Toxicology International
DOI: 10.18311/ti/2026/v33i1/54113
INFORMATICS JOURNALS
eISSN: 0976-5131 | Vol 33(1), 2026 | pp. 109-124

Integrated Biomarker Assessment of Washing Soda Mediated Immune Impairment in a Freshwater Indian Mollusc (*Pila globosa*)

Soumalya Mukherjee and Sourav Sikdar*

Department of Zoology, Brahmananda Keshab Chandra College, 111/2 Barrackpore Trunk Road, Kolkata - 700108, West Bengal, India; souravsikdar@bkcc.ac.in

Abstract

An integrated biomarker assay was employed to evaluate the toxic impacts of washing soda in *Pila globosa*, a common freshwater gastropod of the Indian subcontinent. Component biomarker parameters, both of cellular and biochemical origin, were considered for statistical analyses. Indian freshwater ecosystems, which serve as the natural abode of *P. globosa*, are continuously exposed to anhydrous sodium carbonate (washing soda), an anthropogenically derived chemical contaminant of significant ecotoxicological concern. Washing soda precipitates at the bottom of the ponds and lakes, increases the alkalinity of water, becomes more toxic, and harms the benthic organisms. For integration of analytical endpoints, phagocytosis, superoxide anion, nitric oxide, phenoloxidase, superoxide dismutase, and catalase were considered as component biomarkers of *P. globosa* exposed to 25, 50, 100 and 200 mg/L of washing soda for 1, 2, 4, 8 and 16 days. The objective of the present investigation was to evaluate the immunotoxic responses elicited by washing soda in the hemocytes, the primary immune effector cells of *P. globosa.* Chronic sodium carbonate exposure compromised hemocyte phagocytic potential and altered redox homeostasis, reflecting a progressive decline in immune competence. Unregulated production and unrestricted application of washing soda contaminate the natural habitat of *P. globosa*, impairing cellular, immunological, and reproductive functions, and posing a significant ecotoxicological threat to freshwater molluscan diversity. Hemocytes of *P. globosa* in response to washing soda treatment for 8 and 16 days exhibited higher toxicity and biomarker potential in comparison to that of the 1, 2 and 4 days exposure. Data were suggestive of immunocompromisation of *P. globosa* under prolonged exposure to washing soda. The present study provides insights into washing soda-induced chemical stress in *P. globosa*, highlighting the potential of selected hemocyte-associated endpoints as biomarkers of washing soda toxicity.

Major Findings

1. Washing soda exposure chronically suppressed phagocytosis and impaired pro-antioxidant balance.
2. Integrated Biomarker Response (IBR) star plots identified pro-oxidant and antioxidant components as sensitive indicators of washing soda toxicity.
3. Prolonged exposure elevated IBR, indicating stronger biomarker shifts and impaired immune strength in *P. globosa*.

Keywords: Cytotoxicity, Freshwater Gastropod, Immunotoxicity, Integrated Biomarker Response, Sodium Carbonate

Article Received on: 18.12.2025 **Revised on:** 18.02.2026 **Accepted on:** 20.02.2026

1. Introduction

Molluscan organisms constitute the second-largest animal phylum in terms of species diversity and richness, after Arthropoda. Among molluscs, gastropods constitute the most abundant and diversified group. The pond snail, *P. globosa* (Mollusca: Gastropoda), commonly known as the 'apple snail', is a key member of the Indian freshwater ecosystem. This amphibious mollusc exhibits a dual mode of respiration in both aquatic and aerial environments

*Author for correspondence

through gills and pulmonary sacs, respectively. During summer or prolonged drought, *P. globosa* undergoes aestivation beneath dry soil and resumes its activity with the onset of rain. Being a herbivorous species, *P. globosa* is predominantly distributed in freshwater aquifers and is found attached to succulent aquatic vegetation. This gastropod is widely recognised as an edible variety and represents an affordable source of animal protein for humans and poultry in several countries, including India. The semi-urban and rural sectors of the Indian population consume raw, partially processed, and fully cooked preparations of the flesh of *P. globosa*, and it is also incorporated as a component of feed in prawn and fish aquaculture systems[1]. Beyond its nutritional importance, *P. globosa* holds ethnomedicinal implications. Tribal communities in India use this species for treating conditions like asthma, joint inflammation, ophthalmic diseases, and burns[2,3]. This species is a detritivore, maintaining water quality through filter feeding and controlling algal proliferation. It plays a pivotal role in sustaining ecosystem balance and productivity by promoting nutrient recycling.

Over the last few decades, Indian freshwater ecosystems have experienced contamination from emerging xenobiotics, including different commercial brands of detergents and their related chemical compounds like washing soda[4]. Ecotoxicological impacts of these substances on aquatic invertebrates, despite their widespread applications, remain poorly understood. A marked decline in the natural population of Indian freshwater sponges has been attributed to anthropogenic contamination of waterbodies by washing soda[5]. An extensive field survey conducted in freshwater ponds and lakes of the Kolkata metropolis revealed washing soda (anhydrous sodium carbonate) as a precarious environmental contaminant. In many rural and semi-urban sectors of India, this component of laundry detergent[6] is extensively applied for household activities, such as bathing of humans and livestock, as well as cleaning of apparel and utensils. Nevertheless, contemporary research addressing the toxic impacts of washing soda on freshwater aquatic molluscs remains scarce in available scientific records.

Washing soda, or soda ash, is a widely used constituent of household laundry detergents. It serves as a builder which helps to remove water hardness by chelation, precipitation, and ionic exchange, thereby enhancing the efficiency of the detergents[7,8]. Washing soda-laden precipitates in the bottom layer imposes substantial stress on aquatic ecosystems, exerting negative impacts on aquatic biodiversity[9]. Substantial sodium carbonate release into the environment is largely driven by emissions from industrial sectors like glass production, mining operation, and paper manufacturing, resulting in widespread environmental contamination[10]. Sodium carbonate discharge alters the buffering capacity of freshwater habitats, often increasing alkalinity. Domestic and industrial effluents, along with drainage discharge and other anthropogenic activities, contributed significantly to washing soda contamination of freshwater ecosystems.

Hemocytes, the circulating immunoeffector cells of molluscs, perform a broad spectrum of immunophysiological functions, including phagocytosis, cytotoxicity, cellular aggregation, pathogen encapsulation, and tissue repair[11,12]. In gastropods, these circulating cells are involved in recognition and discrimination of self from nonself particles and are engaged in wound healing, immune defence, nutrient transport, and tissue remodelling[13,14]. Phagocytosis is a classical cell-mediated immune response, during which hemocytes engulf and internalise foreign particulates into phagosomes, followed by enzymatic degradation[15]. Hemocytes are capable of generating cytotoxic molecules, including Superoxide Anions (O_2^-), Nitric Oxide (NO), and Reactive Oxygen Species (ROS) to deactivate and destroy phagocytosed pathogens and parasites[16,17]. Generation of O_2^- in phagocytic cells is closely associated with oxidative burst. Superoxide anion is an established cytotoxic molecule which is involved in the destruction of environmental pathogens[18]. Nitric oxide, another potent killing agent and a signalling molecule, participates in immune defence and may react with superoxide anions to form Peroxynitrite Anion ($ONOO^-$), an effective antimicrobial agent and prooxidant molecule[19]. Phenoloxidase (PO) is another crucial component of invertebrate immune defence that is involved in phagocytosis, self-nonself discrimination, cytotoxicity and melanisation[20]. After activation through a proteolytic cascade, the enzyme PO exhibits bactericidal, fungistatic and antiviral activities[21].

Generation of prooxidant molecules in response to xenobiotic exposure and environmental contamination is generally considered beneficial for the host cell. However, when produced in excess, these reactive molecules overwhelm cellular defences and destabilise redox homeostasis, resulting in oxidative stress and cellular damage[22]. Antioxidant enzymes like Superoxide Dismutase

(SOD) convert superoxide radicals into hydrogen peroxide and oxygen, while Catalase (CAT) subsequently decomposes the hydrogen peroxide into water and oxygen, thereby mitigating oxidative stress[23]. Overall, hemocytes act as multifunctional immune cells, forming the first line of immune protection in molluscs and serving as sensitive indicators of xenobiotic induced immunotoxicity.

In recent years, environmental toxicologists and physiologists have shown a special interest in assessing ecotoxicity in aquatic organisms using the principle of Integrated Biomarker Response (IBR). Several endpoints are solitarily established as effective biomarkers of the toxicity of various contaminants[24,25]. IBR-based analysis of toxicity is a comprehensive means to estimate the toxicological response of a chemical compound in biological organisms[26]. The multiple biomarker approach is an effective tool for toxicity assessment based on central, interdisciplinary, and integrative efforts. The principle of IBR is based on the consideration of multiple experimental endpoints for assessing the toxicity of environmental contaminants present in water. The IBR approach through multi-biomarker response can be considered as a potential tool to study the effect of a toxin on aquatic organisms. The Integrated Biomarker Response version 2 (IBRv2) quantifies the combined effects of multiple biomarkers, enabling effective evaluation of biological impacts in organisms from contaminated environments. In this present study, Phagocytic Index (PI), O_2^-, NO, PO, SOD and CAT were selected as component endpoints for calculation of IBR. It offers both star plot visualisations and a composite numerical index for integrative and comprehensive multi-biomarker analysis.

Derivation of IBR from the studied experimental endpoints may serve as an early diagnostic tool to assess the washing soda toxicity in freshwater ecosystems. The study aims to identify immune parameters in *P. globosa* that can serve as effective indicators and biomarkers of washing soda-induced toxicity in aquatic environments. Moreover, this study presents important ecotoxicological insights into the effects of washing soda in *P. globosa*, an Indian amphibious mollusc performing bimodal respiration.

2. Materials and Methods

2.1 Sampling and Laboratory Rearing of Experimental Specimens

Healthy specimens of adult *P. globosa* were manually sampled from relatively uncontaminated and unpolluted freshwater perennial ponds (22° 51′ 32.42″ N and 88° 25′ 36.37″ E, with an elevation of 16 m above sea level) of the North 24 Parganas district of West Bengal, India. To minimise stress, groups of 10-15 live adult specimens were collected at a time and immediately moved to the laboratory with an adequate amount of pond water. Pre-experimental specimens were maintained in laboratory glass aquaria under continuous aeration and filled with freshly collected pond water for 7 days. The temperature, dissolved oxygen content and pH of the aquarium water were periodically checked at 30 ± 3°C, 14.5 ± 1.5 mg/L and 7.6 ± 0.2, respectively. Throughout the acclimation and experimental period, the aquarium water was renewed at 24h intervals to prevent metabolic waste buildup and minimise residual toxicity. A regular photoperiod of a 12h:12h light-dark cycle was also maintained. The entire study on *P. globosa* was performed in compliance with institutional protocols for animal care and handling.

2.2 Selection of Experimental Concentrations of Washing Soda for Toxicological Analyses

In the present study, 200 mg/L of anhydrous sodium carbonate was selected as the highest experimental concentration, which was less than one-third of the median lethal concentration determined in *P. globosa* after 16 days of treatment. Before selecting the experimental concentration of washing soda, a comprehensive field investigation was conducted in the different freshwater ponds and lakes of the Kolkata and North 24 Parganas districts of West Bengal, India, to evaluate the overall washing soda usage pattern and extent of contamination and quantify the input load per unit time. The available information regarding the environmentally realistic concentrations of washing soda in freshwater aquifers of West Bengal, India, is scanty in published literature. Concentrations of 2, 4, 8 and 16 mg/L of washing soda were selected as experimental concentrations to study the immunotoxicity in the freshwater sponge, *Eunapius carteri*[4,5]. Based on the buffering potential, the acceptable concentrations of washing soda have been estimated to lie between 2 and 20 mg/L in the aquatic ecosystem[10]. However, the actual concentration of washing soda in the freshwater aquifer may surpass the permissible level due to uncontrolled usage or unintended discharge through drains into aquatic basins. Therefore, the concentrations of washing soda employed in this study (25, 50, 100, and 200 mg/L) were considered to be environmentally pertinent and ecologically justifiable.

2.3 Experimental Setup and Treatment Methodology

Solutions of sodium carbonate (CAS No. 497-19-8) at 25, 50, 100, and 200 mg/L were prepared in detergent-free water. Each experimental set consisted of 5 adult and healthy *P. globosa* immersed in a volume of 5L of water obtained from the natural ponds where they reside (Figure 1). For toxicological analyses, specimens were exposed to different sublethal concentrations of washing soda for 1, 2, 4, 8, and 16 days. Regular replenishment of the washing soda solution every 24 hours was performed. Control specimens were kept in washing soda-free water under identical conditions.

2.4 Collection of Hemolymph and Isolation of Hemocytes

Prior to the collection of hemolymphs, the adult specimens of *P. globosa* were cleaned thoroughly by running tap water to remove debris and associated clay particles. Hemolymph (both from control and washing soda-treated specimens) was aseptically collected by the shell puncturing method[27] and stored in prechilled microcentrifuge tubes at 4°C to minimise cell-cell aggregation. Aseptically collected pooled hemolymph from each experimental group (5 healthy and adult *P. globosa*) was centrifuged at 650 × g for 10 min at 4°C. After centrifugation, the hemocyte pellet was resuspended thrice in sterile snail saline (4 mM HEPES, 37 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2.2H_2O$, 4 mM $CaCl_2.2H_2O$; pH adjusted to 7.8)[11] and kept at 4°C for subsequent analyses.

2.5 Screening of Hemocyte Viability

Hemocytes of control and treated *P. globosa* were screened for viability using 0.4% trypan blue following the vital dye exclusion assay protocol[28]. The percentage of stained and unstained hemocytes was enumerated under a light microscope with the help of a Neubauer hemocytometer. More than 90% of viable hemocytes were considered for experimentation.

2.6 Hemocyte Lysate Preparation

Hemocytes were diluted to 10^6 cells/mL, resuspended in 1mL 0.1% Triton X-100, and incubated on ice for 1 hour. After centrifugation at 3500 ×g for 30 minutes, the supernatant was collected at 4°C for downstream enzymatic and biochemical studies.

2.7 Estimation of Phagocytic Response

In vitro phagocytic activity was evaluated using pooled hemocytes from control and washing soda-exposed *P. globosa*, with yeast (*Saccharomyces cerevisiae*) serving as the target particles. Hemocyte suspensions were standardised to 1 × 10^6 cells/mL, and aliquots from each group were spread onto sterile glass slides to form a uniform

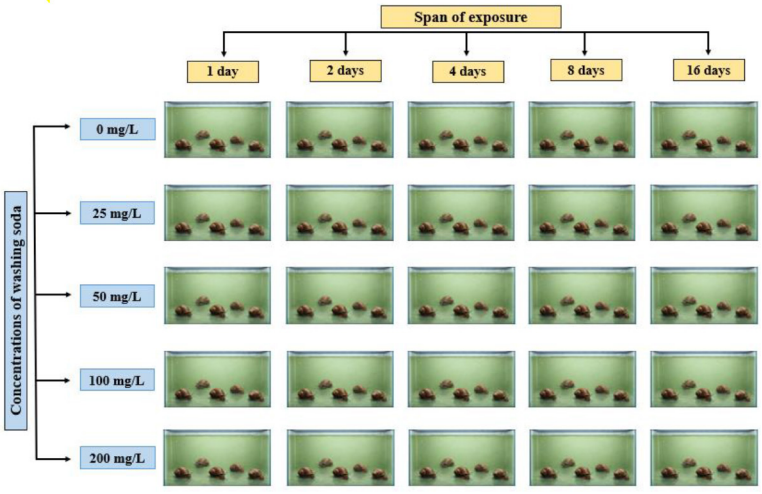


Figure 1. Schematic representation of the experimental design showing concentration and time dependent exposure of *P. globosa* to washing soda. Adult healthy specimens of *P. globosa* (n = 5 per group) were maintained in aerated aquaria containing 5 L of natural pond water and exposed to sublethal concentrations of washing soda (0, 25, 50, 100, and 200 mg/L) for 1, 2, 4, 8, and 16 days. Specimens were collected at each time point for toxicological analyses.

monolayer. The slides were placed in a humid chamber and incubated at 30°C for 60 minutes to allow hemocyte adherence. Subsequently, 10µL of yeast suspension (1×10^7 cells/mL) was added to achieve a hemocyte-to-yeast ratio of 1:10, followed by incubation in a moist chamber for 3 hours. After incubation, excess non-phagocytosed yeast cells were removed by washing with sterile snail saline solution. The slides were air-dried, methanol fixed, and stained with Giemsa. Phagocytic activity was assessed under a light microscope by examining at least 200 hemocytes per slide. The Phagocytic Index (PI) was expressed as a percentage of the hemocytes exhibiting yeast engulfment[29,30].

2.8 Determination of O_2^- Generation

Intrahemocytic O_2^- production was quantified spectrophotometrically using the Nitroblue Tetrazolium (NBT) reduction assay, wherein NBT is reduced to formazan[31]. Hemocyte suspensions were standardised to 1×10^6 cells/mL and incubated with 1mL of 0.03% NBT at 30°C for 30 minutes. Following incubation, cells were centrifuged at 650 × g for 10 minutes at 4°C to eliminate unreacted NBT. The reaction was terminated by the addition of 1mL absolute methanol, and the cell pellet was washed with 70% methanol. The resulting formazan deposits were dissolved in a 4M KOH–DMSO mixture (1:1, v/v), and absorbance was recorded at 630nm using a spectrophotometer. Superoxide anion generation was expressed as OD 10^6 cells^{-1} minute^{-1}.

2.9 Determination of NO Generation

Intraheamocytic NO generation was estimated as nitrite released by *P. globosa* cells upon reaction with Griess reagent[32]. Hemocyte density was adjusted to 1×10^6 cells/ml, and 1ml of hemocyte suspension was incubated with 1ml Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, 5% orthophosphoric acid) for 30 min in the dark at 30°C. The optical absorbance was measured at 550nm against an appropriate reagent blank. Nitric oxide production was calculated from a sodium nitrite standard curve and expressed as µM nitrite 10^6 cells^{-1} minute^{-1}.

2.10 Determination of Phenoloxidase (PO) Activity

PO (EC 1.14.18.1) activity in hemocyte lysates was quantified by a spectrophotometric method employing L-DOPA as the chromogenic substrate[33]. An aliquot of 100 µL lysate was mixed with 200 µL of L-DOPA solution (1.6 mg/mL in cacodylate citrate buffer) and incubated at 30°C for 15 min, after which the reaction was allowed to proceed for an additional 1 min. The assay mixture was subsequently diluted with 200µL of buffer, and absorbance was measured at 490nm. Enzyme activity was expressed as a change in absorbance of 0.001mg protein^{-1} min^{-1}.

2.11 Determination of Superoxide Dismutase (SOD) Activity

SOD (EC 1.15.1.1) activity was assayed following the protocol of NBT photoreduction[34]. The reaction system (2.5mL total volume) comprised 50mM sodium phosphate buffer (pH 7.8), 13mM methionine, 75µM Nitroblue Tetrazolium (NBT), 0.1 mM EDTA, 2µM riboflavin, and 100µL of cell lysate. The mixtures were illuminated using two 15W fluorescent lamps for 15 minutes, and the reaction was terminated by discontinuing illumination. A corresponding non-illuminated mixture served as the blank. Enzyme activity was determined spectrophotometrically at 560nm based on 50% inhibition of NBT photoreduction and expressed as units mg protein^{-1} minute^{-1}.

2.12 Determination of CAT Activity

CAT (EC 1.11.1.6) activity was assayed following the use of hydrogen peroxide as substrate[35]. The reaction mixture contained 40mM H_2O_2 in 50mM sodium phosphate buffer (pH 7.0), to which 100µl of cell lysate was added. Catalase activity was determined by monitoring the decrease in absorbance at 240nm over time using a spectrophotometer. Enzyme activity was expressed as k mg protein^{-1}, where one unit (k) was defined as the amount of enzyme decomposing 1mol H_2O_2 per second under the assay conditions.

2.13 Estimation of Total Protein

The protein concentration in the hemocyte lysate of *P. globosa* was determined using bovine serum albumin as the standard[36].

2.14 Statistical Analyses

Both the control (0 mg/L) and treatment groups (25, 50, 100 and 200 mg/L of washing soda) consisted of five adult and healthy *P. globosa* maintained in aerated glass aquaria containing 5L of pond water. All the experimental

assays were performed in five independent replicates, and the results were presented as mean ± standard deviation (SD). Homogeneity of variance and data normality were evaluated using Bartlett's test[37]. Parametric analyses were performed as all the data sets followed a normal pattern of distribution. One-Way Analysis of Variance (ANOVA) followed by Dunnett's post hoc test was carried out using GraphPad Prism (version 5, GraphPad Software, La Jolla, California, USA) to determine the significant differences between control and treated groups for each experimental day. Statistical significance was considered at $p^{*} < 0.05$, $p^{**} < 0.01$, and $p^{***} < 0.001$. A Pearson correlation matrix was constructed to evaluate the degree of association among immune parameters of *P. globosa* treated with washing soda. Correlation analyses were carried out using Microsoft Excel (Data Analysis ToolPak, 2007), with statistical significance accepted at $p^{*} < 0.05$ and $p^{**} < 0.01$.

2.15 Calculation of Integrated Biomarker Response (IBR)

Integrated Biomarker version 2 (IBRv2) was adopted to evaluate the potential of the experimental endpoints as biomarkers by forming a star plot[38]. Briefly, the comparison of individual biomarker data (X_i) and reference data (X_0) was carried out before application of logarithmic transformation for reducing variance.

$$Y_i = \log \frac{X_i}{X_0} \quad (1)$$

For each concentration, the general mean (m) and standard deviation (s) of each biomarker were calculated, and Y_i was standardised accordingly[24].

$$Z_i = \frac{Y_i - m}{s} \quad (2)$$

A baseline centred at zero was established in the star plot, against which biomarker variation was depicted. The mean standardised biomarker response (B_i) and the mean reference biomarker value (B_0) were then used to derive the biomarker deviation index (D).

$$D = B_i - B_0 \quad (3)$$

IBRv2 was calculated by summing the absolute D values for all biomarkers at each examined concentration.

$$\text{IBRv2} = \Sigma D \quad (4)$$

The biomarker Deviation index (D) for each experimental endpoint was depicted through star plots to evaluate how each biomarker of *P. globosa* deviated from the control. Exposures were conducted at 25, 50, 100, and 200 mg/L of washing soda for 1, 2, 4, 8, and 16 days, with comparisons made against the respective control groups. The area above 0 in the star plot indicated an induction in the component biomarker, while the area below 0 reflected the inhibition in the studied biomarker[39]. All the studied biomarkers were integrated into an IBR index[24,38] to evaluate the overall health of *P. globosa* exposed to varying concentrations of washing soda over 16 days. A radar star plot was constructed to integrate the multi-biomarker immune response of *P. globosa* exposed to 25, 50, 100 and 200 mg/L of washing soda for 1, 2, 4, 8 and 16 days separately.

3. Results

3.1 Phagocytic Index

The Phagocytic Indices (PI) of the pooled hemocytes of *P. globosa* (both control and washing soda treated) were determined under the challenge of yeast particles *in vitro*. The PI of hemocytes of control *P. globosa* ranged between 195.4 ± 3.62 and 195.43 ± 8.75 over a time span of 1 to 16 days (Figure 2). Washing soda treatment resulted in a gradual and overall decrease in the phagocytic potential of hemocytes of *P. globosa*. Exposure to sodium carbonate at 100 and 200 mg/L for 8 and 16 days resulted in a concentration-dependent reduction in phagocytic potential. The maximum decrease of PI was recorded as 47.41 ± 5.93 in the hemocytes of *P. globosa* following 16 days of exposure to 200 mg/L washing soda.

3.2 Superoxide Anion

The generation of intrahemocytic O_2^- in *P. globosa* was assessed under washing soda treatments of 25, 50, 100, and 200 mg/L across different exposure periods, with corresponding controls (Figure 3A). The generation of O_2^- in the hemocytes of control *P. globosa* ranged from 0.005 ± 0.0008 to 0.004 ± 0.0005 O.D. 10^6 cells^{-1} minute^{-1} for different exposure periods. A transient rise in O_2^- production was observed against the washing soda treatment at concentrations of 100 mg/L for 1 day and 50 and 200 mg/L for 2 days. The maximum generation of O_2^- was estimated as 0.021 ± 0.004 O.D. 10^6 cells^{-1} minute^{-1} in hemocytes of *P. globosa* following treatment

with 200 mg/L of washing soda for 2 days. Conversely, prolonged exposure to washing soda at concentrations of 100 and 200 mg/L over 8 and 16 days led to a pronounced suppression of O_2^- production. The maximum reduction in O_2^- generation (0.004 ± 0.0008 O.D. 10^6 cells^{-1} minute^{-1}) was observed after 8 days of exposure to 100 mg/L washing soda.

3.3 Nitric Oxide

Nitric oxide production, expressed as nitrite levels, was quantified in the hemocytes of *P. globosa* following exposure to washing soda at 25, 50, 100, and 200 mg/L for durations of 1, 2, 4, 8, and 16 days (Figure 3B). Nitric oxide generation of untreated *P. globosa* ranged from 0.075 ± 0.01 to 0.068 ± 0.01µM nitrite 10^6 cells^{-1} minute^{-1} under different time spans. Short-term exposure to washing soda at 200 mg/L for 2 days resulted in a statistically significant elevation in NO production, with nitrite levels reaching 0.114 ± 0.002µM 10^6 cells^{-1} minute^{-1}.

However, exposure to washing soda at 100 and 200 mg/L for 4, 8, and 16 days caused a concentration and duration-related suppression of intrahemolytic NO production. The lowest NO level was recorded at 0.008 ± 0.001 µM nitrite 10^6 cells^{-1} minute^{-1} in the hemocytes of *P. globosa* following 16 days of exposure to 200 mg/L washing soda.

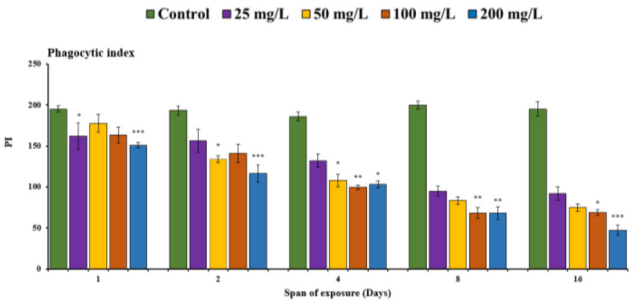


Figure 2. Phagocytic Index (PI) of hemocytes of *P. globosa* experimentally exposed to 25, 50, 100 and 200 mg/L of washing soda for 1, 2, 4, 8 and 16 days, under the challenge of yeast particles *in vitro*. All data were expressed as mean ± Standard Deviation (S.D.), with n = 5 per group. Statistical significance between the control and washing soda-treated groups at each experimental time point was assessed using one-way ANOVA, followed by Dunnett's post hoc test to determine the level of significance. Significant differences from the control group were indicated by asterisks [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)].

3.4 Phenoloxidase

PO activity was quantified in hemocyte lysates of *P. globosa* following exposure to washing soda at concentrations of 25, 50, 100, and 200 mg/L for 1, 2, 4, 8, and 16 days, with corresponding control groups (Figure 3C). PO activity of the control *P. globosa* ranged from 1.62 ± 0.22 to 1.55 ± 0.02 units mg protein^{-1} minute^{-1} over a time duration of 1 to 16 days, respectively. Exposure to washing soda at 100 and 200 mg/L for 1 and 2 days resulted in marked depletion in the activity of PO in *P. globosa*. Prolonged exposure to all experimental concentrations of sodium carbonate for 8 and 16 days yielded a gradual decrease in the activity of PO in the hemocytes of *P. globosa*. The maximum inhibition of PO activity was recorded as 0.18 ± 0.04 unit mg protein^{-1} minute^{-1} in *P. globosa* exposed to 200 mg/L of washing soda for 16 days.

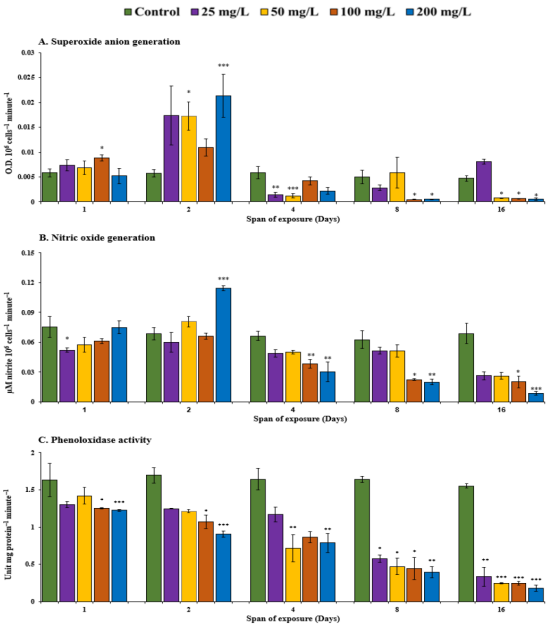


Figure 3. Generation of intrahemocytic superoxide anion **(A)** Nitric oxide **(B)** Activity of phenoloxidase **(C)** Enzyme in the hemocyte lysates of *P. globosa* exposed to 25, 50, 100 and 200 mg/L of washing soda for 1, 2, 4, 8 and 16 days. All data were expressed as mean ± Standard Deviation (S.D.), with n = 5 per group. Statistical significance between the control and washing soda-treated groups at each experimental time point was assessed using one-way ANOVA, followed by Dunnett's post hoc test to determine the level of significance. Significant differences from the control group were indicated by asterisks [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)].

3.5 Superoxide Dismutase

SOD activity was assessed in the hemocyte lysates of *P. globosa* following exposure to washing soda at concentrations of 25, 50, 100, and 200 mg/L, with corresponding control groups (Figure 4A). The SOD activity of hemocytes of control *P. globosa* ranged from 1.40 ± 0.15 to 1.65 ± 0.12 units mg protein^{-1} minute^{-1} over a time duration of 1 to 16 days, respectively. A consistent suppression of SOD activity was observed following sodium carbonate treatment. Exposure to washing soda at 100 and 200 mg/L for 1, 4, and 8 days, as well as at all tested concentrations for 16 days, significantly reduced SOD activity. The most pronounced inhibition (0.29 ± 0.03 unit mg protein^{-1} minute^{-1}) was observed in hemocyte lysates of *P. globosa* following 16 days of treatment with 200 mg/L sodium carbonate.

3.6 Catalase

CAT activity in the hemocyte lysate of *P. globosa* was recorded under experimental exposure to washing soda up to 16 days, with the respective controls (Figure 4B). Treatment with washing soda at concentrations of 50, 100 mg/L for 1 day and 25 and 50 mg/L for 2 days presented a gradual increment in CAT activity. The highest CAT activity was recorded as 1.22 ± 0.01 k mg protein^{-1} in the hemocyte lysate of *P. globosa* treated with washing soda at a concentration of 50 mg/L for 2 days. However, extended exposure to sodium carbonate for 8 and 16 days led to a general reduction in CAT activity that was independent of concentration. The greatest inhibition of intrahemocytic CAT activity (0.07 ± 0.007 k mg protein^{-1}) was observed in *P. globosa* following 16 days of exposure to 200 mg/L washing soda.

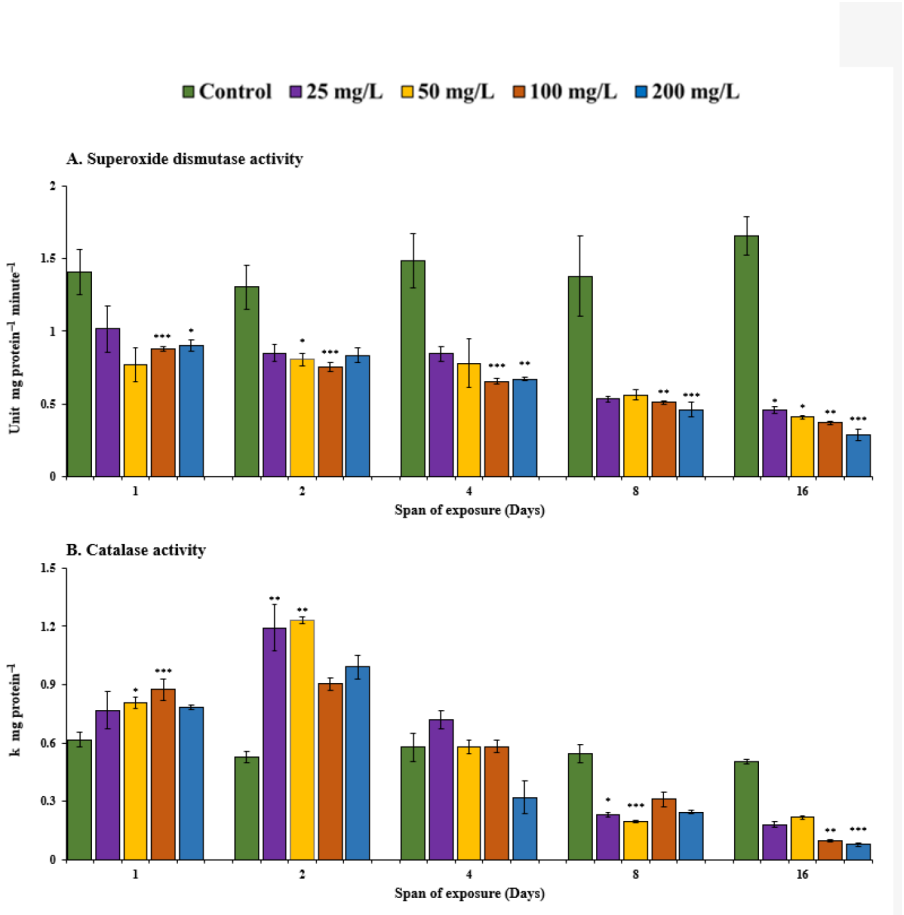


Figure 4. Activities of **(A)** Superoxide dismutase **(B)** Catalase in the hemocyte lysates of *P. globosa* exposed to 25, 50, 100 and 200 mg/L of washing soda for 1, 2, 4, 8 and 16 days. All data were expressed as mean ± standard deviation (S.D.), with n = 5 per group. Statistical significance between the control and washing soda-treated groups at each experimental time point was assessed using one-way ANOVA, followed by Dunnett's post hoc test to determine the level of significance. Significant differences from the control group were indicated by asterisks [$p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)].

Table 1. Pearson correlation matrix for the estimated hemocyte-associated immune parameters of *P. globosa* experimentally exposed to washing soda. * Correlation is significant at the 0.05 level (2–tailed); ** correlation is significant at the 0.01 level (2–tailed).

	Phagocytic index	Superoxide anion	Nitric oxide	Phenoloxidase	Superoxide dismutase	Catalase
Phagocytic index	1					
Superoxide anion	0.346	1				
Nitric oxide	0.683 **	0.735 **	1			
Phenoloxidase	0.977 **	0.361	0.704 **	1		
Superoxide dismutase	0.915 **	0.216	0.641 **	0.910 **	1	
Catalase	0.583 **	0.784 **	0.747 **	0.643 **	0.405 *	1

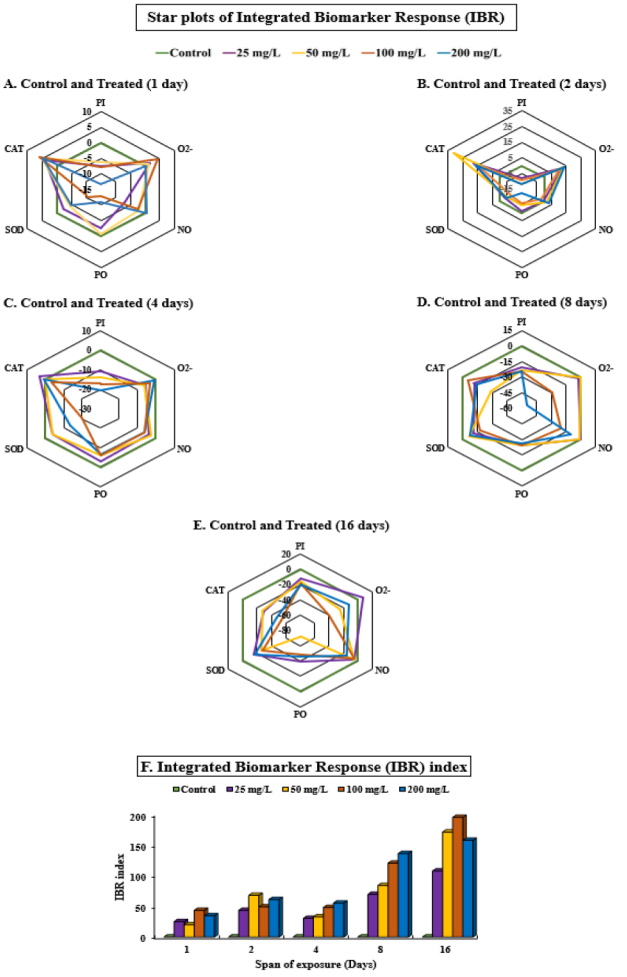


Figure 5. Star plots representing the Integrated Biomarker Response (IBR) of hemocyte-associated immune parameters in *P. globosa* following experimental exposure to washing soda at concentrations of 25, 50, 100, and 200 mg/L over durations of (A) 1, (B) 2 , (C) 4 , (D) 8 , and (E) 16 days. [Phagocytic Index (PI), superoxide anion (O_2^-), Nitric Oxide (NO), Phenoloxidase (PO), Superoxide Dismutase (SOD) and Catalase (CAT)]. Calculated IBR index of hemocytes of *P. globosa* treated with sublethal concentrations of washing soda for (F) 1, 2, 4, 8 and 16 days.

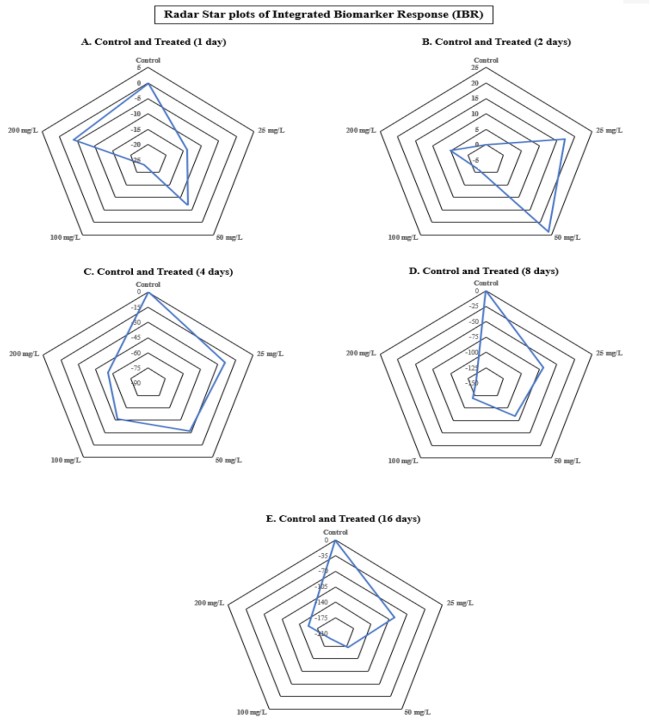


Figure 6. Radar star plots illustrating the Integrated Biomarker Response (IBR) values of hemocyte-associated immune parameters in *P. globosa* exposed to washing soda at concentrations of 25, 50, 100, and 200 mg/L over time periods of (A) 1, (B) 2 , (C) 4 , (D) 8 , and (E) 16 days.

3.7 Pearson Correlation Matrix

A Pearson correlation matrix was constructed for hemocyte-associated immune parameters in *P. globosa* exposed to various concentrations of washing soda over a time period of 16 days (Table 1). A positive relationship was observed between hemocyte phagocytosis and the levels of NO, as well as PO, SOD, and CAT activities in *P. globosa*. The significant correlation between the prooxidant

parameters, like the generation of O_2^- with NO, was very prominent. The activity of CAT was statistically correlated with the rest of the experimental parameters. Generation of NO yielded a positive correlation with the activities of PO and SOD. Moreover, a positive relationship was evident between the activities of PO and SOD.

3.8 Integrated Biomarker Response

The multiparametric approach of different biomarkers of *P. globosa* exposed to different experimental concentrations of washing soda for 1, 2, 4, 8 and 16 days was presented as star plots (Figure 5A-E). In this study, six hemocyte-associated experimental parameters were considered as endpoint components for the calculation of IBR. Hemocyte IBR indices in *P. globosa* were assessed under different washing soda concentrations across 16 days of exposure (Figure 5F). The IBR index revealed distinct differences in the biomarker responses of *P. globosa* across all tested concentrations of washing soda over 1, 2, 4, 8, and 16 days of exposure. Prolonged exposure to washing soda for 8 and 16 days exhibited an overall increase in the IBR index in comparison to that of the biomarker potential calculated for 1, 2 and 4 days. The dynamics and cumulative shift in experimental endpoints were plotted in radar star charts for 1, 2, 4, 8, and 16 days (Figure 6).

4. Discussion

In recent times, wetlands have been rapidly disappearing from metropolitan landscapes due to overexploitation of resources, urbanisation, and industrial expansion. These factors exert an alarming ecotoxicological threat to aquatic ecosystems and their inhabitants. Because of their extensive production and application, detergent derivatives have posed substantial concerns for worldwide ecological and public health[40]. Anhydrous sodium carbonate (washing soda) has been recognised as an aquatoxin capable of altering key physiological parameters in a wide range of invertebrates, including aquatic crustaceans[41], cladocerans[6], and freshwater sponges[4,5,42]. A concentrated solution of washing soda exhibits corrosive characteristics, causing dermal and optic irritation as well as potential bleaching and burning sensations. The toxicity of sodium carbonate on the aquatic ecosystem is largely governed by the alkalinity of the aquatic milieu. The ecological hazards associated with sodium carbonate are primarily dependent on the elevation of aquatic pH levels induced by carbonate ions.

Since washing soda is poorly absorbed onto suspended particles, it tends to precipitate into aquatic sediments, thereby contributing to long-term contamination of benthic environments. Discharge of such agents into aquatic basins silently affects the bottom dwellers, disrupts the trophic dynamics, and thereby destabilises the food web structure. By filtering the ambient water in a dynamic fashion, *P. globosa* regulates the nutrient and mineral distribution in the aquatic ecosystem. As a benthic filter feeder, *P. globosa* tends to accumulate toxins and pollutants inside its body over time, making it a reliable model organism for laboratory-based ecotoxicological assessments[43]. The small body size, limited mobility, broad habitat range, moderate lifespan, and strong bioaccumulation potential of *P. globosa* establish it as a model organism for evaluating contaminant-induced physiological alterations[44]. The degree of contamination in the natural habitat of *P. globosa* is dependent on the nature and intensity of nearby human activities, seasonal variations, and the proximity between pollutant discharge points and the species' habitat.

Hemocytes, the circulating immunocytes of molluscs, perform diverse physiological and defence functions under controlled and stressed conditions. Phagocytosis, an evolutionarily conserved and primitive immune response established in major phyla, serves as a sensitive biomarker of environmental contamination[45]. Sublethal concentrations of copper and mercury altered the biochemical parameters and genome content in the selected tissues of freshwater snail, *P. globosa*[46]. Molluscan hemocytes exhibit strong immunocompetence through phagocytic activity[47], which is often modulated by xenobiotics and pollutants. Fluoride and atrazine exposure decreased phagocytic activity of molluscs, *Venerupis philippinarum*[48] and *Lymnaea stagnali*[49], respectively, whereas *Mytilus edulis* showed an increased activity upon exposure to zinc oxide nanoparticles[50]. Environmental pollutants like Bisphenol A (BPA) and Dichlorodiphenyldichloroethylene (DDE) significantly inhibited the phagocytic potential in the freshwater snail, *Biomphalaria glabrata*[51]. Morphological alteration of hemocytes and shift in phagocytic potential were documented in the freshwater snail, *Biomphalaria* sp., under the face of both conventional and emerging environmental contamination[52]. Similarly, washing soda exposure significantly reduced the phagocytic response

in the freshwater sponge, *Eunapius carteri*[4]. In this present study, 25 and 200 mg/L of washing soda exposure for 1 day and 50 and 200 mg/L of the same toxin for 2 days significantly inhibited the phagocytic potential of hemocytes of *P. globosa* (Figure 2). Treatment with 50, 100 and 200 mg/L of sodium carbonate for 4 days depleted the phagocytic activity. Prolonged exposure (8 and 16 days) at concentrations of 100 and 200 mg/L exhibited a concentration and time-dependent suppression in phagocytic activity. The maximum inhibition of the phagocytic response was observed under the exposure of 200 mg/L of washing soda for 16 days. Persistent exposure to washing soda in freshwater habitats may impair cellular immunity and suppress the phagocytic activity in *P. globosa,* increasing its susceptibility to pathogenic invasion. Prolonged contamination may weaken immune competence, making it vulnerable to environmental stressors and pathogens and, ultimately, threatening population diversity in polluted environments.

The cytotoxic response of immunocytes relies on the capability to generate ROS, including O_2^-, hydroxyl radical, singlet oxygen, hydrogen peroxide, and Reactive Nitrogen Intermediates (RNI), including nitric oxide and peroxynitrite anion[53]. Superoxide production represents a pivotal immune strategy in invertebrates under the exposure to toxins and pathogenic invasion[11], while nitric oxide plays a key role in cytotoxic destruction of internalised pathogens[19]. Hemocytes, which are often regarded as the evolutionary counterpart of mammalian leukocytes, can trigger nitric oxide generation during immune activation[54]. These cytotoxic molecules, along with phenoloxidase, act as potent antimicrobial agents which contribute to cell-mediated innate immunity in both invertebrates and vertebrates[55]. Exposure to tributyltin and benzo[a]pyrene has been shown to enhance O_2^- and NO generation in the gastropod *Haliotis diversicolor*[56]. Contemporary research on oxidative stress physiology in molluscs as biomarkers of aquatic pollution is limited, especially in the Indian apple snail, *P. globosa*[57]. According to them, *P. globosa* can be considered a sensitive sentinel species to detect environmental perturbations in freshwater ecosystems. Experimental exposure to 1.5, 3.6 and 12 mg/L of cadmium chloride for a span of 4 days significantly elevated the generation of O_2^- in the gills of the clam, *Meretrix meretrix*[58]. Treatment with copper oxide nanoparticles and copper sulfate for a span of 14 days significantly inhibited the generation of prooxidant and cytotoxic molecules such as O_2^- and NO in the dissociated cells of freshwater sponge, *E. carteri*, which shares the same habitat with *P. globosa*[37]. An elevation in the generation of NO was recorded in the tissue of the freshwater snail *Bulinus truncatus* under the exposure of saponin, a potent molluscicidal agent[59]. A reduction in pro-oxidant molecules, such as O_2^- and NO, was observed in dissociated cells of *E. carteri* following washing soda exposure[4]. The present study quantified the generation of cytotoxic killing agents like O_2^- and NO in the hemocytes of *P. globosa* following exposure to 25, 50, 100 and 200 mg/L of sodium carbonate over a duration of 1, 2, 4, 8 and 16 days (Figure 3 A, B). Acute exposure to washing soda at the highest experimental concentrations of 200 mg/L for 2 days resulted in an initial spike in the generation of O_2^- and NO. However, sub-chronic exposure for 8 and 16 days at concentrations of 100 and 200 mg/L suppressed the generation of these cytotoxic molecules. Initial rise in intrahemocytic O_2^- served as a marker of oxidative stress induced by washing soda, while the subsequent decline under prolonged exposure reflected immunosuppression and dysfunction of oxidative homeostasis. Such impairment may compromise hemocyte-mediated cytotoxicity and immunological dysfunction in specimens inhabiting polluted environments.

Phenoloxidase is a key component of molluscan immunity. Its inactive precursor, prophenoloxidase, is activated by several factors, including some endogenous enzymes or external elicitors, to generate PO, which catalyses quinone formation leading to melanin synthesis. Melanin is vital for encapsulation, immune recognition, wound healing and melanisation in invertebrates[20]. A strong correlation between inhibition of PO activity and increased disease occurrence and mortality rate was documented in host molluscs[60], highlighting the pivotal role played by PO in the innate immune defence. An increase in the activity of PO was recorded in three species of clam (*Ruditapes philippinarum, Chamelea gallina* and *Tapes decussatus*) under experimental elicitation by Sodium Dodecyl Sulfate (SDS), an anionic surfactant commonly used as a component of laundry detergent[61]. Environmental contaminants such as zinc have been shown to modulate PO activity in marine mollusc *Haliotis tuberculata*[62], while sodium dodecyl sulphate caused time-dependent suppression of enzyme activity in marine gastropod, *Rapana thomasiana*[63]. In this present study, exposure to sublethal concentrations of washing soda up to a span of 4 days yielded a non-linear inhibition in the activity of PO

in the hemocyte lysate of *P. globosa* (Figure 3C). However, prolonged exposure to all experimental concentrations of sodium carbonate presented an overall depletion in the PO activity. The maximum inhibition of PO activity was recorded under the highest experimental concentrations of 200 mg/L of sodium carbonate for 16 days. As PO is principally associated with immune defence in invertebrates, its downregulation compromises host defence, increasing susceptibility to pathogens. Washing soda-mediated depletion in PO activity may impair oxidative balance and deterioration of immune status in contaminated habitats.

Maintenance of cellular oxidative homeostasis primarily depends on the delicate balance between ROS production and the activities of SOD and CAT, central enzymes in antioxidative defence. Prolonged exposure to contaminants and xenobiotics disrupts this balance, leading to excessive ROS production and consequent oxidative stress, including DNA fragmentation and protein denaturation. Hence, alterations in antioxidant enzyme activities can act as a reliable biomarker of pollutant-induced oxidative stress[22]. An alteration in CAT activity was recorded in the clam, *Meretrix meretrix,* in response to invasion of environmental pathogens[64]. Exposure to untreated composite tannery effluent exhibited a significant elevation in the activities of antioxidant enzymes like SOD and CAT in the tissue of the freshwater gastropod *P. globosa*[65]. A significant decrease in the activities of SOD and CAT was recorded in the freshwater mussel, *Dreissena polymorpha*, under the toxic exposure of gamma-cyhalothrin pesticide[66]. In our study, experimental exposure to washing soda resulted in an overall decrease in the activity of SOD in the hemocyte lysate of *P. globosa* (Figure 4A). Whereas, initial exposure of sodium carbonate up to 2 days yielded a significant increase in the activity of CAT, followed by a marked decrease for 8 and 16 days (Figure 4B). The maximum inhibition of both of these antioxidant parameters, like SOD and CAT, was recorded under washing soda exposure at the highest experimental concentrations of 200 mg/L for 16 days. Depletion in antioxidant enzyme activity following washing soda exposure suggests a compromised antioxidative defence and diminished scavenging efficiency against ROS. Such inhibition may impair cellular resilience and subsequent immunosuppression, highlighting the broader ecotoxicological implications of xenobiotic exposure in freshwater biota.

In invertebrates, phagocytosis and the subsequent production of cytotoxic molecules, including O_2^- and NO, represent a primary immune defence strategy[53]. According to them, a direct correlation existed between PI and the generation of NO in the mollusc, *Viviparus ater*. A significant association between PI, O_2^-, and NO production in *E. carteri* under varying thermal conditions was also documented in the report[67]. In this present study, *P. globosa* treated with sodium carbonate presented a positive correlation between O_2^- and NO (Table 1). Exposure to 200 mg/L of washing soda for 2 days yielded an initial increase in the production of intrahemocytic O_2^- and NO in *P. globosa*, followed by a sharp decline in 16 days (Figure 3 A, B). This was indicative of an early adaptive response succeeded by physiological stress and exhaustion under prolonged exposure. These trends are consistent with the classical oxidative stress model, where prolonged xenobiotic exposure suppresses both immune and detoxification pathways. In this study, a positive relationship between the production of free radicals within cellular compartments and antioxidative defence response indicates the detoxification and scavenging potential of SOD and CAT against oxidative stress. This study revealed a positive correlation between SOD and CAT activities (Table 1). A similar pattern of inhibition of SOD and CAT activities was observed in *E. carteri* cells following prolonged (16 days) exposure to washing soda[5].

IBR is the mathematical basis of multiple biomarker responses in an integrated fashion. The current study is aimed at assessing the performance of different immunomarkers of *P. globosa* in response to washing soda treatment to validate and establish a polymarker approach. In this paper, an index termed "IBR version 2"[38] was presented as a star plot to evaluate the ecosystem health and impacts of washing soda in *P. globosa*. Aquatic contaminants exhibit toxicities at different biological levels, affecting multiple cellular parameters. In this paper, several hemocyte-derived experimental endpoints were selected for the calculation of IBR in *P. globosa* exposed to 25, 50, 100, and 200 mg/L of washing soda for a time span of 1, 2, 4, 8 and 16 days (Figures 5 and 6). Exposure to different sublethal concentrations of washing soda upto a span of 16 days exhibited differential variation of scattering of biomarkers in star plots as revealed from IBR analysis. This clearly indicates the differential toxicity of washing soda in *P. globosa* under prolonged exposure. Exposure to sodium carbonate for 8 and 16 days significantly elevated

the IBR index relative to shorter exposure durations (1–4 days) (Figure 5F), indicating enhanced biomarker responsiveness and impaired immunological resilience in *P. globosa* under prolonged toxic insult. Moreover, the gradual increment in the IBR index over 16 days indicated the onset of chronic toxicity in the experimental specimen.

The effect of a toxicant on a single biomarker response does not always reflect significant perturbations; the advent of multiparametric analysis through IBR provides a comprehensive means to evaluate the toxicological response of washing soda in *P. globosa*. IBRv2 can be considered as a prognostic indicator of environmental stress and a useful tool to estimate biomarker response in an integrated fashion[68]. However, there are some inherent drawbacks of IBR[25]. According to the authors, IBR can be demonstrated as a dynamic index rather than a rigid one because it does not provide fixed and directly comparable numerical values to assess the overall health status of an organism at a particular time. The authors demonstrated that increasing the number of experimental endpoints alters the relative contribution of each biomarker to the overall IBR index. Moreover, a marked elevation in a single biomarker has a pronounced effect on IBR value and may influence the overall calculation.

The present investigation was designed to evaluate the immunophysiological effects of washing soda toxicity in *P. globosa*, an indigenous dietary item of tribal populations in India. This important but neglected bioresource in the Indian ecosystem demands special scientific attention from aquaculturists, immunotoxicologists, and malacologists. The inhabitants of freshwater ecosystems across many countries in the Oriental realm are already under stress from anthropogenic influences, making them increasingly vulnerable[69], particularly in the absence of stringent regulatory frameworks and environmental safety protocols regarding the use of washing soda. Unregulated habitat contamination and degradation by washing soda pose significant threats to the survival, fitness, and reproductive efficacy of this freshwater mollusc, resulting in population decline and loss of molluscan biodiversity. Therefore, strengthening public awareness, implementing a molluscan watch programme, and promoting sustainable aquaculture practices are necessary to conserve ecological sanctity and molluscan biodiversity in its natural habitat.

5. Conclusion

The present investigation demonstrates that washing soda exposure resulted in significant time and concentration-dependent alterations in hemocyte-associated immune parameters, including PI, O_2^-, NO, PO, SOD, and CAT, which serve as sensitive biomarkers of immunotoxic stress in *P. globosa*. Prolonged exposure (8 and 16 days) to washing soda elicited markedly stronger immunotoxicological alterations than shorter durations (1–4 days), as revealed by the IBR index. Both pro- and antioxidant parameters emerged as reliable indices of washing soda toxicity in *P. globosa*, reflecting their utility in ecotoxicological surveillance. Collectively, these findings infer that washing soda exerts immunotoxicological stress in freshwater gastropods, highlighting the potential of hemocyte-based immunomarkers as prognostic indicators for environmental risk assessment. Another principal insight of this study is that extended exposure to washing soda initiates oxidative storms; therefore, biomarker-based monitoring frameworks are urgently required to maintain the integrity of freshwater ecosystems.

6. Acknowledgements

The authors gratefully acknowledge the Head of the Department of Zoology, Brahmananda Keshab Chandra College, for providing access to the laboratory and computer facilities. They also acknowledge the use of Artificial Intelligence tools for grammar checking and bibliography preparation.

7. References

1. Baby RL, Hasan I, Kabir KA, Naser MN. Nutrient analysis of some commercially important molluscs of Bangladesh. J Sci Res. 2010; 2(2):390-396. https://doi.org/10.3329/jsr.v2i2.3362
2. Prabhakar AK, Roy SP. Ethno-medicinal uses of some shell fishes by people of Kosi river basin of North Bihar, India. Stud Ethno-Med. 2009; 3(1):1-4. https://doi.org/10.31901/24566772.2009/03.01.01
3. Mahawar MM, Jaroli DP. Traditional knowledge on zootherapeutic uses by the Saharia tribe of Rajasthan, India. J Ethnobiol Ethnomed. 2007; 3(1):25. https://doi.org/10.1186/1746-4269-3-25 PMid:17547781 PMCid:PMC1892771.
4. Mukherjee S, Ray M, Ray S. Immunotoxicity of washing soda in a freshwater sponge of India. Ecotoxicol Environ

Saf. 2015; 113:112-123. https://doi.org/10.1016/j.ecoenv.2014.11.035 PMid:25497767.
5. Mukherjee S, Ray M, Ray S. Shift in aggregation, ROS generation, antioxidative defense, lysozyme and acetylcholinesterase activities in the cells of an Indian freshwater sponge exposed to washing soda (sodium carbonate). Comp Biochem Physiol Part C. 2016; 187:19-31. https://doi.org/10.1016/j.cbpc.2016.05.001 PMid:27178357.
6. Warne MSJ, Schifko AD. Toxicity of laundry detergent components to a freshwater cladoceran and their contribution to detergent toxicity. Ecotoxicol Environ Saf. 1999; 44:196-206. https://doi.org/10.1006/eesa.1999.1824 PMid:10571467.
7. Bajpai D, Tyagi VK. Laundry detergents: An overview. J Oleo Sci. 2007; 56(7):327-340. https://doi.org/10.5650/jos.56.327 PMid:17898499.
8. Burton G A. Assessing contaminated aquatic sediments. Environ Sci Technol. 1992; 26:1862-1863. https://doi.org/10.1021/es00034a613
9. UNEP. Sodium carbonate, SIDS initial assessment report for SIAM 15. Boston, USA. 2003.
10. HERA. Human and environmental risk assessment on ingredients of household cleaning products - Sodium carbonate. Edition 2.0. 2005.
11. Adema CM, Knaap WPW, Sminia T. Molluscan hemocyte-mediated cytotoxicity. The role of reactive oxygen intermediates. Rev Aquat Sci. 1991; 4:201-223.
12. Hillyer JF, Schmidt SL, Christensen BM. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. Cell Tissue Res. 2003; 313:117-127. https://doi.org/10.1007/s00441-003-0744-y PMid:12838409.
13. Martin GG, Oakes CT, Tousignant HR, Crabtree H, Yamakawa R. Structure and function of hemocytes in two marine gastropods, *Megathura crenulata* and *Aplysia californica*. J Molluscan Stud. 2007; 73(4):355-365. https://doi.org/10.1093/mollus/eym032
14. Loker ES. Gastropod immunobiology. In Soderhall, K. (ed.) Invertebrate immunity. 2010; pp. 17-43. Springer New York, NY. https://doi.org/10.1007/978-1-4419-8059-5 PMid:21528689 PMCid: PMC9284565.
15. Ladhar-Chaabouni R, Hamza-Chaffai A. The cell cultures and the use of hemocytes from marine molluscs for ecotoxicology assessment. Cytotechnology. 2016; 68(5):1669-1685. https://doi.org/10.1007/s10616-015-9932-3 PMid:26611734 PMCid:PMC5023553.
16. Manduzio H, Rocher B, Durand F, Gatap C, Leboulenger F. The point about oxidative stress in molluscs. Invertebrate Surviv J. 2005; 2:91-104.
17. Rivero A. Nitric oxide: An antiparasitic molecule of invertebrates. Trends Parasitol. 2006; 22(5):219-225. https://doi.org/10.1016/j.pt.2006.02.014 PMid:16545612.
18. Pipe RK. Generation of reactive oxygen metabolites by the hemocytes of the mussel *Mytilus edulis*. Dev Comp Immunol. 1992; 16(2-3):111-122. https://doi.org/10.1016/0145-305X(92)90012-2 PMid:1323488.
19. Colasanti M, Persichini T, Venturini G. Nitric oxide pathway in lower metazoans. Nitric Oxide. 2010; 23(2):94-100. https://doi.org/10.1016/j.niox.2010.05.286 PMid:20638951.
20. Nappi AJ, Christensen BM. Melanogenesis and associated cytotoxic reactions: Applications to insect innate immunity. Insect Biochem Mol Biol. 2005; 35(5):443-459. https://doi.org/10.1016/j.ibmb.2005.01.014 PMid:15804578.
21. Johansson MW, Söderhäll K. The prophenoloxidase activating system and associated proteins in invertebrates. Prog Mol Subcell Biol. 1996; 15:46-66. https://doi.org/10.1007/978-3-642-79735-4_3 PMid:8963465.
22. Poletta GL, Simoniello MF, Mudry MD. Biomarkers of oxidative damage and antioxidant defense capacity in Caiman latirostris blood. Comp Biochem Physiol Part C: Toxicol Pharmacol. 2016; 179:29-36. https://doi.org/10.1016/j.cbpc.2015.08.003 PMid:26299575.
23. Duan X, Xu L, Song J, Jiao J, Liu M, Hu F, Li H. Effects of benzo[a]pyrene on growth, the antioxidant system, and DNA damage in earthworms (*Eisenia fetida*) in 2 different soil types under laboratory conditions. Environ Toxicol Chem. 2015; 34(2):283-290. https://doi.org/10.1002/etc.2785 PMid:25331318.
24. Beliaeff B, Burgeot T. Integrated biomarker response: A useful tool for ecological risk assessment. Environ Toxicol Chem. 2002; 21:1316-1322. https://doi.org/10.1002/etc.5620210629 PMid:12069320.
25. Broeg K, Lehtonen KK. Indices for the assessment of environmental pollution of the Baltic Sea coasts: Integrated assessment of a multi-biomarker approach. Mar Pollut Bull. 2006; 53:508-522. https://doi.org/10.1016/j.marpolbul.2006.02.004 PMid:16737720.
26. Paul T, Kumar S, Shukla S P, Pal P, Kumar K, Poojary N, Biswal A, Mishra A. A multi-biomarker approach using integrated biomarker response to assess the effect of pH on triclosan toxicity in *Pangasianodon hypophthalmus* (Sauvage, 1878). Environ Pollut. 2020; 260:114001. https://doi.org/10.1016/j.envpol.2020.114001 PMid:32041020.
27. Renwrantz L, Schanke W, Harn H, Erl H, Leibisch H, Gercken J. Discriminative ability and the function of the immunological recognition system of the snail Helix pomatia. J Comp Physiol A. 1981; 141:477-488. https://doi.org/10.1007/BF01101470
28. Mussino F, Pozzolini M, Valisano L, Cerrano C, Benatti U, Giovine M. Primmorphs cryopreservation: A new method for long-time storage of sponge cells. Mar Biotechnol. 2012; 15(3):357-367. https://doi.org/10.1007/s10126-012-9490-z PMid:23151942.

29. Ehlers D, Zosel B, Mohrig W, Kauschke E, Ehlers M. Comparison of in vivo and in vitro phagocytosis in Galleria mellonella L. Parasitol Res. 1992; 78(4):354-359. https://doi.org/10.1007/BF00937096
30. Adamowicz A, Wojtaszek J. Morphology and phagocytotic activity of coelomocytes in *Dendrobaena veneta* (Lumbricidae). Zool Pol. 2001; 46(1-4):91-104.
31. Takahashi K, Akaike T, Sato K, Mori K, Maeda H. Superoxide anion generation by Pacific oyster (*Crassostrea gigas*) hemocytes: Identification by electron spin resonance spin trapping and chemiluminescence analysis. Comp Biochemistry and Physiology Part B. 1993; 105(1):35-41. https://doi.org/10.1016/0305-0491(93)90166-3
32. Green LC, Wagner DA, Glogowski I, Skipper PL, Wishnok IS, Tannenbaum S. Analysis of nitrate, nitrite and (^{15}N) nitrate in biological fluids. Anal Biochem. 1982; 126:131-138. https://doi.org/10.1016/0003-2697(82)90118-X PMid:7181105 PMCid: PMC7905294.
33. Söderhäll K, Unestam T. Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. Can J Microbiol. 1979; 25:406-414. https://doi.org/10.1139/m79-062 PMid:110434.
34. Beauchamp C, Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal Biochem. 1971; 44(1):276-287. https://doi.org/10.1016/0003-2697(71)90370-8 PMid:4943714.
35. Aebi H. Catalase *in vitro*. Methods Enzymol. 1984; 105:121-126. https://doi.org/10.1016/S0076-6879(84)05016-3 PMid:6727660.
36. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193:265-275. https://doi.org/10.1016/S0021-9258(19)52451-6 PMid:14907713.
37. Mukherjee S, Gautam A, Pal K, Karmakar P, Ray M, Ray S. Copper oxide nanoparticle and copper sulfate induced impairment of innate immune parameters in a common Indian sponge. J Hazard Mater Lett. 2021; 2:100036. https://doi.org/10.1016/j.hazl.2021.100036
38. Sanchez W, Burgeot T, Porcher JM. A novel "Integrated Biomarker Response" calculation based on reference deviation concept. Environ Sci Poll Res. 2013; 20:2721-2725. https://doi.org/10.1007/s11356-012-1359-1 PMid:23208755.
39. Cao R, Wang D, Wei Q, Wang Q, Yang D, Liu H, Dong Z, Zhang X, Zhang Q, Zhao J. Integrative biomarker assessment of the influence of saxitoxin on marine bivalves: A comparative study of the two bivalve species oysters, *Crassostrea gigas*, and Scallops, *Chlamys farreri*. Front Physiol. 2018; 9:1-14. https://doi.org/10.3389/fphys.2018.01173 PMid:30246779 PMCid:PMC6110902.
40. Pedrazzani R, Ceretti E, Zerbini I, Casale R, Gozio E, Bertanza G, Gelatti U, Donato F, Feretti D. Biodegradability, toxicity and mutagenicity of detergent: Integrated experimental evaluation. Ecotoxicol Environ Saf. 2012; 84:274-281. https://doi.org/10.1016/j.ecoenv.2012.07.023 PMid:22898309.
41. McKee JE, Wolf HW. Water quality criteria; California State Water Resources Control Board. Publication 3-A. 1963.
42. Mukherjee S, Ray M, Dutta M K, Acharya A, Mukhopadhyay S K, Ray S. Morphological alteration, lysosomal membrane fragility and apoptosis of the cells of Indian freshwater sponge exposed to washing soda (sodium carbonate). Ecotoxicol Environ Saf. 2015; 122:331-342. https://doi.org/10.1016/j.ecoenv.2015.08.011 PMid:26313128.
43. Radwan M, El-Gendy K, Gad A. Biomarker responses in terrestrial gastropods exposed to pollutants: A comprehensive review. Chemosphere. 2020; 257:127218. https://doi.org/10.1016/j.chemosphere.2020.127218 PMid:32497833.
44. Panda F, Pati SG, Das K, Samanta L, Sahoo DK, Paital B. Biochemical and molecular responses of the freshwater snail *Pila* sp. to environmental pollutants, abiotic, and biotic stressors. Front Environ Sci. 2022; 10:1033049. https://doi.org/10.3389/fenvs.2022.1033049
45. Oliver LM, Fisher WS. Appraisal of prospective bivalve immunomarkers. Biomarkers. 1999; 4(10):510-530. https://doi.org/10.1080/135475099230679 PMid:23902396.
46. Patel R, Kurhe A. Alterations in biochemical and genomic content of selected tissues of snail *Pila globosa* under copper and mercury sublethal toxicity. J Stress Physiol Biochem. 2025; 21(2):5–15.
47. Donaghy L, Kim BK, Hong HK, Park HS, Choi KS. Flow cytometry studies on the populations and immune parameters of the hemocytes of the Suminoe oyster, *Crassostrea ariakensis*. Fish Shellfish Immunol. 2009; 27:296-301. https://doi.org/10.1016/j.fsi.2009.05.010 PMid:19490941.
48. Ballarin L, Covre V, Masiero L, Casellato S. Immunotoxic effects of fluoride on the hemocytes of *Venerupis philippinarum*. Invertebrate Surviv J. 2014; 11:22-29.
49. Russo J, Lagadic L. Effects of environmental concentrations of atrazine on hemocyte density and phagocytic activity in the pond snail *Lymnaea stagnalis* (*Gastropoda, Pulmonata*). Environ Pollut. 2004; 127(2):303-311. https://doi.org/10.1016/S0269-7491(03)00269-0 PMid:14568729.
50. Wu F, Kong H, Xie L, Sokolova IM. Exposure to nanopollutants (nZnO) enhances the negative effects of hypoxia and delays recovery of the mussels' immune system. Environ Pollut. 2024; 351:124112. https://doi.org/10.1016/j.envpol.2024.124112 PMid:38705446.
51. Lynch AE, Noble LR, Jones CS, Routledge EJ. Common aquatic pollutants modify hemocyte immune responses in *Biomphalaria glabrata*. Front Immunol. 2022; 13:839746. https://doi.org/10.3389/fimmu.2022.839746 PMid:36159819 PMCid:PMC9493456.
52. Leão GR, Silva LPS, Damacena-Silva L, Rocha TL. Toxicity of environmental chemicals in gastropods' hemocytes: trends

and insights based on investigations using *Biomphalaria* spp. Sci Total Environ. 2024; 957:177522. https://doi.org/10.1016/j.scitotenv.2024.177522 PMid:39561895.
53. Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem. 1995; 64:97-112. https://doi.org/10.1146/annurev.bi.64.070195.000525 PMid:7574505.
54. Franchini A, Fontanili P, Ottaviani E. Invertebrate immunocytes: Relationship between phagocytosis and nitric oxide production. Comp Biochem Physiol Part B: Biochem Mol Biol. 1995; 110:403-407. https://doi.org/10.1016/0305-0491(94)00163-O
55. Nappi AJ, Ottaviani E. Cytotoxicity and cytotoxic molecules in invertebrates. BioEssays. 2000; 22(5):469-480. https://doi.org/10.1002/(SICI)1521-1878(200005)22:5<469::AID-BIES9>3.0.CO;2-4 PMid: 10797487
56. Gopalakrishnan S, Huang W, Wang Q, Wu M, Liu J, Wang K. Effects of tributyltin and benzo[a]pyrene on the immune associated activities of hemocytes and recovery responses in the gastropod abalone, *Haliotis diversicolor*. Comp Biochem Physiol Part C: Toxicol Pharmacol. 2011; 154:120-128. https://doi.org/10.1016/j.cbpc.2011.04.004 PMid:21549218
57. Panda F, Pati SG, Anwar TN, Samanta L, Paital B. Seasonal variation of water quality modulated redox regulatory system in the apple snail *Pila globosa* and its use as a bioindicator species in freshwater ecosystems across India. Water. 2022; 14(20):3275. https://doi.org/10.3390/w14203275
58. Wang J, Deng W, Zou T, Bai B, Chang AK, Ying. Cadmium-induced oxidative stress in *Meretrix meretrix* gills leads to mitochondria-mediated apoptosis. Ecotoxicology. 2021; 30(10):2011-2023. https://doi.org/10.1007/s10646-021-02465-8 PMid:34529205.
59. Ibrahim AM, Al-Fanharawi AA, Dokmak HA. Ovicidal, immunotoxic and endocrine disrupting effects of saponin on *Bulinus truncatus* snails with special emphasize on the oxidative stress parameters, genotoxicological, and histopathological alterations. Environ Sci Pollut Res. 2023; 30(32):78641-78652. https://doi.org/10.1007/s11356-023-27668-w PMid:37273057 PMCid:PMC10313852.
60. Luna-Acostaa A, Breitwiesera M, Renaultb T, Thomas-Guyon H. Recent findings on phenoloxidases in bivalves. Mar Pollut Bull. 2017; 122(1-2):5-16. https://doi.org/10.1016/j.marpolbul.2017.06.031 PMid:28673617.
61. Munoz P, Meseguer J, Esteban MA. Phenoloxidase activity in three commercial bivalve species. Changes due to natural infestation with *Perkinsus atlanticus*. Fish Shellfish Immunol. 2005; 20(1):12-19. https://doi.org/10.1016/j.fsi.2005.02.002 PMid:15979898.
62. Mottin E, Caplat C, Mahaut M L, Costil K, Barillier D, Lebel J M, Serpentini A. Effect of *in vitro* exposure to zinc on immunological parameters of hemocytes from the marine gastropod *Haliotis tuberculata*. Fish Shellfish Immunol. 2010; 29(5):846-853. https://doi.org/10.1016/j.fsi.2010.07.022 PMid:20659566.
63. Idakieva K, Siddiqui N I, Meersman F, De Maeyer M. Influence of limited proteolysis, detergent treatment and lyophilization on the phenoloxidase activity of *Rapana thomasiana* hemocyanin. Int J Biol Macromol. 2009; 45(2):181-187. https://doi.org/10.1016/j.ijbiomac.2009.04.022 PMid:19426758.
64. Wang C, Yue X, Lu X, Liu B. The role of catalase in the immune response to oxidative stress and pathogen challenge in the clam *Meretrix*. Fish Shellfish Immunol. 2013; 34:91-99. https://doi.org/10.1016/j.fsi.2012.10.013 PMid:23079536.
65. Bhattacharya P, Swarnakar S, Mukhopadhyay A, Ghosh S. Exposure of composite tannery effluent on snail, *Pila globosa*: A comparative assessment of toxic impacts of the untreated and membrane treated effluents. Ecotoxicol Enviro Saf. 2016; 126:45-55. https://doi.org/10.1016/j.ecoenv.2015.12.021 PMid:26720808.
66. Aydin AN, Serdar O, Parlak AKT. Determination of toxic effect of gamma cyhalothrin in *Dreissena polymorpha* by some biomarkers. J Limnol Freshw Fisheries Res. 2025; 11(1):20-32. https://doi.org/10.17216/limnofish.1556682
67. Mukherjee S, Bhunia AS, Bhunia NS, Ray M, Ray S. Immunomodulatory effects of temperature and pH of water in an Indian freshwater sponge. J Thermal Biol. 2016; 59:1-12. https://doi.org/10.1016/j.jtherbio.2016.04.005 PMid:27264881.
68. Dzul-Caamal R, Vega-López A, von Osten JR. Distribution of heavy metals in crop soils from an agricultural region of the Yucatan Peninsula and biochemical changes in earthworm *Eisenia foetida* exposed experimentally. Environ Monit Assess. 2020; 192:82. https://doi.org/10.1007/s10661-020-08273-7 PMid:32382918
69. Mukherjee S, Ray M, Ray S. 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. Interdiscip Toxicol. 2018; 11(2):155-168. https://doi.org/10.2478/intox-2018-0012 PMid: 31719787 PMCid:PMC6829681.

Author Contributions

Conceptualisation, S.M. and S.S.; Methodology, S.M.; Software, S.M.; Writing—original draft preparation, S.M.; writing—review and editing, S.M. and S.S.

Access this journal online	
Website: https://www.informaticsjournals.co.in/index.php/toxi/	**Quick Response Code**