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S Deepa Rani
Research Scholar,
PG & Research Department of Advanced Zoology & Biotechnology, Sir Theagaraya College, Chennai, Tamil Nadu, India

R Kavitha
Research Scholar,
PG & Research Department of Advanced Zoology & Biotechnology, Sir Theagaraya College, Chennai, Tamil Nadu, India

M Padmaja
Assistant Professor,
PG & Research Department of Advanced Zoology & Biotechnology, Sir Theagaraya College, Chennai, Tamil Nadu, India

Correspondence
S Deepa Rani
Research Scholar,
PG & Research Department of Advanced Zoology & Biotechnology, Sir Theagaraya College, Chennai, Tamil Nadu, India

Effect of Cadmium Nanoparticle (CdNP) in the gut of mud crab, *Scylla olivacea* (Herbst, 1796)

S Deepa Rani, R Kavitha and M Padmaja

Abstract
Acute lethal toxicity bioassay is useful for providing a measurement of the toxicity of a particular substance, and for determining the chronic toxicity so as to assess water quality criteria. The present work is aims to determine the toxicity of Cadmium Nanoparticle (20ppm) in the mud crab, *Scylla olivacea* with the help of histological and biochemical analysis. The overall histological results revealed that changes in the cell structure, rupture of basal lamina and complete disorganization of the gut region within 10days of exposure. The biochemical constituents total protein, carbohydrate and lipid levels drastically decreased which denotes the energy demand in organism. The antioxidant enzymes such as SOD, CAT and GPx levels were significantly increased in response to oxidative stress in the crab exposed to CdNP and the tissue damaging enzymes such as LDH, SDH, MDH were also significantly increased with increase in the days of exposure which revealed the damage of the tissue exposed to CdNP.

Keywords: cadmium, toxicity, mud crabs, Dehydrogenase, catalase

1. Introduction
Ecotoxicology is the evaluation of risk for an ecosystem exposed to environmental stress, including contamination. Estuaries and coastal zones receive pollutant inputs from both specific and nonspecific sources, especially such ecosystems as seaports, cities, or other industrialized coastal areas that receive chronic inputs of metals. Since many species of crustaceans inhabit estuaries, numerous studies have aimed at examining the bioaccumulation and effects of various toxicants in these animals (Weis *et al.*, 1992; Weis and Weis, 1994) [37, 38]. Acute lethal toxicity bioassay is useful for providing a measurement of the toxicity of a particular substance, and for determining the chronic toxicity so as to assess water quality criteria.

A variety of anthropogenic chemicals, referred to as organic xenobiotics which include aromatic hydrocarbons, organometallics, organohalogens, and various pesticides, all of which have the potential to affect crab growth, reproduction, and development. Although trace metals which are in bulk such as cobalt, copper, and selenium are natural components of aquatic systems and essential for a number of physiological functions, but in nanosclae they become toxic to the aquatic animals and affects all the physiological functions in the aquatic animals (Moore, 2006) [22].

Production of nanoparticles had developed innovative methods of usage of nanoparticle in various fields of agriculture, biomedicine, electronics, cosmetics and pharmaceuticals. As their usage increased in science and technology, it is important to understand the toxic effects of nanoparticle because of its impact on ecosystem as a contaminant of aquatic system (Valeria and Ilaria, 2012) [35].

The heavy metals tend to have a longer residence time in the gut with low permeability coefficient of divalent cations across the lipid bilayer membranes. Profuse binding on to the negative charged sites on the mucosal side of the gut increases the concentrations of heavy metals in the lumen. This interferes with the normal process of nutrient absorption, which explains the need for catabolism of stored energy (Farman Farmaian and Socci, 1984) [7]. Possible mechanisms of NMs include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation for reactive oxygen species, and release of toxic constitutes (Klaine *et al.*, 2008) [15]. Hence the

present work is aimed to determine the toxicity of cadmium nanoparticle (CdNP) on male and female species of mud crab, *Scylla olivacea* with the help of histological and biochemical analysis.

2. Materials and Methods

2.1. Animal Collection

Both male and female species of *Scylla olivacea* are collected from Pulicate Lake, Tamil Nadu, India. They are maintained separately in tanks with aerator which is (capacity of 1000 litres) filled with filtered sea water. The identification of the species is done by a Scientist from Central Institute of Brackish water Aquaculture (CIBA), Santhome, Chennai, India. The crab is acclimatized for ten days before the experiment. Naturally aged estuarine water is used after being shifted through a 0.45 mm pore filter and activated charcoal to remove dissolved organic matter and trace metals. Water temperature is maintained within a range (27.5± 0.5 °C) as recommended for optimal growth of mud crabs (Chen and Jeng, 1980) [5].

2.2. Acute toxicity test

The acute semistatic toxicity test was carried out according to the standard methodology of the *Food and Agriculture Organization* (FAO) (Ward and Parrish, 1982; Reish and Oshida, 1987) [36, 26] and the American Public Health Association (APHA, 1992) [1]. Young crabs are acclimatized for 14 days. Semistatic toxicological bioassays are carried out for 120 hrs. A series of six different concentrations such as 20, 40, 60, 80, 100 and 120ppm of Cadmium nanoparticle (CdNP of 100nm in size) suspension (Sigma and Co) was injected intraperitoneally per kg of crab weight. Three replicates of 10 animals are used for the toxicological studies. Mortality is recorded at every 24 h and the experimental conditions (temperature, salinity, and pH) of the toxicity test are similar to those found in the environment. A probit analysis is used to determine the lethal concentration and 95% confidence limits of SNP that kills 50% of the exposed crabs (LD_{50}).

2.3. Cadmium nanoparticle treatment

After standardization of LD_{50} value, a single concentration of 20ppm/kg of body weight is used for further experiments. Mud crab, *S. olivacea i*s acclimatized in tanks and the temperature is maintained at 27 °C. After acclimatization, healthy adult male and female crabs with a homogeneous size (carapace width 14-16cm, weight 200-300g) are selected for control and Cadmium nanoparticle (20 ppm/kg of crab weight) treatment. The acute exposure lasted for 8 days. During the experiment, crabs are fed and dead animals were removed in time.

2.4. Histological analysis

Cadmium nanoparticle treated and control crabs of both male and female *S. olivacea* are taken from the tank, anaesthetized in ice water for five minutes and sacrificed at every 2 day interval up to 8 days. Gut tissues are removed and then fixed by direct immersion in a 0.1 M, pH 7.4 phosphate buffer with 4% formaldehyde for 24 h at room temperature. Samples are dehydrated with ethanol and toluene series and embedded in paraffin. Serial sections (4 mm) are mounted on gelatin-coated glass slides and stained with hematoxylin and eosin. Slides are examined with a light microscope (Olympus BX51) and the results are documented. Four sections were analyzed from each tissue.

2.5. Biochemical Analysis

2.5.1. Total Protein, Carbohydrate and Lipid analysis

The buffer soluble protein content of Gut tissue is determined by the dye binding method of Bradford (1976) [4] with Bovine Serum Albumin fraction V (Sigma chemical Co., USA) as a standard. The gravimetric, chloroform-methanol extraction method of Folch *et al.* (1957) [8] was followed for lipid estimation in the Gut tissue. Total carbohydrate was estimated by Roe (1955) [27]. 10 % homogenate of tissues was prepared using 5 % TCA and results are expressed as mg/g tissue wet weight.

2.5.2. Antioxidant enzymes analysis

The antioxidant enzymes such as catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) is estimated in the gut to determine the free radical formation in the tissues. Antioxidant activity of gut tissues of *S. olivacea* after exposed to CdNP exposure was assayed colorimetrically. The Assays of Catalase, Superoxide Dismutase (SOD) is performed by following the procedures of Beers and Sizer (1952) [1], Beyer and Fridovich (1987) [3] and Glutathione Peroxidase by Lawrence and Burk (1976) [18].

2.5.3. Tissue damaging enzymes analysis:

After the CdNP exposure the tissue damaging enzymes in gut tissues are colorimetrically assayed. The procedure of Schirawski and Unden **(**1998) [29] is followed for Succinate Dehydrogenase (SDH) and Gloster and Harris (1962) [11] procedure for Lactate Dehydrogenase (LDH) is followed for the estimation of the enzymes.

3. Results

3.1. Histology

3.1.1. Histological changes in the gut of male *S. olivacea*

Results of CdNP induced structural changes of gut of male *S. olivacea* were presented in Figure 1-4. Control crabs showed an epithelium which is cubical or cylindrical in relation to the Content volume (Fig.1A). One cellular type is dominant and extends from the basal lamina to the lumen. These cells have central nuclei, small subapical vacuoles and a well-developed brush border; some of them have a basophilic cytoplasm and are secreting actively. On day 2, minor deformation in striated muscles and epithelial cells (Fig.1B). On day 4, constriction of setae and disorganization of basal lamina (Fig.2B), On day 6, complete damage of gut tissue followed by distortion of setae (Fig.3B) and On day 8, complete disorganization of gut tissues were evident, prominent enlargement of lumen and distortion of mandible (Fig.4B) were observed.

3.1.2. Histological changes in the gut of female *S. olivacea*

Results of CdNP induced structural changes of gut of female *S. olivacea* were presented in Figure 1-4. Control crabs showed a normal epithelium and densely stained dorsal lumen (Fig.1C). On day 2 of CdNP exposure, prominent deformations in both dorsal chamber and epithelial cells were evident (Fig.1D). On day 4, not much tissue disorganization was evident but abnormality was seen compared to control and day 2 (Fig.2D). On day 6, mesh

like deformation of gut tissues and disorganization of basal lamina (Fig.3D), On day 8, complete damage of gut tissue which was compared to their control (Fig.4D) were noted.

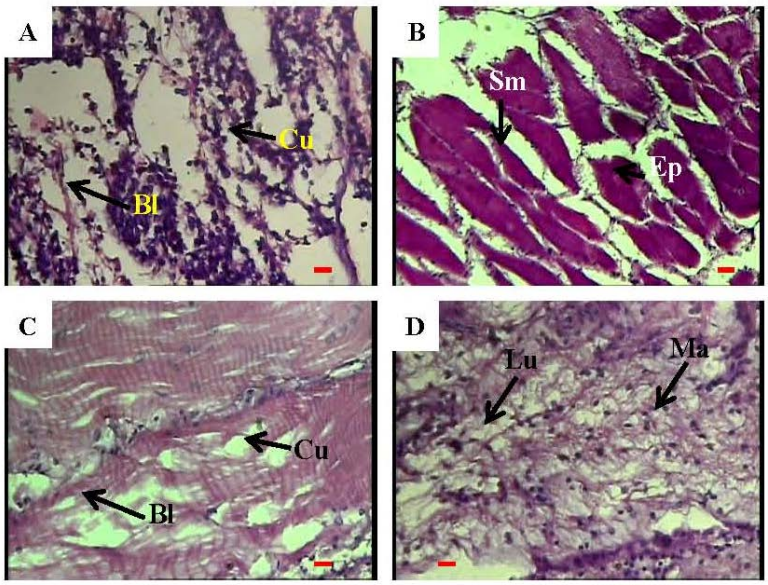

Fig. 1 Effects of CdNP (20ppm) on the anatomical structure of gut in *Scylla olivacea* by light microscope on day 2. Scale bar, 10µm. Ep - Epithelium; Sm - Striated muscles; Ma - Mandible; Fd- Fold; Ex - External elongated setae; Bl- Basal lamina; Lu - Lumen.

A) Control (Male) B) CdNP treated (Male)
C) Control (Female) D) CdNP treated (Female)

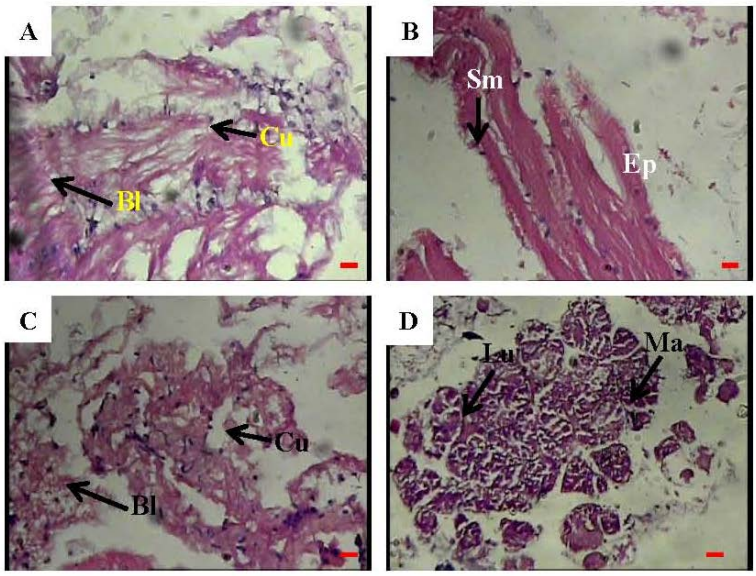

Fig. 2. Effects of CdNP (20ppm) on the anatomy of gut in *Scylla olivacea* by light microscope on day 4. Scale bar, 10µm. Ep - Epithelium; Sm - Striated muscles; Ma - Mandible; Fd- Fold; Ex - External elongated setae; Bl- Basal lamina; Lu - Lumen.

A) Control (Male) B) CdNP treated (Male)
C) Control (Female) D) CdNP treated (Female)

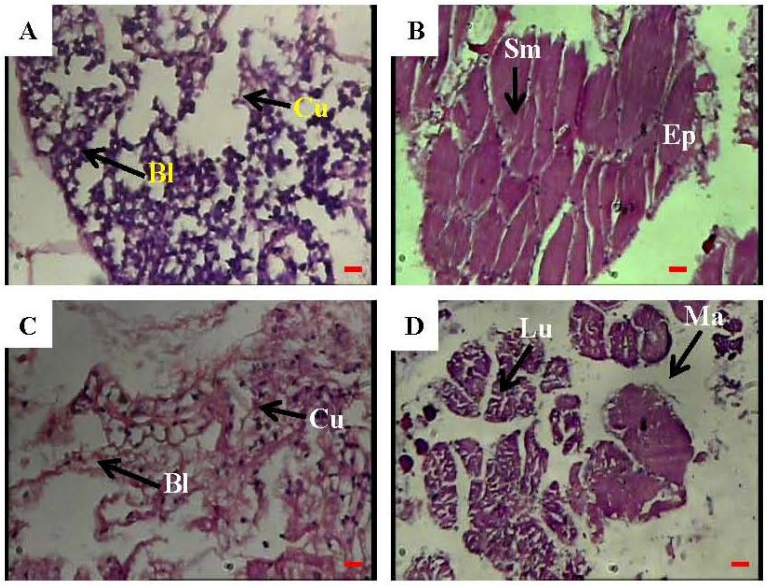

Fig. 3. Effects of CdNP (20ppm) on the anatomy of gut in *Scylla olivacea* by light microscope on day 6. Scale bar, 10µm. Ep - Epithelium; Sm - Striated muscles; Ma - Mandible; Fd- Fold; Ex - External elongated setae; Bl- Basal lamina; Lu - Lumen.

A) Control (Male) B) CdNP treated (Male)
C) Control (Female) D) CdNP treated (Female)

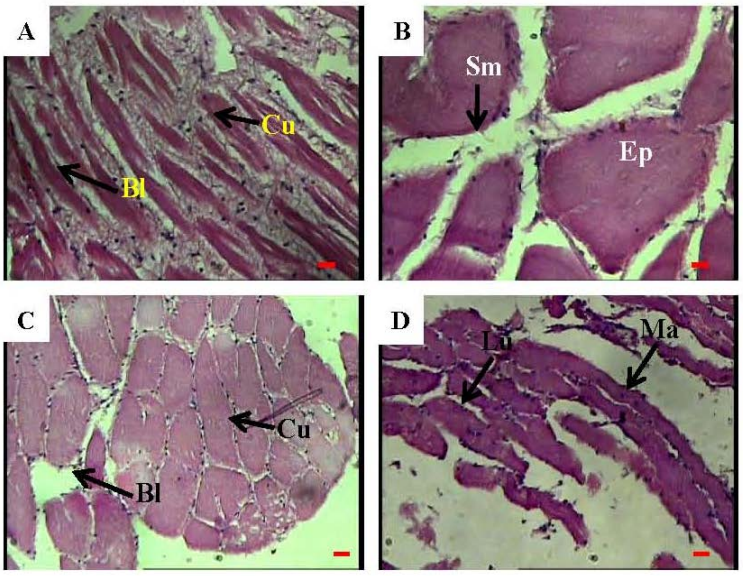

Fig. 4. Effects of CdNP (20 ppm) on the anatomy of gut in *Scylla olivacea* by light microscope on day 8. Scale bar, 10µm. Ep - Epithelium; Sm - Striated muscles; Ma - Mandible; Fd- Fold; Ex - External elongated setae; Bl- Basal lamina; Lu - Lumen.

A) Control (Male) B) CdNP treated (Male)
C) Control (Female) D) CdNP treated (Female)

3.2. Total Protein, Carbohydrate and Lipid content

A time course study in the gut of *S. olivacea* showed an increase in total protein content upon exposure to CdNP (20ppm). Total Protein content started increasing in CdNP treated male and female crabs on day 2 compared to its control, reaching a peak on day 10 compared to its control (Fig.5).

CdNP (20ppm) treatment resulted in increased total carbohydrate content than in control crabs. In males, total carbohydrate content started increasing on day 2 and decreased on day 10. Similarly, in the case of female also carbohydrate content started increasing on day 2 but decreased further on day 4 onwards compared to their respective controls (Fig.6).

Total lipid content started increasing in CdNP treated male crabs on day 2, reached peak on day 8, after CdNP (20ppm) exposure. Similarly, in the case of female also total lipid content started increasing on day 2 and continued to increase up to day 10 compared to their respective controls (Fig.7).

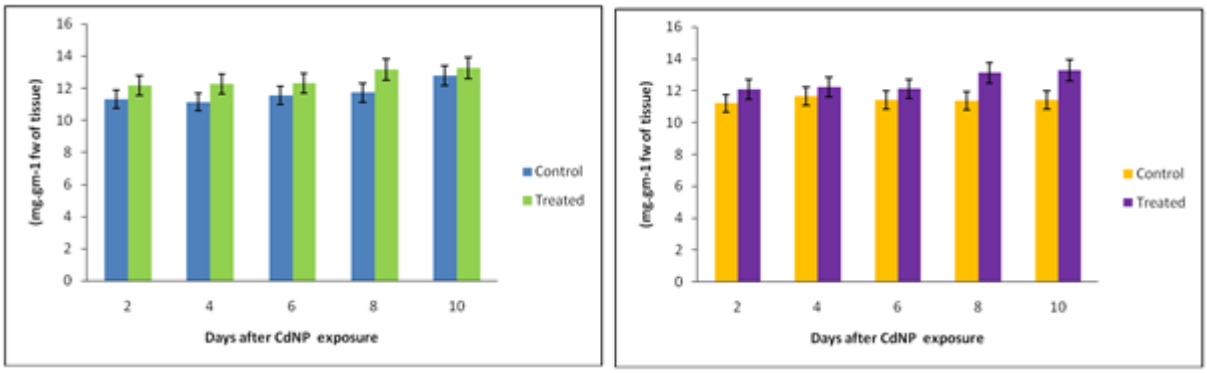

Fig 5: Total protein content in gut of *S. olivacea* after exposure of CdNP

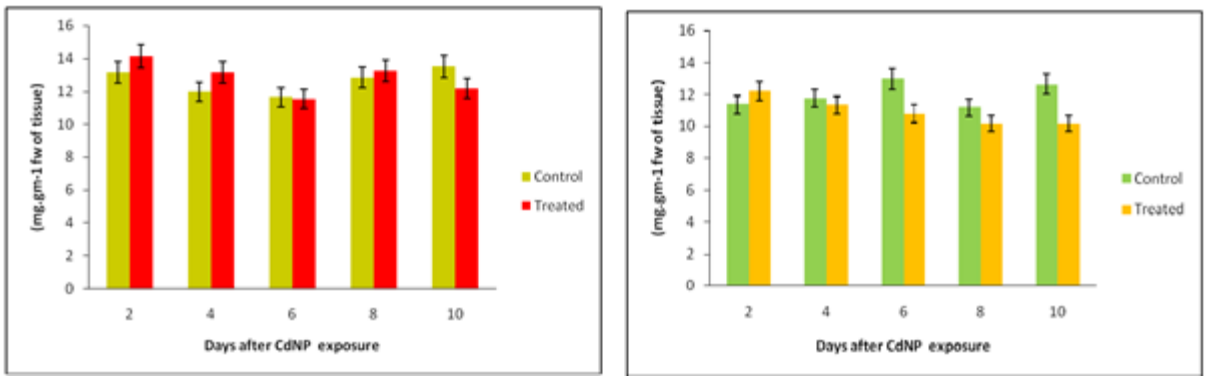

Fig 6: Total Carbohydrate content in gut of *S. olivacea* after exposure of CdNP

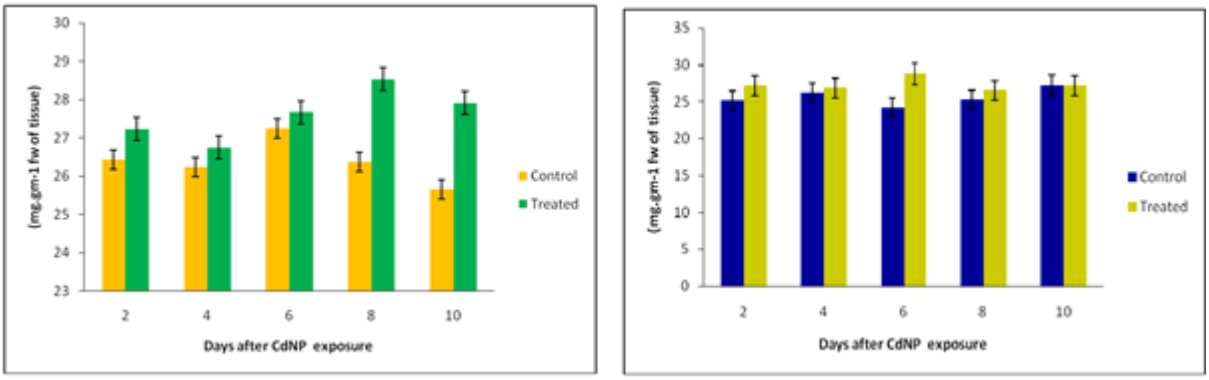

Fig 7: Total Lipid content in gut of *S. olivacea* after exposure of CdNP

3.3. Antioxidant enzyme activity

The gut of both male and female crabs of *S. olivacea* exposed to Cadmium nanoparticle (20ppm) resulted in increased Catalase (CAT) activity than in control crabs. Catalase activity started increasing in exposed crabs right on day 2 and reached peak compared to its control on day 10 of exposure (Fig.9).

Similarly, Cadmium nanoparticle exposure resulted in increased Glutathione Peroxidase (GPx), Superoxide Dismutase (SOD) activity in the gut of *S. olivacea* than in control crabs in both male and female crabs (Fig.8). GPx activity started increasing in exposed crabs right from 2nd day, reached peak on 10th day compared to its control (Fig. 10).

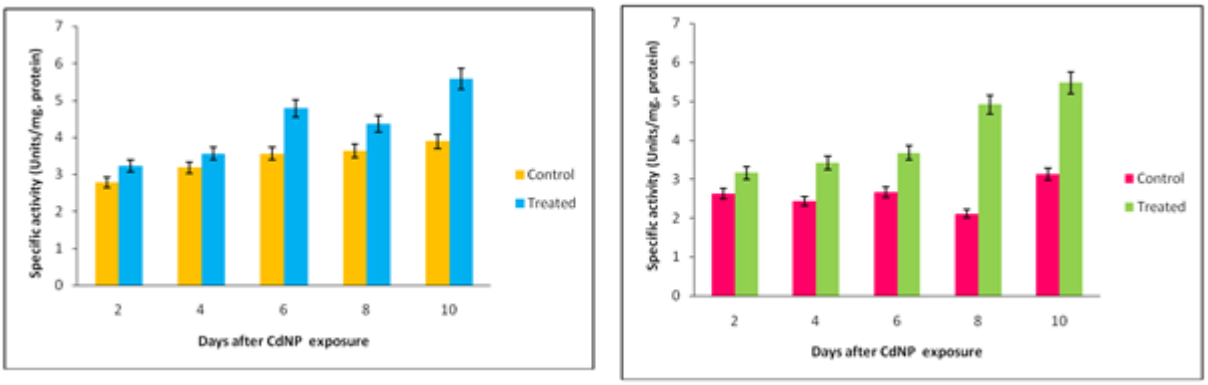

Fig 8: Superoxide dismutase (SOD) activity in gut of *S. olivacea* after exposure of CdNP

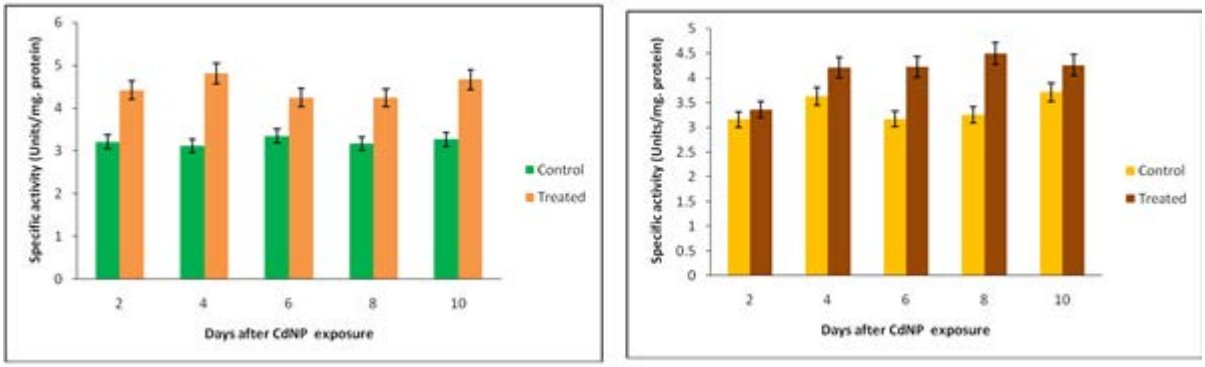

Fig 9: Catalase activity (CAT) in gut of *S. olivacea* after exposure of CdNP

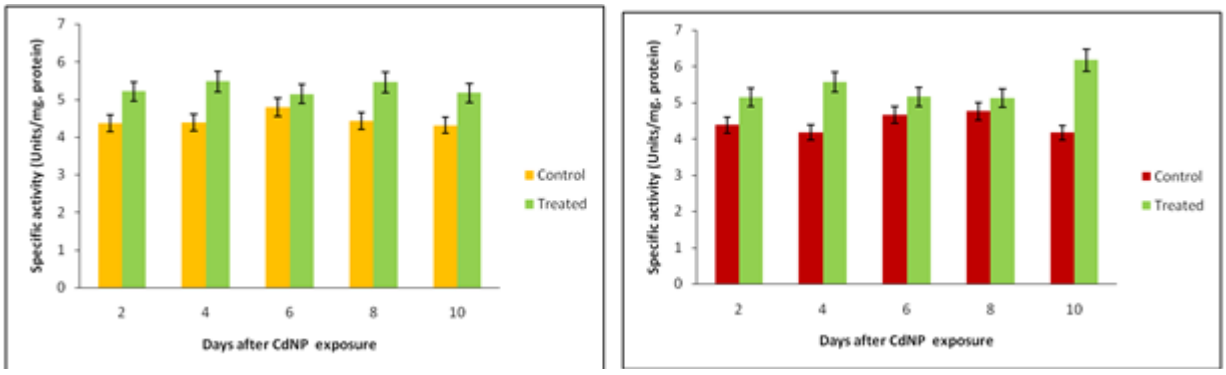

Fig 10: Glutathione peroxidase (GPx) activity in gut of *S. olivacea* after exposure of CdNP

3.4. Tissue damaging enzyme activity

The gut of *S. olivacea* (male and female) exposed to Cadmium nanoparticle (20ppm) showed increase in LDH activity than in control crabs. The LDH activity started increasing right on day 2 compared to control and reached maximum compared to control on day 10 of exposure (Fig.11). Succinate Dehydrogenase (SDH), Malate Dehydrogenase (MDH) activity in the gut of *S. olivacea* exposed to Cadmium nanoparticles (20ppm) increased than in control crabs. The enzyme activities increased right from day 2 compared to control and continue to increase up to 3fold on 10th day compared to their respective controls (Fig.12 & 13).

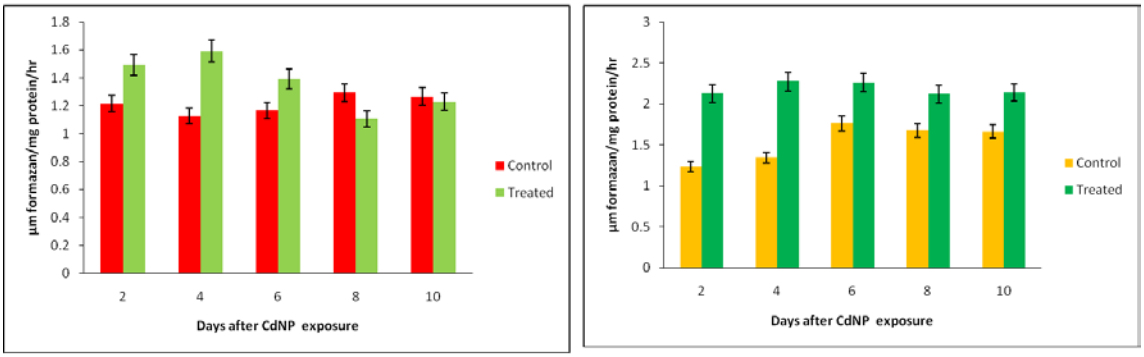

Fig 11: Lactate dehydrogenase (LDH) activity in gut of *S. olivacea* after exposure of CdNP

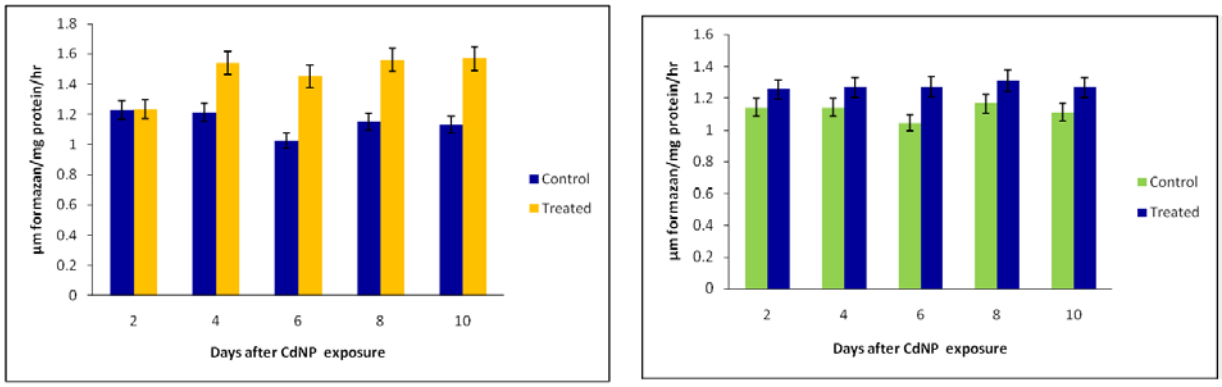

Fig 12: Malate dehydrogenase (MDH) activity in gut of *S. olivacea* after exposure of CdNP

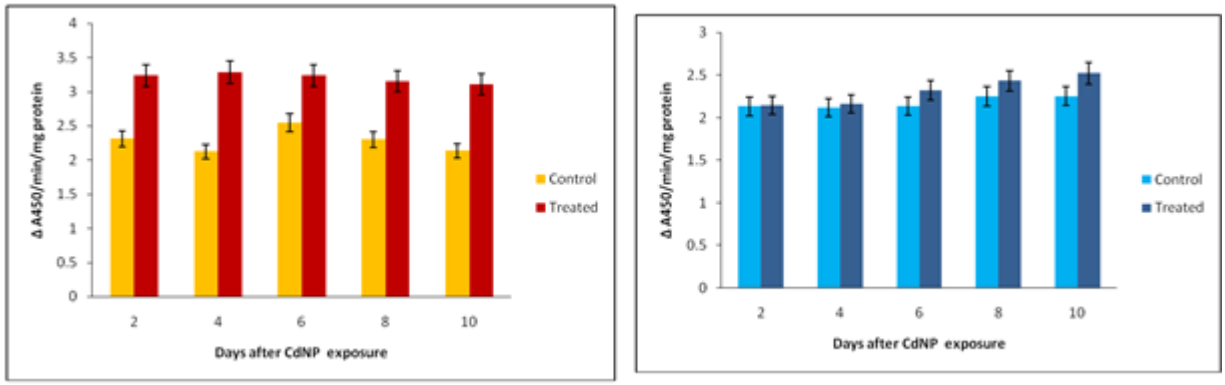

Fig 13: Succinate dehydrogenase (SDH) activity in gut of *S. olivacea* after exposure of CdNP

4. Discussion

Histopathological technique is an important area in the field of research which would help in the assessment of toxicity of heavy metals and their nanoparticles in the aquatic ecosystem. Kale (2002) [13] observed vacuolization in the hepato pancreas of the crab, *Barytelphusa cunicularis* due to cadmium toxicity. The results of Sarojini *et al.* (1990) [28] showed decreased absorptive cells, damaged connective tissue and destruction of hepatopancreatic tissue in the hepatopancreas of freshwater crab, *Barytelphusa guerini* exposed to cadmium chloride. It was confirmed by Krishnaja, *et al.* (1987) [17] in hepatopancreas of the crab, *Scylla serrata* exposed to mercury and cadmium. On exposure to cadmium and zinc, *Litopenaeus vannamei* showed necrosis of tubule of hepatopancreas and rupture of basement membrane (Wu, *et al.*, 2008) [39]. The present results also showed that there was a damage in the basal lamina and complete disorganization in the gut of the *S. olivacea* after 10days of exposure to CdNP.

When an organism exposed to NPs, it can enter into the gut cells by different processes such as diffusion through cell membranes (Lin and Xing, 2007) [19], through endocytosis (Kim *et al.*, 2006) [14] and adhesion (Geiser *et al.*, 2005) [9]. In the works of Sekar *et al.* (2009) [31] total protein, carbohydrate and lipid contents was decreased in *Spiralothelphusa hydrodroma*, when exposed to sublethal (69.66 ppm) concentration of textile dye industry effluent after 30 days of exposure. The results suggested that there is an increased proteolysis and the possible utilization of the products and their degradation of the metabolic process. Sreenivasan *et al.* (2009) [32] studied decreased protein, carbohydrate and lipid content in the Freshwater Field Crab, *Spiralothelphusa hydrodroma* (herbst) on exposure to Cypermethrin from agricultural runoff. In accordance previous findings the present results also suggested that tissues in the gut of *S. olivacea* on exposure to CdNP show decline in the level of biochemical constituent in order to cope up with energy demand the crab showed decrease in the biochemical process such as proteolysis, glycolysis and lipolysis.

On exposure to CdNP toxicity the organs exposed to toxicant show declined enzyme activity against the physiological and biochemical reactions. In the organs affected by toxicant, the enzyme activity may be increased or inhibited to balance energy demand of the organ when it is in denatured or distorted conditions (Valarmathi & Azariah, 2003) [33]. Oxidative stress has been considered as one of the basic events involved in cell and tissue damage. Radicals can cause damage to cardinal cellular components such as lipids, proteins and nucleic acids leading to subsequent cell death by modes of necrosis or apoptosis (Gilgun-Sherki *et al.*, 2002) [10]. Antioxidant enzymes like SOD, CAT and GPx constitute the major defensive system against ROS and the decrease in their activities contribute to the oxidative insult on the tissue. SOD and CAT are the two primary enzymes for radical scavenging, which are involved in protective mechanisms within tissue injury following oxidative process and phagocytosis and their activities are related to the status of the organisms affected by different factors including dietary nutrition, environmental factors etc. Mohamed *et al.* (2014) [21] studied increased SOD, CAT and GPx activities in the tissues of *Artemia salina* on exposure to cadmium toxicity. It was confirmed by the works of Pan and zhang, (2006) [24] in the gills and hepatopancreas of the crab *Charybdis japonica* exposed to sublethal concentration of Cadmium. Kojo (2004) [16] and

Schrauzer (2006) [30] suggested that the enzymatic antioxidant SOD, CAT and GPx are considered as the defence mechanisms of the organisms against oxidative stress and plays an important role in reducing toxicity of heavy metals. In the present investigations significant increment in the enzyme activity of antioxidant enzymes during the treatment of crabs with CdNP were observed which suggested that on exposure to heavy metals either in bulk or nanoparticle form would resulted in oxidative stress in the organism which in turn increases the enzyme activities to balance the energy requirement of the organism on exposure to toxicant.

Dehydrogenase is an important glycolytic enzyme associated with metabolic state which enables energy production in hypoxic (low oxygen) conditions. It is found that under exposure to various concentrations of cadmium telluride (Morgan *et al.*, 1995) [23], cadmium (Hassoun and Stohs, 1996) [12] or oxygen stress (Wu and Lam, 1997) [40], normal LDH, SDH activities was interfered. LDH activity is usually seen as the organism's energy requirement under anaerobic conditions (Moreira *et al.*, 2006) [22], it was proved in *S. plana* in water contaminated by CdNPs and results in significant elevation of LDH level. Devi *et al*. (1993) [6] and Reddy and Bhagyalaxmi (1994) [25] reported that declined LDH activity in the hepatopancreas of fiddler crab, *Uca pugilator* as a result of exposure of CdCl2.The work of Valarmathi and Azariah, (2002) [34] indicated that LDH levels were significantly elevated and SDH activity was suppressed in the muscle, gill and hepatopancreas tissues of the crab *S. quadratum* when exposed to two sublethal concentrations of chlorine. Mayekar *et al.* (2012) [20] studied high SDH activity and declined LDH activity in the female Crab Scylla serrata on treatment with sublethal dose of nickel. From the previous evident the present results also revealed that under exposure to CdNP toxicity the mud crab, *Scylla olivacea* showed increased dehydrogenase activity to balance the metabolic stress induced by the CdNP.

Conclusion

The overall research work conclude that nanoparticles of heavy metals contaminating aquatic system had a hazardous effect on the animals belonging to the aquatic system and thus in turn indirectly affect human health.

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