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Effect of cadmium in the gills of green tiger prawn, *Penaeus semisulcatus*

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Abstract
The aim of this study was to determine the acute toxicity levels of cadmium for *Penaeus semisulcatus* and elucidate the impact of this heavy metal on gills at two sublethal (250 and 500 µg L^{-1}) levels for a period of 14 days. The median lethal concentration level (LC_{50}) of cadmium in 24, 48, 72 and 96 h for *P. semisulcatus* were 8180, 5160, 4000 and 2680 µg L^{-1}. The gills of the shrimps were dissected out and processed for light and electron microscopic studies. The light microscopic studies revealed several alterations in the histoarchitecture of the gills and the prominent changes include deformity of the secondary gill lamellae, infiltration of hemocytes, malformation at the tip of gill filament, lifting of lamellar epithelium, hyperplasia and swelling of the gill lamellae. The ultrastructural examination exposed detachment of gill epithelium, formation of electron dense deposits in the cuticle, disrupted and damaged microvilli, shrunken nucleus, vacuole formation, swollen mitochondria with disoriented cristae, fragmented endoplasmic reticulum and numerous vacuoles with electron dense granules. These alterations impair vital physiological functions, such as respiration and osmoregulation of the gills, which in turn affect the survival of *P. semisulcatus*. Therefore, the present study suggests that efficient remedial measures should be adopted to prevent the occurrence of cadmium contamination in the aquatic environment.

Keywords: *Penaeus semisulcatus,* cadmium, gills, acute toxicity

1. Introduction
Cadmium is a non-essential element that produces profound toxic effects in aquatic organisms [1]. In fishes, elevated levels of cadmium in ambient environment leads to calcium imbalance, damage to the gills and also accumulates in different organs of the animal [2]. Among invertebrates, crustaceans are found to be highly sensitive to this element resulting in damage to the gills and hepatopancreas besides concentrating in various tissues like hepatopancreas, gills, muscle and exoskeleton [3].
The gills play a central role in osmoregulation, respiration and ionic regulation in the Crustacea [4, 5] and are adversely affected after exposing to elevated levels of cadmium [6]. The ultrastructural responses of crustacean gills to trace metals have been reported in *Penaeus duorarum*, *Palaemonetes pugio* and *Palaemonetes vulgaris* [7], *Palaemon serratus* [8], *Crangon crangon* [9], *Eriocher sinensis* [10], *Carcinus maenas* [11] and *Marsupenaeus japonicus* [12, 13] and *Litopenaeus vannamei* [14].
The Green Tiger prawn, *Penaeus semisulcatus* is widely distributed in the Indo-Pacific region and form one of the commercially important shrimp species in India. In addition, they are also a suitable candidate for aquaculture. Extensive studies like biology [15], reproduction, embryonic and post embryionic development [16], feeding habits and digestive physiology [17] have been reported for this animal while information regarding the effects of toxicants are rare [18]. Therefore, the objective of the present study was to determine the acute toxicity of levels of cadmium for 24, 48, 72 and 96 h and the ultrastructural changes in the gills at two sublethal levels.

2. Materials and Methods
2.1 Animals
Live *P. semisulcatus* (6.0 ± 0.25 cm; 4.5 ± 0.6 g) were collected from Palk Bay (9.28°N 79.3°E) and immediately transported to the shrimp hatchery at Mandapam Regional Centre of CMFRI (Tamil Nadu, India).

They were maintained in two-ton capacity fibreglass tanks filled with seawater treated by rapid sand filtration and bio-filtration. The physicochemical parameters of water during the study period were salinity (32±0.5 ppt), temperature (27 ± 1 ºC), pH (8.02 ± 0.1) and dissolved oxygen (6.03 ± 0.2 mg L^{-1}). Adequate aeration was provided using air blowers and the seawater was renewed daily to maintain the water quality at optimum level. A photoperiod of 12:12 h day and night was maintained. The animals were fed with boiled clams ad libitum once in a day at night (21.00 h) during maintenance. Healthy *P. semisulcatus* with no signs of infection or injuries and in the intermoult stage were used for the study [19]. The animals were starved for 24 h in order to remove the stomach contents during acclimatization prior to experiments.

2.2 Bioassay

Acute toxicity (LC_{50}) of cadmium for 24, 48, 72 and 96 h was conducted under laboratory conditions as per the methods recommended for toxicity test with aquatic organisms [20]. The Stock solutions (50 mg L^{-1}) were prepared from cadmium chloride ($CdCl_2$ $2H_2O$; Qualigens, India) with deionised water following the dilution technique adopted by committee on methods for toxicity test [20]. Selected experimental concentrations were made by addition of adequate volumes of stock solution to the seawater.

Twenty numbers of *P. semisulcatus* were released into glass aquaria containing 100 L of seawater for bioassay studies. Simultaneously control shrimps were also placed without the metal exposure. Dead animals were counted and removed and the criteria for death were total lack of movement or lack of response after repeated touches with a probe. LC50's were calculated for 96 h and the Median lethal concentration is estimated by probit analysis [21]. The slope functions (S) and confidence limit (CL) were calculated as per the method of Reish and Oshida [22]. The significant differences between the replicates were tested by the formula developed by Litchfield and Wilcoxon [23]. Morphological discolourations, if any were recorded during the acute bioassay test. All experiments were conducted in triplicate.

2.3 Sub lethal experiment

Sub-acute experiments of cadmium exposure were conducted for 14 days in two different concentrations (250 and 500 µg L^{-1}). Selected experimental concentrations were made by the addition of adequate volumes of stock solution (50 mg L^{-1}) to the seawater in the experimental tanks. The animals were fed with boiled clam meat *ad libitum*, daily at night (21.00 h) during the study period. The treated water was removed daily and refilled with seawater having the same concentration of the cadmium. After 14 days, the gills were dissected from the shrimp and fixed for histopathological examinations.

2.4 Light microscopy

The histological procedure was done by the routine histological method [24]. Briefly, the gills of shrimps from control and experimental group were dissected out and preserved using Davidson's AFA fixative for 48 h. The tissues were dehydrated in alcohol series and embedded in paraffin wax. They were cut into sections of 6 mm thickness by a rotary microtome and stained with hematoxylin and eosin. All histopatholological examinations were done according to Bell and Lightner [24].

2.5 Transmission electron microscopy (TEM)

For TEM, two shrimps each were sacrificed from experimental and control groups. Approximately, 1 mm3 tissues were incised from the gills and fixed in gluteraldehyde (3%) in sodium cacodylate (0.1 M) buffered for four hours in 4 ºC. The samples were subjected to sodium cacodylate buffer wash three times and postfixation was done with 1% osmium tetroxide (OsO_4) for one hour in 4 ºC. The tissues were then washed in buffer, three washes of 30 min duration and left in buffer overnight. After decanting the buffer, the tissues were dehydrated in ascending grades of acetone (30, 50, 70, 80, 90 and 95 %), two changes of 20 min duration and finally in absolute acetone for three changes of 30 min each. The dehydrated tissues were infiltrated and embedded in Spurr's resin. The blocks were trimmed and ultrathin sections were prepared in an LKB Ultramicrotome. The sections were stained with uranyl acetate and lead citrate and observed under Hitachi (H-600) Electron microscope at 50 KV accelerating voltages choosing different magnifications.

3. Results

3.1 Acute toxicity

The 24, 48, 72 and 96 h median lethal concentration level of cadmium for *P. semisulcatus* were 8180, 5160, 4000 and 2680 µg L^{-1} (Table 1). The experiments showed no significant difference between the replicates when tested with 1.96 SEdiff [23]. There was no mortality observed in the control group without the toxicant. Ten percent mortality of shrimps was observed at a level of 500 µg L^{-1} in 96 h and ninety percent mortality were recorded at a dose of 5500 and 6000 µg L^{-1}. At a higher concentration of 7000 µg L^{-1} all the animals were perished within 96 h.

Table 1: Lethal Concentration (LC_{50}) of *Penaeus semisulcatus* exposed to cadmium.

Time in hrs	LC_{50} value (µg L^{-1})	Slope (S)	Confidence limits (CL)	Filucidal Values (95%)	
				Upper (µg L^{-1})	Lower (µg L^{-1})
24	8180	2.34	2.05	16760	4000
48	5160	2.13	1.86	9600	2800
72	4000	1.97	1.72	6900	2320
96	2680	1.58	1.34	3600	2000

3.2 Morphological discolouration

Compared to normal shrimps (Fig 1A-D), those exposed to cadmium during bioassay studies (96 h) develop several morphological discolourations on the exoskeleton, gills and epipodites (Fig 2A-E). The prominent changes were melanisation in the scaphognathite of the antennae and the pereiopods, black deposits on the exoskeleton of thoracic and abdominal regions and blackening of gills and epipodites.

3.3 Light microscopy

The gills of *P. semisulcatus* have regular arrangement of lamellae with uniform interlamellar space. The septum (sep) dividing the afferent (Afs) and efferent vessel (efs), non-branching (NBF) and branching (BFL) secondary gill

filament were very prominent in the gills (Fig 2A). The central axis (CEN), efferent vessel (efp) and afferent vessel of primary gill lamellae (afp) are clearly visible while exploring the histoarchitecture of gills (Fig 2B). The gills of shrimps exposed to cadmium (250 µg L^{-1}) exhibited deformity of the secondary gill lamellae (DL) and necrosis (N) (fig 3A). Infiltration of hemocytes (H), malformation (ML) at the tip of gill filament and swelling (SL) were the major changes observed in the gills (Fig 3B). The shrimps exposed to cadmium (500 µg L^{-1}) showed hemocytic infiltration (H) (Fig 4A), lifting of lamellar epithelium (LLE), malformation at the gill tip (MF) and hyperplasia (HY) (Fig 4B, C).

3.4 Ultra structural changes

The epithelial cells of gills in shrimp develop several alterations after exposure to cadmium (250 µg L^{-1}). The main changes detected at the apical part include; detachment of gill epithelium, formation of few electron dense deposits in the cuticle along with disrupted and damaged microvilli (Fig 5A). The nucleus became shrunken with broken nuclear membrane; numerous vacuoles and unidentified oval accumulations of uniformly dark matter in the cell cytoplasm were also observed in the damaged cell (Fig 5B & C). The mitochondria became swollen with disoriented cristae (Fig 5D). The endoplasmic reticulum was found as fragmented (Fig 5E). The main damage at the basal part of the cell includes vacuolation, loss of cell organelles and disrupted cytoplasm (Fig 5F).

Ultrastructural observation revealed profound damage in the epithelial gill cells of *P. semisulcatus* after treatment with cadmium (500 µg L^{-1}). Prominent variations at the anterior area of the cell include separation of different layers of cuticle, degeneration of microvilli and disrupted endoplasmic reticulum (Fig 6A). The nucleus became shrunken with a wide and broken outer membrane (Fig 6B, C). The external membrane and cristae of mitochondria was fragmented (Fig 6D). In addition to these changes, there was a proliferation of vacuoles near the nucleus, fragmented endoplasmic reticulum and golgi bodies (Fig 6E) and numerous vacuoles with electron dense granules (Fig 6F).

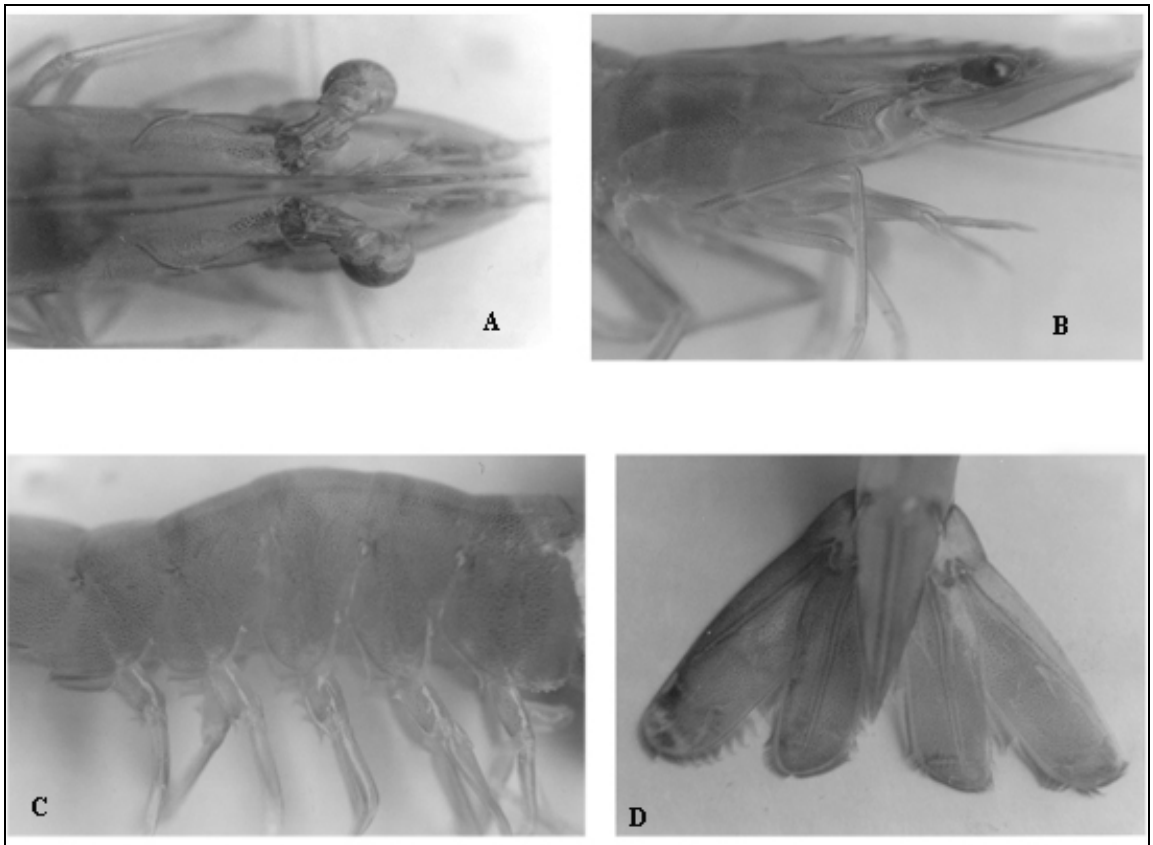

Fig 1. External body parts of *Penaeus semisulcatus*

A. Dorsal view of cephalothorax
B. Lateral view of cephalothorax
C. Lateral view of abdomen
D. Uropod and telson

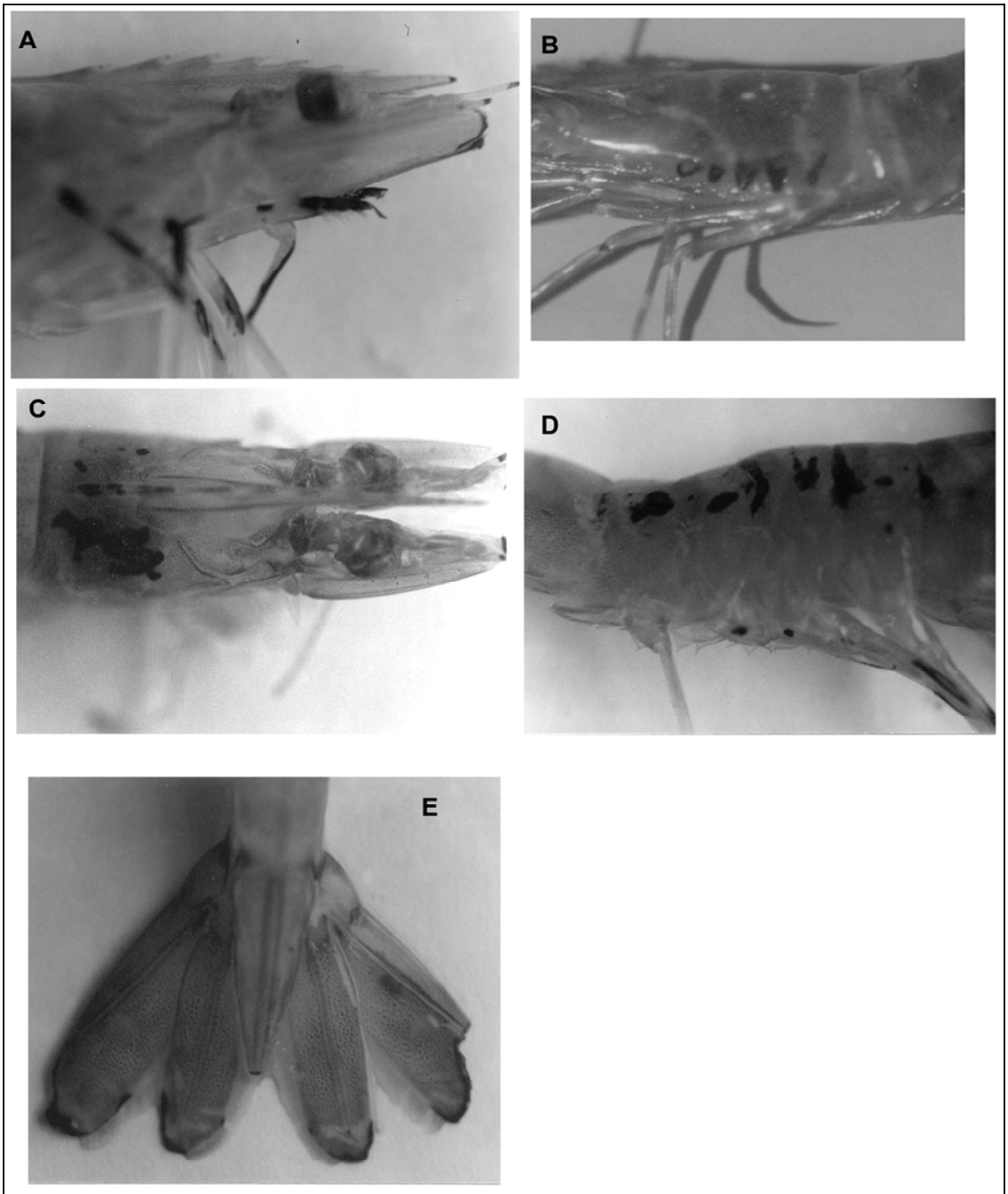

Fig 2. External body parts of *Penaeus semisulcatus* exposed to cadmium

(A) Melanisation of scaphognathite and pareopods
(B) melanisation of gills and epipodites
(C) Black discolouration on the carapace of thoracic region
(D) Darkened areas on the carapace of abdomen
(E) Telson having dark spots

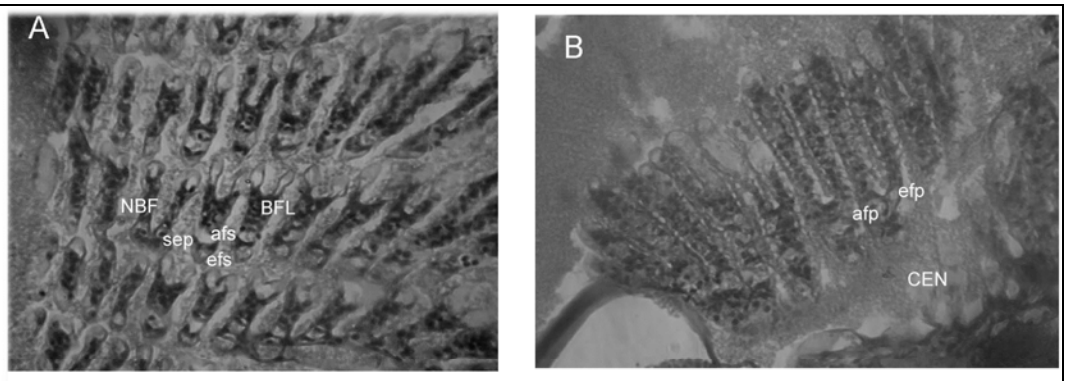

Fig 3. Histoarchitecture of the gills of *Penaeus semisulcatus*
(A) septum (sep) dividing the afferent (afs) and efferent (efs), non braching secondary gill filament (NFL) and branching secondary gill filament (BFL) X 200

(B) the central axis (CEN) of primary gill lamellae, efferent vessel (efp) and afferent vessel (afs) of primary gill lamellae X 200

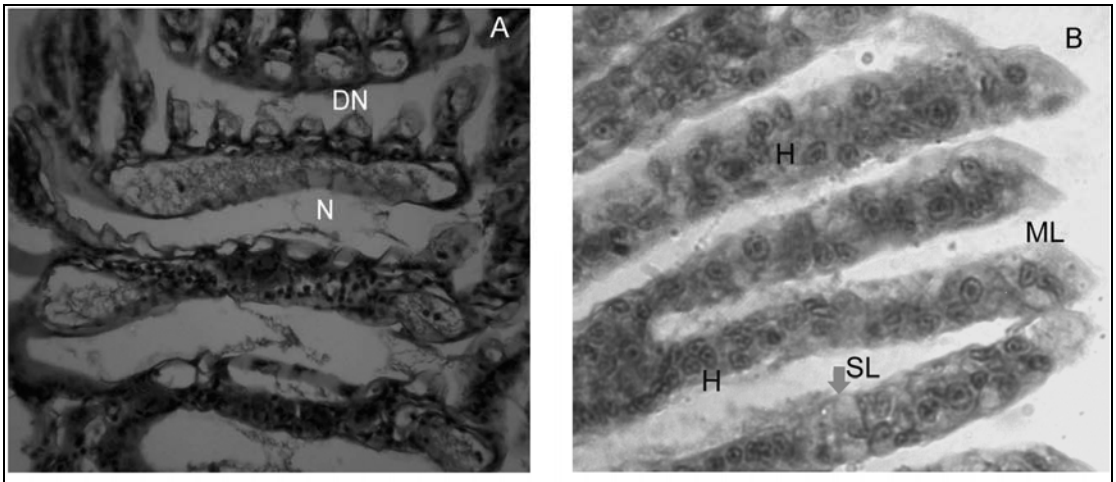

Fig 4. Histoarchitecture of the gills of *Penaeus semisulcatus* exposed to cadmium (250 µg L^{-1})

(A) deformity of the secondary gill lamellae (DN), necrosis of lamellae (N) X 400
(B) hemocytic infiltration (H), swelling of the lamellae (SL) and malformation at the tip of gills (ML) X 400

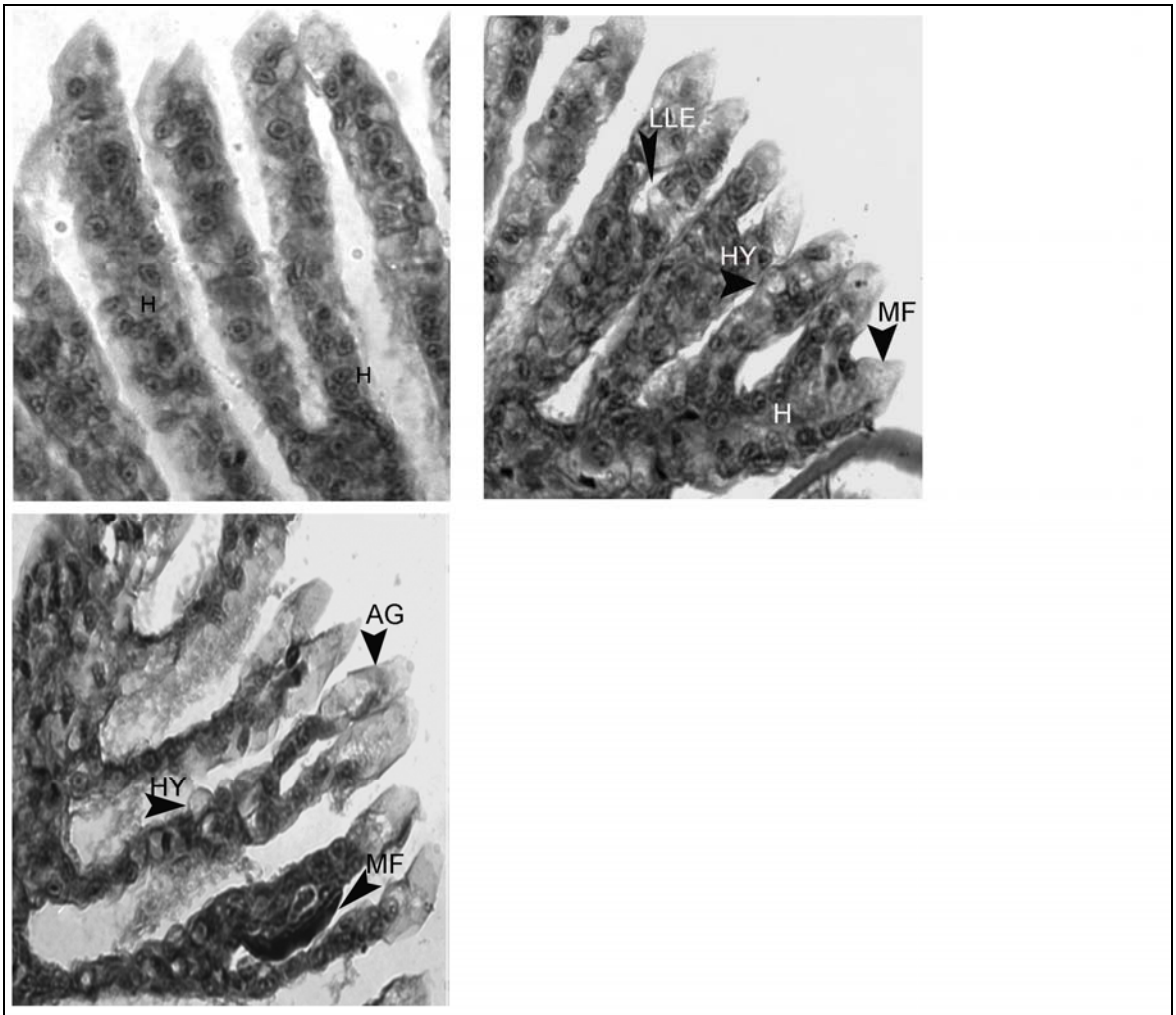

Fig 5. Histoarchitecture of the gills of Penaeus semisulcatus exposed to cadmium (500 µg L^{-1})

(A) Gill lamellae showing hemocyte infiltration (H) X 400

(B) Lifting of lamellar epithelium (LLE), malformation at the tip of gills (MF), hyperplasia (HY) and hemocyte infiltration (H) X 400

(c) abnormal gill tip (AG), hyperplasia (HY) and malformation at the tip of gills (MF) X 400

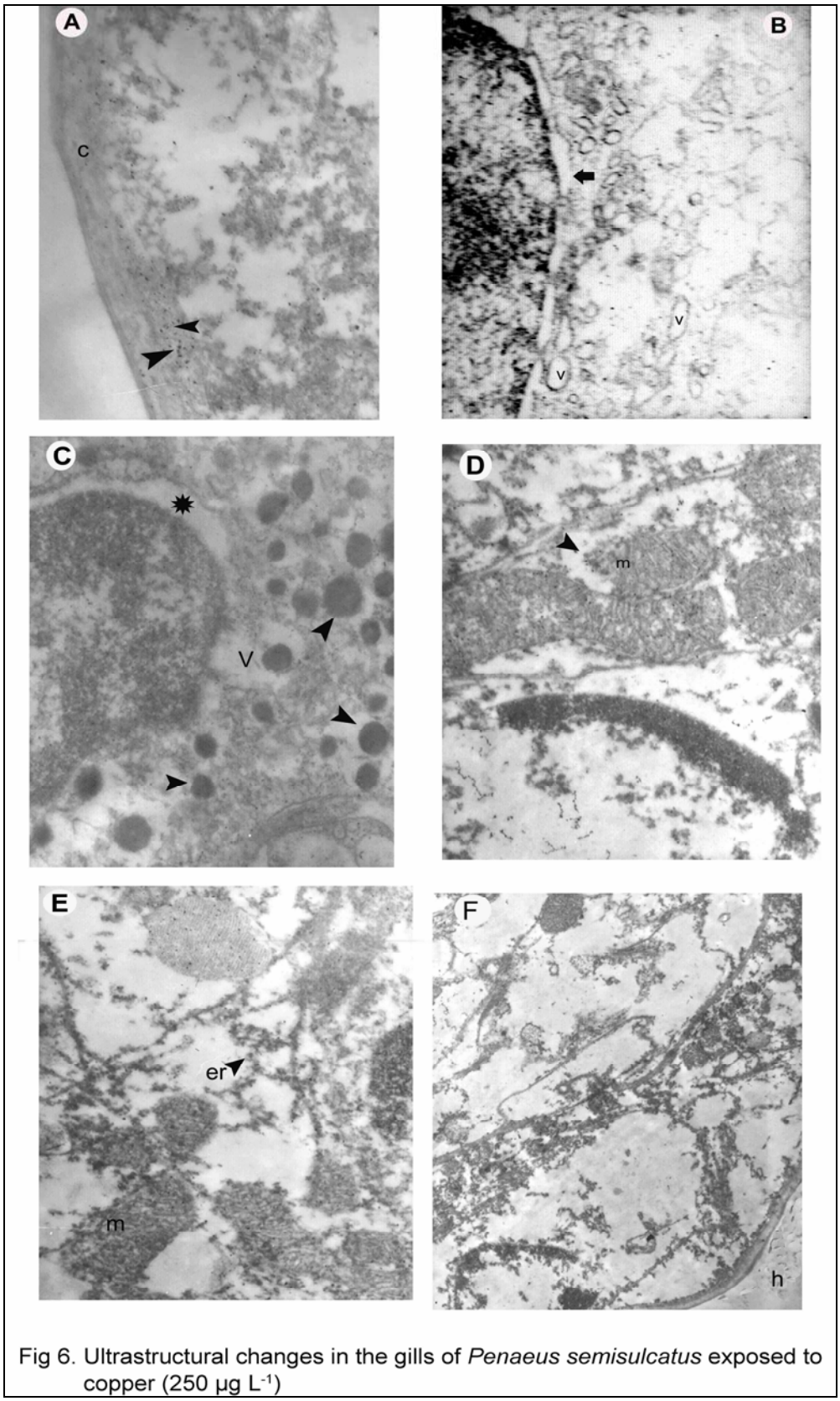

Fig 6: Transmission electron micrographs of epithelial gill cells in *Penaeus semisulcatus* exposed to cadmium (250 µg L^{-1}). (A) Detachment of gill epithelium from the cuticle (c) and few dark electron dense deposits in the cuticle (arrow head) X 35000. (B) Broken outer nuclear membrane (arrow) and numerous vacuoles (v) in the cytoplasm X 12000. (C) Oval accumulations of dark matter (arrow head), increased space between the nucleus and nuclear membrane (star) X 8000. (D) Swollen mitochondria (m) and disoriented cristae (arrow head) X 25000. (E) Fragmented endoplasmic reticulum. (F) Vacuoles, loss of cell organelles and disrupted cytoplasm near to the basal part of the cell X 10000.

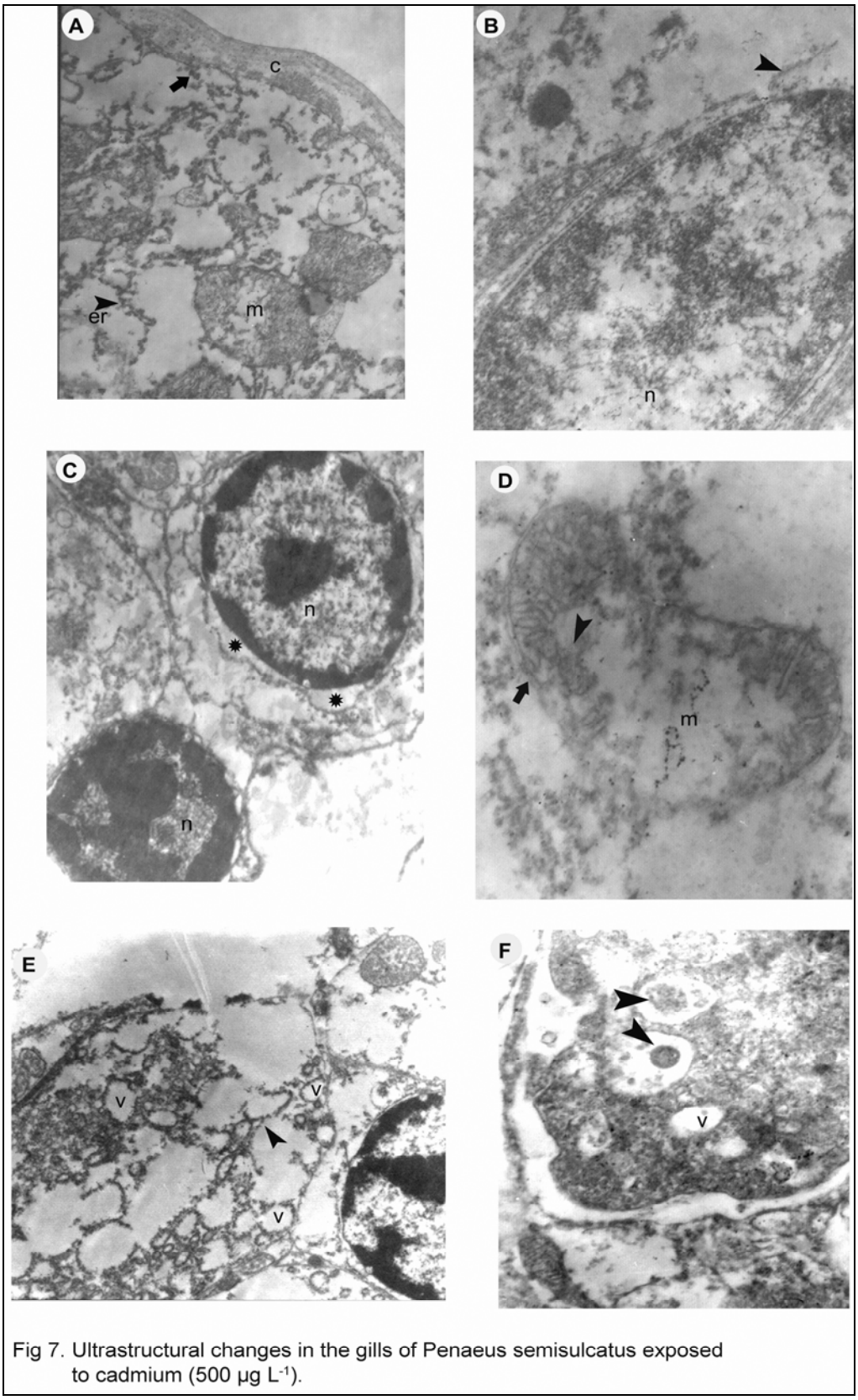

Fig 7. Ultrastructural changes in the gills of Penaeus semisulcatus exposed to cadmium (500 µg L^{-1}).

Fig 7: Transmission electron micrographs of epithelial gill cells in *Penaeus semisulcatus* exposed to cadmium (500 µg L^{-1}). (A) Damaged cuticle (c), microvilli (arrow head), disrupted endoplasmic reticulum (er) and swollen mitochondria (m) X 17000. (B) damaged outer nuclear membrane (arrow head) X 8000. (C) Shrunken nucleus (n) and increased gap between the nuclear membrane (star) X 8000. (D) Damaged mitochondria with broken outer membrane (arrow) and cristae (arrow head) X 12000. (E) Vacuoles (v) and fragmented endoplasmic reticulum (arrow head) X 12000. (F) Vacuoles with dense granules (arrow head) X 20000.

4. Discussion

The 96 h LC_{50} of cadmium for *P. semisulcatus* was found to be 2680 µg Cd L^{-1}. Major studies regarding the median lethal concentrations of toxicants for shrimps are *Penaeus duorarum* (4600 µg L^{-1}) [25], *Marsupenaeus japonicus* (3500 µg L^{-1}) [26], *Palaemon* spp (6600 µg L^{-1}) [27], *P. monodon* (2420 µg L^{-1}) [28] and *Litopenaeus vannamei* (2490 µg L^{-1}) [29]. Gills of crustaceans are known to be the active site for osmoregulation and respiration [4, 5] and also the primary site of waterborne pollutants due to their constant contact with the external environment. The gills, epipodites and exoskeleton of *P. semisulcatus* exposed to cadmium turns black during the bioassay study (Fig. 1A-D). Similar reports of melanised gills and epipodites have been reported in *Carcinus maenas* [11], *Cancer pagurus* [30], *P. vulgaris*, *P. duorarum*, *M. japonicus* [7, 26, 31], *E. sinensis* [10] *and Macrobrachium rosenbergii* [32] and *Litopenaeus vannamei* [14], exposed to heavy metals. The epipodites are elongated and flat, biramous structures attached to the coxopodites of the thoracic appendages and are involved in osmoregulatory mechanism involving both ionic (Na+, K+) and enzymatic (ATPase) activities [33]. The epipodites of *P. semisulcatus* are whitish yellow which turns black after exposure to cadmium. Soegianto [12] reported a similar result in the epipodites of *P. japonicus* after treating the animal with copper for four days at 1000 µg L^{-1}. The melanisation of gills and epipodites observed in present study might result from autolysis of cell gill cells and necrosis leading to the accumulation of black electron-dense material as a means of sequestration of cadmium [34].

Several reasons have been proposed for the blackening of the external body parts of crustaceans after exposing the animal to various environmental contaminants. Bryan [35] proposed that excretion of cadmium occur across the body surface and gills and this process involves the accumulation of metal on the exoskeleton to be discarded from the body during moulting [36]. Moreover, cadmium at high concentrations can cause adverse effect on the endocrine control of pigment migration in crustaceans [37]. Dispersion of the black pigment in *P. semisulcatus* could be due to the effects of cadmium on the neuroendocrine processes that control the melanophores. The neuroendocrine complex in the eye stalk is the source for a black pigment dispersing hormone (BPDH) and the synthesis of this hormone is affected by cadmium resulting in the black pigmentation on various body parts of *P. semisulcatus*.

Toxic substances can easily cause damage to gill tissues, thereby impairing the physiological functions of shrimps [38]. In the present study, *P. semisulcatus* exposed to cadmium at two sublethal levels of cadmium resulted in prominent structural changes of the gill lamellae including necrosis (N), haemocyte accumulation (H) and swelling (SL). Similar alterations in histoarchitecture of gills have been reported in *P. duorarum*, *M. japonicus* [32, 26], *M. rosenbergii* [32] *E. sinensis* [10], *Litopenaeus vannamei* [14]. Further investigation by ultrastructural evaluation revealed several alterations in the gills due to the loss of damaged cuticle, separation of epithelium from the cuticle, disrupted microvilli, shrunken nucleus, swollen mitochondria with disoriented cristae, fragmented endoplasmic reticulum, disintegrated ribosomes and presence of abundant vacuoles in the cytoplasm. These observations are in consistent with the results of previous studies in the gills after heavy metal treatment [8, 10 - 13, 32, 39].

The epithelial layer and microvilli of gills play a prominent role in ionic regulation and respiratory gas exchange. The structural integrity of this membrane is essential for the Na^+ ions to move across the gill epithelium towards the hemolymph by a two-step process involving the antiporter Na^+/H^+ and basolateral Na^+/K^+ pump [4, 5]. Exposure to cadmium resulted in severe disruption of the cell epithelium in *P. semisulcatus*. Heavy metals have the capability to bind with membrane proteins and phospholipids of epithelium and change its structure and function [40, 41]. In addition, the presence of this element stimulates lipid peroxidation process in the gill epithelial membrane [42] helping cadmium to penetrate more easily into the hemolymph space of decapods through the damaged epithelium [43]. Moreover, the disrupted microvilli reduce the available surface area for ion regulatory capability of shrimps.

Osmoregulation is a necessary and fundamental physiological adaptation performed by gills in aquatic animals to actively maintain the ionic concentration of hemolymph. Several enzymes participate in the complex process of osmoregulation and these proteins exhibit altered activity levels after heavy metal exposure [6]. Hansen *et al* [44] have observed reduced expression of glycolytic enzymes like hexokinase and pyruvate kinase in the gills of *Carcinus maenas* due to copper toxicity. Another protein, carbonic anhydrase, catalyses the reversible hydration of CO_2 and water to H^+ and $HCO3^-$ ions for cation and /or anion transport that utilize the ionic products of the hydration reaction as counter ions i.e. Na^+/H^+ and Cl^-/HCO^{3-} exchange [45, 46]. Heavy metals are able to bind with carbonic anhydrase and inhibit its activity in the gills of copper exposed *Callinectes sapidus* and *Carcinus maenas* [47]. In the present study, the various enzymes, believed to be critical for the osmoregulatory and respiratory function in the gills of *P. semisulcatus* might have failed to perform its role as a result of toxic action of cadmium.

Damage to cuticle and extensive vacuolation were observed in the gill cells of shrimp. The cuticle is the most likely site for cadmium accumulation as it is in direct contact with the external medium. As a counter act, the cuticle modifies the epithelium permeability when the external ionic composition changes [48], but this property failed in cadmium treated *P. semisulcatus* leading to profound damage in the external thin barrier of the cell. Additionally, the formation of large vacuoles reflects the severity of the damage caused by osmotic imbalance of the gills during exposure. The necrosis and extensive vacuolation at the apical part of the cell result in the separation of epithelium from the cuticle. Similar changes were observed in the gill tissues of *Palaemon serratus* [8], *Crangon crangon* [9], *Carcinus maenas* [11, 49] after heavy metal exposure. A possible reason for the proliferation of subcuticular vacuoles is that it provides longer diffusion distance for water movement thereby decreasing the rate of entry of water and metals into the cell [11].

Mitochondria, the power house of the cell have become swollen with disoriented and broken cristae. Similar results were observed in shrimps and crabs exposed to elevated levels of cadmium [8] and copper [9, 11-13, 49, 50]. The disruption of mitochondrial membranes reduces its ability to produce ATP by oxidative phophorylation [40], thereby increasing membrane permeability to water leading to the swollen appearance of mitochondria following cadmium exposure. Exposure to cadmium resulted in severe disruption of endoplasmic reticulum and ribosomes in the gills of *P. semisulcatus* Gamulin [51] documented that heavy metals alter the ribosomal distribution between the cytosol and endoplasmic reticulum. Ribosomes produce the Na^+/K^+

ATPase and carbonic anhydrase for osmoregulation, but under the toxic influence of the cadmium the regular manufacture of these enzymes are altered leading to the production of metallothioneins. Metallothioneins are low molecular weight, cysteine rich metal binding proteins that play a primary role in the detoxification of cadmium [52]. Shrunken and broken outer membrane of the nucleus found in the present study could be due to the response of DNA and its associated proteins in the cell to cadmium. Heavy metals create adverse effect on DNA repair and apoptosis through induction of single strand breakage, production of free radicals and inhibition of DNA repair enzymes by displacing metal ions from the active site of proteins involved in repair process [53].

The unidentified, oval accumulations of electron dense matter present in the cell might be lipofuschin granules [54]. In invertebrates, these lipofuchin granules are involved in metal sequestration by rendering them metabolically unavailable [49, 55] and later excreted from the cell exocytotically [56]. Thus the formation of electron dense granules observed in the gill cells of *P. semisulcatus* is a possible method for sequestering cadmium from the animal.

5. Conclusion

In conclusion, the structural alterations observed in *Penaeus semisulcatus* exposed to cadmium (250 and 500 µg L^{-1}) for 14 days leads to malfunction of osmoregulatory and respiratory physiological mechanisms of the gills. The alterations observed in the present study are discussed by correlating with the probable physiological changes in decapods after heavy metal treatment. Since exposure of shrimps to even low levels of cadmium can lead to such harmful changes, it is very important that contamination of the aquatic environment by cadmium should be prevented. The changes observed in the study can also be taken as 'biomarkers' for screening cadmium pollution in aquatic ecosystem.

6. Acknowledgements

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7. References

1. Sorensen EM. Metal poisoning in Fish. Chapter VI. Cadmium. CRC Press, Boca Raton, FL, 1991, 175-234.
2. Rashed MN. Cadmium and lead levels in fish (*Tilapia nilotica*) tissues as biological indicator for lakewater pollution. Environmental Monitoring and Assessment. 2001; 68:75-89.
3. Nuñez-Nogueira G, Rainbow PS. Cadmium uptake and accumulation by the decapod crustacean *Penaeus indicus*. Marine Environmental Research. 2005; 60:339-354.
4. Pequex A. Osmotic regulation in crustaceans. Journal Crustacean Biology. 1995: 15:1-60.
5. Lucu C, Towle DW. $Na^+ + K^+$ - ATPase in gills of aquatic crustacea. Comparative Biochemistry Physiology. 2003; 135A:195-214.
6. Rainbow PS. Physiology, physiochemistry and metal uptake. A crustacean perspective. Marine Pollution Bulletin. 1995; 31:55-59.
7. Nimmo DWR, Lightner DV, Bohner LH. Effects of Cadmium on the Shrimps, *Penaeus duorarum*, *Palaemonetes pugio* and *Palaemonetes vulgaris*. In: Vernberg, F.J., Calabrese, A., Frederick, P., Thurberg, W., Vernberg, B., (Eds.), Physiological Responses of Marine Biota to Pollutants. Academic Press Inc., New York. 1977, 131-183.
8. Papathanassiou E, King PE. Ultrastructural studies on the gills of *Palaemon serratus* (Penant) in relation to cadmium accumulation. Aquatic Toxicology. 1983; 3:273-284.
9. Papathanassiou E. Effect of cadmium ions on the ultrastructure of the gill cells of the brown shrimp *Crangon crangon* Decapoda Caridea. Crustaceana. 1985; 48:6-17.
10. Yang ZB, Zhao YL, Li N, Yang J. Effect of waterborne copper on the microstructures of gill and hepatopancreas in *Eriocheir sinensis* and its induction of metallothionein synthesis. Archives in Environmental Contamination and Toxicology. 2007; 52:222-228.
11. Lawson SL, Jones MB, Moate RM. Effect of copper on the ultrastructure of the gill epithelium of *Carciuns maenas* (Decapoda: Brachyura). Marine Pollution Bulletin. 1995; 31:63-72.
12. Soegianto A, Charmantier MD, Trilles JP, Charmantier G. Impact of copper on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Decapoda). Journal of Crustacean Biology. 1999a; 19:209-223.
13. Soegianto A, Charmantier MD, Trilles JP, Charmantier G. Impact of cadmium on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). Aquatic Living Resources. 1999b; 12:57-70.
14. Wu JP, Chen HC, Huang DJ. Histopathological alterations in gills of White Shrimp, *Litopenaeus vannamei* (Boone) after acute exposure to cadmium and zinc. Bulletin Environmental Contamination and Toxicology. 2009; 82:90-95.
15. Yassien MH. Biology and fishery of the green tiger prawn *Penaeus semisulcatus* de haan (1850) in Bardawil Lagoon, Northern Sinai, Egypt. Egyptian Journal of Aquatic Research. 2004; 30:271-280.
16. Radhakrishnan EV, Maheswarudu G, Arputharaj MR, Ramakrishnan A. Repetitive maturation and spawning of the green tiger prawn *Penaeus semisulcatus* by environmental regulation in closed seawater recirculation systems. Journal of the Marine Biological Association India. 2000; 42:91-100.
17. Al-Mohanna SY, Nott JA. Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda) during the moult cycle. Marine Biology. 1989; 101:535-544.
18. Pourang N, Dennis JH, Ghourchian H. Distribution of heavy metals in *Penaeus semisulcatus* from Persian Gulf and possible role of metallothionein in their redistribution during storage. Environmental Monitoring and Assessment. 2005; 100:71-88.
19. Drach P, Tchernigovtzeff C. Sur la methode de determination des stades d'intermue et son application générale aux Crustacés (in French with English abstract). Vie Milieu. 1967; 18:595-610.
20. APHA (American Public Health Association), Standard Methods for the Examination of Waters and Wastewaters. (17 Edn), American Public Health Association, Washington, DC, 1989,
21. Finney DJ. Probit Analysis. Cambridge, England, Cambridge University Press, 1952, 1-318.
22. Reish DL, Oshida PS. Manual of Methods in Aquatic Environment Research. Part 10. Short-term Static

Bioassays. FAO Fisheries Technical Paper. 1987; 247:1-62.
23. Litchfield JT, Wilcoxon F. A simplified method of evaluating dose effect experiments. Journal of Pharmacology and Experimental Therapeutics. 1949; 96:99-113.
24. Bell TA, Lightner DV. A handbook of normal penaeid shrimp histology. World Aquaculture Society, Baton Rouge, LA, 1988, 1-144.
25. Bahner LH, Nimmo DWR. Methods to assess effects of combinations of toxicants, salinity, and temperature on estuarine animals. In: Hemphill, D.D. (Ed.), Trace substances in environmental health–IX. University of Missouri at Columbia. 1975, 169-177.
26. Bambang G, Charmantier P, Thuet J, Trilles P. Effect of cadmium on survival and osmoregulation of various developmental stages of shrimp *Penaeus japonicus*. Crustacea: Decapoda. Marine Biology. 1995; 123:443-450.
27. Ahsanulla M. Acute toxicity of cadmium and zinc to seven invertebrate species from western port, Victoria. Australian Journal of Marine and Freshwater Research. 1976; 27:187-196.
28. Tangkrock-Olan N, Cheevaporn V. Effects of cadmium on oxygen consumption and tissue accumulation in tiger shrimp *Penaeus monodon* Fabricius. Asian Journal of Biology Education. 2004; 2:65-74.
29. Frías-Espericueta MG, Abad-Rosales S, Nevárez-Velázquez AC, Osuna-López I, Páez-Osuna F, Lozano-Olvera R, Voltolina D. Histological effects of a combination of heavy metals on Pacific white shrimp *Litopenaeus vannamei* juveniles. Aquatic Toxicology. 2008; 89:152-157.
30. Spicer JI, Weber RE. Respiratory impairment in crustaceans and molluscs due to exposure to heavy metals. Comparative Biochemistry and Physiology. 1991; 100C:339-342.
31. Bambang G, Thuet P, Charmantier-Daures M, Trilles-Paul T, Charmantier G. Effect of copper on survival and osmoregulation of various developmental stages of shrimp *Penaeus japonicus* Bate (Crustacea: Decapoda). Aquatic Toxicology. 1994; 33:125-129.
32. Li N, ZhaoY,Yang J. Impact of waterborne copper on the structure of gills and hepatopancreas and its impact on the content of metallothioneins in juvenile giant freshwater prawn *Macrobrachium rosenbergii* (Crustacea: Decapoda). Archives in Environmental Contamination and Toxicology. 2007; 52:73-79.
33. Bouaricha N, Charmantier-Daures M, Thuet P, Trilles JP, Charmantier G. Ontogeny of osmoregulatory structures in the Shrimp *Penaeus japonicus* (Crustacea, Decapoda). Biological Bulletin. 1994; 186:29-40.
34. Nunez-Nogueira G, Mouneyrac C, Amiard JC, Rainbow PS. Subcellular distribution of zinc and cadmium in the hepatopancreas and gills of the decapod crustacean *Penaeus indicus*. Marine Biology. 2006; 150:197-211.
35. Bryan GW. Concentrations of zinc and copper in the tissues of decapod crustaceans. Journal of the Marine Biological Association UK. 1968; 48:303-321.
36. Keteles KA, Fleeger JW. The contribution of ecdysis to the fate of copper, zinc and cadmium in Grass shrimp, *Palaemonetes pugio* Holthius. Marine Pollution Bulletin. 2001; 42:1397-1402.
37. Fingerman M, Jackson NC, Nagabhushanam R. Hormonally-regulated functions in crustaceans as biomarkers of environmental pollution. Comparative Biochemistry and Physiology. 1998; 120:343-350.
38. Anderson JT, Baatrup E. Ultrastructure localisation of mercury accumulations in the gills, hepatopancreas, midgut and antennal glands of the brown shrimp, *Crangon crangon*. Aquatic Toxicology. 1988; 13:309-324.
39. Webb M. Interactions of cadmium with cellular components. In: Webb M., (Ed.), The Chemistry, Biochemistry and Biology of Cadmium. Elsevier/North Holland Press, Amsterdam, 1979, 285-340.
40. Evtushenko ZS, Belcheva NN, Lukyanova ON. Cadmium accumulation in organs of the scallop *Mizuhopecten yessonensis*. II. Subcellular distribution of metals and metal-binding proteins. Comparative Biochemistry and Physiology. 1986; 83C:377-383.
41. Viarengo A, Pertica M, Canesi L, Biasi F, Cecchini G, Orunesu M. Effects of heavy metals on lipid peroxidation in mussel tissues. Marine Environmental Research. 1988; 24:354-358.
42. Silvestre F, Trausch G, Pequeux A, Davos P. Uptake of cadmium through isolated perfused gills of the Chinese mitten crab, *Eriocheir sinensis*. Comparative Biochemistry and Physiology. 2004; 137A:189-196.
43. Hansen JI, Mustafa T, Depledge M. Mechanisms of copper toxicity in the shore crab, *Carcinus maena*s, I. Effects on Na,K-ATPase activity, haemolymph electrolyte concentrations and tissue water contents. Marine Biology. 1992; 114:253-257.
44. Henry RP. Multiple functions of gill carbonic anhydrase. Journal of Experimental Zoology. 1998; 248:19-24.
45. Bottcher K, Siebers D. Biochemistry, localization and physiology of carbonic anhydrase in the gills of euryhaline crabs. Journal of Experimental Zoology. 1993; 265:397-409.
46. Skaggs HS, Henry RP. Inhibition of carbonic anhydrase in the gills of two euryhaline crabs, *Callinectes sapidus* and *Carcinus maena*s by heavy metals. Comparative Biochemistry and Physiology. 2002; 133C:605-612.
47. Lignon JM. Ionic permeabilities of the isolated gill cuticle of the shore crab *carcinus maenas*. Journal of Experimental Biology. 1987; 131:159-174.
48. Hebel DK, Jones MB, Moate RM, Depledge MH. Differing sensitivities of respiratory and osmoregulatory gill tissue of *Carcinus maenas* (Crustacea: Decapoda) to water-borne copper. Marine Biology. 1999; 133:675-681.
49. Doughtie DG, Rao KR. Histopathological and ultrastructural changes in the antennal gland, midgut, hepatopancreas, and gill of grass shrimp following exposure to hexavalent chromium. Journal of Invertebrate Pathology. 1984; 43:89-108.
50. Gamulin S, Car N, Naranosik P. Effect of cadmium on polyribosome structure and function in mouse liver. Experientia. 1977; 33:1144-1145.
51. Wu J, Chen H. Metallothionein induction and heavy metal accumulation in white shrimp *Litopenaeus vannamei* exposed to cadmium and zinc. Comparative Biochemistry and Physiology. 2005; 140C:383-394.
52. Pruski AM, Dixon DR. Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of *Mytilus edulis* L. Aquatic Toxicology. 2002; 57:127-137.
53. Bozzola JJ, Russell LD. Electron microscopy: Principles and techniques for biologists. Jones and Barlett

Publishers, Boston, 1999, 1-670.
54. Viarengo A, Nott JA. Mechanism of heavy metal homeostasis in marine invertebrates. Comparative Biochemistry and Physiology. 1993; 104C:355-372.
55. George SG. Subcellular accumulation and detoxification of metals in aquatic animals. In: Vernberg WB, Calabrese A, Thurberg FP, Vernberg FJ. (Eds.), Physiological mechanisms of marine pollutant toxicity, New York, Academic Press, 1982, 3-52.