Journal of Microscopy and Ultrastructure 4 (2016) 211–221

Contents lists available at ScienceDirect

Journal of Microscopy and Ultrastructure

journal homepage: www.elsevier.com/locate/jmau

Original Article

Electron microscope based X-ray microanalysis on bioaccumulation of heavy metals and neural degeneration in mudskipper [*Pseudapocryptes lanceolatus*]

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ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form 2 March 2016
Accepted 7 March 2016
Available online 16 April 2016

Keywords:
chemosensory
metallobiology
neurodegeneration
Pseudapocryptes lanceolatus
pseudostratified

ABSTRACT

The bioaccumulation of heavy metals and its probable cytological consequences in ciliated olfactory sensory receptor neuron (OSN) of two different groups of *Pseudapocryptes lanceolatus* has been studied using X-ray microanalysis in transmission electron microscopy (TEM-EDX) [i.e., Group I, collected near Kanchrapara (22.56°N 88.26°E) and Group II, collected near Tribeni (22.99°N 88.40°E) of West Bengal, India]. The ciliated OSN is a bipolar neuron and possesses a prolonged dendron with four to six cilia at the olfactory knob, perikaryon, and axon. Excess accumulation of copper (94.50%) and iron (83.81%) was noted under TEM-EDX in the cytoplasm of the olfactory knob as well as nucleoplasm of ciliated OSNs in *P. lanceolatus* (Group II). The degenerating ciliated OSNs show distinct features of lysis of the plasma membrane at the olfactory knob, disintegration of cytoskeletal structures in perinuclear cytoplasm and axoplasm, and fragmented chromatin fibers with granules (diameter, 20–30 nm) in the nucleoplasm. Crowding of acetylcholinesterase-positive vesicles (diameter:, 30–40 nm) at the terminal part of the axoplasm was related to accumulation of heavy metals in degenerating ciliated OSNs of *P. lanceolatus* (Group II). The recorded concentrations of heavy metals in the same organ among different groups of *P. lanceolatus* in varying geographical areas indicates the stress of concerned environmental health. This ultrastructural interpretation on the fish ciliated OSN is a prerequisite for monitoring environmental health as well as metallobiology of several neurodegenerative disorders in fish caused by bioaccumulation of heavy metals.

1. Introduction

The Bhagirathi-Hooghly River is the longest tributary of the River Ganga in West Bengal, India, which is also an important lifeline for the people of the state [1,2]. The physicochemical parameters of this river are altered by different anthropogenic activities and sewage pollution that may adversely affect the biology of fish [3–5]. Olfaction in fish is a special type of chemosensory modality that plays a vital role in various biological functions within the aquatic habitat (namely, searching for food, recognition of sex, avoidance of predators, parental care, and migration) [6]. This sense is largely dependent on the water ventilation mechanism over the olfactory neuroepithelium [7]. The water-soluble chemical odorants are dissolved within the mucous layer, bind to the G-protein-coupled receptors that are present on the kinocilia of olfactory sensory

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http://dx.doi.org/10.1016/j.jmau.2016.03.002
2213-879X/

receptor neurons (OSNs), and are subsequently conveyed to the brain [8,9]. These neurons are also vulnerable when exposed to different organic, inorganic and genotoxic pollutants (including heavy metals) of the aquatic ecosystem, which inhibits olfactory responsiveness [10,11]. The ultrastructural aspects of bioaccumulation of heavy metal contaminants and its effect on olfactory responsiveness through acetylcholinesterase (AChE) activity in fish OSNs are still an obscure part of olfactory research. Therefore, it would be worthwhile to study the bioaccumulation of heavy metals and their effects (both cytological and histochemical) on OSNs by transmission electron microscopy (TEM) in a fish.

Pseudapocryptes lanceolatus (Bloch and Schneider, 1801) is a common teleostean: gobiid of Gangetic Bengal. This species has a well-developed unilamellar olfactory apparatus that is externally lined by pseudostratified neuroepithelium [12,13]. *P. lanceolatus* is mostly abundant on the intertidal habitat on both sides of Hooghly River, that is, Kanchrapara, West Bengal, India (22.56°N, 88.26°E) and Tribeni, West Bengal, India (22.99°N, 88.40°E). Tribeni has many multimodal industrial complexes, including small and heavy industries such as agriculture, tyres, motor vehicles, textiles, and power plants, around the Hooghly River (http://www.hooghly.gov.in/dllro/pdf/district_profile.pdf). This ecosystem near Tribeni is primarily influenced by a large amount of sewage and effluents (including organic and inorganic pollutants and heavy metals), which are responsible for alteration of physicochemical parameters of water quality [14]. The present study focused on the site-specific cytological changes in ciliated OSNs within the olfactory neuroepithelium in *P. lanceolatus*, with special reference to electron enzymology (for AChE activity) and X-ray microanalysis (for bioaccumulation of heavy metals)

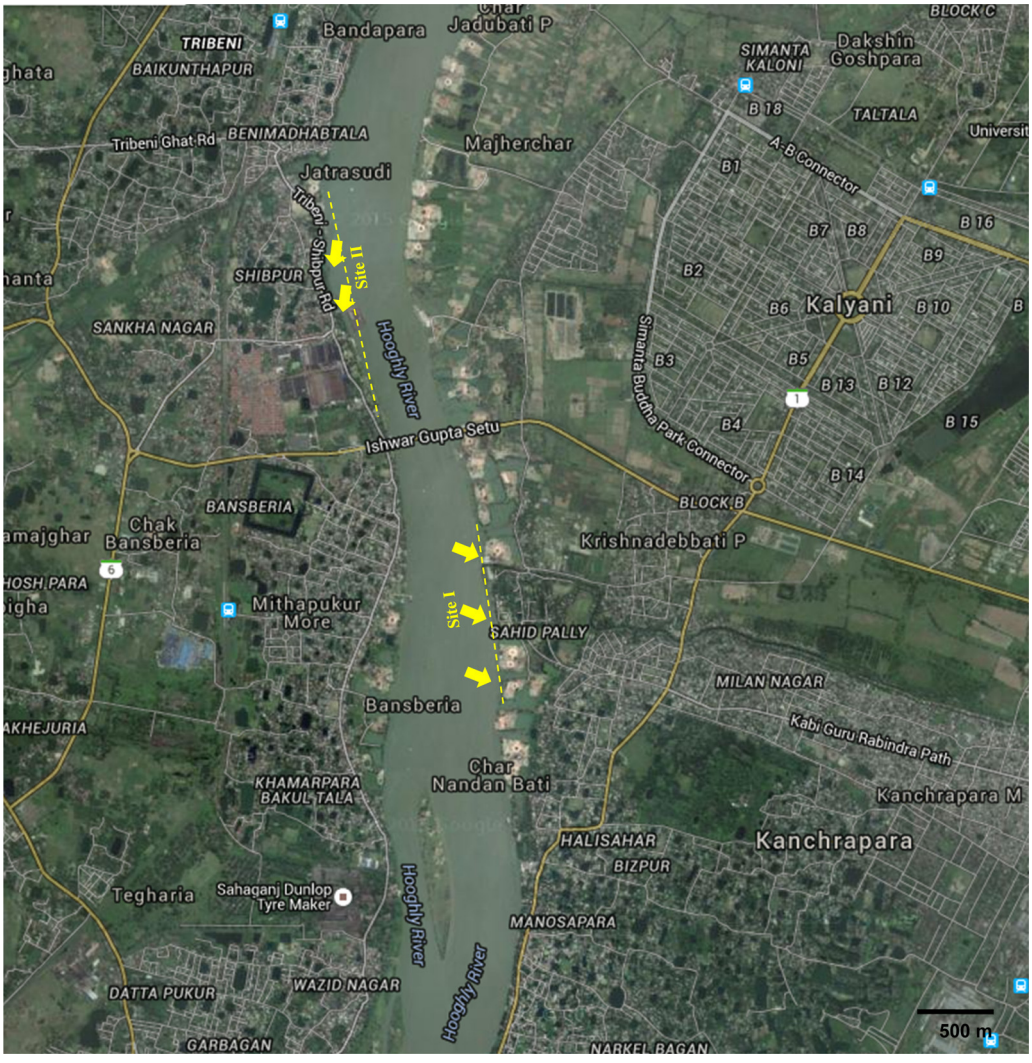


Fig. 1. Satellite image indicating specimen collection sites, that is, Site I (near Kanchrapara, North 24 Parganas, West Bengal, India) and Site II (near Tribeni, Hooghly, West Bengal, India) respectively. (https://www.google.co.in/maps/place/Tribeni,+West+Bengal/@22.9688932,88.4074596,15z/data=!3m1!1e3!4m2!3m1!1s0x39f89356bafff45f:0xf314310b6db40fbb).

under TEM to explore the probable consequences of heavy metal accumulation.

2. Materials and methods

2.1. Selection of study area and specimen collection

Tribeni and Kanchrapara are old and densely populated towns of Hooghly and North 24 Parganas, West Bengal, India, respectively; located on both sides of the Hooghly River. This study was conducted during the summer season in 2013 and 2014 on both sides of the Hooghly riverbank in the adjacent area of Kanchrapara and Tribeni (Figure 1). *P. lanceolatus* is a teleostean: gobiid and considered as "least concerned" according to the Red List Category of the International Union for Conservation of Nature (IUCN; http://www.iucnredlist.org/details/169496/0). Live, adult, and sex-independent specimens of *P. lanceolatus* (total body length: 10–20 cm) were separately collected from the intertidal habitat of Tribeni and Kanchrapara, West Bengal, India and brought to the laboratory (Figures 2A and 2B). Group I (collected form Kanchrapara, West Bengal) and Group II (collected form Tribeni, West Bengal) were acclimatized at 20–25 °C and > 40% humidity for 48 hours.

2.1.1. TEM

Live specimens of *P. lanceolatus* (Groups I and II) were separately anaesthetized with MS-222 (100–200 mg/L) and the olfactory apparatus was dissected out. The olfactory tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) at 4 °C for 2 hours. After primary fixation, the olfactory tissues were rinsed in the same buffer and then secondarily fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2–7.4) for 1 hour at 30 °C. The olfactory tissues were washed in the same buffer and dehydrated using chilled acetone at 4 °C. The tissues were embedded in Araldite CY212 (TAAB, UK) and resin polymerized for 48 hours at 60 °C. The transverse ultrathin sections (70–80 nm) of olfactory lamella were cut by ultramicrotome (Leica Ultracut – UCT, Germany) followed by collection on copper grids, and stained with uranyl acetate and lead citrate. The sections were observed under TEM (Morgagni 268D; Fei Electron Optics, Eindhoven, The Netherlands) operated at 80 kV. Digital images were analyzed using iTEM software (soft imaging system, Olympus Soft Imaging Solutions GmbH, Münster, Germany) attached to the microscope.

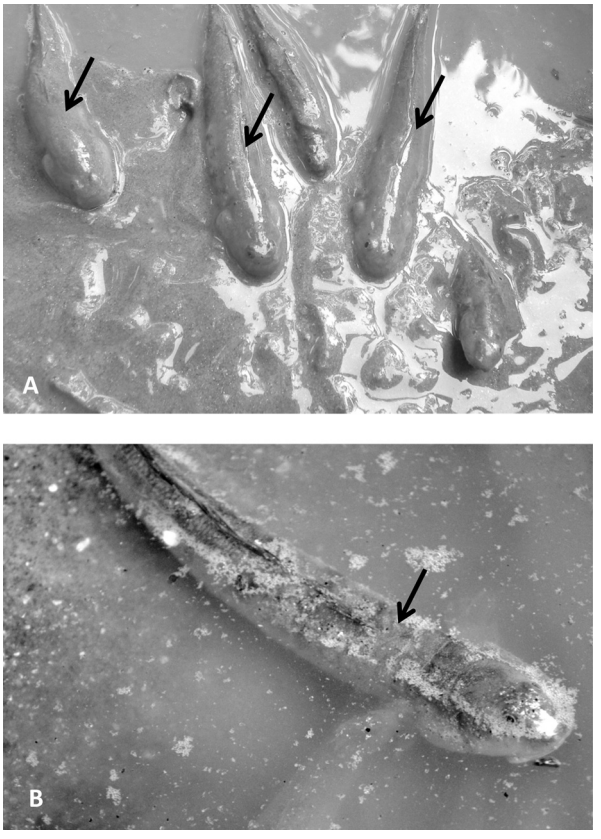


Fig. 2. Photographs showing *Pseudapocryptes lanceolatus* (Bloch and Schneider, 1801) in intertidal habitats of Hooghly River. (A) Specimens of *P. lanceolatus* are present on the intertidal mudflats of Hooghly River, that is, Group I (collected from Site I; arrows). (B) Specimen of *P. lanceolatus* is observed at the surface turbid water of Hooghly River, that is, Group II (collected from Site II).

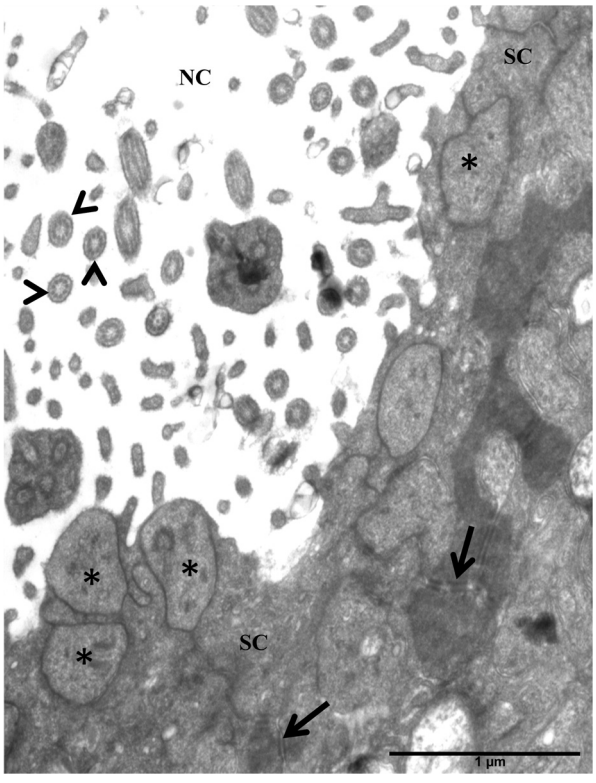


Fig. 3. Apical part of olfactory neuroepithelium in *Pseudapocryptes lanceolatus* (Group I) shows prominent cellular architecture of olfactory sensory receptor neuron and supporting cell (SC). The tip of the dendron formed olfactory knobs (stars) and projected into the lumen of the nasal cavity (NC). (9 + 0) arrangement of microtubules in kinocilia are marked (arrow heads). Desomosomes (arrows) are also demarcated.

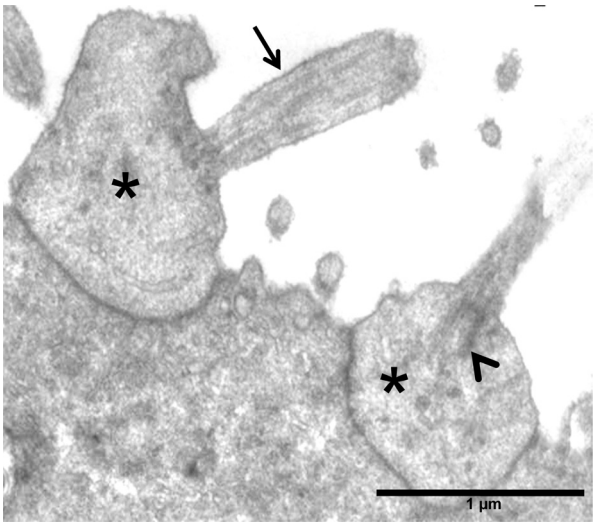


Fig. 4. Olfactory knob of ciliated olfactory sensory receptor neuron in *Pseudapocryptes lanceolatus* (Group I). The cilia along with axial microtubules (arrow), basal body (arrow head), vesicular structures (star) are noted in the olfactory knob of ciliated sensory receptor cell.

2.2. X-ray microanalysis under TEM

For X-ray microanalysis under TEM (TEM-EDX), the transverse ultrathin sections (70–80 nm) of olfactory lamella were cut by ultramicrotome (Leica Ultracut – UCT). The unstained sections of olfactory lamella of *P. lanceolatus* (Groups I and II) were separately collected on aluminum grids and examined under TEM (JEM – 2100; Jeol, Tokyo, Japan) with an EDX attachment (X-Max^n 80 T; Oxford Instruments, United Kingdom).

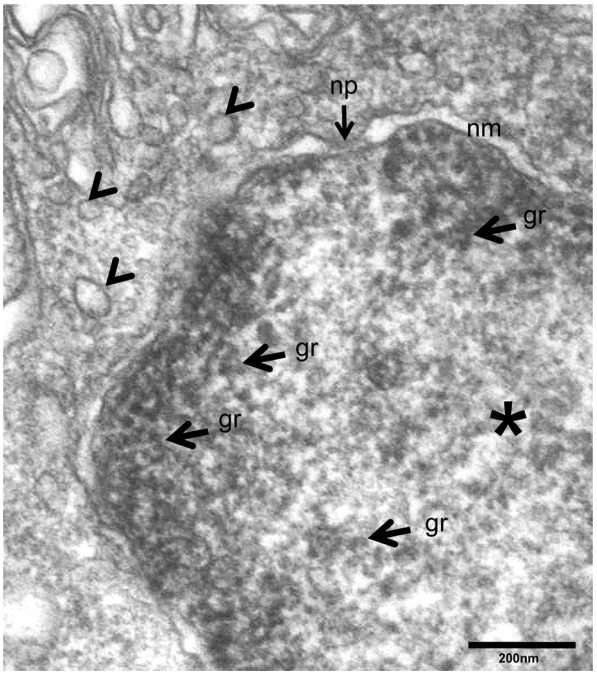


Fig. 5. Perikaryon of ciliated olfactory sensory receptor neuron in *Pseudapocryptes lanceolatus* (Group I). The chromatinized nucleus (star) with prominent nuclear membrane (nm), nuclear pore (np), chromatin granules (gr) are marked. Vesicles with variable diameters (arrow heads) were also frequent in the perinuclear cytoplasm.

2.3. AchE activity under TEM

The olfactory apparatus of *P. lanceolatus* (Groups I and II) was separately dissected out and fixed in 2.5% glutaraldehyde and 4% paraformaldehyde (1:1) in 0.1 M phosphate buffer (pH 7.2–7.4) at 4 °C for 2 hours. The tissues were washed in the same buffer and incubated in the medium described by Karnovsky and Roots [15]. The incubated tissue was rinsed in diaminobenzidine solution and processed for TEM. The processed tissues were secondarily fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2–7.4) for 1 hour at 27 °C. The tissues were rinsed in the same buffer, dehydrated in graded chilled acetone, and embedded in Araldite mixture for 48 hours at 60 °C. The sections (thickness: 70–80 nm) were cut by using an ultramicrotome (Leica Ultracut – UCT), collected on copper grids, and viewed under a transmission electron microscope (Morgagni 268D; Fei Electron Optics) operated at 80 kV.

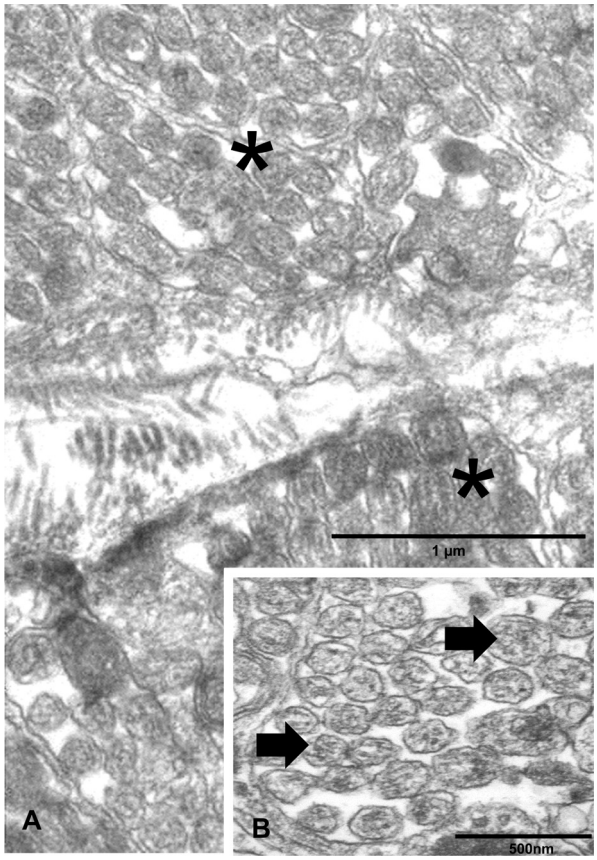


Fig. 6. Photomicrograph shows axonal integration in form of fila olfactoria at the lamina propria of olfactory neuroepithelial structure of *Pseudapocryptes lanceolatus* (Group I). (A) Fila olfactoria shows many axons in OSNs (star). Axonal accumulations are guarded by numerous collagen fibers. (B) The axoplasm of OSNs indicates prominent microtubules and vesicles (arrows).
OSN = olfactory sensory receptor neuron.

3. Results

3.1. Olfactory neuroepithelium in Group I P. lanceolatus

The olfactory neuroepithelium in *P. lanceolatus* showed an intermingled pattern of OSNs and supporting cells forming a pseudostratified architecture (Figure 3). The ciliated OSNs were predominantly distributed throughout the neuroepithelium of *P. lanceolatus* and possessed a dendron, perikaryon and axon (Figures 4, 5, and 6A). The dendron ran towards the surface of the olfactory neuroepithelium and projected into the lumen of the nasal cavity by forming an olfactory knob that had 4–6 cilia supported by microtubules (9 + 0) (Figure 4). The basal body of the cilia was just beneath the plasma membrane and devoid of striated rootlets but associated with centrioles. An association of neurofilaments, microtubules, and numerous vesicular structures was also noted at the adjacent cytoplasmic area of the basal body (Figure 4). The bioaccumulation of heavy metals in the apical part of the olfactory knob adjacent to the ciliary apparatus was mostly recorded as below detection level. A small percentage of iron (0.01%), copper (3.02%), and silver (0.01%) was detected (Table 1). The perikaryon of ciliated OSNs possessed spherical chromatinized nuclei (Figure 5). The heterochromatin materials with large accumulation of condensed chromatin granules (diameter, 20–30 nm) were frequently distributed near the peripheral part of the nucleoplasm, adjacent to the inner nuclear membrane (Figure 5). In the nucleoplasm of ciliated OSNs, bioaccumulation of heavy metals was variable. The percentage bioaccumulation of titanium, iron, silver, cadmium, and lead was less, that is, below detection level. Small percentages of nickel (0.69%) and copper (2.66%) were recorded by TEM-EDX (Table 3). Vesicles with variable diameter (10–50 nm) and mophometry were identified at the perinuclear cytoplasm of ciliated OSNs (Figure 5). The unmyelinated axons of ciliated OSNs invaded the basal lamina, accumulated to form olfactory fascicles or fila olfactoria, and subsequently aggregated into axonal bundles at the lamina propria (Figures 6A and 6B). The axoplasm contained various vesicular structures, neurofilaments (diameter ~10 nm) and microtubules (diameter 20–25 nm; Figures 6A and 6B). Few AChE-positive vesicles of diameter 30–40 nm were identified under TEM (the olfactory lamella was incubated using acetylthiocholine iodide as a substrate according to the Karnovsky and Roots [15] method). These vesicles were mainly distributed near the terminal knob of the axon (Figure 7).

3.2. Olfactory neuroepithelium in Group II P. lanceolatus

The olfactory neuroepithelium in Group II *P. lanceolatus* mostly showed features of degenerating ciliated OSNs due to necrotic cell death (Figure 8). The olfactory knob in degenerating ciliated OSNs was characterized by lysis of the plasma membrane along with disintegrated

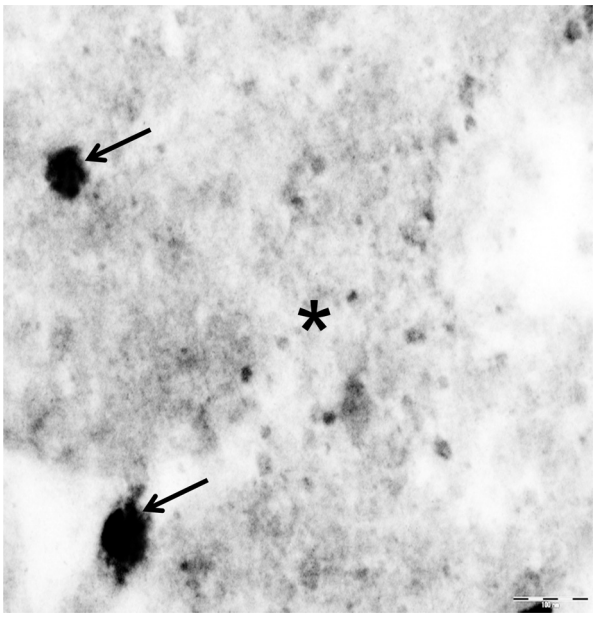


Fig. 7. Arrows within the electron micrograph indicate acetylcholinesterase-positive vesicles (diameter: 30–40 nm) at the axoplasm near the terminal knob of ciliated olfactory sensory receptor neuron in olfactory neuroepithelium of *Pseudapocryptes lanceolatus* (Group I).

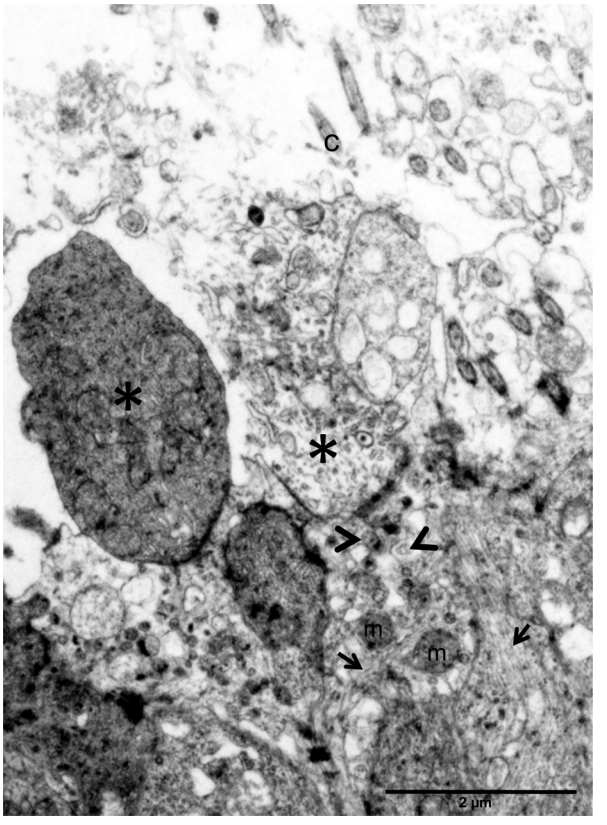
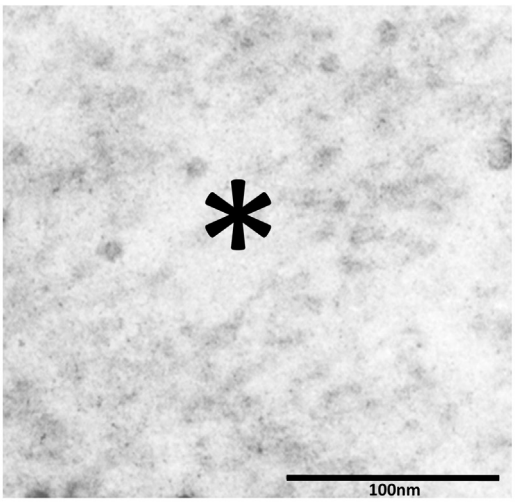


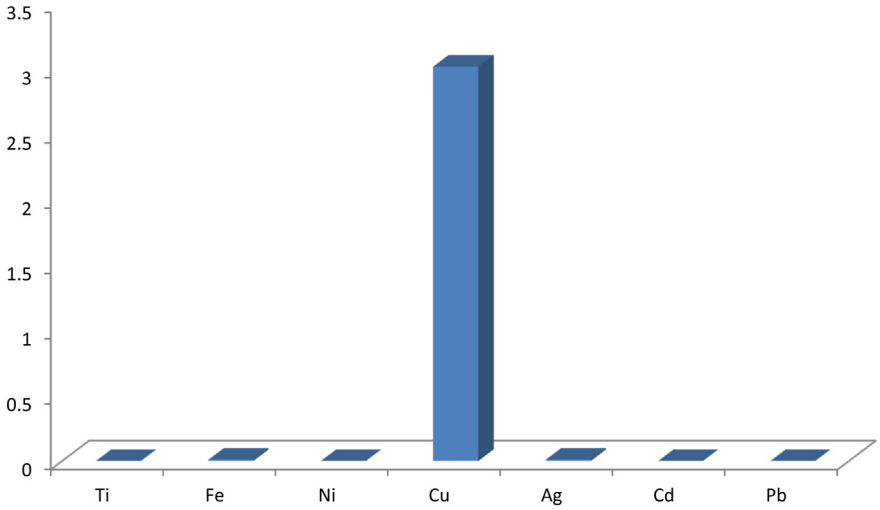
Fig. 8. Olfactory neuroepithelium of *Pseudapocryptes lanceolatus* (Group II) shows necrotic olfactory knob of ciliated OSN (stars). Degenerating cilia (c), microtubules (arrows), mitochondria (m), and various phases of lysosomes (arrowheads) are also marked within the apical cytoplasm of ciliated OSN in *P. lanceolatus* (Group II).
OSN = olfactory sensory receptor neuron.

Table 1
X-ray microanalysis report showing bioaccumulation of heavy metal components in a part of the apical cytoplasmic region (star) near the ciliary apparatus in the olfactory knob of ciliated olfactory sensory receptor neuron in olfactory neuroepithelium of *Pseudapocryptes lanceolatus* (Group I).



Elements	Weight %
Ti	BDL
Fe	0.01
Ni	BDL
Cu	3.02
Ag	0.01
Cd	BDL
Pb	BDL

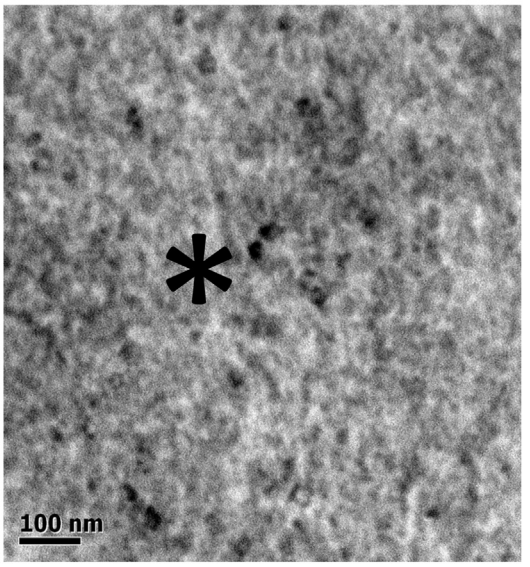
BDL: Below Detection Level



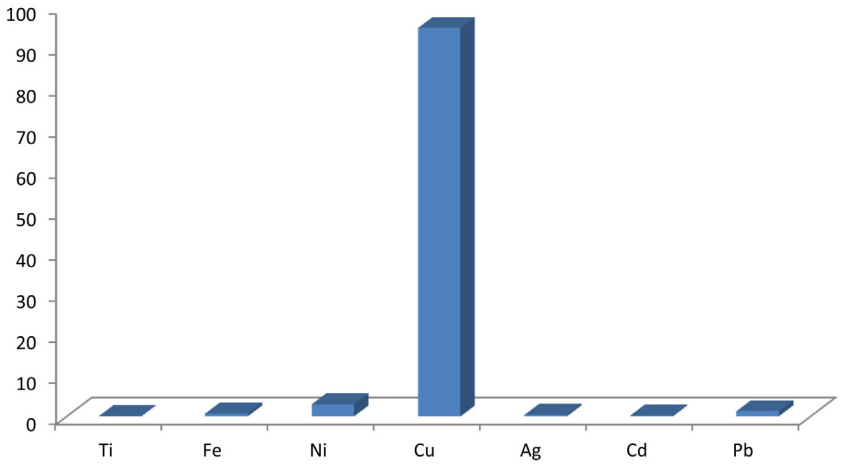
BDL = below detection limit.

subcellular components including microvacuolar deterioration within the cytoplasm (Figures 8 and 9). The cilia were detached from the basal body and consequently shaded off from the terminal part of the olfactory knob. This subcellular region in ciliated OSNs showed a higher concentration of titanium (0.04%), iron (0.68%), nickel (2.92%), copper (94.50%), silver (0.35%), cadmium (0.25%), and lead (1.26%) in *P. lanceolatus*; noted under TEM-EDX (Table 2). The (9 + 0) arrangement of microtubules in olfactory cilia was not well demarcated. The microtubules were also altered in their appearance and loosely arranged within the dendroplasm (Figures 8 and 9). Intertubular spaces were wider than in functional ciliated OSNs (Figure 8). Cluster forms of neurofilaments were also identified within the cytoplasm of degenerating ciliated OSNs (Figure 8). The perikaryon of ciliated OSNs showed swelling of the nuclei. The ultrastructural features of nuclear membranes and nuclear pores were not prominent under TEM (Figure 9). Chromatin fibers were fragmented (Figure 9). The heterochromatin materials with condensed granules (diameter,

Table 2
X-ray microanalysis report indicating part of apical cytoplasmic region (star) near the ciliary apparatus in the olfactory knob and bioaccumulation of heavy metal components in ciliated olfactory sensory receptor neuron in the olfactory neuroepithelium of *Pseudapocryptes lanceolatus* (Group II).



Elements	Weight %
Ti	0.04
Fe	0.68
Ni	2.92
Cu	94.50
Ag	0.35
Cd	0.25
Pb	1.26
Total	**100.00**

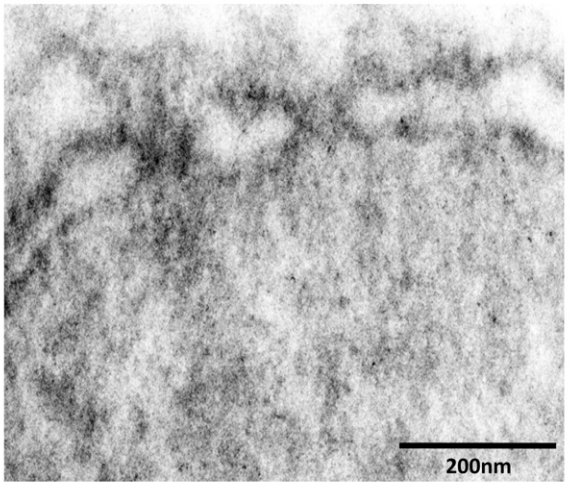


20–30 nm) were clumped at the peripheral part of the nucleoplasm (Figure 9). In the nucleoplasm, titanium was absent. The percentage accumulation of iron (83.81%) in the nucleoplasm was higher than for other metals like nickel (2.80%), copper (11.88%), silver (0.27%), cadmium (0.19%), and lead (1.05%; Table 4). Free unidentified granulated structures were scattered within the perinuclear cytoplasm of ciliated OSNs. Vesicular structures were not well marked within the perinuclear cytoplasm of ciliated OSNs (Figure 9). The necrotic features of fila olfactoria (i.e., swelling of axons, disintegration of cytoskeletal structures in the axoplasm, and loose arrangement of microtubules) are shown in Figure 10. A large accumulation of AChE-positive vesicles was noted at the terminal part of the axons in ciliated sensory receptor cells (the olfactory tissue was incubated against acetylthiocholine iodide as substrate according to the Karnovsky and Roots [15] method; Figure 11).

4. Discussion

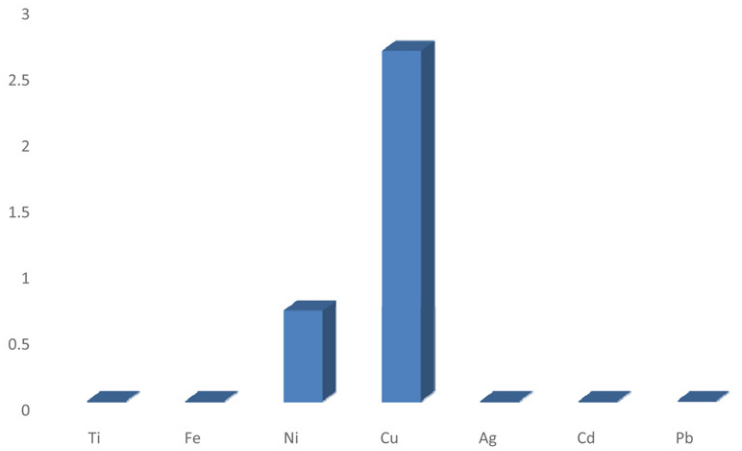
Pollution of the aquatic environment by inorganic chemical compounds is considered to be a major threat to aquatic organisms including fish. Heavy metals are one of

Table 3
X-ray microanalysis report showing bioaccumulation of heavy metal components in part of the nucleoplasm (star) at the perikaryon of ciliated olfactory sensory receptor neuron in olfactory neuroepithelium of *Pseudapocryptes lanceolatus* (Group I).



Elements	Weight %
Ti	BDL
Fe	BDL
Ni	0.69
Cu	2.66
Ag	BDL
Cd	BDL
Pb	BDL

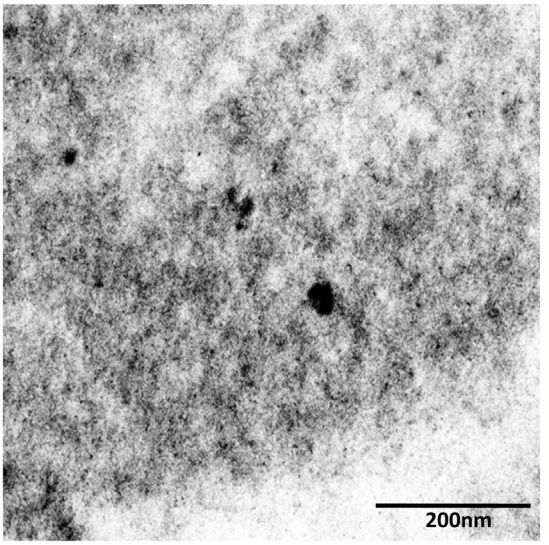
BDL: Below Detection Level



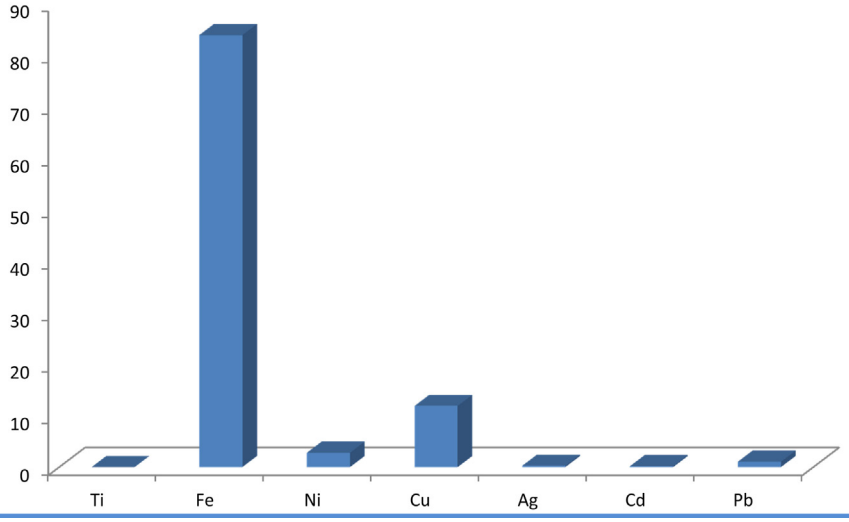
BDL = below detection limit.

the important pollutants of the aquatic environment that are mainly discharged from anthropogenic sources [16]. These elements have an affinity to accumulate within the fish neuronal tissue [17], binding with several ligands and macromolecules to form neurotoxins [18]. Heavy metals such as iron and copper are essential for various biological activities but become toxic at higher concentrations, whereas lead and cadmium are toxic in nature [19]. The perfusion of copper may cause a steady decline of chemoresponsiveness of OSNs in coho salmon (*Oncorhynchus kisutch*) [20]. Olfactory responsiveness is generally caused by contact of water-soluble odorants with the olfactory primary cilia of bipolar olfactory sensory receptor cells. The subcellular organelles like kinocilia, microtubules, neurofilaments, and vesicles in the olfactory knob play important roles in transmission of olfactory signals to the brain in *P. lanceolatus* [21]. The olfactory neuroepithelium of fish is also an excellent passage to transport metals from the olfactory lumen to the brain via olfactory neurons [22]. The cellular junctions between olfactory cellular components probably help to prevent the transport of metals through this passage [23]. Long-term exposure of sublethal doses of heavy metal contaminants may cause histopathological changes and ciliopathy in the fish olfactory neuroepithelial system [24,25]. The loss of kinocilia, lysis of plasma membrane, cluster of neurofilaments, and disintegration

Table 4
X-ray microanalysis report indicating bioaccumulation of heavy metal components in a part of the nucleoplasm (star) at the perikaryon of ciliated olfactory sensory receptor neuron within the olfactory neuroepithelium of *Pseudapocryptes lanceolatus* (Group II).



Elements	Weight %
Ti	0.00
Fe	83.81
Ni	2.80
Cu	11.88
Ag	0.27
Cd	0.19
Pb	1.05
Total	**100.00**



of microtubules in olfactory knobs is clearly indicative of necrosis as well as ciliopathy of olfactory receptor cells in *P. lanceolatus* (Group II). Variable accumulation of heavy metals within the subcellular compartments also demonstrates the comparative environmental conditions of specimen collection sites in the present study. The large accumulation of heavy metals may result in various neurodegenerative disorders [26]. It is widely accepted that excess accumulation of iron is the initial event of age-related neuronal degeneration and its consequent disease processes [27]. Large accumulation of iron causes dramatic mutation of the gene sequences that encode enzymes responsible for iron metabolism [28]. Dysregulation of iron metabolism leads to neuronal death as well as age-related neuronal dysfunction such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [29]. AChE is known to be an inhibitor of nerve impulse transmission by hydrolyzing acetylcholine into acetate and choline, and is synthesized in the perinuclear cytoplasm of olfactory neurons [30,31]. The increasing inhibitory role of AChE is important for indicating different neurodegenerative dysfunctions [32,33]. The excess accumulation of heavy metals in ciliated OSNs may cross the limit of tolerance, causing stress and neural degeneration in Group II *P.*

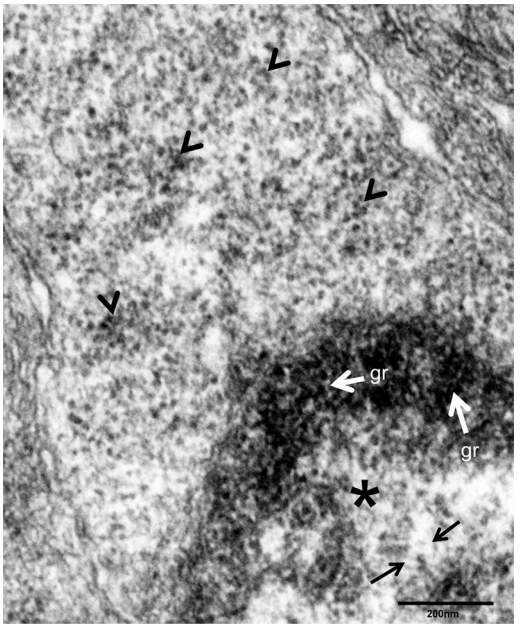


Fig. 9. Perikaryon of ciliated OSN in *Pseudapocryptes lanceolatus* (Group II) shows necrotic features of nucleus (star) under transmission electron microscopy. Condensation of chromatin granules (gr) and fragmented chromatin fibers (arrows) were also noted within the nucleoplasm. Numerous granule-like structures (arrowheads) were also frequently distributed in the peirnuclear cytoplasm of ciliated OSN in *P. lanceolatus* (Group II).
OSN = olfactory sensory receptor neuron.

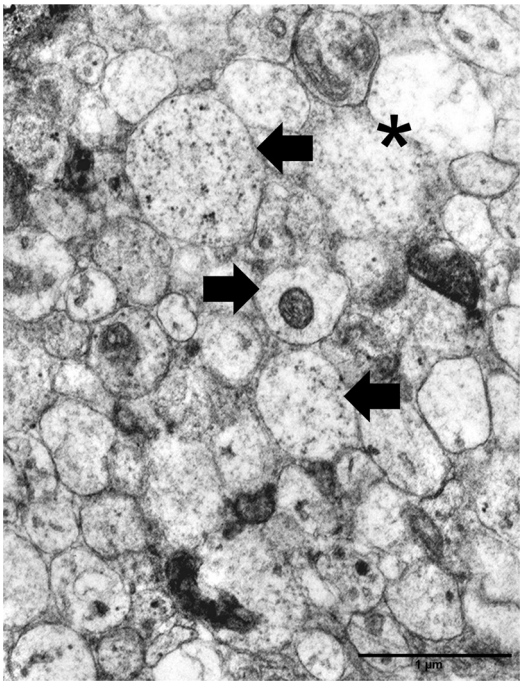


Fig. 10. Electron micrograph shows necrosis of fila olfactoria (star) in the lamina propria of olfactory neuroepithelial system in *Pseudapocryptes lanceolatus* (Group II). The cytoplasm of axons was less granulated with disintegrated cytoskeletal structures (arrows).

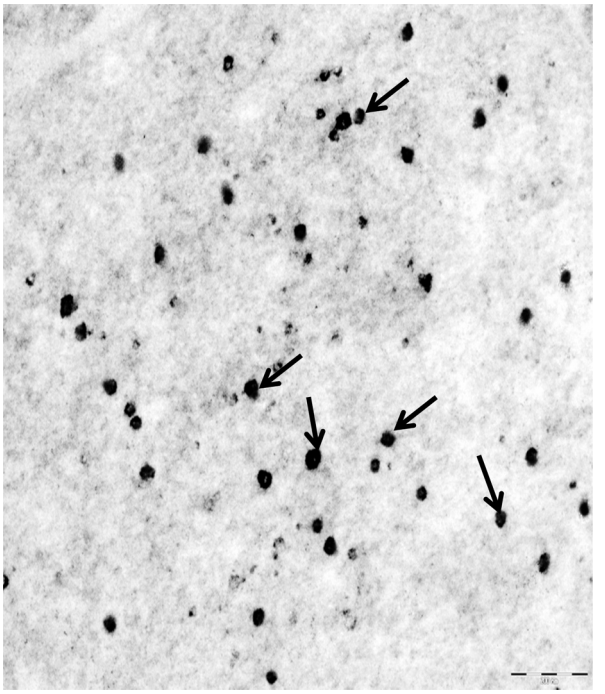


Fig. 11. Photomicrograph indicates large number of acetylcholinesterase-positive vesicles of 30–40 nm diameter, distributed within the terminal part of the axons in degenerating ciliated olfactory sensory receptor neuron.

lanceolatus. Therefore, this EM-based enzymology and X-ray microanalysis focused on significant factors related to monitoring of environmental health as well as metallobiology of neurodegenerative diseases in fish.

Conflicts of interest

We have no conflict of interest to declare.

Acknowledgments

We are thankful to Prof. T.C. Nag, Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi – 110029, India for his kind support and necessary helps.

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