

In vitro effects of methylmercury on ascidian (*Styela plicata*) immunocyte responses

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This study shows that high methylmercury concentrations are cytotoxic for *Styela plicata* hemocytes, whereas sublethal concentrations affect immunocyte responses. Moreover, hemocytes exposed to the xenobiotic present a significantly enhanced phenoloxidase activity as revealed in the hemocyte lysate supernatant compared with the control. Although the cytotoxic activity of *S. plicata* hemocytes toward rabbit erythrocytes is a PO-dependent cell-target reaction due to quinone products, it was significantly decreased by suitable methylmercury concentrations in the medium. The same xenobiotic concentrations decreased the hemocyte phagocytic activity toward yeast. In both the responses cell-target contacts could be affected by methylmercury, whereas the releasing capacity appeared to be unchanged, as indicated by hemocyte PO-release in the medium. Finally, changes in hemocyte shape and spreading capacity were shown. On the basis of the present results, *Styela plicata* hemocyte responses could be an additional immunotoxicology test using a microplate method that reveals cell morphological changes and spreading capacity. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: tunicate; ascidian; hemocytes; toxic metals; methylmercury; phenoloxidase; phagocytosis; cytotoxicity

INTRODUCTION

Pollution by heavy metals is a major risk in aquatic ecosystems, where high concentrations cause adverse biological effects, including changes in immune function of invertebrate and vertebrate species.^{1–3} In the marine environment, although mercury concentration in the water column and sediments may be low, filter feeding invertebrates accumulate this metal in their tissues.⁴ In addition, biological processes mediate mercury methylation, transforming the metal into methylmercury^{5–9} which is the most toxic form due to the methyl group that facilitates cell penetration and interaction with proteins, interfering with their synthesis and leading to lipid peroxidation.¹⁰ Methylmercury is capable of blocking the binding sites of enzymes while they interfere with the incorporation of thymidine into DNA.⁷

Mercury pollution in aquatic systems necessitates knowledge of the biological effects of methylmercury,^{9,11–13} which can be responsible for changes in immune functions^{1–3,14} of animals that accumulate heavy metals. In addition, with the increasing interest in using organisms as bioindicators for marine environmental chronic stress,^{15–17} cellular and functional parameters of the immune system in many sentinel species can represent anticipatory signals in monitoring organisms and their environment.^{18,19} Ascidians filter large amounts of water, accumulate toxicants in their tissues, including heavy metals, and are considered good indicators of water quality.^{20,21} However little is known about the effects of heavy metals on biological functions. Cima *et al.*²² reported that a treatment with organotin compounds decreased the viability of *Styela plicata* embryos, and other authors have shown that copper and tributyltin (TBT) affect cytotoxicity²³ and phagocytosis.^{23–25}

The general functional features of invertebrate and vertebrate phagocytes make phagocytic function attractive for immunotoxicology studies. Phagocytosis can be affected by environmental xenobiotics.^{18,24,26–31} Copper alters actin and fibronectin organization of mussel hemocytes,³² and heavy

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metals impair the phagocytic activity of coelomocytes from terrestrial and aquatic annelid species,^{33–35} chronic exposure to low doses of metallic mercury impaired the chemotactic activity of vertebrate polymorphonuclear leukocytes.^{36,37}

The cascade reaction named 'prophenoloxidase activating system' (proPO) is an invertebrate melanogenic pathway involved in immune responses^{38–40} challenged by bacterial β -1,3 glucans or lipopolysaccharides. A recognition phase starts a protease cascade that activates hemocyte prophenoloxidase, and the subsequent phenoloxidase (PO) pathway produces cytotoxic quinones and radical oxygen intermediates.⁴¹ Ascidian PO is a *o*-diphenoloxidase, detected in hemocyte lysates and identified in hemocytes by cytochemical analysis.⁴²

In ascidians hemocyte, immunoreactivity could be affected by sublethal methylmercury concentration in the organism and/or in sea water. In the present paper, phagocytic and phenoloxidase activities of *Styela plicata* hemocytes appear to be affected by methylmercury. This xenobiotic, at high concentrations, is cytotoxic for hemocytes *in vitro*, whereas at sublethal concentration it affects *S. plicata* immunocyte activities, causing immunosuppression, as revealed by assays of phenoloxidase-dependent cytotoxic activity and phagocytosis. Finally, changes in hemocyte morphology and spreading capacity were shown.

EXPERIMENTAL

Chemicals

Methylmercury was first dissolved at 10^{-3} M concentration in marine solution (MS: 12 mM $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 11 mM KCl; 26 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 45 mM Tris; 38 mM HCl; 0.45 M NaCl; pH 7.4). Stock solutions were then diluted in MS to the final concentrations of 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} M. Preliminary assays showed that the cell viability did not change as an effect of the methylmercury solvent. Unless otherwise reported, all the chemicals used were from Sigma (St Louis, MO, USA).

Tunicates and preparation of hemolymph

Ascidians (25–30 g wet weight) were collected from Mazara del Vallo Harbour (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with a marine invertebrate diet (Hawaiian Marine Imports Inc., Houston, TX, USA).

The tunic was cleaned from epiphytes and sterilized with ethyl alcohol; the incurrent siphon was incised and the exuding hemolymph was collected into sterile tubes containing a 5-fold excess of calcium/magnesium-free artificial sea water (FSW: 9 mM KCl; 0.15 M NaCl; 29 mM Na_2SO_4 , NaHCO_3 , pH 7.4) with 10 mM EDTA (FSW-EDTA) as anticoagulant (1:9 medium:hemolymph ratio), on ice. After centrifuging at 400g for 10 min at 4 °C, the hemocytes were washed three times in sterile FSW-EDTA. Appropriate controls showed that hemocyte mortality evaluated by the Trypan blue test was lower than 5%.

Hemocyte lysate supernatant (HLS)

Hemocytes ($3 \times 10^6 \text{ ml}^{-1}$) in ice-cold FSW-EDTA were centrifuged and suspended in a same volume of 10 mM cacodylate buffer pH 9.0 (CAC) to be sonicated at 4 °C for 60 s (Branson, model B15, Danbury, CT, USA). The cell lysate was centrifuged at 27 000 g for 20 min at 4 °C and the resulting hemocyte lysate supernatant (HLS) used for the assays.

Assay of PO activity

HLS phenoloxidase (PO) activity was measured spectrophotometrically by recording the product obtained from the reaction between DOPA-quinone and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH).⁴³ Briefly, 40 μl of HLS were incubated 20 min at 20 °C with 150 μl cacodylate (CAC) buffer, and 150 μl MBTH reaction mixture, containing 0.49 ml of buffer B (4% *N,N*'dimethylformamide in CAC buffer), 0.2 ml of 5 mM dihydroxyphenylalanine (L-DOPA) and 0.3 ml of 20.7 mM MBTH in buffer B (DOPA-MBTH). After incubation, the reaction product was detected at 505 nm.

The effect of protease as a proPO activator (bovine pancreas trypsin type III) was examined by adding 150 μl of enzyme (1 mg ml^{-1} in CAC) to 40 μl HLS. After 20 min preincubation, 150 μl of L-DOPA were added into the reaction mixture. The spectrophotometric measures were compared with controls, in which protease solutions were substituted by 150 μl of CAC-buffer. The PO activity was expressed as units (U) for min where $1 \text{ U} = 0.001 \text{ DA}_{505} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$.

Protein determination

Protein content was determined using the Bradford method,⁴⁴ with bovine serum albumin (BSA) as a standard. The HLS protein content ranged from 20 to 100 $\mu\text{g ml}^{-1}$.

Cytochemical PO assay

Hemocytes suspended in MS were layered on a cleaned pyrogen-free (heated at 180 °C for 4 h) glass coverslip and incubated for 30 min at 20 °C. Cell monolayers were washed three times with FSW and fixed for 30 min with 1% glutaraldehyde in MS containing 1% sucrose. Finally, after three washings in distilled water, cell monolayers were incubated with DOPA-MBTH and observed after 1–8 h using an inverted microscope. A visible pink product was observed when MBTH reacted with dopaquinones.⁴³

Hemocyte cytotoxic assay (HCA)

The cytotoxic assay against erythrocytes has been described previously.⁴⁸ In brief, 200 μl *S. plicata* hemocyte suspensions (1.5×10^6 cells, Effector) in MS were mixed with an equal volume of freshly prepared rabbit erythrocytes (8×10^6 cells, REs) in the same medium. The mixture was incubated at 20 °C for 1 h, and the amount of the released hemoglobin was estimated by reading the absorbance at 541 nm in the supernatants after mixture centrifugation. To obtain 100% hemolysis, 8×10^6 REs were suspended in 200 μl distilled water and frozen before being centrifuged at 800 g to separate

ghosts. When REs were suspended in MS, the spontaneous hemoglobin release never exceeded 5% of the total release.

The degree of hemolysis was determined according to the equation:

$$\text{percentage hemolysis} = \frac{\text{measured release} - \text{spontaneous release}}{\text{complete release} - \text{spontaneous release}} \times 100$$

Fluorescence quenching for *in vitro* phagocytosis assay

Assays, using *Saccharomyces cerevisiae* (Sigma) as target, were performed according to Cooper *et al.*²⁴ with slight modifications. The yeast cells were prepared in distilled water (d.w.) as a 0.25% (w/v, suspension, approximately 1×10^7 cells ml^{-1}) autoclaved for 15 min, washed twice at 2000g at 4 °C for 5 min, and incubated for 1 h at 20 °C with eosin Y (4-bromo-fluorescein) at 0.05% final concentration. Yeast cells were washed four times, suspended to a final concentration of 0.125% w/v in phosphate buffer saline (PBS, 6 mM KH_2PO_4 , 30 mM Na_2HPO_4 , 0.11 M NaCl, pH 7.4) and stored at 20 °C for a maximum of 2 weeks. Yeast and 100 μl hemocyte suspension in MS (2.5×10^6 cell/ml) were placed (v/v) in a 1 ml plastic tube and incubated (1:4 yeast/hemocyte ratio) for 30 min at 20 °C with gentle stirring, then 50 μl of the quenching solution (2 mg ml^{-1} trypan blue, 2 mg ml^{-1} crystal violet in 0.02 citrate buffer, pH 4.4 containing 33 mg ml^{-1} NaCl) was added. Phagocytes containing fluoresceinated yeasts were observed under a microscope equipped with Nomarski differential interference contrast optics and fluorescent apparatus (450–490 nm filter; Diaplan, Leika, Wetzlar, D); about 200 cells in each slide were counted at 800 \times magnification. The results were expressed as the percentage of cells containing yeasts.

Exposure of hemocytes to methylmercury in microplate

Hemocytes were aliquoted (100 μl for well) into 96-well flat-bottomed cell culture plates, where hemocytes (from 2.5×10^6 cell ml^{-1} to 7.5×10^6 cell ml^{-1}) were maintained at 15 °C for 1 h. For microplate exposures, various concentration of methylmercury (10^{-4} – 10^{-8} M, 10 μl FSW per well) were added to cultured hemocytes. Hemocyte aggregates could be seen on the well bottom following the treatment, and the effect of xenobiotic concentration was evaluated by direct observation.

The hemocyte mortality after 1 h exposure to CH_3HgCl at the concentrations used, as evaluated by the Trypan blue test, was <5%, whereas cell viability, checked by neutral red vital stain,⁴⁵ was >95%.

Hemocytes were layered on a slide and their morphology observed under Nomarski differential interference contrast microscopy (Diaplan, Leika, Wetzlar, D).

Statistical analysis

Unless otherwise indicated, the experiments were repeated three times. The values were the means of three assays

performed in triplicate \pm SD. Significance was determined with Student's *t*-test and differences between results were considered significant at $p < 0.05$.

RESULTS

Nontoxic doses of methylmercury activate hemocyte prophenoloxidase

Since hemocyte exposure to methylmercury concentrations higher than 2×10^{-4} M caused a significant increase in cell mortality (14% dead and 78% living cells at 10^{-3} M) the experiments were performed at sublethal concentrations (10^{-4} – 10^{-8} M) when about 95% viable cells and <5% dead cells were found using neutral red and Trypan blue tests.

Methylmercury activated hemocyte proPO in a dose-dependent fashion, as shown by the increased PO activity found in the hemocyte lysate supernatant (Fig. 1). Significant activation ($p < 0.05$) vs nontreated hemocytes was already evident (65.9 ± 1.32) when the methylmercury concentration was 10^{-6} M. The activity further increased (72.4 ± 1.1) at 10^{-5} mM and reached a high level (79.3 ± 1.3) at 10^{-4} M ($p < 0.01$). This effect was similar to proPO activation due to treatment with trypsin that is known to be the best activator (89.1 ± 5.0 , $p < 0.001$). Quinone production (Abs at 505 nm) became asymptotic within 25–30 min after the addition of DOPA-MBTH mixture (Fig. 1).

Effects of methylmercury on hemocyte cytotoxic activity

Figure 2 shows the methylmercury effect on cytotoxic hemocytes assayed with REs in the presence of increasing

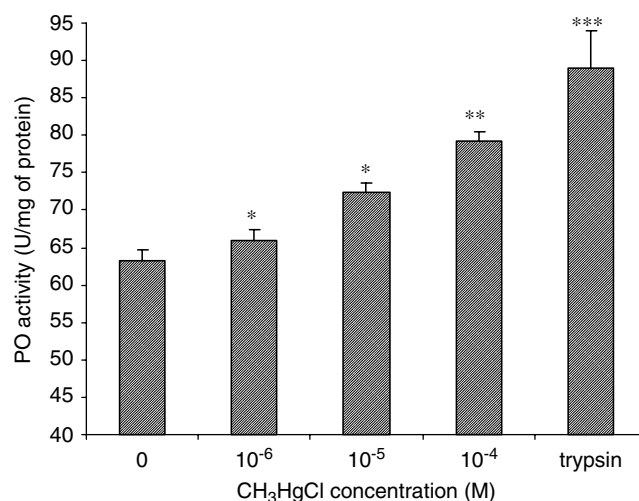


Figure 1. Effect of various methylmercury concentrations on the PO activity (A505) of hemocyte lysate supernatant after *S. plicata* hemocytes were incubated for 1 h *in vitro* with xenobiotic. Bars represent SD, $n = 8$. * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$.

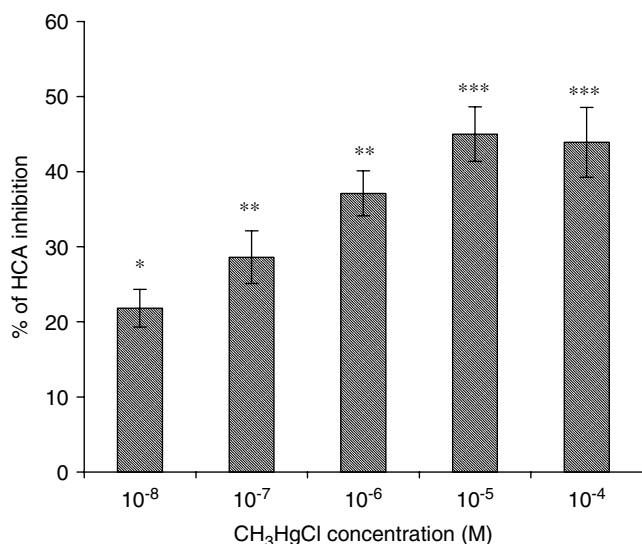


Figure 2. Effect of various methylmercury concentrations on *S. plicata* hemocyte cytotoxic activity (HCA), against rabbit erythrocytes after incubation of hemocytes for 1 h *in vitro* with xenobiotic. Bars represent SD, $n = 8$. * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$.

methylmercury concentrations (10^{-8} – 10^{-4} M). The activity of 7.5×10^6 cells ml^{-1} (effector/target ratio, i.e. E:T = 1:5) was significantly inhibited in a dose-dependent fashion, reaching the lowest level at 10^{-4} M ($p < 0.001$), whereas, at the lowest xenobiotic concentration (10^{-8} M), the inhibition level presented a low significance value ($p < 0.05$; 21.82%) compared with the control. The activity of the cells exposed to higher concentrations (10^{-4} M) decreased by up to 19% ($p > 0.001$). At different hemocyte numbers, the inhibition ranged from about 50% (3.75×10^6 cell ml^{-1} ; E:T = 1:10) to 38% (15×10^6 cell ml^{-1} ; E:T = 1:2.5) depending on the effector:target ratio.

Effect of methylmercury on phagocytic activity

Figure 3(A, B) shows that the highest phagocytic activity was achieved when 2.5×10^6 cells ml^{-1} were incubated with yeast for 60 min at 20 °C. The effects on phagocytic activity of hemocytes exposed *in vitro* to methylmercury are presented in Fig. 3(C). Figure 3(D) shows hemocytes with phagocytized yeast as observed for their fluorescence. After just 1 h of hemocyte incubation with the xenobiotic, the phagocytic activity was significantly lowered ($p < 0.05$) by 10^{-8} and 10^{-7} M methylmercury, reaching the lowest level ($p < 0.001$)

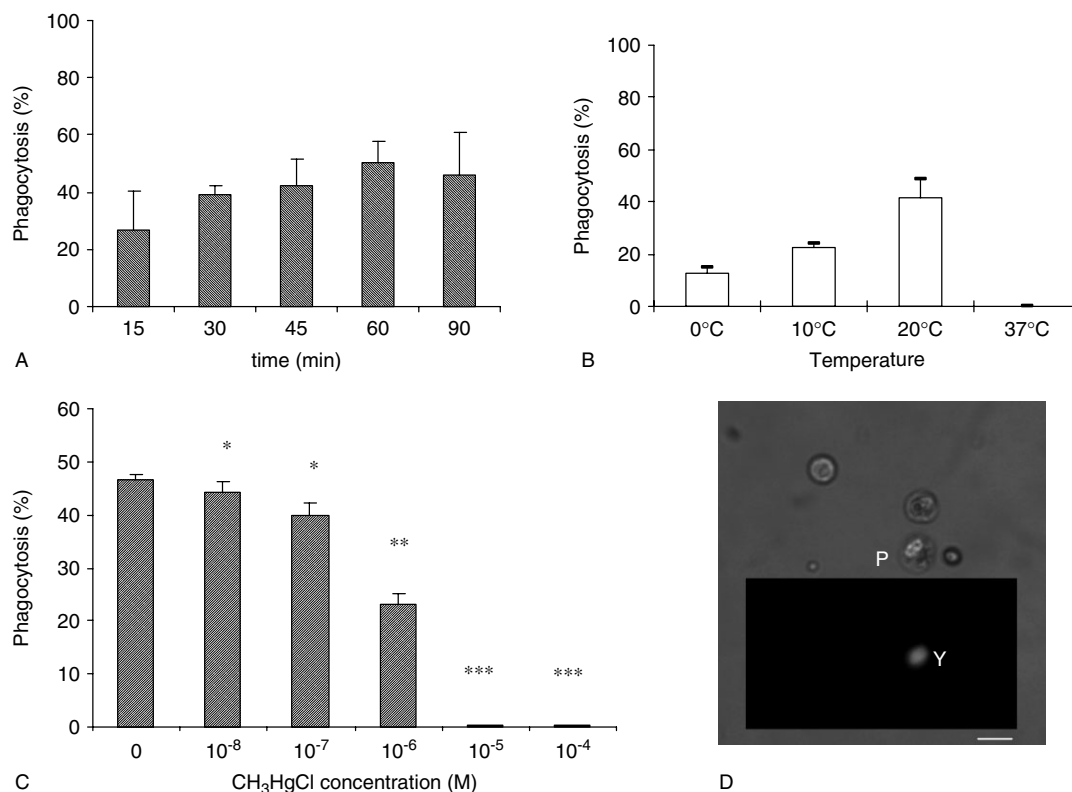


Figure 3. *In vitro* phagocytosis of yeast cells (*Saccharomyces cerevisiae*) by *S. plicata* hemocytes. Phagocytosis was evaluated as the percentage of hemocytes containing yeast (% phagocytosis). (A) Percentage of phagocytes in absence of xenobiotic; (B) phagocytic response of hemocytes at different temperatures; (C) percentage of phagocytes after hemocyte exposure to different methylmercury concentrations; (D) light microscopy observation of phagocytes (P); in the inset fluorescent yeasts (Y) are shown. Bar = 10 μm . * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$.

in the presence of 10^{-6} M, and it was significantly decreased by 10^{-5} M ($p < 0.001$).

Cell morphology modification after methylmercury exposure

When 5×10^6 cell ml^{-1} hemocytes were layered in the wells of a U-bottomed chamber containing decreasing methylmercury concentrations (from 10^{-4} to 10^{-7} M), changes in cell morphology and spreading appeared at a macroscopical level as variously shaped disk of cells [Fig. 4(A)]. Microscopy observations of the hemocytes layered on the well bottom showed that, in the absence of methylmercury, the hemocytes presented a spread shape showing an irregular morphology [Fig. 4(B)]. After treatment with methylmercury at a low concentration (10^{-4}) the hemocytes presented a round shape [Fig. 4(C)]. Similar results were obtained when treatments were directly performed on a slide.

As shown in Fig. 4(D, E), PO-positive morula cells were identified by cytochemical reaction with DOPA-MBTH. An untreated spread morula cell is shown in Fig. 4(D), whereas Fig. 4(E) presents a round methylmercury-treated cell with more intense DOPA-MBTH stain.

Since PO-containing cells could release the enzyme after methylmercury treatment, the PO activity of hemocyte culture medium was compared with that of the cultured hemocytes. Data listed in Table 1 show that the PO activity found in the medium was significantly lower than that of the HLS. In addition, cell treatment with methylmercury significantly ($p < 0.001$) enhanced the activity of both the released (25% enhancement) and cytoplasmic (27% enhancement) PO.

DISCUSSION

Tunicates are filter-feeding marine invertebrates ubiquitous throughout the world. They live along the coast and can be subjected to environmental contaminants such as toxic metals, including the methylated form of mercury, that could affect their innate immune system. According to previous reports, xenobiotics could activate or suppress the immune functions. A decreased immunoreactivity, i.e. phagocytosis and cytotoxicity, affects the preservation of organism health, whereas it is unclear if activation of components of the immune system may affect the organism immunosurveillance

Table 1. PO activity of *S. plicata* hemocytes (10^7 cells ml^{-1}) treated for 1 h with 10^{-5} M methylmercury. Hemocyte culture medium and hemocyte lysate supernatant (HLS) were examined; $n = 8$

	PO activity ($\text{U}_{\text{A505}} \text{mg}^{-1} \text{protein}$)		Increase (%)
	Control	Methylmercury	
Culture medium	35.4 ± 2.9	45.7 ± 2.8 ($p < 0.001$)	(25.9)
HLS	63.3 ± 5.2	80.3 ± 3.2 ($p < 0.001$)	(27.8)

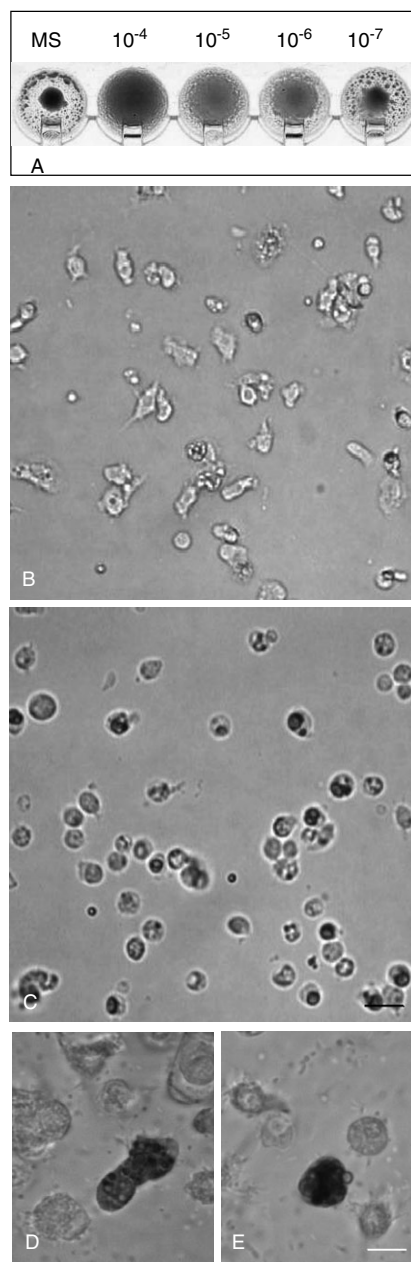


Figure 4. Effect of methylmercury on *S. plicata* hemocyte morphology. (A) Macroscopical test of hemocytes from hemolymph after 1 h of preincubation with different methylmercury concentrations; (B) control: hemocytes maintained for 1 h in marine solution in absence of xenobiotic; (C) light microscopy observations of hemocytes after 1 h treatment with methylmercury (10^{-6} M); (D) phenoloxidase activity of hemocytes maintained in MS in the absence of methylmercury, as revealed by DOPA-MBTH mixture; (E) PO activity of hemocytes treated for 1 h with 10^{-6} M methylmercury, as revealed by MBTH mixture; B and C, bar = 20 μm ; D and E, spot bar = 10 μm .

capacity. In ascidians, phagocytosis,⁴⁶ the prophenoloxidase system⁴² and cytotoxic⁴⁷ activities are the main cellular

immune mechanisms exerted by hemocytes circulating in the hemolymph for defending the organism against several pathogens that could penetrate through pharynx filtering action. Ascidiarians undergo the effect of environmental xenobiotics and bioaccumulate metals, becoming chronically exposed to their action.

In the present paper, we examined the effects *in vitro* of methylmercury, at sublethal concentrations, on *Styela plicata* hemocyte phenoloxidase, cytotoxic and phagocytic activities. In addition, since cell morphology and spreading hemocyte ability can be related to immune functions, these features were taken into consideration. The methylmercury concentrations we used for examining the effect on immunocytes were not toxic, as indicated by Trypan blue dead cell exclusion test, while viability was not affected by the treatment, as shown by the neutral red test. In general terms, 95% hemocytes maintained their main viable properties, whereas immune functions were affected by the xenobiotic compound. The phenoloxidase activity was examined in the lysate supernatant from hemocytes treated with different methylmercury amounts. In the range of 10^{-4} – 10^{-8} M, the xenobiotic enhanced the PO activity in a dose-dependent fashion, suggesting that the pro-phenoloxidase system could be activated even if at a level lower than that observed by a treatment with a serine protease (trypsin), which is known to be a good activator of the proPO pathway.⁴⁸ Similar results have been reported using fruoranthene on *Mytilus edulis* hemocyte PO.⁴⁹

In *S. plicata*, phenoloxidase activity is a property of morula cells⁵⁰ which, after effector–target contacts, display PO-dependent cytotoxic activity against erythrocytes and tumor line K562 cells, probably due to quinones produced by the PO pathway.⁴⁸ When hemocytes were pre-treated with 10^{-5} M methylmercury, in spite of the PO activation, the cytotoxic activity was inhibited, suggesting that the treatment of hemocytes with the xenobiotic affects cell-target contacts, including the recognition mechanism. Accordingly, a similar xenobiotic action could be responsible for phagocytosis inhibition. Phagocytes after 1 h exposition to low xenobiotic concentration (10^{-4} up to 10^{-5} M), were viable but did not phagocytose yeast, whereas their activity was maintained almost at control level when the cells were in the presence of a lower toxicant concentration (10^{-8} M).

The cytotoxicity inhibitory effect could not be imputed to a releasing mechanism, since in the presence of the xenobiotic the enzyme was released into the culture medium showing the same activity level as the controls. However, we do not know if methylmercury could also act on cytoskeletal network, as indicated by changes in hemocyte morphology and spreading capacity. With regard to this, it is known that cytoskeletal alterations lead to reduced phagocytic activity, due to the decreased ability of hemocytes to adhere to the substrate and interact with targets.⁵¹ Finally, a lipid peroxidation¹⁰ could damage the plasma membrane, affecting the activity of both phagocytes and cells of the cytotoxic line.

Effects on the cytoskeleton could be responsible for changes in the hemocyte morphology and spreading capacity, as revealed by the microplate assay we performed. Although the assay cannot reveal the xenobiotic concentration values in water, it appears to be an easy method for indicating that xenobiotics at sublethal toxicant concentration could be present.

In conclusion, we show that high environmental methylmercury concentrations are toxic for tunicate hemocytes, whereas sublethal concentrations affect the *S. plicata* immunocyte activities, causing immunosuppression, as revealed by assaying cytotoxic activity with rabbit erythrocytes and phagocytosis of yeast, which could be used as additional immunotoxicology biomarkers in macrobenthic studies.

Acknowledgments

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