Discussion
6. Discussion

Waterbodies of India exhibit a unique network of ecological fabric with various animals belonging to different taxa reside in their respective niches. The freshwater ecosystem supports a wide variety of biodiversity including the specific members of the Phylum Mollusca. Mollusca is the second largest invertebrate Phylum after Arthropoda, includes varied forms of shelled and shellless organisms including snails, clams, scallops, oysters, squids, octopus etc. B. bengalensis (Figure 2C, D) is an important freshwater gastropod distributed in the littoral zones of ponds, tanks, ‘beels’, reservoirs and rivers in the various districts of the states of Arunachal Pradesh, Bihar, Orissa, Jharkhand and West Bengal (Subba Rao and Dey, 1991) of India. It is a traditional dietary item for the tribal and nontribal populations of eastern India (Subba Rao and Dey, 1991). Baby et al. (2010) reported that the flesh of B. bengalensis contained 8.966 ± 0.26% of proteins, 0.984 ± 0.02% of fats, 4.308 ± 0.17% of carbohydrates, and the source of different minerals and vitamins. Flesh of B. bengalensis is widely used as a supplementary item of the artificial feed of prawns, fishes and fowls (Zaman et al., 2003). B. bengalensis has been widely used as a curative agent of various human diseases like asthma, arthritis, joint pain, rheumatism and conjunctivitis among the tribal and rural population in India (Roy and Singh; 2007). According to Negi and Palyal (2007), molluscs including B. bengalensis are being consumed by tribal populations of India for the purposes of promotion of strength and remedy for various blood borne diseases. However, a thorough ethnomedicinal characterization of the organs and tissues of B. bengalensis is yet to be carried out till date.

B. bengalensis evolved a well developed gill (Figure 66) which is functionally involved in the processes of aquatic respiration, filter feeding and immunosurveillance. By dynamic mode of filtration of the ambient water column, B. bengalensis largely influence the distribution of nutrients and minerals for other aquatic organisms. Through filter feeding, the benthic organisms like B. bengalensis biologically accumulate various environmental toxins within their tissues and maintains the steady health of the aquatic ecosystem and thus bears a special ecotoxicological significance.

In recent years, the range of the natural habitat of B. bengalensis is getting dwindled due to rapid urbanization and industrial development. Additionally, these organisms are randomly harvested from their natural habitat by the skilled collectors for human consumption. Apart from these, B. bengalensis has been encountering physiological adversity due to unrestricted contamination of their habitat by multiple environmental contaminants including the household cleaning agent like washing soda.

Washing soda, chemically identified as anhydrous sodium carbonate, is widely used as a major domestic cleaning agent among the rural and semiurban populations in India. Washing soda is alkaline
cleaning agent and is commercially available at a cheap rate which enables the rural population of India to use this chemical compound for various cleaning and washing purposes at pond and domestic locations (Figure 5B). A field survey carried out in the selected village ponds of the districts of North 24 parganas, South 24 parganas, Purba midnapur and Paschim midnapur of the states of West Bengal revealed that washing soda is in use for the purposes of washing of garments, linens, utensils and bathing of cows, buffaloes and donkeys. Moreover, various waterbodies often receive domestic and industrial effluents contaminated with washing soda and other detergents. Ray et al. (2011) reported detergent as a major contaminant in freshwater ecosystem in India. Kumari (2013) reported detergent induced alteration in the protein content of the foot, mantle and digestive gland of *B. bengalensis*. A significant depletion in the total content of protein was assumed due to increased proteolysis and probable utilization of the degraded product for various metabolic purposes. Report of the toxicity of detergent in molluscs in India is very limited in the current scientific literature. However, the toxicity of detergent in various other organisms is in report. Topale et al. (2013) reported the relative mortality and relative lethality of freshwater crab *Paratelphusa Jacquemontii* under the exposure of detergent and reported detergent as a highly toxic chemical compound for crustaceans. Sodium dodecyl sulphate and tetradecyltrimethylammonium bromide were reported to inhibit the filtering activity of mollusc *Crassostrea gigas* (Ostroumov, 2003). Stefanoni and Abessa (2011) reported genotoxicity of anionic surfactant, linear alkylbenzene sulphonate in brown mussel *Perna perna* by determining the degree of micronucleation. Linear alkylbenzene sulphonate appeared to affect the behavioural profiles like restlessness, swimming and balance in freshwater fish *Clarias gariepinus*. Additionally, detergent exposure resulted in respiratory distress and hemorrhage in the gill filaments in the same species (Ogundele et al., 2004). Screening of toxicity of laundry detergent in *Euglena gracilis* indicated its efficacy as a sensitive indicator of detergent pollution (Azizullah et al., 2011).

Current investigation was aimed to assess the toxicity of washing soda in freshwater mollusc *B. bengalensis* with reference to toxin induced shift in total and differential cell density, nonself adhesion, aggregation, phagocytosis, cytotoxicity, apoptosis-necrosis of hemocytes and transamination, detoxification and antioxidation response in different target tissues. Relative expression of iNOS was estimated in the hemocytes of *B. bengalensis* under the exposure of washing soda. A detailed histopathological analysis of gill, digestive gland and mantle were carried out in the experimental specimens under the sublethal exposures of washing soda. Selected behaviours like relative mobility, foot protrusion an aggregation response of *B. bengalensis* were examined in depth under the environmentally realistic concentrations of washing soda.

Estimation of LC$_{50}$ is toxicologically related with median lethal response; it is a dose response which involves lethality of fifty percent of the test animals (Akhila et al., 2007). Different toxicants like
heavy metals, pesticides and other xenobiotic substances affected the biological systems by producing acute, subacute and chronic states of toxicity. Wallen et al. (1957) reported that the LC$_{50}$ of sodium carbonate for 96 hours as 740 mg/l in mosquitofish. The LC$_{50}$ of sodium carbonate for 96 hours in bluegill sunfish was determined as 300 mg/l by Cairns et al. (1959). In this present study, LC$_{50}$ of washing soda in *B. bengalensis* with an average size of 3 ± 0.5 cm was estimated under different spans of experimental exposures i.e. 24, 48, 96 hours and 7 and 15 days. The highest LC$_{50}$ value of washing soda was determined as 38.92 ± 0.94 g/l for 24 hours of exposure. The lowest LC$_{50}$ value was determined as 19.33 ± 0.83g/l of washing soda for 15 days of exposure (Table 1). LC$_{50}$ values of washing soda were indicative to a high degree of its toxicity in *B. bengalensis*.

The hemolymph of molluscs is a fluid plasma material with specified cells called hemocytes which circulate in the hemolymph following a definite route. Hemocytes are essential cellular components of molluscan blood and are capable of performing diverse physiological and immunological functions (Tripp, 1992; Nakayama et al., 1997; Pipe et al.; 1999, Itoop et al., 2006; Estrada et al., 2013).

Cell viability indicates the magnitude of toxicity of contaminants in test populations. It signifies the impact of the toxicity of a particular chemical compound on test species. Upon treatment with mercury, *Crassostrea virginica* exhibited a decreasing trend of hemocyte viability (Cheng and Sullivan, 1984). Fournier et al. (2001) reported that the viability of hemocytes was suppressed in clams *Mya arenaria* under the exposure of methyl mercuric chloride. A nominal alteration of hemocyte viability was recorded in *B. bengalensis* under exposure of washing soda for various spans of exposure (Table 2).

Morphology of hemocytes represents the physiological as well as immunological status of an organism. Different subpopulations of hemocytes were identified in *B. bengalensis* by using phase contrast, bright field and scanning electron microscopy. Granulocytes and hyalinocytes were reported to be actively involved in phagocytosis and generation of cytotoxic molecules (Söderhäll and Smith, 1983). Pipe et al. (1993) reported that the hyalinocytes were associated in the activity of phenoloxidase. Asterocytes was identified as major phagocytic cells among circulating hemocytes of *Lamellidens marginalis* (Chakraborty et al., 2008). Treatment with 5000 ppm of washing soda for 15 days yielded extensive blebbing of cell membrane associated with rounding up and cytoplasmic shrinkage. These symptoms were reported as diagnostic characteristics of possible apoptotic state of hemocytes (Sokolova, 2004) (Figure 14G). Exposure of washing soda exhibited an apparent loss of cytoplasmic cohesion, disintegration of cell membrane, vacuolations and nuclear aberrations in various subpopulations of hemocytes of *B. bengalensis* (Figure 15F-15J). Hemocytes of *B. bengalensis* treated with washing soda exhibited a relatively smooth surfaced cell membrane with relative reduction in the involutions of cell membrane and rounding up of cells as evident from scanning electron microscopy (Figure 16C, D). Washing soda induced morphological damages of various subpopulations of hemocytes were suggestive
to possible impairment of physiological and immunological activities of hemocytes of *B. bengalensis* inhabiting in the washing soda contaminated habitat.

Hemocytes are circulating blood cells of molluscs involved in multiple physiological functions both in normal and under the condition of environmental stress. The density of circulating hemocyte is reported as an indicator of chemical stress (Chakraborty *et al.*, 2008) and the total hemocyte count is considered as an important experimental tool in monitoring the health status of molluscs distributed in biounsafe environment. The total and differential hemocyte counts are considered as useful cell biological parameters which are related to the immunological reactivity during the period of stress and disease resistance responses (Cima *et al.*, 2000). Increase in the density of hemocytes was reported as a state of immunological stimulation in insect *Drosophila suzukii* (Kacsok *et al.*, 2012). Oubella *et al.* (1993) proposed that the fluctuations of hemocytes density resulted from a mobilization and consecutive migration of resident hemocytes from tissues towards the hemolymph compartment in molluscs *Ruditapes philippinarum* and *Ruditapes decussatus* during host-pathogen interactions. In *B. bengalensis*, a significant and non linear increase in total hemocyte density was recorded against various concentrations of washing soda for 24, 48, 72, 96 hours and 7 days of exposures and a significant decrease in total hemocyte density was recorded against all experimental concentrations of washing soda for 15 days of exposure (Figure 17). This result was suggestive to a state of washing soda induced physiological stress in *B. bengalensis* with reference to hemocyte density and a possible state of immunological alteration due to washing soda exposure.

Differential count of hemocyte of *B. bengalensis* was determined under the sublethal concentrations of washing soda. Hemolymph of *B. bengalensis* contains diverse populations of hemocytes i.e. blast like cells, agranulocytes, granulocytes, asterocytes and hyalinocytes. Morphological classification and allied nomenclature of hemocyte subpopulations raised a confusion and contradiction (Cheng *et al.*, 1995) among the workers of this field. However, we classified the hemocytes on the basis of morphological characters, tinctoriality and size (Ray *et al.*, 2013a). Blast like cells (George and Farguson, 1950) are thought to differentiate into diverse subpopulations with discrete functional significance. Granulocytes are reported to generate cytotoxic responses during pathogenic invasion and chemical stress (Zhang, 2006). Asterocytes, agranulocytes and hyalinocytes are reported as potential phagocytes which are capable of engulfing foreign particulates (Hine, 1999). In this current study, a significant and nondirectional decrease in the density of blast like cells and hyalinocytes, an increase in the density of granulocytes and a minute alteration in the density of agranulocytes and asterocytes were recorded against multiple experimental concentrations of washing soda for various spans of exposures (Figure 18-23). Washing soda affected the relative density of various subpopulations of hemocytes of *B. bengalensis*.
under controlled laboratory condition. Several authors demonstrated that alteration of hemocytes density in bivalves exposed to pollutants could be associated to disease susceptibility (Coles et al., 1994; Pipe et al., 1999). Russo and Lagadic (2004) proposed that the granulocyte density was increased in atrazine exposed mollusc Lymnaea stagnalis. Present data is suggestive to a state of washing soda induced toxic stress in the blood cells of B. bengalensis, resulting in their possible susceptibility to various environmental toxins and pathogens. Moreover, this could result in possible interference in the multiple activities of hemocytes of B. bengalensis distributed in the washing soda contaminated habitat.

Surface adhesion of hemocytes to nonself particulates is regarded as an important immune response of metabolic significance (Chen and Bayne, 1995). Cellular attachment, cytoplasmic spreading and migration of hemocytes are the sequential steps of cellular adhesion (Armstrong, 1980). Hemocytes, the chief immunoeffecter cells of B. bengalensis are consisted of two discrete subpopulations namely adherent and nonadherent types (Guria and Ray, 2002). In this current study, a significant alternation in the percent occurrence of the adherent hemocytes was recorded against almost all concentrations of washing soda. However, the marked inhibition in the occurrence of adherent hemocytes was recorded against 3000, 1500 and 5000 ppm of washing soda for 48 and 72 hours along with 15 days of exposure (Figure 28). Thus, it is apprehended that washing soda exposure may lead to a state of immune impairment in B. bengalensis due to significant shift in the surface adhesion response.

Hemocyte aggregation is considered as an important cellular reaction involved in biological plug formation at wound site, encapsulation reaction and maintenance of physiological homeostasis. Cell-cell aggregation and adhesion are thought to be the important metabolic behaviours of hemocytes (Kenney et al., 1972; Takahashi et al., 1994; Takahashi et al., 1995; Chen and Bayne, 1995). Cell-cell aggregation or clump formation is involved in maintaining the blood homeostasis and wound healing (Sminia, 1981) process in molluscs. The aggregation of hemocyte in molluscs is assumed to be reversible and aggregated hemocytes may disperse and reenter the circulatory system as wound healing progresses. In this study, cell cell aggregation response was examined against the different experimental concentrations of washing soda. The significant suppression in the hemocyte aggregation response was recorded against various concentrations of washing soda for multiple spans of exposure (Figure 30). Hegaret et al. (2003a) reported that temperature fluctuations suppressed the aggregation response of hemocytes in mollusc Crassostrea virginica. This washing soda induced inhibition in the aggregation of hemocytes was suggestive to a possible state of interference of blood homeostasis in B. bengalensis. Washing soda induced damage in hemocyte aggregation may lead to impairment in the physiology of hemocyte plug formation during accidental or natural blood loss. In general, natural organisms are subjected to a continuous state of struggle for food, space, mate and other biological factors. Inter or intraspecific
struggle for existence occasionally may lead to accidental injury of the body surface or internal tissue damage resulting in loss of hemocytes and fluid. In the aquatic environment, the site of loss of blood fluid is needed to be sealed instantly and this situation is assumed to be a life threatening situation. Hemocyte aggregation response is functionally associated with two important physiological functions i.e. formation of hemocyte plug at the site of bleeding and encapsulation of invading microorganisms or parasite for their deactivation. Thus, washing soda induced inhibition of hemocyte aggregation response is assumed to affect adversely the process of arrestation of hemolymph loss and encapsulation response in *B. bengalensis* distributed in the washing soda contaminated habitat.

Phagocytosis of nonself particulates is a classical innate immune response in invertebrates by which the cells engulf relatively smaller particulates (Saha *et al*., 2008; Vijayavel *et al*., 2009). This is an essential strategy of host defense against infections caused by microorganisms (Ratcliffe, 1985). Phagocytic cells are highly conserved throughout the phylogeny and are reported as a classical immunological response against toxins, pathogens and parasites. Cell biologists and immunologists claimed phagocytosis as a biomarker of environmental contamination (Johansson and Söderhäll, 1992). In this present investigation, phagocytic response of hemocytes was estimated in *B. bengalensis* by challenging the cells with yeast particles *in vitro*. Treatment with 700, 1500, 3000 and 5000 ppm of washing soda resulted in a dose dependent decrease in the phagocytic responses of hemocytes under different time spans of treatment. Treatment with 5000 ppm of washing soda for 15 days of exposure yielded a maximum inhibition in the phagocytic response of hemocytes (Figure 32). Toxin induced reduction in phagocytic response in hemocytes are in report in other invertebrate Phyla. Russo and Medac (2007) reported that phagocytic response of hemocytes was decreased in mollusc *Lymnaea stagnalis* against different concentrations of herbicide fomesafen. Sublethal exposures of sodium arsenite resulted in suppression in the phagocytic indices of hemocytes of a freshwater mollusc *Lamellidens marginalis* (Chakraborty *et al*., 2009). Fluoride exposure suppressed the phagocytic efficacy of hemocytes in mollusc *Venerupis philippinarum* (Ballarin *et al*., 2014). According to Mukherjee *et al.* (2015) sodium carbonate exhibited an inhibitory effect on the phagocytic response in freshwater sponge *Eunapius carteri*. Present experimental data suggests that contamination of habitat by washing soda may lead to similar kind of suppression in the phagocytic response of hemocyte of *B. bengalensis*. It is apprehended that continuous and unrestricted contamination of freshwater ecosystem by washing soda may result in an adverse immunological suppression of phagocytosis in *B. bengalensis*. Such a situation might render these organisms vulnerable to invasion of disease producing pathogenic organisms in *B. bengalensi* distributed in a contaminated habitat.
Hemocytes are major immunoactive cells of molluscs which are involved in generating cytotoxic molecules capable of destroying the engulfed pathogens (Adema et al., 1991; Ordas et al., 2007). Cytotoxic agents like superoxide anion, nitric oxide and phenoloxidase are generated within the immunocytes as bacterial “killing agents” (Nappi and Ottaviani, 2000). Chemically, they are highly reactive and functionally contribute to cell mediated immunity of both vertebrates and invertebrates (Conte and Ottaviani, 1995). Ray et al. (2013a) reported that the specific subpopulations of hemocytes of B. bengalensis and Lamellidens marginalis were capable of producing cytotoxic agents like superoxide anion and nitric oxide and phenoloxidase. Xenobiotics induced cytotoxicity was estimated in several invertebrate taxa (Livingstone et al., 1990; Winston and Giulio., 1991; Chakraborty et al., 2009). In this study, generation of cytotoxic agents like superoxide anion, nitric oxide and activity of phenoloxidase were estimated in the hemocytes of B. bengalensis exposed to washing soda along with the respective control.

Oxygen radical mediated killing is based on the premises of toxicity evolved due to high concentration of molecular oxygen. Many of the oxygen derived molecules are immunologically toxic to environmental pathogens. These derived molecules are collectively termed as “reactive oxygen intermediates” or “oxygen radicals” (Nakayama and Maruyama, 1998). Oxygen derivatives are generated at the cell surface when the consumption of oxygen in the cell is increased due to respiratory burst activity following the contact of phagocyte to microorganisms (Manduzio et al., 2005). A significant and dose independent suppression of generation of superoxide anion in the hemocytes of B. bengalensis was recorded against various concentrations of washing soda for different spans of exposure (Figure 33). Washing soda induced suppression of superoxide anion is suggestive to possible impairment of the cytotoxic status of B. bengalensis distributed in the polluted environment.

Nitric oxide or nitrogen monoxide has been identified as a molecule reported initially in the neurotransmission but established later as a participant in diverse physiological processes involving oxidative stress. Nitric oxide plays a significant role in pathogen and parasite destruction in various invertebrate Phyla (Colasanti et al., 2010). However, information of activity and role of reactive oxygen intermediates in the hemocytes of molluscs is limited. In this investigation, generation of nitric oxide in the form of nitrite was recorded in hemocytes of B. bengalensis against different experimental concentrations of washing soda along with control. A significant suppression of generation of nitric oxide in the hemocytes of B. bengalensis was recorded against 700, 1500, 3000 and 5000 ppm of washing soda for 48, 72, 96 hours and 7 and 15 days of exposures (Figure 34). Nitric oxide significantly acts as an effective killing agent of engulfed pathogens. Additionally, nitric oxide in association with superoxide anion biochemically form peroxynitrite on which is reported to be highly toxic reactive intermediate in
mammals. However, a detailed investigation on generation of nitric oxide and peroxynitrite is needed to understand their physiological and cytotoxic roles in invertebrates.

Inducible nitric oxide synthase is the key enzyme to activate L-arginine to produce nitric oxide, an established cytotoxic agent (Gopalakrishnan et al., 2011). In this present study, flow cytometric detection of inducible nitric oxide synthase was carried out in three distinct hemocytes morphotypes of *B. bengalensis* exposed to washing soda and in control. Agranulocytes, semigranulocytes and granulocytes of control hemocytes exhibited strong binding of inducible nitric oxide synthase antibody indicating the existence of inducible nitric oxide synthase in three hemocyte morphotypes. Whereas, upon treatment with 5000 ppm of washing soda for 96 hours resulted in a decrease in the expression of inducible nitric oxide synthase in three principal subpopulations of hemocytes (Figure 35). Conte and Ottaviani (1995) biochemically demonstrated nitric oxide synthase activity in the hemocytes of freshwater snail *Viviparous ater* with lipopolysaccharide pretreatment which was reported to increase nitric oxide synthase activity significantly. de Barros et al. (2009) detected inducible nitric oxide synthase in the ascidian hemocytes by immunohistochemical and western blot analyses using mammalian anti inducible nitric oxide synthase antibody. In this present investigation, washing soda induced suppression of inducible nitric oxide synthase expression justifies the inhibition in generation of nitric oxide in toxin exposed hemocytes of *B. bengalensis*.

Physiological significance of phenoloxidase in different invertebrate Phyla as a defense enzyme has been increased considerably since its localization was confirmed in the hemocytes and hemolymph. The enzyme has been reported in various animal tissues and cell types including circulating blood cells, neurons and melanocytes. A significant and dose independent suppression in the activity of phenoloxidase were recorded against 700, 1500, 3000 and 5000 ppm of washing soda for various spans of exposures in the hemocytes, gills, digestive gland and mantle of *B. bengalensis* (Figure 36-39). The paramyxean protozoan *Marteilia sydneyi*, is the etiological agent of QX disease decreased phenoloxidase activity in mollusc *Saccostrea glomerata* (Peters and Raftos, 2003). Idakieva et al. (2009) reported that sodium dodecyl sulphate influenced time dependent suppression of phenoloxidase activity in mollusc *Rapana thomasiana* hemocyanin. The suppressed phenoloxidase activity in hemocytes due to washing soda treatment was suggestive to a possible impairment of the immunological reactivity and response in *B. bengalensis* inhabiting the washing soda contaminated habitat.

Recovery response of total and differential hemocyte count, nonself adhesion, aggregation response, phagocytosis, generation of superoxide anion and nitric oxide and the activity of phenoloxidase in hemocytes of *B. bengalensis* was recorded after maintenance of pretreated (700, 1500, 3000 and 5000
ppm of washing soda for 15 days) specimens in toxin free water for 7 days. These specimens did not exhibit any recovery of above mentioned hemocytes behaviours and functions (Table 4-15). Chakraborty et al. (2008) reported that the toxicity of sodium arsenite on recovery response of total hemocyte count *Lamellidens marginalis*. Authors reported sodium arsenite induced partial restoration in total hemocyte count in this same specimen. Present investigation indicated that the toxin effect is persistent in the natural habitat of *B. bengalensis*.

Catalase is a heme containing enzyme that catalyzes the conversion of hydrogen peroxide to water and oxygen. Recently, catalase, the pivotal enzyme involved in hydrogen peroxide detoxification, have been shown to be implicated in the disposal of exogenous hydrogen peroxide by asterocytes (Prakash and Rao, 1995). Peroxide, including hydrogen peroxide is one of the main reactive oxygen species leading to oxidative stress. (Gamble et al., 1995). The cytotoxic effect of hydrogen peroxide is thought to be caused by hydroxyl radicals generated from iron catalyzed reactions causing subsequent damages to DNA, proteins and membrane lipids. However, generally, catalase is regarded as being able to catalyse the destruction of hydrogen peroxide. Catalase is reported to bear a special importance in clearing the high load of hydrogen peroxide as and when required (Vijayavel et al., 2005). A significant and nondirectional decrease in the activity of catalase was recorded in hemocytes, gill, digestive gland and mantle of *B. bengalensis* against all experimental concentrations of washing soda (Figure 40-43). Sodium arsenite is reported to suppress the activity of catalase in the hemocytes and digestive gland of bivalve mollusc *Lamellidens marginalis* (Chakraborty et al., 2013). A significant decrease in the activity of catalase was recorded in the gill of fish *Australoheros facetus* against cadmium exposure (Crupkin and Menone, 2013). Copper suppressed the activity of catalase in the hepatopancreas of bivalve mollusc *Scapharca inaequivalvis* (Isani et al., 2003). Inhibition of catalase activity against washing soda exposure was indicative to a possible state of peroxide elevation that may lead to an increase in oxidative stress in different cells and tissue of *B. bengalensis* under the exposure of washing soda. Such a situation may lead to possible impairment of specific cell functions, damage in morphology and histological architecture of target organs in *B. bengalensis*.

Internal defense of molluscs is dependent on circulating hemocytes capable of phagocytizing pathogenic organisms. In performing the intracellular killing of ingested microorganisms, hemocytes are capable of generating reacting oxygen intermediates. In order to protect the tissue of the self from reactive oxygen metabolite produced during respiratory burst, various potentially active antioxidant enzymes are reported in the mussel *Mytilus edulis* (Lesser, 2006). Enzymes like superoxide dismutase are reported to be localized in the hemocytes (Prakash and Rao, 1995). In this present investigation, a significant inhibition in the activity of superoxide dismutase were recorded in hemocytes, gill, digestive gland and
mantle of *B. bengalensis* against 700, 1500, 3000 and 5000 ppm of washing soda for multiple time spans of exposures (Figure 44-47). Almamoori *et al.* (2013) reported that heavy metal exposure bears a role in the inhibition of superoxide dismutase of the gill and digestive gland of two species of molluscs *Viviparrus bengalensis* and *Corbicula fluminea*. Linear alkylbenzene sulphonate suppressed the activity of superoxide dismutase in the digestive gland of mussel *Mytilus sp* (Ros *et al.*, 1995). Washing soda induced inhibition of superoxide dismutase activity was indicative to an increase in the oxidative stress and allied damage of the target cells and organs of *B. bengalensis* leading to possible impairment of the activities of these cells and organs.

The xenobiotics are subjected to chemical alteration within the organisms and are conjugated with glutathione pool through glutathione -S- transferase for detoxification. Glutathione -S- transferase is primarily involved in chemical disposition of toxic substances and have the ability to catalyse the conjugation of glutathione to various toxic electrophiles and inactivate aromatic compounds by non catalytic binding (Yadwad, 1989). Glutathione-S- transferase is involved in the detoxification reaction process of various xenobiotic compounds in the gill, digestive gland and mantle of molluscs (Vidal and Narbonne, 2000). In this present study, a significant alteration in glutathione –S- transferase activity were recorded in hemocytes and gill against all four experimental concentrations of washing soda for multiple spans of exposure (Figure 48, 49). In the digestive gland, treatment with 700, 1500, 3000 and 5000 ppm of washing soda for 24, 48, 72, 96 hours and 7 and 15 days of exposures resulted in a significant inhibition of the activity of glutathione -S- transferase activity in comparison to that of the respective control (Figure 50). In the mantle, a significant, dose independent suppression in the activity of glutathione-S-transferase was recorded against all experimental concentrations of washing soda for 24, 72, 96 hours and 7 and 15 days of exposure and against the treatment of 700, 1500 and 3000 ppm of washing soda for 48 hours of exposure (Figure51). The activity of glutathione -S- transferase had been reported to be increased as a result of exposure to various contaminants (Prakash and Rao, 1995). However, cadmium exposure suppressed the activity of glutathione -S- transferase in the liver and induced its activity in the gill of fish *Australoheros facetus* (Crupkin and Menone, 2013). Current data was suggestive to a possible state of an undesirable alteration in the biochemical efficacy of detoxification in *B. bengalensis* exposed to washing soda.

Phosphatases are pivotal lysosomal enzymes which play an important role in the cytolysis and differentiation processes (Liu *et al.*, 2004). They are reported as accurate and sensitive markers of various environmental stresses in the living organisms (Murti and Shukla,1984). Phosphatases act as reliable index for the assessment of the immune status of the crustaceans (Sarlin and Philip, 2011).
Acid phosphatase is a significant lysosomal enzyme which is functionally involved in autolysis of the degenerated cells and influences cell turnover and immunological defense (Xia et al., 2000). It is reported to destroy and digest the microbial pathogens during immune defense action (Cheng, 1989). In this current study, acid phosphatase activity in the hemocytes of *B. bengalensis* was determined against all experimental concentrations of washing soda along with the respective control. A significant elevation in the activity of acid phosphatase was recorded against almost all experimental concentrations of washing soda for 24 and 48 hours of exposures and a significant suppression in the activity of acid phosphatase was recorded against 700, 1500, 3000 and 5000 ppm of washing soda for 7 and 15 days of exposures (Figure 52). Acid phosphatase activity was reported to be increased in the hemolymph of desert locust *Schistocerca gregaria* on the third day after inoculation with an entomopathogenic fungus *Metarhizium anisopliae* (Xia et al., 2000). Guava leaf extract induced acid phosphatase activity in the serum of prawn *Penaeus monodon* (Yin et al., 2014). Washing soda exposure resulted in a dose independent alteration in the activity of acid phosphatase in hemocytes of *B. bengalensis*. The fluctuated acid phosphatase activity is suggestive to a prenecrotic change in organs. This is indicative to a possible shift in the efficacy of *B. bengalensis* for intralysosomal digestion of the pathogen and parasites in washing soda contaminated habitat.

Alkaline phosphatase is a hydrolytic enzyme which hydrolyses phosphomonoester under alkaline conditions (Miao et al., 2005). It has been used as an important marker enzyme to evaluate the environmental toxicity (Smirle et al., 1996; Koodalingam et al., 2011). Alkaline phosphatase is reported to be involved in carbohydrate metabolism, growth and differentiation, protein synthesis, secretory activity and transport to phosphorylated intermediates across the membranes (Omkar, 1985). This enzyme also bears immunological importance (Sarlin and Philip, 2011). In this present study, alkaline phosphatase activity in the hemocytes of *B. bengalensis* was estimated against all experimental concentrations of washing soda with respective control. A significant inhibition in the activity of alkaline phosphatase was recorded against 700, 1500, 3000 and 5000 ppm of washing soda for 7 and 15 days and against the treatment of 700 ppm of washing soda for 24 and 96 hours of exposures (Figure 53). Saha et al. (2009) reported that the activity of alkaline phosphatase was significantly decreased in the hemocytes of crab *Scylla serrata* exposed to sublethal concentrations of sodium arsenite. Sarlin and Philip (2011) reported that the activities of acid and alkaline phosphatases can be considered as reliable indices of assessment of immune status in prawn. They indicated that the activity of lysosomal phosphatases can be correlated with intracellular lytic potential of the phagocytosed foreign particles. Alkaline phosphatase has been reported as an important regulatory enzyme related with multiple essential functions in the living organisms (Yin et al., 2014). Washing soda induced shift in the activities of acid and alkaline...
phosphatases were indicative to possible impairment of the immune potential and metabolic activities in *B. bengalensis* distributed in the contaminated habitat.

Transaminases such as glutamate oxaloacetate transaminase and glutamate pyruvate transaminase are reported as significant metabolic enzymes in living organisms (Malarvizhi *et al*., 2012) involved in biochemical transamination process. Activities of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase are considered as sensitive indicators to assess cellular and tissue damage (Van der *et al*., 2003). Transaminases are reported to act as important tools to estimate the metabolic adversities due to various environmental pollutants (Nemcsok *et al*., 1981).

Activity of glutamate oxaloacetate transaminase was estimated in hemocytes, gill, digestive gland and mantle of *B. bengalensis* against all experimental concentrations of washing soda along with control. In the hemocytes, a significant suppression in the activity of glutamate oxaloacetate transaminase was recorded against 700, 1500, 3000 and 5000 ppm of washing soda for 24, 48 and 72 hours and against the treatment of 3000 ppm of washing soda for 96 hours of exposure (Figure 54). Sodium arsenite was reported to suppress the activity of glutamate oxaloacetate transaminase in the hemocytes of bivalve *Lamellidens marginalis* (Chakraborty *et al*., 2013). This is indicative to retardation in the transamination reaction thereby affecting the cellular metabolism in molluscs. Tissue damage, loss of cellular integrity associated with elevation of activities of glutamate oxaloacetate transaminase was reported in fish *Cirrhinus mrigala* after heavy metal exposure (Chavan and Muley, 2014). A significant increase in the activity of glutamate oxaloacetate transaminase was recorded in hemocytes, gill, digestive gland and mantle of *B. bengalensis* against all experimental concentrations washing soda for 96 hours and 7 and 15 days of exposures (Figure 54-57). Washing soda induced alteration in the activity of glutamate oxaloacetate transaminase in the hemocytes, gill, digestive gland and mantle indicated a possible state of biochemical alteration in transamination reaction resulting in a possible toxicological stress in *B. bengalensis* distributed in the contaminated habitat.

Activity of glutamate pyruvate transaminase was estimated in the hemocytes, gill, digestive gland and mantle of *B. bengalensis* against all experimental concentrations of washing soda with respective control. In this present investigation, a marked increase in the activity of glutamate pyruvate transaminase was recorded in the mentioned tissues and organs against 700, 1500, 3000 and 5000 ppm of washing soda for multiple spans of exposure (Figure 58-61). The activity of glutamate pyruvate transaminase was reported to increase in the blood serum of freshwater fish *Channa punctatus* against the exposure of a household brand of detergent known as ‘Nirma’ (Choudhary and Jha, 2013). This result is suggestive to glutamate pyruvate transaminase as a marker of cellular and tissue stress. Jia *et al*. (2014) reported that
carbon tetrachloride exposure induced the activity of glutamate pyruvate transaminase in the hepatopancreas of common carp *Cyprinus carpio* which may lead to onset of hepatotoxicity and cellular inflammation in fish. Washing soda induced elevation in the activity of glutamate pyruvate transaminase may be indicative to a state of metabolic stress in *B. bengalensis*.

Lysosomes are organelles which are primarily responsible for intracellular digestive processes and involved in enzyme mediated degradation of foreign particles or pathogens (Moore, 1990; Cesen *et al.*, 2012). Lysosomes play an important role in cell death (Guicciardi *et al.*, 2004) and specific immune responses like phagocytosis and digestion of pathogens by the formation of phagolysosomes. Phagocytosed particulates or pathogens in later stage are subjected to intracellular destruction facilitated by the hydrolytic enzymes of lysosome which act within the subcellular compartments. Structural stability of lysosomal membrane against xenobiotics and other stressors can be quantified by neutral red retention assay (Janeck *et al.*, 1998; Zhao *et al.*, 2011). Neutral red is a membrane permeable cationic dye which accumulates in the acidic lysosomal compartment (Lowe *et al.*, 1992). Determination of neutral red retention time in lysosome is considered as an early warning tool of environmental toxicity (Lee *et al.*, 2009). Linear alkylbenzene sulphonate suppressed the hydrolase enzyme in lysosomes of rat liver (Bragadin *et al.*, 1996). The neutral red retention is either minimally or not at all affected by natural factors, such as temperature and salinity, but is reported to be influenced by environmental toxins and pollutants (Ringwood *et al.*, 1998). Booth *et al.* (2001) reported that the neutral red retention time of hemocytes of earthworm *Aporrectodea caliginosa* was suppressed against organophosphate exposure which indicated its adverse impact on growth and fecundity of the species. Screening of lysosomal membrane stability of molluscs by neutral red retention assay has been proposed as a possible biomarker of toxicity of arsenic (Chakraborty and Ray, 2009). In this present investigation, stability of lysosomal membrane in the hemocytes of *B. bengalensis* exposed to all four experimental concentrations of washing soda for multiple spans of exposure was determined by neutral red retention assay along with a parallel control (Figure 63). Upon treatment with all experimental concentrations of washing soda, neutral red retention time was estimated in hemocytes. Neutral red retention time was recorded to be decreased significantly in washing soda treated hemocytes of *B. bengalensis* in a dose dependent pattern. Experimental result suggested a state of washing soda induced fragility in the membrane of hemocyte lysosome of *B. bengalensis*. It is assumed that similar kind of toxin induced fragility of the lysosomal membrane of hemocyte may occur in the hemocytes of *B. bengalensis* distributed in their natural habitat contaminated with washing soda. This situation may lead to state of undesirable alteration in the pathogen destruction potential of host species inhabiting in a contaminated habitat.
The term ‘apoptosis’ has been coined by Kerr et al. (1972) as programmed cell death. Necrosis has been considered merely as an accidental and uncontrolled form of cell death (Kromer et al., 2009). Hemocyte apoptosis has been established as a cellular immune response of molluscs and biomarker of environmental toxicity (Sweet et al., 1999; Kiss, 2010). Effect of pesticide and other pharmaceuticals on hemocyte apoptosis in pond snail *Lymnea stagnalis* has been studied by Russo and Madec (2004). Foster et al. (2011) explained the effects of copper on hemocyte apoptosis and reported it as an important host defense mechanism. Information of physiological and toxin induced level of hemocyte apoptosis in Indian molluscs is inadequate in the current scientific literature. In this present investigation, the percent distribution of apoptotic and necrotic cells within agranulocytes (P1), semigranulocytes (P2) and granulocytes (P3) in *B. bengalensis* of respective Q4 and Q2 quadrants exhibited variations (Figure 64). Percent apoptosis in agranulocytes (P1) subpopulation exhibited induction against 3000 and 5000 ppm of washing soda for 7 days of exposure in comparison to the general physiological apoptotic level in the control. Whereas, apoptosis level in semigranulocytes (P2) and granulocytes (P3) exhibited depletion under multiple concentrations of washing soda. However, all subpopulations of hemocytes exhibited an increase in percent necrosis upon treatment with 3000 and 5000 ppm of washing soda for 7 days. Sensitivity of immune system of aquatic molluscs to different environmental toxins is in report (Galloway et al., 2001). Report of apoptotic response of hemocytes of *B. bengalensis* exposed to washing soda is absent in current scientific literature. Perry and Lynn (2009) studied physiological and toxin induced apoptosis in bivalves exposed to a molluscicide, baylucide. They also concluded that increased concentration of belucide had decreased the apoptotic response in molluscs. Russo and Madec (2007) described the apoptotic induction in molluscs under the pesticide exposures. Experimental exposure of pesticides like cypermethrin and fenvalerate yielded a significant shift in the density of hemocytes of *B. bengalensis* and *Lamellidens marginalis* (Ray et al., 2013b). Authors reported pesticide induced morphological damage, lysosomal membrane stability and apoptotic response of hemocytes in these same specimens. Present investigation indicated a state of washing soda induced shift in the apoptotic response of hemocytes of *B. bengalensis*. However, the magnitude of washing soda induced apoptotic response of hemocytes was not uniform in all hemocyte subpopulations. Moreover, it is observed that washing soda exposure resulted in modulation in the apoptotic response among multiple subpopulations of hemocytes of *B. bengalensis*. Thus apoptotic response of *B. bengalensis* hemocytes can be considered as a possible marker of washing soda toxicity.

Gill is functionally involved in the physiology of respiration, filter feeding and immunosurveillance (Chakraborty et al., 2010) in aquatic molluscs and acts as a target organ of multiple pollutants (Ogundiran et al., 2009). Anatomically, the gill of gastropods is monopectinate one bearing
parallelly arranged gill lamellae (Aksit and Mutaf, 2007). Molluscs can sieve food particulates by the gill lamellae from water column and are able to discriminate the ingestible and noningestible particulates. Vasanthi et al. (2012) reported that the gill serves as an important histopathological marker for heavy metal induced toxicity. Gill may consider as a significant tool to assess the impact of environmental contaminants in living organisms (Schramm et al., 2000). The histomorphology of gill is an indicator of water quality parameter and the health status of the organism (Peters et al., 1984). The physiological status of the gill was reported to be impaired in molluscs under the exposure of detergents and surfactants (Ostromov, 2003). Gill hemorrhage and respiratory disorder were recorded in Clarias gariepinus fingerlings against linear alkylbenzene sulphonate exposure and is indicative to a physiological threat on aquatic life (Ogundele et al., 2004). B. bengalensis presented distinct water channels and integrated gill lamellae signifying the normal functional status of the tissue. Exposure of 5000 ppm of washing soda for 7 days resulted in morphological swelling of gill lamellae, appearance of hyperchromatic anaplastic cells, dense fibrosis and cellular disintegration (Figure 67). This is indicative to a state of toxicity leading to a possible respiratory blockage, decrease in filtration activity and other gill functions against environmentally realistic concentrations of washing soda. Such a situation may lead to a decrease in the ecological fitness and population size of the species distributed in the washing soda contaminated habitat.

The digestive gland of molluscs is the site of metabolic regulation and the organ of detoxification and elimination of toxins (Moore and Allen, 2002). The morphology of digestive gland was reported to alter in multiple Phyla due to the exposure to several environmental stressors (Sastry et al., 1980; Lomte et al., 1989; Sontakke et al., 1992). Upon treatment with copper sulphate, cellular damage was recorded in the mucous cells of mid gut, epithelial linings and typhosolar cells. Possibility of undesirable change in the digestive mechanism in B. bengalensis exposed to copper sulphate is in report (Kamble and Kamble, 2012). Exposure of 5000 ppm of washing soda for 7 days resulted in infiltration of cellular mass designated as hyperchromatic anaplastic cells, inflammatory lysis of digestive epithelium in comparison to a normal tissue texture comprise of distinct epithelia (Figure 69). Washing soda induced histological damage of the digestive gland of B. bengalensis is suggestive to a state of degeneration and necrosis of the digestive epithelium leading to possible impairment in the digestive physiology in the same species.

Mantle of molluscs is characterized by thin flaps of muscular tissue which externally covers the visceral mass. Mantle forms the perimeter of the body cavity and is actively associated with shell synthesis (Ojima, 1952; Nakahara and Bevelander, 1971; Dix, 1973), secretion of the periostracum (Verheken, 1989) and acts as a nonspecific protective organ. Treatment with 5000 ppm of washing soda for 7 days yielded a rupture of the cuboidal epithelial cells and subsequent damage the muscular fibers of mantle. Cellular disruption and disintegration of mantle appeared to be prominent (Figure 71) due to
washing soda treatment. Heavy metal is reported to disrupt the histological architecture of mantle of mussel *Anodonta cygnea* (Moëzzi et al., 2013). Current result was indicative to a state of cellular disruption in the tissue leading to a possible interference of shell formation and protective function of the organ against mechanical and other stresses. Additionally, the mantle is assumed to act as a physiological barrier against pathogen and toxic entry and acts as a first line of defence. Washing soda induced histological damage of mantle thus be assumed to interfere with the innate immunological efficiency of *B. bengalensis* to evade the toxicity of environmental contaminants and pathogen invasion.

Relative mobility of an organism is functionally associated with various biological activities. Relative mobility of *B. bengalensis* was investigated under the different sublethal concentrations of washing soda along with the control. A significant decrease in trail length was recorded against 700, 1500, 3000 and 5000 ppm of washing soda for 24, 48, 72, 96 hours and 7 days of exposures (Figure 73). Washing soda induced decrease in the relative mobility of *B. bengalensis* is thus assumed to affect the biological activities like food gathering, mate approach and prey escape response (Underwood and Chapman, 1985). Result indicates that with the decrease of relative mobility, *B. bengalensis* may lose a substantial degree of survival fitness in their contaminated habitat.

Gastropod like *B. bengalensis* evolved a well developed muscular foot which occasionally protrudes through its opercular aperture for biological purposes like locomotion, reproduction, food procurement and other sensory activities (Boyden, 1972a; Widdows et al., 1979). Upon exposure of environmental toxins like azadirachtin, bivalve molluscs *Lamellidens marginalis* is reported to reduce the frequency of foot protrusion (Mukherjee, 2010). According to the author, toxin induced physiological irritation might lead to the reduction in the frequency of foot protrusion in *Lamellidens marginalis*. However, sublethal exposures of washing soda yielded a trend of decrease in the protrusion frequency of foot of *B. bengalensis*. Exposure of 700, 1500, 3000 and 5000 ppm of washing soda for 7 days resulted in a marked depletion of foot protrusion response as a symptom of toxicity (Figure 75). Environmental stresses like temperature resulted in an alteration in the valve movement in bivalve mollusc *Corbicula fluminea* (Byrne et al., 1990). Washing soda is thus assumed to act as an interfering agent of behaviour like foot protrusion in *B. bengalensis* which in turn, might affect the related biological activities of this specimen distributed in the contaminated habitat.

Intraspecific aggregation or clumping or grouping is an important behavioural response reported in aquatic molluscs (Casey and Chattopadhyay, 2008). Aggregation behavior is reported to be influenced by various environmental stresses like temperature and desiccation in a high shore mollusc *Littorina unifasciata* (Chapman and Underwood, 1996). According to them, aggregation and dispersal in molluscs
occur in response to various environmental factors like relative humidity, water tide etc. Aggregated snails are thought to preserve larger water reserve and have greater body temperature than the solitary ones. Aggregation in molluscs is reported to serve several ecological functions like defense against predation, physical stabilization and facilitation of reproduction (Huang et al., 2007). Exposure of experimental concentrations of washing soda exhibited a trend of decrease in intraspecific aggregation response in *B. bengalensis*. Toxin induced inhibition in the aggregation response were recorded to be high under the experimental exposure of washing soda for 96 hours and 7 day (Figure 77). Selected ecophysiological functions like water conservation, defense against predation, reproductive potentials and physical stabilization are assumed to be affected due to inhibition in the aggregation response of *B. bengalensis* distributed in the habitat contaminated with washing soda.

A pilot survey carried out by us revealed that washing soda or sodium carbonate acts as a major contaminant of the freshwater ecosystem of the selected geographical regions of the state of West Bengal, India. Domestic effluents, drain water and various human activities in ponds and lakes had been reported as principal routes which caused washing soda contamination of the natural habitat of *B. bengalensis* (Mukherjee et al., 2015). These authors reported immunotoxicological potential of washing soda in *Eunapius carteri*, a common variety of freshwater sponge which shares the habitat with *B. bengalensis* (Figure 2B). Washing soda was found to affect adversely the multiple innate immunological parameters like phagocytosis and cytotoxicity in sponge.

Aim of studying the toxicity of washing soda in *B. bengalensis* was to assess the possible biological and physiological crisis of the specimens exposed to environmentally realistic concentration of washing soda. This organisms itself bears immense ecological and biotechnological importance and a potential bioresource of India. The underprivileged human population inhabiting the rural and urban regions of India largely depends on the edible varieties of molluscs for nutrition and source of ethomedicine. This ageold indigenous item of human diet appears to be a healthy one due to its low content of fat (Baby et al., 2010). Rapid and unrestricted destructions and contamination of the natural habitat of Indian mollusc poses a serious ecotoxicological threat in the process of survival and reproduction of this important bioresource. Report of toxicity of common detergents and their ingredients is grossly insufficient in current scientific literature. Present study indicated a high level of toxicity of washing soda in *B. bengalensis* with reference to hemocyte associated immune parameters, organ toxicities in gill, digestive gland, mantle and selected behavioural profiles. Sublethal exposure of washing soda yielded morphofunctional damage in the different subpopulations of circulating hemocytes and resulted in metabolic and histological damages in the target organs like gill, digestive gland and mantle. Mentioned parameters along with the behavioural alteration are apprehended to reduce the ecological
fitness and reproductive potential of *B. bengalensis* distributed in the habitat contaminated with washing soda and related toxins. Unrestricted contamination of the natural habitat of *B. bengalensis* by washing soda might adversely affect the immunological status and reproductive potential of the same specimens. Such a situation might lead to a shrinkage in the population size of *B. bengalensis* in near future. Formulation of sustainable strategy of conservation of aquatic molluscs, implementation of suitable legislature and public awareness campaign may alleviate this ecotoxicological crisis of *B. bengalensis*. 