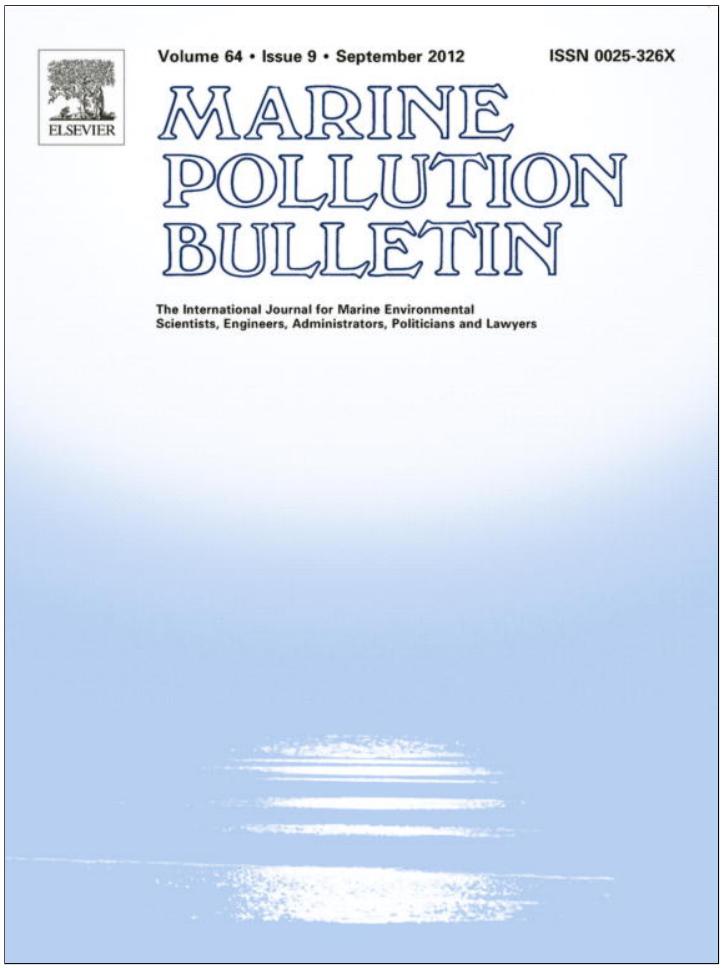

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# Effect of zinc sacrificial anode degradation on the defence system of the Pacific oyster, *Crassostrea gigas*: Chronic and acute exposures

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ABSTRACT

Two types of exposures were performed to assess the effects of zinc released from sacrificial anode degradation: a chronic exposure, in which oysters were exposed to 0.53 ± 0.04 mg Zn $L^{-1}$ for 10 weeks, and an acute exposure, where oysters were exposed to 10.2 ± 1.2 mg Zn $L^{-1}$ for 1 week. At the end of the acute exposure experiment, 81.8% mortality was recorded. In contrast, no mortality was detected after 10 weeks exposure. Moreover, all of the immune system biomarkers studied, except the number of circulating haemocytes, were stimulated by a moderate level of zinc and inhibited by a high level. Our exposure conditions did not induce SOD or MXR mRNA expression in gills and digestive gland. However, an increase of MT mRNA is observed in these tissues. The results indicate that oysters are sensitive to acute zinc toxicity but are only moderately affected by a mild zinc concentration.



## 1. Introduction

Marine ecosystems are continuously subjected to anthropogenic activities that introduce various pollutants into seawater. Among these pollutants, heavy metals, including zinc, lead to pollution all around the world. Zinc concentrations measured in coastal waters of unpolluted and polluted sites range from 0.3 to 300 μg/L (Bird et al., 1996; Terrés-Martos et al., 2002; Baudrimont et al., 2005; Daby, 2006; Hamed and Emara, 2006; El Ati-Hellal et al., 2007). However, extremely high zinc concentrations, ranging from 1.8 mg $L^{-1}$ to 6 mg $L^{-1}$, have been reported in specific areas (Amado Filho et al., 1997; Srinivasa et al., 2005). The anthropogenic sources of zinc are mainly industry, urbanisation and agriculture. In the marine environment, zinc can also be introduced into water by the degradation of sacrificial anodes. To reduce the corrosion of steel materials, such as boats or harbour structures, sacrificial anodes consisting of a metal with a lower electrode potential than iron (e.g., zinc, magnesium or aluminium) are commonly used. Application of zinc sacrificial anodes has been common since the early 1960s, and anodes of zinc are the most often used type of these anodes for marine protection applications (Bird et al., 1996; Wagner et al., 1996). However, this cathodic protection induces the release of dissolved zinc due to degradation of sacrificial anodes. Although the quantity of zinc released from sacrificial anodes into the marine environment is difficult to assess, it is thought to be very large (Wagner et al., 1996). Few studies focused on metal released from sacrificial anode and affecting marine organisms. Recently, Caplat et al. (2010) evaluated the effect of aluminium or zinc released from sacrificial anode by comparison to their traditional sulphate salt. Using sea urchin embryos and sperm bioassays, these authors observed differences in effects and demonstrated a lesser toxicity, up to hormesis, of zinc linked to anode compare to sulphate salt.

Zinc is an essential metal for all living organisms. This metal is a cofactor over 300 enzymes (Vallee and Falchuk, 1993) and plays an important role in immune responses, antioxidant defence systems, energetic metabolism, reproduction, cell division and regulation of DNA transcription (Salgueiro et al., 2000). However, when present in excess, zinc is potentially toxic to organisms, and numerous studies have demonstrated the lethality of zinc in algae (Amado Filho et al., 1997) and in invertebrates (Wiklund et al., 2006). Moreover, it can also have deleterious effects on embryonic development and larval growth of bivalves (Brereton et al., 1973; Bellas et al., 2005; Jorge and Moreira, 2005) as well as on the immune system of vertebrates (Dardenne, 2002).

In molluscs, haemocytes, which are circulating cells present in haemolymph, play a key role in innate immunity. These cells are

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responsible for phagocytosis, pathogen hydrolysis, the production of reactive oxygen species (ROS) and the phenoloxidase cascade (Galloway and Depledge, 2001). In bivalves, some authors have shown that haemocyte functions can be modulated by certain contaminants, including heavy metals (Coles et al., 1995; Auffret et al., 2002; Bouilly et al., 2006; Thiagarajan et al., 2006; Girón-Perez, 2010). For example, Auffret et al. (2002) reported that the phagocytic activity in the total haemocyte population of the European flat oyster *Ostrea edulis* showed a 50% decrease compared to a control when these organisms were exposed to 10 μM cadmium.

To analyse the effects of environmental pollutants, multiparametric approaches have been used. Flow cytometry has classically been used for the analysis of suitable biomarkers, including phagocytosis, ROS production and non-specific esterase activity (Brousseau et al., 2000; Sauvé et al., 2002; Auffret, 2005; Gagnaire et al., 2006; Duchemin et al., 2008). Based on quantification of mRNA levels, some authors have proposed that genes involved in the regulation of oxidative stress (superoxide dismutase (SOD), metallothionein (MT)) (Otero et al., 1996; Cai et al., 1999; Guo et al., 2003) and cell detoxification (multidrug resistance (MDR)) (Farcy et al., 2009) could be suitable biomarkers for metal contamination in field studies addressing molluscs (Bordin et al., 1997; Mouneyrac et al., 1998; Hansen et al., 2006; Saez et al., 2008). MTs are small proteins (~7 kDa) that have the ability to sequester metals, acting in a protective function role (Roesijadi, 1992; Amiard et al., 2006). SOD is responsible for protecting organisms from oxidative stress by catalysing the conversion of superoxide anions into hydrogen peroxide. SOD is used to assess the effects of environmental pollutants on marine organisms, including bivalves (Bebianno et al., 2004). MDR plays an important role in the multixenobiotic resistance (MXR) system, helping to pump contaminants out of the cell to reduce them below toxic levels (Bard, 2000; Minier et al., 2002). This mechanism is similar to the MDR phenomenon first described in mammalian tumour cell lines.

Bivalve molluscs, such as the Pacific oyster, *Crassostrea gigas*, are frequently used in ecotoxicological studies as model organisms because they filter large amounts of water for respiratory and nutritional purposes and are able to accumulate pollutants (Bouilly et al., 2006; Gagnaire et al., 2007; Bado-Nilles et al., 2008; Choi et al., 2008; Jo et al., 2008).

To investigate the effects of zinc resulting from the degradation of sacrificial anodes on *C. gigas*, experiments were performed using two exposure designs: chronic and acute exposures. The chronic exposure experiment consisted of exposing oysters to zinc at a concentration of 0.53 ± 0.04 mg Zn $L^{-1}$ for 10 weeks, which is typical of the pollution levels observed in some anthropogenic areas (Bird et al., 1996; Terrés-Martos et al., 2002; Baudrimont et al., 2005; Daby, 2006; Hamed and Emara, 2006; El Ati-Hellal et al., 2007). The acute exposure was carried out for 7 days at a concentration of 10.2 ± 1.2 mg Zn $L^{-1}$. This experiment mimicked strongly contaminated areas (Amado Filho et al., 1997; Srinivasa et al., 2005). Haemocyte parameters, including phagocytosis capacity, production of ROS and non-specific esterase activity, were monitored using flow cytometry, and phenoloxidase activity was measured by spectrophotometry. mRNAs associated with oxidative stress (SOD and MT) and cell detoxification (MDR1) were quantified using real-time PCR on samples from gills and digestive glands.

## 2. Materials and methods

### 2.1. Animals

Three years old, diploid Pacific oysters, *C. gigas*, were obtained from an oyster farm located in Normandy (France). All of the oysters spent their entire growing life on this farm. The oysters were acclimatised to laboratory conditions for 1 month before the experiments were performed in a tank containing natural seawater. The water was continuously aerated and renewed. During acclimatisation and the experiments, no external food (apart from that provided by natural seawater) was added.

### 2.2. Experimental unit design

Seawater was directly collected in the small fishing harbour of Les Flamands. This harbour is located east of the port of Cherbourg (Basse-Normandie, France). Seawater was decanted twice before being filtered to remove particles in suspension. Then it was transported to two 80 L tanks. One 80 L tank was equipped with an electrochemical device. Each 80 L tank was connected with a high-density polyethylene (HDPE) experimental unit of 500 L that contained oysters. Seawater was continuously renewed (10 L/h) and oxygenated. Physical and chemical parameters (pH, temperature, dissolved $O_2$) were continuously controlled, and salinity was measured one time per day with multi-parametric meters (Orion-5-Star). The temperature of the experimental units followed the temperature of natural seawater; the difference in temperature between natural seawater and the experimental unit was lower than 2 °C.

### 2.3. Electrochemical device

To accelerate anode degradation and simulate the zinc transfer in seawater, an electrochemical device was installed in one 80 L tank, as described previously (Caplat et al., 2010). This device was constructed using one reference saturated calomel electrode (SCE) and one colter electrode (CE) in platinum–titanium alloy. The electrodes were connected to a zinc anode (min. of 99.31% zinc, US-MIL-A1800.1K Norm), with the whole system being electrically linked to a general purpose potentiostat (AMEL instruments, Model 2049, Milan, Italy). The role of the SCE electrode was to stabilise the anode potential; control of the intensity was linked to the total zinc concentration required in the experimental unit (Fig. 1). With this device, the chemical forms of zinc(II) released from anodes were reproduced as in natural conditions.

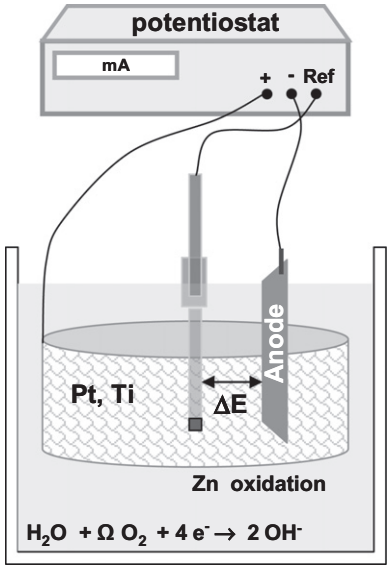


**Fig. 1.** Electrochemical device scheme with zinc anode and potentiostat.

### 2.4. Zinc exposure

Two exposures were performed. The first consisted of a chronic exposure in which 166 oysters were exposed to 0.53 ± 0.04 mg Zn $L^{-1}$ for 10 weeks. The second experiment employed an acute exposure in which 100 oysters were exposed to 10.2 ± 1.2 mg Zn $L^{-1}$ for 1 week. Fifteen oysters per experimental unit (control and exposed) were sampled at different periods during the experiments (0.2, 1, 2, 3, 4, 5, 6, 7 and 10 weeks for the chronic exposure and 24, 48, 96 and 168 h for the acute exposure).

### 2.5. Collection of haemolymph and total haemocyte counts (THC)

For each oyster sampled, haemolymph was drawn from the pericardial cavity using a syringe equipped with a 25-gauge sterile needle. Haemolymph was transferred to a sterile tube and immediately diluted 1:10 in cooled, sterile, anti-coagulant modified Alsever's solution (115 mM glucose, 27 mM sodium citrate, 11.5 mM EDTA and 382 mM NaCl) (Bachère et al., 1988). For each sampling, three pools of five oysters each were used. Each tube containing haemolymph was centrifuged (500×*g*, 10 min, 4 °C) to pellet the haemocytes. Then the supernatant was removed and used for the measurement of phenoloxidase activity. The haemocytes were resuspended in 1 mL of sterile artificial seawater (436 mM NaCl, 53 mM $MgSO_4$, 20 mM HEPES, 10 mM $CaCl_2$, 10 mM KCl).

For each pool, haemocytes were counted using a Thoma counting chamber under an inverted microscope (Leica, DM IRB/E) to determine the haemocyte concentration.

### 2.6. Flow cytometry analysis

Flow cytometry analyses were conducted as previously described (Mottin et al., 2010). Briefly, fluorescence was recorded using an EPICS XL 4 (Beckman Coulter) at 505–545 nm excitation wavelengths corresponding to the FL1 channel. Twenty thousand events were counted for each sample. The results were expressed as cell cytograms indicating the size (FSC value), the complexity (SSC value) and the level of fluorescence of the cell samples using the FL1 channel. For each pool, three replicates were analysed.

#### 2.6.1. Phagocytosis capacity

Phagocytosis activity was measured as the proportion of haemocytes that ingested three or more fluorescent beads (carboxylate-modified FluoroSpheres®, yellow–green fluorescence, 1 μm diameter, Molecular Probes). Haemocytes from each replicate were incubated with bead solution for 1 h in the dark at 17 °C. The final ratio of beads:haemocytes was 100:1.

#### 2.6.2. Reactive oxygen species (ROS) production

ROS production was evaluated using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) method (Bass et al., 1983) as adapted for oyster cells by Lambert et al. (2003). DCFH-DA was added to each replicate at a final concentration of 10 μM, and haemocytes were incubated for 1 h at 17 °C in the dark.

#### 2.6.3. Non-specific esterase activity

Esterase activity was measured using the non-specific liposoluble substrate fluorescein diacetate (FDA, Molecular Probes). Haemocytes were incubated for 15 min at 17 °C in the dark with FDA at a final concentration of 1 μM.

Following the incubations, haemocyte samples were centrifuged (500×*g*, 10 min, 4 °C). The supernatants were removed, and 300 μL of paraformaldehyde was added (3% in phosphate buffer saline, PBS) to fix the cells. Samples were then incubated for 30 min at 25 °C. Finally, 200 μL of PBS was added, and the samples were stored at 4 °C in the dark until analysis.

### 2.7. Phenoloxidase activity

The phenoloxidase activity in cell-free haemolymph was measured by recording the formation of dopachrome produced from 3,4-dihydroxy-L-phenylalanine (L-DOPA, Sigma) (Hernandez-Lopez et al., 2003). Transformation was monitored by spectrophotometry at 492 nm. For a 250 μL sample, 250 μL of Tris (10 mM)/NaCl (150 mM) buffer at pH 8, 250 μL of cacodylate buffer (10 mM, pH 7) and 250 μL of L-DOPA (3 mg/mL) were added. Samples were incubated for 1 h before absorbance was measured. Phenoloxidase activity was expressed as an arbitrary unit.

### 2.8. Quantification of mRNA expression

Quantification of mRNA expression was conducted as previously described (Farcy et al., 2007, 2009).

#### 2.8.1. RNA extraction

For analysis of mRNA expression, gills and digestive glands were dissected at different periods during experiments (see Section 2.4). For each sampling, three pools of four oysters each were used. Total RNA was extracted with Tri Reagent (Sigma) at a concentration of 1 mL/100 mg of tissue. Quality control and quantification of RNA were performed using a Nano-Drop 2000 (Thermo scientific). The RNA was treated with DNase I (1 U/μL, Sigma–Aldrich) at room temperature for 20 min to remove any DNA contaminants.

#### 2.8.2. Real-time PCR and analysis

Reverse transcription was carried out using 700 ng of total RNA treated with DNase I, 500 ng random primers (Promega), 200 U Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT, Promega), 25 U RNasin (Promega) and 0.5 mM dNTPs (Promega). The sequences of the forward and reverse primers were designed using Primer3 software (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesised by Eurogentec (Table 1). Real-time PCR was performed in a MiQ Cycler (Biorad). All procedures were carried out in duplicate. Controls of non-template cDNA were included in the PCR experiments. Ampli-

**Table 1**
Primer sequences and accession number.

| Gene | GenBank accession # | Forward primer | Reverse primer |
|---|---|---|---|
| Actin | AF026063 | 5′ GCCCTGGACTTCGAACAA 3′ | 5′ CGTTGCCAATGGTGATGA 3′ |
| SOD[a] | AJ496219 | 5′ AACCCCTTCAACAAAGAGCA 3′ | 5′ TTTGGCGACACCGTCTTC 3′ |
| MT[b] | AJ243263, AJ242657 | 5′ GGACCGGAAAACTGCAAA 3′ | 5′ CCAGTGCATCCTTTACCACA 3′ |
| MDR1 | AJ422120 | 5′ CCGAGAACATCCGCTACG 3′ | 5′ GCCCTGTGGGAGTTCCTT 3′ |

[a] The amino acid translation of the Genbank #AJ496219 sequence is highly homologous to a Cu–Zn SOD.
[b] This pair of primers did not discriminate between two identified members of oyster MTs: MT1 (accession # AJ243263) and MT2 (accession # AJ242657).

fication was performed in 96-well plates in a total volume of 15 μL containing cDNA samples obtained from reverse transcription of 5 ng of total RNA, 7.5 μL of 2X iQ SYBR Green supermix (Biorad), and both primer (300 nM final concentration of each primer). The amplification conditions were 40 cycles of 15 s at 95 °C and 45 s at 60 °C, followed by the protocol for the melting curve: 80 cycles of 10 s with an increase of 0.5 °C between each cycle from 55 °C to 95 °C. The melting curve was used to check whether the amplification products exhibited the expected Tm.

To analyse gene expression levels, mRNA quantities were normalised to actin mRNA (Farcy et al., 2007).

### 2.9. Data analysis

Values are expressed as the means ± standard deviation of three different pooled oyster samples. Normality (Kolmogorov–Smirnov test) was first checked and the significance of the differences between two samples was determined using the Mann and Whitney U test. These analyses were conducted using Statistica 7.1 software (StatSoft® France, Maison-Alfort, France).

## 3. Results

### 3.1. Effects of zinc exposure on oyster mortality

In the acute exposure experiment, mortality was first observed after 24 h of incubation (3.1% mortality) and reached 81.8% at the end of the experiment; in the control tank, no mortality was observed. No mortality was detected in the chronic exposure tank or in the control tank during the experimental period (data not shown).

### 3.2. Total haemocyte counts (THCs)

Exposure of oysters to zinc (acute and chronic experiments) induced a decrease in the THC (Fig. 2). During acute exposure, a significant difference ($p < 0.05$) was recorded after 24 h and 48 h leading to decreases of 46% and 35%, respectively, compared to the control (Fig. 2A). After 96 h and 168 h, the total haemocyte counts were lower in the treated organisms than the control oysters, but the differences were not statistically significant because of large intra-group variations. In the chronic exposure experiment, haemocyte numbers tended to be lower in the treated group than the control group. These differences were statistically significant ($p < 0.05$) after 5-, 6- and 10-week incubations. In these cases, the variation ranged from 31% to 45% compared to the control (Fig. 2B).

### 3.3. Effects of zinc exposure on immune parameters

Immune parameters were monitored using flow cytometry (phagocytic activity, ROS production and non-specific esterase activity) and spectrophotometrically (phenoloxidase activity). Significant decreases in phagocytosis, ROS production and non-specific esterase activity were detected compared to the controls during acute exposure. From 48 h of incubation, decreases of approximately 16% and 36% occurred in phagocytosis and non-specific esterase activity, respectively, and a decrease of 61% was observed after 24 h in ROS production (Table 2). For the phenoloxidase activity (Fig. 3A), an increase of 111% compared to the control was recorded after 96 h of exposure, followed by a decrease of 53% after 168 h of incubation.

With respect to the effects of zinc during chronic exposure, phagocytic activity was significantly induced after 1 week ($p < 0.001$), 2 weeks ($p < 0.05$) and 4 weeks ($p < 0.05$) of exposure compared to the controls and ROS production was significantly induced after 1 week ($p < 0.05$), 5 weeks ($p < 0.01$) and 10 weeks ($p < 0.01$) of exposure compared to the controls. For non-specific esterase activity, significant increases compared to the control group were detected after 4 weeks ($p < 0.001$), 6 weeks ($p < 0.01$) and 7 weeks ($p < 0.001$) of exposure, whereas a slight, but

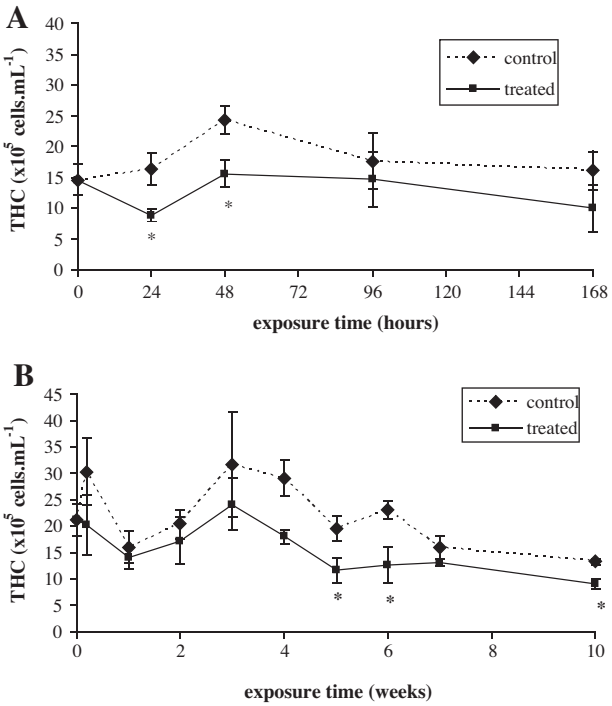


**Fig. 2.** Number of circulating haemocytes during acute (A) and chronic (B) zinc exposure in *Crassostrea gigas*. Each data point represents the mean ± standard deviation of triplicate. Significant differences from zinc treated organisms in comparison to control within sampling times at $p < 0.05$ (*) are indicated.

**Table 2**
Effects of acute exposure to zinc on *Crassostrea gigas* haemocyte immune parameters. For each parameter, the values for the mean ± standard deviation of the samples are indicated as well as the percentage of variation between the treated and control specimens at the same sampling time. The statistically significant differences are indicated (Mann and Whitney U tests).

| Exposure time (h) | | 0 | 24 | 48 | 96 | 168 |
|---|---|---|---|---|---|---|
| Phagocytic activity | Control sample | 19.5 ± 0.5 | 19.5 ± 0.8 | 14.5 ± 0.7 | 19.9 ± 0.7 | 18.0 ± 0.9 |
| | Treated sample | 19.5 ± 0.5 | 18.9 ± 0.5 | 12.1 ± 0.6 | 20.3 ± 0.8 | 13.5 ± 0.7 |
| | Variation (%) | 0 | −3 | −16 ($p < 0.05$) | +2 | −25 ($p < 0.01$) |
| ROS production | Control sample | 34.1 ± 2.1 | 30.6 ± 1.4 | 16.9 ± 0.4 | 37.6 ± 3.7 | 16.2 ± 1.6 |
| | Treated sample | 34.1 ± 2.1 | 11.9 ± 1.3 | 8.9 ± 1.7 | 25.0 ± 3.8 | 9.0 ± 0.7 |
| | Variation (%) | 0 | −61 ($p < 0.001$) | −47 ($p < 0.001$) | −34 ($p < 0.05$) | −44 ($p < 0.01$) |
| Non-specific esterase activity | Control sample | 31.9 ± 3.6 | 23.5 ± 1.8 | 26.3 ± 1.4 | 28.4 ± 2.4 | 22.5 ± 2.2 |
| | Treated sample | 31.9 ± 3.6 | 19.2 ± 1.7 | 16.9 ± 2.5 | 21.7 ± 1.7 | 16.3 ± 0.7 |
| | Variation (%) | 0 | −18 | −36 ($p < 0.01$) | −24 | −28 ($p < 0.05$) |

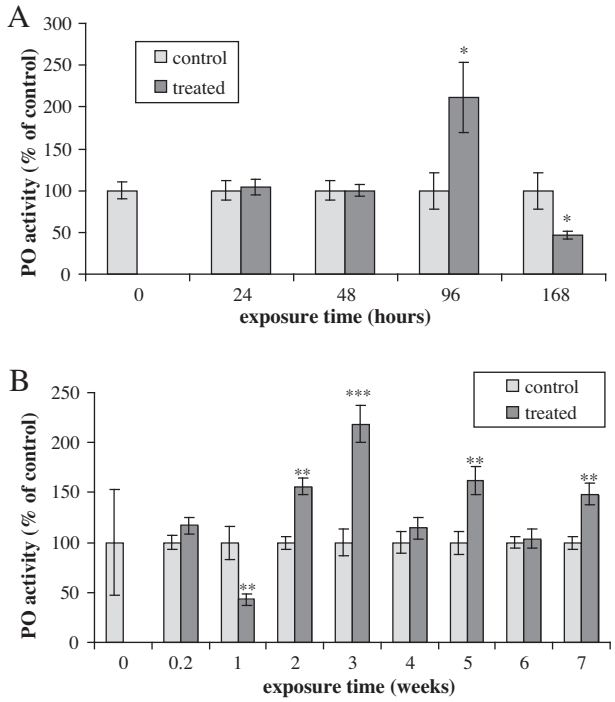


**Fig. 3.** Phenoloxidase activity in haemolymph during acute (A) and chronic (B) zinc exposure in the oyster *Crassostrea gigas*. Each data point represents the mean ± standard deviation of triplicate samples. Significant differences in zinc-treated specimens compared to controls within a particular sampling time at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) are indicated.

significant ($p < 0.05$) decrease compared to the controls was observed after 3 weeks of exposure (Table 3). Phenoloxidase activity significantly ($p < 0.01$) decreased by 57% compared to the control group after 1 week of incubation and then increased (Fig. 3B). The maximum level of induction of penoloxidase activity was observed after 3 weeks of exposure (118% compared to the control).

### 3.4. Effects of zinc on mRNA expression of genes related to stress and detoxification

The effects of zinc on MT, MDR1 and SOD mRNA expression were monitored in two key organs in terms of bioaccumulation: gills and digestive glands. In gills, the main effects of zinc were recorded for MT mRNA expression under both the acute and chronic exposures (Fig. 4). We detected 17-fold ($p < 0.01$) and 74-fold ($p < 0.01$) inductions compared to the control group in the acute exposure experiment after 96 h and 168 h of incubation, respectively. During chronic exposure, MT mRNA expression in the treated group was increased by 5-fold ($p < 0.01$), 8-fold ($p < 0.01$) and 32-fold ($p < 0.01$) compared to the controls after 1, 4 and 7 weeks of exposure, respectively. However, after 10 weeks, the MT mRNA level decreased to the level of the control group. The MDR1 mRNA level was significantly increased by zinc exposure (3-fold compared to the control; $p < 0.01$) after 168 h of exposure in the acute exposure experiment. No significant differences were detected between the treated group and the control group in the chronic exposure experiment. Concerning the SOD mRNA level, a slight, but significant ($p < 0.05$) difference was observed between control and treated samples after 4 weeks of exposure under chronic exposure, whereas no significant differences appeared in the acute exposure experiment.

In digestive glands, the MT mRNA levels were significantly increased compared to the controls in the acute exposure experiment (Fig. 5). Inductions of 2-fold ($p < 0.05$), 4-fold ($p < 0.01$) and 7-fold ($p < 0.01$) were observed after 48 h, 96 h and 168 h, respectively. Under chronic exposure, the MT mRNA level in the treated group was induced ($p < 0.01$) by 2.7-fold compared to the controls after 4 weeks of exposure. Additionally, the level of MDR1 expression was significantly ($p < 0.05$) elevated by 1.7-fold compared to the controls after 48 h of exposure in the acute experiment, whereas no significant differences were recorded between the treated group and the control group under chronic exposure. In the chronic exposure experiment, a slight (1.5-fold) significant ($p < 0.05$) increase in the SOD mRNA level compared to the control was detected after 1 week of exposure, whereas a significant ($p < 0.01$) decrease (1.9-fold) compared to the control was observed after 7 weeks of exposure. No significant differences were recorded between the treated group and the control group with respect to the SOD mRNA levels under acute exposure.

## 4. Discussion

In this study, we performed chronic and acute exposure experiments to monitor the effects of zinc released due to the degradation of a sacrificial anode on stress mRNA expression in both gills

**Table 3**
Effects of chronic exposure to zinc on *Crassostrea gigas* haemocyte immune parameters. For each parameter, the values for the mean ± standard deviation of the samples are indicated as well as the percentage of variation between the treated and control specimens at the same sampling time. The statistically significant differences are indicated (Mann and Whitney U tests).

| Exposure time (weeks) | | 0 | 0.2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phagocytic activity | Control sample | 20.1 ± 0.7 | 21.4 ± 0.7 | 19.8 ± 0.6 | 21.5 ± 1.1 | 21.9 ± 0.7 | 16.5 ± 0.6 | 16.3 ± 1.0 | 22.6 ± 0.7 | 21.1 ± 0.6 | 19.6 ± 0.6 |
| | Treated sample | 20.1 ± 0.7 | 22.9 ± 1.1 | 24.4 ± 0.4 | 25.3 ± 0.9 | 23.2 ± 0.5 | 19.0 ± 0.6 | 18.1 ± 1.0 | 21.1 ± 0.5 | 19.9 ± 0.9 | 21.4 ± 0.8 |
| | Variation (%) | 0 | +7 | +24 ($p < 0.001$) | +18 ($p < 0.05$) | +6 | +15 ($p < 0.05$) | +11 | −7 | −6 | +9 |
| ROS production | Control sample | 14.1 ± 3.3 | | 15.7 ± 2.3 | 38.0 ± 5.5 | 17.3 ± 1.5 | 15.5 ± 2.6 | 16.0 ± 2.1 | 47.7 ± 4.9 | 44.0 ± 2.3 | 23.3 ± 0.9 |
| | Treated sample | 14.1 ± 3.3 | | 25.2 ± 2.5 | 47.8 ± 2.4 | 21.9 ± 2.4 | 22.0 ± 2.7 | 29.5 ± 2.1 | 42.1 ± 1.5 | 36.6 ± 2.6 | 29.5 ± 1.6 |
| | Variation (%) | 0 | −11 | +60 ($p < 0.05$) | +26 | +27 | +41 | +85 ($p < 0.01$) | −12 | −17 | +27 ($p < 0.01$) |
| Non-specific esterase activity | Control sample | 76.3 ± 2.2 | 82.1 ± 2.2 | 68.5 ± 4.4 | 66.7 ± 2.7 | 82.5 ± 1.1 | 57.3 ± 2.0 | 67.6 ± 2.0 | 77.2 ± 1.2 | 65.9 ± 2.1 | 68.6 ± 2.3 |
| | Treated sample | 76.3 ± 2.2 | 79.1 ± 3.0 | 75.4 ± 2.2 | 66.4 ± 2.4 | 76.4 ± 2.3 | 79.9 ± 2.6 | 71.0 ± 1.8 | 82.7 ± 1.0 | 79.2 ± 1.6 | 74.7 ± 1.9 |
| | Variation (%) | 0 | −4 | +10 | 0 | −7 ($p < 0.05$) | +39 ($p < 0.001$) | +5 | +7 ($p < 0.01$) | +20 ($p < 0.001$) | +9 |

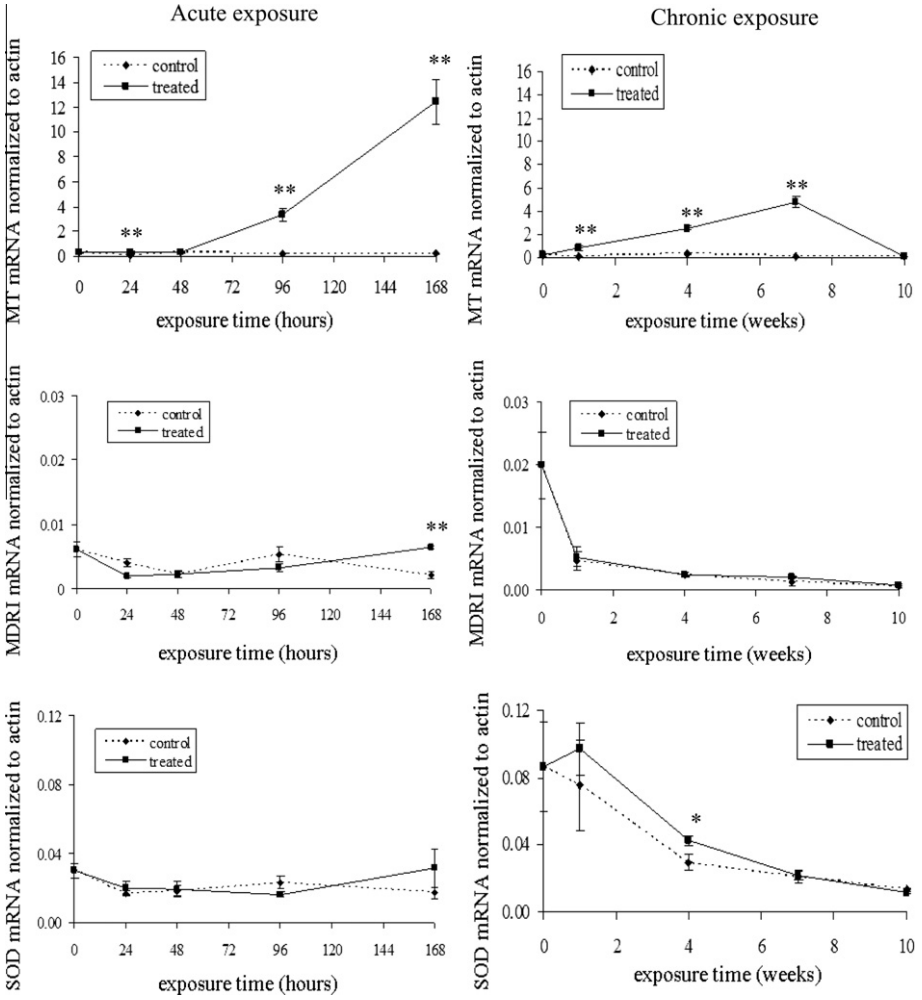


**Fig. 4.** Expression of MT, MDR1 and SOD mRNA in gills in response to acute or chronic zinc exposure in *Crassostrea gigas*. Each data point represents the mean ± standard deviation of triplicate samples. Significant differences between zinc-treated specimens compared to controls within a particular sampling time at $p < 0.05$ (∗), $p < 0.01$ (∗∗), and $p < 0.001$ (∗∗∗) are indicated.

and digestive glands as well as immune parameters of the Pacific oyster, *C. gigas*. Haemocytes constitute the cellular defence system of invertebrates, and the total number of circulating haemocytes (THC) is an appropriate biomarker for monitoring pollution. In this study, we showed that zinc induced a decrease in THCs during both exposure conditions. Our results are in accordance with results published by Lorenzon et al. (2001). These authors exposed the shrimp *Palaemon elegans* to different heavy metals, including zinc, and concluded that heavy metal exposure provokes a THC decrease. Decreases in the number of circulating haemocytes due to heavy metals have also been detected in the mussel *Mytilus edulis* (Parry and Pipe, 2004), in two crab species, *Scylla serrata* and *Paratelphusa hydrodromous* (Victor, 1993; Vijayavel et al., 2009), and in the ant *Formica aquilonia* (Sorvari et al., 2007). Different hypotheses have been proposed to explain this phenomenon. A decrease in haemocyte numbers could be a consequence of cell lysis, reduced haemopoiesis or migration of haemocytes from the circulatory system into tissues (Pipe and Coles, 1995; Parry and Pipe, 2004; Hannam et al., 2009). Considering the results of the present study, we are not able to reach a conclusion regarding the reasons that decreased numbers of haemocytes were observed, and further experiments should be conducted to test these hypotheses.

Phagocytosis, ROS production and non-specific esterase activity are also good biomarkers allowing the assessment of the immunotoxicity of xenobiotics. These haemocyte parameters are frequently studied using flow cytometry (Gagnaire et al., 2007; Bado-Nilles et al., 2008; Morga et al., 2011). Our results showed increases in haemocyte parameters when oysters were exposed to 0.5 mg Zn $L^{-1}$ for 10 weeks. In contrast, significant decreases were observed under exposure to 10 mg Zn $L^{-1}$ from 48 h onward. Some studies in bivalves have demonstrated that haemocyte activity may be stimulated by moderate levels of heavy metals and inhibited by high levels (Perez and Fontanetti, 2010). This phenomenon could be termed hormesis, as hormesis is the name given to phenomena in which opposite effects are observed at low compared to high doses for a given measured parameter (Stebbing, 1982; Calabrese and Blain, 2011). A hormetic effect of zinc has previously been observed by Pagano et al. (1986) and more recently by Caplat et al. (2010) in the sea urchin. Moreover, some studies have demonstrated that phagocytosis may be stimulated by heavy metals in bivalves (Cheng and Sullivan, 1984; Cheng, 1988; Pipe et al., 1999; Brousseau et al., 2000; Sauvé et al., 2002; Parry and Pipe, 2004; Duchemin et al., 2008). For example, haemocytes of the mussel *M. edulis* exposed *in vivo* to concentrations of copper and mercury chloride exhibit stimulated phagocytic activity (Pipe et al., 1999; Duchemin et al., 2008). Similar results have also been observed for haemocytes of various invertebrates exposed to zinc (Brousseau et al., 2000; Sauvé et al., 2002; Filipiak et al., 2010). For all living organisms, zinc is an essential trace element that plays a key role in the immune system. It is possible that a low

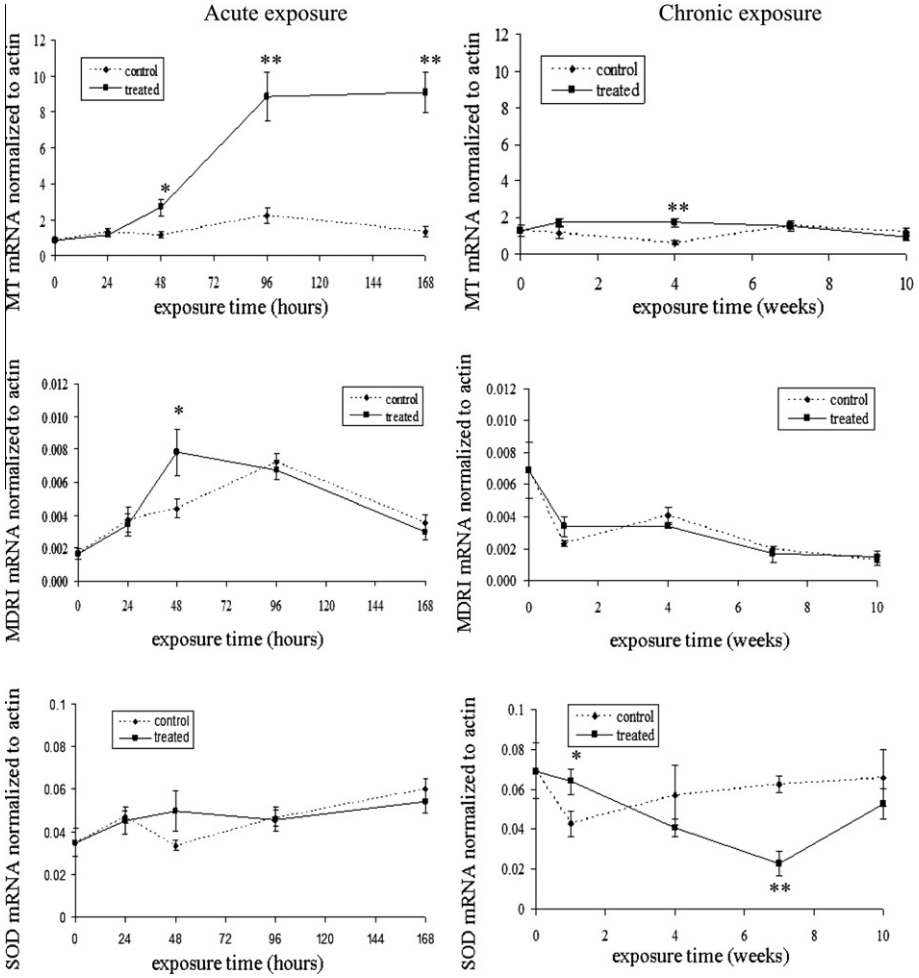


**Fig. 5.** Expression of MT, MDR1 and SOD mRNA in digestive gland in response to acute or chronic zinc exposure in *Crassostrea gigas*. Each data point represents the mean ± standard deviation of triplicate samples. Significant differences between zinc-treated specimens compared to control within a particular sampling times at $p < 0.05$ (∗), and $p < 0.01$ (∗∗) are indicated.

level of this metal directly stimulates the process of phagocytosis. In contrast, our results showed inhibition of phagocytosis when oysters were exposed to 10 mg Zn $L^{-1}$. Some authors have shown a decrease of this activity in haemocytes of different bivalve species exposed to heavy metals (Coles et al., 1995; Fournier et al., 2001; Auffret et al., 2002; Thiagarajan et al., 2006; Duchemin et al., 2008). Bouilly et al. (2006) have observed that cadmium also induces a reduction of phagocytic activity in haemocytes of *C. gigas* exposed to a concentration of 50 ng $L^{-1}$ for 21 days. The process of phagocytosis is dependent on membrane properties, and some authors have suggested that the inhibition of phagocytosis may thus be due to alterations in the cell membrane (Grundy et al., 1996; Hannam et al., 2009). It has been shown that heavy metal exposure can lead to cytoskeletal alterations (Cima et al., 1998). This disorganisation of the cytoskeleton can result in inhibition of the mobility of haemocytes. Moreover, zinc and other heavy metals are known to exhibit a high affinity for the SH groups of proteins, including $Ca^{2+}$-ATPases (Viarengo and Nicotera, 1991). In haemocytes of *M. edulis*, this high affinity between metals and $Ca^{2+}$ channels can also impair $Ca^{2+}$ homeostasis (Viarengo et al., 1994), which could be responsible for the decrease of phagocytic activity.

Measurement of ROS production using flow cytometry frequently employs 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Hégaret et al., 2003; Lambert et al., 2003; Delaporte et al., 2007). After diffusion into cells, DCFH-DA is hydrolysed to 2′,7′-dichlorofluorescein (DCFH), which is then oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS, such as $H_2O_2$, and released within phagocytic blood cells (Hégaret et al., 2003). Therefore, it has been suggested that ROS production is positively correlated with phagocytosis capacity. Our results confirmed this hypothesis, as both phagocytosis capacity and ROS production increased during chronic exposure but decreased during acute exposure. In addition, a hormesis-like effect on ROS production by haemocytes has also been observed by Auffret et al. (2002) in the oyster *O. edulis* under exposure to cadmium.

Flow cytometry analysis allows the examination of non-specific esterase activity, but few authors have used this biomarker (Gagnaire et al., 2007; Bado-Nilles et al., 2008), and Morga et al. (2011) have reported that this non-specific esterase activity can be recognised as reflecting the global activity of haemocytes. In this study, the activity of this enzyme was also stimulated by a moderate zinc level but inhibited by a high zinc level. Similar to zinc, cadmium is able to modulate non-specific esterase activity in haemocytes of *C. gigas* (Bouilly et al., 2006). Esterases are involved in the degradation of internalised particles after phagocytosis. Consequently, it was not surprising to find in the present study that non-specific esterase activity evolved concomitantly with phagocytic activity.

Zinc is an essential metal necessary for the functioning of numerous enzymes. In various invertebrates, phenoloxidase (PO) represents an important humoral defence system, which is also

found in *C. gigas* (Hellio et al., 2007). PO is a copper-containing enzyme and is present in haemocytes as an inactive form of prophenoloxidase (ProPO). In the present work, PO activity varied with exposure time and zinc concentration employed. During chronic exposure, PO activity increased globally compared to the control group. Some authors have described an increase in PO activity after cadmium exposure in Pacific oysters (Bouilly et al., 2006), copper exposure in *M. edulis* (Pipe et al., 1999) and nickel exposure in the mud crab *S. serrata* (Vijayavel et al., 2009). Thiagarajan et al. (2006) suggested that increased levels of PO activity due to metal exposure could indirectly result in elevated ROS levels because PO intermediates generate superoxide anions during redox cycling of these intermediates. In contrast, in the present study, PO activity was found to increase at 96 h and decrease at 168 h during acute exposure. Some other authors have also shown that PO activity varies according to exposure time. PO activity in the plasma of *Perna viridis* was observed to decrease after 5 days of copper exposure but increase after 15 and 25 days (Thiagarajan et al., 2006). Thus, our results appeared to be in agreement with those obtained by these authors.

Metallothioneins (MTs) are low-molecular-mass cysteine-rich metal-binding proteins with a high affinity for heavy metal ions that are found in a wide variety of organisms. MTs display antioxidant functions and are involved in zinc homeostasis (Amiard et al., 2006). Our results showed that zinc treatment significantly increased MT mRNA expression in both the gills and the digestive glands under acute exposure and in the digestive gland during chronic exposure. These increases were time and dose dependent. In addition, MT mRNA expression levels were higher during acute exposure than chronic exposure in both gills and digestive glands. These observations are in accordance with the findings of Choi et al. (2008), who showed that cadmium treatment increases MT mRNA expression in a dose- and time-dependent manner in both the gills and the digestive gland of *C. gigas*. Marie et al. (2006a) have also shown that zinc exposure leads to a significant increase in MT concentrations in *Corbicula fluminea*. In contrast, these authors did not observe MT gene induction in *Dreissena polymorpha*. In the present study, it appeared that MT mRNA expression was correlated with zinc bioaccumulation in tissues, which also appears to be time and dose dependent (Caplat et al., 2012). Correlations between zinc concentrations and MT levels in tissues of some species of bivalves have been reported (Irato et al., 2003; Marie et al., 2006b). However, during chronic exposure, expression of MT mRNA was increased in gills, but not in the digestive gland, whereas the zinc concentration was higher in the digestive gland than in gills (Caplat et al., 2012). It therefore appeared that zinc accumulation in the digestive gland did not lead to MT induction. There are species-specific differences between the two tissues. For example, Irato et al. (2003) measured higher MT concentrations in the digestive gland of *Tapes philippinarum*, whereas they recorded higher MT concentrations in gills in *Mytilus galloprovincialis* and *Scapharca inaequivalvis*. Moreover, in these last two bivalve species, a good correlation between zinc and MT concentrations in gills has been reported (Irato et al., 2003). In addition, some authors have suggested that gills constitute an appropriate tissue in which to analyse the expression of the MT gene to evaluate metal pollution (Geret and Cosson, 1999; Irato et al., 2003; Marie et al., 2006a). The results of our study were in accordance with these results, demonstrating that MT is an appropriate biomarker for monitoring zinc pollution caused by a sacrificial anode. Unlike MT, the expression of MDR1 and SOD mRNA were not altered by zinc exposure. Some authors have reported MXR induction in the presence of zinc. Three days of exposure to 500 μg Zn $L^{-1}$ (the concentration that we used for chronic exposure) were found to induce MXR protein in the gills of *C. fluminea* (Achard et al., 2004). In *Perna perna*, zinc also provokes activation of MXR activity (Franco et al., 2006). These results differ from those found in this study, in which we analysed mRNA levels, not protein levels. In future investigations, it could therefore be of interest to monitor the evolution of MDR1 protein quantities or activity in the gills or the digestive glands of oysters exposed to chronic and acute treatment of zinc in our experimental system to evaluate whether differences can be detected between mRNA levels and protein levels.

SOD is involved in an antioxidant defence system, as this enzyme catalysis $O_2^-$ into $H_2O_2$ (which is ultimately transformed by catalase to reduce toxicity) (Marklund and Marklund, 1974). We demonstrated that zinc modulates the production of ROS. Nevertheless, the expression of SOD mRNA did not vary in the presence of zinc. In the literature, it is generally reported that metal exposure results in upregulation of SOD mRNA expression or enzyme activity, although the effect of each metal is quite variable among tissues, times of exposure, organisms and pollutants (Regoli and Principato, 1995; Doyotte et al., 1997; Regoli et al., 1998; Company et al., 2004; Jing et al., 2006; Kim et al., 2007). For example, Cho et al. (2006) and Kim et al. (2007) have shown that zinc induces an increase of SOD mRNA expression in the fish *Oplegnathus fasciatus* and in the disk abalone *Haliotis discus discus*, respectively. SOD mRNA expression was found to increase in the gills and digestive gland of *H. discus discus* after 24 h and 72 h exposure to copper, cadmium and zinc (Kim et al., 2007). In contrast to the results of our study, this work has recorded an approximately 2-fold induction of SOD mRNA expression after 72 h of exposure to the same concentration used in our chronic exposure (10 μM ≈ 0.65 mg $L^{-1}$). In our experiments, the first endpoint analysed correspond to 1 week, which is much longer than the 3 days of analysis used in the work by Kim et al. (2007). The difference observed in these two studies could be explained by this difference in timing. Therefore, it would be of interest to monitor the SOD mRNA level in our experimental device for shorter time lapses during the first week of exposure to more precisely analyse the evolution of mRNA expression.

We hypothesise that during exposure to a moderate zinc concentration, oysters present an adaptative metabolism leading to induction of MT, which protects organisms from mortality to some degree because no mortality and the highest zinc concentrations in the organs were recorded under these conditions (Caplat et al., 2012). Inversely, when zinc was present in excess, zinc accumulation was too rapid to allow MT to sequester this metal. As a consequence, zinc exposure resulted in a lower zinc concentration in the organs (Caplat et al., 2012), decreases in immune system parameters and high rates of oyster mortality under these conditions.

## 5. Conclusions

In summary, the present work demonstrated that zinc released from sacrificial anodes had an impact on *C. gigas*. This pollution source was able to modulate immune system activities. Exposure to a concentration of 0.5 mg $L^{-1}$ stimulated all of the immune system biomarkers studied (phagocytosis, ROS production, non-specific esterase activity and phenoloxidase activity). In addition, no mortality was observed in this chronic exposure experiment. However, the number of circulating haemocytes decreased. Even so no drastic effect of zinc has been demonstrated in this study, we keep in mind that chronic exposure does stress the oyster possibly making them more vulnerable to disease. In contrast, exposure to a zinc concentration of 10 mg $L^{-1}$ resulted in a high level of mortality, a decrease in the number of circulating haemocytes and an inhibition of immune parameters. Zinc is therefore an essential metal that can become toxic for oysters if present in high levels. Our exposure conditions did not induce SOD or MXR mRNA expression. However, a dose- and time-dependent increase of MT mRNA

expression was observed in the gills and the digestive gland of exposed oysters, which is probably correlated with zinc accumulation in these tissues. Metallothioneins are able to sequester metals and regulate zinc homeostasis.

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