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Lead (Pb) Induces Paraptosis Like Cell Death in Hemocytes of *Lamellidens* Sp: A Preliminary Study

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ABSTRACT

Molluscs represent a simple and significant model system to test how specific metals can simultaneously affect development and putative mechanisms of defense and/or cell death. *Lamellidens marginalis* (Bivalvia: Eulamellibranchiata) are the molluscs of Indian freshwater ecosystem and important biological resources. Lead (Pb) is widespread heavy metal that is released into the environment from different sources. Their accumulation in the soils can become dangerous to all kinds of organisms, including plants, animals and human life, causing many adverse effects. *Lamellidens* are used as reliable markers and bioindicators highly sensitive to environmental changes. The aim of the present work was to determine the toxic effect of lead on the cytomorphology of hemocytes of *Lamellidens marginalis* and to evaluate its potential as a bio monitor for detecting a heavy-metal polluted environment. Treated cells showed apoptosis and paraptosis (vacuolation) like features. Morphological analyses suggested an irretrievable destruction of normal morphology of hemocyte. However further studies are needed to investigate this topic, particularly in relation to the pollutants reported in this paper.

Keywords: Hemocytes, Molluscs, Cellular Death, Lead (Pb), Apoptosis, Paraptosis, *Lamellidens marginalis*

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1. INTRODUCTION

Toxicants like arsenic, lead were reported to affect immunocytes (Guria S *et al*., 2016). This often leads to impairment in the efficiency of invertebrates to destroy pathogen under the exposure of pollutants. Selected toxin-induced morphological changes may result in the overall impairment in homeostatic levels of invertebrates inhabiting the polluted environment. Lead (Pb) occurs naturally in the environment. However, most Pb concentrations that are found in the environment are the result of human activities. Pb accumulates in the bodies of water and soil organisms and it is a bio-persistent pollutant that accumulates at the top of the food chain (Scheifler R *et al*., 2006). Heavy metals are persistent in the environment for long periods, cause serious eco-toxicological problems. Pollutant contamination from industries has a high negative impact on the physico-chemical and biological quality of the water.

Lamellidens marginalis (Bivalvia: Eulamellibranchiata) are the molluscs of Indian freshwater ecosystem and important biological resources. *Lamellidens marginalis* are known to accumulate high levels of heavy metals in their tissues and yet survive in polluted environments (Mansoori A *et al*., 2013). They are used as markers and bio-indicators highly sensitive to environmental changes. Molluscs represent a simple and significant model system to test how specific metals can simultaneously affect the mechanisms of defense and/or cell death (Chiarelli R *et al*., 2014). The aim of the present study was to determine the toxic effect of lead (Pb) on the cytomorphology of hemocytes of *Lamellidens marginalis* and to evaluate its potential as a bio monitor for detecting a heavy-metal polluted environment. In view of this, in the present research attempt has been made to unify the classification of various cell deaths.

2. MATERIALS AND METHODS

2. 1. Treatment

Lamellidens marginalis (Mollusca: Bivalvia: Eulamellibranchiata) were manually collected from selected habitats of West Bengal and acclimatized in aerated glass containers at an ambient temperature of 25–30 °C for 7 days. None of these experimental animals had the previous history of exposure to toxicant. Control specimens were untreated by any toxic metals. Some (n=16) specimens were exposed to 50 mg/L of Lead Nitrate in water medium and maintained for 20 days (after Jantataeme S *et al*., 1996).

2. 2. Hemolymph collection

In case of bivalve molluscs, *Lamellidens marginalis* hemolymph was collected by gently prying the shell to open approximately 5 to7 mm. with a thin knife. The shell was held open with tissue forceps. The foot was visible between slightly gaping of shell valves, as a highly muscular white surface, then it was gently penetrated with a needle and hemolymph was easily collected using gentle intermittent suction (after Kambale and Potdar, 2010). The drop of hemolymph was then drawn into a thin film by the edge of another slide and the film air - dried before staining.

2. 3. Fixation and staining of hemocytes

Hemolymph was placed and smeared directly on sterilized glass slides and were fixed by methanol and stained by Giemsa, Methylene blue and observed under light microscope. Cellular morphology was examined.

2. 4. Counting of hemocytes

Hemolymph was used for cell counting by hemocytometer.

2. 5. Trypan blue dye exclusion test

Cells were treated with 50 µl of 0.25 % trypan blue dye solution for 5 minutes. Cells that have taken up the dye are dead, since the dye is normally excluded by the membranes which maintain their semi permeability intact and therefore, the percentage of blue-stained cells represents a mortality index (Guria S *et al*., 2016).

$$\text{Mortality index} = \frac{Number\ of\ cells\ with\ blue\ stained\ cytoplasm}{Total\ number\ of\ cells} \times 100$$

2. 6. Study of phagocytosis

Hemocyte suspension of 100 µl collected from each specimen was smeared on glass slides and incubated for 1 h at 37 °C in a humid chamber for cell adherence. The phagocytic efficiencies of hemocytes were determined by challenging the hemocytes with activated charcoal particles and were incubated for 1 h at 37 °C. After incubation, the cells were washed with saline, air dried and fixed with methanol, stained with Giemsa and examined under light microscope. Different stages of phagocytosis of charcoal particles by hemocytes were determined in both control and treated molluscs. The phagocytic index was calculated as per the following formula.

$$\text{Phagocytic index} = \frac{Number\ of\ cells\ with\ phagocytosed\ charcoal\ particle}{Total\ number\ of\ cells\ counted} \times 100$$

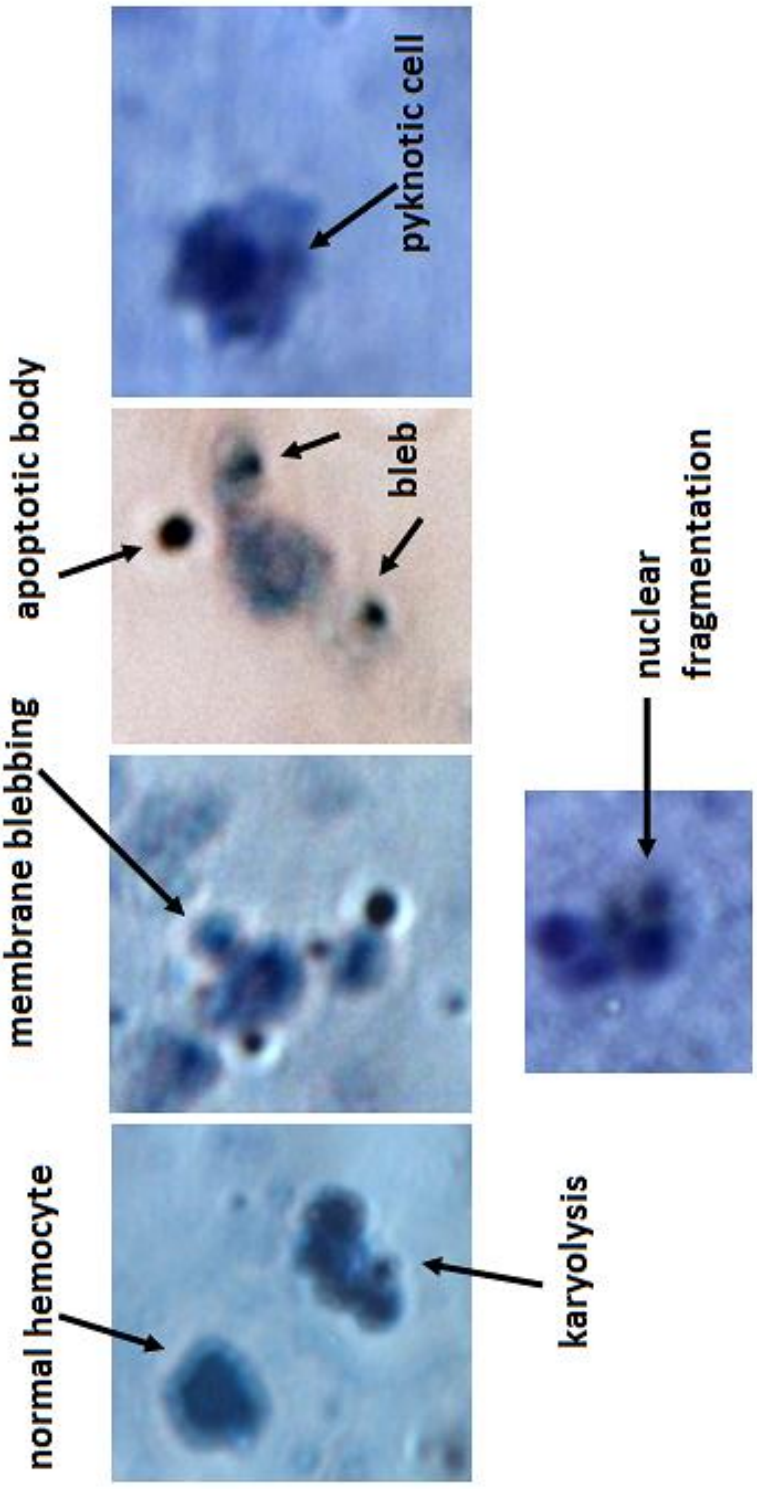
3. RESULTS

3. 1. Normal cytomorphological profile of hemocyte

Normal cell morphotypes were noticed in control *Lamellidens marginalis*. Blast like cells, round hyalinocytes, spindle hemocytes, asterocytes were categorically recognised (figure not shown).

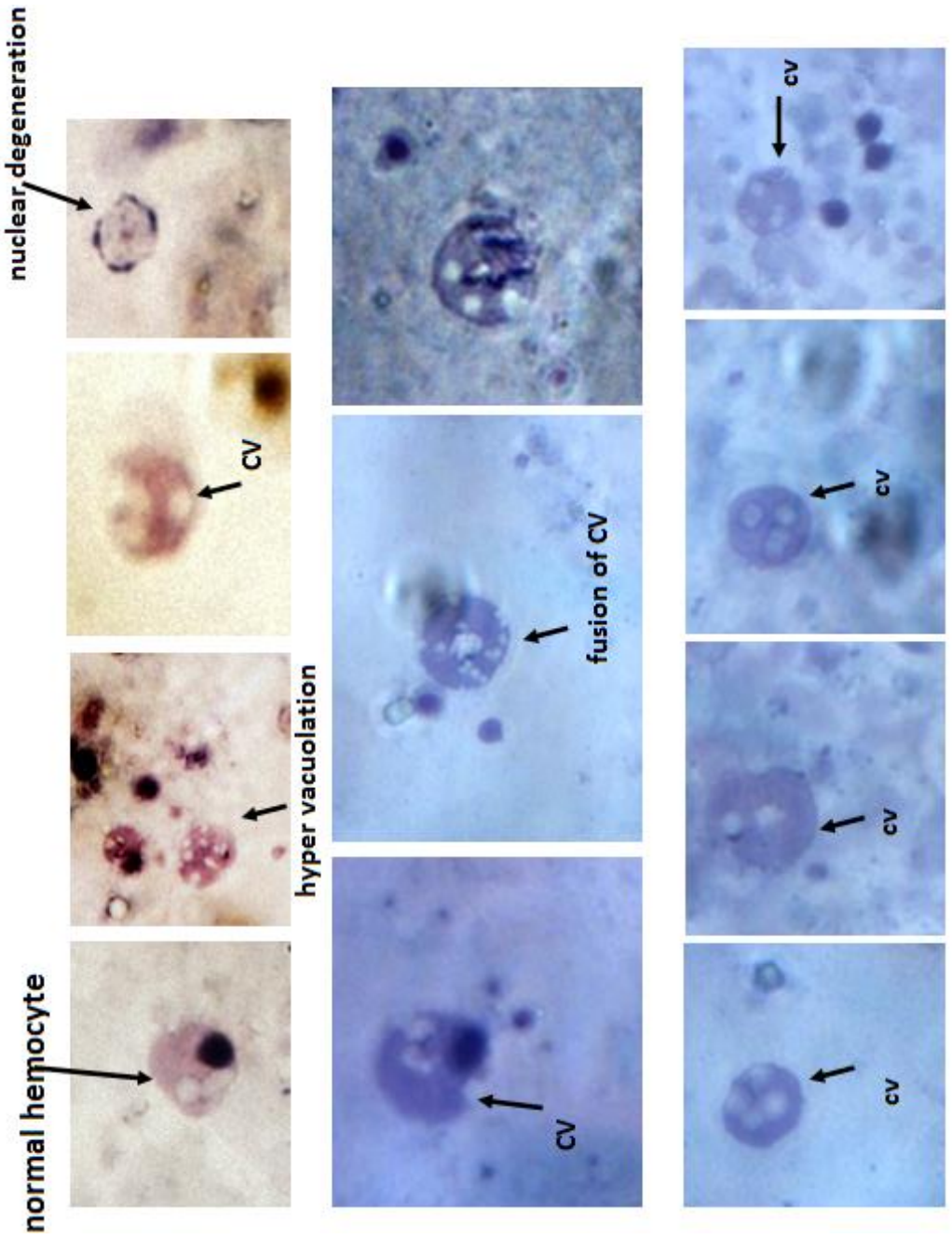
3. 2. Effect of lead (Pb) on cell-structure of hemocytes

Lead (Pb) treatment led to vacuolization in the cytoplasm of hemocytes. Significant changes were observed in the cytomorphology of hemocytes when compared with the control group under light microscopy.



(Fig. 1A)

Figure 1A. Lead treated hemocytes showed altered cell surface indicating membrane blebbing, cellular apoptosis like features and nuclear degeneration.



(Fig. 1B)

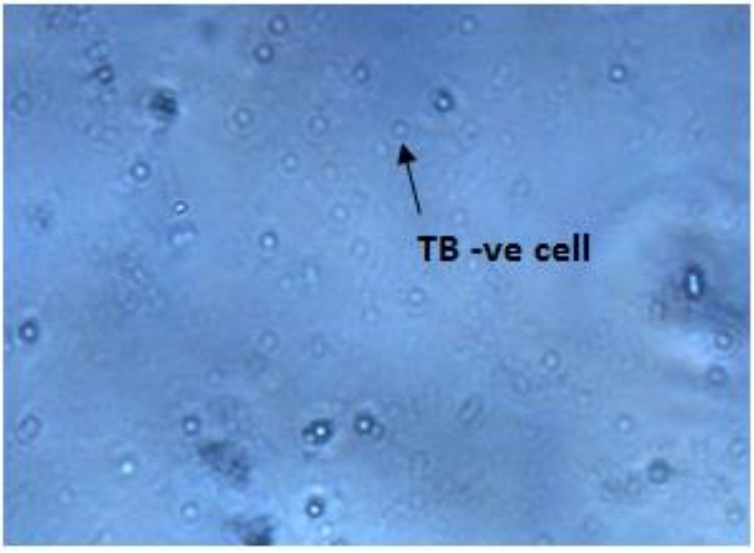
Figure 1B. Lead treated hemocyte with different phases of cytoplasmic vacuole formation (CV = Cytoplasmic Vacuole).

Treated molluscs exhibited cellular damage. Higher magnification of lead treated cells showed different phases of cellular death like formation of membrane blebs, rupture of plasma membrane and degeneration of nuclei (Fig. 1A). A significant percentage of

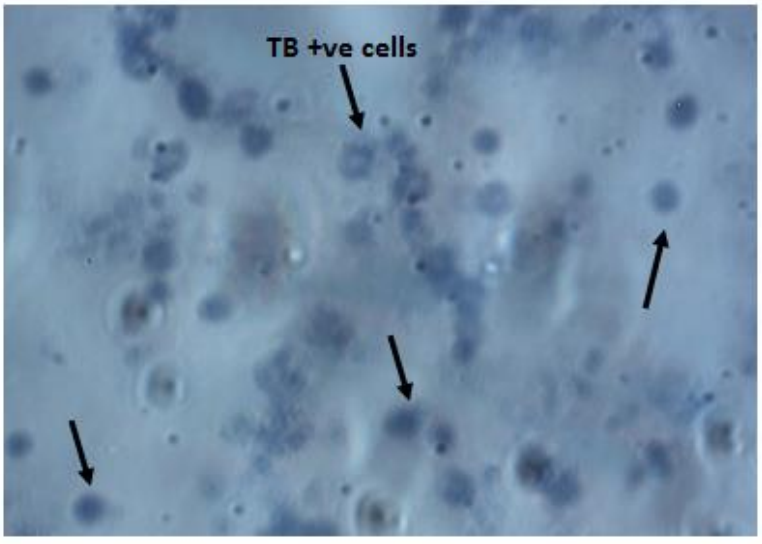
hemocytes become pyknotic. Note the large cytoplasmic vacuoles found in Pb treated hemocytes. Normal ultra- structural morphology was predominately found in the control cells, showing a well-defined plasma membrane and intact nucleus. After Pb treatment, the cell cytoplasm displayed vacuoles (Fig. 1B).

3. 3. Trypan blue staining of hemocytes

Significant number of treated hemocytes showed Trypan blue positive response. Dead cells were blue in colour whereas the viable cells of controls were white (Fig. 2A and B).



(Fig. 2A)



(Fig. 2B)

Figure 2A and B. Control hemocytes (Fig. 2A) and lead treated dead hemocytes (Fig. 2B)

3. 4. Phagocytic index

Mean phagocytic index was significantly reduced in treated group (Fig. 3).

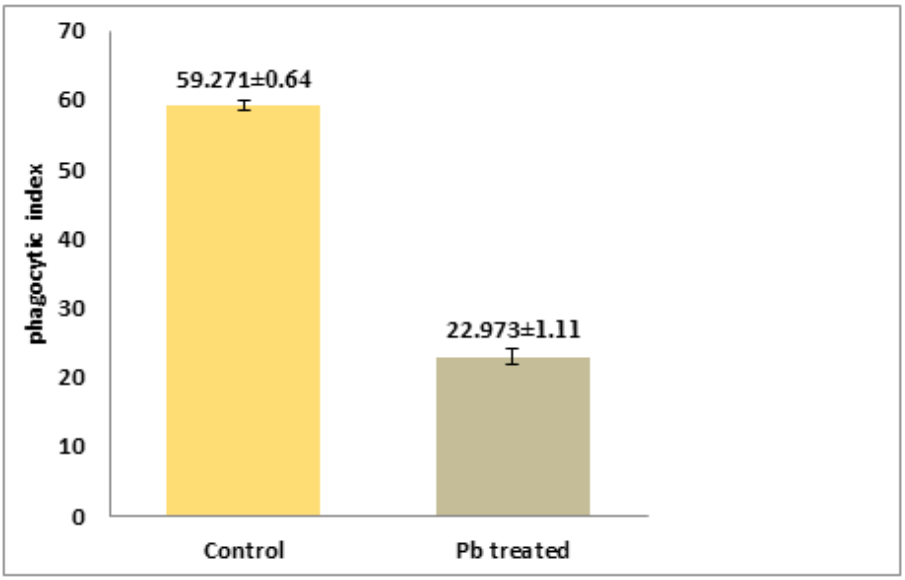


Figure 3. Mean phagocytic index in normal and treated group. Values are expressed as Mean ± SEM.

3. 5. Calculation of mortality index

Mean mortality index was significantly increased in treated group (Fig. 4).

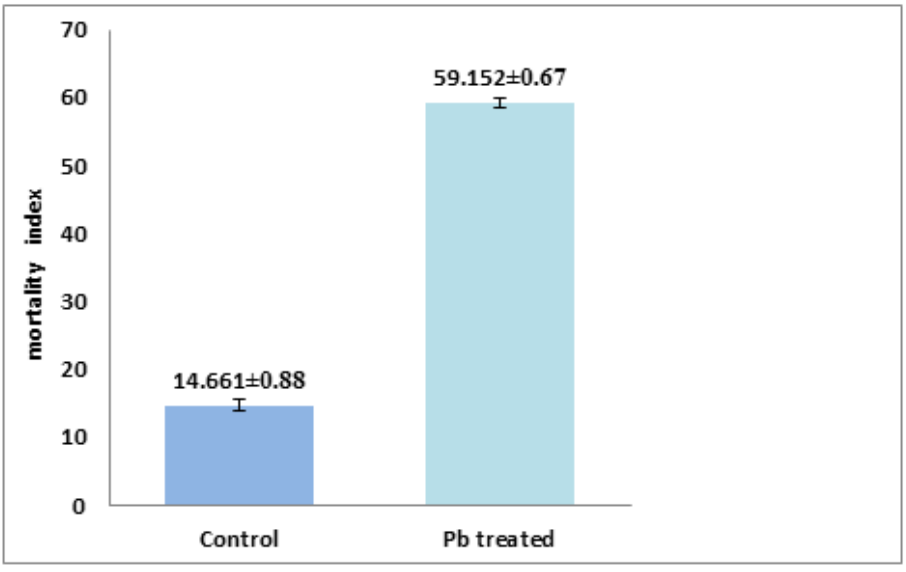


Figure 4. Mean mortality index in normal and treated group. Values are expressed as Mean ± SEM.

4. DISCUSSION AND CONCLUSION

During last few decades, continuous release of toxicants into the global environment has been expected to yield an eco-toxicological challenge for aquatic organisms. Molluscs, in general, depend on innate immune system to combat invading parasites, pathogens and toxin exposure (Ottaviani, 2006). Hemocyte is the major functional component of cell mediated immunity of molluscs and present responsiveness to different environmental toxins. Hemocytes are capable of performing diverse immunological functions like phagocytosis, encapsulation and cytotoxicity under the exposure of toxins and pathogens (Cheng, 1977 and Cesen *et al*., 2012). Hemocytes of a wide range of species exhibit immunological reactivity against environmental toxins (Galloway *et al*., 2001).

According to Ray *et al*., (2013), in *L. marginalis*, round granulocytes act as principal immunoeffector cells exhibiting strong phagocytic efficiency and generation of high level of nitric oxide and superoxide anion. However, both the vertebrate macrophage and the invertebrate hemocyte phagocytic systems are homologous in being concerned with recognition and engulfment of foreign material. The mechanism whereby invertebrate phagocytes inherently recognise 'foreignness' in the absence of immunoglobulin is unknown (Roitt I *et al*., 2000).

Due to continuous use of lead contaminated water, it may accumulate in soils, can be taken up by plants and there by enter the food chain. Lead affected molluscs may play a significant role in accumulating and further transferring toxic metals to higher trophic levels in the food chain.

A previous study reported the toxic effect of lead on the cytomorphology of hemocytes of *Gesonula sp* (Guria S *et al*., 2016). The present study corroborated the earlier study. In the present study lead (Pb) treatment led to vacuolization in the cytoplasm of hemocytes. Higher magnification of lead treated cells showed different phases of cellular death like formation of membrane blebs, rupture of plasma membrane and degeneration of nuclei (Fig. 1A). A significant percentage of hemocytes become pyknotic. After Pb treatment, the cell cytoplasm displayed vacuoles (Fig. 1B)**.** Mean phagocytic index was significantly reduced in treated group (Fig. 3). Mean mortality index was significantly increased in treated group (Fig. 4).

Cell death images in present study showed nuclear degeneration, membrane blebbing which is the indication of apoptosis. After lead treatment, the cell cytoplasm displayed vacuoles (Fig 1B). These features resemble another type of programmed cell death (PCD), called paraptosis. It is characterized by the appearance of large vacuoles. Paraptosis, in contrast to apoptosis, does not show activation of the caspase-3 cascade, followed by chromatin condensation (Guria S and Das M, 2016; Sperandio, 2000). Paraptosis is a form of type III programmed cell death with a unique combination of certain apoptotic and necrotic characteristics. Paraptosis does not reveal nuclear fragmentation, membrane blebbing, formation of apoptotic bodies, or definitive demonstration of chromatin condensation – all seen in apoptosis. The number and size of vacuoles increases over time. Eventually, the vacuole sizes reach a point of no return and the cell can not recover (Guria S and Das M, 2016; Cagle and Allen, 2009).

One of the most important goals of toxicological studies is to determine whether heavy metal pollution causes adverse effects on organisms. Although concentrations of priority metals in the aquatic system are regularly monitored worldwide, great effort is being made towards the application of biomarkers that indicate an early response in selected target

organisms that finally provide evidence of the exposure to the chemical pollutants and may indicate a toxic effect (Chiarelli R *et al*., 2014). Biomonitoring of heavy metals and effect studied on natural populations of organisms must take into account the pollution-induced tolerance in the communities that are exposed to particular pollutants for a long time (Chiarelli R *et al*., 2014). Molluscs, crustaceans and other aquatic invertebrates are known to accumulate high levels of heavy metals in their tissues and yet survive in polluted environments. On the contrary, non-essential heavy metals (e.g. Cd, Hg, Ag and Pb) are toxic for living organisms even at low concentrations (Mansoori A *et al*., 2013).

Molluscs represent a simple and significant model system to test how specific metals can simultaneously affect development and putative mechanisms of defense and/or cell death (Chiarelli R *et al*., 2014). However further studies are needed to investigate this topic, particularly in relation to the pollutants reported in this work.

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