Fish & Shellfish Immunology 29 (2010) 846–853

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Effect of *in vitro* exposure to zinc on immunological parameters of haemocytes from the marine gastropod *Haliotis tuberculata*

Elmina Mottin [a,b], Christelle Caplat [b,c], Marie-Laure Mahaut [d], Katherine Costil [a], Daniel Barillier [b], Jean-Marc Lebel [a], Antoine Serpentini [a,*]

[a] *UMR 100 IFREMER « Physiologie et Ecophysiologie des Mollusques Marins », IFR 146 ICORE – IBFA, Université de Caen Basse-Normandie, Campus 1, Science C, Esplanade de la Paix, 14032 Caen cedex, France*
[b] *Equipe de Recherche en Physico-Chimie et Biotechnologie, EA 3914, IUT – UFR de Sciences, Université de Caen Basse-Normandie, Campus 2, Sciences 2, Boulevard du Maréchal Juin, 14032 Caen cedex, France*
[c] *CORRODYS, 145 Chemin de la Crespinière, BP 48, 50130 Cherbourg-Octeville, France*
[d] *CNAM-Intechmer, Digue de Collignon, BP 324, 50103 Tourlaville, France*

ARTICLE INFO

Article history:
Received 10 March 2010
Received in revised form
2 July 2010
Accepted 19 July 2010
Available online 24 July 2010

Keywords:
Zinc
Immune parameters
Abalone
Haliotis tuberculata
Haemocytes

ABSTRACT

Environmental pollutants such as heavy metals exert immunotoxic effects on aquatic organisms. The immune defence of molluscs is comprised of cell-mediated and humoral mechanisms, in which haemocytes play a key role. In this study, a model based on primary cultured haemocytes from the gastropod mollusc *Haliotis tuberculata* was established to investigate the effects of zinc *in vitro*. Cells were exposed for 24 h to $ZnCl_2$ concentrations of 0, 10, 100 or 1000 μM. The effects of zinc on haemocyte parameters were investigated using morphological, spectrophotometric and flow cytometry analysis. Immunotoxicity was reflected by a significant decrease in the number of viable haemocytes (LC_{50}(24 h) = 314 μM). Moreover, the cell area was dramatically reduced, and the percentage of rounded cells increased with increasing zinc concentrations. Exposure to 1000 μM zinc induced a significant reduction in acid phosphatase activity, phagocytic activity and reactive oxygen species production in haemocytes. However, several haemocyte parameters increased significantly after 24 h of zinc exposure. In response to a 1000 μM exposure, the phenoloxidase level was 26-fold higher than that of the control, and non-specific esterase activity was increased by 69% above that of the control. These results suggest a relationship between zinc exposure and alterations in the functional responses of haemocytes from *H. tuberculata*.

1. Introduction

Heavy metals are continually discharged into the marine environment by human activities. Anthropogenic sources of metals include urban runoff, sewage, traffic emissions, coal and oil combustion, and industrial production [1]. Among these metals, zinc is one of the most widely spread environmental toxicants [2].

Zinc is an essential trace element for all living organisms. This metal is a constituent of a large number of zinc-dependent enzymes that are important for cellular function and zinc plays a crucial role in stabilising membranes [2–6].

Zinc is also known to play a central role in the immune system. In vertebrates, this metal is crucial for the normal development and functioning of cells that mediate immunity, such as neutrophils and natural killer cells [7]. For example, zinc deficiency affects the development of acquired immunity by preventing both the outgrowth and various functions of T lymphocytes, such as activation, Th1 cytokine production, and B lymphocyte assistance. Macrophages, which are pivotal cells in many immunological functions (such as intracellular killing, cytokine production and phagocytosis), and their activation can be deregulated by zinc deficiency [7]. However, zinc can also be potentially toxic to organisms if it occurs at high concentrations [8]. When present in excess, Zinc can have deleterious effects on the immune system of vertebrates [9].

In molluscs, the cellular defence system is represented by haemocytes, which are responsible for phagocytosis, pathogen hydrolysis, production of reactive oxygen species and the phenoloxidase cascade [10-13]. Numerous studies have been conducted on bivalve haemocytes, and these studies demonstrate that haemocytes functions (cell viability, cytoskeletal organisation, phagocytic activity) can be affected by several contaminants, including

* Corresponding author. Tel.: +33 2 31 56 56 80; fax: +33 2 31 56 53 46.
E-mail address: antoine.serpentini@unicaen.fr (A. Serpentini).

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doi:10.1016/j.fsi.2010.07.022

heavy metals [11,14–18]. In gastropods, and in particular in abalone, several studies have reported that haemocytes can be affected by environmental factors such as abiotic stresses [19–21], infections [22] and organic compounds [23], but little is known about the effect of heavy metals.

To assess the effect of zinc on abalone immune response, an *in vitro* approach was chosen. During the past decade, invertebrate cell culture had been developed to study physiological processes [24–27] and pollutant effects [28]. In ecotoxicology, this approach is an alternative to animal testing because of the reduced use of experimental animals, low cost and rapid performance [29]. Moreover, an *in vitro* approach allows the use of specific endpoints to determine the targets of toxic effects with great precision and reproducibility. The major disadvantage of *in vitro* analysis systems is the difficulty of extrapolating the results to the *in vivo* condition.

Although numerous studies have already been conducted on the toxic effects of zinc on marine animals [30,31] and, more specifically, on molluscs [16,18], few investigations have reported the effects of heavy metals on marine gastropods at the cellular level. The aim of the present study was to investigate the *in vitro* effects of zinc on several haemocyte parameters in the European abalone, *Haliotis tuberculata*. Cell viability and morphology, phagocytic activities, and production of reactive oxygen species were analysed.

2. Materials and methods

2.1. Animals

Living adult abalone, *H. tuberculata*, 9–11 cm in shell length, originating from the northern Cotentin peninsula (France) were purchased. They were then maintained in natural and continuously aerated seawater at 17 °C at the Centre de Recherche en Environnement Côtier (C.R.E.C., Luc-sur-Mer, Lower-Normandy, France). The studied abalones were regularly fed with a mixture of *Laminaria sp*. and *Palmaria palmata*.

2.2. Primary cell cultures

Haemocytes were cultured as previously described [24,26,32]. Briefly, after an incision in the foot, haemolymph was collected (10–15 ml per animal) using a 20-ml syringe fitted with a 25-gauge hypodermic needle. Haemolymph was transferred to a sterile tube and immediately diluted 1:4 in cooled, sterile, anti-coagulant modified Alsever's solution (115 mM glucose, 27 mM sodium citrate, 11.5 mM EDTA, 382 mM NaCl) [33]. Haemocytes were rapidly plated at $1.5.10^6$ cells in 6-wells plates (MTT assay or enzymatic analysis) or at $0.5.10^6$ cells in 12-wells plates (flow cytometry analysis) into which three volumes of sterile artificial water were added. Cultures were maintained at 17 °C in an incubator (CO_2 free). After 90 min of incubation, cells were covered with Hank's sterile 199 medium modified by the addition of 250 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM Hepes, 2 mM L-glutamine, 100 μg/ml streptomycin and 60 μg/ml penicillin G at a final pH of 7.4. The cells were then kept at 17 °C for 24 h before beginning the experiments. Then, cells were exposed for 24 h to a range of $ZnCl_2$ concentrations: 0, 10, 100, or 1000 μM. Zinc concentrations ranging from 7–46 μM (0.47–3000 μg Zn/L) have been reported in coastal waters worldwide that are inhabited by *Haliotis spp*. [34].

2.3. MTT assay

Cell viability was evaluated by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay [35] adapted to molluscan cell cultures [24,36] and LC_{50}(24 h) was used as an index of cytotoxicity. The MTT assay is a colorimetric metabolic assay based on mitochondrial dye conversion to assess viability. Briefly, 10% (v/v) of the MTT stock solution (5 mg MTT/ml of PBS) was added to the culture dishes. After 24 h of incubation, an equal volume of isopropanol containing 0.04 N HCl was added to each culture to dissolve the converted formazan. Absorbance was then measured at a wavelength of 570 nm with a 630 nm reference.

2.4. Analysis of cellular morphology

To test the potential effects of zinc on cell morphology, haemocytes were cultured as described above on coverslips. Haemocyte monolayers were fixed in a solution of methanol for 10 min at room temperature. The coverslips were placed for 1 min in hematoxylin to stain nuclei, then briefly rinsed in distilled water and incubated for 1 min in light green dye to stain the cytoplasm. Haemocytes were then incubated for 10 min in acetone, and finally, the coverslips were mounted in Roti-Histol® (Roth, Karsruhe, Germany). Observations were carried out with a Nikon Eclipse 80i light microscope. The cell and nuclear areas were measured using a computer-assisted microscopic image analysis system, the NIS-elements D 2.30 software (Nikon).

2.5. Flow cytometry analysis of abalone haemocytes

Haemocyte analysis was performed using an EPICS XL 4 (Beckman Coulter), and 20 000 events were counted for each sample. Results are expressed as cell cytograms indicating the size (FSC value), the complexity (SSC value) and the level of fluorescence using the FL1 channel.

2.5.1. Phagocytosis capacity

Phagocytosis activity was measured by quantifying the ingestion of fluorescent beads (carboxylate-modified FluoroSpheres®, yellow-green fluorescence, 1 μm diameter, Molecular Probes). In each culture well, 7 μL of bead solution were added, and cells were incubated for 60 min at 17 °C in the dark.

2.5.2. Reactive oxygen species (ROS) production

ROS production was evaluated using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA, sigma) method [37] as adapted to mollusc cells by Lambert et al. [38]. Haemocytes were incubated for 20 min at 17 °C in the dark with DCFH-DA to a final concentration of 100 μM. Then, 6 μL of phorbol 12-myristate 13-acetate (PMA, 1 mg/ml, Sigma) were added to each well, and cells were incubated for 60 min at 17 °C in the dark.

2.5.3. non-specific esterase activity

Esterase activity was measured using the non-specific liposoluble substrate fluorescein diacetate (FDA, Molecular Probes). The FDA probe was added to each well at a final concentration of 5 μM, and cells were incubated for 60 min at 17 °C in the dark.

2.5.4. Cell fixation

After the incubation, the wells were gently scraped, and haemocyte samples were centrifuged (500×*g*, 10 min, 4 °C). Then, the supernatants were removed, and cells were fixed with paraformaldehyde (2% in distilled water). Samples were stored at 4 °C until analysis.

The results of the phagocytosis experiments are expressed as percentages of haemocytes containing three beads or more, according to Delaporte et al. [39], Hegaret et al. [40] and Labreuche et al. [41]. For the ROS production experiments, lysosome-positive cell assays and non-specific esterase activity studies, results are expressed as the percentage of cells containing fluorescence.

2.6. Enzymatic activities

Acid phosphatase (AcP) activity was evaluated as previously described by González et al. [42]. Briefly, the culture medium was removed, and the cells were scraped and lysed in 50 mM Tris buffer (pH 7.4). An equal volume of 1.5 M Tris buffer (pH 5) supplemented with 5 mM *p*-NPP (*para*-nitrophenyl phosphate, Sigma) as a substrate, 1 mM $ZnCl_2$, and 1 mM $CaCl_2$ were added to each sample. The samples were then incubated at 37 °C for 30 min. The reaction was stopped with 0.3 N NaOH, and the absorbance was measured at 410 nm. The AcP activity was estimated with a standard curve of *p*-nitrophenol.

Detection of phenoloxidase activity in the haemocyte culture medium was carried out by measurement of 3,4-dihydroxy-L-phenylalanine (L-DOPA) transformation in dopachromes [43]. Transformation was monitored by spectrophotometry at 492 nm. Briefly, 250 µl of Tris (10 mM)/NaCl (150 mM) buffer at pH 8, 250 µl of cacodylate buffer (10 mM) and 250 µl of L-DOPA (3 mg/ml) were added to a 250 µl sample. Absorbance was then measured after 1 h of incubation at 25 °C. Phenoloxidase activity was expressed as an arbitrary unit.

The amount of protein in each cellular extract and in the medium of each primary culture was determined by the Bradford method [44] using bovine serum albumin as a standard.

2.7. Data analysis

Results are expressed as means ± S.D. Each experiment was repeated at least three times, and the means were calculated from triplicates for each experiment. The significance of the differences between mean values was estimated using student's *t*-tests

3. Results

3.1. Effects of zinc on haemocyte viability

A dose–response cytotoxicity curve, as quantitated by an MTT assay after 24 h of exposure of haemocytes to zinc, is shown Fig. 1. No significant change was observed at 10 µM Zn. At zinc concentrations of 100 µM and higher, haemocyte viability decreased, and maximum toxicity was observed when cells were exposed to 1000 µM zinc. The LC_{50}(24 h) value, was determined graphically from the semilogarithmic plots representing the zinc concentration required to reduce cell viability to 50%; the LC_{50}(24 h) for Zinc was approximately 314 µM for haemocytes in culture.

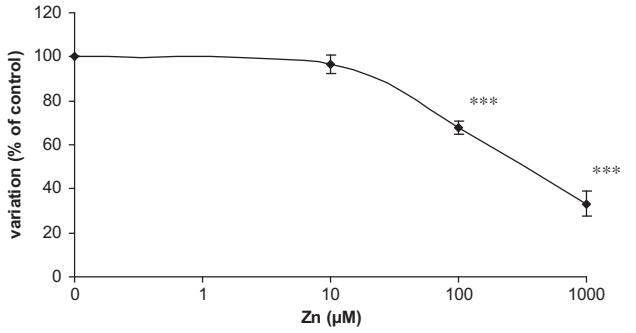

Fig. 1. Dose-response effect of zinc on cell viability as determined by the MTT reduction assay. Haemocytes were plated at a density of $1.5.10^6$ cells per well in 6-well culture plates and cultured at 17 °C in modified Hank's-199 medium. 24 h after the beginning of cultures, the medium was renewed. Cells were exposed for 24 h to $ZnCl_2$ and MTT test was performed. Each data point represents the mean percentage ± standard deviation of triplicate cultures. Significant difference from control cells at $P < 0.001$ (***).

3.2. Effects of zinc on haemocyte morphology

The morphological effect of zinc on haemocytes after 24 h of exposure is illustrated in Fig. 2. Haemocytes were noticeably affected by the presence of zinc. In the absence of zinc (control), cells were interconnected and elongated with large pseudopods. In the presence of zinc, haemocytes became isolated and more rounded. This change was quantified by the measurement of haemocyte area, nucleocytoplasmic ratio and a shape factor (Fig. 3). In the control preparation of *Haliotis* haemocytes, the mean cellular area measured 210.4 ± 5.5 μm^2 (range 84.3–384.6 μm^2). The means of haemocyte area in zinc-treated cells decreased in a dose-dependent manner, resulting in cell areas that were decreased by 36% (133.4 ± 4.6 μm^2 vs. 210.4 ± 5.5 μm^2) and by 52% (99.9 ± 3.4 μm^2 vs. 210.4 ± 5.5 μm^2) in the presence of 100 µM and 1000 µM zinc, respectively. Concurrently, the nucleocytoplasmic ratio significantly increased by 17% (0.23 ± 0.01 vs. 0.20 ± 0.01) and 36% (0.27 ± 0.01 vs. 0.20 ± 0.01) when haemocytes were exposed to 100 µM and 1000 µM zinc, respectively.

3.3. Zinc effects on haemocyte cellular activity

The influence of zinc on non-specific esterase and phagocytic activities as well as ROS production was assessed by flow cytometry after 24 h of exposure (Fig. 4). The percentage of haemocytes presenting phagocytic and a non-specific esterase activities as well as ROS production were 32.6 ± 5%, 34.9 ± 2% and 31.5 ± 4%, respectively, for each control. At low concentration (10 µM), zinc stimulated esterase and phagocytic activities slightly, but not significantly. Compared to the control, the highest zinc concentration (1000 µM) decreased phagocytic activity and ROS production by 22% (78.82 ± 3.08% in treatment vs. 100% for the control) and 35% (65.65 ± 12.01% in treatment vs. 100% for the control), respectively. For non-specific esterase activity, the percentage of fluorescent cells increased by 69% (169.60 ± 5.07% in treatment vs. 100% for the control) after exposure to 1000 µM zinc.

3.4. Zinc effects on haemocyte enzymatic activity

After 24 h of zinc exposure, phenoloxidase activity was stimulated in a dose-dependent manner compared to the control. A 6-fold and a 26-fold induction of phenoloxidase activity were observed with treatments of 100 µM and 1000 µM zinc, respectively (Fig. 5A). For AcP activity, no significant effects were recorded after an incubation with 10 µM or 100 µM zinc. When cells were exposed to 1000 µM zinc, a decrease in AcP activity of 77% was observed in comparison with the control (Fig. 5B).

4. Discussion

The results obtained in this study demonstrate the toxic effects of zinc on a primary culture of haemocytes from the gastropod *H. tuberculata*. After 24 h of exposure, the observed viability was not more than of 68% and 33% compared to the control for treatments of 100 µM and 1000 µM $ZnCl_2$, respectively. Decreases in the viability of various cell types, including haemocytes from *Mya arenaria*, coelomocytes from *Lumbricus terrestris*, interrenal cells from the trout *Oncorhynchus mykiss* and cells of the human lineage ARPE-19, have been reported after 18 h or 24 h of cell exposure to zinc at concentrations ranging from 10^{-5} M to 10^{-3} M [45,46]. Zinc is not the only metallic compound able to induce mortality in haemocytes in primary culture. For example, $AgNO_3$, $CdCl_2$, CH_3HgCl and $HgCl_2$ also cause a decrease in the viability of haemocytes from several bivalves [16,45]. In our study, the LC_{50} value (representing the zinc concentration at which 50% of cells died after

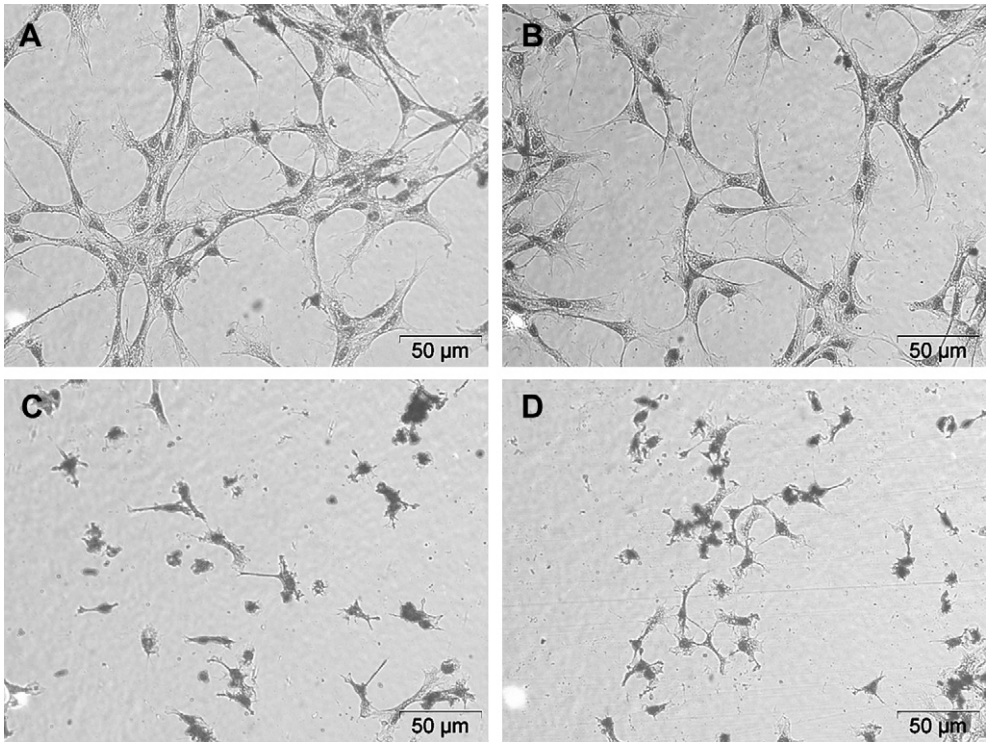

Fig. 2. Light microscopic pictures showing morphology of *Haliotis tuberculata* haemocytes. Cells were seeded on coverslips at $0.5.10^6$ cells per well and grown at 17 °C in modified Hank's-199 medium in absence (A) or presence to a range of zinc chloride concentrations: 10 µM (B), 100 µM (C), 1000 µM (D). Cells were exposed for 24 h to $ZnCl_2$

24 h of exposure) was calculated at 314 µM. Brousseau et al. [45] reported a decrease of more than 50% in the viability of haemocytes from *M. arenaria* exposed *in vitro* for 18 h to $ZnCl_2$ at concentrations between 100 and 1000 µM. Thus, our results appear to be in agreement with previous reports.

In addition to haemocyte viability, the extension of pseudopods by haemocytes and their capacity to adhere play key roles in cellular migration and the processes of immune defence of molluscs. The morphological results obtained in this study highlight significant physical changes in the cells exposed to zinc. The morphological changes observed with increasing concentrations of zinc were characterised by both a decrease in the spreading of haemocytes and a decrease in the number of cytoplasmic extensions. A concomitant increase in the number of rounded cells and a decrease in the cell area values (by ~52%, 1000 µM zinc vs. control) were observed. These modifications were accompanied by an increase of the nucleocytoplasmic ratio. These results are in agreement with data from the literature. For example, Olabarrieta et al. [15] and Gómez-Mandikute and Cajaraville [47] showed that haemocytes from mussels (*Mytilus galloprovincialis*) in primary culture exposed to cadmium or to copper became rounded and did not present additional cytoplasmic extensions when the concentration of the metals increased. Such morphological modifications of haemocytes in response to metallic treatments are commonly associated with disturbances of the cytoskeleton, such as an intracellular disorganisation of actin [14,15].

The analysis by flow cytometry allowed us to study complementary immune parameters, such as phagocytosis, lysosome-positive cells, production of ROS and non-specific esterase activity. Phagocytosis is generally considered as the first line of defence in molluscs. We showed that phagocytic activity decreased when the haemocytes were cultured in the presence of 100 µM Zn. These results are in agreement with previously reported studies that showed, in primary culture, an inhibition of phagocytic activity in the haemocytes of several species of bivalves, including *M. arenaria*, *Crassostrea virginica*, *Mytilus edulis*, exposed to different metallic

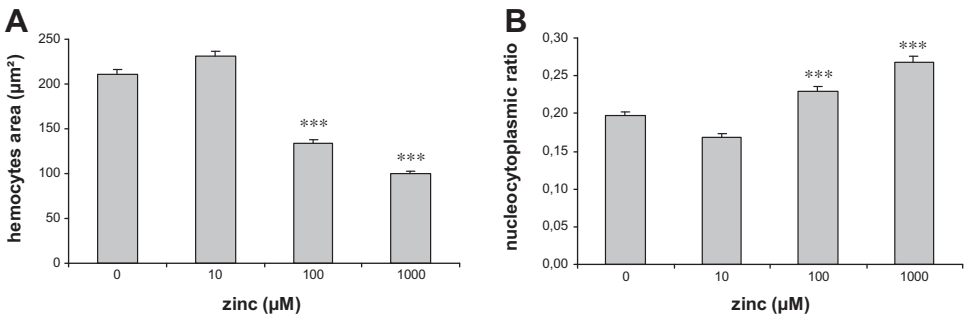

Fig. 3. Effect of zinc on haemocyte morphological parameters after 24 h exposure. Variation in cellular area (A), nucleocytoplasmic ratio (B) and in cell shape factor (C). Each data point represents the mean percentage ± standard deviation of triplicate cultures. Significant difference from control cells at $P < 0.001$ (***).

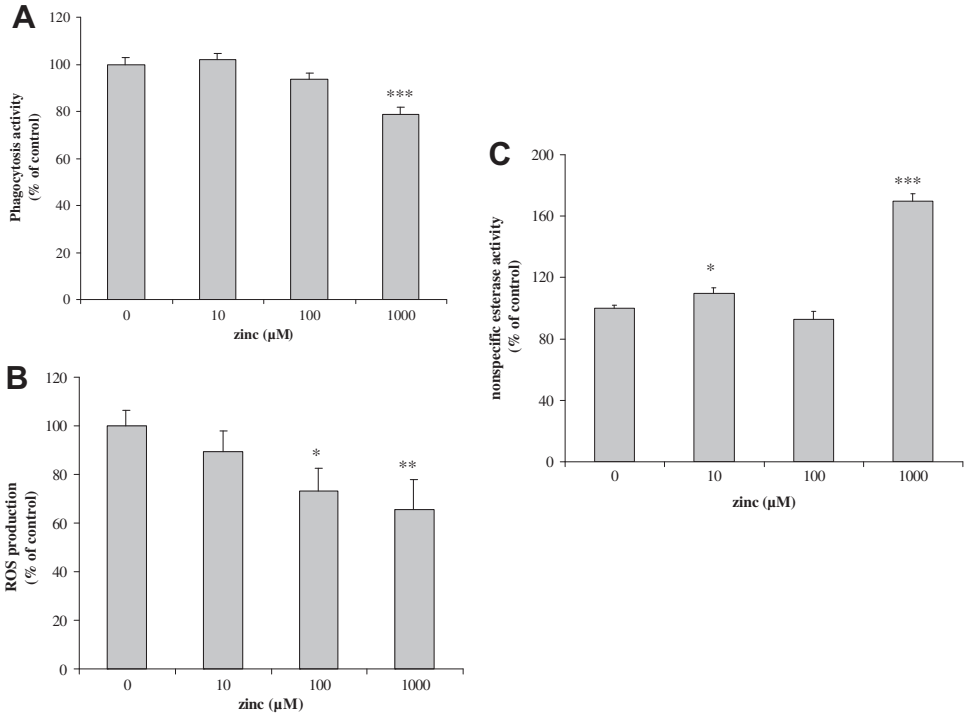

Fig. 4. Effect of zinc on immune parameters after 24 h incubation. Phagocytic activity (A), lysosome-positive cells (B), ROS production (C), and non-specific esterase activity (D). Each data point represents the mean percentage ± standard deviation of triplicate cultures. Significant differences from control cells at $P < 0.05$ (*), $P < 0.001$ (***).

components, such as Ag, Al, Cd, Cu, Hg [18,51] and especially $ZnCl_2$ [16,45,48].

A decrease in phagocytic activity can be related to a disorganisation of the cytoskeleton, with an inhibition of the mobility of haemocytes as a consequence. Indeed, *in vitro* studies applied to bivalve cells showed that morphological and functional modifications of haemocytes could be responsible for the observed decrease in the phagocytic activity [14,49–51]. In the present study, the increase of the proportion of rounded cells observed during treatment with zinc suggests a direct or indirect effect of zinc in the disorganisation of the cytoskeleton that is characterised by a decrease of the phagocytic activity of haemocytes.

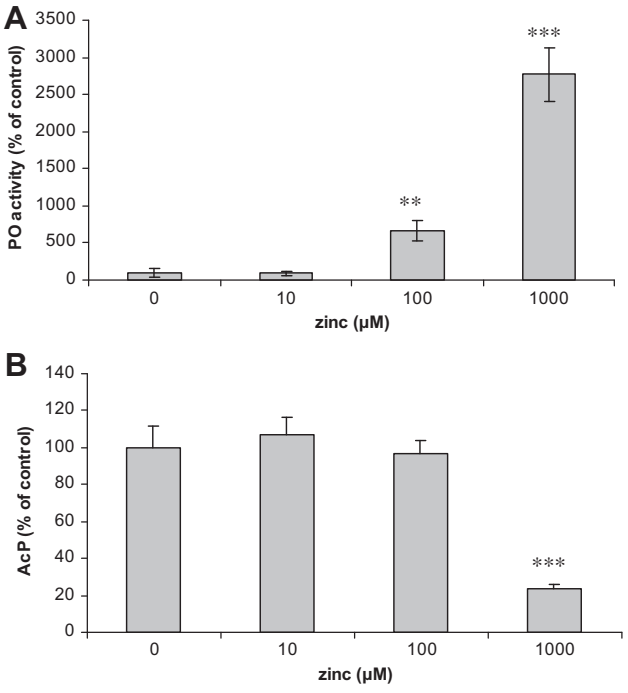

Fig. 5. Effect of zinc on enzymatic activity after 24 h incubation. Phenoloxidase activity (A), Acid phosphates activity (B). Each data point represents the mean percentage ± standard deviation of triplicate cultures. Significant difference from control cells at $P < 0.01$ (**), $P < 0.001$ (***).

Flow cytometry allowed us to follow the production of reactive oxygen species (ROS). As was observed for phagocytosis, zinc induced an inhibition of ROS production, which, at a concentration of 1000 µM zinc, was reduced by 34% of that in the control. Gómez-Mendikute and Cajaraville [47] also noticed a decrease in ROS production by haemocytes of *M. galloprovincialis* exposed *in vitro* to Cu for 24 h. Our study was in agreement with these results. However, these same authors indicated no modification of the ROS production by these cells during exposures to Cd. On the other hand, several authors observed an increase of the ROS production by different cell types exposed to Zn in culture. As an example, 100 µM Zn^{2+} cation stimulated ROS production in cells of the human lineage ARPE-19 treated for 24 h [46]. Similarly, the production of O^{2-} was stimulated in haemocytes from *M. galloprovincialis* exposed to 50 µM $ZnCl_2$ [51]. The difference between these latter results and ours could be explained by the different exposure times; the Zn exposure time was only 30 min for the experiments carried out by Kaloyianni et al. [51], whereas it was 24 h in our study.

Our *in vitro* experiments exposing cultured cells to Zn demonstrate a correlation between phagocytic activity, morphological changes and the ROS production. The decrease in ROS production could be explained by a disorganisation of the cytoskeleton. As suggested by Thiagarajan et al. [52], the disturbance of the cytoskeleton could be correlated to the NADPH-oxidase complex contained in the plasma membrane. This complex is responsible for the formation of O^{2-} in haemocytes and could be inhibited in response to a treatment by zinc.

The presence of zinc (1000 μM) in the cellular culture medium significantly stimulated non-specific esterase activity in the haemocytes. The activity of these enzymes, which are involved in the intracellular degradation of foreign bodies, was studied by flow cytometry. Our results are in agreement with those obtained by Bado-Nilles et al. [53] who observed a stimulation of non-specific esterase activity in haemocytes from *Crassostrea gigas* that were exposed *in vitro* to naphthalene (HAP). However, Gagnaire et al. [17,54] showed that the non-specific esterase activity was not modified by mercury, but it was inhibited by some pesticides (paraoxon and chlorohalonil) and HAP (benzo[a]pyrène, phenanthrene, anthracene and fluoranthene). Mazón et al. [55] and Zvereva et al. [56] reported an *in vitro* inhibition of the non-specific esterase activity in cells (exposed to Ni, Cd and Cu) from *Littorina littorea* and *Chrysomela lapponica*. The results published in the literature on non-specific esterase activity report different effects according to the studied metallic species. Zinc is an essential metal necessary for the function of numerous enzymes including some esterases, such as phosphodiesterases [57]. Therefore, it is possible that the activity of these enzymes may be stimulated by the introduction of metal into the culture medium.

Our enzymatic analyses focussed on the activity of phenoloxidase (PO) and acid phosphatase (AcP). Phenoloxydase is a copper-dependent enzyme directly implicated in mechanisms of immune defence. PO has been detected in the haemolymph of several invertebrates [58–60]. To our knowledge, the pro-phenoloxydase system has not yet been characterised in abalones. However, recent studies relative to the effects of environmental parameters and pathogens in the immune response of *Haliotis diversicolor supersexta* and *H. tuberculata* measured PO activity [19–21]. These studies indicated that abiotic factors such as temperature, salinity, and levels of ammonium or nitrite present in the water, as well as biotic factors (such as spawning) induced a decrease of PO activity. Our study showed that PO activity increased in a dose-dependent manner when haemocytes were exposed to zinc. In the literature, few studies have reported an increase in the PO activity of haemocytes in culture in the presence of anthropogenic contaminants. Nevertheless, Bado-Nilles et al. [53] showed an increase in PO activity *in vitro* in haemocytes from *C. gigas* exposed for 24 h to various concentrations of benzo[b]fluoranthene. In the ascidian *Styela plicata*, the *in vitro* exposure of haemocytes to 1, 10, 100 or 1000 μg/L of $ZnSO_4$ for 72 h did not affect the PO activity [61], whereas the Zn^{2+} cation (5 mM) inhibited the activity of this enzyme in the crab *Charybdis japonica* [59]. Mercury also inhibited the PO activity of haemocytes from *C. gigas* in culture after 21 h of exposure [17]. Therefore, the response of PO activity to contaminants appears to vary according to the animal species and the type and form of contaminants.

Contrary to the PO activity, the activity of acid phosphatase was inhibited by zinc. This inhibition was by more than 70% of the control activity for the highest concentration. These results are in agreement with those obtained by Mazorra et al. [62] who also demonstrated a decrease in the activity of this enzyme from *Scrobicularia plana* exposed for 48 h to different heavy metals, including Cd (0.05–1 mM), Cu (0.1–1 mM), Hg (0.05–1 mM) and particularly Zn (0.05–1 mM). Some metals are capable of stimulating the activity of phosphatases. Thus, the AcP activity is up-regulated in haemocytes from *M. galloprovincialis* after *in vitro* exposure to numerous concentrations of Cd [15]. A similar result was obtained in gills and digestive glands from *Pinctada fucata* exposed to copper [63]. However, the response of phosphatase to metallic contamination can differ according to the organ in which it is measured. For example, Rajalakshmi and Mohandas [64] observed a stimulation of AcP activity in gills and an inhibition of this activity in the digestive gland from *Lamellidens corrianus* exposed to copper.

Acid phosphatase is a lysosomal enzyme involved in the degradation of pathogenic or foreign elements. Therefore, the decrease of its activity in the presence of metals, and particularly of zinc, could be due either to a specific inhibition and/or to a more general change of the lysosomal system. Recent studies have been focused on the analysis of AcP activity in the haemolymph of *Haliotis diversicolor supertexta* infected by pathogenic bacteria [65]. In this study, AcP activity was increased in the haemolymph of abalone infected by *Vibrio parahaemolyticus*. Such a result shows that this enzyme might be secreted into the extracellular fluid by haemocytes in response to bacterial infection. In our work, AcP activity was analysed at the cellular level. Therefore, it is possible that the modulations of haemocytes exposed to a strong zinc concentration that we observed are caused by the same processes as in the case of infection by a pathogenic organism. Thus, the decrease measured during our experiments would reflect a more important secretion of this enzyme by haemocytes and a decrease of the stock of this protein inside haemocytes.

5. Conclusions

In summary, our *in vitro* experiments demonstrated that contamination by zinc could induce important physiological effects in haemocytes. Exposure to 100 μM Zn decreased cell viability and induced morphological and physiological modifications in haemocytes. Furthermore, this contamination decreased ROS production and increased PO activity. For the highest concentration we tested (1000 μM Zn), exposure induced an inhibition of phagocytic capacity, a decrease in acid phosphatase activity, and an increase in the non-specific esterase activity in addition to the effects already observed at 100 μM. Except for cell viability and morphology, the most sensitive haemocyte parameters to zinc are PO activity and ROS production. It was not surprising to observe an association between PO and ROS production. Indeed, it has been established that, to fight against infections, the activation of PO catalyses the production of ROS during the processes of repair, wound healing or encapsulation [66].

Increased mortality of haemocytes is associated with a change of cell shape, which becomes rounded, a massive secretion of PO, a decrease of AcP activity and an inhibition of phagocytic capacity and ROS production. Zinc is a trace element essential to cell functions, but it was shown in this study that it could become toxic if present in high levels (100 and 1000 μM). Thus, zinc induced a general disturbance of the immune capacities of haemocytes. In conclusion, it can be speculated that environmental contamination by zinc can render abalone more vulnerable to infections or environmental stresses.

Acknowledgements

E. Mottin's work was financially supported by a fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. The authors thank L. Poulain and M. Duval (IFR 146 ICORE) for their help in flow cytometry studies, the technical staff of the C.R.E.C. for their assistance in animal care and Elsevier Language Editing Services for English revision.

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