Immunotoxicity of heavy metals (silver, cadmium, mercury and lead) on marine bivalve *Mytilus edulis*: in vitro exposure of hemocytes

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Introduction

Anthropogenic pressures lead to profound deleterious effects on coastal aquatic ecosystems. Many chemicals released in the environment generate toxicity in aquatic organisms. In response to these environmental disturbances, there is a growing concern about the consequences of environmental contaminants on the immune system of aquatic species.^{1,2} Various environmental surveys have been set up to monitor these changes. They allow medium and long-term monitoring of coastal ecosystems exposed to chemical contamination through the study of selected sentinel species. As sessile filter-feeder, bivalve mollusks are directly exposed to environmental contaminants. They bioaccumulate most environmental pollutants, including heavy metals.^{3,4,5} Therefore, they reflect the surrounding contamination by integrating the environmental exposure. Immune defense in bivalves is coordinated primarily by circulating hemocytes. Those multifunctional cells are able to recognize foreign particles and respond through phagocytic ingestion.⁶ In mussels, hemocytes circulating in the hemolymph represent the main component of their immune system.^{7,8} Hence, the blue mussel, *Mytilus edulis*, is considered as interesting sentinel specie in immunotoxicological studies.

The use of hemocytes and the monitoring of their phagocytic activity may represent an ideal biological endpoint.⁹ The present study aims to validate the use of phagocytic responses in *Mytilus edulis* as biomarker of heavy metals contamination. The purpose of this present study is to establish dose-response curves of viability and phagocytic activity on marine bivalve *Mytilus edulis* exposed to various heavy

metals [cadmium (Cd), lead (Pb), mercury (Hg), silver (Ag)].

Materials and Methods

Animals

Mytilus edulis were collected in the Baie de Plaisance from Îles de la Madeleine (47°29'N, 61°87'W). They are located in the center of Gulf of St Lawrence, a place exempt from industrial activities. Upon arrival to the laboratory, all bivalves were transferred and maintained in aquaria filled with salted artificial marine water (15°C, salinity 31-1 psu) and fed *ad libitum*.

Hemolymph collection and viability of cells

Hemolymph was collected individually from the posterior adductor muscle using a 3 mL syringe with a 23G gauge needle. Immediately after the hemolymph collection, hemocytes viability was evaluated by flow cytometry using the Guava PCA flow cytometer (Guava Technologies, Hayward, CA, USA) and the Viacount kit (Guava technologies) according to the supplier's instructions. Briefly, an aliquot of 50 µL of hemolymph was mixed with 200 µL of Viacount and at least 1000 events were recorded. Only individuals whose cell viability was higher than 75% were kept.

Heavy metals and exposure protocol

Heavy metals used in this study were reagent grade: cadmium chloride (CdCl₂), lead chloride (PbCl₂), mercuric chloride (HgCl₂) and silver nitrate (AgNO₃) (Sigma, ON, Canada). The concentrations tested were the same for all four metals, ranging from 10⁻³ to 10⁻⁹ mol. Working solutions of each metal were then prepared by serial dilutions in distilled water.

Phagocytosis and cell viability

For each metal, dose-response curves were performed on nine different mussels. Phagocytosis was assessed by determining the ingestion of latex fluorescent beads by flow cytometry according to Brousseau *et al.*¹⁰ Briefly, 500 µL of hemolymph containing 100,000 cells were mixed with 25 µL of each metal working solution and exposed during 3 h at 15°C under constant agitation. After the incubation, latex fluorescent beads (Yellow-green Fluoresbrite, Polysciences®) were added to each tube at a ratio of 100:1 beads/cell and suspensions were re-incubated for another 18 hours. At the end of the exposure period, the cells were layered over a 3%

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bovine serum albumin gradient in RPMI-1640 medium containing 31 g.l⁻¹ of marine salt and centrifuged at 138 x g for 8 min at 4°C to remove free beads. The cell pellets were then re-suspended in 0.5 mL of 0.5% formaldehyde and 0.2% sodium azide in 31 g.l⁻¹ salt water. The number of engulfed beads was determined using a FACSCalibur (Becton-Dickinson, CA, USA) flow cytometer. Fluorescence emission was read in FL1 (λ=530 nm), with at least 10,000 events in the region of interest.

Statistical analysis

Data are presented as mean standard deviation. The results were tested for homogeneity using an analysis of variance (ANOVA). Significant differences between treatments and control were determined using the SigmaStat™ Software version 3.5. (Jandel, Scientific Software, San Rafael, CA, USA). Then, we used the Holm-Sidak method to perform multiple comparisons versus control group to identify significant differences (P<0.05).

Results

Effects of heavy metals on cell viability

Regarding hemocyte viability, as illustrated in Figure 1, silver nitrate appeared to be more cytotoxic than mercury for this population of cells. We noticed a significant decrease in viability at 10⁻⁵ M for Ag (Figure 1D) and Hg (Figure 1C). Cadmium (Figure 1A) and lead (Figure 1B) showed similar results on cell viability with a significant decrease at 10⁻³ M.

Effects of heavy metals on phagocytosis

In vitro analysis of metal toxicity on hemocytes ranked the metals as follows: mercury > silver > lead > cadmium. These heavy metals also lead to a significant increase in the immune response at low concentrations. These concentrations tested are much higher than those found in the ocean. Silver induced a significant decrease of phagocytosis at concentrations ranging from 10^{-5} M to 10^{-3} M (Figure 1D). Dissolved Ag concentrations in seawater ranging from $1\text{--}20 \cdot 10^{-12}$ M ($0.1\text{--}2.0$ ng.L⁻¹) in pristine offshore waters up to $300 \cdot 10^{-12}$ M (30 ng.L⁻¹) in estuaries close to point source discharges.¹¹ A significant increase of phagocytosis at 10^{-7} M is noticed. The IC₅₀ value obtained (calculated with a linear regression) for the hemocytes exposed to silver nitrate is $9 \cdot 10^{-6}$ M (Table 1). Mercury induced a significant decrease of phagocytosis at concentrations ranging from 10^{-6} M to 10^{-3} M (Figure 1C). We can also notice a significant increase of phagocytosis at concentrations ranging from 10^{-9} M to 10^{-7} M. The IC₅₀ value

calculated for the hemocytes exposed to mercury chloride is $3 \cdot 10^{-6}$ M (Table 1). Total Hg concentrations are low and range from 0.2 to $4 \cdot 10^{-12}$ M (40 to 802 pg.L⁻¹), with the higher concentrations found near the coast likely due to riverine inputs.¹² Cadmium and lead induced a significant decrease of phagocytosis at 10^{-3} M (Figure 1A and B). For both metals the IC₅₀ values calculated are greater than 10^{-3} M (Table 1). CdCl₂ and PbCl₂ compounds significantly increased the immune response at low concentrations ranging from 10^{-9} M to 10^{-6} M. In seawater, dissolved lead is between $7,2$ and $72 \cdot 10^{-11}$ M ($15\text{--}150$ ng.L⁻¹) in the North Atlantic and total dissolved Cd exists at low concentrations usually less than $1.8 \cdot 10^{-6}$ M (0.2 mg.L⁻¹).¹³

Discussion

Environmental concentrations do not induce short-term responses, so we tested higher concentrations ranges. These concentrations test-

ed are found in oceans for cadmium, while silver, lead and mercury are present in lesser amounts. Hemocyte cells of *Mytilus edulis* showed a classical pattern of the *in vitro* toxicity of heavy metals. At low concentrations, there is a lack of cell cytotoxicity and stimulation of the phagocytosis then followed with normal phagocytosis. At moderate concentrations, phagocytosis is dramatically reduced which is accompanied by low changes in cell viability. At high concentrations, cytotoxicity is relatively high, accompanied by a drastic inhibition of the phagocytosis. Similar results support the observation that exposure to low levels may stimulate phagocytic activity whereas increasing concentrations induce suppression.^{9,14} The average sensitivity for phagocytosis can be ranked in decreasing order of toxicity: HgCl₂, AgNO₃, and CdCl₂. The impact of lead on phagocytosis was never analysed in the blue mussel and was ranked between Hg and Cd. In *Mya arenaria*, it was suggested by others⁹ that hemocyte mortality only partially explained the decrease of the phagocytic index. Similarly, for the blue mussel, cytotoxicity is not the sole mechanism for phagocytosis inhibition. For example, cells incubated with HgCl₂ at 10^{-5} M show an almost normal level of viability (80% of control), while phagocytosis is strongly inhibited with only 20% of the control value. The phagocytosis dose-response curves for AgNO₃, HgCl₂ suggest that the toxicity tends to be threshold rather than linear. For example, AgNO₃ concentrations lower than 10^{-6} M, HgCl₂ lower than 10^{-7} M had no apparent negative effect on phagocytosis. Yet, next higher dose shows inhibition levels sometimes exceeding 60%. Compared to lethal or acute bioassays, the measurement of phagocytosis by flow cytometry in bivalves represents a sensitive endpoint to study the adverse effects of heavy metals at sublethal concentrations.¹⁵ However, exposure was done under acute and short-term conditions which differ from environmental exposures *i.e.*, under chronic and low concentrations.

Figure 1. Phagocytosis and viability of hemocytes from *Mytilus edulis* exposed at different concentrations ranging from 10^{-9} M to 10^{-3} M. Gross values of phagocytosis's control for 1 bead and more: A) Cd 48.3%; B) Pb 44.0%; C) Hg 41.7%; D) Ag 44.7%. *P<0.05.

Table 1. Nominal metal concentrations inducing 50% inhibition of phagocytosis relative to controls.

Metal	EC ₅₀ M	EC ₅₀ (mg metal.l ⁻¹)
CdCl ₂	> 10^{-3} M	>112
PbCl ₂	> 10^{-3} M	>207.2
AgNO ₃	$9 \cdot 10^{-6}$ M	0.97
HgCl ₂	$3 \cdot 10^{-6}$ M	0.6

EC₅₀, effective concentration; CdCl₂, cadmium chloride; PbCl₂, lead chloride; AgNO₃, silver nitrate; HgCl₂, mercuric chloride.

References

1. Fisher WS, Oliver LM, Walker WW, Manning CS, Lytle TF. Decreased resistance of eastern oysters (*Crassostrea virginica*) to a protozoan pathogen (*Perkinsus marinus*) after sublethal exposure to tributyltin oxide. *Mar Environ Res* 1999;47: 185-201.
2. Fournier M, Cyr D, Blakley B, Boermans H, Brousseau P. Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics. *Am*

- Zool 2000;40:412-20.
3. Zatta P, Gobbo S, Rocco P, Perazzolo M, Favaro M. Evaluation of heavy metal pollution in the Venetian lagoon by using *Mytilus galloprovincialis* as biological indicator. *Science of the Total Environ* 1992; 119:29-41.
 4. Phillips DJH. The chemistries and environmental fates of trace metals and organochlorines in aquatic ecosystems. *Mar Pollut Bull* 1995;31:193-200.
 5. Gunther AJ, Davis JA, Hardin DD, Gold J, Bell D, Crick JR, et al. Long-term bioaccumulation monitoring with transplanted bivalves in the San Francisco Estuary. *Mar Pollut Bull* 1999;38:170-81.
 6. Pipe RK, Coles JA. Environmental contaminants influencing immunefunction in marine bivalve molluscs. *Fish Shellfish Immunol* 1995;5:581-95.
 7. Cheng TC. Biochemical and ultrastructural evidence for the double role of phagocytosis in mollusks: defense and nutrition. *Comparat Pathobiol* 1977;3:21-30.
 8. Adema CM, Van der Knapp WPW, Sminia T. Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. *Rev Aquat Sci* 1991;4:201-23.
 9. Fournier M, Pellerin J, Lebeuf M, Brousseau P, Morin Y, Cyr D. Effects of exposure of *Mya arenaria* and *Mactromeris polynyma* to contaminated marine sediments on phagocytic activity of hemocytes. *Aquat Toxicol* 2002;59:83-92.
 10. Brousseau P, Payette Y, Tryphonas H, Blakley B, Boernaus H, Flipo D, et al. Manual of immunological methods. Boca Raton, FL: CRS Press; 1999.
 11. Kramer JR, Benoit G, Bowles KC, DiToro DM, Herrin RT, Luther GW III, et al. Environmental chemistry of silver. In: Andren AW, Bober TW, eds. Silver in the environment: transport, fate, and effects. Pensacola, FL: Society of Environmental Toxicology and Chemistry (SETAC); 2002. pp 1-25.
 12. Mason RP, Gill GA. Mercury in the marine environment. In: Parsons MB, Percival JW, eds. Mercury Sources, measurements, cycles and effects. Mineralogical Association of Canada Short Course Series, Vol. 34. Halifax: Mineralogical Association of Canada; 2005. pp 179-216.
 13. Pan J, Plant JA, Voulvoulis N, Oates CJ, Ihlenfeld C. Cadmium levels in Europe: implications for human health. *Environ Geochem Health* 2010;32:1-12.
 14. Brousseau P, Pellerin J, Morin Y, Cyr D, Blakley B, Boermans H, et al. Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam *Mya arenaria* hemocytes following in vitro exposure to heavy metals. *Toxicology* 2000;142:145-56.
 15. Sauvé S, Brousseau P, Pellerin J, Morin Y, Sénécal L, Goudreau P, et al. Phagocytic activity of marine and freshwater bivalves: In vitro exposure of hemocytes to metals (Ag, Cd, Hg and Zn). *Aquat Toxicol* 2002;58:189-200.