Toxicology International, *Vol 27(3&4), 168-173, 2020, DOI: 10.18311/ti/2020/v27i3&4/25917*

Original Research
ISSN (Print): 0971-6580
ISSN (Online): 0976-5131

Impact of Cu and Hg on Biochemical Parameters of Bivalve, *Villorita cyprinoides*

Ranjusha Nair* and Rajathy Sivalingam

School of Environmental Studies, Cochin University of Science and Technology, Kochi – 682022, Ernakulam, Kerala, India; ranjushamanoj@gmail.com

Abstract

Heavy metal pollution is a growing environmental problem in marine coastal waters; for this reason the monitoring of water pollution needs the availability of test-species able to furnish reliable and cheap results. From this point of view, bivalves are important test organisms owing to their wide distribution and for their key-position within the food web. As *Villorita cyprinoides* is a promising target-species in ecotoxicology, in this study the toxicity of different concentrations of two heavy metals (copper and mercury) was evaluated, 4 mg/l and 9 mg/l of Cu were taken. Treatments were performed after 10 and 20 days of exposure on adult *Villorita cyprinoides.* The biochemistry of the animals was evaluated. There was marked decrease observed in the stored glycogen level in the hepatopancrease of the organism, 35.77 mg gm^{-1} after 20 days with a concentration of 4 ppm and 30.57 at 9 ppm after 20 days exposure to Cu. There was a significant decrease in the level of protein content in the foot and hepatopancrease in presence of Cu. The protein content of the foot, after 20 days, in 4 ppm and 9 ppm was 15.62 and 10.25 mg/l respectively. The level of protein decreased to 13.37 and 14.12 mg gm^{-1} respectively in the hepatopancrease after 20 days of exposure to 4 ppm and 9 ppm. The results showed that mercury (0.1 and 0.3 mg/l) was toxic for *Villorita cyprinoides.* Animals exposed to Hg alone as well as in combination with Cu and Hg exhibited high mortality rate. The synergistic effect of the metals was more lethal. The study shows that the prolonged exposure of the heavy metals resulted in anoxic condition. The stress condition resulted in the utilization of stored energy.

Keywords: Biochemistry, Hepatopancrease, Heavy Metals, Synergistic effect

1. Introduction

India has rich molluscan resources all along the coast, in inshore waters, bays, backwaters and estuaries[1]. In India, bivalves are largely found in the southeast and southwest coasts of India. Bivalves are mostly harvested for meat and lime-based industries utilization. The Kerala State is one of the richest clams producing state in India. Clam resources are most abundant in the Vembanad Lake of the state, with annually estimated landings of 66,000 t. In Vembanad Lake, the molluscan bivalve; *Villorita cyprinoides* (Black mollusk) and *Etroplus suratensis* (Pearlspot) fish, contribute around 99% of the aggregate fisheries[13]. Several investigations were done on Vembanad Clam shells resources[13–14–18].

The use of aquatic organisms as bio-indicators for trace metal pollution is very common these days. Molluscs are among the organisms most used for this purposes. It is widely observed that various species of molluscs are the effective sentinel organisms and can achieve high concentrations of metals and metalloids relative to concentrations gradient of these substances in the surrounding environment[13–18].

Molluscs are basically a marine group of animals, but gastropods and bivalves are also found in fresh water environment. They occur in nearly all latitudes from polar to the tropical temperatures. Bivalves exhibit sedentary lifestyle, filter feeding habit, abundance, ease of identification

*Author for correspondence

and tolerance to pollution[2]. Bivalves have an extensive resource of antioxidant defenses and variations in these were found to be useful for bio monitoring[12]. There has been a search for sub lethal effects that would occur at much lower concentrations of the toxic substances than those causing death so these concentrations can be monitored. The present study was done to understand the impact of Copper and Mercury (Cu and Hg) at varying concentrations on the organism.

2. Materials and Methods

2.1 Study Area and Procurement

Bivalves, *Villorita cyprinoides* (75 ± 5 gm.) were collected from the Cochin estuary, Ernakulam District, Kerala, India (N 10°66'45.1and E 076°2'37.4" to N 10°03'16.1" and E 076°15'43.4") and transported to the departmental laboratory under suitable conditions. The animals were collected with the help of Van-veen grab and local fishermen. Animals were acclimatized to the laboratory conditions for 15 days with filtered sea water medium.

2.2 Experimental Regime

20 numbers of bivalves were exposed to the sub-chronic concentrations 4 and 9 mg/l of Cu and 0.1 mg/l and 0.3 mg/l Hg for 10 and 20 days respectively. They were also treated in combination of the two metals with same concentration. A group of bivalves unexposed served as control. The bivalves were reared in tanks of size 60 cm x 30 cm x 30 cm. The organisms were treated with $CuSO_4.5H_2O$ and $HgCl_2$. The experiment was performed in triplicate.

2.3 Biochemical Analysis

After the completion of the exposure period, the animals were sacrificed and different tissues like muscles, gills, hepatopancrease, mantle and foot were taken out. Total proteins were estimated by Folin-Phenol reagent method using bovine serum albumin as standard[14]. To 1 ml tissue homogenate (1:10 W/V) 5 ml of alkaline solution was added. After 10 min of incubation at room temperature, 0.5 ml of diluted Folin-Phenol reagent was added and the contents were thoroughly mixed. The color developed was read after 30 min using spectrophotometer at a wavelength of 750 nm.

Glycogen from the selected tissues was estimated by Anthrone reagent method[16–18]. Freshly excised tissues of bivalves were digested in 30% KOH. After digestion, the neutralization was done by glacial acetic acid. Each KOH digest was diluted to 100 ml with distilled water. To a 5 ml aliquot of this solution, 10 ml of freshly prepared Anthrone reagent (0.2% Anthrone in concentrated H_2SO_4 was added gradually. Similarly blank and standard tubes were also prepared using distilled water and glucose solution respectively.

The mixture was then boiled for 8 min by keeping the tubes in boiling water bath. After cooling the optical density of the mixture was read at 625 nm using spectrophotometer. Standard calibration curve was prepared for calculating glucose values. The glucose values thus obtained were converted into glycogen using Morrison's factor (1.11).

Lactic acid content in the tissues of bivalves was estimated by Hawk and Oser method[11]. The excised tissues were accurately weighed and homogenized in 10% TCA. After centrifugation, the clear supernatant was used for estimating lactic acid. 2 ml aliquot of the protein free filtrate was mixed with 1 ml of 20% copper sulphate solution. The mixture was diluted to 10 ml and 1 gm. of calcium hydroxide pellets was added to it. It was mixed on cyclometer for 1 min till the powder was uniformly dispersed and then allowed to stand for half an hour. The mixture was then centrifuged and 1 ml of clear supernatant from each tube was transferred to wide mouthed test tube to which 0.05 ml of 4% copper sulphate solution was added followed by 6 ml of concentrated sulphuric acid. Then the tubes were kept in a boiling water bath for 5 min. After cooling, 0.1 ml of p-hydroxydiphenyl reagent was added drop by drop in each tube. The tubes were kept at 30°C for half an hour. After placing the tubes in boiling water bath for 90 sec and cooling, the optical density of the solution was measured at 560 nm.

2.4 Statistical Analysis

Data presented in this study are mean of 5 samples with SD calculated on Microsoft Excel by standard statistical methods. The student's t-test was used for determining the significance of difference between mean value of control and experimental groups.

3. Results

In the present study, the bivalves exposed to 4 and 9 ppm of Cu for 10 and 20 days showed significant decrease in glycogen content in all the tissues as compared to control (Table 1). Mechanisms of bioaccumulation, storage and loss of accumulating substances in bivalves consist of several alternative pathways, varying with species, and chemical form of substance and mode of assimilation[9]. At the concentration of 4 ppm, after 10 days of exposure no significant decrease in glycogen content was found in mantle, gills, foot and hepatopancrease (Figure 1). Muscles showed significant decrease in glycogen content from 26.77 to 23.81 mg gm^{-1} weight. After 20 days of exposure at 4 ppm no significant decrease was found in foot and hepatopancrease but all the other tissues showed significant

decrease in glycogen. Glycogen content in gills was found to be decreased from 23.95 to 19.84 mg gm^{-1} weight (Table 1). Similar results were found in muscles and mantle at 4 ppm after 20 days of exposure.

At the sub-lethal concentration of 9 ppm after 10 days of exposure muscle and mantle indicated a significant decrease in glycogen while in other tissues it decreased insignificantly. Muscles showed decrease in glycogen content from 26.77 to 20.73 mg gm^{-1} weight. Mantle showed decrease from 48.79 to 33.66 mg gm^{-1} weights respectively. The long term exposure to 9 ppm showed significant decrease in glycogen content in all the tissues except foot (Table 1). All organisms died when treated with Hg at concentration of 0.1 mg/l and 0.3 mg/l on the fourth day. 100% mortality was observed in day one in the experimental setup with the combination of metals Cu and Hg.

Table 1. Glycogen (mg gm^{-1}) content in tissue of bivalve, *Villorita cyprinoides* after exposure to different concentration of Cu

Tissue	Control	4 mg /l		9 mg/l	
		10 days	20 days	10 days	20 days
Muscle	26.77±2.25	23.81*±1.9	21.10*±0.32	20.73*±0.30	19.17*±1.4
Mantle	48.79±7.98	48.57±0.24	38.99*±1.5	33.66*±0.47	31.82*±4.14
Gills	23.95±3.11	22.76±1.4	19.84*±1.4	21.73±1.39	17.37*±0.41
Foot	23.33±6.07	23.09±0.3	18.56±1.1	17.90±0.70	16.49*±0.62
Hepatopancrease	54.98±17.34	52.72±2.2	35.77±2.0	35.58±3.19	30.57*±2.44

Values are mean of five replicates ± SD *significantly different from control (P<0.05)

Table 2. Protein (mg gm^{-1}) content in tissue of bivalve, *Villorita cyprinoides* after exposure to different concentration of Cu

Tissue	Control	4 mg /l		9 mg/l	
		10 days	20 days	10 days	20 days
Muscle	22.5±1.58	20.25±1.9	16.75*±2.1	15.5*±1.08	16.62*±1.8
Mantle	31.87±1.7	26.37*±1.9	22.5*±2.12	17.0*±1.2	13.37*±1.75
Gills	28.75±2.3	24.5*±3.4	19.37*±3.5	17.75*±1.5	15.37*±2.13
Foot	21.75±1.8	18.87*±1.3	15.62*±1.7	12.5*±1.2	10.25*±1.3
Hepatopancrease	30.0±1.6	26.37±3.7	13.37*±2.2	13.87*±1.6	14.12*±1.6

Values are mean of five replicates ± SD; *significantly different from control (P<0.05)

Table 3. Lactic acid (mg gm^{-1}) content in tissue of bivalve, *Villorita cyprinoides,* after exposure to different concentration of Cu

Tissue	Control	4 mg /l		9 mg/l	
		10 days	20 days	10 days	20 days
Muscle	0.48±0.02	0.53±0.04	0.56*±0.007	0.72*±0.04	0.83*±0.003
Mantle	0.38±0.01	0.56*±0.04	0.67*±0.04	0.75*±0.04	0.81*±0.01
Gills	0.75±0.23	0.79±0.03	0.97±0.07	1.026±0.02	1.13*±0.07
Foot	0.40±0.05	0.67*±0.19	0.82*±0.02	0.80*±0.018	0.96*±0.005
Hepatopancrease	0.47±0.02	0.52*±0.03	0.56*±0.03	0.69*±0.008	0.76*±0.008

Values are mean of five replicates ± SD; *significantly different from control (P<0.05).

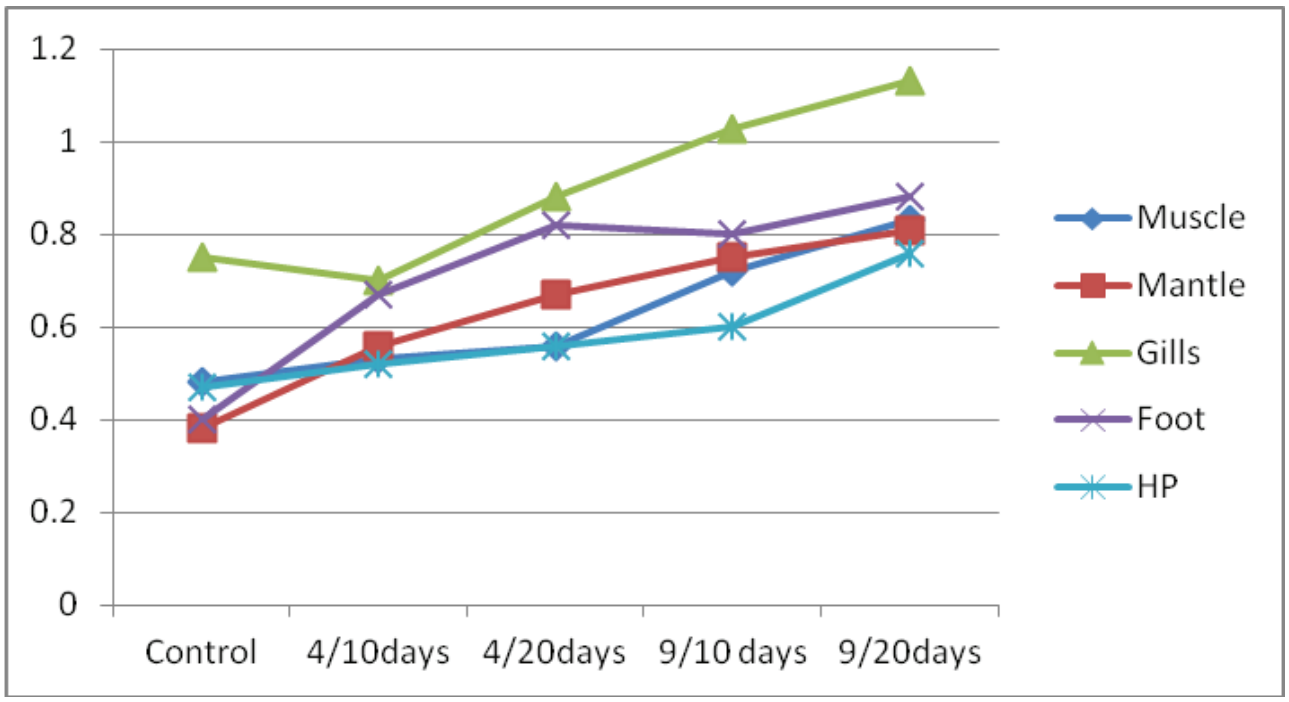


Figure 3. Lactic acid (mg gm^{-1}) content in the tissue of bivalve, *Villorita cyprinoides* after exposure to different concentrations of Cu.

4. Discussion

A marked decrease in Hepatopancrease (HP) glycogen in the present study indicates an extensive utilization of stored glycogen to meet the extra demands of energy necessitated under toxic stress[2–4]. A decrease in glycogen was due to the high energy demands of the organism under toxic stress which leads to hyperglycemia. Protein which serves as an energy source during stress conditions decreased from control as the concentration of the toxicant and the exposure period increased[5]. More energy to mitigate any stress conditions may be obtained from carbohydrates, proteins and lipids. Organisms try to detoxify the toxicant by spending more energy and thereby showed reduction in protein level[5–7]. At 4 mg/l after 10 days protein content of all the tissues decreased significantly except muscles and hepatopancrease (Figure 2). Gills showed a decrease in protein content from 28.75 to 24.5 mg gm^{-1} weight. Mantle and foot showed decreased protein content from 31.87 and 21.75 to 26.37 and 18.87 mg gm^{-1} weight respectively (Table 2). At the same concentration for 20 days of exposure all the tissues showed significant reduction in their protein content. At 9 mg/l for both the exposure periods, protein content showed significant decrease in all the tissues. The progressive decrease in protein content of the tissue with duration of exposure indicates a gradual accumulation of toxicant resulting in gradual increase in toxicity[11]. Proteins may be channelized into TCA cycle through aminotransferase system to cope up with excess demand of energy during toxic stress[9]. Lactic acid content in mantle, foot and hepatopancrease showed significant increase in 4 ppm concentration while in muscle and gills increased insignificantly after 10 days. Hepatopancrease showed increase in lactic acid content from 0.47 to 0.52 mg gm^{-1} weight (Table 3). Foot and mantle indicated rise in lactic acid levels from 0.40 and 0.38 to 0.67 and 0.56 respectively. After 20 days of exposure at 4 mg/l lactic acid content of all the tissues except gills increased significantly. At 9 mg/l lactic acid was increased significantly except gills and gonads after 10 days. After 20 days of exposure period at 9 mg/l, lactic acid content in muscles increased up to 0.83 weight mg gm-1 which is significant. Mantle showed increase in lactic acid up to 0.81 from 0.38 weight mg gm^{-1}. Gills and foot indicated increase in lactic acid from 0.75 and 0.40 to 1.13 and 0.96 mg gm^{-1} weight respectively. Lactic acid contents in hepatopancrease were found to be increased up to 0.76 from 0.47 mg gm^{-1} weight (Figure 3). The enhanced glycolytic mechanism would indicate the animal's dependency on the anaerobic breakdown of glucose to alternate the increased anoxic condition[8–11]. Conversion of glucose to lactic acid leads to increase in lactic acid content6. The increase in lactic acid content is possibly due to glycogenolysis and operation of glycolytic pathway in liver and muscle accounting for hyperglycemia. Excess utilization of these metabolites indicates the possible arrival of anoxic conditions[8]. Glycogenolysis was also

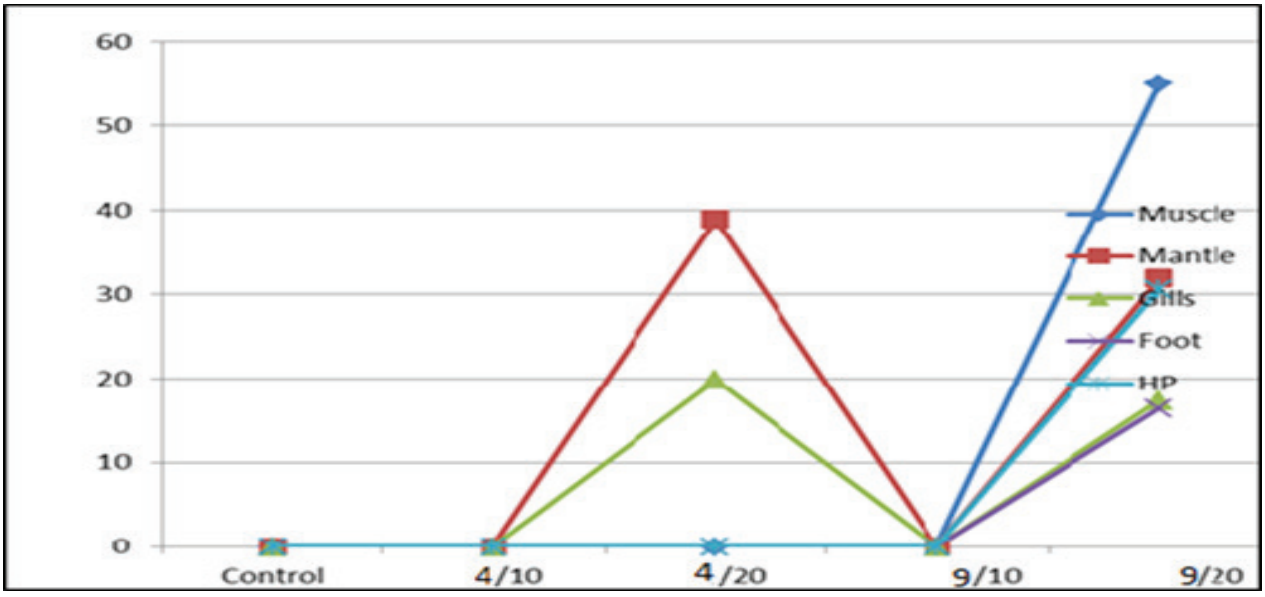


Figure 1. Glycogen (mg gm^{-1}) content in the tissue of bivalve, *Villorita cyprinoides* after exposure to different concentrations of Cu.

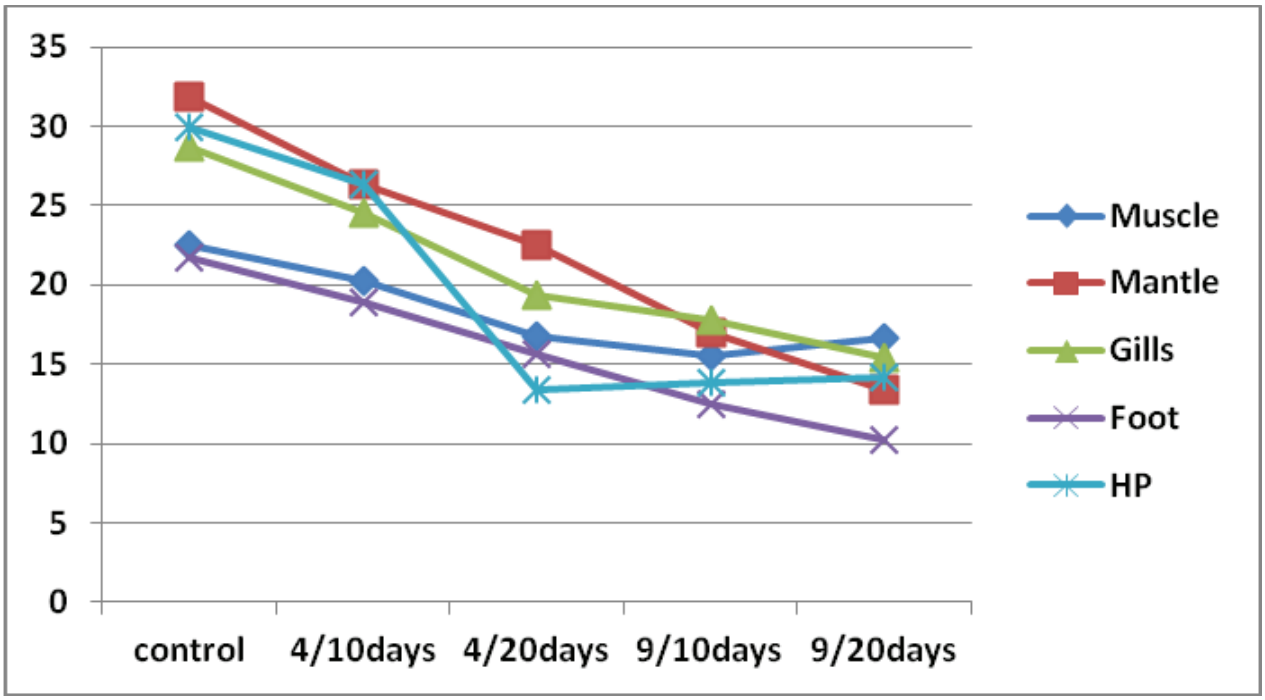


Figure 2. Protein (mg gm^{-1}) content in the tissue of bivalve, *Villorita cyprinoides* after exposure to different concentrations of Cu.

reported during exposure to various pollutants considered stressful due to hypoxic situation[10].

5. Conclusion

The results obtained in this study indicate that the Cu affects the metabolism in fresh water bivalve, *Villorita cyprinoides* by reducing glycogen due to stress conditions and protein levels due to proteolysis and impairment in synthesis. Lactic acid contents increased due to anoxic conditions forced on the animals. Thus, Cu shows synergistic effects on the physiology of the bivalve, *Villorita cyprinoides* at sub-lethal concentration after long term exposure. The toxicity impact of mercury was very lethal for the organism. The synergistic effect of Cu and Hg was highly lethal for the organisms.

6. Acknowledgment

The authors acknowledge the Department of Environmental Science CUSAT, India for providing facilities to carry out research work. We are also thankful to the Department of Chemical Oceanography for providing facilities to carry the research work.

7. References

1. Andhale AV. Effect of nickel induced biochemical alterations in fresh water bivalve *Lamellidens marginalis*. J Ecobiotechnol. 2011; 3:18–24. http://updatepublishing.com/ journal/index.php/jebt/article/view/149
2. Bellotto VR, Miekeley N. Trace metals in mussel shells and corresponding soft tissue samples: A validation experiment for the use of Perna shells in pollution monitoring. Anal Bio Anal Chem. 2007; 389:769–76. PMid: 17619864. https://doi.org/10.1007/s00216-007-1420-y
3. Bhavan SP, Geraldine P. Carbaryl induced alterations in biochemical metabolism of the prawn, *Macrobrachium malcolmsonii*. J Environ Biol. 2002; 23-2:157–62. PMID 12602852.
4. Chaudhari T, Patil P, Rao K, Subhash M. Effect of sodium pentachlorophenate on protein metabolism of the gastropod, *Indoplanorbis exustus*. Uttar Pradesh Journal of Zoology. 1998; 18(2):87–90. https://mbimph.com/index.php/UPJOZ/article/view/112
5. Dhote S, Varghese B, Mishra SM. Impact on idol immersion on water quality of Twin Lakes of Bhopal. Indian J Environ Porte. 2001; 21:998–1005..
6. Durairaj S, Selvarajan VR. Influence of quinolphos, an organo phosphorus pesticide on the biochemical constituents of tissue of fish *Oreochronus mossambicus*. J Environ Biol. 1992; 13:181–5
7. Ferrando MD, Andrew-Moliner. The effect of time on physiological changes in eel, *Anguilla anguilla* induced by Lindane. Comparative Biochemistry and physiology. Comp Pharmacol Toxicol. 1991; 100(1-2): 95–8. PMID: 1713818. https://doi.org/10.1016/0742-8413(91)90130-L
8. Ghosh R, Shrotri RV. Blood glucose and tissue glycogen interrelationship in *Scylla serrata* (Forskal) chronically exposed to Thiodan. J Environ Biol. 1992; 13:233–7.
9. Gokhale AA, Mane UH. Acute fluoride toxicity to the freshwater bivalve mollusc, *Lamellidens marginalis* (Lamarck). Indian J Inviron Zool Aqua Biol. 1990; 2:11–4.
10. Gupta K, Sharma A. Macro invertebrates as indicators of pollution. J Environ Biol. 2005; 26:205–11.
11. Hawk PB, Oser BL. Hawk's physiological chemistry. 14th Edn. McGraw-Hill, New York. Heath, A.B. and A.W. Pritchard: Effects of severe hypoxia on carbohydrate energy stores and metabolism in two species of freshwater fish. Physiol Zool. 1965; 88:325. https://doi.org/10.1086/physzool.38.4.30152409
12. Kumar AA, Dipu S, Sobha V. Seasonal variation of heavy metals in cochin estuary and adjoining Periyar and Muvattupuzha Rivers, Kerala, India. Global J Environ Res. 2011; 5(1):15–20.
13. Prahash NT, Rao KSJ. Modulations in antioxidant enzymes in different tissues of marine bivalve *Perna viridis* during heavy metal exposure. Mol Cell Biochem. 1995; 146:107–13. PMid: 7565639. https://doi.org/10.1007/BF00944602
14. Lowry OH, Rosenbrough NJ, Farr AZL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biological Chem. 1951; 193:265–75.
15. Rodriguez-Ariza A, Alhama J, Diaz-Mendez et. al. Content of 8-oxod G in chromosomal DNA of *Sparatus aurata* fish as a biomarker of oxidative stress and environmental pollution. Mutat Res. 1999; 438:97–107. https://doi.org/10.1016/S1383-5718(98)00156-9
16. Sathyanathan B, Nair SM, Chacko J, Nambisan PNK. Sublethal effects of copper and mercury on some biochemical constituents of the estuarine clam *Villorita cyprinoides* var. cochinensis (Hanley). Bull Environ Contam Toxicol. 1988; 40:510–6. PMid: 3370345. https://doi.org/10.1007/BF01688374
17. Seifter S, Dayton S, Novic B, Muntwyler E. The estimation of glycogen with the Anthrone reagent Arch Biochem 1950; 25:191–200.
18. Viarengo A. Biochemical effects of trace metals. Mar Poll Bull. 1985; 16:153–8. https://doi.org/10.1016/0025-326X(85)90006-2