strong with 1 μg L^{-1} as with 10 μg L^{-1}. While Cd accumulation is correlated to MT protein accumulation in the gills and mantle of mussels (Li et al., 2015), this suggests that there is a transcriptional level of MT regulation under the effect of the dose of Cd. In addition, it indicates a translational level of regulation, as has also been suggested in *C. gigas* (Marie et al., 2006). The up-regulation of MT in *P. margaritifera* persists at least 8 days after the cessation of the contamination at the low Cd concentration, due to the persistence of cadmium accumulated in the flesh. An important result of this study is obtained with the establishment of the relationship between Cd accumulated in tissue and relative MT expression. This could also suggest that the detoxification capacity is limited as soon as the Cd content accumulated in the tissues exceeds the 25 mg kg^{-1} dw, and beyond this level of contamination, mortality occurs.

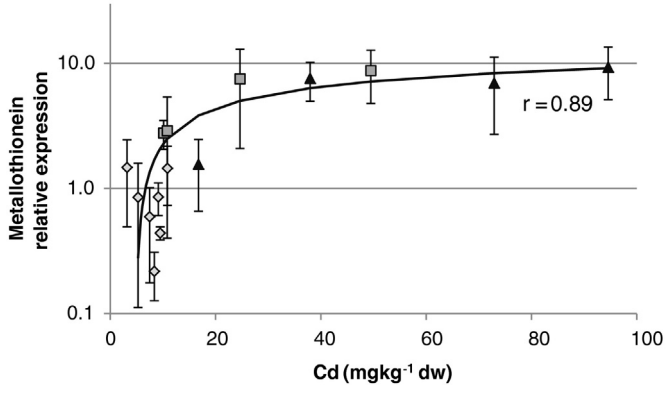

Fig. 5. Effect of cadmium (Cd) contamination in tissue on the relative expression of metallothionein (MT). Light grey rhombuses correspond to control, dark grey square to low contamination and black triangle to high contamination.

Cadmium (Cd) is known to influence the oxidative status of marine organisms and can induce the formation of reactive oxygen species (ROS). Lipid peroxidation by ROS is considered to be a major mechanism by which oxyradicals can cause tissue damage, leading to impaired cellular function and alteration in physicochemical properties of cell membranes (Vlahogianni and Valavanidis, 2007). It has been reported that Cd affects the functioning of antioxidant enzymes such as CAT and SOD (Wang et al., 2013), but there is a level of transcriptional regulation in an attempt to neutralise the effects on enzyme activity by increasing the level of expression of the SOD and CAT genes, as found in the octopus (Nicosia et al., 2015). This compensation mechanism does not seem to be present in *P. margaritifera* because the SOD and CAT genes are strongly down-regulated by Cd, reducing the corresponding enzymes' synthesis capability. In addition to the antioxidant enzymatic system that represents the SOD and CAT genes, exposure of *P. margaritifera* to a Cd dose of 10 μg L^{-1} caused a negative response of a non-enzymatic system represented by the gene coding for the GPX, GSTO and GSTM genes. Literature describes the effect of low concentrations of cadmium, which increase the GSH concentration in cells (Pathak and Khandelwal, 2006), allowing the cell to implement defense mechanisms. Indeed, the exposure of *P. margaritifera* to a low dose of Cd has not disrupted the GPX and GST systems. However, at high concentrations of toxic substances, antioxidant systems are repressed, and synthesis decreases reflected the response of GPX, GSTO and GSTM of *P. margaritifera* exposed to the highest Cd concentration.

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Heat shock proteins are involved in protection against a wide range of environmental stressors, and their expression is triggered by denatured/misfolded proteins in the cell. Cytosolic HSP70 and HSP90 are among the most abundant cellular proteins protecting against stress-induced damage (Mayer and Bukau, 2005). Most commonly, HSP70expression in bivalve molluscs is up-regulated by exposure to low concentrations of metals, whereas that of HSP90 is unchanged (Taylor et al., 2013). However, Werner and Hinton (1999) found that the expression of HSP70 and HSP90 was down-regulated in contaminated field sites compared to controls. They hypothesised that this might be due to the disruption of the protein synthesis in highly contaminated environments. Thompson et al. (2011) also found a reduced level of HSP70 protein expression in response to Cd, Pb and Zn, whereas no significant difference in the expression of HSP90 was observed. The response pattern expressed by *P. margaritifera* to contamination by Cd seemed atypical for the HSP. Indeed, at high doses, greater expression of HSP70 and HSP90 were observed only 1 day after the contamination at high concentrations of Cd (10 $\mu g\ L^{-1}$), but this over-expression did not persist, which is sufficient to protect cells with cellular repair mechanisms. The detoxification genes' encoding, multidrug resistance protein (MDR1) and cytochrome *c* oxidase (CytC), as well as that of inhibitor of apoptosis (IAp1), a gene involved in the synthesis of proteins associated with apoptosis, showed negatively modified expressions. Like the caspase (CASP) down-regulation with high Cd levels, this could correspond to a weakening of the anti-inflammatory response (McIlwain et al., 2013), and this response would be independent of apoptosis (Lemarié et al., 2004; Shih et al., 2004).

This study demonstrates that exposure to Cd elicits significant changes in the transcription of individual genes in *P. margaritifera*. Among the 12 candidate genes potentially involved in the response of the pearl oyster, *P. margaritifera*, to heavy metal contamination, eight were potentially biomarkers of a heavy metal contamination in the environment. Two of them were the most sensitive, MT and CAT genes were respectively up-regulated and down-regulated, and both sensitive at low doses of Cd. MT and CAT gene could be relevant biomarkers for 3 reasons: (1) their sensitivity to low dose of metal, (2) their graduated response to the metal dose and (3) and their ability to retain the memory of intoxication during the phase of decontamination. The others genes (SOD, GSTO, GPX, CASP, GSTM, MDR) were all down-regulated at high metal dose during exposure time, therefore they could be used in case of high contamination.

Further studies should evaluate the whole pearl oyster response to confirm the observed data or to highlight tissue-specific differences in expression. Based on the development of these molecular tools, the pearl oyster *P. margaritifera* could be used as a sentinel species for the health of the Polynesian lagoon ecosystem. Such a surveillance and monitoring network could be easily implemented. Indeed, pearl oysters are already grown in most of the important islands of French Polynesia.

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References

Amiard, J.-C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202 (PMID: 16289342).

Bridges, C.C., Zalups, R.K., 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicol. Appl. Pharmacol. 204, 274–308.

Chakraborty, S., Bhattacharya, T., Singh, G., Maity, J.P., 2014. Benthic macroalgae as biological indicators of heavy metal pollution in the marine environments: a biomonitoring approach for pollution assessment. Ecotoxicol. Environ. Saf. 100, 61–68.

Ciacci, C., Barmo, C., Gallo, G., Maisano, M., Cappello, T., D'Agata, A., Leonzio, C., Mauceri, A., Fasulo, S., Canesi, L., 2012. Effects of sublethal, environmentally relevantconcentrations of hexavalent chromium in the gills of *Mytilus galloprovincialis*. Aquat. Toxicol. 120–121, 109–118.

Conti, M., Cecchetti, G., 2003. A biomonitoring study: trace metals in algae and mollucs from Tyrrenian coastal areas. Environ. Res. 93, 99–112.

Eisler, R., 1981. Trace Metal Concentrations in Marine Organisms. Pergamon Press, Oxford, p. 685.

Eisler, R., Hennekey, R., 1977. Acute toxicities of Cd^{+2}, Cr^{+6}, Hg^{+2}, Ni^{+2}, and Zn^{+2} to estuarine macrofauna. Arch. Environ. Contam. Toxicol. 6, 315.

Ercal, N., Gurer-Orhan, H., Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: mechanisms involved in metal induced oxidative damage. Curr. Top. Med. Chem. 1, 529–539.

Espinoza, H., Williams, C., Gallagher, E., 2012. Effect of cadmium on glutathione S-transferase and metallothionein gene expression in coho salmon liver, gill and olfactory tissues. Aquat. Toxicol. 110–111, 37–44.

Fu, F., Wang, Q., 2011. Removal of heavy metal ions from wastewaters: a review. J. Environ. Manag. 92, 407–418.

Giarratano, E., Duarte, C.A., Amin, O.A., 2010. Biomarkers and heavy metal bioaccumulation in mussels transplanted to coastal waters of the Beagle Channel. Ecotoxicol. Environ. Saf. 73, 270–279.

Hosono, T., Su, C., Delinom, R., Umezawa, Y., Toyota, T., Kaneko, S., Taniguchi, M., 2011. Decline in heavy metal contamination in marine sediments in Jakarta Bay, Indonesia due to increasing environmental regulations. Estuar. Coast. Shelf Sci. 92, 297–306.

Izagirre, U., Errasti, A., Bilbao, E., Múgica, M., Marigómez, I., 2014. Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70 in mussels, *Mytilus galloprovincialis*. Aquat. Toxicol. 149, 145–156.

Joubert, C., Piquemal, D., Marie, B., Manchon, L., Pierrat, F., Zanella-Cleon, I., Cochennec-Laureau, N., Gueguen, Y., Montagnani, C., 2010. Transcriptome and proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization. BMC Genomics 11, 613–626.

Kim, S.H., Kim, S.J., Lee, J.S., Lee, Y.M., 2014. Acute effects of heavy metals on the expression of glutathione-related antioxidant genes in the marine ciliate *Euplotes crassus*. Mar. Pollut. Bull. 85, 455–462.

Lemarié, A., Lagadic-Gossmann, D., Morzadec, C., Allain, N., Fardel, O., Vernhet, L., 2004. Cadmium induces caspase-independent apoptosis in liver Hep3B cells: role for calcium in signaling oxidative stress-related impairment of mitochondria and relocation of endonuclease G and apoptosis-inducing factor. Free Radic. Biol. Med. 36, 1517–1531.

Li, Y., Yang, H., Liu, N., Luo, J., Wang, Q., Wang, L., 2015. Cadmium accumulation and metallothionein biosynthesis in cadmium-treated freshwater mussel *Anodonta woodiana*. PLoS ONE 10 (2):0117037. http://dx.doi.org/10.1371/journal.pone.0117037.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta DeltaC(T)) method. Methods 25, 402–408.

Ma, W.L., Wang, L., He, Y.J., Yan, T., 2008. Tissue-specific cadmium and metallothionein levels in freshwater crab *Sinopotamon henanense* during acute exposure to waterborne cadmium. Environ. Toxicol. 23:393–400. http://dx.doi.org/10.1002/tox.20339 (PMID: 18214890).

Ma, W.L., Yan, T., He, Y.J., Wang, L., 2009. Purification and cDNA cloning of a cadmium-binding metallothionein from the freshwater crab *Sinopotamon henanense*. Arch. Environ. Contam. Toxicol. 56:747–753. http://dx.doi.org/10.1007/s00244-008-9224-4 (PMID: 18846314).

Maanan, M., 2008. Heavy metal concentrations in marine molluscs from the Moroccan coastal region. Environ. Pollut. 153, 176–183.

Marie, V., Gonzalez, P., Baudrimont, M., Boutet, I., Moraga, D., Bourdineaud, J.-P., Boudou, A., 2006. Metallothionein gene expression and protein levels in triploid and diploid oysters *Crassostrea gigas* after exposure to cadmium and zinc. Environ. Toxicol. Chem. 25, 412–418.

Marie, B., Joubert, C., Tayalé, A., Zanella-Cléon, I., Belliard, C., Piquemal, D., 2012. Different secretory repertoires control the biomineralization processes of prism and nacre deposition of the pearl oyster shell. PNAS 109 (51), 20986–20991.

Mayer, M.P., Bukau, B., 2005. Hsp70 chaperones: Cellular functions and molecular mechanism. Cell. Mol. Life Sci. 62, 670.

Mc Innes, J.R., Calabrese, A., 1978. Response of embryos of the American oyster, to heavy metals at different temperatures. In: Lucky, D.S., Berry, A.J. (Eds.), Physiology and Behaviour of Marine Organisms. Pergamon press, New York, pp. 195–202.

McIlwain, D.R., Berger, T., Mak, T.W., 2013. Caspase functions in cell death and disease. Cold Spring Harb. Perspect. Biol. 5, a008656.

Medina, J., Hernandez, E., Pastor, A., Beferull, J.B., Barbera, J.C., 1986. Determination of mercury, cadmium, chromium and lead in marine organisms by flameless atomic absorption spectrophotometry. Mar. Pollut. Bull. 17, 41–44.

Mohan, C.V., Menon, N.R., Gupta, T.R.C., 1984. Acute toxicity of cadmium to six intertidal invertebrates. Fish. Technol. 21, 1–5.

Moore, M.N., Depledge, M.H., Readmen, J.W., Leonard, D.R.P., 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutat. Res. 552, 247–268.

Naser, H.A., 2013. Assessment and management of heavy metal pollution in the marine environment of the Arabian Gulf: a review. Mar. Pollut. Bull. 72, 6–13.

Nicolaidou, A., 1994. Identification of biological indicators of heavy metals at thesite of a smelting factory. UNEP/FAO, Final Reports on Research Projects Dealing With the Effect of Pollutants on Marine Organisms and Communities. MAP Technical Reports Series N° 80. Athe, pp. 25–38.

Nicosia, A., Salamone, M., Mazzola, S., Cuttitta, A., 2015. Transcriptional and biochemical effects of cadmium and manganese on the defense system of *Octopus vulgaris* paralarvae. Biomed. Res. Int., 437328 http://dx.doi.org/10.1155/2015/437328 (11 pp.).

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Pathak, N., Khandelwal, S., 2006. Oxidative stress and apoptotic changes in murine splenocytes exposed to cadmium. Toxicology 220, 26–36.

Salvat, B., Aubanel, A., Adjeroud, M., Bouisset, P., Calmet, D., Chancerelle, Y., Cochennec, N., Davies, N., Fougerousse, A., Galzin, R., Lagouy, E., Lo, C., Monier, C., Ponsonnet, C., Remoissenet, G., Schneider, D., Stein, A., Tatarata, M., Villiers, L., 2008. Le suivi de l'état des récifs coralliens de Polynesie francaise et leur récente évolution. Rev. Ecol. (Terre Vie) 63, 103–135.

Shih, C.-M., Ko, W.-C., Wu, J.-S., Wei, Y.-H., Wang, L.-F., Chang, E.-E., Lo, T.-Y., Cheng, H.-H., Chen, C.-T., 2004. Mediating of caspase-independent apoptosis by cadmium through the mitochondria-ROS pathway in MRC-5 fibroblasts. J. Cell. Biochem. 91:384–397. http://dx.doi.org/10.1002/jcb.10761.

Sidoumou, Z., Gnassia-Barelli, M., Siau, Y., Morton, V., Roméo, M., 2005. Heavy metal concentrations in molluscs from the Senegal coast. Environ. Int. 32, 384–387.

Sommanee, P., 1980. Toxicity of heavy metals on *Donax faba* Chemnitz. Thai. Fish Gazette. 32, pp. 391–402.

Taylor, D.A., Thompson, E.L., Nair, S.V., Raftos, D.A., 2013. Differential effects of metal contamination on the transcript expression of immune and stress-response genes in the Sydney rock oyster, *Saccostrea glomerata*. Environ. Pollut. 178, 65–71.

Thompson, E.L., Taylor, D.A., Nair, S.V., Birch, G., Haynes, P.A., Raftos, D.A., 2011. A proteomic analysis of the effects of metal contamination on Sydney rock oyster (*Saccostrea glomerata*) haemolymph. Aquat. Toxicol. 103, 241–249.

Tiapa, J., Vargas-Chacoff, L., Bertrán, C., Carrasco, G., Torres, F., Pinto, R., Urzúa, S., Valderrama, A., Letelier, L., 2010. Study of the content of cadmium, chromium and lead in bivalve molluscs of the Pacific Ocean (Maule Region, Chile). Food Chem. 121, 666–671.

Usero, J., Morillo, J., Gracia, I., 2005. Heavy metal concentrations in molluscs from the Atlantic coast of southern Spain. Chemosphere 59, 1175–1181.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., Koehler, A., 2007. The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp. Biochem. Physiol. C 146, 281–300.

Vlahogianni, T.H., Valavanidis, A., 2007. Heavy-metal effects on lipid peroxidation and antioxidant defense enzymes in mussels *Mytilus galloprovincialis*. Chem. Ecol. 23, 361–371.

Wang, J., Zhang, P., Shen, Q., Wang, Q., Liu, D., Li, J., Wang, L., 2013. The effects of cadmium exposure on the oxidative state and cell death in the gill of freshwater crab *Sinopotamon henanense*. PLoS ONE 8 (5), e64020. http://dx.doi.org/10.1371/journal.pone.0064020.

Werner, I., Hinton, D.E., 1999. Field validation of hsp70 stress proteins as biomarkers in Asian clam (Potamocorbula amurensis): Is downregulation an indicator of stress? Biomarkers 4, 473–484.

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