UTTAR PRADESH JOURNAL OF ZOOLOGY

42(5): 1-8, 2021
ISSN: 0256-971X (P)

EFFECT OF FENVALERATE ON SUPEROXIDE ANION AND NITRIC OXIDE GENERATION IN THE JUVENILES OF *Bellamya bengalensis* AN EDIBLE GASTROPOD OF WEST BENGAL

CHIRANJIB MANDAL[1*] AND SUMAN MUKHERJEE[2]

[1]Department of Biological Science and Environmental Studies, Ghatakpur Swamiji Vidyapith High School (H.S), Village and P. O.- Ghatakpur, Dist- South 24 Parganas, West Bengal, India.
[2]Department of Zoology, Bidhannagar College, SaltLake City, Kolkata-700064, West Bengal, India.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Author CM designed study, literature searches, wrote the protocol and performed the experiment. Author SM performed the statistical analysis of data. Both authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Dr. Juan Carlos Troiano, Professor, University of Buenos Aires, Argentina.
Reviewers:
(1) Rozane Maria Restello, Brazil.
(2) Luidi Eric Guimarães Antunes, State University of Rio Grande do Sul, Brazil.

Received: 30 December 2020
Accepted: 07 March 2021
Published: 18 March 2021

Original Research Article

ABSTRACT

Mollusc represents a vital component of freshwater ecosystem of our country. *Bellamya bengalensis* is an edible viviparous gastropod constitutes a traditional food item of human, poultry and fish. It demands a special importance in ecology, ethnomedicine and economy. Natural habitat of *B. bengalensis* is under ecological risk due to indiscriminate and unrestricted use of a synthetic pyrethroid pesticide fenvalerate by Indian farmers. Mollusc in general, depends on hemocytes in elicitation of immunological responses including production of cytotoxic agents like superoxide anion and nitric oxide against environmental xenobiotics and pathogen. Present study is aimed to assess cytotoxic response with superoxide anion and nitric oxide generation in hemocytes of juvenile specimens of *B. bengalensis* to examine any immune alteration of the edible species.. Present study would provide an important information base of immunotoxicity of fenvalerate in juvenile specimens of *B. bengalensis*, which can be utilized in formulating a sustainable strategy of conservation and culture of aquatic mollusc in their natural habitat for human consumption.

Keywords: Pesticide; mollusc; hemocyte; superoxide anion generation; nitric oxide.

**Corresponding author: Email: chiranjib.san@gmail.com;*

ABBREVIATIONS

NBT : *Nitro blue tetrazolium*
ROS : *Reactive oxygen species*
OD : *Optical density*
E.C : *Emulsifiable concentrate*

1. INTRODUCTION

Molluscs play a significant role in freshwater ecosystem. Freshwater molluscs like *Bellamya bengalensis* (Gastropoda:Prosobranchia) is an economically, nutritionally and medicinally important species of India [1]. Well organized markets of the edible freshwater molluscs like *B. bengalensis* and *Lamellidens marginalis* are in report in the state of West Bengal, India. Molluscs evolved filter-feeding mechanism and bioaccumulation on potential in aquatic environment [2]. Due to rapid increase of human population and urbanization, habitats of the molluscs have been restricted to a limited area and facing the risk of chemical contamination. For controlling insect pest, pesticide is used in agriculture and horticulture sectors. Indiscriminate use of synthetic pyrethroid like fenvalerate is considered as a serious concern of aquatic environment. Pyrethroid adversely affects the nontarget organisms like crustaceans [3], bivalve [4], fish [5] and mammal [6].

Information on pyrethroid toxicity in *B. bengalensis* is scanty in the present scientific study. Ray et al..(2013b) [7] reported that fenvalerate and cypermethrin exposure created structural damage and density shift in the hemocytes of bivalve *L. marginalis* and gastropod *B. bengalensis* respectively. Molluscan immune system depends on hemocytes. Hemocytes are the main immunoeffector cells of molluscan blood and also responsive to the toxic challenge of different types of toxins. Hemocytes act as a sensitive target for multiple environmental contaminants. They are involved in various types of physiological functions such as cell aggregation, self-nonself discrimination, wound repairing and various cytotoxic responses. In this study, cytotoxic agents like generation of superoxide anion and nitric oxide were estimated in the hemocytes of *B. bengalensis* exposed to fenvalerate along with respective control. The basic defence mechanism is involved with release of various types of cytotoxic molecules and phagocytosis, encapsulation [8]. Hemocytes produce defence molecule superoxide anion against intruding pathogenic microorganisms. Production of the superoxide anion by NADPH oxidase is a cascade of enzymatic reaction which is involved with reactive oxygen species (ROS) generation. Superoxide anion generation in the hemocytes can be determined by reduction of nitro blue tetrazolium (NBT) to a blue coloured compound formazan. We estimated the rate of superoxide anion generation in the hemocytes of juvenile specimens of *B. bengalensis* under the exposure of various experimental concentrations of fenvalerate respectively. Nitric oxide is a vital intrahemocytic cytotoxic molecule generated in response to oxidative stress and provides immunological defence to the host by deactivating foreign microorganisms [9]. Nitric oxide itself is reported as a cytotoxic agent, but in combination with superoxide anion produces a highly toxic substance the peroxynitrite anion ($ONOO^-$) [10]. At the exterior of the cell, in the dismutation reaction, superoxide anions get converted into hydrogen peroxide. Ions may react with hydrogen peroxide to produce oxygen singlet. In addition to cytotoxicity, nitric oxide involved in the physiology of neurotransmission, muscle contraction, mucus secretion, excretion and triggering the feeding behaviour [11]. Nitric oxide had been reported as a biomarker of arsenic toxicity in *L. marginalis* [12]. In this study intrahemocytic generation of nitric oxide of *B. bengalensis* was determined by exposing them to sublethal concentrations of fenvalerate in controlled laboratory conditions. In current years, juvenile toxicity of chemical toxins like fenvalerate has been emerging as a subject of special interest in many invertebrate species. Freshly hatched juveniles of *B. bengalensis* are subjected to investigation of cytotoxic molecule generation of superoxide anion, nitric oxide in the hemolymph of juvenile *B. bengalensis* under the sublethal exposure of fenvalerate.

2. MATERIALS AND METHODS

2.1 Collection, Laboratory Acclimation and Maintenance of Juvenile *B. bengalensis* in Controlled Laboratory Condition

Fresh live specimens adult and gravid females of *B. bengalensis* with shell lengths (35- 44 mm) was collected from freshwater ponds of South 24 Parganas district of West Bengal. The collected specimens were transported to the laboratory in rectangular plastic containers as moist heaps within 2 hours of collection. Brood sac dissection of adult gravid female *B. bengalensis* (35- 44mm) was de-shelled mechanically to expose intact brood sac with eggs and juveniles (Fig.1A and B). Brood pouch contains unfertilized egg, fertilized egg with embryo and live juveniles measuring shell length (2-4 mm). Membranous covering of the brood sac was carefully dissected and the released eggs and juveniles were collected in a sterile watch glass with 3ml of sterile normal saline. Tissue debris was separated carefully with forceps.

The juvenile specimens were acclimatized in the borocilicate glass jars for 8-10 days. Replenishment of freshwater was carried out every 24 hours to avoid residual toxicity. Juvenile specimens of *B. bengalensis* were maintained according to the protocols and methods of Raut (1991) [13].

2.2 Treatment with Fenvalerate

After 8-10 days of laboratory acclimation, both sexes of juvenile specimens of *B. bengalensis* (Fig. 1) were exposed to 0.125, 0.25, 0.5 and 1ppm of fenvalerate (Fenvalerate, 20 % E.C., Cas Number- 51630-58-1) for various spans of time i.e. 24,48,72,96 hours and 15 days for cytotoxicity of hemocyte along with the control.

2.3 Hemolymph Collection

Water present over the shell of juvenile was soaked with filter paper, air dried and subjected to hemolymph collection. Hemolymph from each 30-40 live specimens was collected by manual shell crushing method and smeared in sterile grease free glass slides for cellular study and pooled hemolymph was collected in prechilled microfuge tube at 4^0c for biochemical analysis.

2.4 Hemocyte Isolation

Collected hemolymph, prior to hemocyte isolation [14] was observed under the microscope (OlympusBX2, India) after smearing on glass slide. The hemolymph was centrifuged at4^0c at 3000 rpm for 10 minutes and resuspended thrice. The density of hemocyte was maintained with unit volume of sterile snail saline [15].

2.5 Cell viability Assay

The viability of hemocytes was screened by staining the cells with 0.4% (1:1) trypan blue (E. Marck, Germany) for 2-5 minutes.

2.6 Cytotoxicity Assay of Hemocyte

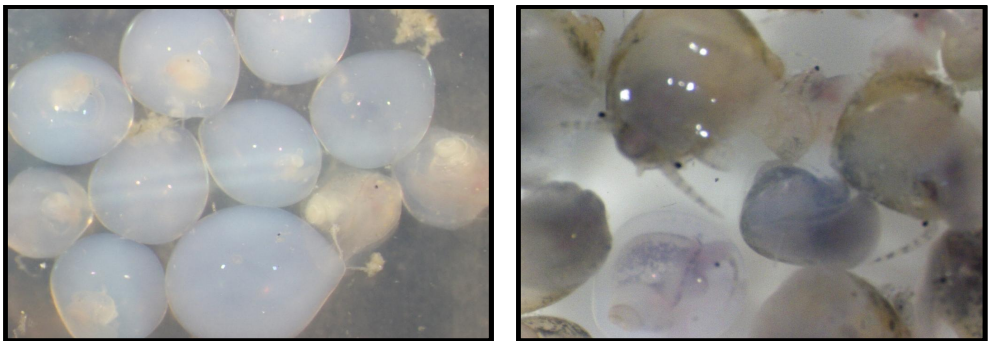
Production of cytotoxic agents like superoxide anion and nitric oxide were examined in the hemocytes of juvenile *B. bengalensis* under backdrop of fenvalerate exposure against untreated specimen.

2.6.1 Superoxide anion estimation

Production of superoxide anion was estimated spectrophotometrically by the principle of nitro blue tetrazolium reduction [15]. Sorted hemocytes were washed and adjusted to the density of 10^6cells/ml. An equal volume of nitro blue tetrazolium (NBT) solution (0.03%) was added with it to incubate for 30 minutes at 37^0C. The reaction was ended by removing the NBT solution by centrifugation and adding absolute methanol. The hemocytes were cleaned with 70% methanol and suspended in a KOH solution (1 ml, 2 M) and DMSO (1 ml) to dissolve the blue coloured cytoplasmic formazan. The optical density of dissolved formazan was estimated spectrophotometrically (CECIL-CE 4002, Germany) at 630nm.The production of superoxide anion was expressed as OD 630nm/min/10^6 cells. The entire experiment was repeated for at least 5 times.

2.6.2 Nitric Oxide Estimation

Isolated hemocytes were washed with sterile saline. The cell density was adjusted to 10^6cells/ml and 1ml of the hemocyte suspension was incubated with equal volume of Griess reagent (1%Sulphalinamide, 0.1% naphthyl ethylene diamine dihydrochloride and 5% orthophosphoric acid) at 37^0 c for 30 minutes in humid chamber. The absorbance was determineds in a spectrophotometer (CECIL- CE 4002, Germany) at 550 nm against a standard blank. The generation of nitric oxide was expressed in terms of formation of nitrite in µM/106cells/min. Production of nitric oxide was estimated spectrophotometrically at 550 nm following the principle of Griess reaction [16].



A **B**

Fig.1. A. Egg and B. Juveniles of *Bellamya bengalensis* after de-shelled condition

3. RESULTS

3.1 Superoxide Anion

Generation of superoxide anion was determined in the hemocytes of two separate sex of juvenile specimen of *B. bengalensis* in all four experimental concentrations in comparison to the respective control. The superoxide anion generation in the hemocytes of untreated juvenile specimen of male *B. bengalensis* ranged from 0.05±0.01 OD/10^6 cells/ml/min to 0.10±0.01 OD/10^6 cells/ ml/min under different spans of exposure (Fig. 2). A significant alteration of superoxide anion generation of hemocyte was observed under various experimental concentrations of fenvalerate. Generation of superoxide anion of juvenile specimen of male *B. bengalensis* was recorded as high in the hemocytes of 0.21, 0.19, 0.20 OD/10^6 cells against 0.5ppm for 72 hours, 0.25ppm for 15 days and 1ppm for 15 days respectively (Fig. 2).

The generation of superoxide anion in the hemocytes of untreated juvenile specimen of female *B. bengalensis* ranged from 0.11±0.03OD/10^6 cells/ml/min under various spans of exposure (Fig. 3). However, the generation of superoxide anion was recorded as high in the hemocytes of juvenile specimen of female *B. bengalensis* as 0.248±0.01, 0.16±0.02 OD/10^6 cells/ ml/min against 0.5ppm for 72hours, 0.25ppm for 15days fenvalerate respectively (Fig. 3). Pattern of fenvalerate induced variation in the generation of superoxide anion in the hemocytes of juvenile specimens of male and female *B. bengalensis* appeared to be similar pattern.

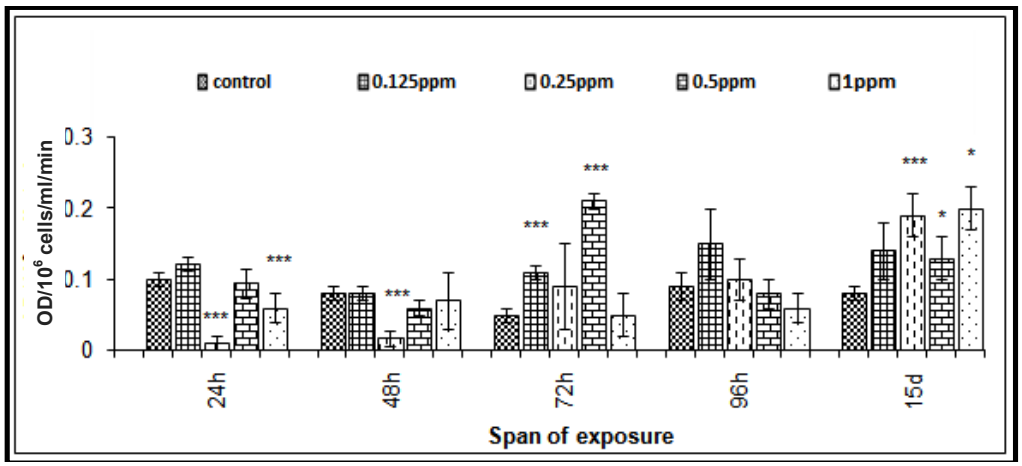


Fig. 2. Generation of superoxide anion in hemocytes of juvenile male of *B. bengalensis* exposed to fenvalerate, Data expressed as mean ± S.D. (n = 5) *p<0.05, **p<0.01, ***p<0.001

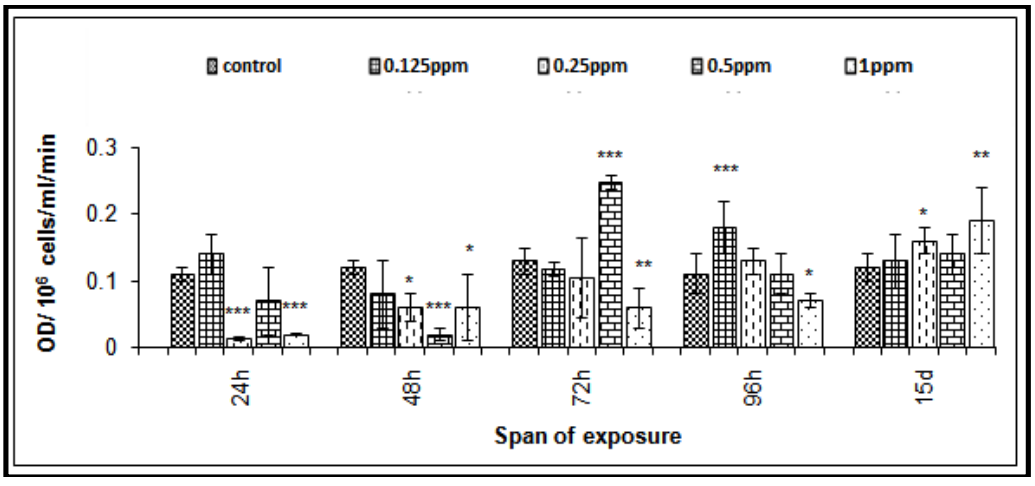


Fig. 3. Generation of superoxide anion in hemocytes of juvenile female of *B. bengalensis* exposed to fenvalerate, Data expressed as mean ± S.D. (n = 5) *p<0.05, **p<0.01, ***p<0.001

3.2 Nitric Oxide

Intrahemocytic generation of nitric oxide was determined in the form of nitrite formation in the hemocytes of two separate sexes of juvenile specimen of *B. bengalensis* against different experimental concentrations of fenvalerate for multiple span of exposure along with respective control. Generation of nitric oxide exhibited by the untreated hemocytes of juvenile specimen of male *B. bengalensis* ranged between 0.013±0.001 µM nitrite/10^6cells/min and 0.022±0.002 µM nitrite/10^6 cells/min under various spans of experiment (Fig. 4). A significant, nonlinear inhibition of the generation of nitric oxide was recorded against all experimental concentrations of fenvalerate for all spans of exposure in comparison to the respective control. The highest inhibition of nitric oxide generation was recorded as 0.008±0.002µM nitrite/10^6cells/min against the concentration of 1ppm of fenvalerate for 48 hours of exposure. The maximum increase in the generation of nitric oxide was recorded as 0.046±0.002µM nitrite/10^6cells/min against 0.25ppm fenvalerate for 72 hours exposure (Fig. 4).

Generation of nitric oxide exhibited by the untreated hemocytes of juvenile specimen of female *B. bengalensis* ranged between 0.017±0.003 µM nitrite/10^6cells/min to 0.024±0.007 µM nitrite/10^6cells/min under different spans of experimental exposure (Fig. 5). The highest inhibition of nitric oxide generation was recorded as 0.0074±0.002 µM nitrite/10^6cells/min against the concentration of 1ppm fenvalerate for 48 hours exposure. The maximum increase of generation of nitric oxide was recorded as 0.049±0.003 µM nitrite/10^6cells/min against 0.25ppm of fenvalerate for 72 hours exposure (Fig. 5). Pattern of fenvalerate induced alteration in the generation of nitric oxide in the hemocytes of juvenile specimens of male and female *B. bengalensis* exhibited similar pattern.

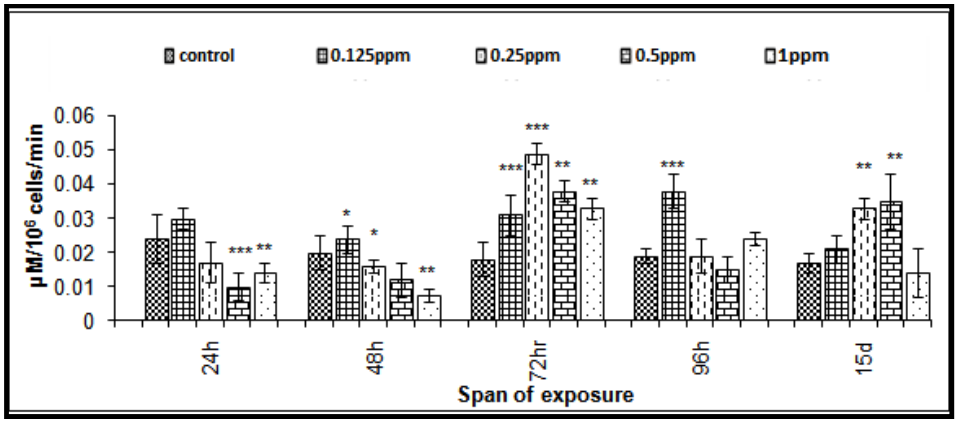


Fig. 4. Intrahemocyte nitric oxide generation in juvenile male of *B. bengalensis* under the exposure of fenvalerate, Data expressed as mean ± S.D. (n = 5) *p<0.05, **p<0.01, ***p<0.001

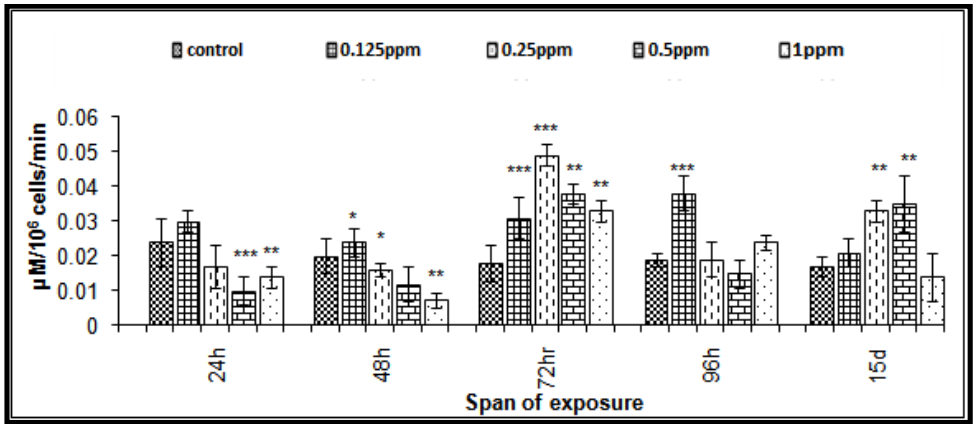


Fig. 5. Intrahemocyte nitric oxide generation in juvenile female of *B. bengalensis* under the exposure of fenvalerate, Data expressed as mean ± S.D. (n = 5) *p<0.05, **p<0.01, ***p<0.001

4. DISCUSSION

Molluscs in ecotoxicology occupy a distinct place and often considered as agents for passive and active biomonitoring for environmental risk assessment [17]. *B. bengalensis* bears commercial prospect and hold significance in ethnomedicine, nutrition, biotechnology and pharmaceutical industry. This benthic organism accumulates various environmental toxins within tissues and maintaining the steady and sustainable health of aquatic ecosystem. Report of toxicity of fenvalerate in juvenile *B. bengalensis* is absent in literature. Juveniles in general are more susceptible chemical toxicity. In this article, selected cytotoxic responses are reported in hemocytes of *B. bengalensis* exposed to fenvalerate respectively with reference to generation of superoxide anion and nitric oxide. Hemocytes are capable of generating various types of cytotoxic compounds under the exposure of environmental toxins and microorganisms. Superoxide anion and nitric oxide are the cytotoxic chemicals reported both in invertebrate and vertebrate [18,19]. A significant non directional alteration of generation of superoxide anion in the hemocytes of juvenile specimens of both male and female *B. bengalensis* were recorded against the exposure of 0.125,0.25,0.5 and 1ppm for 24,48,72,96 hours and 15 days (Figs. 2 and 3). Pattern of alteration in the generation of superoxide anion appear to be a dose independent and nonlinear and indicative to a state of a toxin induced cellular stress. Hemocytes release cytotoxic substances for elimination of phagocytosed materials during adverse physico-chemical environmental conditions [20]. This result is suggestive to a possible state of immunological alteration due to fenvalerate induced immunological stress in the hemocytes of *B. bengalensis.*

Nitric oxide is an active free redical which performs a fundamental physiological role in the neuronal, cardiovascular and immune systems of different organisms. Nitric oxide generation was significantly decreased in both juvenile specimens of male and female *B. bengalensis* under the exposure of 0.25, 0.5 and 1 ppm fenvalerate with time span of 24 and 48 hours (Figs. 4 and 5). But nitric oxide generation was significantly increased the exposure of 0.125,0.25,0.5 and 1ppm fenvalerate with time span of 72, 96 hours and 15 days. Maximum generation of nitric oxide in the hemocytes of both juvenile male and female *B. bengalensis* was noted against 0.25 ppm of fenvalerate for 72 hours (Figs. 4 and 5). Superoxide and nitric oxide generation act as an important role in the innate immune response of organism [21]. The nitric oxide generating system might be a sensitive biomarker of stress in invertebrate. Nitric oxide is an important cytotoxic factor that elicits host defence reaction under the exposure of various toxins and invasion of pathogens. The result was suggestive to a state of fenvalerate induced immunological stress in *B. bengalensis* with reference to alteration of nitric oxide generation in hemocyte. Such a situation may lead to affect different physiological functions like elicitation of cytotoxic substances and immunological activity of hemocytes of *B. bengalensis* inhabiting a contaminated habitat. This investigation would provide important malacological information with reference to the functional attributes of hemocytes and its cytotoxic response against pyrethroid pesticides. In a contaminated environment juveniles population is assumed to be maximally affected by toxins. Subadult immatured stages of *B. bengalensis* is thought to be heavily affected by fenvalerate. Toxicity of fenvalerate in juveniles of both sexes would adversely affect the population size and population structure of adult organisms. Unrestricted contamination of the natural habitat of *B. bengalensis* may shrinkage of population size of this gastropod. Situation may lead to a state of ecological crisis and loss of traditional dietary item for the human population of rural India.This report is thus expected will provide a novel base of information regarding pyrethroid toxicity in *B. bengalensis* an economically important edible mollusc of India.

5. CONCLUSIONS

B. bengalensis, a prosobranch mollusc is widely distributed in the freshwater ecosystem of our country. Natural habitat of this species bears the risk of contamination by fenvalerate, a synthetic type II pyrethroid pesticide. Fenvalerate exposure yielded in a general increase in the generation of superoxide anion in the hemocytes of juveniles of both male and female *B. bengalensis* against all experimental concentrations of fenvalerate for 72, 96 hours and 15 days exposure. It is reported that fenvalerate exposure led to cause in a general increase in the generation of nitric oxide in the hemocytes of juveniles of both male and female *B. bengalensis* against all experimental concentrations of fenvalerate for 72, 96 hours and 15 days exposure. Current investigation would provide a vital information base of the toxicity of fenvalerate in gastropod mollusc *B. bengalensis.* Unrestricted use of fenvalerate and the contamination of freshwater reservoirs by pyrethroids may result in a gradual decrease in the population of *B. bengalensis* and allied species in future. *B. bengalensis* is reported to be economically and medicinally important species and important bioresource of India.

ACKNOWLEDGEMENTS

We are thankful to the Postgraduate Department of Zoology, Bidhannagar College and Management

committee of Ghatakpur Swamiji Vidyapith High School (H.S) for providing support to complete the research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Khalua RK, TRIPATHY S, Paul B, Bairy D. Seasonal variation of carbohydrate, protein and lipid of common freshwater edible gastropod (*Bellamya bengalensis*) of Medinipur district, West Bengal. Research Journal of Biology. 2014;2:49-52.
2. Prabhakar AK, Roy S P, Ethnomedicinal uses of some shell fishes by people of Kosi river basin in North Bihar, India. Ethno Medicine. 2009;3(1):1-4.
3. Rittschof D, McClellan GP. Molluscs as multidisciplinary models environment toxicology. Marine Pollution Bulletin. 2005;5:369-373.
4. Sreenivasan RS, Moorthy PK, Deecaranan M. Variations in the enzyme activity of gills and hemolymph in cypermethrin, a pesticide treated freshwater female field crab, *Spiralothelphusa hydrodroma* (Herbst). International Journal of Sustainable Agriculture. 2010;2(1):10-15.
5. Das K, Ray M, Ray S. Shift in foot protrusion frequency, cholinergic impairment in pedal ganglia and histopathology of labial palp in freshwater mussel exposed to cypermethrin. In: Perspectives in Animal Ecology and Reproduction (Gupta VK. Ed.) Daya Publishing House: India, 2015;123-136.
6. Muller-Mohnssen H. Chronic sequelae and irreversible injuries following acute pyrethroids intoxication. Toxicology Letter. 1999;107:161-76.
7. Ray M, Bhunia AS, Bhunia NS, Ray S. Density shift, morphological damage, lysosomal fragility and apoptosis of hemocytes of Indian molluscs exposed to pyrethroid pesticides. Fish Shellfish Immunology. 2013b; 35:499-512.
8. Carballal MJ, Lopez C, Azevedo C, Villalba A. *In-vitro* study of phagocytic ability of *Mytilus galloprovincialis* Lmk. hemocytes. Fish Shellfish Immunology.1997b; 7:403-416.
9. Radomski MW, Martin JF, Moncada S. Synthesis of nitric oxide by the hemocytes of the American horse shoe crab (*Limulus polyphemus*). Philosophical Transactions B. 1991;334:1269.
10. Beckman JS, Koppenol W. Nitric oxide, Superoxide and peroxynitrite: The good, the bad and the ugly. American Journal of Physiology-Cell Physiology. 1996;40c:1424-1437.
11. Moroz LL, Cheng D, Gillette MU, Gillette R. Nitric oxide synthase activity in the molluscan CNS. Journal of Neurochemistry. 1996;66:873-876.
12. Chakraborty S, Ray M, Ray S. Evaluation of phagocytic activity and nitric oxide generation by molluscan hemocytes as biomarkers of inorganic arsenic exposure. Biomarkers. 2009b;14(8):539-546.
13. Raut SK. Laboratory rearing of medically and economically important molluscs. In Snails, flukes and man (Jairajpuri MS, Ed.). Zoological Survey of India Publication. 1991;79-83.
14. Brousseau P, Payette Y, Tryphonas H, Blackey B, Boernans H, Flipo D, Fournier M. Manual of Immunological methods. Boca Raton, 1999. FL: CRC Press.
15. Adema CM, Van der Knaap WPW, Sminia T. Molluscan hemocytes mediated Cytotoxicity: Role of reacting oxygen intermediates. Reviews in Aquatic Science. 1991b;4:201-223.
16. Green LC, Wagner D A, Glogowski I, Skipper PL, Wishnok IS, Tannenbaun S. Analysis of nitrate, nitrite and (N^{15}) nitrate in biological fluids. Analytical Biochemistry. 1982;126:131-8.
17. Salanki J. Biological monitoring of the state of the environment: Bioindicators. An overview of the international union for biological science programme on bioindicators. IRL Press Limited, Oxford; 1986.
18. Nappi AJ, Ottaviani E. Cytotoxicity and cytotoxic molecules in invertebrates. Bioassays. 2000;22:469-480.
19. Vijayavel K, Gopalakrishnan S, Thiagarajan R, Thilagam H. Immunotoxic effects of nickel in the mud crab *Scylla serrata.* Fish and Shellfish Immunology. 2009;26(1):133-139.
20. Anderson RS, Oliver LM, Brubacher LL. Superoxide anion generation by *Crassostrea virginica* hemocytes as measured by nitro blue

tetrazolium reduction. Journal of Invertebrate Pathology. 1992;59:303-307.

21. Wong JM, Billiar TR. Regulation and function of inducible nitric oxide synthase during sepsis and acute inflammation. Advances in Pharmacology. 1995;34:155-170.