



The ascidian *Styela plicata* hemocytes as a potential biomarker of marine pollution: *In vitro* effects of seawater and organic mercury



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ABSTRACT

Toxic metals, such as mercury, contribute substantially to anthropogenic pollution in many estuarine environments. Animals living in those environments, particularly invertebrate filter feeders like tunicates, can be used as bioindicators. In an attempt to identify cellular markers for revealing pollution, this study examined *in vitro* the effects of different concentrations of methyl mercury on *Styela plicata* hemocytes. The harvested hemocytes from *S. plicata* that were exposed to the metal had a significant mortality, cellular count and morphometric alterations. These findings provided evidence of MeHg immunotoxic effects on *S. plicata*, resulting in hemocyte death and morphological changes induced by cytoskeleton alterations. Thus, a morphometric cellular parameter, such as spreading ability, was used as a complementary method for differentiation between hemocytes treated with a marine solution (as a negative control) and hemocytes incubated with methylmercury and/or Sicilian seawater samples.

1. Introduction

In the last few decades, human activities have increased the flux of many naturally occurring chemicals, such as trace metals, in marine ecosystems (Bellante et al., 2012). They can enter into marine waters from different sources such as mining, wastes, sludge residues, oil burning and atmospheric deposition (Singh et al., 1997). Due to the increased concern about trace metals contamination, a great interest in marine pollution monitoring and assessment has arisen. Bioindicator organisms accumulate trace metals in their tissues and may therefore be used to monitor contaminants in the ecosystem (Rainbow, 2002). Moreover, the measurement of biochemical, cellular and physiological responses (biomarkers) to pollutants developed by bioindicator organisms (Nicosia et al., 2014), is considered as a useful tool to evaluate contaminant exposure and effects (Leomanni et al., 2016). Ascidiaceans are filter-feeding organisms occupying littoral and estuarine habitats that are exposed to a wide range of pollutants.

The evaluation of immunotoxicity has been well established in the colonial ascidian *Botryllus schosseri*, on which the effects of organotin compound on the phagocytosis process have been clarified (reviewed in Cima and Ballarin (2000)).

The species *Styela plicata* is particularly able to accumulate a certain amount of metals *via* filter-feeding mechanisms, and can thus

be used as a bioindicator of water quality (Bellante et al., 2016). Some trace metals, such as mercury, do not have any biological function and appear to be potentially toxic, even at low concentrations. Methylmercury (MeHg) is the most toxic and bio-accumulative form of Hg in marine organisms. It is noteworthy that MeHg can adversely affect organism health, either by neurotoxic effects or by immunotoxic effects that alter homeostasis and the immune system functions (Haggqvist et al., 2005; Krey et al., 2015). Thus, MeHg immunotoxicity could affect the capacity of animal survival by reducing resistance to environmental stress. Hemocytes in the circulating hemolymph of invertebrates represent the primary line of defense *via* phagocytosis, nodulation, encapsulation, cytotoxicity and hemolymph coagulation activities (Falleiros et al., 2003; Lavine and Strand, 2002; Parrinello et al., 2016; Perez and Fontanetti, 2011). In this regard, hemocyte viability, physiology, the total cell number and frequency of certain cell types play a fundamental role in organism homeostasis.

In ascidiaceans, hemocyte types were distinguished according to Radford et al. (1998), Wright (1981) and De Leo (1992). Based on their morphological characters and cytoplasmic granules, cells were classified into two main categories: (1) agranular hemocytes, including hemoblasts, lymphocytes-like cells (LLC) and hyaline amoebocytes (HA); (2) granular hemocytes including granulocytes with small granules (sG), granulocytes with large granules (lG), signet ring cells

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that present a single vacuole with granules, and morula cells (MC) that may assume a berry like appearance (Wright, 1981, De Leo, 1992, Arizza and Parrinello, 2009).

Hemoblasts are hemopoietic stem cells in the hemopoietic tissue and hemolymph; LLCs, also considered as stem cells, are smaller than hemoblasts and do not have a nucleus, and HA contain fine electron-dense granules of uniform size in their cytoplasm.

Styela plicata hemocytes showed several crucial immune reactions including cytotoxic activity (Cammarata et al., 1995, 1997; Lipari et al., 1995), phagocytosis (Cammarata et al., 2007), allograft rejection (Raftos et al., 1990) and phenoloxidase-dependent cytotoxicity (Cammarata et al., 1997).

Hemocytes represent one of the first targets of immunotoxic MeHg (methylmercury II) action in invertebrates (Calisi et al., 2009; Nigro et al., 2006). Thus, physiological alterations in these immune cellular types have been extensively used as pollutant biomarkers (Calisi et al., 2008; De Ros and Nesto, 2005). Bivalves, such as oysters and mussels, are the marine species most commonly used for immunotoxic tests (Company et al., 2004; Dyrzynda et al., 1998; Gómez-Mendikute and Cajaraville, 2003; Lowe and Pipe, 1994; Pipe, 1992).

In spite of this, little is known about the immunotoxic MeHg effects specifically linked to the hemocytes of Ascidians (Galloway and Depledge, 2001). In a previous paper, we reported that methylmercury sublethal concentrations quickly affect *in vitro* *Styela plicata* hemocyte innate immune activities such as phenoloxidase activity, cytotoxicity and phagocytosis, suggesting an immunosuppressive effect (Cammarata et al., 2007). Exposure to pollutants can also affect the number and the morpho-functional properties of these cells (Radford et al., 2000). For example, an increase in immune cell rounding with a reduction in pseudopod numbers has been previously reported in hemocytes of different invertebrate species (such as snail and mussel) exposed to Hg (Leomanni et al., 2016; Marchi et al., 2004). Thus, evaluation of hemocyte numbers and their morphometric alterations has been retained as a tool to reveal sublethal stressing conditions caused by Hg. Hg and MeHg may also have different effects on hemocytes.

In the present paper, the *Styela plicata* hemocytes exposed to MeHg were investigated by assessing the immunotoxicity. Total Hg concentrations in seawater samples and *Styela plicata* tissues were also measured, to evaluate bioaccumulation of trace metals. In addition, the hemocytes were exposed to marine waters collected at different sites along the Sicily coasts. In particular, mortality and morphometric alterations were microscopically analyzed by SEM to evaluate morphological change and used as indicators of seawater pollution.

2. Materials and methods

2.1. Sample collection

2.1.1. Water and tissue collection

Samples of water were collected from different locations around Sicily to assess the effects of seawater on hemocyte mortality and morphology (Table 1). The water samples were transported to the laboratory inside tubes, filtered and kept at 4 °C until use for the assays. Fifty specimens of *Styela plicata* (20–25 g wet weight) and three samples of water were also collected from one site (Cala, Pa7) in the Gulf of Palermo (Sicily, Italy) during one sampling session in May 2015 to assess Hg concentrations. Samples of water were immediately acidified with 1 mL of concentrated nitric acid. In order to minimize contamination risks, acid-cleaned laboratory materials were used during sample collection and analytical determination. The tissue samples were immediately removed from each individual and stored at –20 °C. The hepatopancreas and the branchial basket tissues of *Styela plicata* were selected to monitor Hg accumulation. For each analysis, a pool of five branchial baskets and hepatopancreas tissues were combined, with the aim of providing sufficient amounts of

samples for the analysis. The tissues were dried for 48 h at 40 °C in an oven and their dry weights were determined. The dried samples were ground with a mortar and pestle for subsequent analysis.

2.1.2. Hemocyte collection

Specimens collected weekly in the Gulf of Palermo were maintained in tanks with aerated seawater at 18 °C and fed every second day with a marine invertebrate diet (Hawaiian Marine Imports Inc., Houston, TX, USA). Animals were blotted dry and rinsed briefly with absolute ethanol. Each specimen was incised across the upper stolon to collect the hemolymph. Samples of hemolymph were conserved on ice in polystyrene tubes containing an equal volume of calcium/magnesium-free artificial seawater (FSW: 9 mM KCl; 0.15 M NaCl; 29 mM Na₂SO₄, NaHCO₃, pH 7.4) with 10 mM EDTA (FSW-EDTA) as anticoagulant. The hemolymph was diluted on ice to recover approximately 4×10⁶ hemocytes per specimen. After centrifuging at 400×g for 10 min at 4 °C, the hemocytes were washed in marine solution (MS: 12 mM CaCl₂; 11 mM KCl; 26 mM MgCl; 45 mM Tris; 38 mM HCl; 0.45 M NaCl, pH 7.4). Appropriate controls showed that hemocyte mortality, evaluated by the Trypan blue exclusion assay, was lower than 5%.

2.2. Trace metals analysis

Approx. 200 mg of each oven-dried and homogenized tissue sample were digested under pressure in 1 mL of ultra-grade HNO₃ and 0.5 mL of H₂O₂ in Teflon vessel liners using a microwave digestion system (CEM MARS-5). Samples were prepared and analyzed to minimize contamination from glassware and reagents, all of which were of Suprapur quality. The concentrations in Hg(II) solution were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES Optima 2100), equipped with an auto-sampler model AS90. Analyses were carried out by external calibration using standard solutions in the same acid matrix of samples, prepared by diluting the ICP High-Purity Standard Solutions. Reagent blanks and controls were also taken into account to monitor the appropriateness of the analytical procedures. All the individual data were calculated as an average of 3 replicates. Analytical precision, measured as relative standard deviation, was routinely between 5% and 6% and never higher than 10%. All results were calculated with respect to dry weight (dw). The instrument ICP-OES optima 2100's automatic dual viewing system ensures very low detection limits for trace metals analysis. The instrument detection limit for selected elements ranged from 0.2 to 0.9 µg/kg dw.

2.3. Experimental design

2.3.1. Exposure of hemocytes to MeHg *in vitro*

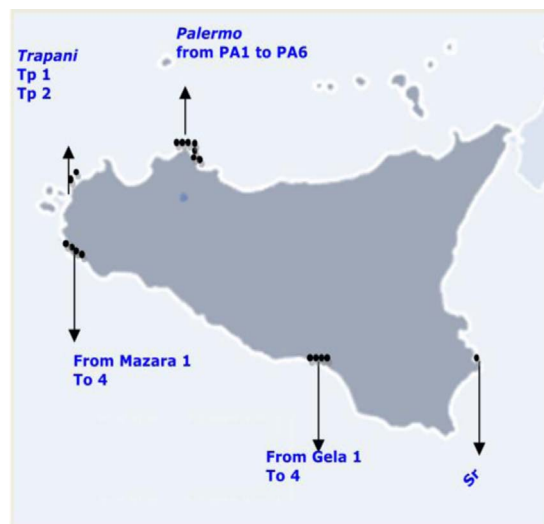
Methylmercury was dissolved at 10^{–3} M in MS, and then diluted in MS to reach 10^{–6}, 10^{–5}, and 10^{–4} M final concentrations. Hemocytes (2×10⁶ cells/mL), prepared in MS, were aliquoted (500 µl) in Eppendorf tubes and centrifuged at 400×g, at 4 °C. The pellet was then suspended in MS containing methylmercury (10^{–6} M, 10^{–5} M, 10^{–4} M). For *in vitro* exposure, MeHg at 3 different concentrations was added to the cultured hemocytes in MS. After 30 min or 1 h, the treated hemocytes were layered on a slide and their morphology was observed under Nomarski differential interference contrast microscopy (Diaplan, Leika, Wetzlar, D). Hemocyte mortality was evaluated by the Trypan blue test.

2.3.2. Exposure of hemocytes to various seawater samples

Hemocytes were aliquoted (about 4×10⁶ cells in 100 µl/well) into 96-well flat-bottomed cell culture plates, and maintained at 15 °C for 1 h. The cultured hemocytes were exposed to each seawater sample from various sites along the Sicily coasts. Two controls were performed: cells in 10^{–5} M MeHg (positive control) and cells in MS (negative control). After 1 h incubation, the effect of the treatments

Table 1
Sites of sampling along the Sicily coast selected for sea water sampling.

Name	Locality
Pa 1	Mondello (PA)
Pa 2	Vergine Maria (PA)
Pa 3	Sferracavallo (PA)
Pa 4	Addaura (PA)
Pa 5	Barcarello (PA)
Pa 6	CALA 1 (PA)
Pa 7	CALA 2 (PA)
Mazara 1	Tonnarella (Mazara)
Mazara 2	Porto Nuovo (Mazara)
Mazara 3	Torretta Granitola
Mazara 4	Porto Turistico (Mazara)
Tp 2	San Giuliano (TP)
Tp 1	Nobia (TP)
Gela	ENI (Gela)
Gela 2	Porto Rifugio (Gela)
Gela 3	Spiaggia Macchitella
Gela 4	Manfria (Gela)
Siracusa 1	Erg Petrolchimico (SR)



was evaluated by macroscopic and microscopic observation of the well bottom and by microscope observation.

2.4. Microscopic observations

2.4.1. Detection of cell morphology by light microscopy

Treated hemocytes were placed on microscope slides and observed under light microscope (LM), without any cytological treatment. A cell count was performed on five fields for each slide. The morphological changes were detected by comparing them with the morphological features of the hemocytes cultured in MS as a negative control.

2.4.2. SEM

For scanning electron microscopy (SEM), thin blood smears were prepared on a coverslip by mixing a drop of hemocyte in MS or in methylmercury solution, fixed in cacodylate buffer (0.1 M, pH 7.3) containing 2.5% glutaraldehyde, post-fixed in osmium tetroxide 1%, dehydrated in graded alcohol and dried at the critical point. The preparations were mounted on stubs, gold coated in a sputter coater and analyzed by SEM (LEO 420). *S. plicata* cell types were identified by SEM observations of hemocytes washed with the MS negative control.

2.5. Measurement of cell roundness by Image J processing

"ImageJ" is a freely available java-based public-domain image processing and analysis program developed at the National Institute of Health (NIH), using the Image J website (ImageJ) folder. This application can calculate area and pixel value statistics of user-defined selections. It is able to measure distances and angles allowing density histograms and line profile plots to be created. The circularity parameter formula used was $4\pi(\text{area}/\text{perimeter})$. A value of 1.0 indicates a perfect circle, whereas larger values indicate oblong and non-circular objects. As the circularity value approaches 0.0, it indicates an increasingly elongated polygon. Invalid values due to very small particles were removed by excluding particles that were below a specific minimum area. To obtain circularity data, the images obtained by OM and SEM were converted into 8-bit jpeg format and subsequently digitized by Image J. The evaluation was repeated three times for each image.

2.6. Statistical analysis

All experiments were repeated three times. The values of mortality, roundness and morphological changes were the means of three assays performed in triplicate \pm SD and are expressed as mean \pm SD. Multiple comparisons were performed with a one-way analysis of variance (ANOVA) and different groups were compared using Tukey's *t*-test. A *p*-value lower than 0.05 was considered statistically significant.

3. Results

3.1. Hg bioaccumulation in the body

Hg concentrations in water samples and tissues of *S. plicata* from Site Pa7 are shown in Table 2. Hg concentration in seawater range from < dl to 0.001 mg/kg dw. Significantly higher Hg concentration was found in the branchial basket (range: 0.001–0.053 mg/kg dw; mean=0.016) with respect to hepatopancreas tissue (range: 0.001–0.008 mg/kg dw; mean=0.004). The bioaccumulation factor (BAF) for Hg was calculated as the ratio of metal concentration in the tissue to

Table 2

Mercury amount in tissues of *S. plicata* specimens (Id) collected at site Pa7, and Hg Bio Accumulation Factor (BAF) in pharynx and hepatopancreas. Concentrations are expressed in mg/kg dry weight.

Id	Branchial basket	Hepatopancreas
	Hg (mg/kg dw)	Hg (mg/kg dw)
1	0.002	< dl
2	< dl	< dl
3	< dl	< dl
4	< dl	< dl
5	< dl	< dl
6	< dl	< dl
7	< dl	0.008
8	0.002	< dl
9	0.053	0.003
10	0.007	0.001
Mean	0.016	0.004
BAF	16	4

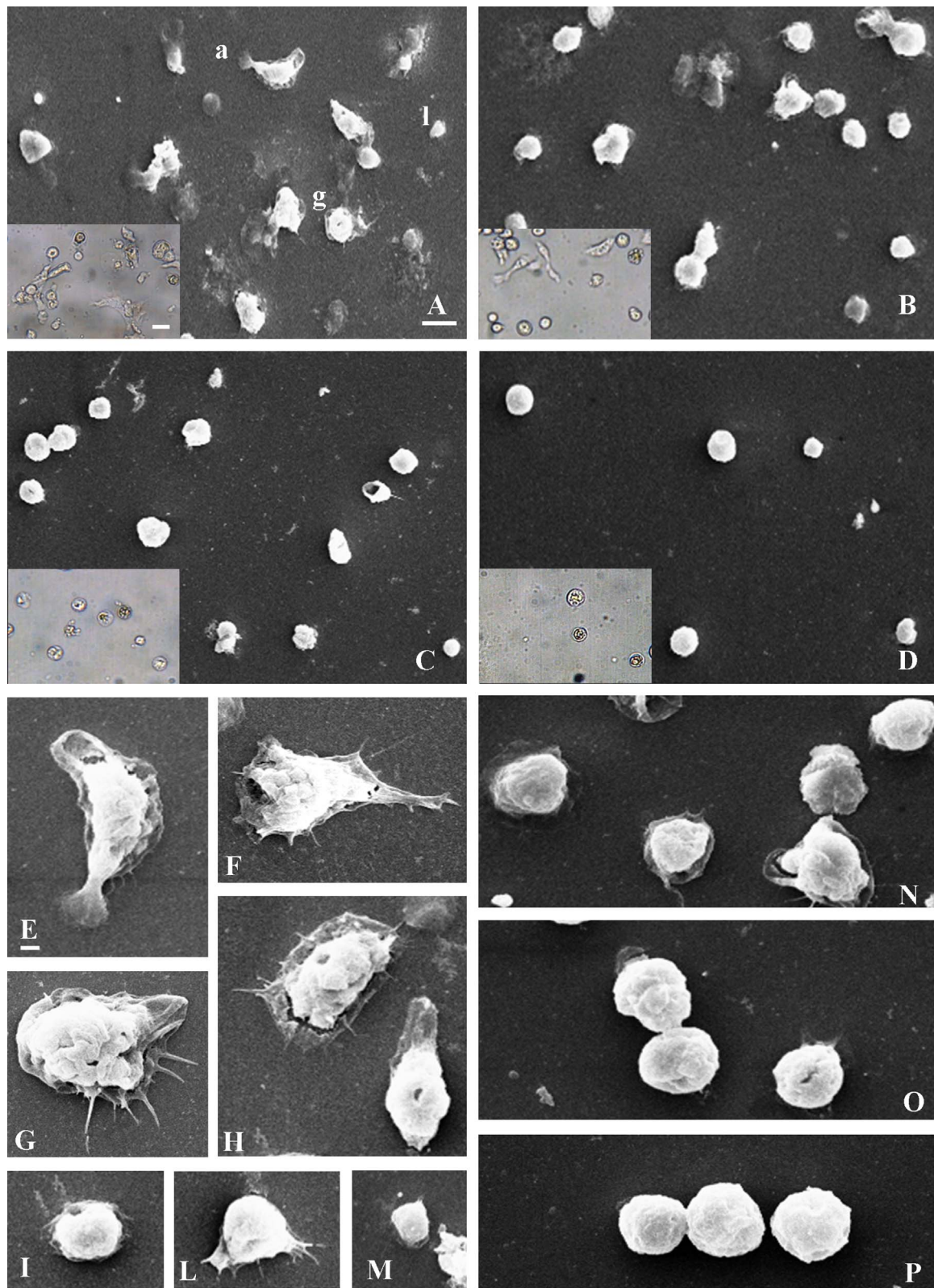


Fig. 1. SEM observations of *Styela plicata* hemocytes treated with marine solution (A) or marine solution enriched with MeHg (10^{-6} M, B; 10^{-5} M, C; 10^{-4} M, D). Spots in A-C: Light microscopy observations. E-M: magnification of hemocytes, in marine solution, spread on a glass support. E: hyaline amebocyte; F: amebocyte with fine granules; G: large granulocytes; H: granulocyte with large granules; I and L: spreading features of small granulocytes; M: lymphocyte-like cell. N-P: magnification of hemocytes treated with MeHg (10^{-6} M, N; 10^{-5} M, O; 10^{-4} M, P). N: granulocytes with impaired spreading ability. O and P: roundish feature of hemocytes without any cell extrusions. a: hyaline amebocyte; g: granulocyte; l: lymphocyte-like. A-D: Bar 5 μ m; spots: Bar 5 μ m; E-P: Bar 2 μ m.

that in the water. As shown in Table 2, higher BAFs for Hg were recorded in the branchial basket compared to hepatopancreas tissues.

3.2. Hemocyte morphology and cell type identification

SEM observations and comparison with light microscopy observations led us to distinguish three main hemocyte types as they appeared when treated with MS, and loaded on the corresponding support (Fig. 1A). They were sorted on the basis of their size, surface features and amoeboid morphology. Small (3.4 μm in diameter) lymphocyte-like cells appeared smooth in their surface (Fig. 1M); amoebocytes, lengthened and spread on the support (major axis 16 μm), mainly presented large cytoplasmic extrusions (Fig. 1E), and the rough surface of some indicated that they contained very fine granules (Fig. 1F); roundish granulocytes showed a diameter of 14 μm and were characterized by a rough surface with short phylopodia (Fig. 1G and H); the degree of surface globosity could be imputed to the amount and size of the granules. Numerous granulocytes, loaded on glass slides (LM) or coverslips (SEM) appeared as elongated cells (14–16 μm major axis) with large granules (morula cells) that spread on the support (Fig. 1A and E–H). Phylopodia and large cytoplasmic protrusions were suggestive of an amoeboid behavior.

3.3. SEM observations of the MeHg effect on hemocyte morphology

After *in vitro* treatment with 10^{-6} , 10^{-5} or 10^{-4} M MeHg, spread hemocytes did not show the large cytoplasmic extensions (Fig. 1B and C) observed when hemocytes were cultured in MS. Amoeboid cells were difficult to observe because they tended to take on a rounded appearance. After treatment with 10^{-4} M MeHg, the hemocyte number for slide area appeared to be decreased, and only rounded cells were observed (Fig. 1D).

3.4. Effects of MeHg solutions and seawater on hemocytes of *S. plicata*

3.4.1. Hemocytes clumped on plate wells

Fig. 2 shows that hemocytes form clumps on the bottom of plate wells. In the controls (MS treatment as negative control), the hemocytes formed a regular small clump on the surface of the well bottom (Fig. 2A). Direct observations of hemocyte distribution in the plate wells after treatment with MeHg 10^{-5} M showed that hemocytes lost their aggregation state and formed a wide clump that lay on the entire surface available (Fig. 2G). Seawater from different sites and the negative controls in MS medium are reported in Fig. 2. In the controls, hemocytes clump regularly at the central surface of the well (Fig. 2A). Similar to the effect of MeHg, seawater samples collected from different sites changed the hemocyte aggregation state and are missing in the well (Fig. 2B–F).

3.4.2. Hemocyte mortality and roundness evaluation

The percentage levels of hemocyte mortality (mean \pm SD) after treatment with 10^{-4} M, 10^{-5} M MeHg solutions and seawater samples are shown in Fig. 3A. Mortality was low in the control and in samples

containing 10^{-5} M MeHg (1% and 3%, respectively); conversely, it reached 18% after treatment with the highest MeHg concentrations (10^{-4} M). Mortality was at a low level (2–4%) when cells were treated with seawater from the majority of sites, whereas non-significant higher values were found when assaying the samples from PA7 (PA) (7.5%), Nobia (TP) (about 6.5%), San Giuliano (TP) (about 5%) and Cala 1 (PA). Light microscopy observations showed that hyaline amoebocytes and lymphocyte like-cells were missing after the treatment with the highest MeHg concentration (10^{-4}), whereas granulocytes with granules of various sizes were found.

Generally, after *in vitro* treatment with MeHg 10^{-6} and 10^{-5} M MeHg, hemocytes did not show the large cytoplasmic extensions previously observed in Fig. 1. In Fig. 3B, the percentage of hemocyte morphological alterations after treatment with different seawater samples was reported and compared with morphological alterations after treatment with MeHg 10^{-5} M and negative control solutions. The control cells featuring roundness did not exceed $9 \pm 0.5\%$, whereas values significantly increased to $65 \pm 2\%$ when the hemocytes were treated with 10^{-5} M MeHg and control MS. The percentage of hemocytes with roundness features after treatment with seawater samples ranged from 9% to 30%, and the highest value was observed after treatment with seawater samples from Site Pa7 (Fig. 3B). The average values of quantification of cell roundness, calculated by Image J software, are reported in Fig. 4. The cells held in the control marine solution presented the lowest values of roundness (0.163 and 0.173; Fig. 2C). At 10^{-6} , 10^{-5} and 10^{-4} M MeHg concentrations, the value of the rounded cell, in both sets of data processed, increased in a dose-dependent manner (0.3 and 0.35 at 10^{-6} MeHg; 0.4 and 0.45 at 10^{-5} MeHg; 0.5 and 0.6 at 10^{-4} MeHg). In SEM images a higher rate of hemocyte roundness was observed than that found by LM observations (Fig. 4C).

4. Discussion

4.1. Bioaccumulation of mercury

Although the concentrations of trace elements in water column and sediments may be low, it has been demonstrated that ascidians are capable of accumulating trace elements in their tissues up to several orders of magnitude above natural seawater concentrations (Monniot et al., 1994; Rainbow, 1990). As shown in Table 2, low Hg concentrations were reported in both hepatopancreas and branchial basket tissues of *S. plicata*. Statistical significant differences of Hg concentrations in the different tissues analyzed suggest their preferential accumulation in the branchial basket with respect to hepatopancreas, as confirmed by the higher BAF value. Since the main vital functions such as absorption, circulation, storage, breeding, etc. take place predominantly in the branchial basket, it is not surprising that the accumulation of metals could be higher in this tissue. Comparable Hg concentrations have been found previously in the same tissues of *S. plicata* collected in the harbor of Termini Imerese, Sicily (Bellante et al., 2016). These concentrations were lower than those found in tissues of *M. galloprovincialis* and *S. spallanzanii* collected in the same study area (Bellante et al., 2016). Trace metal concentrations in

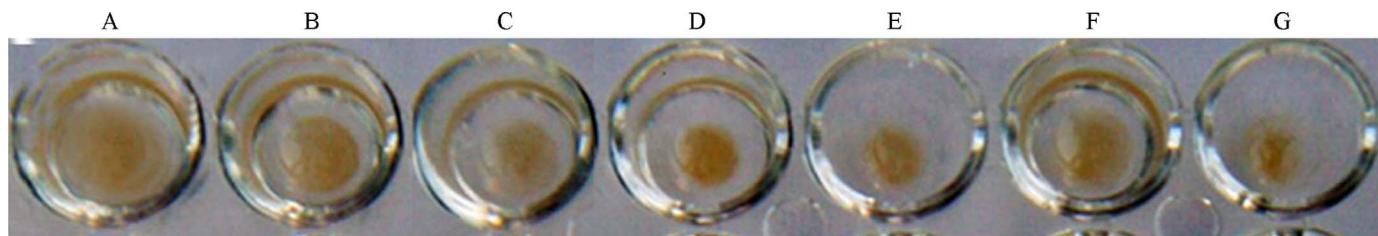


Fig. 2. Macroscopic test in well plate of hemocytes after 6 h of incubation with 10^{-5} MeHg or in marine solution as a control, and seawater samples from several geographical sites along the Sicily coasts. (A) MEHg; (B) Mazara 3; (C) PA 7, (D) Mazara 4; (E) PA 6; (F) Gela 4; (G) Marine solution as a control.

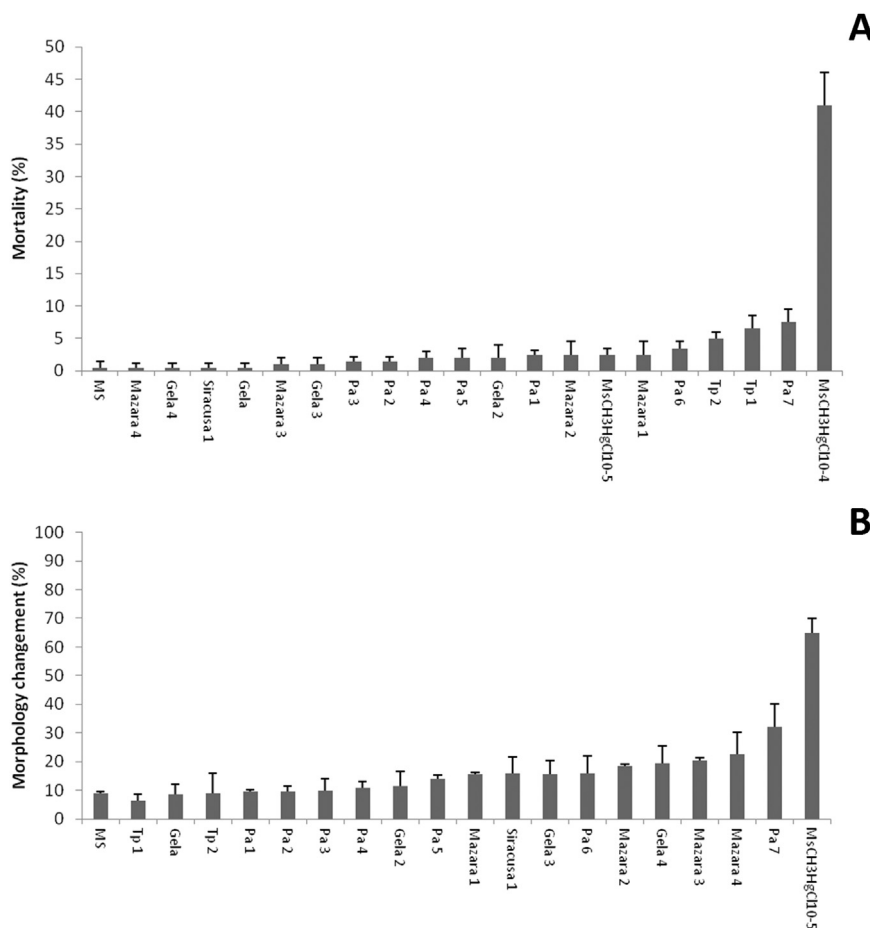


Fig. 3. (A) Mortality of *S. plicata* cells after incubation with different marine seawater samples, 10^{-5} and 10^{-4} MeHg M solutions (Trypan Blue Dye Exclusion Assay). (B) Effect of treatment with marine seawater sample on morphology cell. Histogram showing the average (\pm S.D., $n=6$) values of morphology change of the *S. plicata* hemocytes after incubation with different marine seawater samples and 10^{-5} MeHg M solution.

organism tissues are the result of metabolically available accumulated metal and the concentration of metal in its detoxified form (Rainbow, 2002). Chelating-Hg molecules have never been identified in Ascidians species. Therefore, we speculate that the relatively low Hg concentrations in tissues of *S. plicata* could be due to the absence of storage and detoxification mechanisms. In spite of that, our results confirm that *S. plicata* specimens are able to accumulate small amounts of metabolically available Hg in branchial basket tissue, which could result in immunotoxic effects (Table 3).

4.2. Immunotoxic effects of methylmercury on hemocytes of *S. plicata*

The evaluation of metal concentration in tissue samples was carried out in terms of total Hg concentrations (inorganic plus organic mercury compounds). Nevertheless, considering that methylmercury (MeHg) is the most immunotoxic form of Hg in organisms (Lohren et al., 2015), we only investigated its effect on *Styela plicata* hemocytes. As shown in Fig. 3, a significant increase in mortality was observed in hemocytes of *S. plicata* after treatment with 10^{-4} M MeHg, the lower 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 (Cammarata et al., 2007).

The literature reports increasing hemocyte mortality in other invertebrate species (*M. edulis*, *M. arenaria* and *M. truncata*) at the same MeHg concentration (10^{-4} M), suggesting similar mechanisms of

toxicity (Brousseau et al., 2000; Fournier et al., 2001; Sauve et al., 2002). SEM observations showed the morphology of the *S. plicata* hemocytes, as distinguished by surface globosity indicating cytoplasmic granules of various sizes, and by the cell extrusions (lamellipodia and filopodia) indicating the spreading capacity on a glass support. Small lymphocyte-like cells, spread hyaline amebocytes and amebocytes with fine granules, and spread granulocytes with large granules could also thereby be distinguished. The treatments with MeHg diluted in artificial seawater altered, *in vitro*, the cell surface features and the spreading ability of *S. plicata* hemocytes, even at low concentrations. In a dose-dependent fashion, the hemocyte surface appeared to be smooth, few and short filopodia were found at the lowest MeHg concentration (10^{-6} M), rare extrusions were seen at 10^{-5} M and no filopodia were observed at the highest concentration. The SEM observations also highlight that, after the xenobiotic treatment, the hemocyte number decreased. Due to the common roundness of the cells, therefore, we were not able to point out a differential mortality of hemocyte types as an effect of the xenobiotic concentrations. Presumably, lymphocyte-like cells may be less affected by the treatments. These results were supported by well plate experiments and roundness evaluations by image J processing. Particularly, our observations suggest that MeHg contamination induces a reduced capability of cell attachment, spreading ability and changes in cell morphology. Changes in spreading ability have been previously observed in *S. plicata* hemocytes after treatment with increasing MeHg concentrations (from 10^{-7} to 10^{-4} M) (Cammarata et al., 2007). These findings are congruent with the already known effects of MeHg on phenoloxidase, cytotoxicity and phagocytosis activities of hemocytes.

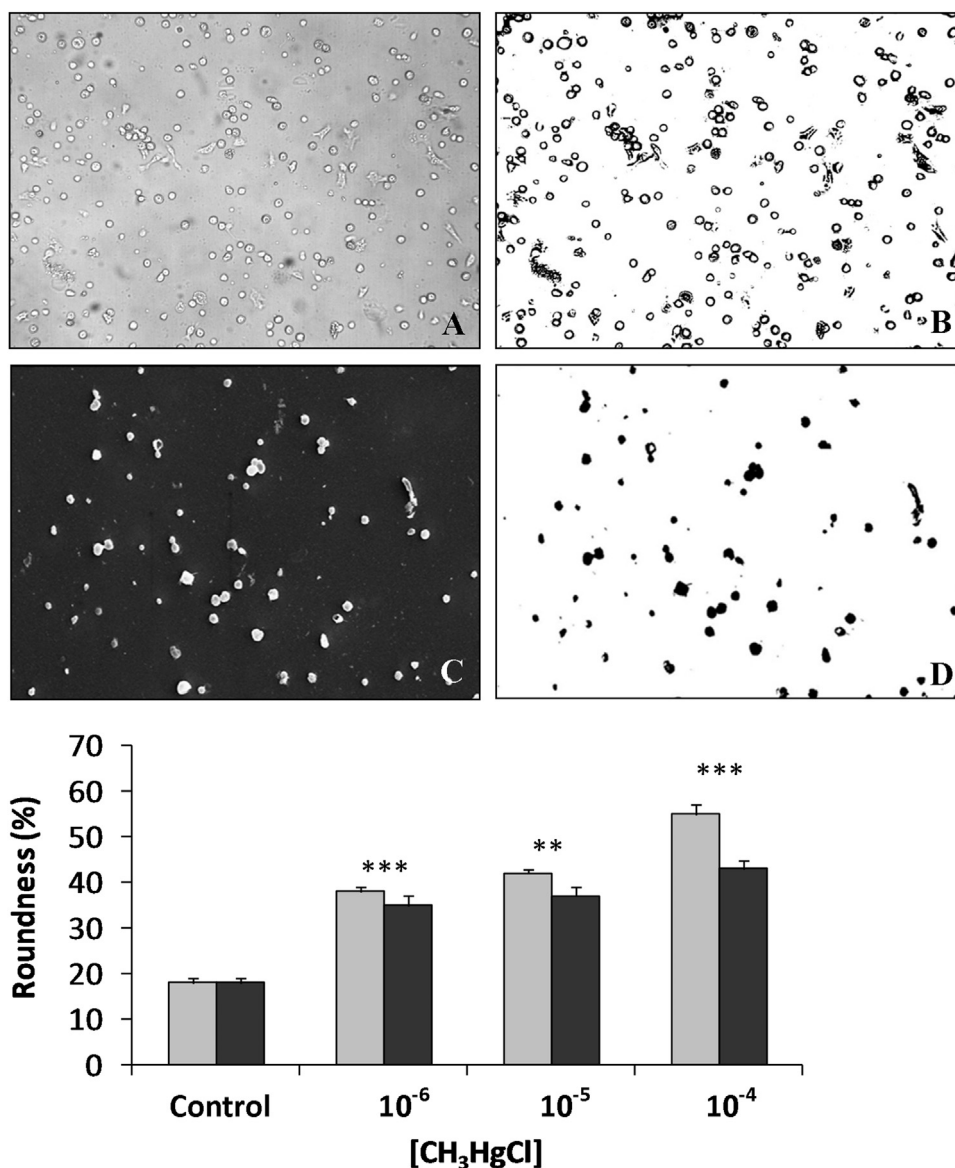


Fig. 4. *S. plicata* hemocytes in culture and percentage of hemocyte roundness calculated by Image J software. 10^{-6} , 10^{-5} and 10^{-4} M MeHg concentrations.

Table 3

Trace elements concentration in sediment samples collected at site Pa7.

	As	Cd	Cr	Cu	Hg	Ni	Pb	Zn
Not polluted ^a	< 3	< 0.3	< 45	< 178	< 1.2	< 28	< 94	< 474
Moderately polluted ^a	3–8	> 0.3	25–75	25–50	> 1	20–50	40–60	90–200
Heavy polluted ^a	> 8	> 0.68	> 75	> 50	> 1	> 50	> 60	> 200
TEL	7.24	0.68	52.3	18.7	0.13	15.9	30.2	124
PEL	41.6	4.21	160	108	0.7	42.8	112	271

^a USEPA sediment quality guidelines; TEL: Threshold Effects Level; PEL: Probably Effects Level.

Of note is that MeHg could affect target contact, recognition, release of active factors and enzyme/substrate reaction (Cammarata et al., 2007). Here, exposure of hemocyte lysate supernatant to 10^{-4} – 10^{-8} M of MeHg increased the PO activity in a dose-dependent fashion, presumably affecting the prophenoloxidase activation pathway (Cammarata et al., 2007). Biological processes, such as differentiation, growth and apoptosis, depend on cell shape and cytoskeleton organization directly determined by cell/surface interaction (Boudreau and

Bissell, 1998; Schwartz and Ginsberg, 2002). Therefore, measurements of pollutant-induced hemocyte morphological alterations can represent a biomarker that may be directly linked to organism health. Pertaining thereto, in the exposed organisms, we observed a significant increase in hemocyte rounding with a reduction in pseudopod numbers after treatment with 10^{-4} MeHg M. These effects suggest Hg induced cytoskeletal alterations. Pollutants are known to induce alterations in a number of cytoskeletal components. Particularly, Hg is able to inhibit tubulin polymerization and also induce microtubule disassembly (Liliom et al., 2000). A plausible explanation for these findings is that Hg modifies the SH groups of tubulin involved in MT assembly. Mercury is also involved in the decrease of cellular F-actin content within 20 s of exposure (Sweet et al., 2006) and disruption of the balance between the phosphorylated and non-phosphorylated forms of cofilin, which regulates actin dynamics and facilitates actin filament turnover (Vendrell et al., 2010). Moreover, in mussel hemocytes, Hg has been demonstrated to alter intracellular Ca^{2+} concentration, which is a prominent regulator of the structure and dynamics of cytoskeletons (Marchi et al., 2004). Therefore, the interference of Hg with the microtubule and/or microfilament cytoskeletal components in *S. plicata* hemocytes is consistent with the alteration in hemocyte shape

observed in exposed organisms. The effects on cell surface and cytoskeletal networks could explain the inhibitory effect of 1 h exposure to methylmercury (from 10^{-3} up to 10^{-5} M) on *in vitro* *S. plicata* hemocyte phagocytosis previously observed (Cammarata et al., 2007). These results confirm that MeHg is toxic for *S. plicata* hemocytes, causing immunosuppression either by cell death or morphological changes.

4.3. Effects of seawater samples on hemocytes of *S. plicata*

The effect of seawater from various geographical sites along the Sicily coasts on *S. plicata* hemocytes cultures was evaluated to check the potential use of hemocyte alterations as biomarkers in biomonitoring studies. Low percentages of hemocyte mortality were recorded after treatment with seawater samples, suggesting the presence of pollutants at sub-lethal levels. As shown in Fig. 2, treatment with seawater samples caused changes in *S. plicata* hemocytes aggregation ability, as demonstrated by the spread distribution of the clumped cells on well bottoms, similar to those of the cells treated *in vitro* with methylmercury. This result was supported by light microscope observations of hemocytes that showed similar changes in cell morphology with respect to those treated *in vitro* with methylmercury. The percentage of hemocytes with roundness features after treatment with seawater samples ranged from 9% to 30%, with the highest value observed after treatment with the seawater sample from Site Pa7 (Fig. 3). This led us to suggest the site as most contaminated by xenobiotics, probably due to its proximity to the urban center and confirmed by direct measurement (Bellante et al., in preparation). The analysis of trace metals in marine water samples is not suitable for assessing anthropogenic pressure of marine coastal environments. This is because the metal concentrations in water often lie near or below the detection limit of instruments and fluctuate drastically, depending on water flow and intermittence of discharge (Morillo et al., 2005; Rainbow, 1995). Thus, the pollutant concentrations in the water column are not representative parameters. Unpublished data show high trace metal concentrations in sediment samples previously collected in the same study area. Chemical contaminations in the sediments were evaluated by comparison with the sediment quality guidelines (SQGs) proposed by USEPA (United States Environmental Protection Agency). The threshold effect level (TEL) and probable effect level (PEL) of the SQGs were also applied to assess the degree to which the sediment-associated chemical status might adversely affect aquatic organisms (Long et al., 1995, 1998; Macdonald et al., 1996). The TELs were interpreted to represent chemical concentrations below which adverse biological effects rarely occur, and the PELs were intended to represent chemical concentrations above which adverse biological effects are more frequently observed (Long et al., 1998; Macdonald et al., 1996). Sediment samples from Site Pa7 were heavily polluted with As, Cu, Hg, Pb and Zn. In particular Cu, Hg and Zn concentrations exceeded the PELs values. In mussel hemocytes, Cu^{2+} has been demonstrated to alter intracellular Ca^{2+} concentration, thus altering the structure and dynamics of cytoskeletons, but with an intensity of approx. 50% with respect to Hg^{2+} (Marchi et al., 2004). Cu and Zn also showed significant immunosuppressive effects, with concentrations inducing 50% inhibition of phagocytosis ranging from 10^{-5} to 10^{-4} M, in the species *Eisenia fetida*, *Lumbricus terrestris*, *Aporrectodea turgida* and *Tubifex tubifex* (Sauve et al., 2002). The inhibition of phagocytosis suggests the same effects of these trace metals on the morphological features of hemocytes. These findings suggest that morphological changes observed in *S. plicata* hemocytes after treatment with seawater samples from Site Pa7 are due to the synergic toxic actions of Cu, Hg and Zn. Despite this assertion, the immune toxic effects of other xenobiotics not analyzed here must be taken into account. For example, it is interesting that Tributyltin (TBT) has detrimental effects on the immune system of the colonial ascidian *Botryllus schlosseri*, through interaction with calmodulin and alteration of Ca^{2+} homeostasis (Cima and Ballarin, 2000).

These results support the finding that *S. plicata* hemocyte morphological changes may be used as biomarkers in biomonitoring studies aimed at assessing trace metals contamination even at sub-lethal concentrations.

5. Conclusions

In the present paper, for the first time, we investigated pollutant-induced mortality and morphometric alterations in *S. plicata* hemocytes with possible applications as a sensitive, simple, and quick biomarker for monitoring and assessment purposes. Our study provides evidence of the MeHg immunotoxic effects on the immune system of *S. plicata*, resulting in hemocyte death and morphological changes induced by cytoskeleton alterations. Due to the important immunological role of these cells, which mediate many of the innate immune responses in invertebrates, the observed adverse effects of MeHg on *S. plicata* hemocytes may increase the susceptibility of animals to diseases and reduce their survival ability. Moreover, the present study demonstrated that the immunological responses of *S. plicata* hemocytes after *in vitro* treatment with seawater samples represent a useful biomarker that could be used as an indicator of trace metals pollution.

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