SHIFT IN CYTOARCHITECTURE OF IMMUNOCYTES OF MUDCRAB EXPOSED TO ARSENIC

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ABSTRACT: Estuarine mudcrab, *Scylla serrata* is an economically important species and inhabitant of Sundarban ecosystem. This intertidal species is under constant exposure of diverse xenobiotics including toxic metalloid arsenic. Arsenic, being a major environmental contaminant of selected districts of the state of West Bengal poses a serious toxicological threat to this edible species. Toxicity of arsenic was investigated in the cytoarchitecture of hemocyte of *S. serrata* by exposing the animals to the sublethal concentrations of 1, 2 and 3 ppm sodium arsenite for a maximum period of 30 days in controlled laboratory conditions. Arsenic induced hemocytic disruption in relation to shape, size of cell and nuclear morphology is indicative to possible dysfunction of blood cell. Blood cells or hemocytes are reported as chief immunoeffector cells of invertebrates and are capable of performing multiple immunological functions including nonself adhesion, aggregation, phagocytosis and generation of cytotoxic agents. The toxic exposure may impaired or altered the innate immune response of hemocyte of the animal that may lead to decline of this economically important species in Sundarbans Biosphere Reserve. This study would help to be selected *S. serrata* as bioindicator species and also formulate sustainable strategy of conservation of this important species inhabiting the biologically unsafe habitat of Sundarbans.

Keywords: *Scylla serrata*, Sodium arsenite, Hemocyte, Cytoarchitecture, Bioindicator.

INTRODUCTION

S. serrata (Arthropoda: Crustacea: Decapoda) is an important member of the estuarine ecosystem of Sundarbans Biosphere Reserve of West Bengal, India. Due to its high economical and nutritive value, the species is of great demand in the local and export market. *S. serrata* is indiscriminately harvested from its natural habitat and selected areas of the estuarine for human consumption. They are widely distributed in the intertidal mudflat of Sundarbans and feed on the flesh of mussel, fish and other crustacean species (Ali *et al.* 2004; Saha and Ray, 2006). They are specially adapted to a wide range of salinity and tidal fluctuation. *S. serrata* constitutes a large population of crab in Sundarbans mangrove swamp (Chaudhuri and Choudhury, 1994). Mudcrab culture has evolved from low-density pond polyculture with fish using wild seeds. Crab is a nutritious and indigenous palatable food item of our country. The crabmeat is rich in calcium, magnesium, phosphorus, iron and copper. The protein content of crab meat is 18-20 %. Gravid female mud crabs with their orange-red egg attached to the carapace are of great demand as seafood. *S. serrata* is also commonly known as green or mangrove crab which constitutes an important aquacrop for its size, meat quality and high export potential. Presently in India, the mudcrabs have come into prominence and crab meat, cut crab and live crabs are being exported to foreign countries like, Singapore, Hong Kong, Japan, USA, France etc. Natural habitat and growing pond of crabs also bears the risk environmental threat due to exposure of environmental contaminant including arsenic.

Arsenic is an important toxic metalloid which is potentially capable of creating physiological stress both at cellular and sub-cellular levels of invertebrates. Arsenic is a contaminant not only to the ground water but to the surface water of selected plains of the state of West Bengal too. Arsenic originates from chemical weathering of natural rocks (Das and Roy Chowdhury, 2006) and selected anthropogenic activities. Arsenic is the main constituent of more than 200 mineral species, of which about 60% are arsenate, 20% are sulfide and sulfosalts and the remaining 20% includes arsenides, arsenites, oxides and elemental arsenic (Onishi, 1969). The most common of the arsenic mineral is arsenopyrite which is found to be associated with many types of mineral deposits (Boyle and Jonasson, 1973). Concentrations of arsenic in open ocean water are typically 1-2 µg/liter. The concentrations of arsenic in unpolluted surface water and groundwater are typically in the range of 1-10 µg/liter. Elevated concentrations in surface water and ground water of up to 100 – 5000 µg / liter can be found in areas of sulfide mineralization. Microbial oxidation of organic carbon depleted the dissolved oxygen in the ground water. The highly reducing nature of the ground water explains the presence of arsenite (< 50 %) in the water. The "Pyrite oxidation" hypothesis is to be a major process and the "Oxyhydroxide reduction" hypothesis (Acharyya *et al.*, 1999) is probably the main cause of arsenic contamination in ground water. Preservation of timber, purifying industrial gases (removal of sulfur), electronics manufacturing (microwave devices, lasers, light-emitting bodies, photoelectric cells and semiconductor devices), mining, smelting of non-ferrous metals and burning of fossil fuels are the major industrial processes that contribute to anthropogenic arsenic concentration of air, water and soil (Das and Roy Chowdhury, 2006). Many of the districts of the state of West Bengal are under the potential threat of arsenic toxicity (Das *et al*., 1995; Acharyya, 2002).

In nature, arsenic-bearing minerals undergo oxidation and release arsenic to water. This could be one explanation for the problems of arsenic in ground water of West Bengal. The excessive withdrawal and lowering of the water table for rice irrigation and other requirements lead to the exposure and subsequent oxidation of arsenic-containing pyrite in the sediment. As the water table recharged after rainfall, arsenic leaches out of the sediment into the aquifer (Gomez- Caminero, 2001). In West Bengal region almost 6 million people in 9 of 18 districts are endemically exposed to arsenic. It was estimated that over 1 million people consume drinking water containing up to 3.2 mg/liter arising from normal geochemical processes (Das and Roy Chowdhury, 2006).

Invertebrates including crustaceans lack adaptive immunity and mostly rely on innate immune defences (Galloway and Depledge, 2001). They have complex and efficient host defence systems that can identify and eliminate potential pathogens efficiently. According to Holmblad and Soderhall (1999), innate immune response includes the activities of external physico-chemical barrier of hard cuticle, mucous layer, hemolymph and hemocytes. In Crustacea, hemocytes are considered as the immunocytes which are capable of mounting immunological response through aggregation, adhesion, phagocytosis, generation of cytotoxic agents and production of antioxidant enzymes in biounsafe aquatic environment (Factor, 1995; Johansson *et al.*, 2000; Iwanaga and Lee, 2005). Studies on cell function, cytochemistry and ultrastructure suggest that there are two major types of hemocytes are present in the decapods. Hyaline hemocytes have a high nucleo-cytoplasmic ratio without or scanty electron dense granules. Granulocytes, on the other hand, have a low nucleo-cytoplasmic ratio. In most species, granulocytes may be subdivided into small and large granule hemocytes (Hose *et al*., 1990). Hyalinocytes have primary role in clotting and phagocytosis; semigranulocytes are involved in encapsulation, phagocytosis, cytotoxicity, storage and release of the proPO system. Granulocytes are involved in cytotoxicity, storage and release of the proPO system (Johansson *et al.*, 2000).

Saha and Ray (2006) already identified three types of hemocytes namely hyalinocyte, semigranulocyte and granulocyte in the mudcrab, *S. serrata* on the basis of cytoplasmic granulation. Arsenic induced alteration of hemocyte density and its restoration in mudcrab is in report by Saha *et al.* (2009a). Phagocytosis is a classical innate immune response in invertebrates by which cells engulf smaller particulates of foreign origin (Saha *et al.*, 2008). Saha *et al*. (2009b) reported arsenic induced dose dependent decrease in the activity of the alkaline phosphatase and proposed the parameter as a possible biomarker of aquatic pollution in Sundarbans mangrove. Saha *et al.* (2009c) raised antisera in rabbit against arsenic exposed crab hemocyte and murine lymphocyte and reported high titer of hemocyte agglutination. A shift in agglutination response of arsenic exposed hemocytes was indicative of arsenic induced possible alteration of hemocyte surface characters and reactivity. Saha *et al*. (2010) estimated the phagocytic response and intrahemocytotoxicity of *S. serrata* under the exposure of sodium arsenite. In this present study, we studied cellular as well as nuclear anomalies against sublethal concentrations of sodium arsenite exposure in the hemocyte of *S. serrata*. These cellular responses can be used as early warning signals of environmental arsenic contamination before whole organism become extinct. Moreover the investigation would evaluate the credibility of *S. serrata* to be chosen as a model species for bioindicator for surface water arsenic toxicity.

2. Materials and Methods

2.1. Collection, transportation, acclimation and maintenance of *S. serrata*

Live intermolt adult specimens of *S. serrata* weighing 65-75 gm were collected throughout the year manually from selected habitats of the district of south 24 Parganas of West Bengal, India which are free from arsenic contamination. Length and breadth of the carapace of experimental animals were 6 ± 0.5 cm and 9 ± 0.5 cm respectively. The crabs were collected alive and transported immediately to the Aquatic Toxicology Laboratory of department of Zoology of University of Calcutta in moist jute bags. In the laboratory, live crabs were maintained in large rectangular glass aquaria containing simulated marine salt water in controlled laboratory condition under constant aeration (Ali *et al.*, 2004; Heasman and Fielder, 1983). They were fed with fresh flesh of prawn and aquatic molluscs once a day. The water of glass aquaria was routinely replenished in every 24 hours to avoid residual toxicity. Animals were acclimated in laboratory condition for 6-8 days prior to experimentation. Principal water quality parameters were kept constant. Temperature and acidity were monitored routinely and dissolved oxygen, hardness and salinity were estimated after Barnes (1954). A photoperiod of 12 hr light and 12 hr darkness was provided. Tidal conditions were simulated in laboratory by increasing and decreasing the water level in every 12 hours of duration.

2.2. Treatment with sodium arsenite

After acclimation to the laboratory conditions, acute toxicity study was carried out to determine the lethal (LC_{100}), median lethal (LC_{50}) and safe sublethal (LC_0) concentrations of sodium arsenite in *S. serrata*. Determination of LC_{50} was carried out by 'Behrens – Karber method' after Klassen (1991) whereas LC_0 and LC_{100} were determined after Hart *et al.* (1948) and Shibu Vardhannan and Radhakrishnan (2002) respectively. The crabs were divided into multiple groups and each group contained 10 acclimated crabs of same age and sex in triplicate which were introduced in the glass aquaria containing 10 liters of water. From the stock solution, desired concentrations of sodium arsenite were prepared for determining the toxicity of *S. serrata*. During acclimation and treatment, crabs were routinely checked for morbidity, mortality and dead crabs were instantly discarded to avoid additional aquatic toxicity (Saha *et al*, 2008 and 2010).

In static water environment, median lethal concentration (LC_{50}) of sodium arsenite is determined as 15 ppm and 10 ppm in adult and juvenile crabs for 96 hours of span respectively. In this present study, all the experimental concentrations of sodium arsenite were selected to be less than 50% of LC_{50} value. According to Das and Roy Chowdhury (2006), concentration of arsenic in aquatic ecosystem of West Bengal ranges from 0.5 – 3.2 ppm in different seasons. Dynamics of concentration of dissolved arsenic is resulted due to several environmental factors i.e. desiccation, dilution and natural availability. All the experimental concentrations of sodium arsenite were less than the highest reported natural concentration of arsenic.

For *in vivo* treatment, crabs were exposed to desired concentrations i.e. 1, 2 and 3 ppm of sodium arsenite (E. Merck, Germany; 99% pure; CAS number 7784-46-5) in marine salt water taken in borosilicate glass containers. During treatment, mouths of test containers were partially covered by glass lids to prevent atmospheric desiccation.

2.3. Collection of hemolymph

Hemolymph of *S. serrata* was collected aseptically from the base of one of the second walking legs using a sterile syringe fitted with a 23-gauge needle in 2 ml. of ice-cold citrate EDTA buffer (0.45 M NaCl; 0.1 M glucose; 30 mM trisodium citrate; 20 mM citric acid; 100 mM EDTA, pH 4.6) as anticoagulant that arrests instant melanization (Bell and Smith, 1994). The volume of the collected blood never exceeded the 2 ml. of volume at a time (Yildiz and Atar, 2002; Saha and Ray, 2006). Collected hemolymph was stored in prechilled sterile glass vials and routinely examined under microscope (Axiostar Zeiss, Germany) by smearing on glass slide. Hemocytes were sedimented by cold centrifugation (Hermle, Germany) of the hemolymph at 3000 rpm for 5 minutes and cell density was adjusted with unit volume of sterile phosphate buffered saline (PBS) as and when necessary. Free and live hemocytes in suspension were observed and screened under inverted microscope (Axiovert, Zeiss, Germany).

2.4. Testing of hemocyte viability

Cell viability was tested by staining the cell with vital dye trypan blue (SRL, India) following the principle of dye exclusion. For this experiment, 100 µl of freshly collected hemolymph was mixed on a glass slide with 20 µl of a 1.2% solution of trypan blue (Hose *et al.*, 1990). The total number of blue stained nuclei and colourless cells were determined microscopically (Sauve *et al.*, 2002).

2.5. Light microscopy

Freshly fixed hemocytes were examined under light microscope for analysis of micrometry and morphology. Collected hemocytes were subjected to cytofixation by addition of 2.5% glutaraldehyde (SRL, India) in a ratio of 1:1 (v/v) (Hose *et al.*, 1990). Fixed hemocytes were stained with Wright's stain (SRL, India) for 5 – 10 minutes and were examined under light microscope ((Axiostar Zeiss, Germany) after proper cytopreparation. Live and unstained hemocytes were instantly examined under phase contrast microscope (Axiovert, Zeiss, Germany). Cytoimages were digitally recorded by camera (Prog Res C_5, Germany) attached with microscope (Axiostar Zeiss, Germany).

2.6. Micrometry of hemocytes under the sodium arsenite exposure

Live and stained hemocytes of *S. serrata* were undergone micrometry by calibrated occular micrometer (Erma, Japan). Micrometric analyses were carried out for at least five times with the samples of each experiment.

3. Results

Median lethal concentration of sodium arsenite in adult and juvenile were estimated against different spans of exposure i.e. 24, 48, 72 and 96 hours in static water environment (Figure 1). In adult crab, the LC_{50} values of sodium arsenite against 24 and 96 hours of span were determined as 100 ppm and 15 ppm respectively (Table I). Safe concentration and safe application rate of sodium arsenite in adult and juvenile *S. serrata* were determined as 0.8, 12.18 and 0.6, 6.6 ppm respectively in static water environment (Table I). The average viability of hemocytes ranged between 97.91% and 95.15 % under the experimental exposure of 1 and 3 ppm sodium arsenite respectively.

Figure: 1. Acclimation of *S. serrata* in controlled laboratory condition

Table: I. Median lethal concentration, safe concentration and safe application rate of sodium arsenite in *S. serrata*.

Span of exposure (h)	LC_{50} (ppm)		Safe conc. (ppm)		Safe application rate (ppm)	
	Adult (6X9±0.5 cm)	Juvenile (2.5X3.5±0.5 cm)	Adult (6X9±0.5 cm)	Juvenile (2.5X3.5±0.5 cm)	Adult (6X9±0.5 cm)	Juvenile (2.5X3.5±0.5 cm)
24	100	60	0.8	0.6	12.18	6.6
48	30	20				
72	20	15				
96	15	10				

Hemolymph of *S. serrata* exhibits three discrete types of hemocyte subpopulations i.e. hyalinocyte, semigranulocyte and granulocyte with cell diameter as 18.05 ± 0.5 x 6 ± 0.5 µm, 12.05 ± 0.5 x 12 ± 0.5 µm and 14.05 ± 0.5 x 14 ± 0.5 µm respectively (Figure 3, Table II). The micrometric values of hyalinocyte, semigranulocyte and granulocyte under 3ppm arsenic exposure were recorded as 20 ± 0.5 x 8 ± 0.5 µm, 16 ± 0.5 x 16 ± 0.5 µm and 16 ± 0.5 x 15 ± 0.5 µm respectively (Figure 3, Table II) at largest axis or length of the cell. Hyalinocytes are spindle shaped cells occasionally characterized by one or two tail extensions discernable under light microscopy and phase optics (Figure 2 and 3).

Table: II. Cytometry of hemocyte subpopulations of *S. serrata*.

Hemocyte subpopulations	µm (length X width)	
	Control	Treated
Hyalinocyte	18 ± 0.5 X 6 ± 0.5	20 ± 0.5 X 8 ± 0.5
Semigranulocyte	12 ± 0.5 X 12 ± 0.5	16 ± 0.5 X 16 ± 0.5
Granulocyte	14 ± 0.5 X 14 ± 0.5	16 ± 0.5 X 15 ± 0.5

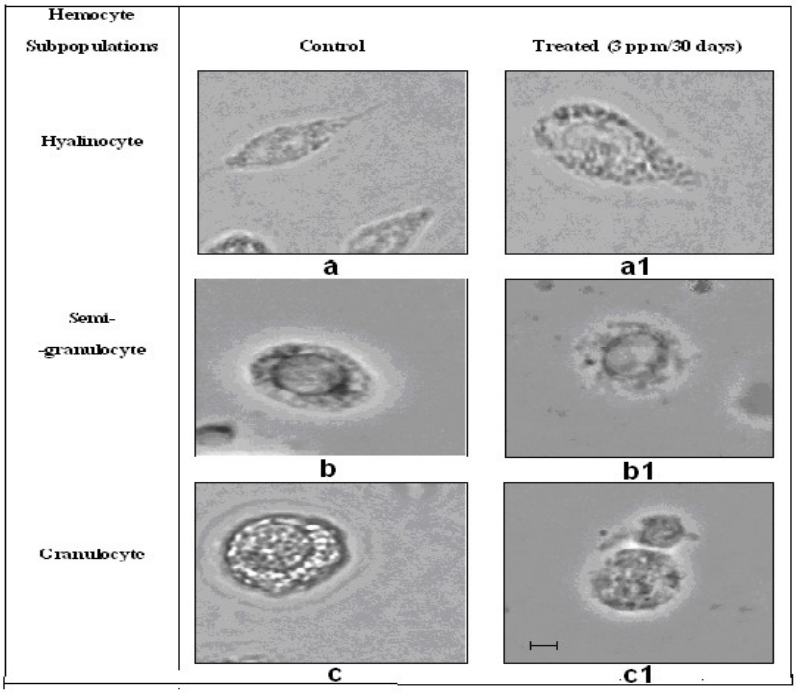

Figure: 2 (a, a1, b, b1, c, c1). Phase contrast image of hemocyte subpopulations. (a) and (a1) spindle shaped hyalinocytes; (b) and (b1) oval semigranulocytes; (c) and (c1) round granulocytes. Scale bar = 10 µm.

Nucleus is round or ovoid in shape and cytoplasm is well differentiated under stained condition (Figure 2 and 3). Semigranulocytes are round shaped in appearance with a large centrally placed nucleus and a thin rim of cytoplasm encircling the nuclei is characteristic to this cell type (Figure 2 and 3). Granulocytes are round shaped cells with large nucleus and the cytoplasm bears rounded granules (Figure 2 and 3). Treatment with 3 ppm of arsenite for 30 days resulted a swollen appearance of hyalinocyte (Figure 2 and 3). The micrometric values of hyalinocyte, semigranulocyte and granulocyte under 3ppm of arsenic were recorded as 20 ± 0.5 x 8 ± 0.5 µm, 16 ± 0.5 x 16 ± 0.5 µm and 16 ± 0.5 x 15 ± 0.5 µm respectively (Table II) at the largest axis or length of the cell.

Table: III. Percentage occurrence of nuclear anomalies of hemocytes of *S. serrata* exposed to 3 ppm sodium arsenite / 30 days.

Nuclear anomalies	Control (%)	Treated (3 ppm/ 30 days) (%)
Binucleation	0.6	15
Smudged cell	0.3	6
Karyorrhexis	0.2	5
Karyolysis	0.2	6
Nuclear fragmentation	0.2	3
Total anomalies	1.5	35

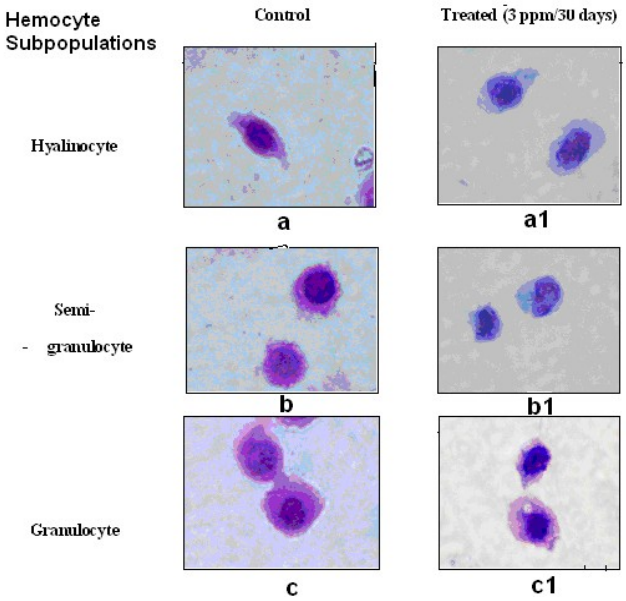

Figure: 3 (a, a1, b, b1, c, c1). Light micrograph of hemocytes of *S. serrata* stained with H & E. (a) and (a1) spindle shaped hyalinocytes; (b) and (b1) oval semigranulocytes; (c) and (c1) round granulocytes. Scale bar = 10 µm.

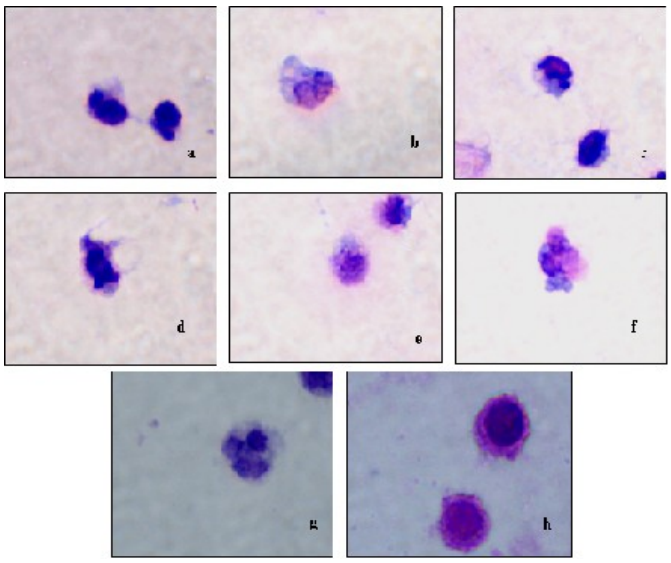

Figure: 4 (a, b, c, d, e, f, g, h). Morphological variations of hemocytes nuclei under sodium arsenite exposure (3 ppm/30 days): (a) and (b) binucleation, (c) smudged cell, (d) karyorrhexis, (e) and (f) karyolysis (g) nuclear fragmentation (h) normal hemocytes. H&E; Scale bar = 10 µm.

Exposure of 3 ppm of sodium arsenite resulted an alteration in morphology of hemocyte nuclei. Treatment of sodium arsenite (3 ppm / 30 days) resulted in diverse nuclear anomalies like binucleation (15%), smudged cell (6%), karyorrhexis (5%), karyolysis (6%) and nuclear fragmentation (3%) (Figure 4, Table III). A total of 35% of hemocytes exhibited morphological aberrations of nuclei against a control value of 1.5% (Table IV).

4. Discussion

Sundarbans Biosphere Reserve is a unique ecosystem and characteristic of diverse types of habitats ranging from mudflat to sandy beaches. Estuaries play an important role in maintenance of the aquatic life by providing the necessary supplies for their growth and survival. Tidal submergence and periodic inflow of nutrients make this ecosystem dynamic in relation to physiological status of the animals. Generally, Sundarbans estuary plays as a link between estuarine and marine ecosystems and the estuarine ecosystem receives diverse environmental toxins of known and unknown chemistry. These toxins are transported through water and silt and creates ecological crisis (Sarkar *et al.*, 2002 and 2004). Arsenic, a toxic metalloid of the environment, bears the potentiality to affect the normal physiology of crustaceans (Vijayavel and Balasubramanian, 2006). Mudflats are the natural habitat of mudcrab including *S. serrata*, an important inhabitant of this ecosystem (Naskar and Ghosh, 1989; Chaudhuri and Choudhury, 1994; Ali *et al.* 2004).

Arsenic is a naturally occurring metalloid toxin distributed widely in ground and surface water and get concentrated in rivers and lakes upto a concentration of 5 ppm due to summer desiccation (Galloway and Depledge, 2001; Gomez – Caminero, 2001).Trivalent arsenite is more mobile and stable than pentavalent arsenate especially at pH greater than 7 and the toxicity of arsenate is linked with reduction of arsenate to arsenite (Panthi *et al.*, 2006). During summer, the arsenate is chemically reduced to a highly toxic arsenite due to arsenite's ability to react with sulfhydryl groups of the compound present in the mud-water interface (Chakrabarti *et al.*, 2008). It was found that a large amount of these arsenic residues have been accumulated in the aquatic organisms namely fish and crustaceans upto a concentration of 100 µg / g dry weight (Gomez – Caminero, 2001). Marine algae are capable of accumulating arsenate from sea water and reduce arsenate to arsenite and oxidize the arsenite to a large number of organoarsenic compounds. These compounds are bioaccumulated in the seafood products i.e. crabs, shellfish, prawn etc (Neff, 1997). As a potent sulphydryl-reactive compound, arsenic has been reported to affect various cellular functions. No suitable biomarker of arsenic toxicity is available in Crustacea. Currently, the concentration of arsenic in blood or urine is considered for determination of the tissue burden of arsenic in human (Yoon *et al.*, 2008). Mudcrabs are immunologically protected against foreign invaders by internal defence system (Vijayavel *et al.*, 2005). Report on toxicity of arsenic in relation to hemocyte morphology is absent in current literature with reference to Indian mudcrab, *S. serrata* of Sundarbans delta.

In static water environment, median lethal concentration for 96 hours in adult and juvenile *S. serrata* was determined as 15 and 10 ppm respectively. In this present study, the highest experimental concentration (i.e. 3ppm) was selected as one fifth of the 96 hours of LC_{50} value of adult whereas it was less than one third of 96 hours of LC_{50} of juveniles (Table I). The highest experimental concentration was lower than the reported safe application rate for adult and juvenile. The morphological characterization and classification of different types of hemocytes in crustaceans was achieved through phase contrast microscopy, light microscopy (Saha and Ray, 2006; Saha *et al.*, 2009). The common criterion considered was the presence or absence of refractile granules of hemocytes (Soderhall and Smith, 1983; Hose *et al.*, 1990) as well as their size. For crustacean blood cells, most scientists follow the classification of Bauchau (1981), who classified hemocytes as three main categories i.e. hyalinocyte, semigranulocyte and granulocyte which supported our observation. Exposure of sodium arsenite resulted disintegration of cell membrane in hyalinocyte as evident from phase optics, light microscopy analyses (Figure 2 and 3). Semigranulocyte and granulocyte exhibited the arsenic induced sign of cellular degradation and damage as evident from phase optics analysis, light microscopy and transmission electron microscopy (Figure 2 and 3). Morphological analyses suggest an irrepairable destruction of normal morphology of hemocyte.

Experimental observation is suggestive of possible breakdown of homeostasis with reference to the physiology of blood of mudcrab. Shift in the morphology of nuclei of hemocytes were investigated under the exposure of sodium arsenite.

Alteration of size and shape of cellular nuclei were identified as binucleation, smudged appearance, karyorrhexis, karyolysis and nuclear fragmentation which were studied under light microscope (Figure 4 and Table III). Exposure to arsenic resulted a severe insult over the morphology of hemocyte nuclei. Data indicates a persistent level of genotoxicity of sodium arsenite in crab which may lead to a precarious physiological state of the animal in its contaminated habitat. In many of the cases, genotoxicity lay parallel with carcinogenicity of a compound leading to a serious damage of chromosome and nucleus (Dixon *et al.*, 2002). Significant increase of morphological distortion of nuclei of hemocyte indicates a possible state of severe genotoxicity in mudcrab.

Sundarbans biosphere reserve is an ecologically sensitive geographical zone and natural habitat of diverse flora and fauna. Chronic exposure of arsenic might have a long term toxic effect in mudcrab in relation to their physiology and immunity in their natural habitat. Result indicates an alteration and impairment of physiological, biological, immunological and behavioural activity and dysfunction of tissues like hemocytes. Sublethal toxicity of arsenic is reported to affect the mudcrab population by reducing its environmental fitness thus increasing its vulnerability to higher degree of disease, parasitamia and predation. Aquatic ecosystem of Sundarbans supports a wide range of biodiversity which is under the threat of environmental contamination including arsenic. Additionally, present study is also aimed to establish an effective bioindicator by which the health of the aquatic ecosystem of Sundarbans can rapidly and accurately be screened to protect its important bioresource. From present investigation and generated data, we suggest to adopt necessary measure to minimize the degree of aquatic contamination for protection of various economically and ecologically important species including mudcrab of Sundarbans Biosphere Reserve.

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