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Histopathological changes and presence of rodlet cells in different organs of common finfish *Liza tade* in brackish water of the Chilika Lagoon

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Abstract
Chilika Lagoon, the largest brackish water lagoon in Asia, is a prominent biodiversity hotspot along the Indian east coast. Heavy metals can be accumulated by marine organisms like finfish (*Liza tade*) through a variety of pathways. A specific type of cells called rodlet cells are found in teleosts, either in fresh or marine fishes, as an endogenous cell. The aim of these studies was to examine the localization of rodlet cells in organs of finfish exposed to heavy metals in a brackish water environment. We analyzed the essential (Cu, Zn) and non-essential (Cd, Pb) trace metals and studied the existence of rodlet cells in kidney, brain eyes and gills of *Liza tade* collected from three different sites (Kalupadaghat, Balungaon, and Keshapur) of Chilika lagoon by microscopic observations. The tissue samples were prepared from gills, kidney, brain and eyes of the finfish for heavy metal analysis by AAS (Perkin Elmer, Model 5000). The metal concentration of analyzed elements (Cd, Zn, Cu and Pb) was highest in the gills (0.53, 0.59 and 0.43 µg/g) for Kalupadaghat. The histological sections collected from intact gill, kidney, brain and eye of *Liza tade* were observed microscopically for existence of rodlet cells due to pollution. Trace metals content was detected in the organs like gill, kidney, brain and eyes and morphology was found to be normal. The present findings state that heavy metal pollution induces stress in fishes with presence of rodlet cells in the histological characteristics of vital organs like gills and kidney.

Highlights
- Analyses of water quality of Chilika Lagoon suggests need for necessary actions to prevent decay of coastal ecosystem with all its genetic diversity.
- The measurement of heavy metals in fish tissues for health risk calculations showed that consumption poses no threat for the population.
- Histopathological changes in gills, kidney, brain and eyes varied based on the heavy metal pollution load.

The occurrence of rodlet cells in the gills and kidney of finfish shows its special function as biomarkers of exposure to contaminants.

Keywords: Heavy metals, finfish, Chilika Lagoon, Sensory system, Rodlet cells

1. Introduction
Chilika Lagoon, the largest brackish water lagoon of Asia located in Odisha in east coast of India, is a prominent biodiversity hotspot in the subcontinent. It is situated between latitude 19° 28′-19° 54′N and longitude 85° 05′-85° 38′E along the Odisha coast [1]. The lake encompasses a combination of fresh water, brackish water and marine components and receives significant amount of pollutants through Daya and Bhargabi rivers [2, 3]. Pollution from domestic, industrial, agriculture and aquaculture wastes, over exploitation of fishery resources, agriculture and aquaculture, deforestation in the catchment area, had aggravated the environmental problems [4, 5, 6]. The mullets belonging to the family mugilidae comprises mainly of coastal marine species and are widely distributed in all tropical, subtropical and temperate seas like Chilika lagoon [7]. Fish have the ability to accumulate heavy metals in their tissues by absorption along gills surface and kidney, liver and gut tract wall to higher levels than environmental concentration [8]. Heavy metals, such as copper (Cu), Zinc (Zn), and cadmium (Cd), lead (Pb) are of the most important trace metals which effect aquatic environment and fish fauna [9, 10]. One of the most important mullet species, Tade mullet

(*Liza tade*) is vulnerable to this heavy metal pollution [11].
Fish like mullets being the staple diet of the people, may accumulate in large amounts of certain metals above the standard levels [12]. Heavy metals in fish come mainly from their diet, and levels of bioaccumulation of contaminants are higher in fish which comes higher in food chain [13] and consequently accumulate in different organisms positioned in various trophic levels. The transfer of heavy metals from mothers to foetus through placenta may cause neuronal damages during development and increase the risk of abortion in pregnant women [14, 15]. Fish have been shown as indicators of heavy metal contamination [16] and studies showed heavy metals like Pb, Cd, Cu and Zn have toxic effects, altering physiological activities of fish [17]. The finfish that grow in Chilika area could be a potential source of heavy metals intake for human consumers especially when it is frequently consumed. The metals, such as Cd and Pb, are toxic to living organisms even at quite low concentrations, whereas others, such as Zn and Cu, are biologically essential and natural constituents of aquatic ecosystems, and only become toxic at very high concentrations [18].
Heavy metals are known to induce oxidative stress [19, 20] by mediating free radical formation and adversely affect various metabolic processes in developing fish. Rodlet cells were detected in the epidermis of carp (*Cyprinus carpio*) and trout (*Oncorhynchus mykiss*) exposed to stressors (e.g. acid water, heavy metals, thermal elevation, polluted water of the Rhine river, water loaded with organic manure, and distilled water) [21]. These cells have been found in a wide range of tissue of fresh water and marine teleosts [22]. Unique morphological features enable rodlet cells to be easily distinguished in different tissues and organs. Morphologically, they are characterized by a distinctive cell cortex and conspicuous inclusions, called rodlets.
Rodlet cells are pear-shaped cells with a basal nucleus, fibrous layer in the inner aspect of the plasma membrane, club or rod-shaped granules, mitochondria, endoplasmic reticulum; Golgi [23-25]. Rodlet cells are described in different organs in most species of teleosts. They were detected in the digestive tract [26], pancreases [27] vascular system [28], the bulbus arteriosus [29], kidney tubules [23, 25, 29] the gonads [30] the skin [31], the epithelium of the operculum and gills [32]. The secretary function of a rodlet cell is mostly described in the epithelium where these cells discharge their contents via holocrine mode [3, 34]. They are also attributed in the mediation of immune response and considered as a type of the granulocytes [35]. Rodlet cells act as ion transporting cells and have a role in osmoregulation [36]. Involvement in sensory function is mentioned for rodlet cells [37] and it might be candidate quantitative and qualitative biomarkers for stressors and chemical agents. Hence, exposure to heavy metals may induce an early response as defense in the finfish evidenced by alterations in both at structural and functional levels of different organs including endogenous origin of rodlet cells, which can act as biomarkers of exposure and biomarker of effect to contaminants.
However, earlier studies in Chilika Lagoon recorded on the levels of contamination of heavy metal concentrations, especially chromium, Cu and Pb, resulting in degenerative and neoplastic diseases in target organs [38]. Since, this study area is being considered as an important source for fishery, the presence of toxic heavy metals in lake water would be the primary source for the bio magnifications of metals in fish.
The objective of present study was to investigate the variation in water quality parameters in different locations of Chilika Lake in order to establish new information about heavy metals level in different organs of *Liza tade* and to elucidate the role of different cellular components like presence of rodlet cells, involved in fish inflammatory response as a protective measure to combat heavy metal contamination.

2. Materials and methods
2.1 Study areas
The present study water samples were collected in the morning hours between 9AM 10 AM from three different sites (Kalupadaghat, Balungaon, and Keshapur), where Kalupadaghat in freshwater region and Balugaon are in brackish water zone of Chilika Lagoon in 250 ml polypropylene bottle and analyzed for water quality parameters. Physical parameters like temperature and pH of the samples were determined on the spot and chemical parameters of collected water samples by following standard methods as prescribed by National Institute of Oceanography [39]. All the protocols followed in the present experiments were approved by the University ethics committee (Ravenshaw University, Odisha, India) in accordance with the guidelines of the 'committee for the purpose of control and supervision of experiments on animals' by Institutional Animal Ethics Committee (Regd. No. 1927/Go/Re/S/16/ CPCSEA). The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

2.2 Field sampling and sample analysis
The concentration of heavy metals (Cd, Pb, Zn and Cu) was studied in tissues of *tade* mullet collected from three different locations of Chilika Lagoon. The collection sites are noted for intense industrial and other activities and are basically a fish culture HUB in the northeast coast of India. The collected tissue samples were stored in a container, preserved in crushed ice, and brought to the laboratory for further analysis.
2.3 The commonly edible finfish (*L. tade)* was caught during high tide condition from the lower stretch of the Lagoon (22° 00' 26. 07"N and 88° 03' 29.64"E) during a rapid EIA study from 5th June, 2016 to 15th July, 2019. Specimens of each species were sorted out for analyzing the metal level in different tissues of finfish. The fishes were dissected into separate organs gills, kidney, eyes, and brain using stainless steel instruments and digested by the method as described in the procedure. About 1g of the samples was digested with perchloric acid and nitric acid (65%, Merck) ratio (1:1) HNO_3-$HCLO_4$, followed by sulphuric acid, and the mixture was heated at 20ºC for 30 minutes. Then the remaining digested solution was made up to certain volume with double distilled water.
A standard torch for this instrument was used with an outer argon gas flow rate of 15 L/min and an intermediate gas flow of 0.9 L/min. The applied power was 1.0 kW. The ion settings were standard settings recommended, when a conventional nebulizer/spray is used with a liquid sample uptake rate of 1.0 mL/min. After complete digestion it was then cooled down to room temperature and made up to 50 ml scale with distilled water and analysed for Cd, Cu, Zn, and Pb ($\mu g/m^3$) using Atomic Absorption Spectrophotometer (Perkin Elmer, Model 5000). An analytical blank was prepared in a similar manner and the prepared blanks were used to calibrate the instrument.
All reagents used were of high purity available and of

analytical grade. High purity water was obtained with a Nanopure II water-purification system. All glassware was soaked in 10% (v/v) nitric acid for 24 hours and washed with de-ionised water prior to use. The control blank process was prepared with distilled water in place of biological samples following all the treatment steps as described above. The final volume was made up to 50ml. Finally, the samples and blank solutions were analysed by Spectrophotometer. All analyses were done in triplicate and the results were expressed with standard error of mean.

2.4 Histopathological study

Gills, kidney, brain and eyes of finfish were dissected for the Haematoxylin and eosin (H&E) staining using standard protocols [40]. The tissue sections were immersed in a mixture of 20 mL of 2.5% glutaraldehyde and 80 mL 0.1 M Na-phosphate buffer pH -7.4). Then specimens were washed by 0.1 M Na-phosphate buffer (pH-7.4) then immediately fixed in Bouin's fluid for 2 h. Bouin`s fixed samples were extensively washed in 70% ethanol (3×24 h) to get rid of the fixative. Serial sections were obtained from fish at 5µm and then sections were cleared in methyl benzoate and embedded in paraffin wax for 8h and sections were stained with H&E for observation of morphological changes and localization of rodlet cells through histological examination. The sections were dewaxed (2x30 min), rehydrated in a descending series of ethanol (100%, 95%, and 70%) and with distilled water. After staining, the sections were dehydrated again in an ascending series of ethanol (70%, 95%, and 100%), cleared in xylene (2×10 min) and mounted with DPX. Stained sections were observed under microscope (x80 magnification) and photos were taken using a CCD camera attached to a microscope (Olympus, CX31, Japan).

2.5 Statistical analysis

A logarithmic transformation was done on the data to improve normality. Analysis of variance (ANOVA) was performed to assess whether heavy metal concentrations varied significantly between the cooking process; possibilities less than 0.01 ($p < 0.01$) were considered statistically significant. All statistical calculations were performed with SPSS 9.0 software for Windows.

3. Results and Discussion

3.1 Physico-chemical parameters

In present study all the water samples were collected from different stations of Chilika Lake and found to have dust particles in most of the water samples. All the physicochemical parameters studied showed noticeable spatial variations, which may be attributed to the local climatic conditions and exchange mechanism between lagoon and the sea. The salinity values were ranged from 5.75 to 13.87 for whole lake and the water temperature was observed in all the sectors with maximum of 30.1ºC in Kalupadaghat and minimum of 28.1ºC in Balugaon. The pH of all the study areas were ranged from 8.13 to 8.91 and appeared as slightly alkaline in nature due to presence of salt in water by mixing of river and sea. Ammonia becomes more toxic at higher values of pH than the normal range. Thus, pH is very important for fish distribution and abundance. Normally high dissolved oxygen was encountered in unpolluted areas while at polluted areas level of dissolved Oxygen (DO) is very less. The DO in sea mouth area is due to less solubility of oxygen in salt saturated water which depends on water temperature, water movement and salinity. Hence, the DO concentration varies widely throughout the lake. The overall chloride (Cl^-) content was found to be very high as well as the salinity of the water. The Cl^-content of the lake also varies in all three different sites of the lake. Total dissolved solid (TDS) was found to be very high in Kalupadaghat sites of the lake. High TDS was found in these areas due to high rate of evaporation which causes accumulation of salt and no inflow of fresh water to the lake. The nitrate (NO_3) and sulphate (SO_4) content varied aberrantly throughout the three different location sites, whereas the phosphate (PO_4) content was found to be very low in Keshapur areas in comparison to Kalupadaghat and Balugaon sites. The mixing of sea water with lake water helps in increasing the nitrate content in the water and concentration of nitrogen also gives useful indication of level of micronutrients in the sea water of Chilika Lake (Table 1).

Table 1: The water quality parameters of three different locations in Chilika Lagoon

Parameters	Kalupadaghat	Balugaon	Keshapur
Salinity	5.75-9.05	7.97-15.2	9.34-13.87
Temperature (°C)	28±1°C	30± 1°C	29±1°C
pH	8.1 ± 0.2	8.5 ± 0.2	8.9± 0.2
Dissolved oxygen (mg/l)	8.2-8.9	7.9-8.2	7.5-8.6
Total Hardness (mg/l as $CaCO_3$)	106.2	98.0	91.3
Total alkalinity (mg/l as $CaCO_3$)	42.8	46.7	53.0
Total dissolved solids (mg/l)	173	167	195
Cl-(mg/l)	10.5	12.2	14.5
NO_3 (mg/l)	0.034	0.039	0.085
SO_4 (mg/l)	13.0	15.0	18.3
PO_4 (mg/l)	0.03	0.1	0.1

3.2 Heavy metals content in organs of finfish

3.2.1 Copper (Cu) accumulation in different organs of finfish

Heavy metals accumulate in gills, kidneys, brain and eyes of finfish collected from three different sites of Chilika Lagoon (Fig. 3). The maximum concentration of Cu was detected in the gills and kidney tissues of finfish (0.98 µg/g) and (0.78 µg/g) from Kalupadaghat and Balugaon (Fig. 3A). At Chilika Lagoon, the maximum level of Cu was recorded as (0.59 µg/g) in kidney of fish collected from Kalupadaghat whereas the minimum value of (0.19 µg/g) was observed in eyes samples in finfish collected from the Keshapur. Among the heavy metals analyzed, the Kalupadaghat hat showed the maximum Cu level it might be due to land run off and the mechanical and chemical weathering of rocks, the components also washed from the catchment areas through runoff and windblown dust (Nayak *et al.*, 2010). Excessive intake of Cu may lead to liver cirrhosis, dermatitis and neurological disorders [41, 42] and Cu toxicity in fish is taken up directly from the water via gills. Effects of high concentrations of Cu on fish are not well established, however, high concentrations of Cu in finfish may cause toxicity. It can combine with other contaminants such as ammonia, mercury and zinc and can produce an additive toxic effect on finfish [43]. The heavy metal is believed to have been released in the environment mostly from industrial and domestic waste and carried by the rivers to the mouth of the Lake.

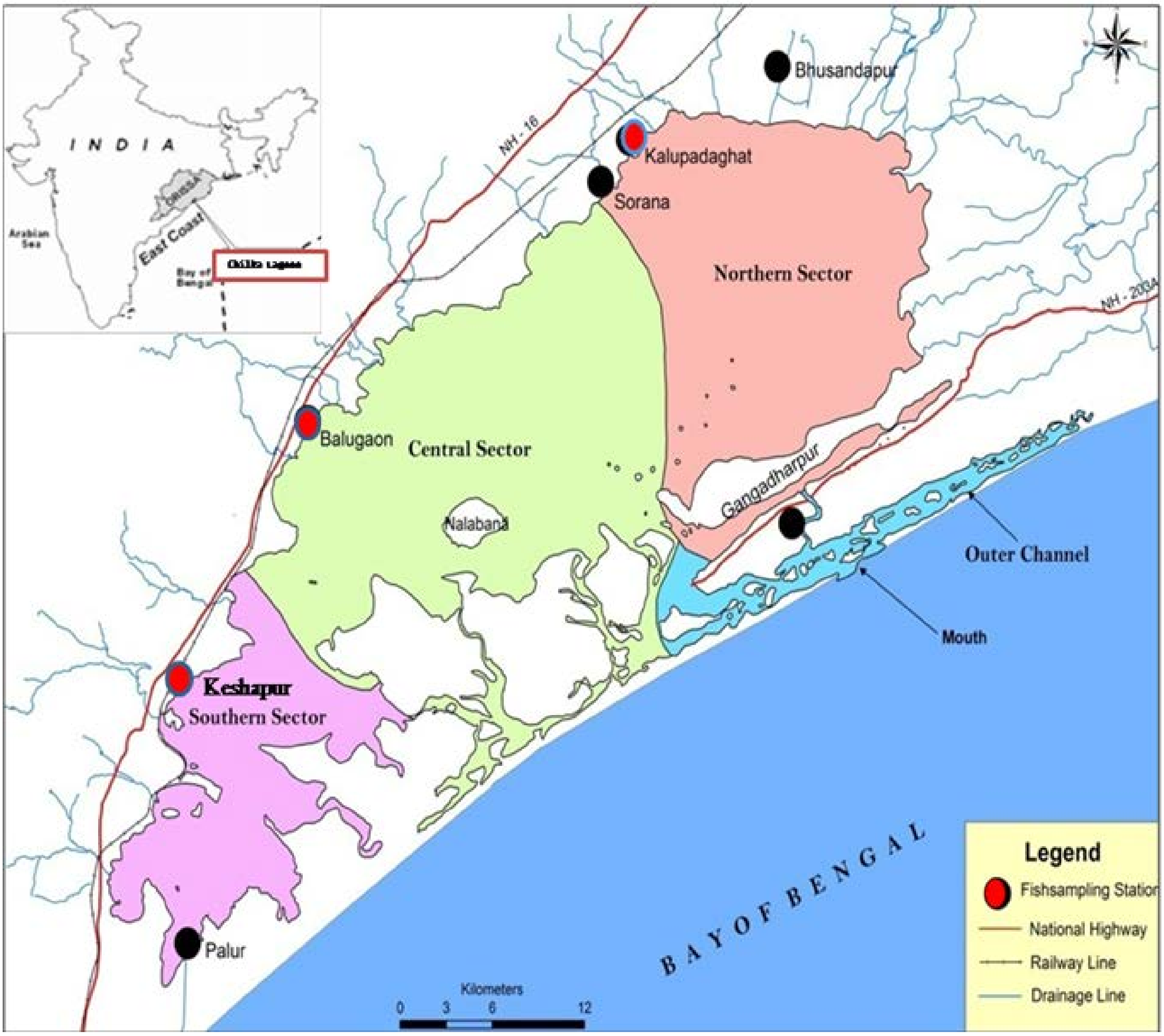
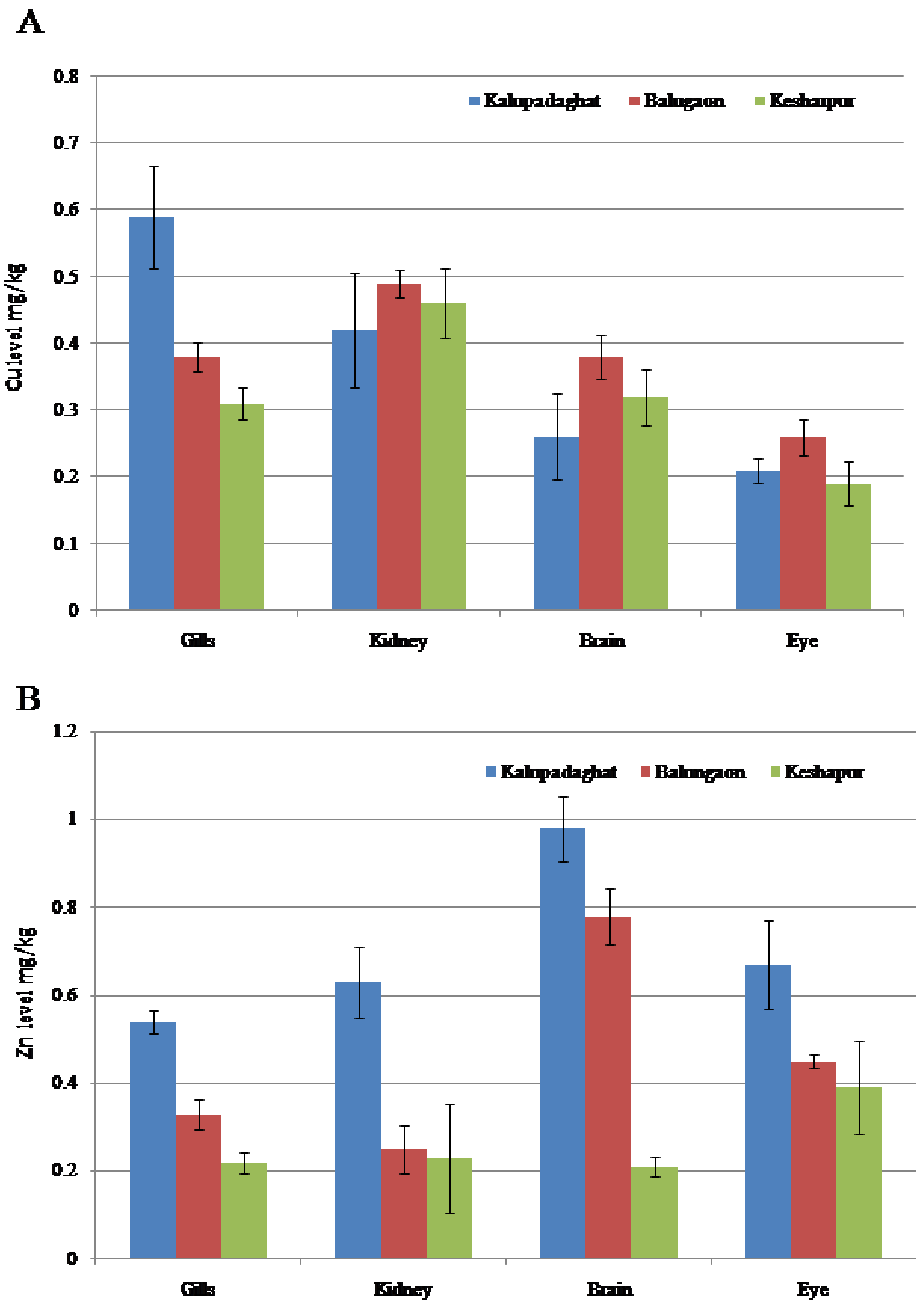


Fig1: Map of Chilika Lake broadly divided into four zones on the basis of water salinity values, namely the southern, central, northern, and outer channels. Finfish were collected from three different locations of the sampling areas (Kalupadaghat, Balugaon and Keshapur).

3.2.2. Zinc (Zn) accumulation in different organs of finfish

The concentration of Zn was found to be highest in brain tissues of finfish collected from Kalupadaghat and Balugaon of Chilika Lagoon (Fig. 3B). In Kalupadaghat, the highest concentration of Zn was observed to be (0.98, 0.67, 0.63 and 0.53µg/g) in kidney and gills of finfish respectively. The highest concentration of Zn can cause vomiting, stomach cramps, nausea and skin problems. Kidney is engaged in excretion of metals ion out of the body; hence the marked concentration of metals found in this tissue could be related to accumulative capacity of this organ. Gills are the main place for gas exchange in fish and in this organ, because of the short distance between blood and surrounding seawater, heavy metals ions may directly take up from the passing water. The lowest concentration of Zn was observed in brain, kidney and eyes of finfish collected from Keshapur, whereas Balugaon showed the lowest Zn concentration in kidney and gills, which was found to be 0.25 and 0.33µg/g respectively.


A
Kalupadaghat
Balugaon
Keshapur
Cu level mg/kg
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Gills
Kidney
Brain
Eye
B
Kalupadaghat
Balugaon
Keshapur
Zn level mg/kg
1.2
1
0.8
0.6
0.4
0.2
0
Gills
Kidney
Brain
Eye

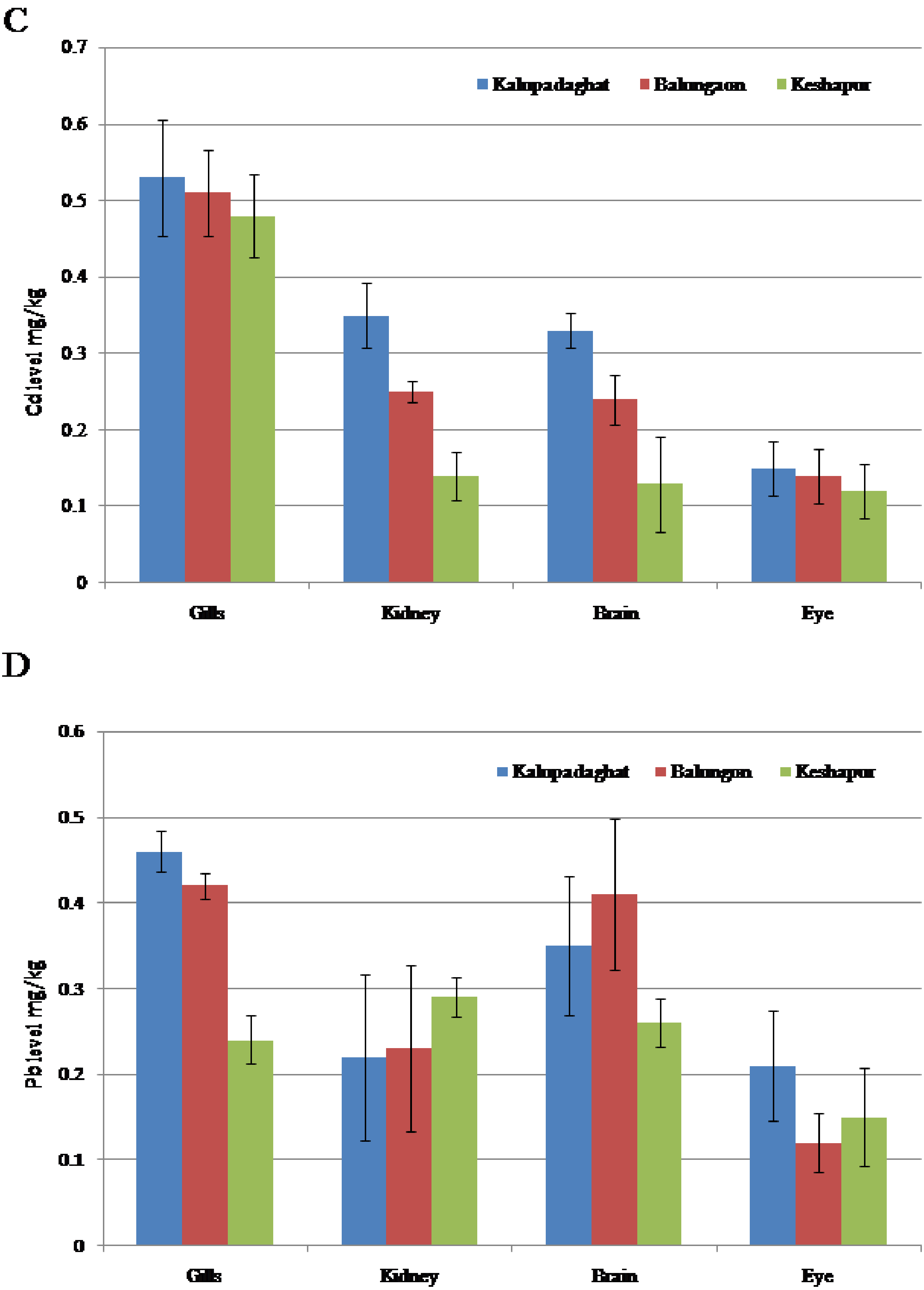


Fig 2: Histogram showed data obtained for heavy metals content analyzed in fishes and presented for the metal contents (µg/g) of gills, kidneys, brain and eyes of finfish (*Liza tade)* collected from three different locations (Kaludaraghat, Balugaon and Keshapur) of Chilika Lagoon (Fig 2). Heavy metal Cd (Fig 2, A), Zn (Fig 2, B), Cu and Pb concentration (Fig 2, C and D) in the gills, kidney, brain and eyes of finfish. Values are expressed as mean ± SEM, n=6.

3.2.3. Cadmium (Cd) accumulation in different organs of finfish

The maximum concentration of Cd (0.53 µg/g) and Pb (0.51 µg/g) were obtained in the finfish from Kalupadaghat and Balugaon areas respectively (Fig 3C). The concentration of Cd (0.53, 0.51 and 0.48 µg/g) in gills of finfish was high at all three different sites. Gill is the main place for gas exchange in fish. In this organ, because of the short distance between blood and surrounding seawater, heavy metals, ions may be directly taken from the passing water [9]. On the hand, it is also engaged in excretion of metals ion out of the body. Another explanation for the findings with high concentration in gills may be due to mucous excretion by this organ. Thus, the marked concentration of metals found in this tissue could be related to accumulative capacity of this organ during its role in depuration and absorption of up-taken heavy metals [12]. The excreted mucous has affinity to be bound with metal ions. Therefore, even when heavy metals accumulation is low in the in the tissues of target organs like kidney, brain and eyes, it is possible that continuous exposure to heavy metals can cause deleterious health effects in humans [44]. Hence, the finfish and its products should be tested for their heavy metal content prior to export so that heavy metals remain within permissible limits.

3.2.4. Lead (Pb) accumulation in different organs of finfish

The lowest concentration of Pb (0.21 µg/g), (0.12 µg/g) and (0.15 µg/g) were found in the eye tissue of the fish from Kalupadaghat Balugaon and Keshapur sites of Chilika Lake (Fig 3D). The maximum Pb concentration (0.42 and 0.45 µg/g) was found at Kalupadaghat and Balugaon sites, whereas Keshapur showed the minimum (0.24 µg/g), though the station near the sea mouth was not affected by sediment carried by the rivers. The higher Pb contamination is believed to have been caused by natural sources. The present findings also indicate that contamination factor and enrichment factor values for lead were higher at Kalupadaghat, while Pb concentration at Keshapur could be categorised as unpolluted, Kalupadaghat and Balugaon could be considered as an unpolluted to moderately polluted zone. The present study also indicates that contamination factor and enrichment factor values for Pb concentration were higher at Kalupadaghat and Balugaon. The maximum metal concentration was found at Kalupadaghat and the variations observed in the metals concentration in three different locations could be attributed to the different physicochemical factors of Chilika Lagoon.

3.3. Histological studies for observation of rodlet cells

The current study was also carried out to explore the existence of rodlet cells and identification of rodlet cells, by (H&E) staining. Rodlet cells have been observed in the sections of kidneys and gills, located in vascular walls (Fig 3). The present study revealed their presence in variable number in gills, localized near the vessels and in kidney, close to vessels and eosinophilic granular cells but absent in brain and eyes of finfish collected from three different sites of Chilika Lagoon. The different localizations of rodlet cells within the body observed in finfish could be due to an adaptation to the environment of each fish family. The population of rodlet cell was found in gills as the organs constantly under pathogen attack. The large vesicular rodlet cells accumulated cytoplasmic small fibrils occurred as individual fibers or well organized in bundles in kidney tubules. Many authors, favorable to the hypothesis of teleostean origin of rodlet cells due to lack of a tissue-specificity, thought them as leukocytes granular cells.

Some researchers also hypothesized that rodlet cells could be a glandular component of many epithelial tissues [26]. The heavy metal induced histological characteristics in finfish lead to understanding the adverse impact of pollution on aquatic ecosystem. Our present study is the first to document the presence of rodlet cells near the tubule cells of kidney and epithelial cells of gills in fish (Fig 3). These cells have been described in different tissues of marine and freshwater teleosts. The rodlet cells are mostly characterized by conspicuous inclusions called ‘rodlet’ and interpreted as endogenous cells. Studies also highlighted the relation between a higher number of rodlet cells and stressing factors, such as water temperature changes, other medium condition, crowding in farming condition, toxicants and polluted water [45]. Moreover, rodlet cells apparently start to develop from the basal layers of the epithelium moving forward to the surface, where they discharge their content, most likely a mucous cell [22], apparently contracting the fibrillary cytoplasmic border [45]. The rodlet cells mainly posses’ mitochondria very similar to those of epithelial cells of the same organ. Other functions ascribed to rodlet cells are an osmoregulatory function and ion transports [37]. The present study pointed the presence of rodlet cells in gills and kidney that it may be involved in secretary functions. In the aquatic environment gills and kidneys are the first organ to be affected by contaminants inducing structural changes.

4. Conclusion

The Chilika having variety of lagoon habitats is of great value in preserving genetic ecological diversity. Chilika Lagoon brackish water quality is presently facing serious challenges because of environmental pollution. The concentrations of heavy metals in the finfish at different locations in the brackish water system are consistent with the local industrialization levels. Kalupadaghat of the lagoon is found to be the most polluted compared to other part of the estuary, the results clearly indicated that the concentration of all the four heavy metals analysed were below the prescribed limit for human consumption. The heavy metals like Cd can affect calcium balance and induces damage in the structure of gills and kidney of finfish. But it can be elevated through food chain, hence it is necessary to give more attention to accumulation of heavy metals in organisms as far as the sea food industry and public health is concerned. The role of rodlet cells seems to take part in the inflammatory responses is not completely understood, it may appear in organs like kidneys and gills after exposure to toxicants. Further lines of research are suggested that might elucidate the true function of these enigmatic cells.

Conflict of Interest

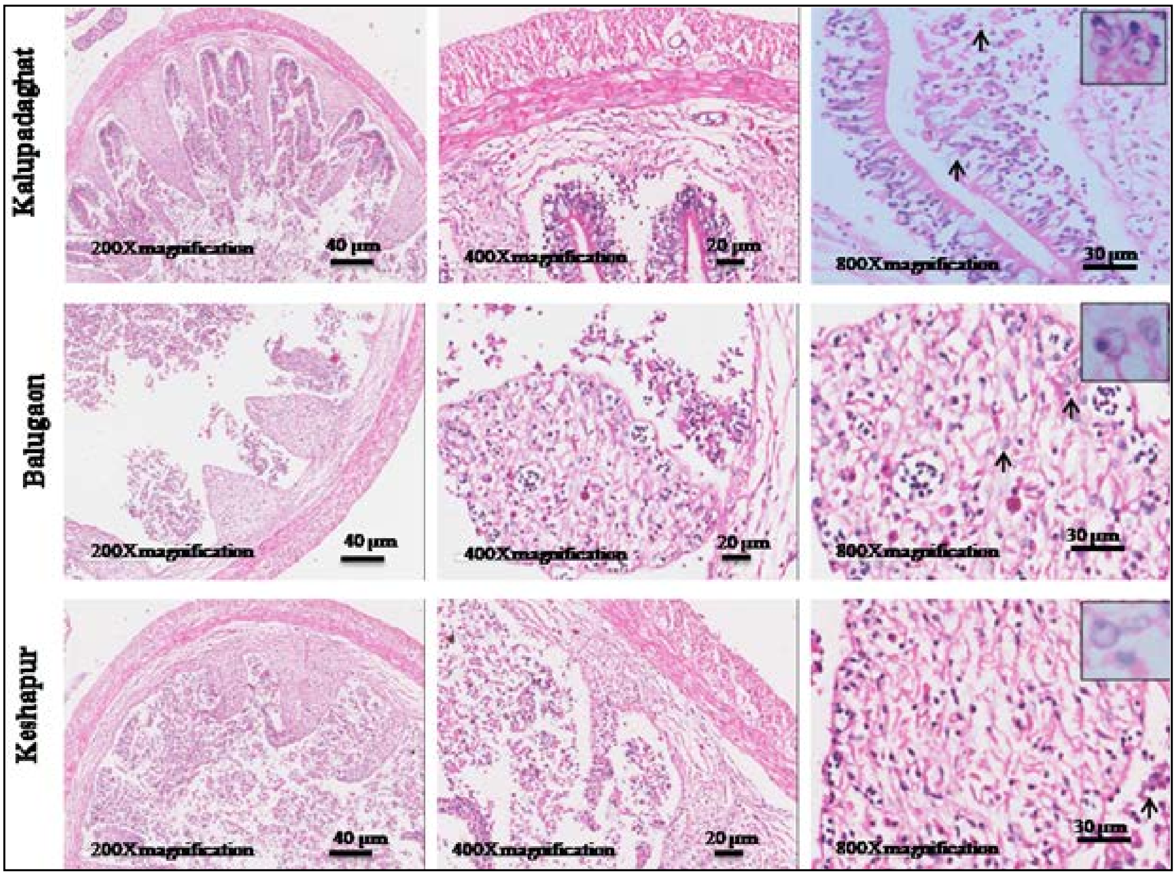
The authors declare that there is no conflict of interest.

Authors’ contributions

MP designed the protocol, performed the experiments and interpreted the data with the image analysis. AP prepared the images and also drafted the manuscript.

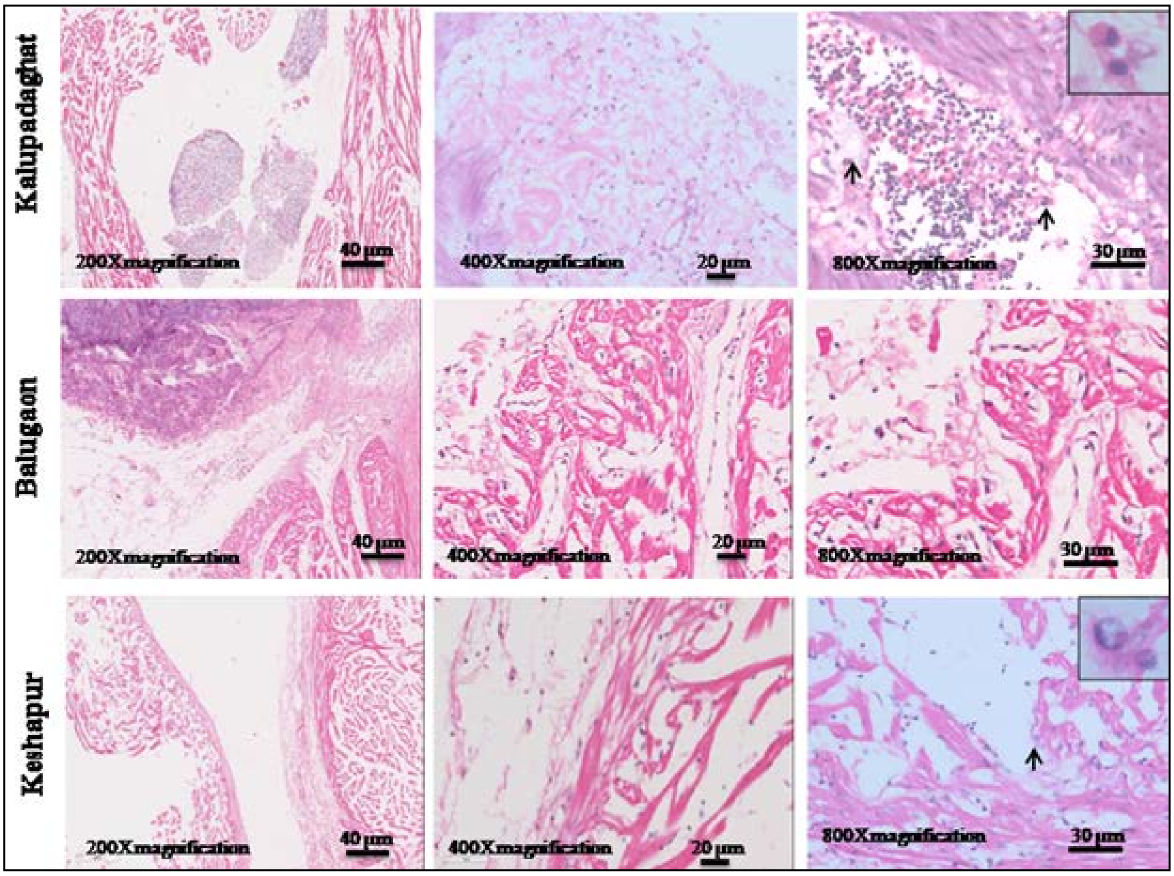
Data availability statement

No additional unpublished data are available.



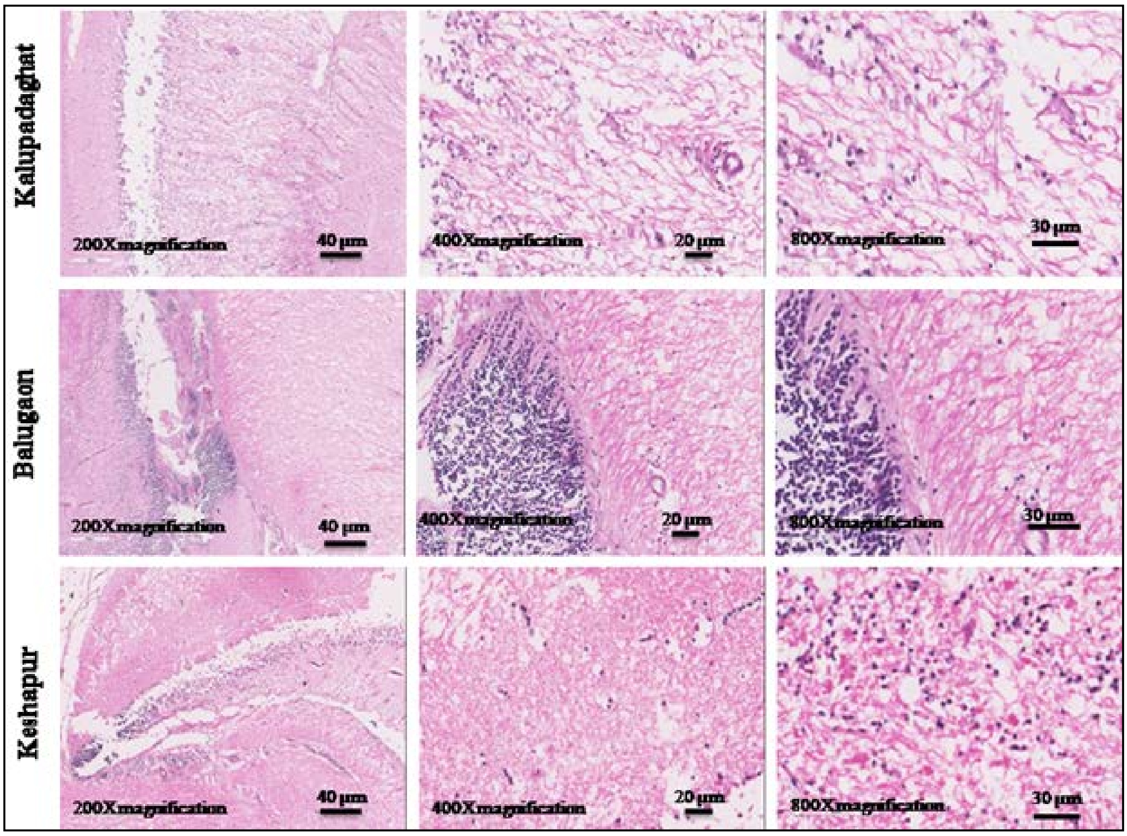
Gills

Gills: Rodlet cells (arrows) were observed in Paraffin sections stained by H & E staining in the finfish with an ellipsoidal to oval morphology near the mesothelium of gills and localized near the vessels with rod shaped inclusions parallel to cell long axis (Fig 3).



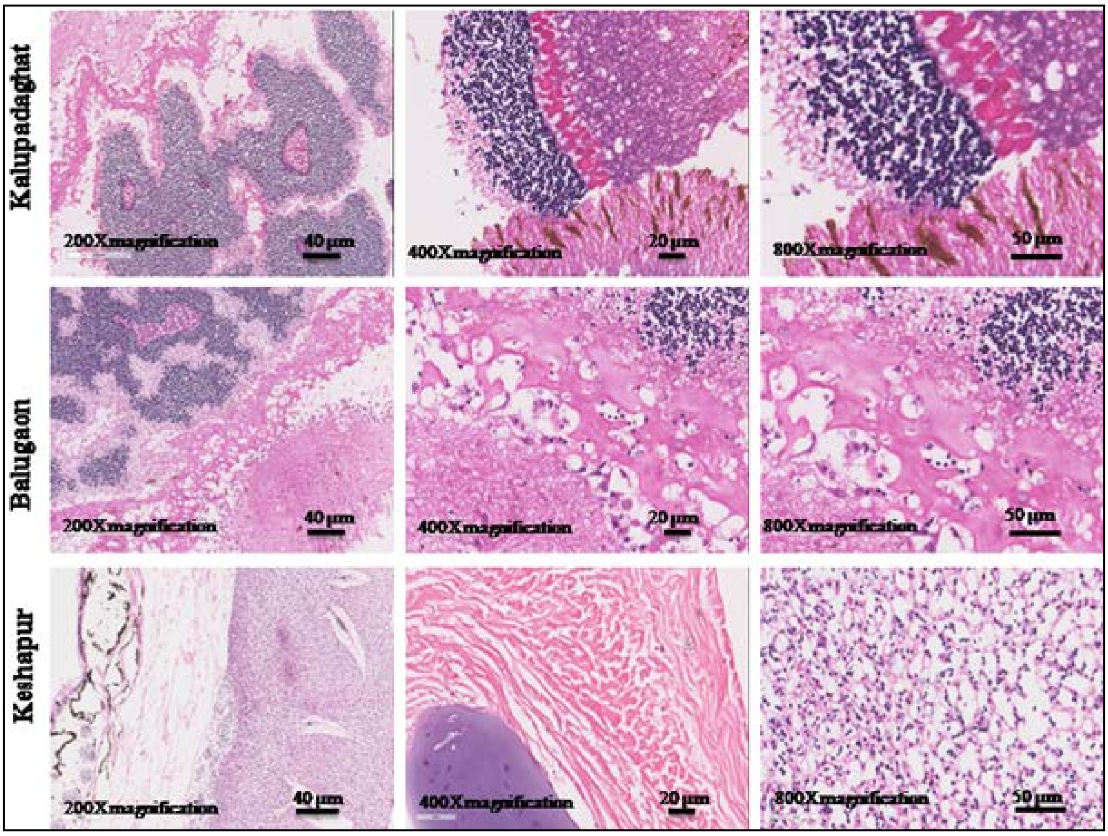
Kidney

Kidney: Rodlet cells (arrows) observed in Paraffin sections stained by H & E staining showed a plasma lemma in close contact with a thick-layered capsule was found in kidney close to eosinophilic granular cells, creating an encircling sac around the tubules (Fig 3).



Brain

Brain: Transverse section of brain sections showing (Fig 3) neuronal cells in finfish collected from different locations of the sampling areas (Kalupadaghat, Balugaon and Keshapur).



Eye

Eyes: Transverse section of finfish eyes showing different layers of retina with lumen of a large blood vessel behind the eye (Fig 3).

Fig 3: Alterations in the histology of finfish with presence of rodlet cells were observed through photomicrograph sections of different organs like Gills, Kidney, Brain and Eyes (Fig 3) collected from polluted waters of Balugaon and Kalupadaghat and compared with that of finfish collected from less polluted areas like Keshpur. Values are expressed as mean ± SEM, n=3.
Rodlet cells in different organs of mullets.

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