Ecotoxicology and Environmental Safety 136 (2017) 126–134

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

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

D. Parrinello[a], A. Bellante[b], M.G. Parisi[a], M.A. Sanfratello[a], S. Indelicato[b], D. Piazzese[b], M. Cammarata[a,*]

[a] Marine Immunobiology Laboratory, Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche University of Palermo, Palermo, Italy
[b] Dipartimento di Scienze della Terra e del Mare, University of Palermo, Palermo, Italy

ARTICLE INFO

Keywords:
Tunicate
Ascidian
Hemocytes
Toxic metals
Mercury
Seawater
Pollution
SEM

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). Ascidians 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 ascidians, 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 amebocytes (HA); (2) granular hemocytes including granulocytes with small granules (sG), granulocytes with large granules (lG), signet ring cells

* Correspondence to: Marine Immunobiology laboratory, Department of STEBICEF, Via Archirafi 18, 90123 Palermo, Itay.
E-mail address: matteo.cammarata@unipa.it (M. Cammarata).

http://dx.doi.org/10.1016/j.ecoenv.2016.11.001
Received 2 August 2016; Received in revised form 2 November 2016; Accepted 4 November 2016
Available online 12 November 2016
0147-6513/

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; Dyrynda 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 Na_2SO_4, $NaHCO_3$, pH 7.4) with 10 mM EDTA (FSW-EDTA) as anticoagulant. The hemolymph was diluted on ice to recover approximately 4×10^6 hemocytes per specimen. After centrifuging at 400×*g* for 10 min at 4 °C, the hemocytes were washed in marine solution (MS: 12 mM $CaCl_2$; 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 HNO_3 and 0.5 mL of H_2O_2 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 (2×10^6 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 4×10^6 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