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Effect of exposure to cadmium on the tropical freshwater prawn *Macrobrachium rosenbergii*

HA Kaoud* and A Rezk

*Department of Veterinary Hygiene and Environmental Pollution, Faculty of Veterinary Medicine, Cairo University, Egypt * Corresponding author, email: ka-oud@link.net*

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The effect of cadmium on mortality, resistance and bioaccumulation in the tropical freshwater prawn *Macrobrachium rosenbergii* from Egypt were studied. Survival of prawns exposed to cadmium doses over 60 μg l_{-1} were significantly lower than of those exposed to lower doses. After 96 hours, prawns exposed to >40 μg l^{-1} of Cd had a greater reduction in total haemocyte count and phagocytic activity than those exposed to lower concentrations. Bioaccumulation of Cd in the gills, hepatopancreas and muscles was variable. Cd accumulated significantly in gills and hepatopancreas, but Cd accumulation in the muscles increased only marginally. *Macrobrachium rosenbergii* manifested histopathological alterations in gills, hepatopancreas and muscles when exposed to various concentrations of Cd.

Keywords: haemocyte count, survival, toxicity

Introduction

Metals are considered a major source of environmental pollution. Cadmium (Cd), which is one of these pollutants, has received considerable attention for its toxic effects on living individuals. Metal contamination is typically derived from different sources: mining, industrial waste discharges, sewage effluent, harbor activities and agrochemicals. Cd is unique among the other metals because of its toxicity at a very low dosage, long half-life (30 years in humans) and its low rate of excretion from the body (Jones and Cherian 1990).

The contamination of natural waters by heavy metals affects aquatic biota and poses considerable risks and concerns to the environment (Otchere 2003, Amisah et al. 2009) and human health.

Metals such as Cd are known to accumulate in marine organisms, and cause rapid genetic changes (Nimmo et al. 1978, Nevo et al. 1986). It is also possible that environmental toxicants may increase the susceptibility of aquatic animals to various diseases by interfering with the normal functioning of their immune, reproductive and developmental processes (Couch 1978, Brock 1997). In decapod crustaceans three types of circulating haemocytes are recognized: hyaline, semi-granular and large granular cells (Tsing et al. 1989). They are involved in cellular immune responses that include phagocytosis and constitute the primary method of eliminating microorganisms or foreign particles (Bayne 1990). In addition to phagocytosis, haemocytes are involved in coagulation and in the production of melanin via the prophenoloxidase system (Söderhäll et al. 1996). Enzymes for the prophenoloxidase system are contained in the granular haemocytes, released as proenzymes upon stimulation by microbial cell components such as 1 ,3-glucan or lipopolysaccharide from fungal cell walls, and activated by a serine protease (Söderhäll et al. 1993). The activity of phagocytosis has been reported for many crustaceans (Söderhäll et al. 1996) including the brown shrimp *Penaeus californiensis* (Hernández-López et al. 1996), the tiger shrimp *P. monodon* (Sritunyalucksana et al. 1999) and *Macro brachium rosenbergii* (Cheng et al. 2002). Several physico-chemical parameters and environmental contaminants have been reported to affect the immune response in crustaceans and these have been reviewed by Le Moullac and Haffner (2000). Environmental toxicants have been reported to cause a reduction in haemocyte count in the common shrimp *Crangon crangon* (Smith and Johnston 1992).

Pollution of aquatic environments with metals is common worldwide and, under certain environmental conditions, aquatic fauna may concentrate large amounts of some metals from the water in their tissues. Metals such as Cd are potentially harmful to most organisms, even in very low concentrations, and have been reported as hazardous environmental pollutants that may bioconcentrate in aquatic organisms. Neurotoxicity can cause a variety of neurochemical and behavioural changes (Desi et al. 1998). Cd can enter into the brain parenchyma and neurons causing neurological alterations in humans (Rose et al. 1992) and animal models (Lukawski et al. 2005). It is listed by the U.S. Environmental Protection Agency (1984) as one of 126 priority pollutants; is carcinogenic to a number of tissues (Waalkes 2000); and is classified by IARC (1993) as a human carcinogen. In laboratory animals, acute Cd poisoning produces primarily hepatic and testicular

injury, whereas chronic exposure results in renal damage, anaemia, and immuno- and osteotoxicity (Goering et al. 1995, Klaassen et al. 1999). It has been suggested that the mechanism of Cd toxicity involves production of reactive oxygen species and free radicals (Manca et al. 1994, Stohs et al. 2001).

The aim of this study was to investigate the effect of Cd toxicity on mortality and resistance in the giant freshwater prawn *M. rosenbergii* and also to investigate the bioaccumulation of Cd residues in its tissues.

Material and methods

Experimental design

Fresh water was adjusted to the desired parameters as follows: temperature 20–28 °C, pH 7–7.8, dissolved oxygen 5–8 mg l^{-1}, salinity 2, hardness 100–150 ppm $CaCO_3$, total ammonia <10 ppm, nitrate 20 ppm and nitrite 1 ppm, after New (1995).

A stock cadmium solution of 100 mg $CdCl_2$ salt dissolved in a solution composed of 20 ml water plus 5 ml concentrated HCl and made up to 1 000 ml with water was prepared (1.00 ml = 100 µg Cd). Nine different concentrations of Cd were then prepared from the stock solutions (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg l_{-1} Cd). Exposure solutions were not analysed, therefore all concentrations referred to hereafter are nominal.

Macrobrachium rosenbergii were obtained from commercial farms in Alexandria and Al-Kalubia, Egypt, and acclimated in the laboratory for two days before experimentation.

Toxicity tests were conducted according to the standard procedures of FAO (1985). Ten concentrations (4 replicates) of Cd were set up, ranging between 10 and 100 µg l^{-1}, plus a control. Ten shrimps of the same size (ranging from 13.2 to 16.5 g body weight with an average body weight of 15.32 ± 0.15 g) were randomly transferred from the holding tanks into the control and experimental tanks. The aquaria were aerated continuously, while the test solution in each tank was changed with the appropriate fresh solution every 24 hrs to maintain the definite concentration of Cd for 96 h. Observations for mortality were made twice daily at 10:00 and 18:00.

Cell counts

Haemolymph (100 µl) was sampled individually at the beginning of each test and at 96 h post exposure to Cd. It was withdrawn from the ventral sinus of each prawn into a 1 ml sterile syringe (25 gauge) containing 0.9 ml anticoagulant solution (trisodium citrate 0.114 M, sodium chloride 0.1 M, pH 7.45, osmolality 490 mOsm kg^{-1}). A drop of the anticoagulant-haemolymph mixture was placed on a haemocytometer to measure THC using an inverted-phase contrast microscope.

Culture of Lactococcus garvieae

The bacterial strain *L. garvieae* isolated from diseased *Macrobrachium rosenbergii* after artificial infection was used in this study. The bacterium was cultured on tryptic soy agar (TSA) for 24 h at 28 °C before being transferred to 10 ml of tryptic soy broth (TSB) for 24 h at 28 °C as a stock culture. The stock cultures were then centrifuged at 7155 × g for 15 min at l4 °C. The supernatant fluid was removed and the sediment resuspended in saline solution (0.85 NaCl) and adjusted at 10^{10} cfu ml^{-1} as stock bacterial suspensions for testing.

Phagocytic activity of M. rosenbergii *to* L. garvieae

After 72 h of Cd exposure in each treatment, prawns were injected in the cephalothorax with 20 µl of the bacteria suspension (10^{10} cfu ml^{-1} in 0.85% NaCl) resulting in 2 × 10_8 cfu prawn l^{-1}. After injection, the prawns were held in their respective solutions for 3 h (s). Haemolymph (200 µl) was collected from the ventral sinus and mixed with 200 µl of sterile anticoagulant containing sodium citrate (0.8 g), EDTA (0.34 g), Tween 80 (10 µl) and distilled water (100 ml with pH of 7.45).

Phagocytic activity was measured using the method described by Weeks-Perkins et al. (1995) where 200 µl of diluted haemolymph sample was mixed with 0.2 ml of 0.1% paraformaldehyde for 30 min at 4 °C to fix the haemocytes. They were then centrifuged at 800 × g at 4 °C, washed and resuspended in 0.4 ml of sterile phosphate buffer solution. The suspension (50 µl) was spread onto a slide glass and air-dried and stained with Diff-Quick stain. 200 haemocytes were counted using light microscope and the phagocytic rate was estimated as follows:

PR = [(phagocytic haemocytes) / (total haemocytes)] × 100.

Preparation and analysis of tissue samples

Procedure A: Each sample was represented by one gram of tissue dissected from the gills, hepatopancreas, and muscles, then placed in a clean screw-capped tube and digested according to the method described by Finerty et al. (1990).

Procedure B: The obtained solutions were then analysed using an Air/Acetylene Flame Atomic Absorption Spectrophotometer (UNICAM 696 AA Spectrometer) for determination of Cd levels in examined samples.

Histopathological examination

Tissue specimens from experimental *M. rosenbergii* were taken (gills, hepatopancreas and muscles) and fixed in 10% buffered neutral formalin. They were processed to obtain five micron thick paraffin sections then stained with haematoxylin and eosin, (H&E) according to Bancroft et al. (1996) and examined under light microscope.

Statistical analysis

Data were analysed using analysis of variance (ANOVA) and means were separated by the Duncan *post hoc* test at a probability level of <0.05 (SAS 2000).

Results

After 96 h, mean (± SD) survival of prawns in control tanks (0 Cd) was 94 ± 2.2% and significantly higher ($P < 0.05$) than that of prawns in all other treatments (Table 1). After 96 h, survival of prawns exposed to 10–50 µg l_{-1} concentrations of Cd was significantly greater ($P < 0.05$) than for prawns exposed to higher concentrations (60 µg l_{-1} or greater), but were not significantly different from each other ($P < 0.05$). Survival of prawns exposed to 60, 70, 80, 90

Table 1: Effect of cadmium on survival, total haemocyte count (THC) and phagocytic % of freshwater prawns *Macrobrachium rosenbergii* exposed to cadmium at different concentrations for 96 hours post-treatment. Values are means ± SD *(n* = 4 prawns in each case). * = significant *(P* < 0.05)

Cd conc. (Cd^{2+} µg l^{-1})	Survival %	Immune response	
		THC (ml^{-1} ×	Phagocytic %
0	94± 2.20	196 ± 70	90 ± 7.7
10	86± 1.70*	195 ± 16	90 ± 8.7
20	86± 1.60*	199 ± 12*	84 ± 7.0
30	70± 1.67*	170 ± 9.0*	70 ± 7.0*
40	63± 0.87*	170 ± 8.0*	62 ± 7.0*
50	60± 0.30*	145 ± 11*	50 ± 2.7*
60	57± 0.70*	138 ± 9.0	40 ± 0.7*
70	50± 0.70 *	136 ± 8.0*	40 ± 0.7*
80	50± 0.70 *	130 ± 12*	40 ± 3.0*
90	40± 0.20*	130 ± 8.0*	40 ± 0.0*
100	40± 0.21*	120 ± 0.0*	30 ± 3.0*

and 100 µg l_{-1} of Cd was significantly lower *(P* < 0.05), with means (± SD) of 57 ± 0.70%, 50 ± 0.70%, 50 ± 0.70%, 40 ± 0.20% and 40 ± 0.21%, respectively (Table 2). The regression analysis of prawn survival (%) on Cd concentration was highly significant *(P* < 0.001; r_2 = 0.964).

Table 1 and Figure 1 show that, at 96 h, prawns exposed to 40, 50, 60, 70, 80, 90 and 100 µg l_{-1} concentrations of Cd had significantly greater reductions in THC and phagocytic activity than for prawns exposed to lower concentrations (10–30 µg l^{-1}), *(P* < 0.05).

The $_{LC50}$ of Cd on M. rosenbergii

The 96-hour $_{LC50}$ of Cd in *M. rosenbergii* was calculated to be 74 µg l^{-1}, Cd using the resulting regression equation.

The bioaccumulation of Cd in different tissues of M. rosenbergii

The highest bioaccumulation of Cd was observed in the organs mainly implicated in metal intoxication. Cd in tissues was high in the gills > hepatopancreas > muscles (Figure 2).

Gills: The rate of accumulation of Cd was maximum in gills of exposed prawns and no detectable amount of Cd was observed in the gills of control prawns, as well as at the lowest concentration of Cd (10 µg l^{-1}). The rate of accumulation increased along with the increasing of Cd concentration, reaching 1.1 ± 0.02 at 100 µg l_{-1} (Table 2).

Hepatopancreas: As with the gills, Cd could not be traced in the hepatopancreas of the control fish, as well as at the lowest concentration 10 µg l^{-1}. Even though the quantity of accumulated Cd was less in the case of hepatopancreas when compared to gills, the pattern of accumulation showed a more or less continuous increasing trend (Table 2).

Muscles: The rate of accumulation of Cd in muscle increased along with exposure concentration. The mean rate of accumulation at 100 µg l_{-1} was 0.06 5 ± 0.008. The rate of accumulation was less as compared with other tissues (Table 2).

Histopathological alterations in the tissues of M. rosenbergii

M. rosenbergii manifested histopathological changes in the gills, hepatopancreas and muscles.

Table 2: The residual analysis of cadmium in freshwater prawns, *Macrobrachium rosenbergii,* exposed to cadmium at different concentrations for 96 hours post-treatment. Values are means ± SD

Cd conc.	Accumulation in tissues Cd^{2+} µg g^{-1}(dry weight)		
(Cd^{2+} µg l^{-1})	Gills	Hepatopancreas	Muscles
0	–	–	–
10	–	–	–
20	0.05± 0.008	0.02± 0.006	0.005± 0.001
30	0.05± 0.009	0.025± 0.008	0.01± 0.001
40	0.06± 0.018	0.03± 0.012	0.02± 0.003
50	0.065± 0.021	0.04± 0.009	0.02± 0.005
60	0.08± 0.022	0.06± 0.011	0.03± 0.01
70	0.90± 0.011	0.065± 0.011	0.05± 0.009
80	1± 0.011	0.08± 0.012	0.055± 0.011
90	1.1± 0.02	2± 0.01	0.06± 0.022
100	1.1± 0.025	2.2± 0.02	0.065± 0.008

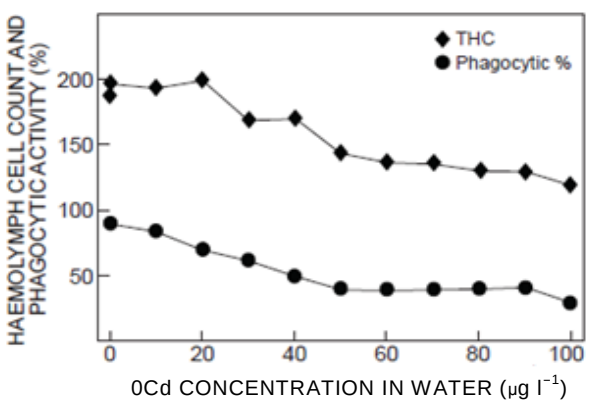

Figure 1: Effect of 96 hours exposure to cadmium on immune response in *M. rosenbergii*

Gills: showed mild congestion, swelling and edema at low doses of Cd intoxication. Severe edema, hyperplasia, at highest doses was observed (Figure 3).

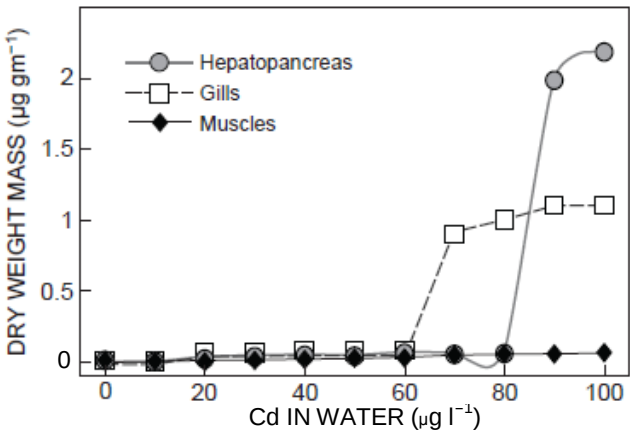

Figure 2: Accumulation of cadmium in gills, hepatopancreas and muscles of *M. rosenbergii* after 96 hours exposure

Hepatopancreas: showed degeneration of the hepatocytes, haemolysis, haemocytic infiltration in the interstitial sinuses, an increased number of haemocytes, thickening and ruptures of the basal laminae, and necrosis of the tubules (Figure 4).

Muscular tissues: Figure 5 shows the normal structures of the muscles. Several histopathological alterations were seen in the muscles *M. rosenbergi.* The pathological findings included degeneration in muscles with infiltration and aggregations of haemocytes between them, and focal areas of necrosis. Atrophy of muscle bundles, edema, hyaline degeneration and splitting of muscle fibres were also observed.

Discussion

At 96 h, survival of prawns exposed to 10–50 µg l_{-1} concentrations of Cd was significantly greater *(P* < 0.05) than for prawns exposed to higher concentrations (60 µg l_{-1} or greater). Cheng (1979) tested Hg, Cu, Cd and Zn in *Penaeus monodon* and found that Hg was the most toxic of all metals, followed by Cu, Cd and Zn, and added that Cd toxicity was the most rapid one. Kuo et al. (1984) suspected that Cd and Cu were the cause of mortalities in prawn hatcheries in Taiwan in 1980–1981, with the metals coming from waste water discharged by nearby industries. Prawns exposed to 40, 50, 60, 70, 80 , 90 and 100 µg l_{-1} concentrations of Cd had significantly greater reduction in THC and phagocytic activity than prawns exposed to lower concentrations (10–30 µg l^{-1}), *(P* < 0.05).

Several researchers have investigated the effects of environmental parameters on crustacean defence mechanisms. Dean and Vernberg (1966) reported that temperature affects haemolymph clotting time, haemocyte counts and serum protein concentration in the hermit crab *Uca pugilator.* Truscott and White (1990) found tide-associated rhythms in the total haemocyte count for freshly captured shore crab *Carcinus maenas,* with peak count occurring at high tide. Increased haemocyte numbers provide an enhanced immune capability during periods of high activity. Hauton et al. (1995) reported a significant negative correlation between phenoloxidase activity and tidal height in *C.*

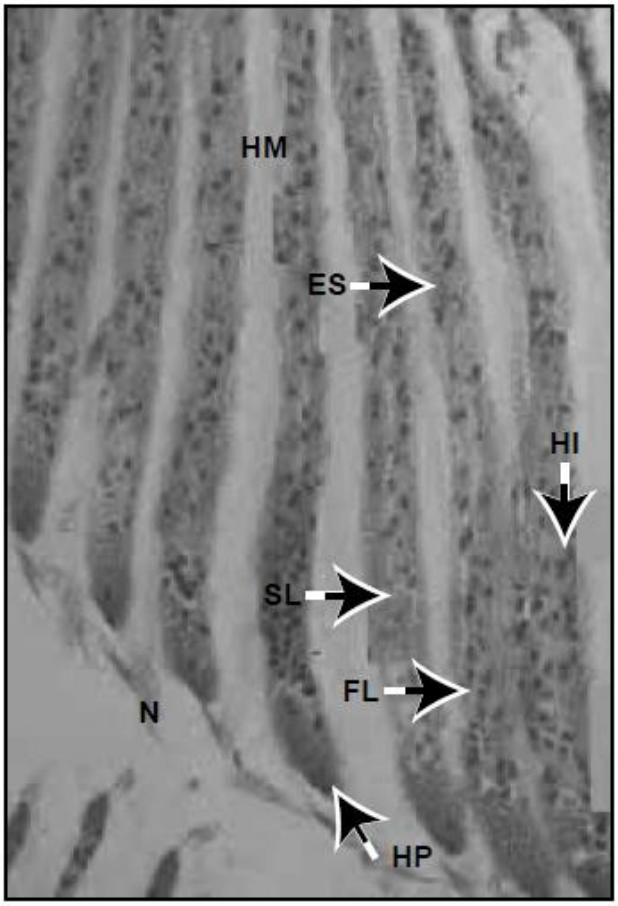

Figure 3: Gills showing congestion and haemocytic infiltration (HI), swelling (SL), edema, necrosis (N), hyperplasia (HP) and fused (FL) and enlargement of the lamellar sinuses (ES). H&E stain (×200)

maenas, and this indicated cyclical changes in immunocompetence. An increased prevalence in the shell disease of marine decapods crustaceans has been reported to result from polluted environments, also suggesting a decrease in immunocompetence (Gopalan and Young 1975, Young and Pearce 1975).

Carolina (2009) studied the effect of magnesium on the immune system of marine invertebrates and found that it severely suppressed the number of circulating haemocytes in *Nephrops norvegicus* by inducing apoptosis. However, Mn increased the number of circulating haemocytes in *Asterias rubens,* and at the same time affected their ability to phagotize. The sensitivity of exposed gills to bacterial infection has been previously described in other shrimps exposed to Cd (Couch 1977, Darmono et al. 1990). Their presence has also been observed in gills of *P. japonicus* (Souheil 1995) and of the crayfish *Astacus leptodactylus* (Maestracci and Vey 1987) infected by fungi.

A significant reduction in phagocytosis of *Bacillus cereus* was observed in the shore crab *Carcinus maenas* following 14 days exposure to 500 µg l_{-1} Cd (Truscott and White 1990).

The 96-hour $_{LC50}$ for Cd in *M. rosenbergii* was calculated to be 74 µg l^{-1}. Fafioye and Ogunsanwo (2007) found that the

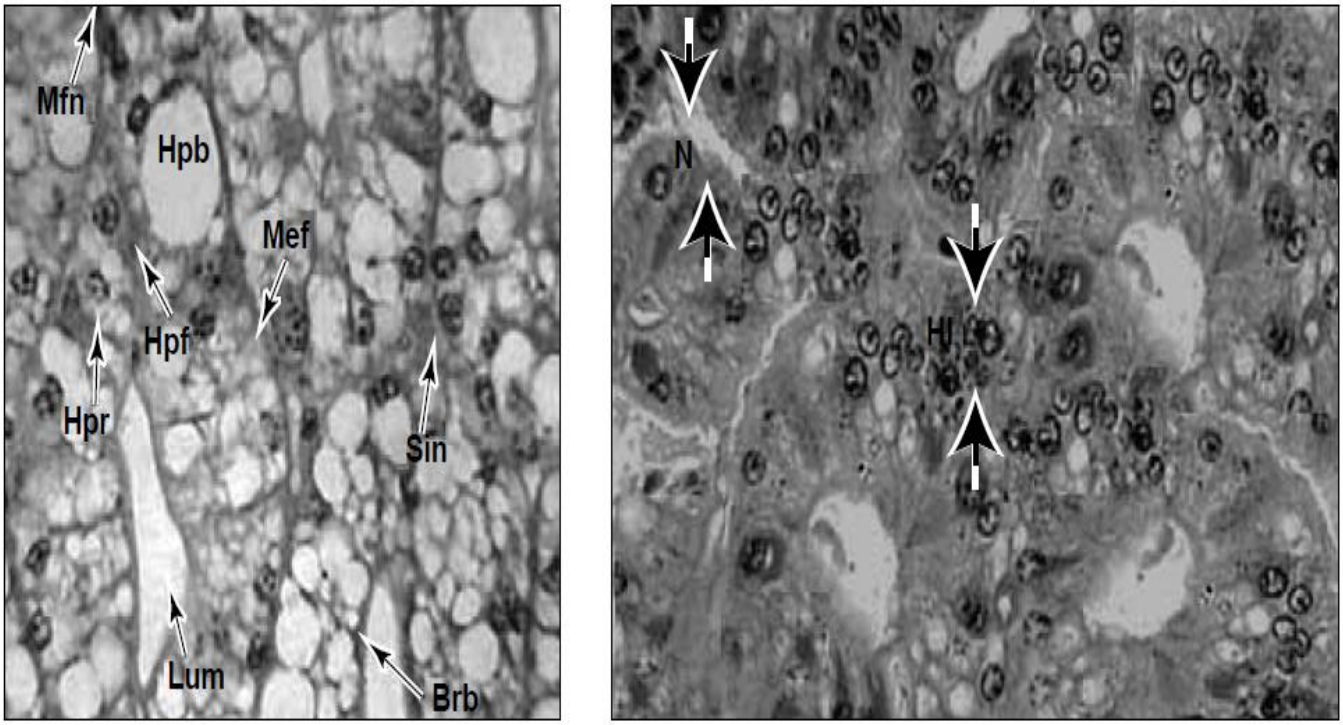

Figure 4: Cross-sections of hepatopancreatic tubules: left — normal lumens (Lum), tubule tissues (Hpf: F-cell; Hpb: B-cell; Hpr: R-cell; Mfn: myoepithelial cell nuclei; Mef: myoepithelial layer; Brb: microvillus brush borders) and hemal sinuses (Sin) between tubules; right — hepatopancreas showing degeneration of hepatocytes, haemolysis and haemocytic infiltration (HI) and necrosis (N)

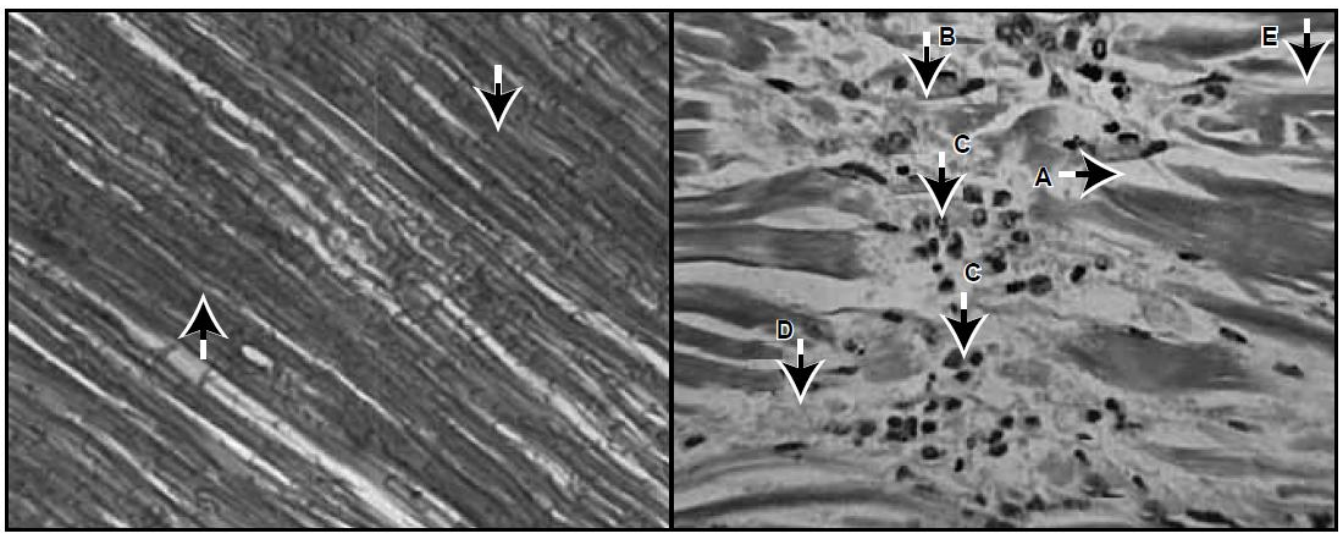

Figure 5: Muscle tissues: left — normal muscle bundles; right — bundles showing pathological alterations; including degeneration in muscles, with infiltration and aggregations of haemocytes between them, and focal areas of necrosis. Also, atrophy of muscle bundles, edema, hyaline degeneration and splitting of muscle fibres. A; splitting of muscle fibres, B: hyaline degeneration, C: infiltration of haemocytes, D: focal areas of necrosis, E: atrophy of muscle bundles and edema.

lethal concentration (LC_{50}) at 96 h of exposure to Cd for *M. rosenbergii* post larvae was 323 µg l_{-1} which is a higher toxicity than the $_{LC50}$ obtained in our experiment. 96 h $_{LC50}$ values of 288, 302 and 311 µg l_{-1} of Cd, have been reported for *Penaeus monodon* (Diaz 1995), *P. pencilatus* (Gao and Zou 1995) and *P. indicus* (Chinni and Yallapragda 2000), respectively.

The highest bioaccumulation of Cd was observed in the organs mainly implicated in metal intoxication. Cd in tissues was high in the gills > hepatopancreas > muscles.

The highest Cd concentration in gills might be related to the important quantity of this metal in the haemolymph and or the necrosed tissues, or these organs might constitute

the entry sites of the metal and act as a transient store for accumulated Cd (Martin and Rainbow 1998). The relatively higher Cd concentration in gills than the hepatopancreas could originate from a progressive transfer of Cd from gills to the hepatopancreas via the haemolymph (Bjerregaard 1990), and/or from a process of differentiation of hepatopancreatic epithelium as observed by AliKhan (1989) in the isopod *Porcellio spinicornis,* leading to transfer of the metal into the intestinal lumen and from this site to the exterior as observed by Brown (1982) in crayfish. However, the higher Cd concentration in the hepatopancreas suggested that this organ plays a role in metal storage and or in detoxification process by a metal binding component (White and Rainbow 1986)

Mean Cd accumulation in muscles of *M. rosenbergii* ranged from 0.005–0.065 (μg g_{-1} dry mass) and the maximum permissible limits recommended by WHO (1984) is 0.005 ppm (5 μg g_{-1} dry mass). The recorded results of Cd concentrations in muscles of *M. rosenbergii* were higher than the permissible limits intended by Boletin Oficial del Estado (1994) in Spain (l.0 pg or 0.001 μg g^{-1}) and FAO/ WHO (1992) (0.05 ppm or μg g_{-1} dry mass) but lower than the Egyptian Organization for Standardization and Quality Control (EOSQC) (1993) (100 μg g^{-1}).

The bioconcentration factor (BCF) is the ratio of a substance's concentration in tissue of an aquatic organism to its concentration in the ambient water, in situations where the organism is exposed through the water only and the ratio do not change substantially over time. BCF is a dimension-less number representing how much of a chemical is in a tissue relative to how much of that chemical exists in the environment. The BCFs were increased in gills, hepatopancreas and muscles, with increasing exposure concentrations, respectively. Frías-Espericueta et al. (2009) found that the mean contents of Cd, Cu, Pb and Zn of the white shrimp (Litopenaeus vannamei) were lower in the muscle than in the corresponding hepatopancrease samples, which is in agreement with most literature on the metal contents in the tissues of different aquatic organisms because the hepatopancreas is the main organ for metal accumulation (Roesijadi and Robinson 1994, Yang et al. 2007).

The histopathological alterations in different tissues of M. rosenbergii

Gills showed mild congestion, swelling and edema at low doses of Cd intoxication. Severe edema and hyperplasia at highest doses of intoxication. Similar effects such as necrosis, cell proliferation, epithelial lifting and dilated lamellae were observed in gills of fish exposed to metals, including Cd as observed by Malia (1985). Since high Cd concentrations result in serious damage to the gills, the metal may consequently inhibit the physiological functions of these organs. As the gills of the shrimp are probably involved in gas exchange, we suppose that these alterations could result in disruption of respiration (Thurberg et al. 1973). The effects of Cd on fish gill morphology have been studied in some species (Gardner and Yevich 1970, Karlsson-Norrgren et al. 1985, Pratap and Wendelaar Bonga 1993, Thophon et al. 2003).

The hepatopancreas showed degeneration of the hepatocytes and haemolysis (Figure 4). These findings were apparent as the hepatospleen is considered the organ of detoxification, excretion and binding proteins such as metallothionein (MTs). The metal-binding proteins were present in the nuclei of hepatocytes, which suggested an increase in cell damage similar to that reported by De Smet and Blust (2001). Similar results were observed by van Dyk (2003) and Mela et al. (2007).

Frías-Espericueta et al. (2008) studied the effect of three concentrations of Cu (3.512, 1.756 and 0.877 mg l^{-1}) on juvenile *Litopenaeus vannamei* and found that there were severe time- and dose-dependent structural damages, such as necrosis, loss of regular structure and infiltration of haemocytes in the gill tissues, as well as atrophy, necrosis and irregular tubular structure in the hepatopancreas. Cadmium is a highly toxic non-essential metal and it does not have a role in biological processes in living organisms. Thus, even in low concentrations, Cd could be harmful to living organisms (Burden et al. 1998). High accumulation of Cd in the liver, similar to that seen in the hepatopancreas in this study, may be due to its strong binding with cystine residues of metallothionein (Klaassen et al. 1999).

Conclusion

This study reveals an important precaution for prawn cultivation. Knowledge of the toxicity of cadmium will be helpful for water quality management in fish farms with reference to prawn culture, since it affected the immune response and caused a reduction in haemocyte count in *Macrobrachium rosenbergii.* Caution should be exercised against water source contamination and exposure to fertilizers like superphosphate with Cd and industrial pollution. For this reason, the assessment of risk and the safe levels of toxic substances added to any natural environment through human or natural sources should not neglect the effects on biological systems caused by exposure to minute amounts of toxicants.

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