

## ORIGINAL ARTICLE

# Washing soda induced alteration of the differential cell count, nonself surface adhesion efficacy and nuclear morphology of the polyphenotypic cells of a freshwater sponge of India

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## ABSTRACT

Washing soda has been identified as a precarious contaminant of the freshwater ponds and lakes, the natural habitat of *Eunapius carteri*. Treatment of sublethal concentrations of washing soda for 384 hours exhibited a significant decrease in the densities of blast like cells, small and large amoebocytes. The percentage occurrence of granular cells and archaeocytes yielded a marked increase against the experimental concentrations of washing soda. Washing soda mediated alterations in the differential cell densities of *E. carteri* indicative of a state of physiological stress and an undesirable shift in the cellular homeostasis of the organism distributed in polluted environment. Experimental exposure of washing soda yielded a significant increase in the cellular dimensions of large amoebocytes and archaeocytes. Prolonged treatment with washing soda presented a gross reduction in nonself surface adhesion efficacy of *E. carteri* cells. Experimental concentrations of washing soda resulted in a dose dependent increment in the frequencies of binucleation and micronucleation in the cells of *E. carteri*. The data were indicative of a high level of genotoxicity of washing soda in *E. carteri*. The present investigation provides an important information base in understanding the toxin induced chemical stress on the archaic immune defense of a primitive urmetazoa.

**KEY WORDS:** cellular adhesion; differential cell count, *Eunapius carteri*, genotoxicity, sodium carbonate

## Introduction

Sponges, the members of the phylum Porifera, evolved before Cambrian and overcame the harsh and stressful conditions of the environment (Müller *et al.*, 2007). Freshwater sponges of the Indian subcontinent are a relatively little researched group of metazoans and demands a special scientific attention of toxicologists and environmentalists. The freshwater ecosystem of India is an abode of a wide range of aquatic bioresources, including members of freshwater sponge. *Eunapius carteri* (Porifera: Demospongiae: Spongillidae), an inhabitant of Indian freshwater ecosystem, is reported as an effective

biomonitoring organism of aquatic pollution (Kakavipure and Yeragi, 2008) and an important component of the biota (Mukherjee *et al.*, 2015a). It is considered a neglected bioresource of Indian freshwater ecosystem which bears the potential to act as a source of bioactive and biomimetic molecules (Manconi *et al.*, 2013). Porous mode of structural organization and ability to generate microcurrent by the ciliary beating of the flagellated choanocytes permit sponges to filter a large volume of water that corresponds to several times their own body volume (Leys *et al.*, 2011). Nonselective filter feeding mode of adaptation enabled sponges to distribute food particles, minerals and gases and thus subjected to exposure of environmental contaminants of natural and anthropogenic origin and can accumulate toxins within their body.

The freshwater ecosystem of India has been facing a serious ecotoxicological risk of contamination by diverse groups of environmental compounds of anthropogenic origin including washing soda, chemically identified as

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anhydrous sodium carbonate (CAS registry number: 497-19-8) (Ray *et al.*, 2015; 2017). Washing soda, synonymous to 'soda ash', is a precipitating builder (Bajpai and Tyagi, 2007) and is a component of laundry detergent (Warne and Schiffko, 1999) which is capable of increasing the alkalinity of water. Washing soda has been reported as a popular brand of cleaning agent used by the rural and semiurban human populations of India for the purposes of cleaning of apparels, washing of utensils and bathing of cattles (Mukherjee *et al.*, 2015b). Glass, paper, metal and mining industries have been identified as major sources of washing soda which contaminates the global environment (UNEP, 2003). Household cleaning products containing washing soda effluents are generally disposed through drain (HERA, 2005) and silently contaminate the freshwater ponds and lakes, the natural habitat of *E. carteri*. On the basis of buffering potential of the aquatic ecosystem, the acceptable concentration of sodium carbonate which can be discharged to the environment varies from 2 to 20 mg/L (HERA, 2005). Thus our experimental concentrations appear to be rational and environmentally realistic. The report of toxicity of washing soda in the aquatic invertebrates is scanty in current scientific literature (Mckee and Wolf, 1963; Warne and Schiffko, 1999). However, washing soda has been identified as an 'immunotoxin' in *E. carteri* (Mukherjee *et al.*, 2015b; 2015c; 2016c). Authors reported washing soda mediated alteration in the phagocytic response, generation of cytotoxic molecules, activities of antioxidant enzymes, lysozyme activity and apoptotic response in the cells of *E. carteri*.

Poriferans mostly depend on the innate immune system (Wiens *et al.*, 2007) to combat the invasion of environmental pathogens, parasites and toxins. For their evolutionary primitiveness, sponges are often considered a model group to study the evolution of metazoan immunity (Wiens *et al.*, 2005). Unlike the vertebrate blood cells, identification, classification and morphofunctional characterization of sponge cells appeared to be non uniform and raised confusions and contradictions among different spongologists and evolutionary biologists. Differential cell density of aquatic invertebrates has been reported as an established immunotoxicological marker for the evaluation of health status of an organism distributed in contaminated environment (Oliver and Fisher, 1999; Chakraborty *et al.*, 2008). Mookerjee and Ganguly (1964) microscopically identified several morphological variants such as archaeocytes, amoebocytes, choanocytes, scleroblasts, pinacocytes and reproductive cells in the freshwater sponge *Ephydatia* sp. While examining the internal defense mechanism of the marine sponge *Terpios zeteki*, Cheng *et al.* (1968) classified the cell types as collencytes, archaeocytes, chromocytes, thesocytes and scleroblasts. Smith and Hildemann (1991) microscopically recorded pinacocytes, choanocytes, archaeocytes, spherulous cells, acid mucopolysaccharide-positive cells, acidophilic granulated cells, sclerocytes and germ cells as the major cell categories of Indo-Pacific marine sponge, *Callyspongia diffusa*. While studying the morphofunctional characterization of *E. carteri* cells, Mukherjee *et*

*al.* (2015a) microscopically identified the archaeocytes as the predominant cell population followed by large amoebocytes and granular cells in the dissociated cell suspension. However, report of toxin mediated alteration in the differential density of sponge cells is almost absent in current scientific literature.

Cellular adhesion is considered a fundamental prerequisite for the establishment of tissue and organ architecture in metazoans. Shift in the adhesive property of the cells might lead to migration of cells from one place to another leading to metastasis (Zetter, 1990; Albelda *et al.*, 1990). Studies on the mechanism of cellular adhesion in sponge revealed that sponge cell adhesion is mediated by large, calcium dependent proteoglycan like molecules termed as "aggregation factors" (Varner, 1995). Fernandez-Busquets *et al.* (2002) demonstrated the role of cellular adhesion in the allogenic recognition process in a marine sponge, *Microcionia prolifera*. Sponges have been considered 'biotools' to study cellular recognition process in biomedical research (Fernandez-Busquets *et al.*, 2002) and model organisms to study cellular adhesion response (Fernandez-Busquets and Burger, 1999). Moreover, the self-nonsel self recognition ability of sponges has been presented as a window to speculate the evolution of histocompatibility system in metazoans. However, a report of toxin mediated alteration of the nonself surface adhesion efficacy of sponge cells is absent in the existing scientific literature.

The micronuclei assay has been considered a biomarker of environmental genotoxicity in monitoring the health of aquatic organisms (Dailianis *et al.*, 2003; Chakraborty and Ray, 2009). Micronuclei are generated from chromosome fragments which may occur due to the problem in cytokinesis or damage in the centrometric region (Heddle *et al.*, 1991). According to Hsu (1982), toxic chemicals can exhibit genotoxic impacts on biological organisms by altering the structure of the DNA, thus resulting in irreversible damage to the structural integrity of chromosome. Correia *et al.* (2017) reported a significant increase in the frequency of micronuclei formation in the coelomocytes of the earthworm *Eisenia andrei* under experimental exposure of titanium silicon oxide nanomaterials, indicating its genotoxicity. Furthermore, report of toxin induced dose dependent augmentation of the frequency of micronucleation is in report in molluscs (Scarpato *et al.*, 1990; Wrisberg *et al.*, 1992), with no information available in sponge. In the present study, genotoxic effects of washing soda were assessed by enumerating the frequencies of binucleation and micronucleation in the cells of *E. carteri*.

The aim of the present investigation is to analyze and quantitate the magnitude of washing soda induced physiological stress in *E. carteri* at cellular and subcellular levels. The toxicity of washing soda in *E. carteri* was examined with reference to differential cell density, nonself surface recognition efficacy, micrometry of cells and genotoxicity. Furthermore, the current analyses would provide an important set of ecotoxicological information regarding the physiological stress of washing soda in *E. carteri* and evaluation of toxicity of washing soda in the freshwater ecosystem of West Bengal.

## Materials and methods

### Collection, transportation and laboratory maintenance of experimental sponge specimens

Live specimens of *E. carteri* were manually collected from the selected freshwater ponds (22° 86'N, 88° 40'E) of the state of West Bengal of India without a history of pisciculture, anthropogenic activities and toxin contamination. Pieces of freshwater sponge were surgically dissected from the submerged plant twigs by sterile scalpel and were immediately transported to the laboratory with ample volume of freshly collected pond water obtained from its natural habitat. The dissolved oxygen, pH and temperature of the pond water were routinely screened during the collection of sponge specimens and maintained accordingly during acclimation of sponge specimens in the controlled laboratory condition (Mukherjee *et al.*, 2015b). Sponges were acclimated in glass aquaria fitted with electrically operated aerator for 7 days and the water of the experimental glass aquaria was replenished routinely at every 24 hours with freshly collected pond water to supplement suspended food and for avoidance of toxicity due to accumulation of excretory products and metabolites (Mukherjee *et al.*, 2015c). Proper illumination and a uniform light rationing of 12:12 hours dark-light cycle were monitored throughout the experiment. The entire experiment on *E. carteri* was designed according to the guidelines and institutional norms of animal ethics and maintenance of the Department of Zoology of the University of Calcutta.

### Experimental design and treatment methodology of *E. carteri* with washing soda

The body mass of experimental *E. carteri* was dissected into pieces each with an approximate dimension of 2 cm<sup>3</sup> containing at least one osculum (Hansen *et al.*, 1995). The dissected body masses of *E. carteri* were maintained in aerated glass aquaria in controlled laboratory conditions for 7 days to minimize the physiological stress and to reorganize their aquiferous system (Duckworth and Pomponi, 2005). Each experimental set consisted of 5 replicates of *E. carteri*, immersed in a volume of 20 l of pond water taken in different glass aquaria (Mukherjee *et al.*, 2016c). Specimens of *E. carteri* were treated with sublethal and environmentally realistic concentrations of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours for toxicological analyses. Parallel control sets with similar replicates of healthy *E. carteri* were maintained in sodium carbonate free water. The highest experimental concentration of 16 mg/L of washing soda was less than one third of the median lethal concentration of the toxin determined in *E. carteri* for 384 hours of treatment. Hydrological parameters like pH, total alkalinity, carbonate ion, bicarbonate ion, calcium, magnesium, sodium and chloride ions of the experimental pond water with and without sodium carbonate were assessed following APHA (1998) (Mukherjee *et al.*, 2015c).

### Mechanical dissociation of *E. carteri* cells and preparation of free cell suspension

Pieces of *E. carteri* with an approximate dimension of 0.5 cm<sup>3</sup> were surgically excised from the healthy specimens and subsequently rinsed with sterile phosphate buffered saline (PBS, pH7.4) to remove clay, sand and other adhered particles prior to experimentation. Dissociated cell suspension of *E. carteri* was prepared by mechanical squeezing of the dissected body fragment through a meshed cloth (Ganguly, 1960) into sterile mineral medium (M-medium: 1 mM CaCl<sub>2</sub>·H<sub>2</sub>O, 0.5 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 mM NaHCO<sub>3</sub>, 0.05 mM KCl, 0.25 mM Na<sub>2</sub>SiO<sub>3</sub>·9H<sub>2</sub>O; pH7.5) (Funayama *et al.*, 2005; Mukherjee *et al.*, 2015a) taken in a prechilled glass vial. The resultant cell suspension was passed through a 50 µm nylon mesh to eliminate spicules and other undissociated masses (Chernogor *et al.*, 2011). The filtrate with suspended cells, was centrifuged (Hermle Z323 K; Hermle Labortechnik, Wehingen, Germany) at 650×g for 10 min, pellets were resuspended in sterile M-medium and stored at 4°C to minimize cellular aggregation. The viability of sponge cells was routinely examined by staining the cells with 0.4% trypan blue (HiMedia, India) employing the principle of vital dye exclusion (Mukherjee *et al.*, 2016a) and did not exhibit significant alteration.

### Enumeration of differential cell density

An aliquot of cell suspension (both control and washing soda treated *E. carteri*) was smeared on separate glass slides using micropipette and incubated for 1 hour. Postincubated cells were air dried for 10 min, fixed in methanol and stained with Giemsa. Differential cell count of washing soda treated *E. carteri* along with the control was carried out microscopically following the field count method and was expressed as relative percentages of the various cell types (Mookerjee & Ganguly, 1964; Mukherjee *et al.*, 2015a). At least 200 sponge cells per glass slide were examined under bright field optics for determination of percentage of different cell types.

### Micrometric analyses of *E. carteri* cells

The micrometric analyses of the different types of cells (*E. carteri* exposed to 8 and 16 mg/L of washing soda for 384 hours along with the control) and their nuclei were determined by estimating the longest axis using an ocular micrometer (Erma, Tokyo, Japan) attached with the microscope (BH-2; Olympus, Tokyo, Japan) (Mukherjee *et al.*, 2015a).

### Estimation of nonself surface adhesion efficacy of cells

The nonself surface adhesion efficacy of *E. carteri* cells was assessed after the protocol of Chen and Bayne (1995) with necessary modifications. Freshly prepared sponge cell suspension with a density of 10<sup>6</sup> cells/ml from control and washing soda treated *E. carteri* was placed on clean and sterilized glass slides and incubated in a humid chamber at 25°C for 180 minutes for adequate adhesion. Supernatants of postincubated cell suspensions were carefully removed by a micropipette and the number of

non-adherent cells was enumerated using a Neubauer hemocytometer attached to the microscope (Axiostar Plus; Zeiss Microscopy, Jena, Germany).

For analysis of the morphology of adherent sponge cells, the *E. carteri* cells were examined under a scanning electron microscope (Zeiss EVO 18 special edition, Germany). The sponge cells were fixed in 3% glutaraldehyde (Sigma, USA) dissolved in 0.1 M sodium cacodylate buffer containing 12% glucose (pH 7.8) for 2 h at 4°C (Mukherjee *et al.*, 2015b). This was followed by postfixation in 1% osmium tetroxide (Sigma, USA) for 1 h. Fixed cells were dehydrated with graded ethanol, air dried, sputter coated with gold in an ionic coater for scanning electron microscopy.

#### Detection of nuclear anomalies in cells of *E. carteri*

Relative abundance of micronucleated and binucleated cells of *E. carteri* was estimated after the modified protocol of Fenech *et al.* (2003) and Bolognesi and Fenech (2012). The Giemsa stained cells were examined under light microscope (Axiostar Plus; Zeiss Microscopy, Jena, Germany) for different kinds of nuclear aberrations. Digital photodocumentation and analyses of different nuclear anomalies of *E. carteri* cells was carried out using a CCD camera (ProgRes C5; Jenoptik, Jena, Germany) attached to the light microscope (BH-2; Olympus, Tokyo, Japan). Round or ovoid shaped non-refractory particles with the color and structure identical to chromatin with diameter equaling 1/3–1/20 of the main nucleus and its clear detachment from macronucleus were interpreted as ‘micronuclei’ (Chakraborty & Ray, 2009). On the other

hand, binucleated cells consisted of two nuclei with intact nuclear membrane and identical staining intensity and were found within the same cytoplasmic boundaries. At least 450 cells were examined per slide for the enumeration of micronucleated and binucleated cells in *E. carteri* treated with washing soda.

#### Statistical analyses

The experimental data were initially checked for normality and homogeneity using Bartlett’s test (Mukherjee *et al.*, 2016c). Since all data were normal, parametric statistics were applied, following one-way analysis of variance (ANOVA). The statistical data analysis was carried out using one way ANOVA followed by Dunnett’s post hoc test (GraphPad Prism version 5 for windows, GraphPad Software, La Jolla California, USA) among the samples of each experimental hour to determine the level of significance between control and treatment sets (Gopalakrishnan *et al.*, 2009; Mukherjee *et al.*, 2015c). Each experiment was repeated 5 times. Data were presented as the mean  $\pm$  standard deviation (S.D). Differences were considered significant at \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Results

#### Washing soda mediated shift in the differential cell count of *E. carteri*

Light microscopical analyses of dissociated *E. carteri* cells revealed the existence of eight distinct cellular variants, *i.e.* blast like cells, choanocytes, small amoebocytes, granular cells, pinacocytes, large amoebocytes,

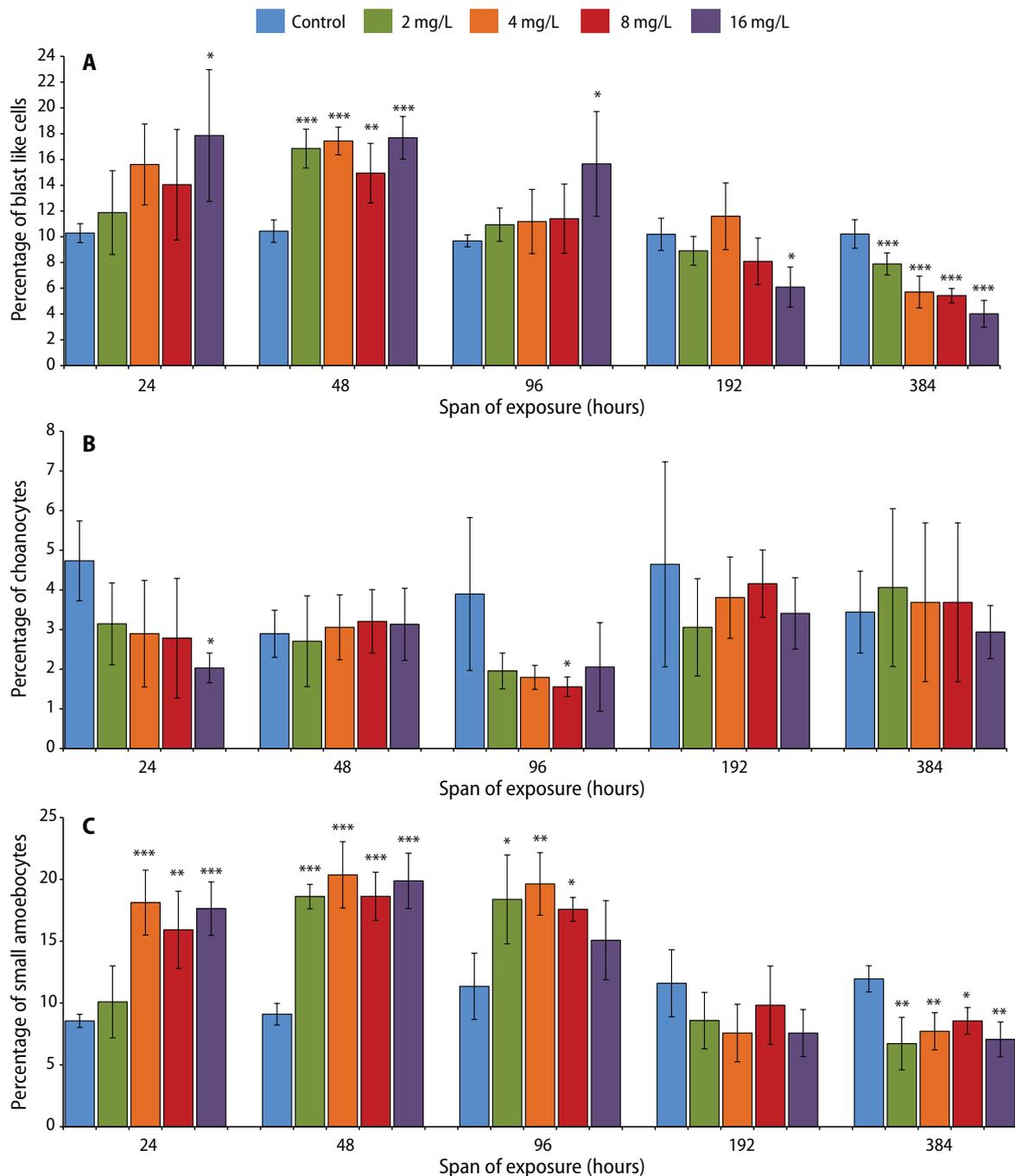
**Table 1.** Micrometric estimations of the cell types of *E. carteri* exposed to 8 and 16 mg L<sup>-1</sup> of washing soda for 384 hours.

Cell types	Dimensions	Exposure to washing soda for 384 h		
		Control for 384 h	8 mg/L	16 mg/L
Blast like cells	Cell (C) diameter; $\mu\text{m}$	5.85 $\pm$ 0.36	6.11 $\pm$ 0.32	6.09 $\pm$ 0.51
	Nuclear (N) diameter; $\mu\text{m}$	3.91 $\pm$ 0.22	4.01 $\pm$ 0.59	3.67 $\pm$ 1.14
	N/C ratio	0.66 $\pm$ 0.05	0.65 $\pm$ 0.02	0.58 $\pm$ 0.08
Choanocytes	Cell (C) diameter; $\mu\text{m}$	5.52 $\pm$ 1.16	5.66 $\pm$ 0.73	5.74 $\pm$ 0.45
	Nuclear (N) diameter; $\mu\text{m}$	1.65 $\pm$ 1.05	1.29 $\pm$ 0.11	1.78 $\pm$ 0.33
	N/C ratio	0.28 $\pm$ 0.10	0.23 $\pm$ 0.06	0.31 $\pm$ 0.03
Small amoebocytes	Cell (C) diameter; $\mu\text{m}$	7.67 $\pm$ 2.98	8.97 $\pm$ 2.00	9.45 $\pm$ 2.27
	Nuclear (N) diameter; $\mu\text{m}$	2.93 $\pm$ 1.50	3.93 $\pm$ 0.48	3.91 $\pm$ 0.53
	N/C ratio	0.38 $\pm$ 0.01	0.43 $\pm$ 0.01	0.39 $\pm$ 0.06
Granular cells	Cell (C) diameter; $\mu\text{m}$	18.79 $\pm$ 3.15	19.11 $\pm$ 0.68	21.46 $\pm$ 1.71
	Nuclear (N) diameter; $\mu\text{m}$	5.68 $\pm$ 0.89	5.90 $\pm$ 1.86	5.92 $\pm$ 1.61
	N/C ratio	0.30 $\pm$ 0.01	0.31 $\pm$ 0.04	0.29 $\pm$ 0.08
Pinacocytes	Cell (C) diameter; $\mu\text{m}$	16.44 $\pm$ 5.57	15.14 $\pm$ 2.72	16.98 $\pm$ 1.31
	Nuclear (N) diameter; $\mu\text{m}$	6.49 $\pm$ 0.56	6.78 $\pm$ 0.66	7.13 $\pm$ 0.95
	N/C ratio	0.43 $\pm$ 0.11	0.44 $\pm$ 0.15	0.42 $\pm$ 0.12
Large amoebocytes	Cell (C) diameter; $\mu\text{m}$	20.81 $\pm$ 0.92	22.49 $\pm$ 0.80	23.45 $\pm$ 0.50*
	Nuclear (N) diameter; $\mu\text{m}$	6.16 $\pm$ 1.15	7.26 $\pm$ 1.29	6.91 $\pm$ 1.32
	N/C ratio	0.30 $\pm$ 0.02	0.32 $\pm$ 0.03	0.28 $\pm$ 0.04
Archaeocytes	Cell (C) diameter; $\mu\text{m}$	22.35 $\pm$ 0.96	22.32 $\pm$ 0.88	24.9 $\pm$ 0.30*
	Nuclear (N) diameter; $\mu\text{m}$	6.75 $\pm$ 0.84	6.53 $\pm$ 1.11	7.10 $\pm$ 0.95
	N/C ratio	0.31 $\pm$ 0.05	0.29 $\pm$ 0.01	0.28 $\pm$ 0.03
Sclerocytes	Cell (C) diameter; $\mu\text{m}$	11.02 $\pm$ 1.81	10.48 $\pm$ 1.48	11.21 $\pm$ 1.28
	Nuclear (N) diameter; $\mu\text{m}$	3.12 $\pm$ 0.43	2.86 $\pm$ 0.47	3.10 $\pm$ 0.44
	N/C ratio	0.28 $\pm$ 0.07	0.25 $\pm$ 0.11	0.24 $\pm$ 0.09

Data were presented as mean  $\pm$  S.D. (n=5). The asterisks indicate the values that were significantly different from the control (\* $p < 0.05$ ).

archaeocytes and sclerocytes (Mukherjee *et al.*, 2015a). The authors microscopically identified and characterized the sponge cells on the basis of their shapes, dimensions, cytoplasmic inclusions and nuclear-cytoplasmic ratios. Differential count of cellular subpopulations in *E. carteri* exposed to sublethal concentrations of washing soda along with the control was enumerated microscopically employing bright field optics. Upon treatment with 2, 4, 8 and 16 mg/L of washing soda for multiple spans

of time, *i.e.* 24, 48, 96, 192 and 384 hours, the cellular subpopulations exhibited differential changes in density distribution (Figures 1–3). The relative percentage of blast like cells of control *E. carteri* ranged from  $10.28 \pm 0.73$  to  $10.21 \pm 1.10$  over a time span of 24 to 384 hours respectively (Figure 1A). The density of blast like cells was significantly increased against all the experimental concentrations of washing soda for 48 hours and 16 mg/L of toxin for 24 and 96 hours. The highest density of blast like cells was

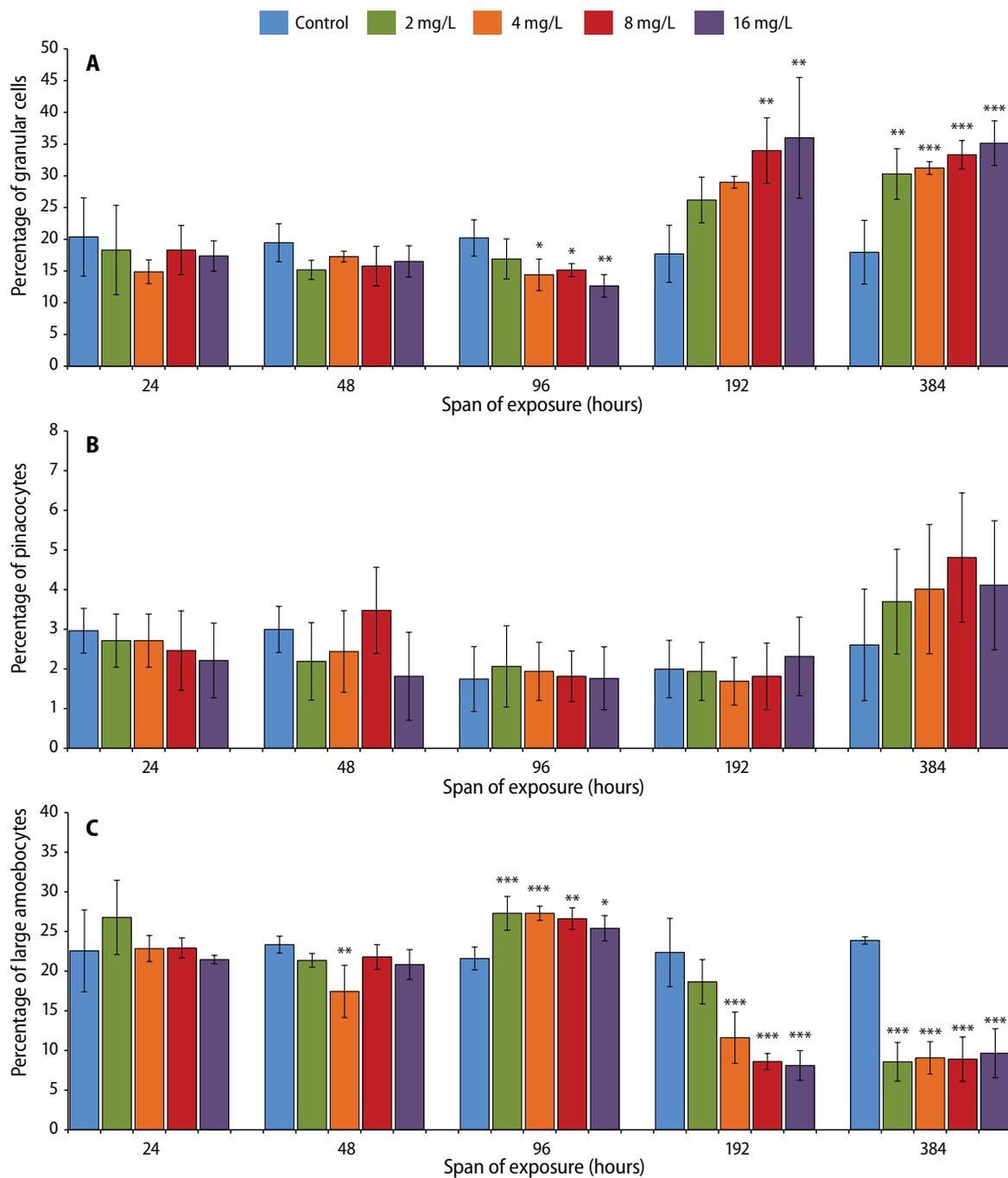


**Figure 1.** Dynamics of densities of blast like cells (A), choanocytes (B) and small amoebocytes (C) of *E. carteri* under the exposure of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean  $\pm$  S.D. (n=5). The asterisks indicate the values that were significantly different from the control (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

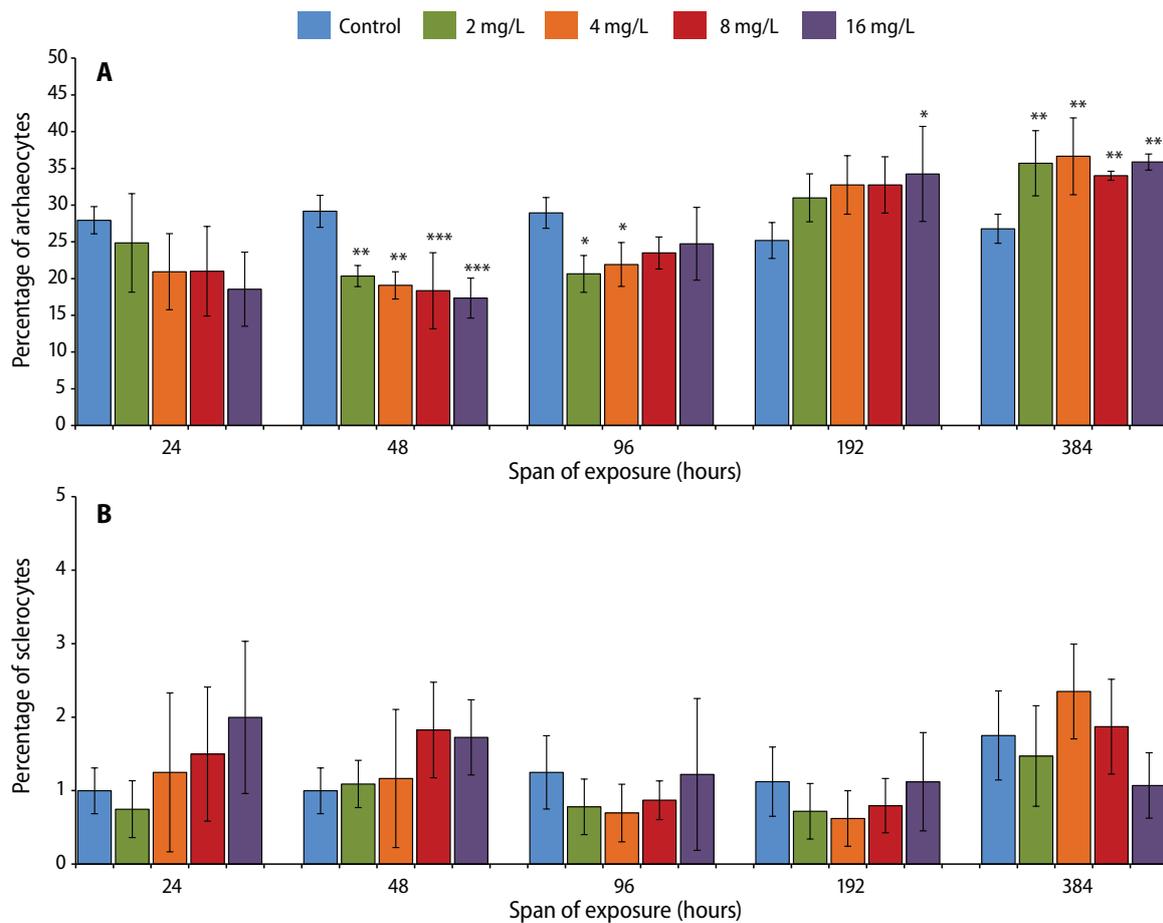
recorded as  $17.85 \pm 5.11\%$  under 16 mg/L of washing soda treatment for 24 hours. However, prolonged exposure of washing soda for 384 hours exhibited a dose dependent decrease in the density of blast like cells. The maximum inhibition in the relative percentage of blast like cells was recorded to be  $4.01 \pm 1.04$  under 16 mg/L of washing soda for 384 hours.

The relative density of choanocyte subpopulation of control *E. carteri* ranged from  $4.73 \pm 1.01\%$  to  $3.44 \pm 1.03\%$

over a time span of 24 to 384 hours (Figure 1B). A nonlinear dose independent fluctuation in the density of choanocytes was recorded under all experimental concentrations of washing soda for different time spans. Experimental exposure of 16 mg/L of washing soda for 24 hours and 8 mg/L for 96 hours exhibited a significant decrease in the relative percentage of choanocyte subpopulation. In control *E. carteri*, the percent density of small amoebocyte subpopulation varied from  $8.56 \pm 0.52$  to  $11.95 \pm 1.06$



**Figure 2.** Dynamics of densities of granular cells (A), pinacocytes (B) and large amoebocytes (C) of *E. carteri* under exposure to 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean  $\pm$  S.D. (n=5). The asterisks indicate the values that were significantly different from the control (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 3.** Dynamics of densities of archaeocytes (A) and sclerocytes (B) of *E. carteri* under the exposure of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean  $\pm$  S.D. (n=5). The asterisks indicate the values that were significantly different from the control (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001).

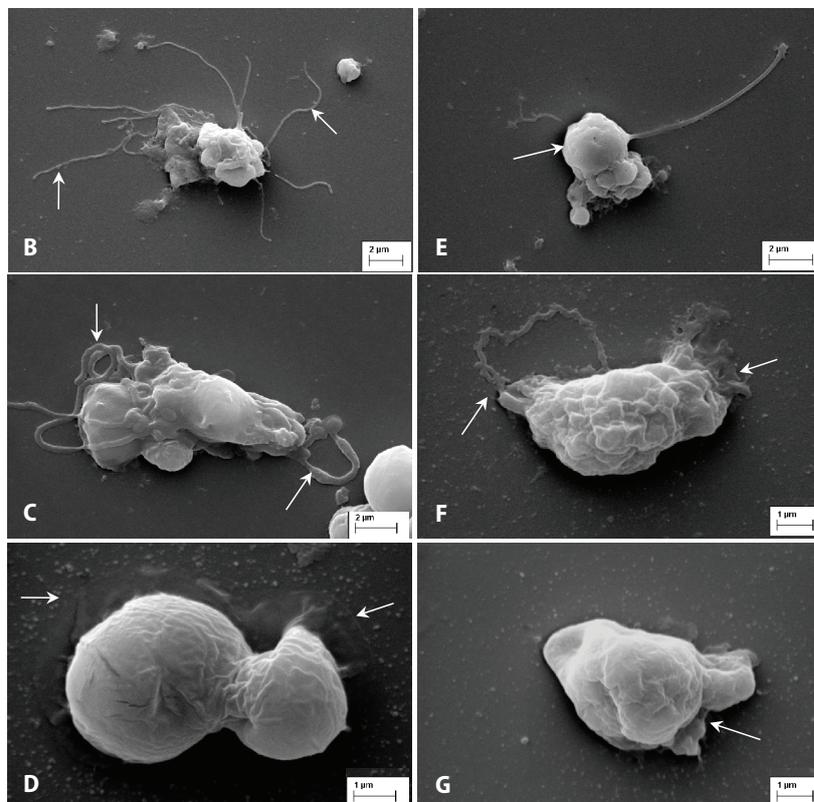
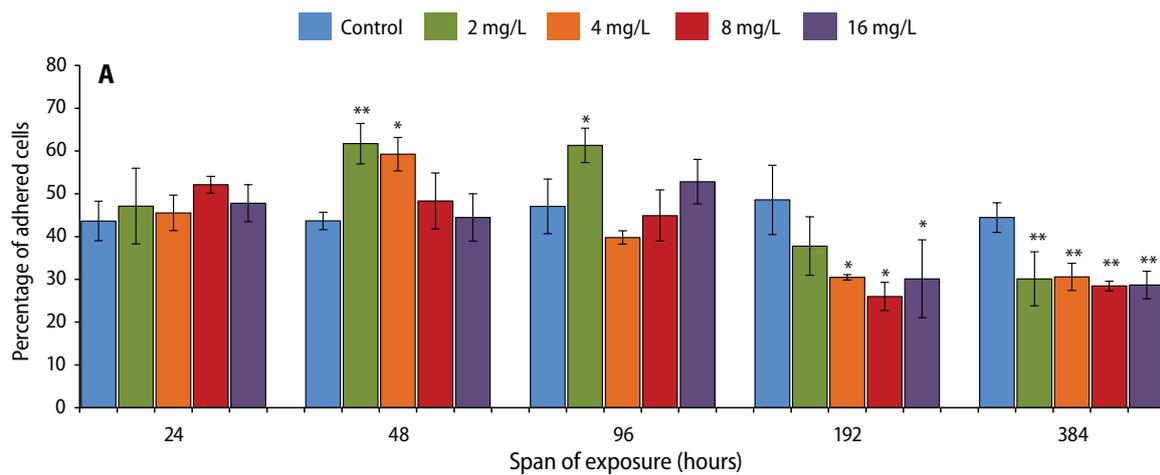
for different time spans (Figure 1C). A trend of increase in the percentage occurrence of small amoebocytes was recorded against the experimental concentrations of washing soda up to 96 hours. The highest density of small amoebocyte subpopulation was recorded as  $20.36 \pm 2.68\%$  in *E. carteri* treated with of 4 mg/L of washing soda for 48 hours. However, long-term exposure of washing soda for 384 hours presented an overall decrease in the relative density of small amoebocyte subpopulation against all experimental concentrations. The highest inhibition in the percentage of small amoebocytes was recorded as  $6.71 \pm 2.11$  against 2 mg/L of washing soda for 384 hours of exposure. The percentage of granular cells ranged from  $20.36 \pm 6.17$  to  $17.96 \pm 5.02$  in untreated *E. carteri* over a time span of 24 to 384 hours respectively (Figure 2A). A trend of decrease in the density of granular cells was recorded under 4, 8 and 16 mg/L of washing soda treatment for 96 hours. The lowest density of granular cells was recorded as  $12.64 \pm 1.78\%$  in *E. carteri* exposed to 16 mg/L of washing soda for 96 hours. However, treatment of sublethal concentrations of washing soda for 192 and 384 hours exhibited a trend of increase in the density

of granular cells. The maximum density of granular cells was recorded as  $35.98 \pm 9.52\%$  under 16 mg/L of washing soda exposure for 192 hours. The percent density of pinacocyte subpopulation of untreated *E. carteri* ranged from  $2.96 \pm 0.56$  to  $2.60 \pm 1.40$  for multiple time spans (Figure 2B). Experimental exposure of washing soda did not exhibit any significant alteration in the percentage occurrence of pinacocytes.

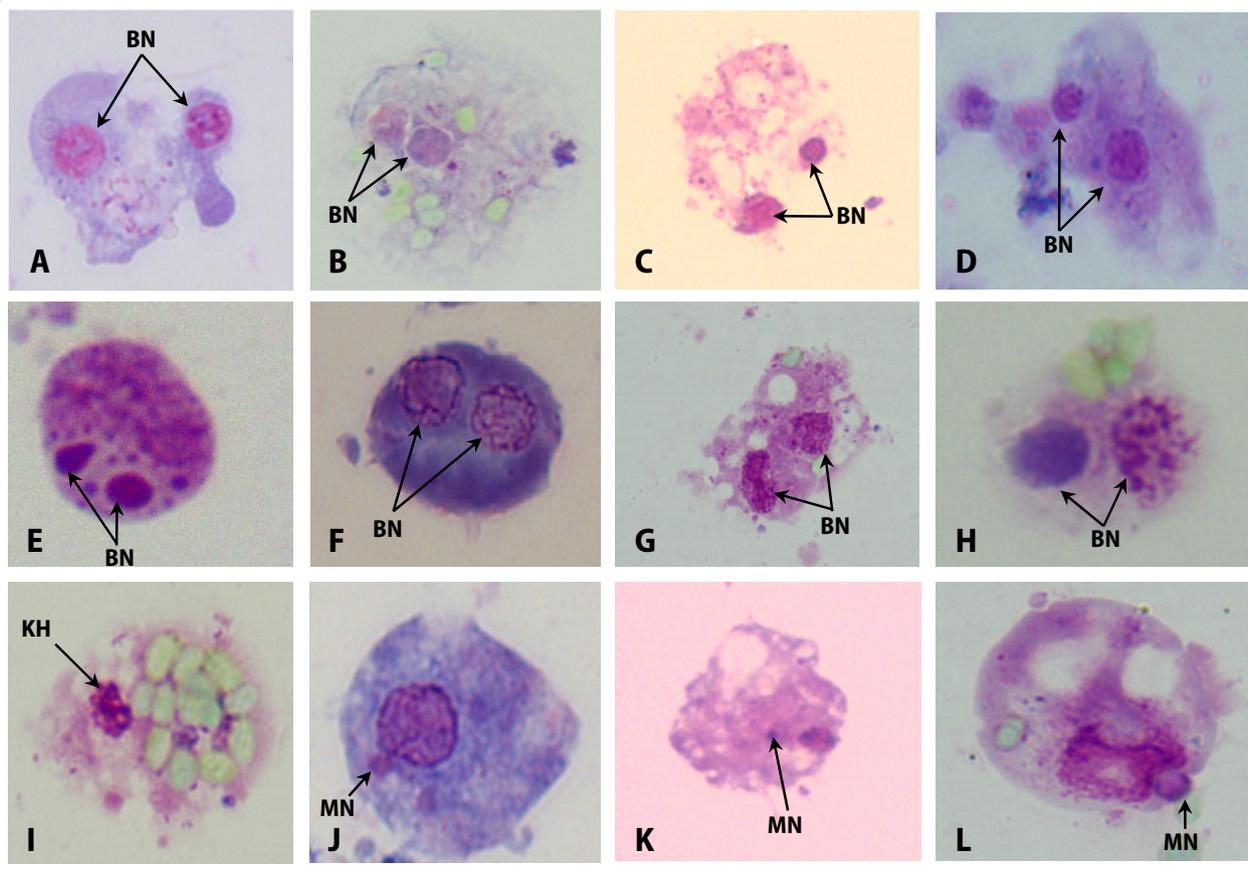
The relative density of large amoebocytes in the control *E. carteri* ranged from  $22.56 \pm 5.16\%$  to  $23.87 \pm 0.45\%$  over a time span of 24 to 384 hours (Figure 2C). Experimental exposure of washing soda up to 48 hours did not exhibit any significant alteration in the percentage occurrence of large amoebocytes except in *E. carteri* treated with 4 mg/L of washing soda for 48 hours which exhibited a significant decrease in comparison to that of the control. A significant increase in the percentage of large amoebocytes was recorded under 2, 4, 8 and 16 mg/L of washing soda for 96 hours. The maximum density of large amoebocytes was recorded as  $27.31 \pm 2.13\%$  under the experimental exposure of 2 mg/L of washing soda for 96 hours. A dose dependent decrease in the relative percentage of large

amoebocytes was estimated in *E. carteri* for 192 hours of washing soda exposure. However, prolonged treatment of sodium carbonate for 384 hours presented an overall depletion in the percentage of large amoebocytes in all experimental concentrations. The maximum inhibition in the relative percentage of large amoebocytes was recorded as  $8.11 \pm 1.86$  against 16 mg/L of washing soda for 192 hours of exposure. The percent density of archaeocytes of

untreated *E. carteri* varied from  $27.94 \pm 1.85$  to  $26.77 \pm 1.97$  for different time spans (Figure 3A). An inhibition in the percentage of archaeocyte subpopulation was recorded under the exposure of all experimental concentrations of washing soda for 48 hours and 2, 4 mg/L of toxin for 96 hours. The maximum inhibition in the percentage occurrence of archaeocytes was recorded as  $17.34 \pm 2.72$  under the treatment of 16 mg/L of washing soda for 48 hours.



**Figure 4.** Percentage of adherent cells of *E. carteri* exposed to 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours (A). Data were presented as mean  $\pm$  S.D. (n=5). The asterisks indicate the values that were significantly different from the control (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). Scanning electron micrographs of adherent cells of *E. carteri* on the glass surface (B–G). B, C, D represent the cells of the control *E. carteri* and E, F, G exhibit the sponge cells treated with 16 mg/L of washing soda for 384 hours. The cells of the control *E. carteri* exhibited extensive membrane involutions (B) as well as longer cytoplasmic projections (B, C, D). Exposure of washing soda yielded membrane smoothing (E) and relative decrease of pseudopodial projections (E, F, G) in the cells of *E. carteri* as evident from ultrastructural analyses.



**Figure 5.** Light microscopical identification of nuclear anomalies in the cells of *E. carteri* exposed to 16 mg/L of washing soda for 384 hours. Washing soda yielded binucleation (BN) (A–H), karyorrhexis (KH) or nuclear fragmentation (I) and micronucleation (MN) (J–L) in the cells of *E. carteri*. Scale bar: 10  $\mu$ m.

However, a significant increase in the relative percentage of archaeocytes was recorded under 16 mg/L of washing soda for 192 hours and all experimental concentrations of sodium carbonate for 384 hours. The maximum elevation in the density of archaeocyte subpopulation was recorded as  $36.64 \pm 5.22\%$  against 4 mg/L of washing soda for 384 hours of exposure. The relative density of sclerocyte subpopulation was recorded to be the least among other subpopulations of cells. Sclerocytes were rarely encountered in the dissociated cell suspension of *E. carteri*. The percent density of sclerocytes of control *E. carteri* ranged from  $0.99 \pm 0.31$  to  $1.75 \pm 0.60$  over a time span of 24 to 384 hours respectively (Figure 3B). Treatment of washing soda did not present any significant alteration in the relative density of sclerocyte subpopulation.

#### Micrometric alteration of *E. carteri* cells under the exposure of washing soda

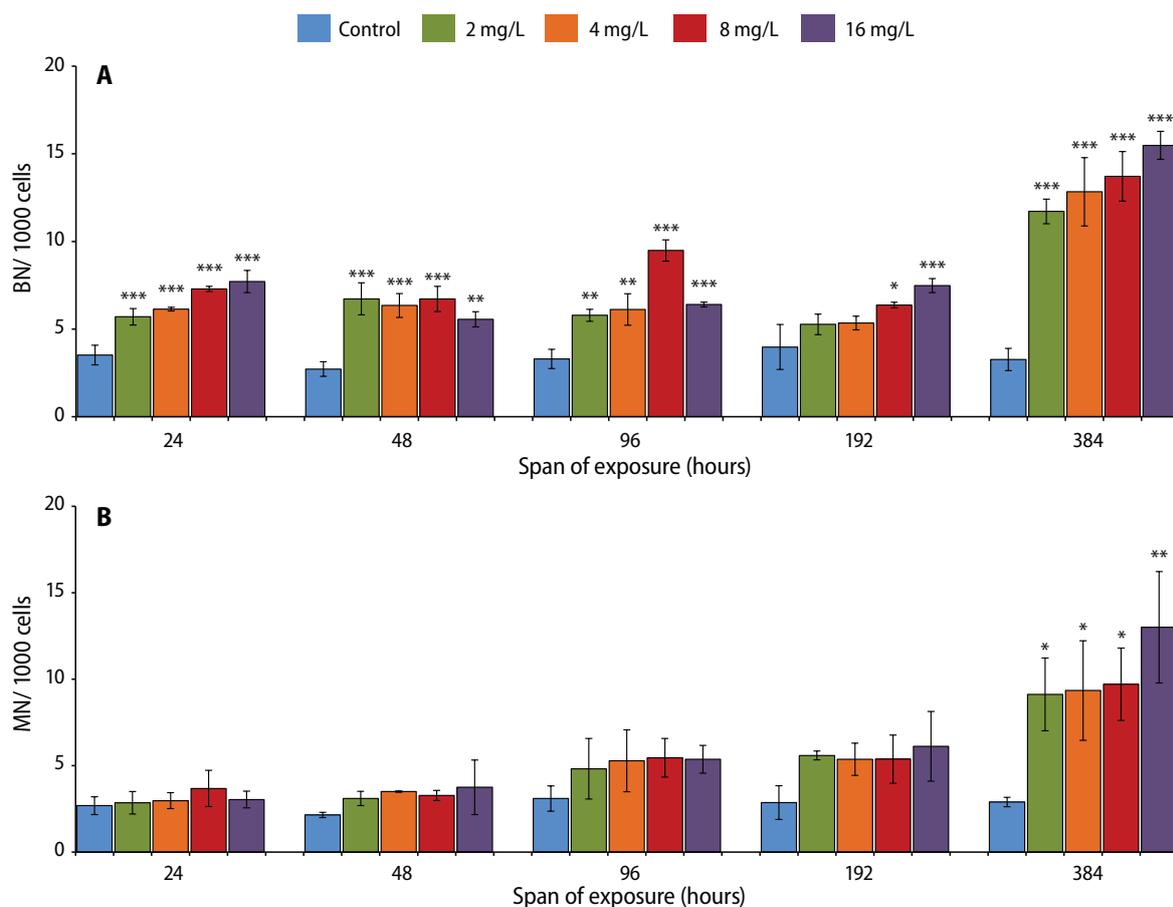
Micrometric analyses of different types of cells were estimated in *E. carteri* treated with 8 and 16 mg/L of washing soda for 384 hours along with controls (Table 1). Cell and nuclear diameters and nuclear-cytoplasmic (N/C) ratios were microscopically estimated in blast like cells, choanocytes, small amoebocytes, granular cells, pinacocytes, large amoebocytes, archaeocytes and sclerocytes of

control and washing soda exposed specimens employing bright field optics. Micrometric analyses of sponge cells revealed significant swelling in the cellular dimensions of large amoebocytes and archaeocytes of *E. carteri* exposed to 16 mg/L of washing soda for 384 hours in comparison to the respective controls.

#### Washing soda mediated shift in the nonself surface adhesion efficacy of *E. carteri* cells

When the cells of *E. carteri* were allowed to settle over the glass surface, cells got adhered, flattened and exhibited pseudopodial extensions as evident from their scanning electron microscopic images (Figure 4B–G). Morphologically, the adherent cells of control *E. carteri* were characterized by their pseudopodial projections over the glass surface (Figure 4B–D). Cells exposed to sublethal concentrations of washing soda exhibited distinctive morphological alterations and relative reduction in the cytoplasmic projections, membrane involutions and natural spreading behavior (Figure 4E–G).

Percentage occurrence of adherent cells of *E. carteri* was microscopically enumerated under the experimental exposure of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours of time span (Figure 4A). The percentage of adhered cells of control *E. carteri* ranged



**Figure 6.** Frequencies of binucleation (A) and micronucleation (B) in the cells of *E. carteri* treated with 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean  $\pm$  S.D. ( $n=5$ ). The asterisks indicate the values that were significantly different from the control (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ).

from  $43.63\pm 4.62$  to  $44.64\pm 3.46$  over a time span of 24 to 384 hours respectively. A significant increase in the percentage of adherent cells was recorded in *E. carteri* treated with 2 mg/L of washing soda for 48 and 96 hours and 4 mg/L of toxin for 48 hours. The highest percentage of adherent cells was recorded to be  $61.71\pm 4.72$  in *E. carteri* exposed to 2 mg/L of washing soda for 48 hours. A significant decrease in the percentage of adherent cells was recorded in *E. carteri* treated with 4, 8 and 16 mg/L of washing soda for 192 hours. Long-term exposure of washing soda for 384 hours yielded an overall depletion in the cellular adhesion response of sponge cells under all experimental concentrations. The maximum inhibition in the percentage of adherent cells was recorded as  $26\pm 3.28$  against 8 mg/L of washing soda for 192 hours of exposure.

#### Washing soda induced nuclear anomalies in the cells of *E. carteri*

Light microscopical observation revealed that experimental exposure of washing soda appears to be genotoxic in *E. carteri* as evident from the appearance of nuclear anomalies, i.e. binucleation (Figure 5A–H), micronucleation (Figure 5J–L) and karyorrhexis or nuclear fragmentation

(Figure 5I) in the sponge cells. The frequency of binucleation (Figure 6A) and micronucleation (Figure 6B) in the cells of *E. carteri* were estimated microscopically under 2, 4, 8 and 16 mg/L of washing soda for multiple spans of exposure along with the respective controls. Normal physiological levels of binuclei frequencies ranged from  $3.51\pm 0.55$  to  $3.26\pm 0.63$  per 1000 cells during 24 to 384 hours of observations made in the control *E. carteri*. A marked increase in the frequency of binuclei was recorded against the exposure of all experimental concentrations of washing soda for 24, 48 and 96 hours in comparison to the controls. Washing soda exposure of 8 and 16 mg/L for 192 hours exhibited a significant increase in the frequency of binucleation in the cells of *E. carteri*. However, all the experimental concentrations of washing soda for 384 hours of treatment presented a dose dependent increase in the frequency of binucleation in the cells of *E. carteri*. The highest frequency of binuclei was recorded as  $15.48\pm 0.79$  per 1000 cells in *E. carteri* treated with 16 mg/L of washing soda for 384 hours. The micronuclei frequencies in the cells of control *E. carteri* ranged from  $2.89\pm 0.27$  per 1000 cells over a time span of 24 to 384 hours. Experimental exposures of washing soda up to a

span of 192 hours did not yield any significant alteration in the frequencies of micronuclei. However, long term exposure of washing soda for 384 hours exhibited a significant increase in the frequency of micronucleation under all experimental concentrations. The highest frequency of micronuclei was recorded as  $13.01 \pm 3.21$  per 1000 cells in *E. carteri* treated with 16 mg/L of washing soda for 384 hours.

## Discussion

For their developmental characteristics, sponges are often considered candidate species in evaluating the toxicity of aquatic ecosystem (Goh, 2008). From a scientific point of view, sponges are important because of their unusual cellular organization, their ability to regenerate the damaged parts and their biochemical uniqueness. Wagner *et al.* (1998) proposed sponges as a model organism to evaluate the toxicity of environmental compounds of anthropogenic origin. *E. carteri* is considered an “ecological hotspot” (Mukherjee *et al.*, 2016b) and is able to establish a wide array of functional relationships with other aquatic organisms, including small crabs, shrimps, insect larvae, water mites, nematodes, oligochaetes, polychaetes, ciliates, molluscs (Soota, 1991) in their shared natural habitat. Many of these organisms utilize the colony of *E. carteri* as suitable sites for reproduction, parental care and shelter.

The impact of detergents and their allied compounds on the aquatic ecosystem and human health have been an issue of concern for many decades due to their widespread production and use (Pedrazzani *et al.*, 2012). Warne and Schifko (1999) revealed the toxicity of sodium carbonate in the freshwater cladocera, *Ceriodaphnia dubie*. Azizullah *et al.* (2011) reported the toxicity of laundry detergent in the freshwater flagellate *Euglena gracilis*. Sublethal concentrations of pesticides, rogar and endosulfan were reported to increase the protein content of the freshwater sponge *Spongilla lacustris* (Ingle *et al.*, 2003). According to Cebrian *et al.* (2003), Mediterranean sponge *Crambe crambe* has been reported to accumulate moderate concentrations of lead, copper and vanadium within its body. Contamination of natural habitat under the face of metal pollution might lead to an alteration in the physiological growth, fecundity and survival of the experimental species.

Due to apparent nonexistence of well constructed tissues and organs, sponges present morphofunctional diversities of heterogenous populations of cells to perform diverse physiological functions including nonself surface adhesion, cell-cell aggregation, phagocytosis and generation of cytotoxic molecules, etc. (Smith & Hildemann, 1991; Varner, 1995; Philip, 1997; Peskin *et al.*, 1998; Leys & Eerkes-Medrano 2006; Mukherjee *et al.*, 2015a). Russo and Lagadic (2000) reported hexachlorobenzene and atrazine mediated induction in the hyalinocyte population of mollusc, *Lymnaea palustris*. Chakraborty *et al.* (2008) reported inorganic arsenite induced modulation in the relative densities of hemocyte subpopulations of freshwater bivalve *Lamellidens marginalis*, which shares

the same habitat with *E. carteri*. In the present investigation, treatment of 2, 4, 8 and 16 mg/L of washing soda for 48 hours yielded a significant increase in the percentage occurrence of blast like cells (Figure 1A). However, persistent exposure of washing soda for 384 hours yielded a dose dependent decrease in the relative percentage of blast like cells. Treatment of sublethal concentrations of washing soda up to a span of 96 hours resulted in an overall increase in the relative density of small amoebocytes of *E. carteri* followed by a decrease at 384 hours (Figure 1C). Washing soda exposure for 192 and 384 hours resulted in a dose dependent induction in the percentage occurrence of granular cells in *E. carteri* (Figure 2A). Treatment of all experimental concentrations of washing soda for 96 hours exhibited a significant increase in the density of large amoebocyte subpopulation (Figure 2C). However, washing soda exposure for 192 and 384 hours resulted in a gradual depletion in the percentage occurrence of large amoebocytes in comparison to that of the controls. Washing soda treatment of 2, 4, 8 and 16 mg/L for 384 hours yielded a significant increase in the relative percentage of archaeocytes in *E. carteri* (Figure 3A).

The blast like cells are relatively smaller in size, with large central nuclei which resemble mammalian lymphocytes. Funayama *et al.* (2010) proposed the blast like cells as immature choanocytes or choanoblasts and demonstrated their involvement in the development and differentiation processes in the freshwater sponge *Ephydatia fluviatilis*. The archaeocytes, on the other hand, played a pivotal role in the reconstitution and reorganization of the dissociated cells to form an adult functional sponge and bore the potential of stemness (Funayama, 2010). The granular cells are a special type of sponge cells with intense cytoplasmic granulation and play a pivotal role in glycogen synthesis. Sclerocytes are responsible for the formation of siliceous sponge spicules by accumulating and depositing silicate in an organized fashion. Archaeocytes, large amoebocytes and granular cells were identified as chief phagocytes of *E. carteri* which were capable of engulfing foreign particles (Mukherjee *et al.*, 2015a). Archaeocytes and large amoebocytes were recorded as major generators of superoxide anion and nitric oxide in *E. carteri*. Washing soda mediated undesirable shift in the differential cell density of *E. carteri* might adversely affect the cell mediated innate immunological status and physiological homeostasis of the organism distributed in a polluted environment. Moreover, the present data are indicative of possible interference of multiple functional attributes like phagocytosis, nonself recognition efficacy and generation of cytotoxic molecules in different cell types of *E. carteri* distributed in washing soda contaminated habitat.

Sublethal and environmentally realistic concentrations of washing soda yielded substantial morphological damage in the heterogenous cell populations of *E. carteri* (Mukherjee *et al.*, 2015c). The authors identified cytoplasmic disintegration, nuclear dissolution, cell surface smoothening, hypervacuolation and membrane blebbing as principal morphological damage of *E. carteri* cells

under washing soda exposure. According to them, washing soda induced morphological damage of *E. carteri* cells was indicative to impairment of overall morpho-functional status of the organism inhabiting detergent contaminated habitat. Mukherjee *et al.* (2015a) reported the micrometric data of different cell types of control *E. carteri*. However, report of toxin induced micrometric alteration of the cell types of sponge is absent in current scientific literature. Chakraborty *et al.* (2013) reported micrometric alteration and significant swelling in the hemocytes of freshwater bivalve, *L. marginalis* under sublethal concentrations of sodium arsenite, a geogenic toxin. Calisi *et al.* (2008) recorded cadmium chloride induced morphological alterations in the granulocytes of mollusc, *Mytilus galloprovincialis*. The authors reported enlargement of granulocyte morphology and rounding up of cells due to loss of pseudopodial projections in *M. galloprovincialis* under the experimental exposure of cadmium. According to them, pollutant induced morphometric alterations in the granulocytes of mollusc might function as a biomarker for monitoring and assessment of the degree of environmental contamination. In a separate study, Calisi *et al.* (2009) demonstrated the enlargement of granulocytes of the earthworm, *Eisenia fetida*, under exposure to copper sulfate and methiocarb. The authors claimed the studied parameter as a sensitive biomarker for monitoring the health of earthworm distributed in the contaminated habitat. In this present investigation, washing soda exposure of 16 mg/L for 384 hours resulted in a significant increase in the cellular dimensions of the large amoebocytes and archaeocytes (Table 1). The washing soda induced micrometric alterations of the cells were suggestive of an impairment of the structural integrity and physiological activity of cells of *E. carteri* distributed in polluted environment.

Throughout the course of evolution, cell-cell and cell-substratum adhesion play a significant role for the purposes of different physiological functioning including food uptake, epithelium formation and self-nonsel recognition, development as well as maintenance of body plan in metazoans (Varner, 1995). The degree of cell spreading and nonself surface adhesion efficacy of molluscan hemocytes had been modulated by environmental chemicals (Chen & Bayne, 1995). Canesi *et al.* (2001) demonstrated the surface interaction between the bacteria *Escherichia coli* and the hemocytes of the Mediterranean mussel, *M. galloprovincialis*. Anderson *et al.* (2011) reported modulation in hemocyte number and cellular adhesion in  $\beta$ -1, 3 glucan treated mollusc, *Crassostrea virginica*. Experimental exposure of cholera toxin modulated the hemocyte adhesion efficacy of wax moth, *Galleria mellonella* (Lapointe *et al.*, 2012). According to Wootton and Pipe (2003), phagocytosis is principally dependent on the membrane properties of hemocytes and alteration in the adhesive property of hemocytes may lead to a decrease in phagocytic activity. In the present investigation, sublethal concentrations of washing soda for 384 hours resulted in a significant inhibition in the nonself surface adhesion efficacy of cells of *E. carteri* (Figure 4A). The result was

indicative to an undesirable shift in cellular integrity, morphogenesis and maintenance of body plan in *E. carteri* distributed in washing soda contaminated habitat.

The micronuclei assay is considered an established method to study the genotoxicity and mutagenicity of a chemical compound by estimating the structural and numerical aberrations of chromosomes (Bolognesi and Cirillo, 2014). An increase in the frequency of micronucleation was recorded in the hemocytes of moth, *Galleria mellonella* exposed to deltamethrin, a pyrethroid insecticide used in agricultural fields (Kurt and Kayış, 2015). Experimental treatments of organotin compounds, arsenic, polyaromatic hydrocarbons and polychlorinated biphenyl exhibited a significant alteration in the micronuclei frequency of clam, *Mya arenaria* (Debenest *et al.*, 2013). According to them, augmentation in micronuclei frequency was physiologically associated with oxidative stress, which in turn affected the phagocytic potential of hemocytes of clams. Several xenobiotics can cause oxidative stress in living organisms through the generation of reactive oxygen species (ROS) and alter the antioxidant buffering potential of the cells in invertebrates (Livingstone, 1993). ROS can react with biological macromolecules to cause lipid peroxidation, DNA damage and protein oxidation (Shi *et al.*, 2005). Thus, it can be inferred that oxidative DNA damage under the exposure of environmental pollutants can be considered to be the most putative mechanism of genotoxicity (Hu *et al.*, 2010).

While studying the toxicity of sodium arsenite in freshwater bivalve *Lamellidens marginalis*, Chakraborty and Ray (2009) identified micronucleation, binucleation, pyknosis and nuclear disintegration as principal nuclear aberrations of hemocytes. The authors proposed the nuclear anomalies of molluscan hemocytes as an effective biomarker of environmental genotoxicity and an “early warning tool” to assess the health status of freshwater ecosystem. Stefanoni and Abessa (2011) reported genotoxicity of anionic surfactant, linear alkylbenzene sulphonate in brown mussel *Perna perna* by determining the degree of micronucleation. In the present study, treatment of washing soda resulted in an overall increase in the frequency of binucleation in *E. carteri* cells (Figure 6A). On the other hand, all experimental concentrations of washing soda for 384 hours yielded a significant increase in the frequency of micronucleation in the cells of *E. carteri* (Figure 6B). The frequencies of micronucleation and binucleation were recorded to be the highest in the cells of *E. carteri* treated with 16 mg/L of washing soda for 384 hours. Washing soda induced induction in the frequencies of both binuclei and micronuclei formation were suggestive to a high degree of genotoxicity of this aquatic contaminant in *E. carteri*. The result was indicative of a substantial damage in the genetic makeup of *E. carteri* distributed in washing soda contaminated waterbodies.

Unrestricted contamination of the natural aquifers by washing soda is thus apprehended to cause physiological and immunological stresses and adversities in diverse groups of aquatic invertebrates, including the sponge. Such a situation might lead to a shrinkage in the

population density and depletion in reproductive efficacy and survival fitness of *E. carteri* inhabiting the polluted environment. A special “sponge watch programme” (Hansen *et al.*, 1995) was launched in USA to monitor the health of aquatic ecosystem. Similarly, initiation of *E. carteri* as a biomonitoring organism might be an effective biological measure for evaluating the toxicity of environmental xenobiotics in the freshwater ecosystem of India. Furthermore, the cellular parameters like differential count, nonself surface adhesion and nuclear aberrations appeared to bear the potential to qualify as biomarkers of washing soda toxicity. The present investigation might thus help spongologists, environmental managers and aquaculturists to conserve this important bioresource for its rational utilization in a chemically safe environment.

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