Alteration of total count, hemocyte density and morphology of hemocytes of *L. marginalis* treated with washing soda

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ABSTRACT

Lamellidens marginalis (Mollusca: Bivalvia: Eulamellibranchiata) is an important representative of filter feeding, bivalve mollusc which finds its habitat in the freshwater ecosystems of India. It feeds by filtering phytoplanktons, bacteria and other particulate organic matter suspended in the natural water body. Washing soda have been chemically identified as anhydrous sodium carbonate, is particularly used as a cleaning agent in India among the rural and urban populations, which frequently contaminates the freshwater lakes and ponds, the natural habitat of freshwater mussel *Lamellidens marginalis.* Hemocytes which circulates in the hemolymph of bivalves, act as blood cells and are functionally principal immunoeffector cells when comes in contact to various contaminants, xenobiotics or parasites. The various hemocytes of *Lamellidens marginalis* have been morphologically characterized and ascribed to be treated as a biomarker to ascertain the level of freshwater pollution by sublethal concentrations of washing soda, a natural contaminant of freshwater ecosystem. The diverse hemocyte subpopulations were morphologically characterized as blast-like cells, agranulocytes, granulocytes, hyalinocytes and asterocytes. A depression in the total hemocyte count (THC) and a gradual elevation in the percent of blast-like cells was observed under the long term exposure to experimental washing soda concentrations. Long term exposure to washing soda suppressed the relative densities of hyalinocytes, asterocytes, granulocytes and agranulocytes compared to the control animals.The present data indicates a shift in the immunological parameters of *L. marginalis* in washing soda contaminated freshwater ecosystems. The present study is aimed at estimating the degree of washing soda induced stress in *L. marginalis* and enumerating the hemocyte densities to establish it as a biomarker of aquatic pollution in several geographical regions of India. Long term exposure of hemocytes to experimental sublethal washing soda concentrations inflicted several morphological and nuclear damages like cell membrane disruption, hypervacuolation, membrane blebbing, micronucleation, binucleation, cytoplasmic smoothening. All these physical alterations in cellular morphology in its turn negatively affected phagocytic ability which is a clear manifestation of depressed cell mediated immune response of *L. marginalis* hemocytes.

Index terms: Lamellidens marginalis, washing soda, biomarker, hemocytes, hemolymph, hypervacuolation, total count, differential count, micronucleation, binucleation.

1. INTRODUCTION

Freshwater ecosystem provides the natural habitat for the myriad of aquatic invertebrates including members of the phylum mollusca. Many of the molluscan species bears ecological, economical, nutritional, medicinal, industrial and biotechnological importance and considered as an important biological resource. *Lamellidens marginalis* are bivalve molluscs are inactive, stationary, molluscs that clings to its substratum. They are filter feeding aquatic invertebrates which can bioaccumulate pollutants and store them in their tisssues, thus they are considered an ideal species for monitoring the effects of environmental pollutants and contaminants on aquatic invertebrates (Wootton et al., 2003). Therefore they gained a priority for acting as bioindicators of chemical contaminants present in aquatic ecosystems (Fournier et al., 2001 Zhou et al., 2008). Bivalves like *L. marginalis* are sedentary filter-feeding organisms, which are able to bioaccumulate and concentrate most water pollutants even when they are present at fairly low concentrations (Niyogi *et al*., 2001). Burrowing bivalves have the ability to increase sediment homogenization, oxygen penetration and are capable of providing clear substratum for the colonization of epiphytic and epizoic biota (Beckett *et al*., 1996). There are certain selected body parts of *L.marginalis* which are used as medicine to cure a number of diseases (Prabhakar and Roy, 2009). *L. marginalis* is an edible species, which are considered to be a rich and cheap source of dietary protein for rural human population of eastern India and used as an ingredient in the artificial food supplement for poultry and fishes (Chakraborty *et al.*, 2008).

In recent times Indian freshwater ecosystem are facing the threat of contamination by diverse environmental toxins including various commercial brands of detergents (Ray et al., 2011) and allied compounds. Washing soda, is a component of laundry detergent (Warne and Schifko, 1999) which acts as a water softening “precipitating builder” (Bajpai and Tyagi, 2007) and iscapable of increasing the alkalinity of water. Field survey which were carried out in the North and South 24 parganas of West Bengal revealed that the commercial brands of detergents including washing soda i.e. anhydrous sodium carbonate (Na_2CO_3), (CAS number : 497-

19-8) acts as as a major aquatic toxicant of freshwater ecosystem. Washing soda causes acute and chronic toxicity in aquatic molluscs like *L. marginalis* at different concentrations. Unrestricted anthropogenic use of washing soda and their subsequent dischargeinto the freshwater ecosystem has been drastically reducing the population of freshwater molluscs like *L. marginalis, Bellamya bengalensis and Pila globosa* etc.

Hemocytes of bivalves has been reported as the principal immunoeffector cells of bivalves which are capable of performing diverse physiological functions like adhesion, aggregation, phagocytosis and generation of several cytotoxic agents (Pinto et al., 1994, Sami et al., 1992, Chu, 1988., Hubert et al., 1996, Feng, 1988., Cheng, 1996., Lopez et al., 1997a, Allam and Paillard., 1998, Millar and Ratcliffe, 1994). The estimation of physiological and biochemical responses of molluscs in contaminated ecosystem may be considered as indicators of the health of the larger population and community (Bayne *et al.,* 1978). Marked variations in the total count (TC), and differential count (DC) and the density of hemocytes may be related to irregular hemocyte release from hemocytopoietic organs into the open circulatory system (Hoffmann, 1973; Crossley, 1975). The open circulatory system of bivalve molluscs like *L. marginalis* are continually exposed to several fluctuating environmental factors and to a diverse array of several chemical contaminants suspended in polluted water bodies. Within the molluscan body a body fluid called hemolymph circulates within the open circulatory system that transports nutrients, respiratory gases, metabolic wastes and toxic substances throughout its body. Humoral defence factors and nucleated cells remains suspended in the hemolymph. Hemocytes circulating in the hemolymph of *L. marginalis* constitutes the major component of innate immunity (Auffret et al., 2002; Mukherjee et al., 2006).Thus subtle fluctuations in the major components of hemolymph might have an adverse effect on the physiology and behavior of molluscs. Thereby molluscs garnered an importance as bioindicators to assess and monitor the extent of chemical contamination in their aquatic habitat. The study of morphology, total and differential counts of the hemocyte subpopulations reflects the basic responses of hemocytes to environmental fluctuations (Pampanin et al., 2002).

In*L. marginalis* five major types of hemocytes were characterized. The blast-like cells are characterized by high nuclear to cytoplasmic ratios. The nuclei of these cells occupies most of the cellular space and reaches upto the periphery of the plasma membrane. The marginal basophilic cytoplasm are devoid of any granular structure. Granulocytes are identified by the presence of large number of eosinophilic granules in the cytoplasm and the presence of basophilic granules are rare. Spindle shaped conformation characterize the hyalinocytes with central dense nuclei and sharp pseudopodial cytoplasmic extensions. Agranulocytes are identified by their concave or lobed nucleus with or without the presence of very scarce cytoplasmic granules. Agranulocytes are comparatively larger in size. The asterocytes are characterized by the presence of very sharp cytoplasmic projections with dense nuclei. Asterocytes contains mainly agranular cytoplasm.

2. MATERIALS AND METHODS:

2.1. Collection, maintenance and exposure of the animals :

Fresh specimens of *L. marginalis* with the average dimensions of 7cm×4 cm were collected from the selected freshwater ponds of the district of 24-Parganas (South) of West Bengal in India. The harvested animals were transferred to the laboratory and acclimated for 7 days in well aerated fish tanks (90×cm ×90 cm×30 cm) in batches of 15 per tank. The animals were fed with finely chopped lettuce leaves. The water of these fish tanks was replaced with fresh pond water at an interval of every 12 hrs to avoid toxicity due to accumulation of excretory products. The average dissolved oxygen content and hardness of the water were maintained at 14.1 mg/l and 457 mg/l respectively according to (Raut, 1991). The experiment on *L. marginalis* was designed complying with the guidelines of institutional norms of animal handling and care of the University of Calcutta. The West Bengal Biodiversity Board of the Department Environment of Government of West Bengal, India permitted us to collect the specimens of *L. marginalis* from their natural habitat.

Each experimental set consisted of 4 animals of same size exposed to a volume of 2 L of sodium carbonate solutions. The exposures were at concentrations of 20, 40, 80 and 160 mg/l for 1, 2, 4, 8, 16 and 32 days. As a control, 5 sets of animals were kept in sodium carbonate free fresh analytical grade water. The experiments were carried out in a stagnant aquatic environment and fresh solutions of sodium carbonate were replaced in every 12 hrs. The temperature of the water was maintained between 24^0- 26^0C. During experimental chronic exposure in sodium carbonate solutions the animals were supplemented with standard feed to avoid stress due to starvation. The pH of the control aquatic environment i.e. the water that is free from the contamination of sodium carbonate solutions or other aquatic contaminants is maintained at 7.2.

2.2. Collection of hemolymph and estimation of total count of hemocytes:

Hemolymph was collected aseptically from the posterior adductor muscle (Adema et al., 1991) with the help of a hypodermic syringe slowly to eliminate the risk of bubbling along hemolymph in prechilled glass vials. The hemolymph samples collected are resuspended in sterile snail saline (SSS) (5mM HEPES, 3.7mM NaOH, 36mM NaCl, 2mM KCl, 2mM $MgCl_2$. $2H_2O$ and 4mM $CaCl_2.2H_2O$ at pH 7.8) (Chakraborty et al., 2008) and stored at 4^0C. The total hemocyte count was estimated with the help of Neubauer hemocytometer (Brousseau et al., 1999). Data was presented in mean ± standard deviation (S.D.).

2.3. Differential count of hemocyte subpopulations :

The differential count of hemocytes of *L. marginalis* exposed to sublethal experimental washing soda concentrations along with respective control was estimated by enumerating the subpopulation densities following field count method. Freshly collected aliquot of hemocytes were placed on sterilized glass slides, allowed to settle, adhere and fix for 10 minutes with the help of micropipette. Methanol was used to fix the adherent hemocytes. The fixed uniform monolayer of the hemocytes were stained with Giemsa (Himedia, India).The Giemsa stained monolayer of the hemocytes wereexamined under bright field microscope (Olympus BX2, India) to ascertain the percentage of different subpopulation of hemocytes. Areas for microscopic analysis were selected such a way where more than 100 cells were observed per slide to determine the hemocyte types (Chakraborty *et al*., 2008).Differential count of hemocytes was expressed as the percentage abundance of respective morphotypes of hemocytes (Chakrabort*yet al*., 2008)..

2.4. Microscopical analysis of hemocyte morphology under the exposure of washing soda :

The analysis of the morphology of hemocytes of *L. marginalis* was carried out microscopically using scanning electron microscopy and bright field microscopy exposed to different experimental sublethal washing soda concentrations along with the control. The morphology of Giemsa stained hemocyte was studied under bright field optics. The ultrastructural analysis of the hemocytes was carried out under scanning electron microscope.

2.5a. Scanning electron microscopy :

The analysis of the ultrastructure of the hemocytes of *L. marginalis* under scanning electron microscope(Zeiss EVO 18, USA) was carried out after Gagnaire (2004) with some minor modifications. For the photodocumentation of cells under scanning electron microscopy, the hemocytes freshly withdrawn from the adductor muscles were fixed in 1% glutaraldehyde (Sigma, USA) along with 0.2M phosphate buffer consisting of monobasic sodium phosphate and dibasic sodium phosphate (pH 7.2) for 1 hour. After fixation of the hemocytes, the cells were rinsed for 3-4 times in 0.2M phosphate buffer and then dehydrated through alcohol grades. Once the hemocyte samples are properly dried were fixed physically on aluminium stubs with the aid of silver DAG-915. For scanning electron microscopy the samples were then coated with gold-palladium (thickness 180Å).

2.5b. Bright field microscopy :

The suspension of hemocytes collected having more 95% viable cells were considered suitable for the experiment (Bryan, 1971).The viable hemocytes were placed over sterile glass slides in a moist chamber for 30 minutes for adhesion. The adhered hemocytes were then dried, fixed with methanol and stained with Giemsa (Himedia, India) for 15 minutes. The stained cells were then washed and air dried and were analyzed and documented microscopically employing light microscope (Olympus BX51, Japan) having digital image capturing facility (Zenoptic, Japan) at different magnifications. The morphology of various control and washing soda treated hemocyte subpopulations were then carefully analyzed. At least 200 hemocytes were microscopically examined per slide to analyze the morphology of different subpopulations of hemocytes.

2.6. Statistical analysis:

The data were checked for normality and homogeneity applying Barlett's test. Since all the data were normal, parametric statistics were applied, following one-way analysis of variance (ANOVA) (Singaram et al., 2013). The statistical data analysis was performed using one way ANOVA followed by Dunnett's multiple comparison post hoc test to compare the significant difference between active and other groups. Data was presented as the mean ± standard deviation (SD). Each experiment was repeated for 10 times (n = 10).Differences were considered to be significant at $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

3. RESULTS:

3.1. Total hemocyte count :

The total hemocyte count (THC) of different hemocyte subpopulations are the most widely used parameters to assesss bivalve health status which changes from time to time under stressful condition (Koprucu, 2010). A nonlinear fluctuation in the value of THC was recorded under exposure of 20, 40, 80 and 160 mg/l of washing soda for a time span of 1to 8 days compared to the control value. The highest total count under the chronic exposure of sodium carbonate was recorded as 2.68 ± 0.110065 in specimens exposed to 160 mg/l of sodium carbonate for a time span of 8 days. The lowest total count was recorded as 0.53 ± 0.023905 in specimens exposed to 160 mg/l of washing soda for a time span of 32 days. A steady decrease in the total count of haemocytes were observed in specimens exposed to 20, 40, 80 and 160 mg/l of washing soda for a time span of 16 and 32 days. The value of THC in washing soda unexposed specimens ranged between 2.08 ±0.058554004 for 1day to 2.62 ± 0.0659 for 32 days.

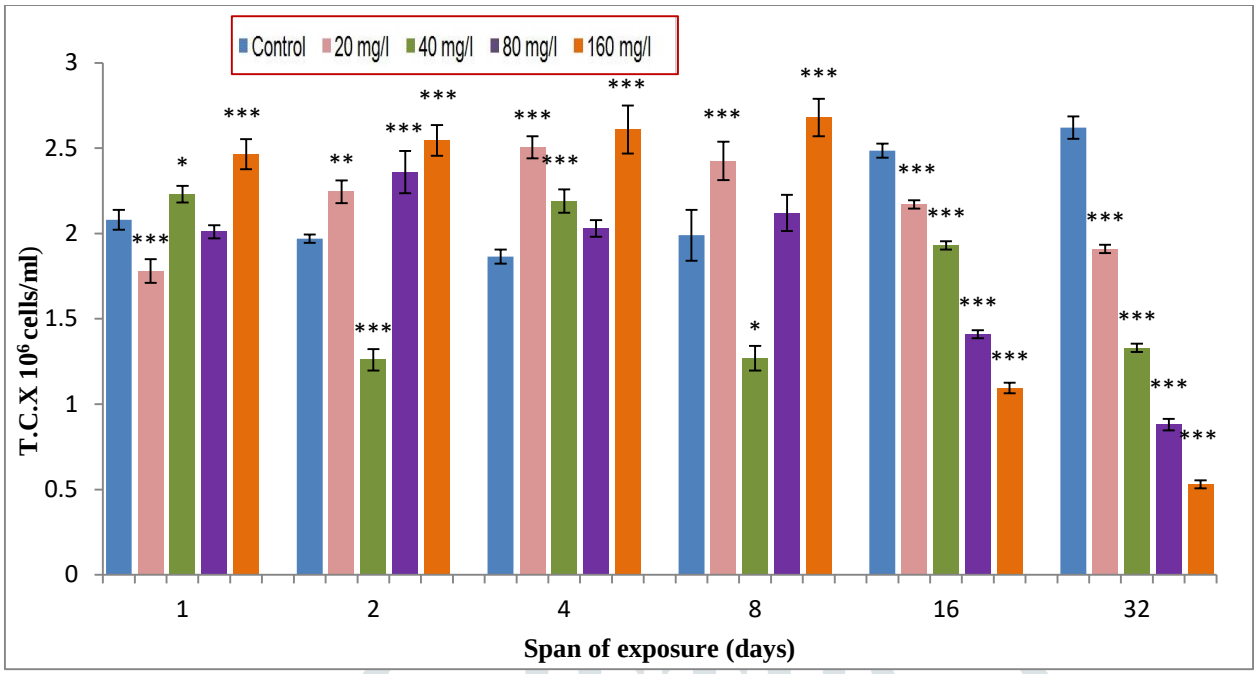

figure1 : total count (t.c.) of hemocyte of *l. marginalis* at 1, 2, 4, 8, 16 and 32 days of exposure to 20, 40, 80, 160 mg/l of washing soda. statistical analyses were performed using one-way anova followed by dunnett's post hoc test at each experimental hour for means separation. data were presented as mean ± s.d. (n = 10). theasterics (*) indicated the values that were significantly different from the control ($p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$)

3.2. Differential hemocyte count :

The differential count of five different morphotypes of hemocyte subpopulations of *L. marginalis* was microscopically carried out exposed to experimental sublethal washing soda concentrations along with control.In our study exposure to experimental sublethal washing soda concentrations had resulted a significant shift in the hemocyte subpopulation dynamics in comparison to respective controls.

The percentage distribution of different hemocyte variants of washing soda unexposed population of *L. marginalis* have manifested the most densely populated cells to be agranulocytes followed by blast like cells, granulocytes, hyalinocytes and asterocytes. The animals on being exposed to sublethal concentrations of washing soda the granulocytes and blast like cells exhibited a significant rise in the population, whereas the agranulocytes showed a marked decline in their number. The differential count of hyalinocytes reached to a highest number at 8 days of exposure to different sublethal washing soda concentrations, compared to the control value. But the hyalinocyte number declined sharply on being exposed for 16 and 32 days to sublethal washing soda concentrations. The differential count of asterocytes significantly increased on being exposed for 1, 2 and 4days to different washing soda sublethal concentrations, compared to the control value. But on prolonged exposure for 8, 16 and 32 days to different sublethal concentrations of washing soda the asterocyte number dwindled to a remarkable extent compared to the control value. The highest inhibition in the percentage of asterocytes was noted as 0.783494 ± 0.01387 compared to the control value of 4.559326 ± 0.750115 under 40 and 160mg/l of washing soda exposure for a time span of 32 days.The relative percentage of blast like cells of control *L. marginalis* was ranged from 21.75303 ± 3.63606 to 20.29997 ± 1.347774 over a time span of 1day to 32 days. A steady trend of increase in the density of blast like cells was recorded from 8 to 32 days of exposure to washing soda. The highest increase in the percentage of blast like cells was recorded as 41.97899 ±4.612093 under exposure of 1600mg/l of washing soda for 32 days. The relative percentage of granulocytes of control *L. marginalis* ranged from 9.128788 ± 2.240898 to 11.6141 ± 1.735286 over a time span of 1 day to 32 days. The percentage of granulocytes increased steadily from 1 to 8 days of exposure under different sublethal washing soda concentrations compared to control value but on prolonged exposure for 16 and 32 days their percentage declined compared to control value. The highest inhibition in the percentage of granulocytes was noted to be 6.347212 ± 1.141013 against 160mg/l of washing soda for 32days of exposure. The relative percentage of agranulocytes control *L. marginalis* ranged from 61.95909 ± 9.764612 to 61.62171± 4.728789 over a time span of 1day to 32 days. A trend of increase in the percentage of occurrence of agranulocytes was recorded upto 4 days of exposure to different sublethal washing soda concentrations. On prolonged exposure

from 8 to 32 days agranulocyte percent declined compared to control value. The highest inhibition in the percent of agranulocytes was recorded as 38.41189 ± 3.819708 against 160mg/l of washing soda for 32days of exposure. A dose dependent nonlinear fluctuation in the percentage of hyalinocytes was noted from 1 to 8 days of exposure to different sublethal washing soda concentrations. But on prolonged exposure for 16 and 32 days under different washing soda concentrations the percent of hyalinocytes declined markedly relative to the control value. The highest inhibition in the percent of hyalinocytes was noted as 0.603429 ± 0.467544 under exposure of 80mg/l of washing soda for 32 days against a control value of 5.447283 ± 0.507517.

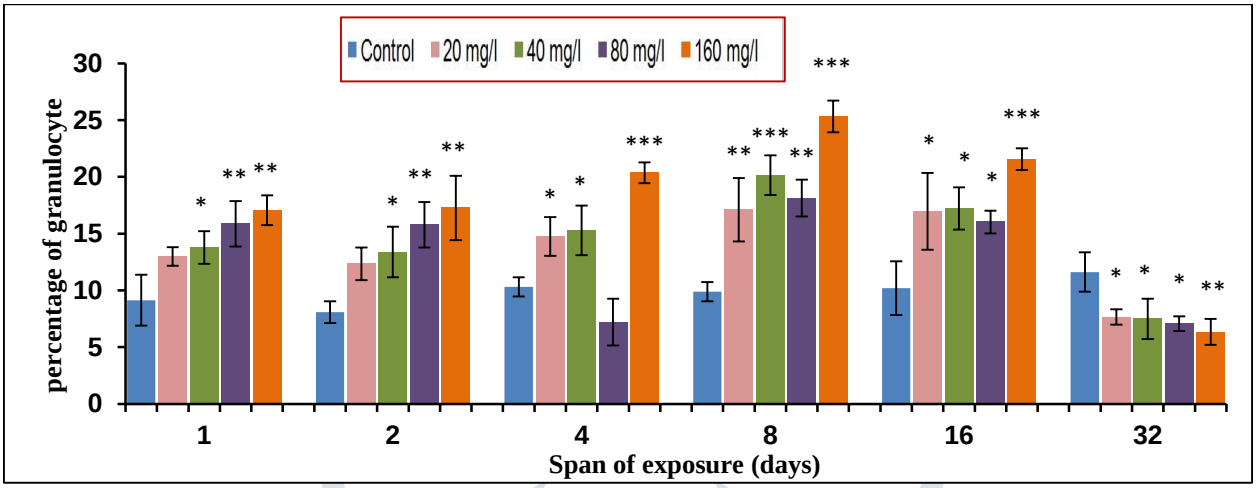

figure 2 : frequency of granulocytes of *l. marginalis* at 1 , 2 , 4 , 8 , 16 and 32 days of exposure to 20 , 40 , 80 , and 160 mg/l of washing soda. data represented is the mean ± standard deviation (n=10). the asterics (*) indicate the values that are significantly different (*p<0.05, **p< 0.01, ***p<0.001) from the control.

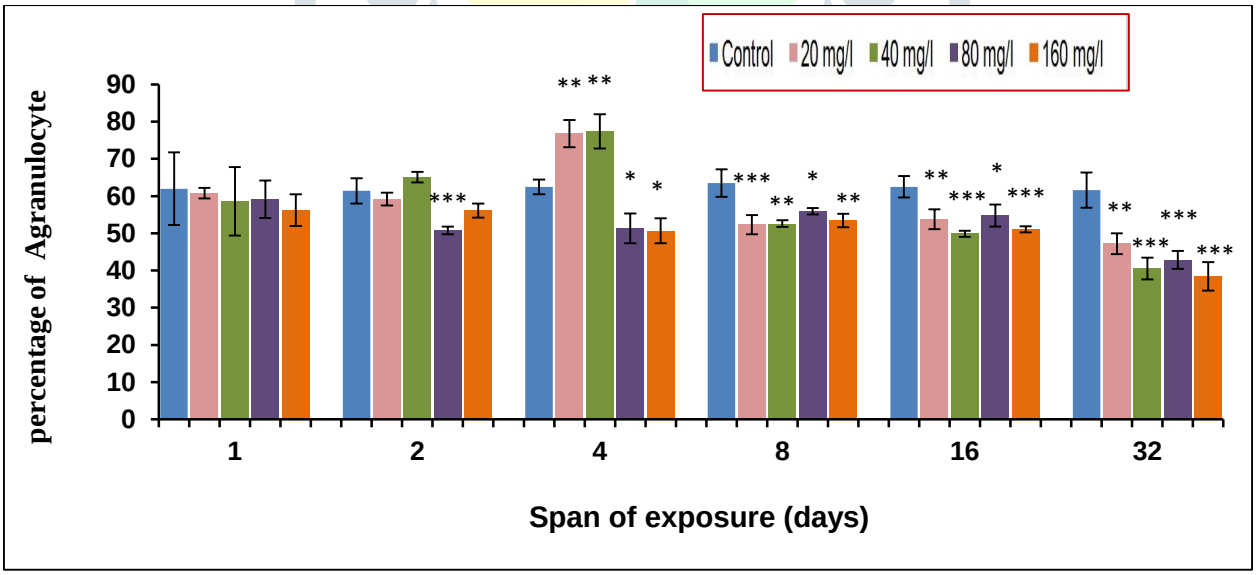

figure 3: frequency of agranulocytes of *l. marginalis* at 1 , 2 , 4 , 8 , 16 and 32 days of exposure to 20 , 40 , 80 , and 160 mg/l of washing soda. data represented is the mean ± standard deviation (n=10). the asterics (*) indicate the values that are significantly different (*p<0.05, **p< 0.01, ***p<0.001) from the control.

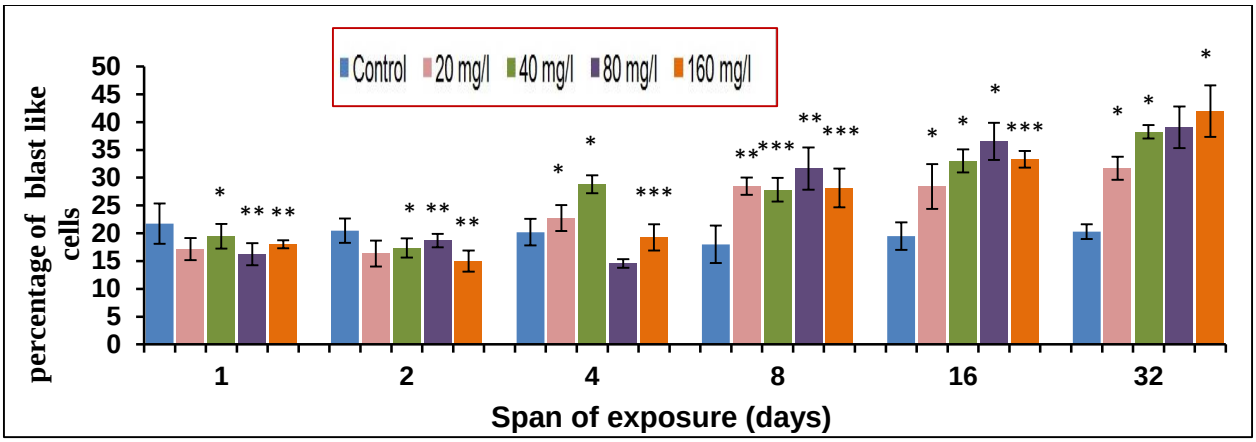

figure 4: frequency of blast-like cells of *l. marginalis* at 1 , 2 , 4 , 8 , 16 and 32 days of exposure to 20 , 40 , 80 , and 160 mg/l of washing soda. data represented is the mean ± standard deviation (n=10). the asterics (*) indicate the values that are significantly different (*p<0.05, **p< 0.01, ***p<0.001) from the control.

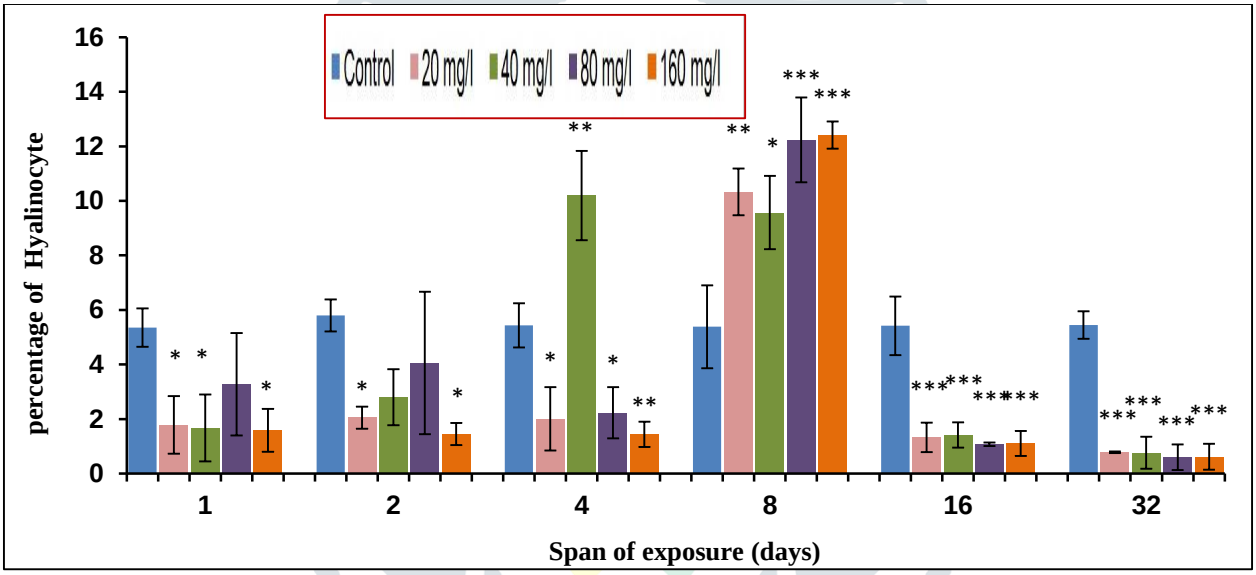

figure5: frequency of hyalinocytes of *l. marginalis* at 1 , 2 , 4 , 8 , 16 and 32 days of exposure to 20 , 40 , 80 , and 160 mg/l of washing soda. data represented is the mean ± standard deviation (n=10). the asterics (*) indicate the values that are significantly different (*p<0.05, **p< 0.01, ***p<0.001) from the control

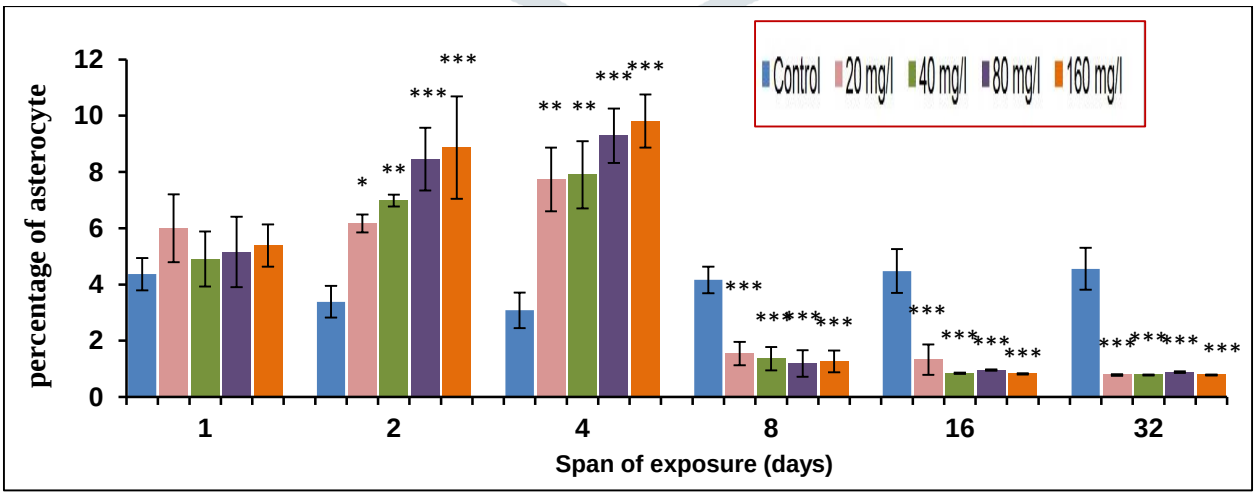

figure 6: frequency of astrocytes of *l. marginalis* at 1 , 2 , 4 , 8 , 16 and 32 days of exposure to 20 , 40 , 80 , and 160 mg/l of washing soda. data represented is the mean ± standard deviation (n=10). the asterics (*) indicate the values that are significantly different (*p<0.05, **p< 0.01, ***p<0.001) from the control.

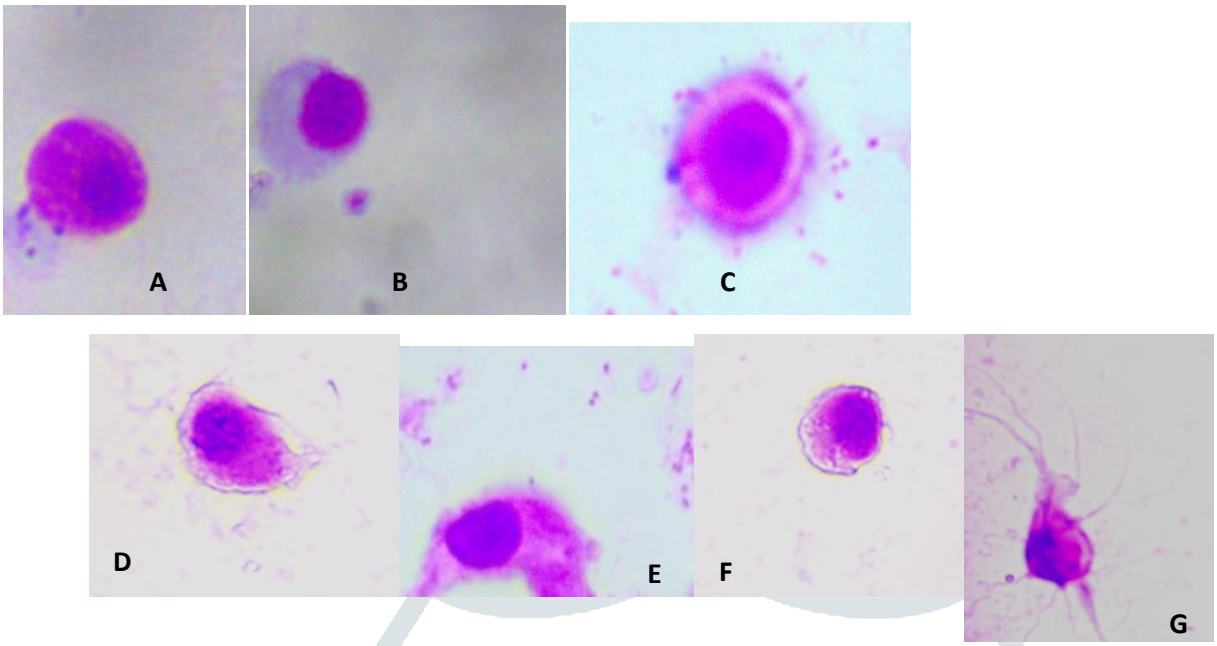

figure 7: bright field microscopic images of hemocyte subpopulations of *L. marginalis* stained with giemsa: A) round granulocyte; B) round hyalinocyte; C) blast like cell; D-E) spindle granulocytes; F) round semigranulocyte, G) asterocyte

3.3. Haemocyte morphology from SEM images:

The scanning electron micrograph (SEM) images of untreated control hemocytes of *L. marginalis* revealed the existence of longer membranous extensions (Figure: 4A) and membrane involutions (Figure:4B).On exposure to 160mg/l of washing soda for 32days exhibited corroded plasma membrane surface (Figure:4C) membrane blebbing (Figure: 4E) of hemocytes. On exposure to 40mg/l of washing soda for 16days the hemocytes of *L. marginalis* exhibited cytoplasmic smoothening (Figure: 4D) and relative reduction of membranous extensions compared to the untreated hemocytes.

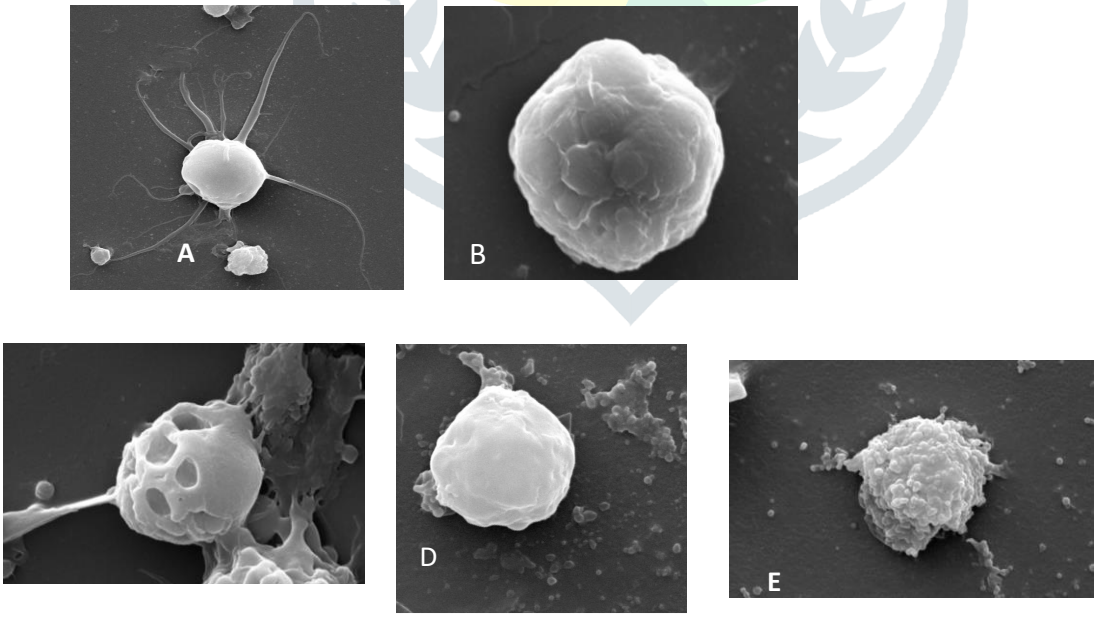

figure 8: scanning electron microscopic images of cells of *l. marginalis* treated with washing soda.scanning electron micrographs of control cells revealed the existence of membrane involutions (figure-8B) and multiple longer membrane projections (figure-8A). treatment of washing soda yielded increased corrosion of the plasma membrane(figure-8C),cytoplasmic smoothening relative reduction of the membrane projections(figure-8D) and membrane blebbing (mb) indicating the sign of apoptosis(figure-8E) in *l. marginalis* cells.

3.4 Morphological damages of hemocyte morphotypes in bright field microscopic images :

A large array of the morphotypes of hemocytes are circulating in the hemolymph of *L.marginalis* and were recognized as granulocytes, semigranulocytes and agranulocytes. Each of these morphotypes are comprised of several discrete submorphotypes like blast like cells, spindle and round hyalinocytes (agranulocytes), spindle and round granulocytes, granular asterocytes (granulocytes), round semigranulocytes and semigranular asterocytes (semigranulocytes). Morphology of the normal untreated hemocyte subpopulations of *L. marginalis* was reported by Ray et al. (2013). Exposure of *L. marginalis* to washing soda for a span of 16 and 32 days exhibited many morphological damages to the hemocytes, in respect to the control hemocytes observed through bright field microscope. Washing soda induced cellular damages involved change in cellular shape, lobed nucleus, hypervacuolation, membrane disintegration, nuclear disintegration, binucleation.

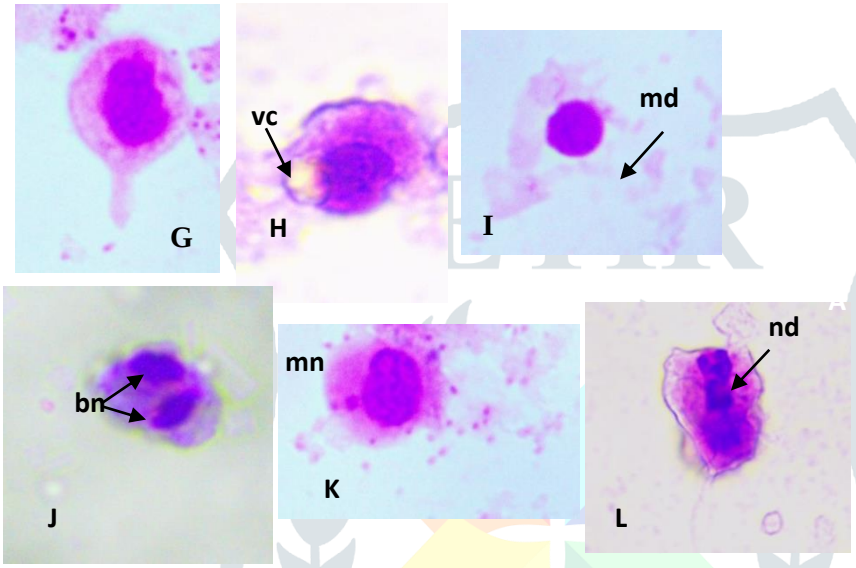

figure 9: bright field microscopic images of washing soda treated hemocyte subpopulations of *L. marginalis* stained with Giemsa: G) mb (membrane blebbing); H) vc (vacuolation); I) mb (membrane disintegration); J) bn (binucleation); K) mn (micronucleation); L) nd (nuclear disintegration).

4. Discussion :

Bivalve molluscs like *L. marginalis* are often identified as bioindicator organisms in invertebrate immunotoxicology. Bivalves have a worldwide distribution, enjoys a sedentary mode of life, and are prone to bioaccumulate environmental pollutants due to their filter-feeding behaviour, they are garnered as ideal species for the assessment of environmental pollution (Wade et al., 1998; Wootton et al., 2003). By studying the modulation of immune system in many freshwater and marine species of molluscs has become one of the emerging subject for evaluating the physiological responses of the animal to the different environmental factors and contaminants (Oubella and Auffret, 1997). Physiological responses of bivalve molluscs to various environmental contaminants and biological agents are mediated in part byhemocytes, circulating within the open vascular system (Cheng, 1981). Hemocytes are responsible for recognition, phagocytosis and elimination of any foreign particlesincludingbacteria, virus and parasites (Cheng, 1981). It has been well established in the bivalve molluscs, that hemocytes can be affected by environmental contaminants, stresses and (Lacoste et al., 2002) or pathogenic challenges (Anderson et al., 1994; Oubella et al., 1993).

Sodium carbonate is a white, crystalline amorphous powder which is used as a builder in detergent powders and for water softening processes during washing purposes. Solutions of sodium carbonate are being used for soaking of clothes, washing of utensils, bathing of cattles (HERA). Massive quantities of detergents and surfactants which are being used in various household cleaning and industrial processes, ultimately find their sink in different environmental compartments (Chaturvedi and Tiwari, 2013). In water sodium carbonate dissociates into sodium and carbonate ion and does not gets adsorbed to a significant level. When sodium carbonate gets discharged to aquatic environment, it tends to raise the alkalinity and pH value of water. Discharge of 100- 1000mg/l

of sodium carbonate into water bodies increases the pH value of water to 10-11, and these pH values have toxic effects on various aquatic organisms(HERA).

The internal defence mechanism of bivalve molluscs are based on innate non-lymphoid system, which involves both cellular and humoral components (Cheng; 1983 and Hine; 1999). The effectiveness and efficacies of bivalve hemocytes may be suppressed by various chemical contaminants, and xenobiotics dispersed in aquatic systems. Total hemocyte count (THC) have been reported (Chakraborty *et al*., 2012) as one of the important immune response of invertebrates to different stress factors. Total hemocyte count acts as a pivotal immuno marker of environmental stress (Mello et al., 2010). Our present study demonstrates the THC significantly altered (Akinrotimi, 2012) under the sublethal exposure of washing soda. Initial trend in the increase of hemocyte population is followed by a dose dependent decrease pattern. Decline in THC on prolonged washing soda exposure are suggestive to reduced mitotic activity in a dose and time dependent manner. Washing soda have an effect on the hematopoietic tissue thus the total count of hemocytes. Initial increase in hemocyte population facilitates the infiltration of hemocytes in affected tissues (Oliveira *et al*., 2010). Decline in the hemocyte population for the prolonged period of exposure leads to depression in the defence activity (Fisher et al., 1988).

The differential densities of cells is considered to be an important immunomarker of toxicity of environmental xenobiotics (Oliver and Fisher, 1999). The densities of different hemocytes function in a coordinated way to maintain blood homeostasis. The total and differential count of hemocytes are considered to be an effective cellular parameters correlated to the immunological responses during the period of active stress and disease resistance (Cima*et al*., 2000).Increase of hemocyte density in insect *Drosophila suzukii* had been reported as a state of immunostimulation (Kacsoh *et al*., 2012).Fluctuations in hemocyte density in mollusc *Ruditapes decussates* during host-pathogen interactions is a consequence of mobilization and migration of resident hemocytes from tissues towards the hemolymph Oubella *et al*. (1993).According to Ray et al., (2013) round granulocytes acts as principal immunoeffector cell in *L. marginalis*. Granulocyte performs major immune responses through phagocytosis (Donaghy et al., 2009) and by synthesizing and generating phenoloxidase, superoxides, peroxides (Aladaileh et al.,2007).In our present study washing soda mediated depletion of granulocyte populations implies a possible suppression of phagocytic and cytotoxic activities, which in turn makes the animal vulnerable to bacterial and pathogenic invasions. Potential phagocytic activities are manifested by agranulocytes, asterocytes and hyalinocytes which are efficient in engulfing foreign particles (Hine, 1999). In *Lamellidens marginalis* the chief phagocytic cells are reported to be asterocytes (Chakraborty *et al*., 2008).

Alterations in the morphology of invertebrate cells on exposure to environmental toxins reflects a pivotal role of cell-mediated immunity in the host organisms (Ray *et al*., 2015). The granulocyte of mollusc *Mytilus galloprovincialis* under sublethal concentration of cadmium chloride exhibited morphological alterations, which inflicted rounding up and cell enlargement due to loss of pseudopodia. (Calisi et al., 2009) reported pollutant inflicted alterations in the morphology of granulocytes of earthworm, *Eisenia foetida*. They investigated the correlation between the environmental toxicity of methiocarb and copper sulphate to the morphological damages of earthworm coelomocytes. Microscopical recording of blebbing of *L. marginalis* hemocyte membrane had been identified as a sign of cellular apoptosis (Sokolova et al., 2004) as recorded in other molluscs. Similar kind of membrane blebbing of sponge cells indicated the state of washing soda induced apoptosis in the cells of *E. carteri (*Mukherjee *et al*., 2015c*).* Scanning electron microscopic recording has exhibited extensive membrane involutions of the control hemocytes of *L. marginalis* can be correlated with the cells' phagocytic efficiency under normal physiological condition. Treatment of washing soda yielded a relative reduction of the surface involutions in *L. marginalis* hemocytes. This observation may be correlated with the washing soda induced inhibition in the phagocytic response of the cells of *L. marginalis.*This observation is in accordance with the washing soda induced inhibition in the phagocytic efficiency of the cells of *E. carteri* (Mukherjee et al., 2015b). Thus washing soda treatment resulted in cytoplasmic smoothening of hemocyte surface, as a result of reduction of membranous projections and increased the corrosion of plasma membrane.

The morphologically discrete multiple hemocyte subpopulations of *L. marginalis* like blast like cells, round granulocytes, spindle and round hyalinocytes, granular asterocytes as revealed through bright field microscopy reflects their specific immunological roles. Chakraborty and Ray (2009) reported aberrations in the morphology of hemocyte nuclei induced by arsenic. Exposure to experimental concentration of washing soda inflicted several morphological and nuclear damages to *L. marginalis* hemocytes like hypervacuolation, changes in cell shapes, binucleation, membrane disintegration, membrane blebbing, degranulation, lobulated nuclei.The appearance of membrane blebs during washing soda treatment implies possible cytoskeletal disruption. The various hemocyte morphotypes of *L. marginalis* exert their cytotoxic activities by generating phagocytosis mediated nitric oxide, super oxide anion. Washing soda induced alteration in phagocytic efficacy thus suggestive to possible suppression of cell mediated immune response of hemocytes.

Washing soda exposure is thus assumed to affect adversely the overall morphofunctional status of cells of *L. marginalis*. Sodium carbonate induced morphological alterations of the various populations of cells were suggestive to the possible impairment of physiological and immunological activities of cells of *L. marginalis* distributed in washing soda contaminated habitat.

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References

Adema, C. M., Van der Knaap, W. P. W. and Sminia, T. (1991a). Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. Rev. Aquatic Sci. 4, 201–223

Akinrotimi, O. A., Gabriel, U. U., Ariweriokuma, S. V. 2012. Haematotoxicity of cypermethrin to African *Catfish Clarias gariepinus* under laboratory conditions. *J. Environ. Eng. Tech.*, **1 (2):** 20- 25.

Allam, B., Paillard, C., Auffret, M. 2000. Alterations in hemolymph and extrapallial fluid parameters in the manila clam, Ruditapes philippinarum, challenged with the pathogen Vibrio tapetis. J. Invertebr. Pathol., 76: 63-69.

Anderson, R. S. 1994. Hemocyte derived reactive oxygen intermediate production in four bivalve mollusks. *Dev.Comp. Immunol*., **18:**89-96.

Auffret, M., Oubella, R. 1997. Hemocyte aggregation in the oyster Crassostrea gigas: In vitro measurement and experimental modulation by xenobiotics. Comp. Biochem. Physiol., 118A(3): 705-712.

Auffret, M., Mujdzic, N., Corporeau, C. Moraga, D. 2002. Xenobiotic-induced immunomodulation in the European flat oyster, *Ostrea edulis. Mar. Environ. Res*., **54**: 585-589.

Bajpai, D., Tyagi, V.K., 2007. Laundry detergents: an overview. J. Oleo Sci. 56 (7), 327–340.

Bayne, B.L., Widdows, J. 1978. The physiological ecology of two populations of Mytilus edulis *L. Oecologia* (Berlin). *Oceologia*, **37(2):** 137-162.

Brousseau, P., Payette, Y., Tryphonas, H., Blakley, B., Boermans, H., Flipo, D., Fournier, M., 1999. Manual of Immunological Methods. CRC Press, Boca Raton, FL. Butt, D.,

Chakraborty, S., Ray, M., Ray, S. 2008. Sodium Arsenite Induced Alteration of Hemocyte Density of *Lamellidens marginalis* – An Edible Mollusk from India. *Clean Soil Air Water*, **36 (2):**195 – 200.

Cheng, T. C. 1981. Bivalves. In: Invertebrate Blood Cells, N. A. Ratcliffe and A. F. Bowley, eds. Academic Press, New York. Pp. 233-300.

Cheng, T. C. 1996. Hemocytes: forms and functions. In: *The Eastern oyster Crassostrea virginica.* (Kennedy, V. S. and Newell, A.F. Ed.) Maryland sea grant college: College Park. pp. 236-299.

Chu, F. L. E. 1988. Humoral defense factors in marine bivalves. *American Fish. Soc. Bethl.*, **18:**178–88.

Cima, F., Matozzo, V., Maria, M. G. 2000. Hemocytes of the clam *Tapes philippinarum* (Adams and Reeve, 1850) : morphofunctional characterisation. Fish Shell fish Immunol., 10:677-693.

Donaghy,L., Hong, H. K., Lambert, C., Park, H. S., Shim,W. J., Choi, K. S. 2009. Flow cytometry studies on the populations and immune parameters of the hemocytes of the Suminoe oyster, *Crassostrea ariakensis*. Fish Shellfish Immunol., 27: 296-301.

HERA, 2005. Human and Environmental Risk Assessment on ingredients of household cleaning products – Sodium carbonate. Edition 2.0. April 2005.

Hine, P.M. 1999. The Interrelationships of Bivalve Hemocytes. *Fish Shellfish Immunol.*,**9**: 367.

Kacsoh, B. Z., Schlenke, T. A. 2012. High hemocyte load is associated with increased resistance against parasitoids in *Drosophila suzukii*, a relative of *D. melanogaster*. *PLoS One*, **7(4)**: e34721.

Mello, D.F., Proenc¸a, L.A., Barracco, M.A., 2010. Comparative study of various immune parameters in three bivalve species during a natural bloom of *Dinophysis acuminata* in Santa Catarina Island, Brazil. Toxins 2 (5), 1166–1178.

Mukherjee, S., Ray, M., Dutta, M.K., Acharya, A., Mukhopadhyay, S.K., Ray, S., 2015c. Morphological alteration, lysosomal membrane fragility and apoptosis of the cells of Indian freshwater sponge exposed to washing soda (sodium carbonate). Ecotoxicol. Environ. Saf. 122, 331–342.

Niyogi, S., Biswas, S., Sarkers, M. & Datta, A.G. 2001. Antioxidant enzymes in brackishwater oyster, *Sacostrea cucullata* as potential biomarkers of polyaromatic hydrocarbon pollution in Hoogly Estuary (India): Seasionality and its consequences. *Sci. Total Environ, 281*, 237-246.

Oliveira, A. L., Levada, P. M., Zanotti-Magalhaes, E. M., Magalhães, L. A., Ribeiro-Paes, J. T. 2010. Differences in the number of hemocytes in the snail host *Biomphalaria tenagophila*, resistant and susceptible to *Schistosoma mansoni* infection. Genet. Mol. Res., 9:2436–2445.

Prabhakar, A. K., Roy, S. P. 2009. Ethno-medical uses of some shell fishes by people of Kosi river basin of North-Bihar, India. *Ethno. Med.*, **3(1)**: 1-4.

Raut, S. K. 1991. Laboratory rearing of Medically and Environmentally Important Molluscks.In: *snails, Flukes and Man.* (Jairajpuri, M. S. Ed.) Zoolocial Survey of India Publication. pp. 79-83.

Ray, S., Ray, M., Chakraborty, S. Mukherjee, S. 2011. Immunotoxicity of environmental chemicals in the pearl forming mussels of India – a review, In: McGevin, L.E. (Eds.), Mussels: anatomy, habitat and environmental impact. Nova Science Publishers Inc., USA, pp. 429–440.

Ray, M., Bhunia, A. S., Bhunia, N. S., Ray, S. 2013b. Density shift, morphological damage, lysosomal fragility and apoptosis of hemocytes of Indian molluscs exposed to pyrethroid pesticides. *Fish Shellfish Immunol*.,**35:**499-512.

Ray, S., Mukherjee, S., Bhunia, N. S., Bhunia, A. S., Ray, M. 2015. Immunotoxicological threats of pollutants in aquatic invertebrates. *Intech*, DOI: 10.5772/60216.

Singaram, G., Harikrishnan, T., Chen, F., Bo, J., Giesy, J.P., 2013. Modulation of immune associated parameters and antioxidant responses in the crab (*Scylla serrata*) exposedto mercury. Chemosphere 90, 917–928.

Sokolova, I.M., Evans, S., Hughes, F.M., 2004. Cadmium induced apoptosis in oyster hemocytes involves disturbance of cellular energy balance but no mitochondrial permeability transition. *J. Exp. Biol*. **207**, 3369–3380.

Feng, S. Y.1988. Cellular defense mechanisms of oysters and mussels. Am.Fish. Soc. Spec. Publ. **18:**153–168.

Fournier, M., Cyr, D., Blackley, B., Boermans, H., Brousseau, P. 2000. Phagocytosis as a biomarker of immunotoxicity in wild life species exposed to environmental xenobiotics. *Am. Zool.*, **40**: 412-420.

Koprucu, K., Yonar. S. M., Seker, E. 2010. Effects of cypermethrin on antioxidant status, oxidative stress biomarkers, behaviour, and mortality in the freshwater mussel *Unioelongatulus eucirrus*.*Fisheries Sci.*, **76**:1007-1013.

Lacoste, A., Malham, S.K., Gelebart, F., Cueff, A., Poulet, S. A. 2002. Stress-induced changes in the oyster *Crassostrea gigas*. *Dev. Comp. Immunol.*, **26**: 1-9.

Lopez, C., Carballal, M. J., Azevedo, C., Villalba, A. 1997a. Differential phagocytic ability of the circulating haemocyte types of the carpet shell clam *Ruditapes decussatus* (Mollusca: Bivalvia). *Dis. Aquat. Org.*, **30**: 209- 215.

Mukherjee, S., Ray,M. and Ray,S. 2006. Azadirachtin induced modulation of total count of hemocytes of an edible bivalve *Lamellidens marginalis.P.Zool.Soc.Calcutta*, **59(2)**: 203-207.

Oliveira, A. L., Levada, P. M., Zanotti-Magalhaes, E. M., Magalhães, L. A., Ribeiro-Paes, J. T. 2010. Differences in the number of hemocytes in the snail host *Biomphalaria tenagophila*, resistant and susceptible to *Schistosoma mansoni* infection. Genet. Mol. Res., 9:2436–2445.

Pampanin, D. M., Marin, M. G., Ballarin, L. 2002. Morphological and cytozymatic characterization of hemocytes of the venus clam *Chamelea gallina*., *Dis. Aquat. Org.*, **49**: 227 – 234.

Warne, M.S.J., Schifko, A.D., 1999. Toxicity of laundry detergent components to a freshwater cladoceran and their contribution to detergent toxicity. Ecotoxicol. Environ. Saf.44, 196–206.

Wootton, E. C., R. K. Pipe. 2003. Structural and functional characterisation of the blood cells of the bivalve mollusc, *Scrobicularia plana*. *Fish Shellfish Immunol.*, **15**: 249-262 .