Research Article

In vitro Analysis of Heavy Metal Interference in Immune System of Estuarine Black Clam *Villoritta cyprinoides* (Gray).

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ABSTRACT

Deposition of metals in aquatic organisms leads to a long-standing burden on biogeochemical cycling in the environment. Bioaccumulation of heavy metals becomes an environmental problem, when chemicals accumulated are poisonous. Toxicity may take place along the food chain when the infected species or a material is consumed. In this study we exposed the clams with sublethal concentration of cadmium and lead, and analyzed the consequence of heavy metals on immune molecules, viability, hemocyte count, some important enzymes and DNA damage of the cell. After analysing the heavy metals in *V. cyprinoides*, two heavy metals Cadmium and Lead were selected for the supplementary studies as per the AAS analysis. Metals were tested its sublethal concentration after the exposure of 24 hr in laboratory condition. Lethal and sublethal concentrations of cadmium and lead were determined after the acclimatization of clams. Three replicates were done and the 24 hr LC_{50} value was recorded and statisticaly analysed. The sublethal concentration (Cd 0.030 ppm and Pb 0.020 ppm) of heavy metals also were recorded. The activity of agglutinin molecule in the serum of heavy metal treated *V. cyprinoides* is one fold lesser than the non-treated animal and the lethal concentration of Cd and Pb affect the activity of agglutinin molecule.

Key words: Heavy metals, Immune response, Black clam, *Villoritta cyprinoides,* Bioaccumulation.

INTRODUCTION

The pollutants pollute the normal environment; that influences our regular lifestyles destructively which is called as environmental pollution. Pollutants are considered as the basics of contamination and disturb our ecosystem and the environmental balance. With the improvement and progress in our lives, pollution has reached its maximum level; causes global warming and diseases. Bivalves are helpful biomonitors and are generally used for heavy metal monitoring program in worldwide.[1-4] Bivalves are the sentinel organisms for analyzing the range of pollutants in aquatic environment. From the last two decade, biomonitoring program was developed to assess the quality of an environment withstanding determined incoming of pollutants mainly of trace metals in coastal and estuarine waters.[5-9] The heavy metals like copper, selenium and zinc are important for the metabolism of human beings. At the same time, the metals such as cadmium, lead, arsenic and chromium causes chronic disorder to human beings. Bryan *et al.*[7] and Akberali and Trueman[10,11] have been reported that the toxicity and biological effects of heavy metals. In this current research work we are concentrating only with heavy metals because of the unavailability of pesticides. Cadmium (Cd) is one of the most toxic heavy metal which has been toxic to all type of aquatic organisms.[12,13] Friberg *et al.*[14] and Herber *et al.*[15] reported that the consequence of Cd toxicity includes kidney damage and pains in bones. It has a mutagenic, carcinogenic and teratogenic effect.[14,16-18]

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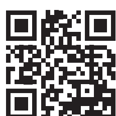

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According to United States Environmental Protection Agency (USEPA) the lead is potentially harmful to most forms of life, and poisonous to aquatic organisms.[19] Mercury is unsafe to humans in different ways. Zinc is an important metal which is used in current civilization, widely used as a coating for iron to prevent corrosion and also a significant nutrient for the humans.[20] The most important resource of zinc in the environment is particles coming out from vehicle tyres.[21] Deavaux *et al*.[22] and Lewis and Galloway[23] have been reported that great number of environmental pollutants, directly or indirectly distressing DNA of organisms, such as carcinogenesis and reproductive effects. Disclosure to these toxicants can lead to irregular physiological responses and cause unfavorable effects on the growth, behavior, development and reproduction.[24-28] Epizootic neoplasms were found in various aquatic species, such as shellfish, echinoderms, and fish[29-31] in connection with the disclosure to particular classes of DNA damaging xenobiotics.[32-34]

A variety of humoral factors naturally occurring have been detected in the serum of non-chordates which is including agglutinin, lysine, antibacterial and antifungal proteins, phenoloxidase system, 1,3-β glucon binding proteins.[35-37] Renwrantz[38] and Mullainathan[39] explained that in some invertebrate mucus and tissues the agglutinin molecules were detected. According to Cheng,[40] bivalve hemocytes distinguish and respond to foreign substances and microorganisms by phagocytosis or encapsulation even though this recognition process is generally mediated by specific or non-specific particles such as soluble lectins[41] or opsonins.[42,43]

MATERIALS AND METHODS

Collection of *V. cyprinoides*

V. cyprinoides (50±5 g) collected from Atholi, Kozhikode were transported along with the water in to laboratory (temperature 24 ± 2°C), after the acclimation, 0.03ppm of Cd and 0.02 ppm of Pb were added to the water. The analyses were conducted after 24 hr of exposure.

Collection of hemolymph and preparation of serum

Hemolymph samples collected as described in 5.3.1 was centrifuged (400 x *g*, 15 min, 4°C) and the clear supernatant (=serum) were used for phenoloxidase assays. For treated animals the clams were treated with 30 µL cadmium and 20 µL lead respectively. Hemolymph were collected from this treated clams as mentioned above.

Oxidation of phenolic substrates by serum

On checking the oxidizing activity of serum with different phenolic substrates L-DOPA was found to be effective. This was done by incubating 100 µl of serum with 2 ml of L-DOPA or DL-DOPA for 15 min at 25°C. The color developed was calculated at 480 nm in spectrophotometer (Shimadzu UV-2450, Japan) against a reagent blank in which Tris-HCl buffer (350 mM, pH 7.5) was substituted for serum sample.

Assay of PO activity

PO activity in haemolymph (serum) was determined by incubating the sample with L-DOPA as described above and the formation of dopa chrome was measured at 480 nm. Phenoloxidase activity is expressed as units min^{-1} mg $protein^{-1}$.[44]

Activation of *pro*PO

Activation of inactive *pro*PO into active PO was done by incubating enzyme samples with different activators namely trypsin, α-chymotrypsin, SDS and Triton X-100. It was performed by incubating 100 µl of serum with equal volume of activators. Control consisted of Tris-HCl buffer (350 mM, pH 7.5) instead of activators. All the reaction mixtures were incubated for 15 min, followed by addition of 2 ml of L–DOPA. After 15 minutes of incubation the OD was read at 480 nm against a suitable reagent blank.

Effect of inhibitors on *pro*PO activation

Inhibition of *pro*PO was performed by incubating enzyme samples with different inhibitors namely DTT, EDTA and Dexamethosone. It was performed by incubating 100 µl of serum with equal volume of inhibitors. Control consisted of Tris-HCl buffer (350 mM, pH 7.5).

Protein estimation

Total protein in serum was determined following Lowry *et al*.[45] 50 µl of serum were precipitated with 20 volumes of 80% ethanol and centrifuged (800 x *g*, 5 min, R_t). Surplus the supernatant and the pellet was dissolved in 1 ml of 1N NaOH. Simultaneously, 40 µl of standard (BSA) was diluted to 1 ml using 1N NaOH. To the sample and standard 5 ml of reagent C was added. After 10 min of incubation, 0.5 ml of 1N phenol reagent was added and mixed thoroughly. The blank consisted of 1 ml of 1N NaOH, 5 ml of reagent C and 0.5 ml of 1N phenol reagent. After 20 min, the intensity of blue color developed in the sample and

standard were measured against the blank at 500 nm in spectrophotometer.

Statistical analysis

Each experiment was performed six times using samples from different preparations. Difference between treatments of serum samples was analyzed statistically by Student *t*-test.

Analysis of glutamate oxalo acetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)

Preparation of tissue homogenate

Tissues were removed from the shells of the controlled and treated animals and washed with 0.9% saline without sodium aside. It was weighed and homogenized with equal volume of 0.1 N phosphate buffer saline. The homogenized samples were centrifuged at 5000 rpm for 5 min. The supernatant was collected and refrigerated until use.

Preparation of substrates and analysis of enzymes

Glutamate oxalo acetate transaminase (GOT)

Substrate: 0.2 M D, L-aspartic acid (0.2662g) and 2 mM ketoglutarate (0.0029) in 0.05 M phosphate buffer (pH 7.4). 0.1 ml of tissue homogenate was added followed by the addition of 0.5 mL of substrate in to the experimental and control tubes. This mixture was incubated at 37°C for 30 min and the reaction was terminated by adding 0.5 mL of 1mM 2,4- DNPH solution. This was held on room temperature for 20 min with infrequent shaking. After that 5 mL of 0.4 N NaOH was added and mix well. After 10 min the OD was noted at 540 nm.

Glutamate pyruvate transaminase (GPT)

Substrate: 0.2 M D,L-alanin (0.1781) and 2 mM ketoglutarate (0.0029) in 0.05 M phosphate buffer (pH 7.4). The procedure adopted for GOT was similar to that used for GPT activity with the exception of the substance comprised alanin instead of aspartic acid.

Alkaline phosphatase (ALP) and Acid phosphatase (ACP)

Presence of acid and alkaline phosphatase in the hemolymph (two point method) were analyzed by the method described by Bergmeyer, 1983 with the automate apparatus, Metrolab 2300 plus, Argentina, with Darman Kave and Pars Azmoon kit at 37°C and 410 nm and 405 nm respectively.

Lipase

Activity of Lipase was analyzed Photometrically in Pars-Azmoon Diagnostics Infinity reagent kit (Procedure No. 15024) at 580 nm.

Cytochemical analysis

Total hemocyte count (THC)

1 mL of hemolymph was extracted from the adductor muscle of the clam using a syringe containing 1 mL of iso osmotic Tris HCl buffer. The hemocyte suspension was filled in hemocytometer by capillary action and was held in moist chamber for 5 min in order to allow the hemocytes for settling. The hemocytes in the four large corner squares were counted. The experiment was conducted for the control and 24 hr heavy metal treated animals. The THC in 1 mL hemolymph was calculated using the standard formula.

Flow cytometry

1 ml of hemolymph was collected from control and treated clams to 1 ml of PBS buffer which is held on ice. From this 50µl suspension was mixed with 50µl of Anexin (FITC and PI). This mixture was incubated in dark at room temperature for 30 min. The sample was analyzed using flow cytometer. The experiment was done for the control and 24 hr heavy metal (sub lethal concentration) exposed organisms. Propidium iodide binds to double-stranded DNA and fluoresces at wavelengths above 630 nm; it enters and stains nonviable cells but cannot cross the membrane of viable cells, thereby marking the dead hemocytes as more fluorescent in the flow cytometer's orange light detector. Based on this we can able to assess the percentage of dead hemocytes.

Trypan blue dye exclusion test

Using the hemocyte suspension (from control and treated) six monolayers were made using 50 µl of suspension for each monolayer. The monolayers were left in the moist chamber up to 2 h and the viability of cells was tested at 60 and 120 min with various concentrations of cadmium and lead. For viability assay, before 5 min of the specific time interval 50 µl of trypan-blue dye solution was overlaid on the monolayer and left in moist chamber for 5 minutes. After 5 min, the slide was viewed under bright-field at 20 X for the presence of any dead cells. The percentage of the viable cells for control and treated was calculated using the standard formula.

In vitro phagocytosis assay

Preparation of target cells

1gm of yeast (*Saccharomyces cerevisiae*) was dissolved in 250 ml of saline, the yeast cells were then heat killed by autoclaving. This yeast cells was washed in saline (2000rpm, 5 min) for 6 times and stored in saline. Keep in refrigerator until use.

Preparation of monolayers

Five monolayers (each 50 µl) were made using the hemocyte suspension obtained as described above. The monolayers were left in the moist chamber for 15 minutes at 27°C. Then, the monolayers were lightly washed with TBS-VIII and left in the moist chamber for 3 to 5 min for rounding up. The monolayers were over laid with 100 µl of 0.5% yeast cells pretreated with serum. The monolayer overlaid with untreated yeast cell suspended in TBS-VII served as control. All the five monolayers were left in the moist chamber for 30 min at 27°C. After 30 min, the monolayers were gently washed to remove the non-phagocytosed yeast cells and fixed with 1% glutaraldehyde in TBS. Then the slides were observed under microscope at 20 X by counting 250 to 300 cells in each slide.

Comet assay

Hemolymph was taken from the control and treated clams as described above. 100 µl of 1% normal melting agarose in PBS were evenly spread in to a frozen microscopic slide. On that 100 µl of 75% LMA in PBS was added after drying. Covered the slide with a cover slip and solidified at 4°C for 10 min, 70 µl of LMA was mixed with 30 µl of single cell solution and from this 70µl of solution was quickly added on the first LMA layer after removing the cover slip. 100 µl of LMA was added to act as a protective layer, solidified for 10 min after covering with cover slip. After the gel had set the cover slip were removed and the cells were lysed in a higher salt lysis buffer. After lysis the slides were placed in to a horizontal electrophoresis tank covered with electrophoresis buffer for 30 min. 4°C in dark for unwinding DNA (25V, 300 MA, 25 min). The samples were neutralized three times with tris buffer at 5 min intervals. After neutralization the slides were washed with distilled water and stained using 2-3 drops ETBR for 5 min. Finally slides were washed with distilled water, cover slip were placed over the gel and visualized using an epifluorescence microscope.

RESULTS

Levels of PO and *pro*PO activation

Spectrophotometric analysis revealed the oxidation of L-DOPA by the serum of black clam *V. cyprinoides* In the present study the pro-enzymes were activated and the heavy metal treated sample shows maximum level of PO activation (Figure 3). Incubation of serum with active serine proteases significantly enhanced the PO activity of which α-chymotrypsin was more effective in all these hemolymph fractions (Figure 1).

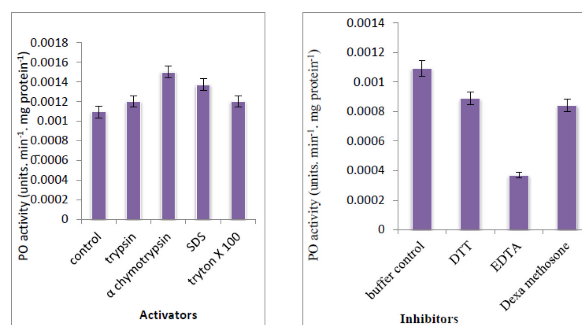


Figure 1: Levels of PO activity in the serum of *V. cyprinoides* using L-DOPA as substrate. Values are based on mean ± SD of six determinations.

Table 1: Enzyme/Protein portion levels in the hemolymph of *V. cyprinoides*.

Analysis (U/mg Protein)	Control	Cd treated	Pb treated
GOT	51.33±1.154	93.33±5.77	76.66±2.309
GPT	16.0±3.605	36.0±7.63	24.2±3.51
ALP	14.4±2.34	18.6±3.81	17.5±2.64
ACP	1.68±0.7	2.24±0.02	2.02±0.01
Lipase	2.12±0.14	2.31±0.12	2.02±0.11

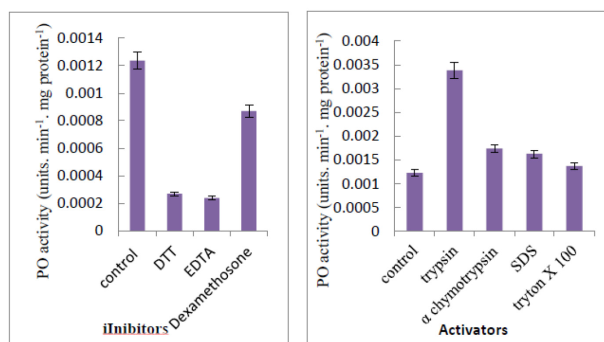


Figure 2: Effect of serine proteases, non-ionic and anionic detergents on the activation of prophenoloxidase in the hemolymph fractions of *V. cyprinoides* after exposing to sublethal concentration of cadmium. Values are based on mean±SD of increase in OD.

Analysis of enzymes

The activity of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase was amplified in heavy metal treated sample when compared to the control. Any

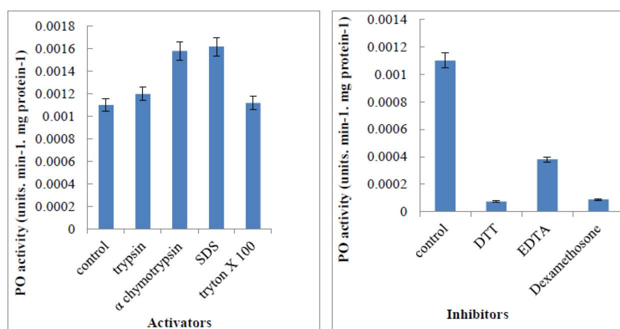


Figure 3: Effect of serine proteases, non-ionic and anionic detergents on the activation of *pro*phenoloxidase in the hemolymph fractions of *V. cyprinoides* after exposing to sublethal concentration of lead. Values are based on mean±SD of increase in OD.

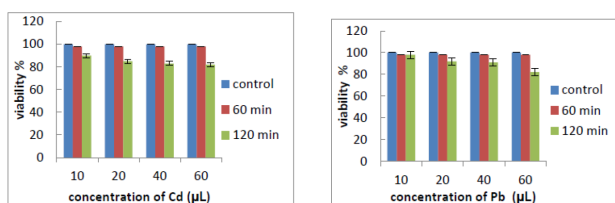


Figure 4: Viability of hemocyte in the hemolymph of *V. cyprinoides* to exposure of Cd and Pb.

changes occurring in the enzyme level of an organism is an exact index for the analysis of quantity and quality of unwanted materials. In the case of other enzymes such as alkaline phosphatase, acid phosphatase and lipase the activity was slightly increased than the control (Table 1). GPT is found in many tissues but the large concentration is in hepatocytes. The scale of serum level increase is proportional to the number of affected hepatocytes and is not indicative of the reversibility of the lesion.

Total hemocyte count (THC) and viability

The THC in the hemolymph of *V. cyprinoides* ranged between 320 and 365 x 104 cells per mL hemolymph (Figure 4) THC was also analyzed the clams which was exposed to cadmium and lead at various concentrations and the results shows that the reduction in total hemocyte count and cell viability.

Flow cytometry

There is a disparity was found in the percentage of dead hemocytes with propidium iodide both before and after exposing to sublethal concentration of cadmium and lead. Nevertheless, there is a significant increase in dead hemolytic after the animals exposed to heavy metals were found (Figure 5) cadmium shows more activity than lead in the hemocyte of *V. cyprinoides.*

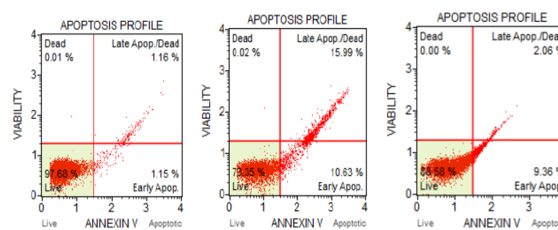


Figure 5: Apoptotic profile of the hemocytes of *V. cyprinoides* after the exposure of cadmium and lead for 24 hr by Flow Cytometry.

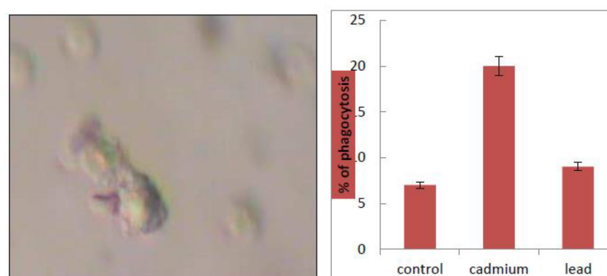
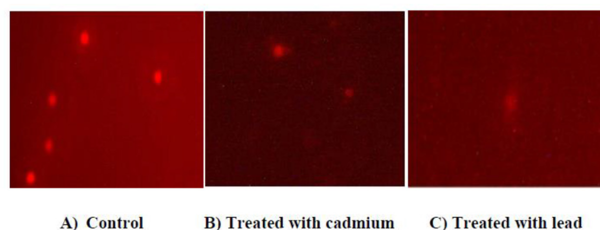


Figure 6: *In vitro* phagocytosis of glutaraldehyde-fixed human A RBC by the hemocyte of *V. cyprinoides.*



A) Control B) Treated with cadmium C) Treated with lead

Figure 7: Evaluation of genotoxic effect of cadmium and lead treated *V. cyprinoides* (24 hr).

In vitro phagocytosis

The current study reveals that the *in vivo* exposure of cadmium and lead affects the viability of cells and the hemocyte count of this black clam. Percentage of *in vitro* phagocytosis become high while exposing the clams in to cadmium (Figure 6).

Comet assay

Due the contact of Cd and Pb on the hemolymph of *V. cyprinoides*a medium damage in the DNA was observed in the Comet assay (Figure 7) and the results were tabulated.

DISCUSSION

Activation of phenoloxidase (PO) is the one of the vanguard defense mechanism in molluscs. The non-self-molecules are determined by pattern recognition (cell wall compositions) and immediately encountered. Infiltration of pathogens leads to serine protease cascade activation then followed by activation of phenoloxidase. Due to presumed cytotoxicity of these compounds, PO activity and its proteolytic activation are regulated as a local transient reaction against non-self in order to minimize damage to the host tissues and cells. *pro*PO activation observations clearly demonstrated that PO exists as a proenzyme in the hemolymph of clam *V. cyprinoides*. On the other hand, detergents failed to activate the proenzyme in all the serum. In certain bivalve species such as *Mytilus edulis, Perna viridis, Modiolus demissus and Crassostrea gigas*, PO activity has been found or and in some species like *Tridacna crocea*, *Perna perna*, are not. The levels of total *pro*PO were determined by substracting the PO activity of buffer-control (0.0176±0.0007) from that of trypsin (0.0210±0.0005) or α-chymotrypsin (0.0266±0.0009) treated HLS samples. These observations clearly showed that the *pro*PO is hemocyte-associated are soluble and is located within the hemocytes in *V. cyprinoides*. Inhibitors such as DTT, EDTA, Dexamethosone inhibit the activity of phenoloxydase (Figure 2).

Phenoloxidase activity in clams after exposing heavy metals at sub lethal level

The present study demonstrated that the heavy metals such as cadmium and lead affect the activity of phenoloxidase enzyme. PO activity was increased with activators when the clams treated with Cd and Pb while comparing to control (Figure 3). While trypsin was used as elicitor the PO unit increased significantly compared with control (untreated). Previous reports demonstrated that the PO system was involved in the reaction of carpet shell clams to parasitic infestation by *Perkinsus atlanticus*. Several earlier studies have shown that β-1,3 glucan and bacterial LPS trigger activation of *pro*PO in the whole hemolymph of invertebrate, implicating the role of hemolymph *pro*PO system in the immuno-recognition process of invertebrates. In the case of Cd treated clams trypsin enhances the activity of PO comparing to the other activators with significance. In the case of inhibitors EDTA inhibit the activation of PO. Clams exposed to Pb shows α chymotrypsin and SDS enhances the PO activity. DTT and Dexamethosone significantly inhibit the PO activity.

Analysis of enzymes

Proteins are the important factor in the metabolism of organisms. In this current study, after exposing the clams in to sub lethal concentration of heavy metals the activity of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase was drastically augmented when compared to the control

Increase in the level of GOT is due to the hepatotoxicity due to heavy metals. Cajaraville[46] reported for the detection of lysosomes in cell fractions acid phosphatases act as marker enzymes and are able to change by the presence of toxic substances.

Total hemocyte count (THC) and viability

Results show that while increasing the concentration of heavy metals, the total hemocyte count was decreased. Similar interpretations were reported by Gijo *et al.*[47] in *Crassostrea madrasensis* which was treated with copper. The same outcome was there by George *et al.*[48] in *Ostrea edulis* which is exposed to cadmium. Studies results that the oyster *Crassostrea virginica* exposed to cadmium causes stimulation in hemopoieses. Studies on the effect of sub lethal concentration of copper in *V. cyprinoides* by Spada *et al.*[49] reported that the reduction in total cell counts in 0.15 and 0. 45 ppm Cu^{2+} dosed clams were less than that in those dosed with 0.30 ppm Cu^{2+}. On testing the viability of hemocytes by trypan blue dye exclusion technique revealed that viability of cells become decreased while increasing the concentration of heavy metal and time (Table 2). It has been reported that exposure of 1 mg L^{-1} concentration cadmium on *Crassostrea gigas* results decrease in hemocyte viability after 24 hr contact. But at the same time in *Mytilus edulis* the total hemocyte count was increasing after the exposure of 400 µg L-1 cadmium.[50] It clearly illustrated that the exposure of higher concentration of heavy metal does not affect the hemocyte viability. Studies show that, apart from the wandering hemocyte, heavy metals accumulate in the hemolymph from gills, labial palp and digestive tract walls of clams. As a result we can articulate that the movable hemocyte may not be

Table 2: Total cell count on exposure of *V. cyprinoides* to Cd and Pb for 24 hr.

Concentration (ppm)	control	THC (× 104 cells/ ml)	
		Cd	Pb
0.01	336±16	322±4	320±7
0.02	335±15	288±5	319±3
0.04	333±15	279±2	317±6
0.06	333±14	209±8	245±2

the key sector for heavy metal accumulation. Friberg *et al*.[14] reported that the cadmium accumulation of *Ostrea edulis* is higher in uncontaminated area while comparing the contaminated one. It is reported that on marine prosobranch *Nerita saxtilis* the concentration of lead and cadmium up to 50 folds of that of surrounding marine water does not show any histopathological changes in its body.[51] On the basis of the current study we can suggested that the black clam *V. cyprinoides* can be considered as a good bio indicator for heavy metal pollution.

Flow cytometry

Allam *et al*.[52] reported that there is no considerable dissimilarity in the percentage of dead hemocyte calculated by flow cytometrically and microscopically. In the current study, the total number of hemocye in controles animal was 97.68 % and in cadmium and lead treated animals it is 73.35%and 88.58% respectively after the 24 hr disclosure of heavy metals. Here we can also report that there is not much variation of viability of hemocyte analyzed from flow cytometrically and microscopically.

In vitro phagocytosis

According to Allam *et al*.[52] the concentration of cells in the haemolymph was expressed as total haemocyte count (THC), is influenced by different pathogens or external factors. Disclosure of adult animals to lethal environmental contaminants such as heavy metals can lead to immune suppression.[14] Hemocytes of bivalve molluscs are concerned in different homeostatic functions such as wound healing, transportation of calcium and protein for the repair of the shell, intracellular digestion, and the elimination of non-self-materials in internal defense. *V. cyprinoides* (Gray) was a typical euryhaline clam bring into being all over West coast of India. It is suitable for human consumption and is rich with proteins and its shell used for the preparation of lime and cement.

Comet assay

Comet assay is a highly perceptive technique for the recognition of damage of DNA in bivalves. DNA damage was reported in the snail *Potamopyrgus antipodarum* when it was exposed to 10 μg/L of cadmium. Hotz *et al*.[20] reported increase in bioaccumulation of cadmium in the tissues of mussels exposed to CdCl2 in a time-dependent manner indicating that cadmium exposures produces damage in the DNA.

Aquatic habitats are contaminated by heavy metals and pesticides, via run-off, leaching, human activities such as agriculture, irrigation, mining, spray drift or accidental spills. By analyzing water, sediment and samples, pesticides pollution of the marine environment has been monitored. However, sub lethal undesirable belongings may result from disclosure to these products at environmentally significant concentrations. *Villorita cyprinoides* is one of the most imperative clam species in India. Kerala has been considering as the major producer of the species. Nearly in Vembanad Lake, a largest estuary on the west coast of India, nearly 35,000 tons of black clams [t/year] harvested. Molluscs are considered as a sensitive indicator of heavy metal pollution in aquatic ecosystem.[53] At the present time, over use of xenobiotics and agricultural drainage systems stand for the most dangerous form of heavy metal pollution. Renwrantz (1986)[38] reported that the most considerable heavy metals of aquatic pollution are zinc (Zn), copper (Cu), lead (Pb), cadmium (Cd), mercury (Hg) etc. After entering the heavy metals in to the aquatic environment, it will react with constituents of water and settle to the base and respond with the sediments. Metals precipitate as oxides or hydroxides at various pH regions and the amphoteric elements return to solution at higher pH. Alloway and Ayres[54] reported some factors also have an effect on the metal ions like redox condition and in the presence of adsorbent sediments.

CONCLUSION

Analysis of heavy metals from water samples and tissue of *V. cyprinoides* from three diverse locations shows these black clams are capable to accumulate heavy metals in their body. Exposure of these clams to sub lethal concentration of heavy metals results decrease in cell viability and total cell count. But the rate of phagocytosis was increased while comparing to the control using human A RBC as target.

Phenoloxidase activity was detected in the hemolymph of *V. cyprinoides* and pro-enzymes were activated *in vitro* by α-chymotrypsin (proteases) and heavy metas such as well as Cd an Pb showed highest level of PO activation. The PO activating cascade was interfered and concealed by DTT and PTU. The activity of enzymes such as Glutamate oxaloacetate transaminase, Glutamate pyruvate transaminase, alkaline phosphatase, Acid phosphatase and Lipase were increased when the clams exposed to heavy metals. This indicates the heavy metals affect the enzyme activity of black clams.

Based on these findings, we can strongly suggest the greatest need to conserve our water bodies harboring aquatic organisms as well as implementation of stringent regulations regarding industrial disposal and

use of heavy metals and we can utilize the black clam *V. cyprinoides* as a sentinel organism for heavy metal pollution.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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