Mar Biol (2006) 150:197–211
DOI 10.1007/s00227-006-0350-0

RESEARCH ARTICLE

Subcellular distribution of zinc and cadmium in the hepatopancreas and gills of the decapod crustacean *Penaeus indicus*

G. Nunez-Nogueira · C. Mouneyrac · J. C. Amiard · P. S. Rainbow

Received: 19 July 2005 / Accepted: 28 April 2006 / Published online: 9 June 2006
© Springer-Verlag 2006

Abstract The decapod crustacean *Penaeus indicus* accumulated Cd and Zn in different subcellular compartments of hepatopancreas and gill cells. Most of the Cd and part of the Zn accumulates within the soluble fraction of the cells, while the remainder of the Zn is found in insoluble inclusions, associated with P, Ca, Mg and Si in B-, F- and R-cells in the hepatopancreas, and haemocytes, nephrocytes and epithelial cells in the gills. No presence of Cd was observed in metal-rich inclusions in any cell analysed. Metallothionein-like proteins (MTLP), analysed by differential pulse polarography, were present in the hepatopancreas (12–18 mg g^{-1}) and gills (7–8 mg g^{-1}) of metal-exposed prawns. Binding to MTLP is the detoxification mechanism for cadmium, while the detoxification of zinc involves both binding to MTLP and incorporation into insoluble metal-rich inclusions.

Communicated by J.P. Thorpe, Port Erin

G. Nunez-Nogueira · P. S. Rainbow (✉)
Department of Zoology, The Natural History Museum,
Cromwell Road, London, SW7 5BD, UK
e-mail: psr@nhm.ac.uk

C. Mouneyrac
Centre d'étude et de Recherche sur les écosystèms
aquatiques, Université Catholique de l'ouest,
44 Rue Rabelais, BP 808, 49008 Angers
Cedex 01, France

J. C. Amiard
Service d'Ecotoxicologie, CNRS-GDR 1117, ISOMer,
SMAB, 2 Rue de la Houssinière, BP 92208,
44322 Nantes Cedex 3, France

G. Nunez-Nogueira
Laboratorio de Contaminación Marina, Instituto de
Ciencias del Mar y Limnología, UNAM,
Circuito Exterior S/N. 04510, México DF, Mexico

Introduction

The capacity of the decapod crustacean *Penaeus indicus* to accumulate zinc and cadmium, either from solution or food, indicates the presence of physiological detoxificatory processes at the cellular level to retain these metals for a shorter (in the case of the essential metal zinc) or longer period (non-essential cadmium) (Nunez-Nogueira and Rainbow 2005a, b; Nunez-Nogueira et al. 2006). The body distributions of newly taken up zinc and cadmium in this decapod show that the hepatopancreas is strongly involved in the accumulation of these metals, and also indicate that the gills play an important role in trace metal uptake from solution and in any loss from the body (Nunez-Nogueira and Rainbow 2005a, b; Nunez-Nogueira et al. 2006).

The detoxification of accumulated trace metals in cells can involve their binding to both insoluble and soluble subcellular components. Several ultrastructural studies have been performed on invertebrates to investigate the cellular detoxification of trace metals, indicating that different metals may be present in more than one subcellular compartment or form in different tissues and cells (Al-Mohanna and Nott 1987a, b, 1989; Hopkin 1989; Vogt and Quinitio 1994; Mason and Jenkins 1995; Nassiri et al. 2000; Marigomez et al. 2002). The chemical composition and appearance of these metal-rich deposits vary widely. Hopkin (1989) classified metal-containing granules into four different categories: *type A,* consisting of concentric layers of calcium and magnesium phosphates which may contain

other class A and borderline metals such as manganese and zinc; *type B*, more heterogeneous in shape and always containing sulphur in association with class B and borderline metals including cadmium, copper and zinc; *type C*, often polyhedral with a crystalline form, mainly containing iron, probably derived from ferritin; and *type D*, larger (extracellular) granules composed of concentric layers of calcium carbonate. In crustaceans, including decapods, the most commonly reported metal-rich granules in the hepatopancreas (or equivalent organs) are type A and B granules (Al-Mohanna and Nott 1985, 1987a, 1989; Correa-Junior et al. 2000; Nassiri et al. 2000).

Particular trace metals (Zn, Cu, Cd, Ag, Hg for example) have been found to be associated with, and induce, metallothioneins, low molecular weight cytosolic proteins involved in the cellular regulation and detoxification of these metals (Roesijadi 1981; Engel and Roesijadi 1987; Viarengo et al. 1999). The presence of thiolic groups in cysteine residues in the proteins provides the high metal affinity of the molecule (Roesijadi 1981; Engel and Roesijadi 1987), sequestering metals in the cytoplasm and reducing their metabolic availability. Induced metallothioneins can bind copper and zinc already present in the cell and not react only to newly taken up metals. Zinc and cadmium are among the trace metals reported to induce metallothionein production in the gills and digestive glands (or hepatopancreas) of different invertebrates exposed to raised concentrations of these metals (Rainbow and Scott 1979; Roesijadi 1981; Carpene 1993; Canli et al. 1997), indicating that these proteins could be involved in zinc and cadmium detoxification within the cells of the hepatopancreas and gills of decapod crustaceans exposed to raised zinc or cadmium availabilities.

The hepatopancreas of decapod crustaceans plays an important role in trace metal accumulation (Bryan 1976; Gibson and Barker 1979; Dall and Moriarty 1983; Rainbow 1998). Detoxified copper or zinc-rich granules have been described from the hepatopancreas of *P. semisulcatus* (Al-Mohanna and Nott 1985) and *P. monodon* (Vogt and Quinitio 1994). Zinc is stored in phosphorus-rich granules in hepatopancreas cells of *P. semisulcatus*, and granule abundance and hepatopancreas zinc concentration vary with the moult cycle (Al-Mohanna and Nott 1985). It has been suggested that copper is accumulated in the hepatopancreas of decapods before use in the synthesis of haemocyanin (Johnston and Barber 1969), and the hepatopancreas also stores high concentrations of copper in decapods exposed to raised dissolved copper concentrations, prior to elimination from the body through the faeces (Gibson and Barker 1979).

The cellular accumulation of metals in the hepatopancreas varies according to the cell involved (Gibson and Barker 1979). The epithelial cells of the decapod crustacean hepatopancreas have been classified by either their form or function. The four cell types reported are Embryonic (E-cells), Fibrillar (F-cells), Blister-like (B-cells) and Resorptive (R-cells) respectively [after Jacob (1928) and Hirsch and Jacob (1930) in Gibson and Barker 1979]. A fifth cell type has been described by Al-Mohanna and Nott (1987), denominated "midget or M-cell", although later studies only report the presence of four different cell types in the decapod hepatopancreas as described previously (Caceci et al. 1988; Andersen and Baatrup 1988; Vogt and Quinitio 1994). The abundance of one particular cell type in the hepatopancreas is related to the moulting cycle and the state of starvation of the decapod (Papathanassiou and King 1984; Al-Mohanna and Nott 1989).

Gills are important permeable areas in the body of a decapod, involved in the chemical exchange of molecules such as respiratory gases, water, ammonia and metals, between the body and the external medium. Decapod gills are involved in respiration, osmoregulation, excretion and pH regulation (Foster and Howse 1978; Gilles and Pequeux 1983; Taylor and Taylor 1992). Foster and Howse (1978) analysed the structural organization and morphology of gills of the brown shrimp *P. aztecus*, establishing the penaeid gill as dendrobranchiate and describing the presence of different cell types. Five different epithelial cell types are reported to be present in decapod gills, denominated as thin cells, thick cells, attenuated cells, pillar cells and flange cells; haemocytes and nephrocytes are found in the blood spaces in the gills (Taylor and Taylor 1992). Only the last four gill epithelial cell types have so far been reported in *Penaeus* prawns (Foster and Howse 1978).

The biology of trace metals in the gills of *Penaeus* prawns has not been studied in as much detail as in the hepatopancreas, although studies have analysed the role of the gills in ion uptake (Arruda-Freire and Campbell-McNamara 1995), in addition to osmotic regulation and respiration (Gilles and Pequeux 1983). Gills have been shown to be vulnerable to trace metal exposure, with ultrastructural changes reported (Gilles and Pequeux 1983). Soegianto et al. (1999a, b) observed such effects in *P. japonicus* exposed to copper and cadmium in solution at relatively high concentrations (greater than 500 μg Cu l^{-1} and 2,000 μg Cd l^{-1}, respectively).

Penaeid prawns are important subjects of mariculture and coastal fisheries activities throughout the tro-

pics and subtropics (Holthuis 1980; Grey et al. 1983; Perez-Farfante and Kensley 1997). They are common in estuaries and are therefore potentially affected by anthropogenic metal contamination. This study is part of a wider investigation of the biology of the trace metals zinc and cadmium in a model penaeid prawn *P. indicus* (Nunez-Nogueira and Rainbow 2005a, b; Nunez-Nogueira et al. 2006). It specifically investigates the subcellular detoxification of accumulated zinc and cadmium in the hepatopancreas and gills of *P. indicus*, after sublethal exposure of the prawns to dissolved zinc and cadmium. The study incorporates an ultrastructural investigation of the nature and localisation of any metal-rich inclusions in the cells of the hepatopancreas and gills, and a study of metallothionein-like proteins (MTLP) binding zinc and cadmium in the same cells.

Material and methods

Juveniles of *P. indicus* [between 1 and 2.5 cm total length (Le Reste 1978); mean dry weight 31.16 ± 17.96 mg] were obtained from cultures at the School of Ocean Sciences, University of North Wales, Bangor, Wales from stocks originally from the Gulf of Aden. No distinction between genders was as yet discernible.

Prawns were maintained in artificial sea water (Tropic Marine New, TMN; Aquarientechnick, Watenberg®-Germany) at 15 salinity (pH 7.8), 12:12 light/dark periods and 25°C as previously described (Nunez-Nogueira and Rainbow 2005a, b). The use of artificial seawater in all experiments provided physico-chemical stability, insignificant background dissolved trace metal concentrations, and replicability for trace metal uptake studies (Rainbow 1997).

The experimental design involved seven separate experimental groups containing ten prawns each: controls (not metal exposed; three groups), prawns exposed to 100 μg l^{-1} zinc (two experimental groups), and cadmium (two experimental groups) in TMN at 15 salinity at 25°C. The hepatopancreas and gills were removed from one control group at the beginning of the experiment, as described by Nunez-Nogueira and Rainbow (2005a). Prawns from the remaining two control and metal-treated groups were dissected after 5 or 10 days of exposure.

The dissolved metal concentrations are environmentally realistic for metal contaminated estuaries (Bryan et al. 1985; Law et al. 1994) and are below dissolved zinc and cadmium concentrations toxic to penaeids (McClurg 1984; Joseph et al. 1992; Chinni and Yallapragada 2000). In order to maintain the nominal metal concentrations in the experimental media (confirmed by atomic absorption spectrophotometry), all experimental glassware was pre-soaked in the appropriate experimental medium to saturate any adsorption onto the vessels. Thereafter the experimental media were changed every 2 days immediately after feeding the prawns for 30 min (see Nunez-Nogueira and Rainbow 2005a, b).

X-ray microanalysis and electron microscopy analysis

Hepatopancreas and gills samples destined for ultrastructural study were transferred into individual vials to be fixed in 2.5% glutaraldehyde solution (0.2 M Sorensen's buffer, pH = 7.2 at 2°C) immediately after dissection, for 120 min. Samples were then rinsed with buffer, and dehydrated by immersion in a series of diluted ethanols up to 100% ethanol (BDH Lab, England), using cold (2°C) ethanol over a period of 3.5 h. Dehydrated samples were transferred into propylene oxide (PO) and distilled water solution for 5 min. Samples were then transferred into a PO solution mixed with TAAB resin (50% v/v) and rotated overnight, before final transfer into 100% TAAB resin for 10 h. At the end of the day, samples were embedded in fresh TAAB resin within BEEM capsules and dried at 90°C for 8 h for solidification.

Ultrathin (between 0.5 and 0.8 nm thickness) and semithin sections (between 0.5 and 0.9 μm thickness) were cut on an ultramicrotome (Reichert Ultracut S®) with glass knives or diamond knife (Microstar Technologies®) and collected on uncoated nickel (Agar®) or aluminium (Polaron Equipment Ltd.) grids (3.05 mm size and 200 square mesh). Ultrathin sections were stained with an alcoholic solution of uranyl acetate (1% uranyl acetate in 50–100% methanol; 4 min) and Reynold's lead citrate (35 mg lead citrate ml^{-1} in 1 N NaOH, 7 min) for observation of subcellular structures. Semithin sections were kept unstained for X-ray microanalysis. Both sections were analysed in a TEM-R200 EXII Electron Microscope with STEM attachment and LINK QX 2000 Energy Disperser and X-ray microanalysis system. X-ray analyses were performed at 100 keV and specimen tilted 30°.

Metal and MTLP analyses

Samples destined for biochemical analysis were transferred into plastic vials to be immediately frozen in liquid nitrogen for a few minutes. After freezing, samples were freeze-dried overnight and their dry weight measured. Then, samples were homogenized in a buffer solution (20 mM TRIS, 10–5 β-mercaptoethanol, 150 mM NaCl solution adjusted to pH 8.6). The soluble

and insoluble fractions resulting from this homogenization procedure were separated by centrifugation at 25,074*g* for 55 min at 4°C (Biofuge 28 RS, Heraeus Sepatech®). The cytosolic heat-stable compounds including metallothionein were isolated by centrifugation of the soluble fraction (12,000*g* for 10 min at 4°C) after heat-treatment (75°C for 15 min). In the heat-denatured cytosol, the amount of MT was determined by differential pulse polarography (DPP) according to Thompson and Cosson (1984) and Olafson and Olsson (1991). A MDE 150 Stand Polarographique (Radiometer Copenhagen) Tracelab™ 50, controlled by the computer software Tracemaster 5 through a Polarographic analyser POL 150 was used. The temperature of the cell was maintained at 4°C. The method of standard addition was used for calibration with rabbit liver MT (SIGMA Chemical Co., St Louis, MO, lot. 20 k7000; M-7641 code) in absence of a shrimp MT standard. Polarographic determination in heat-denaturated cytosol is an analytical procedure based on several characteristics of MTs, but it does not allow, with certainty, the assertion that the target molecule is a true MT unless purification and sequencing are carried out. Strictly, therefore, what has been measured is the concentration of proteins with metallothionein properties, ie. MTLP.

Metal analysis was carried out on the soluble and insoluble fractions. Nalgene bottles were used to store all reagents. All glass labware was soaked in 10% HCl, rinsed three times with deionized water and dried in a desiccator protected from atmospheric dust. The insoluble and soluble fractions were heated (75°C, 12 h) with suprapure HNO_3 acid (Carlo Erba). After digestion, metal concentrations in these acid digests were determined after dilution with deionized water by flame AAS (Zn) or electrothermal atomic absorption spectrophotometry (EAAS) (Cd) using the Zeeman effect (Hitachi Z 8200 spectrophotometer). The analytical method has been described previously by Amiard et al. (1987).

Standard addition analyses were performed in an iso-medium and concentrations of each element were +125, +250, +500 ng Zn ml^{-1} for FAAS and +0.25, +0.5, +1 ng Cd ml^{-1} for EAAS. The analytical methods were validated by external intercalibrations (Coquery and Horvat 1996; Campbell et al. 2000). Total metal concentrations were recalculated from summation of quantities of trace elements in soluble and insoluble fractions determined previously, by the combined means procedure (Williams 2000). Results are expressed in μg g^{-1} dry weight of the organ.

Student's *t* test ($P < 0.05$) were performed for protein and metal concentration comparisons. Tests were developed according to Williams (2000), and carried out using STATISTICA 5.1 for Windows (StatSoft Inc.).

Results

Subcellular partitioning

Table 1 shows the concentrations (and percentage distributions) of Zn and Cd in the soluble and insoluble components of the cells of the hepatopancreas and gills of *P. indicus* exposed or not to raised availabilities of Zn and Cd. Percentage distributions of Zn was majority accumulated in the soluble fraction (between 76 and 83%) in the hepatopancreas, while the gills showed a similar distribution between these two fractions (40–55% insoluble fraction, 45–60% soluble). The percentage distribution of Cd was always higher in the soluble fraction in both tissues (70–92% in the hepatopancreas; 73–90% in the gills). The tissue zinc concentrations did not differ significantly among groups, while cadmium was clearly accumulated in both the hepatopancreas and the gills during cadmium exposure (Table 1).

The total cadmium concentration in the hepatopancreas (48.0 ± 12.6 μg g^{-1}) increased after exposure in prawns exposed for 10 days to 100 μg Cd l^{-1}. Of this, 85% was found in the soluble fraction of the tissue and only 15% in the insoluble fraction (Table 1). Gills of cadmium-exposed prawns had the same pattern, with 90% of cadmium in the soluble fraction and 10% in the insoluble fraction. The total cadmium concentration in this tissue was 36.3 ± 1.9 μg g^{-1}. Exposure of *P. indicus* to 100 μg Zn l^{-1} for 10 days did not produce significant changes in the tissue metal concentrations. Metal-exposed prawns showed an average concentration of 121.2 ± 24.5 μg Zn g^{-1} (Cd-exposed) and 115.1 ± 28.1 μg Zn g^{-1} (Zn-exposed), which were not significantly different from Zn concentrations in the control group (150.3 ± 59.5 μg Zn g^{-1}) (Table 1).

Hepatopancreas ultrastructure

The four hepatopancreas cell types (Embryonic, E-cells; Fibrillar, F-cells; Blister-like, B-cells; and Resorptive, R-cells) reported by Gibson and Barker (1979) were recognised in *P. indicus* in this study. The fifth cell type (Midget or M-cell) described by Al-Mohanna and Nott (1987) could not be distinguished.

The distribution of the cells along a blind-ending tubule of the hepatopancreas of *P. indicus* (Fig. 1) clearly follows the previous description of Al-Mohanna

Table 1 Zinc and cadmium concentrations (μg g^{-1}, mean ± SD) and percentage distributions between soluble and insoluble moieties (mean ± SD) in hepatopancreas and gills of *Penaeus indicus* juveniles after separate Cd and Zn exposures (100 μg l^{-1}) for 10 days at 15 salinity and 25°C

Metal	Tissue	Experimental group	Insoluble fraction	Soluble fraction	Whole tissue
Zinc	Hepatopancreas ($n = 4$)	Controls	25.1 ± 6.9 (16.7 ± 4.6%)	125.2 ± 59.1 (83.3 ± 39.3%)	150.3 ± 59.5
		Cd-exposed	26.6 ± 14.3 (21.9 ± 11.7%)	94.6 ± 19.9 (78.1 ± 16.4%)	121.2 ± 24.5
		Zn-exposed	27.4 ± 7.9 (23.8 ± 6.8%)	87.8 ± 27.0 (76.2 ± 23.5%)	115.1 ± 28.1
	Gill ($n = 2$)	Controls	43.4 ± 19.3 (55.4 ± 24.6%)	35.0 ± 0.8 (44.6 ± 1.1%)	78.4 ± 19.3
		Cd-exposed	33.3 ± 15.3 (41.3 ± 18.9%)	47.3 ± 7.5 (58.7 ± 9.3%)	80.6 ± 17.0
		Zn-exposed	28.6 ± 20.0 (40.4 ± 28.3%)	42.1 ± 15.9 (59.6 ± 22.5%)	70.7 ± 25.5
Cadmium	Hepatopancreas ($n = 4$)	Controls	0.2 ± 0.1 (30.1 ± 16.9%)	0.6 ± 0.0 (69.9 ± 2.4%)	0.8 ± 0.1
		Cd-exposed	7.1 ± 5.0 (14.8 ± 10.4%)	41.0 ± 11.6 (85.2 ± 24.1%)	48.0 ± 12.6
		Zn-exposed	0.4 ± 0.7 (8.6 ± 14.8%)	4.8 ± 5.2 (91.6 ± 99.2%)	5.2 ± 5.3
	Gill ($n = 2$)	Controls	0.1 ± 0.2 (26.8 ± 37.5%)	0.4 ± 0.2 (73.2 ± 39.3%)	0.6 ± 0.3
		Cd-exposed	3.5 ± 1.0 (9.8 ± 2.7%)	32.8 ± 1.7 (90.3 ± 4.6%)	36.3 ± 1.9
		Zn-exposed	0.4 ± 0.1 (17.5 ± 6.0%)	1.9 ± 1.3 (82.6 ± 55.3%)	2.3 ± 1.3

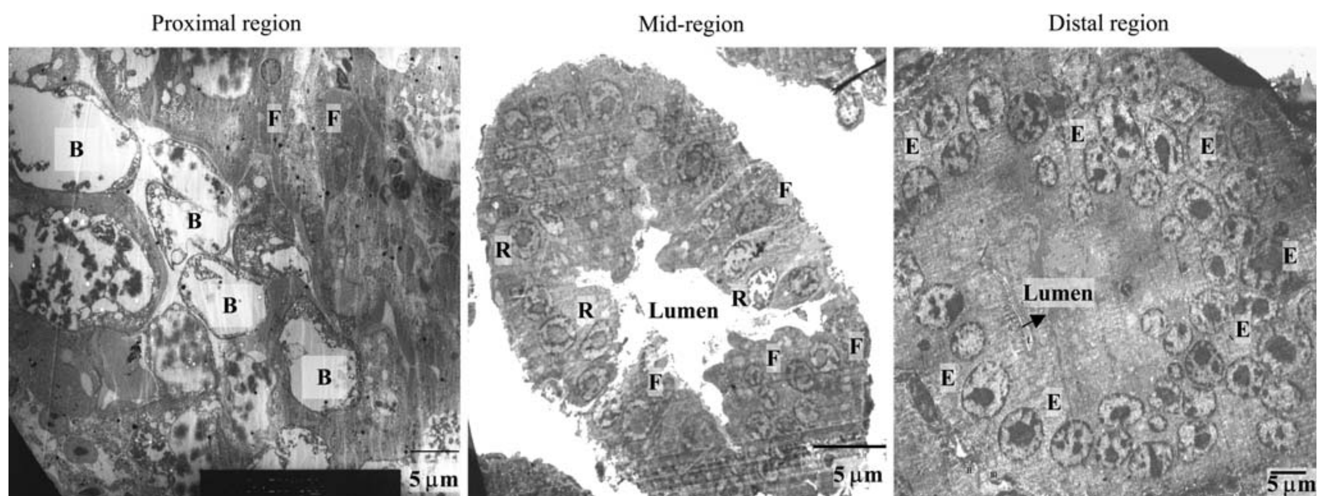

Fig. 1 Cellular distribution in a blind-ending tubule (*transverse view*) of the hepatopancreas of *P. indicus* at inter-moult stage. *B*, B-cells; *F*, F-cells; *R*, R-cells, and *E*, E-cells

and Nott (1989) for *P. semisulcatus*. Sub-mature and mature cells were mainly localized in the middle or proximal regions, while the distal region contained embryonic cells. The morphological structure of the cells from metal-exposed prawns remained unchanged in comparison to those of unexposed prawns in both regions of the tubule, showing no sign of cellular damage after zinc or cadmium exposure. The presence of different electron dense metal-rich inclusions was observed in B-, R- and F-cells from prawns in all the experimental groups, including controls.

The apical region of B-cells showed the presence of electron dense metal-rich deposits inside individual vacuoles or forming part of a big central vacuole (defined as a digestive vacuole, Icely and Nott 1992) that dominates the cytoplasm of the cells (Fig. 1). The presence of zinc, phosphorus, chlorine, sulphur, silicon and some traces of calcium were detected in these deposits

by X-ray microanalysis. Zinc peaks were correlated with phosphorus peaks in the analytical spectra (Fig. 2). In zinc-exposed prawns, B-cells showed similar spherical deposits within the digestive vacuole as in the control group (Fig. 2). The electron dense contents were distributed equally through the vacuole, in close contact with the enclosing membrane (Figs. 2, 3). On occasions the vacuole contents showed a more diffuse shape, not being clearly spherical, although equally electron dense. Slightly different metal-rich deposits were observed in R- and F-cells. Several of these cells were observed in the hepatopancreatic tubules, decreasing in number towards the proximal region. Both R- and F-cells contained a very well developed supranuclear vacuole, cytoplasmic processes and concentric deposits of electron dense material, and X-ray analysis showed the presence of chlorine, sulphur, phosphorus, and copper mainly in these deposits (Fig. 4). Although traces of zinc were detected (on a few occasions) associated with these concentric granules, its signal was always negligible (Fig. 4), compared to the signal obtained from the deposits in B-cells.

It is important to highlight that there was no detection of cadmium in any electron dense deposit in any group of samples, even those prawns exposed to 100 μg Cd l^{-1} for 10 days, although cadmium was accumulated in the cells (Table 1).

Gill ultrastructure

Only four of the five gill epithelial cell types (thick cells, attenuated cells, pillar cells and flange cells) were found to be present in *P. indicus* in this study, the thin cells being absent (in agreement with Foster and Howse 1978).

Electron microscopical analysis of the gills showed no alteration of the ultrastructure of cells of either zinc- or cadmium-exposed prawns. No evidence of necrosis nor cell damage was detected when compared with controls. The presence of granular cells containing multiple dense bodies was observed in all the samples in the epithelial layer or in the lacuna, either free or in close contact with the basal lamella, beneath the gill epithelium, but they were more frequent in prawns exposed to cadmium (Fig. 5a). These granular cells were localized below the epithelium layer, in close contact with nephrocytes and in the haemolymph. The analysis of the multiple deposits within these granular cells showed the presence of zinc, sulphur, chlorine, silicon, phosphorus, calcium and traces of copper (Fig. 5b).

Individual round electron dense deposits of about 0.9 μm in diameter were also observed in epithelial cells (pillar and flange cells) from all groups (Fig. 6a), normally orientated towards the inner side of the cell (facing the basal lamella). Zinc was the main constituent detected, but traces of iron and copper were also present (Fig. 6b).

Inside the haemolymph vessels it was possible to observe nephrocytes and haemocytes. Nephrocytes were in close contact with the epithelial cell basal lamellae, while haemocytes were localized at different levels inside the haemolymph space (lacuna) and were more cylindrical in shape (Fig. 7a). The electron dense

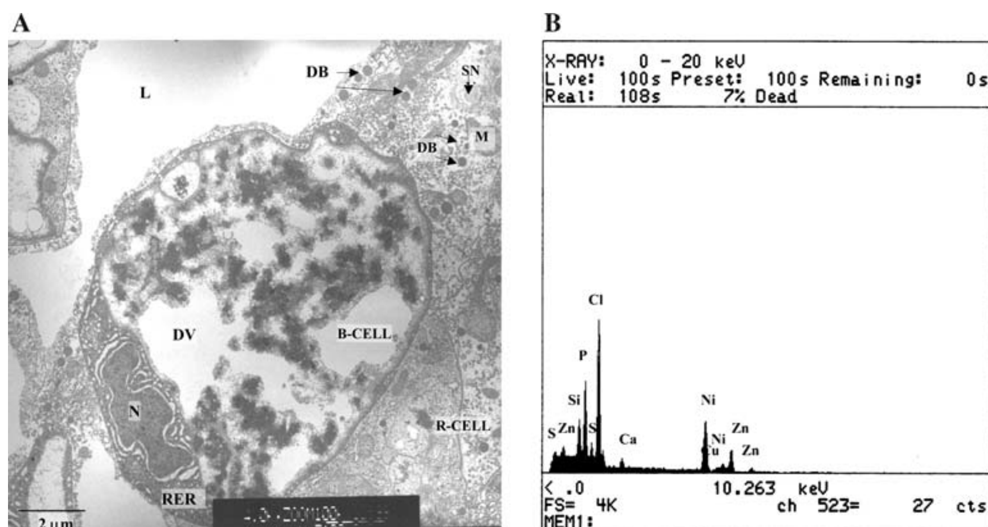

Fig. 2 **a** B-cell after 10 days of exposure to 100 μg Zn l^{-1}. **b** X-ray spectrum derived from digestive vacuole (*DV*) electron dense material, showing peaks of Cl, P, Si, Zn and Ca. Ni is derived from section-support grid. *L*, lumen; *N*, nucleus; *RER*, rough endoplasmic reticulum; *DB*, dense body; *M*, mitochondria; *SNV*, supranuclear vacuole

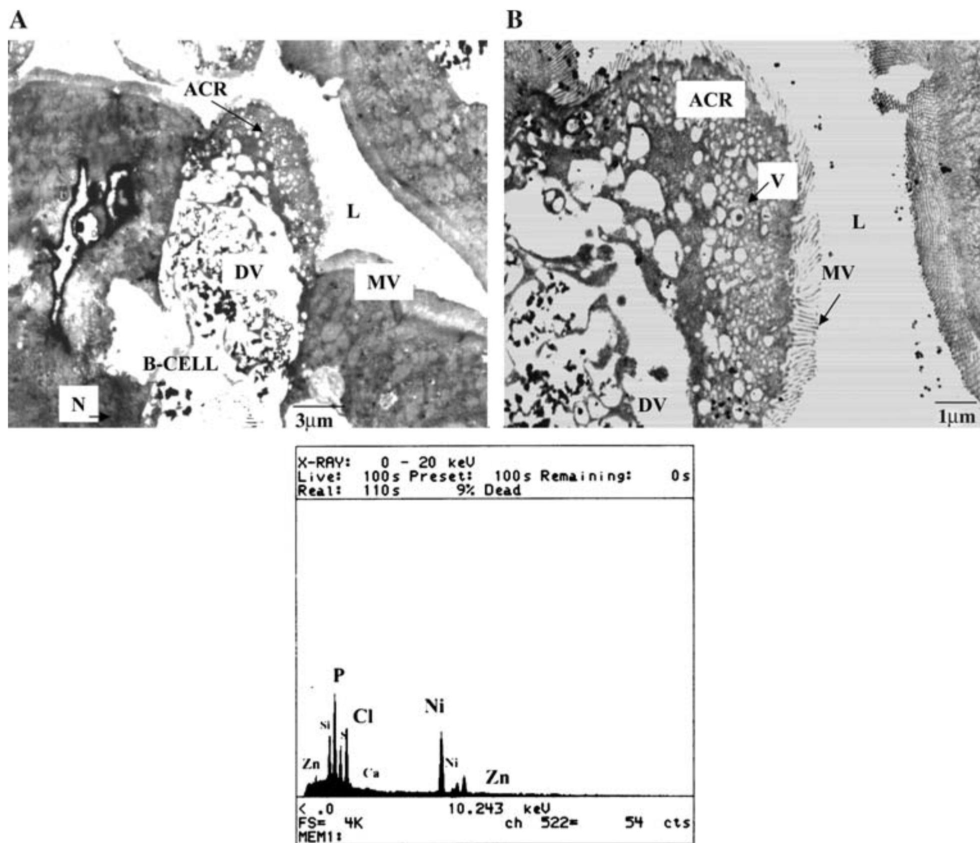

Fig. 3 **a** B-cell in the hepatopancreas of control prawns showing a digestive vacuole (*DV*) and apical complex region (*ACR*) in extrusion phase. **b** ACR with electron dense material. **c** The electron dense deposits within the digestive vacuole and ACR contain Zn, P, Cl, S, and Si mainly. *L*, lumen; *MV*, microvilli; *N*, nucleus; *V*, vacuole

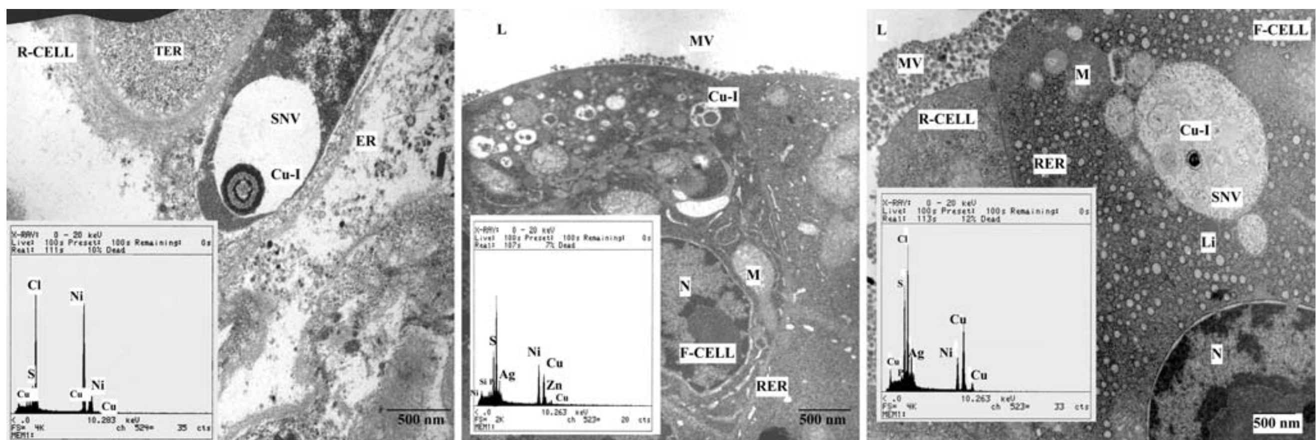

Fig. 4 Copper-rich inclusions (*Cu-I*) present in F- and R-cells, and X-ray spectra derived from these inclusions showing peaks of Cu, Cl, P, Si, Zn and Ag. Ni is derived from section-support grid. *L*, lumen; *N*, nucleus; *RER*, rough endoplasmic reticulum; *MV*, microvilli; *M*, mitochondria; *SNV*, supra nuclear vacuole; *Li*, lipid; *TER*, transitional elements of the rough endoplasmic reticulum

deposits inside these cells varied in size and number, and gave the cytosol a granular appearance. Their contents varied little, X-ray microanalysis showing peaks of zinc, calcium, chlorine, phosphorus, sulphur, magnesium, iron and copper (Fig. 7b). Their presence was detected equally in metal-exposed and control prawns.

The last type of electron dense deposits observed in gill tissue is shown in Fig. 8a. This deposit consisted of multiple dense bodies with a circular shape in general, localized in the cytosol of epithelial cells. Calcium was the main element detected, but traces of other metals including zinc and magnesium were also observed (Fig. 8b).

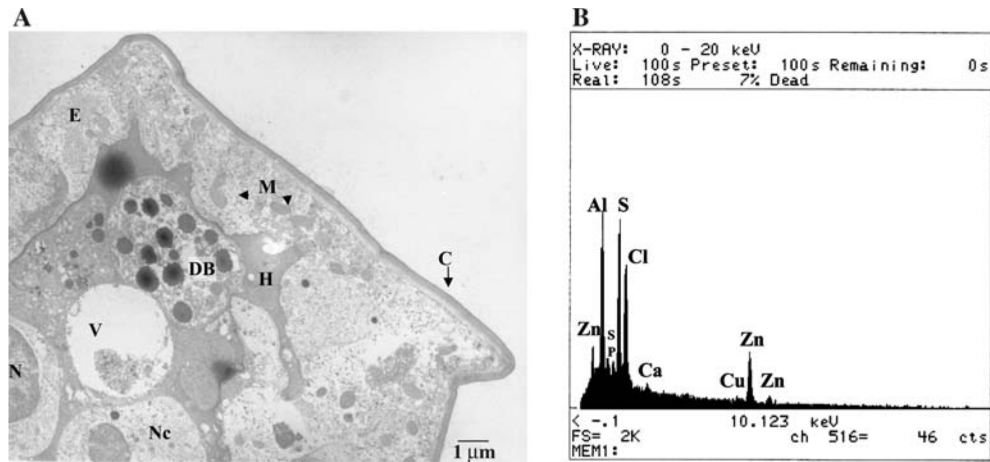

Fig. 5 **a** Granular cell below to the gill epithelium (*E*) in the haemolymph space, from prawns exposed to cadmium (100 µg Cd l^{-1} for 10 days). **b** These cells showed multiple dense bodies (*DB*) containing Zn, S, P, Cl, Cu, and Ca. Al peaks are derived from the supporting grid. *C*, cuticle; *E*, epithelium; *H*, haemolymph; *M*, mitochondria; *N*, nucleus; *Nc*, nephrocyte; *V*, vacuole. Aluminium peaks produced from section-grids

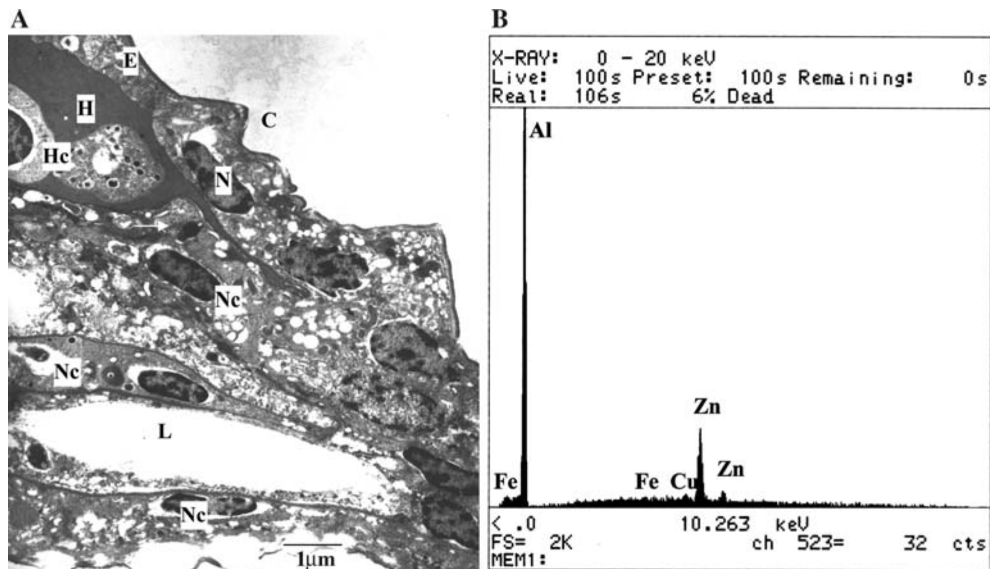

Fig. 6 **a** Electron dense deposit (*arrow*) present in gill epithelial cell (*E*) from *P. indicus*. **b** X-ray analysis from these deposits (*arrow*) showing the presence of zinc. Aluminium peak is derived from the supporting grid. *C*, cuticle; *H*, haemolymph; *L*, lacuna; *M*, mitochondria; *N*, nucleus; *Nc*, nephrocytes, and *Hc*, haemocytes

In metal-exposed prawns, no increase in the number of nephrocytes was observed compared to controls. As was specified for the hepatopancreas cells, no cadmium-rich inclusions were detected by X-ray analysis of gill cells.

Metallothionein-like proteins

DPP analyses showed the presence of MTLP reduction waves (Fig. 9) in hepatopancreas and gill samples from control and metal-treated prawns, between 1.45 and 1.55 V, within the range observed for rabbit liver metallothionein.

MTLP concentrations did not differ significantly between either tissue samples from control and experimentally exposed prawns, although a small (non-significant) increase in the mean concentration of MTLP was found in the hepatopancreas of cadmium-treated prawns (Fig. 10). The concentrations observed were 18.1 ± 3.9 mg g^{-1} (cadmium-treated prawns), 12.6 ± 4.2 mg g^{-1} (zinc-treated prawns) and 13.5 ± 4.3 mg g^{-1} (control prawns). Similar results were observed in the gills, where metal-exposed prawns showed a mean concentration greater than in the controls, but not significantly so (Fig. 10). The concentrations of MTLP in the gills were 7.1 ± 1.2 mg g^{-1} in cadmium-exposed

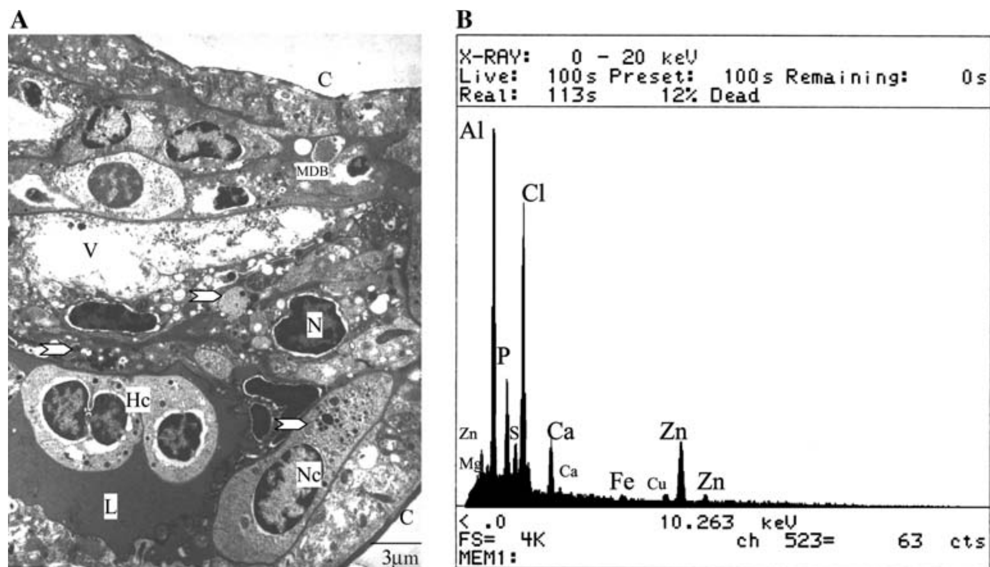

Fig. 7 **a** Electron dense deposits present in nephrocytes (*Nc*) and haemocytes (*Hc*) cells from gills of *P. indicus*. **b** X-ray analysis from dense round deposits in nephrocytes (*arrows*) contained Zn, Cl, and P mainly. *C*, cuticle; *H*, haemolymph; *L*, lacuna; *MDB*, multiple dense bodies; *N*, nucleus; *V*, vacuole

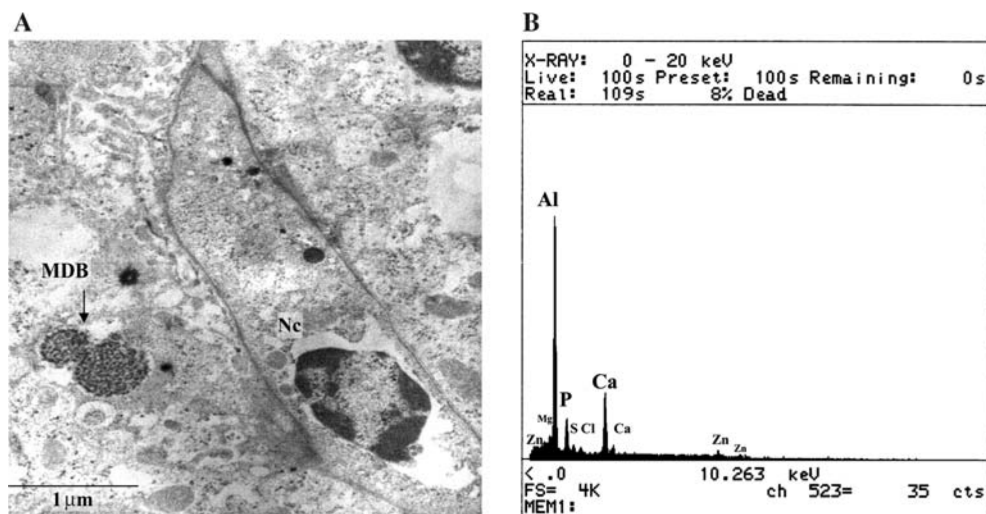

Fig. 8 **a** Multiple dense bodies (*MDB*, *arrow*) in epithelial cells from gills of *P. indicus*, with Ca, P and Zn peaks after X-ray microanalysis. Aluminium peak from uncoated grid (**b**). *Nc*, nephrocytes

prawns, 8.1 ± 2.2 mg g^{-1} in zinc-exposed prawns and 5.5 ± 0.7 mg g^{-1} in controls.

Discussion

Subcellular insoluble fraction

The formation of metal-rich insoluble deposits or granules in the hepatopancreas and gills of *P. indicus* varied between tissues and among cells within each tissue. No cadmium-rich deposits were identified but zinc-rich deposits were present in B-, R- and F-cells of the hepatopancreas, and in epithelial cells, haemocytes and nephrocytes in the gills.

In the particular case of the hepatopancreas, the B-cells contained the main zinc deposits, storing this essential metal in lysosomal residual bodies, which congregated in a big supranuclear vacuole which is eventually extruded into the lumen following the normal turnover process of the cells (Al-Mohanna and Nott 1985; Andersen and Baatrup 1988). Similar reports of zinc deposits in hepatopancreatic cells (including B-cells) have been made in the case of *P.*

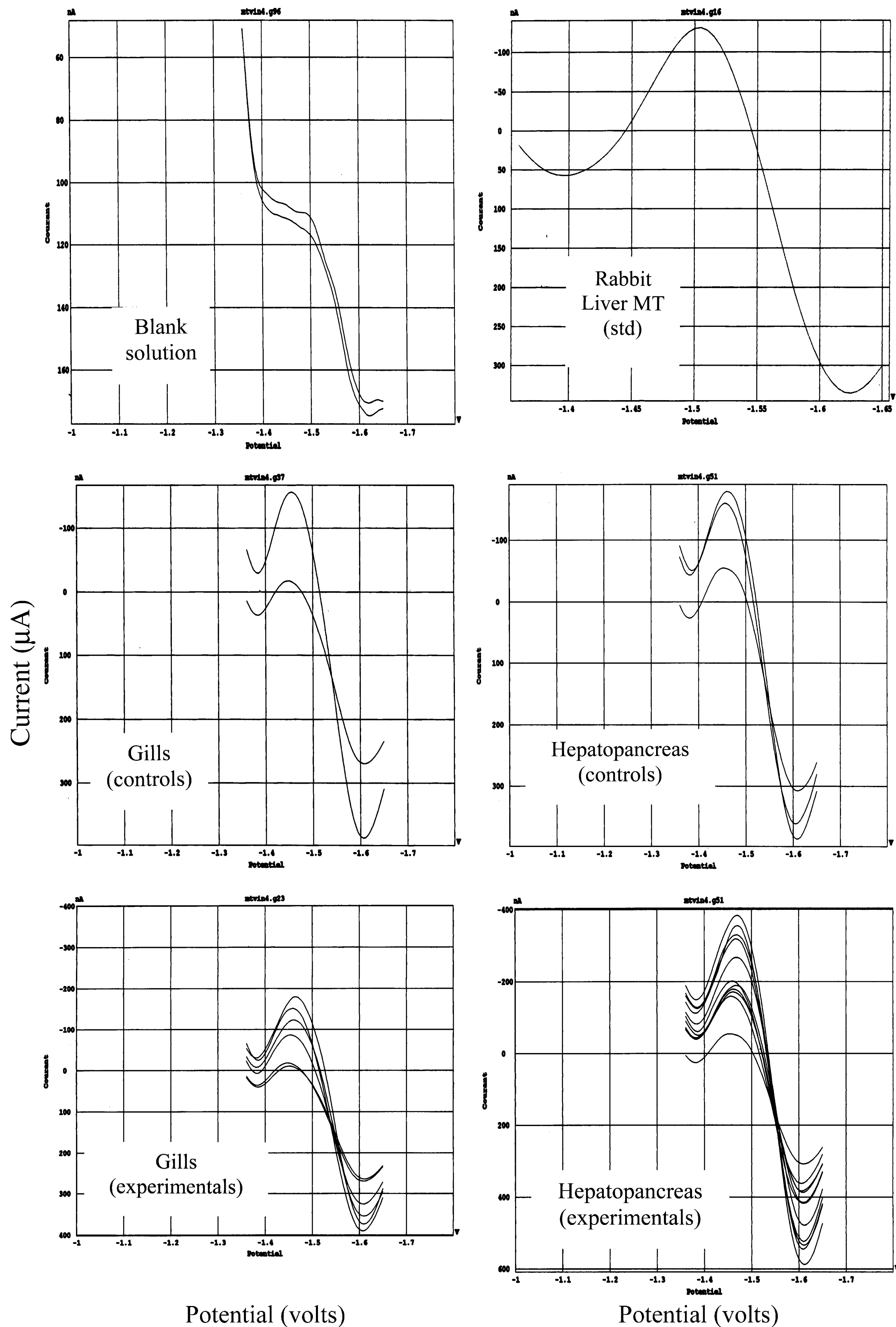

Fig. 9 Differential pulse polarograms showing B reduction waves of the rabbit liver MT and MTL proteins of gills and hepatopancreas from *P. indicus* exposed to 100 µg l^{-1} of zinc or cadmium for 10 days, and controls. B waves (presence of MTLP) observed in all the tissues analysed

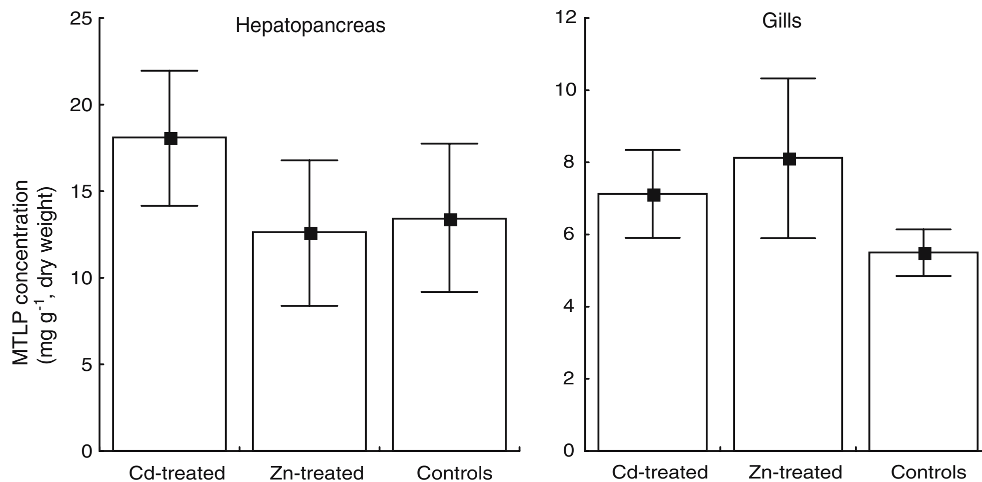

Fig. 10 Metallothionein like protein concentrations (mean ± sd, $n = 3$) detected in the soluble fraction of hepatopancreas and gills from prawns exposed for 10 days to 100 μg l^{-1} of cadmium or zinc

semisulcatus, in vacuoles (lysosomes) also containing copper (Al-Mohanna and Nott 1987a). Al-Mohanna and Nott (1987a) proposed that metals are taken up from haemolymph through the basal membranes of the R-cells and carried via small cytosolic inclusions to be then incorporated into supranuclear vacuoles. Metal inclusions have also been detected in F-cells and B-cells in *P. semisulcatus*, and these metal incorporations are considered to occur by the same processes (Al-Mohanna and Nott 1987a).

Vogt and Quinitio (1994) analysed the accumulation of copper, lead, iron and calcium in the hepatopancreas of *P. monodon*, and concluded that metal granule formation and excretion varied greatly according to the metal tested. This was also observed in *P. indicus* in the present study, where zinc deposits found in B-cells showed variability in appearance, sometimes forming small circular deposits or with a more diffuse shape. Copper-rich deposits in R- and F-cells showed a more stable appearance, with a concentric round shape, but the presence of zinc in these copper-rich deposits was minimal. It is possible that these copper-rich deposits play a more important role in zinc sequestration at higher concentrations of zinc exposure, as a result of greater zinc accumulation, as has been observed in R-cells of other crustaceans (Al-Mohanna and Nott 1985; Nassiri et al. 2000). This particular cell type (the R-cell) has been shown to accumulate copper from water exposure, diverting the metal into concentric deposits for detoxification (Vogt and Quinitio 1994).

The presence of detoxified metal granules has been observed in crustaceans from both polluted and non-polluted areas (Al-Mohanna and Nott 1985; Icely and Nott 1992; Nassiri et al. 2000). In the amphipod *Orchestia gammarellus*, Nassiri et al. (2000) considered the presence of such metal-rich deposits as a physiological adaptation to the accumulation of toxic metals, representing a major detoxification pathway. In *P. indicus*, the fact that these zinc deposits were also present in the B-cells from control prawns, suggests that these metal deposits are also formed during normal regulation of cell zinc concentrations. Zinc is temporarily stored in the hepatopancreas as a normal process of zinc accumulation in *P. indicus*, and some newly accumulated zinc can be excreted from the body (Nunez-Nogueira and Rainbow 2005a). Al-Mohanna and Nott (1985) proposed that the metal regulatory capacity of the hepatopancreas is affected by the moult cycle. In *P. semisulcatus*, the zinc concentration in the hepatopancreas rises when the prawns move into the intermoult stage, the zinc being stored within membrane-limited granules. During this period the density of B-cells also increases (Al-Mohanna and Nott, 1989), presumably contributing to the detoxification of any increased stored zinc concentration observed in *P. indicus* (Nunez-Nogueira and Rainbow 2005a).

Zinc in *P. indicus* was found in phosphorus-rich deposits in the hepatopancreas, classified as Type A granules according to Hopkin (1989). These zinc inclusions are similar to metal "type I" deposits reported previously in *P. semisulcatus* by Al-Mohanna and Nott (1985). Pinocytosis is the process considered to be involved in metal uptake from the lumen by B-cells (Caceci et al. 1988; Al-Mohanna and Nott 1989). Al-Mohanna and Nott (1989) considered that this uptake route occurs mainly during pre-moult stages, after differentiation of the F-cells into B-cells. It has also been suggested that the material diverted into these vacuoles developed in B-cells may have a "storage" role during starvation and after ecdysis (Al-Mohanna

and Nott 1989), but it is difficult to conclude this in *P. indicus* from microscopic observations alone.

Transport of cell contents into, and from, the haemolymph has been attributed mainly to R-cells, during intermoult and premoult stages (Al-Mohanna and Nott 1987b). Metal-phosphate deposits found in R-cells of *P. semisulcatus* are considered to be resorbed during and immediately after ecdysis, their calcium content being required for hardening the exoskeleton (Al-Mohanna and Nott 1987b). The absence of significant zinc deposits in R-cells of *P. indicus* suggests that B-cells are the predominant cell type involved in zinc sequestration at sub-lethal concentrations of zinc exposure under the experimental conditions employed, most probably as zinc phosphate.

Penaeus indicus when exposed to 10 μg labelled zinc l^{-1} at 15 salinity and 25°C accumulated labelled Zn in the hepatopancreas at a rate of 4.9 μg Zn g^{-1} day^{-1} (Nunez-Nogueira and Rainbow 2005a). During a subsequent 10 day depuration phase, the labelled zinc concentration of the hepatopancreas decreased at 4.7 μg g^{-1} day^{-1}. On the other hand, the total zinc concentration of the hepatopancreas of *P. indicus* did not change, suggesting equal influx and efflux of metal into and out of the hepatopancreas. Metal inclusions in the hepatopancreas of *Penaeus* prawns have been reported to be expelled into the lumen together with epithelial cells which undergo lysis once in the lumen, and then all the debris is excreted through the faeces (Vogt and Quinitio 1994). This seems to explain, at least partially, the zinc loss observed in *P. indicus* (Nunez-Nogueira and Rainbow 2005a).

This form of discharge is apparently related not only to the moult cycle as mentioned above, but also to the hepatopancreas cell cycle and feeding cycle (Hopkin and Nott 1980; Al-Mohanna and Nott 1989). Total renovation of the hepatopancreatic epithelium requires a few days (5 days in adult decapods; Davis and Burnett 1964), being faster in juveniles, and probably promoted in well fed organisms (Vogt and Quinitio 1994). According to Al-Mohanna and Nott (1989), this cell extrusion into the lumen occurs during the beginning and final stages of the pre-moult period for F- and B-cells and at the end of post-moult for R-cells. The fact that accumulation and loss rates of Zn in the hepatopancreas were similar (Nunez-Nogueira and Rainbow 2005a) explains the observation that the total zinc concentration of the hepatopancreas of *P. indicus* showed no significant change in the experiments conducted here with no net zinc accumulation in the hepatopancreas (Table 1). If most of the senescent B-cells are excreted before moulting, this could be the explanation for part of the loss of labelled zinc of *P. indicus* during the depuration phase observed previously (Nunez-Nogueira and Rainbow 2005a). Under the same experimental conditions as employed in the present study, the prawns showed a mean period of 10 days between moults (Nunez-Nogueira and Rainbow 2005a), implying that cellular discharges could have contributed during the depuration phase because most of the prawns in that previous study (11 out of 13) were in the late premoult stage (between 6 and 18 days after the last moult) when dissected.

Gills also showed the presence of zinc deposits in epithelial cells, nephrocytes and haemocytes. These deposits varied in appearance and content. Haemocytes found in the gill lacunae showed a granular cytoplasm in all the experimental groups. The spherical granules showed considerable zinc and sulphur peaks after X-ray microanalysis. Haemocytes in gills of decapods have been proved to be involved in metal sequestration (e.g. mercury) after uptake from solution (Andersen and Baatrup 1988). The epithelial layer is considered to be the first barrier against toxic metal exposure, while cells in the haemolymph are the site of main phagocytic activity and are capable of fast sequestration of particulates within the gills (Martin and Hose 1992). Sequestration of metals by these granulocytes probably occurs during their passage through the open and closed vascular system of the gills (Foster and Howse 1978). The presence of single electron-dense deposits containing zinc facing the haemolymph in epithelial cells (Fig. 6) suggests a temporary storage of the metal in the epithelial layer before transfer to the blood, although zinc excretion through the gills has been considered as a possible adaptation or reaction to metal pollution (Gilles and Pequeux 1983).

Nephrocytes within the gills also showed the presence of zinc deposits. This cell type contains a vacuolated cytoplasm, with light granular contents. These granules seem to be accumulated gradually according to the moult stage, being released into the blood during the next moult (Taylor and Taylor 1992). Nephrocytes, although not forming an epithelium, can appear in groups of several cells in the septum or lying free surrounded by the haemolymph (Taylor and Taylor 1992).

In the case of cadmium, no cadmium-rich deposits were observed in any sample, either of hepatopancreas or gills. Nevertheless, confirmation of cadmium accumulation in the tissue was obtained by AAS (Table 1), indicating that this non-essential metal remains in the cytosol (soluble fraction of the tissue) in a different form. Compartmentalization analysis showed that 86% of cadmium was present in the soluble fraction of the hepatopancreas, indicating that cadmium is being handled and detoxified in a different way from zinc, most

probably by soluble metal-chelating molecules. One of the strategies for soluble trace metal detoxification in aquatic invertebrates involves metallothioneins (or strictly MTLP) (Roesijadi 1981, 1993; Engel and Roesijadi 1987; Carpene 1993; Viarengo 1999).

Subcellular soluble fraction

Metallothioneins (MTs) or MTLP have been found in the hepatopancreas and gills of decapod crustaceans (Rainbow and Scott 1979; Wong and Rainbow 1986; Engel and Brouwer 1993; del Ramo et al. 1995; Canli et al. 1997; Legras et al. 2000; Mouneyrac et al. 2001; Pourang et al. 2004), including *Penaeus* species (Moknes et al. 1995). In the crab *Carcinus maenas*, Rainbow and Scott (1979) observed that cadmium-binding proteins can be present in the hepatopancreas of field-collected specimens as well as in crabs exposed to high concentrations of cadmium in solution. Similar results have been observed in other decapods including the blue crab *Callinectes sapidus* (Engel and Brouwer 1993), the crayfish *Procambarus clarkii* (Del Ramo et al. 1995), and the Norway lobster *Nephrops norvegicus* (Canli et al. 1997). Moksnes et al. (1995) observed that metallothionein concentrations in the hepatopancreas of *P. vannamei* increased by more than 100% in a dose-dependent response when the prawns were exposed to an abnormally high cadmium concentration (1.5 mg Cd l^{-1}) for 9 days.

The kinetic study of newly taken up cadmium in the hepatopancreas and gills of *P. indicus* showed that the hepatopancreas accumulated labelled cadmium at a rate of 8 ng day^{-1}, when exposed to 10 μg labelled Cd l^{-1} (at 25°C and 15 salinity), while the gills showed an uptake rate of 6 ng day^{-1}, without significant loss during the depuration phase (Nunez-Nogueira and Rainbow, 2005b), showing that cadmium is stored in these tissues. During this study, 85% of total cadmium burden is localized in the soluble fraction of the hepatopancreas after 100 μg l^{-1}cadmium exposure for 10 days, while 90% was found in the soluble fraction of the gills.

MTLP were found in hepatopancreatic and gill tissue from *P. indicus* (an average of 18 mg g^{-1} in hepatopancreas and 7.1 mg g^{-1} in gills, respectively), with the potential to play a role in cadmium sequestration and detoxification in this soluble cellular. The conclusion that Cd is predominantly detoxified by MTLP in *P. indicus* is also supported by the absence of cadmium-rich insoluble subcellular inclusions.

MTs have been found in high concentrations (compared to other organs) in the hepatopancreas of decapod crustaceans (Chavez-Crooker et al. 2003). Chavez-Crooker et al. (2003) considered that hepatopancreatic E-cells in the lobster *Homarus americanus* play a significant role in heavy metal dietary uptake and sequestration, based on their high metallothionein (MT) concentration with MT translocation during cell division. These authors also suggest that MTs may be involved only in metal sequestration in hepatopancreatic R- F- and B-cells.

The presence of MTLP in gill tissue suggest a role for the gills in detoxification mechanisms and not only in transitory metal uptake and loss. Nimmo et al. (1977) considered that gills in *P. duorarum* could be a site for cadmium excretion, collecting the metal from circulating haemocytes to be accumulated within the gills for later elimination by the sloughing off of affected portions of the gills. An increase in the number of nephrocytes in gill filaments after cadmium exposure has also been observed in *P. japonicus* exposed to 200 μg Cd l^{-1} for 15 days (Soegianto et al. 1999b), suggesting that this type of response occurs at acute metal exposure. It is highly probable that most part of the metal accumulated in the gill of *P. indicus* is related to the presence of nephrocytes, with a smaller part stored temporarily in the cytoplasm of the gill epithelial cells, bound to MTLP (Chavez-Crooker et al. 2003).

It is concluded that in *P. indicus* binding to MTLP is the detoxification mechanism for cadmium, while the detoxification of zinc involves both binding to MTLP and incorporation into insoluble metal-rich subcellular inclusions. Zinc-rich inclusions are developed within B-, R- and F-cells in the hepatopancreas. Traces of zinc are mainly localized within the digestive vacuole in B-cells associated with phosphorus, while R- and F-cells contain zinc associated with sulphur and phosphate in supranuclear vacuoles. These latter deposits were less common compared to those in B-cells. Subcellular zinc deposits in gills were detected in granular and epithelial cells, and within capillary vessels, particularly in nephrocytes and haemocytes. Cadmium, on the other hand, is only detected in the soluble fraction of the cells in both tissues and is not stored in metal-rich cellular inclusions. MTLP in the hepatopancreas and gills are also involved in the sequestration and storage of zinc, perhaps transporting zinc for deposition within insoluble deposits.

Acknowledgments This work was supported by funding to GN-N from CONACyT and SEP-Mexico, and by funding to Dr. C. Amiard-Triquet (Service d'Ecotoxicologie, ISOMer, Nantes, France). It is a pleasure to acknowledge the considerable technical support of Keith Pell (Queen Mary College, University of London), and Dr. Alex Ball (The Natural History Museum, London), and for training and advice on the X-ray microanalysis and TEM analysis, respectively. We are also grateful for the technical support provided by CEREA-UCO (Angers, France).

References

Al-Mohanna SY, Nott JA (1985) The accumulation of metals in the hepatopancreas of the shrimp *Penaeus semisulcatus* de Haan (Crustacea: Decapoda) during the moult cycle. In: Halwagy R, Clayton D, Behbehani M (eds) Marine environment and pollution. University of Kuwait, Kuwait pp 195–207

Al-Mohanna SY, Nott JA (1987a) R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). Mar Biol 95:129–137

Al-Mohanna SY, Nott JA (1987b) M-'Midget' cells and moult cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). J Mar Biol Assoc UK 67:803–813

Al-Mohanna SY, Nott JA (1989) Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda) during the moult cycle. Mar Biol 101:535–544

Amiard JC, Pineau A, Boiteau H, Metayer C, Amiard-Triquet C (1987) Application of atomic absorption spectrophotometry using Zeeman effect to the determination of eight trace elements (Ag, Cd, Cr, Cu, Mn, Ni, Pb, and Se) in biological materials. Water Res 21:693–697

Andersen JT, Baatrup E (1988) Ultrastructural localization of mercury accumulation in the gills, hepatopancreas, midgut, and antennal glands of the brown shrimp, *Crangon crangon*. Aquat Toxicol 13:309–324

Arruda-Freire C, Campbell-McNamara J (1995) Fine structure of the gills of the fresh-water shrimp *Macrobrachium olfersii* (Decapoda): effect of acclimation to high salinity medium and evidence for involvement of the lamellar septum in ion uptake. J Crust Biol 15:103–116

Bryan GW (1976) Some aspects of heavy metal tolerance in aquatic organisms. In: Lockwood APM (eds) Effects of pollutants on aquatic organisms. Cambridge University Press, London pp 7–34

Bryan GW, Langston WJ, Hummerstone LG, Burt GR (1985) A guide to the assessment of heavy-metal contamination in estuaries using biological indicators. Occ Publ Mar Biol Assoc UK 4:1–92

Caceci T, Neck KF, Lewis DH, Sis RF (1988) Ultrastructure of the hepatopancreas of the Pacific white shrimp, *Penaeus vannamei* (Crustacea: Decapoda). J Mar Biol Assoc UK 68:323–337

Canli M, Stagg RM, Rodger G (1997) The induction of metallothionein in tissues of the Norway lobster *Nephrops norvergicus* following exposure to cadmium, copper and zinc: the relationship between metallothionein and the metal. Environ Pollut 96(3):343–350

Chavez-Crooker P, Pozo P, Castro H, Dice MS, Boutet I, Tanguy A, Moraga D, Ahearn GA, (2003) Cellular localization of calcium, heavy metals, and metallothionein in lobster (*Homarus americanus*) hepatopancreas. Comp Biochem Physiol C 136:213–224

Campbell MJ, Radecki Z, Trinkl A, Burns KI (2000) Report on the intercomparison runs for the determination of trace and minor elements in cabbage material. Rep. IAEA/AL/123, IAEA-359, Vienna

Coquery M, Horvat M (1996) The analytical performance study for the MED POL area: determination of trace elements in marine sediments SD-MEDPOL-1/TM and fish homogenate MA-MEDPOL-1/TM. Report IAEA, Monaco

Carpene E (1993) Metallothionein in marine molluscs. In: Dallinger R, Rainbow PS (eds) Ecotoxicology of metals invertebrates. Lewis Publishers, Boca Raton pp 55–72

Chinni S, Yallapragada PR (2000) Toxicity of copper, cadmium, zinc and lead to *Penaeus indicus* postlarvae: effects of individual metals. J Environ Biol 21:255–258

Correa-Junior JD, Allodi S, Amado-Filho GM, Farina M (2000) Zinc accumulation in phosphate granules of *Ucides cordatus* hepatopancreas. Braz J Med Biol Res 33:217–221

Dall W, Moriarty DJ (1983) Functional aspects of nutrition and digestion. In: Mantel LH (eds) The biology of Crustacea, internal anatomy and physiological regulation. Academic Inc. NewYork pp 215–261

Davis LE, Burnett AL (1964) A study of growth and cell differentiation in the hepatopancreas of the cryfish. Dev Biol 10:122–153

Del Ramo J, Torreblanca A, Martinez MAP, Diaz-Mayans J (1995) Quantification of cadmium-induced metallothionein in crustaceans by the silver-saturation method. Mar Environ Res 39:121–125

Engel DW, Brouwer M (1993) Crustaceans as models for metal metabolism: I. Effects of the molt cycle on blue crab metal metabolism and metallothionein. Mar Environ Res 35:1–5

Engel DW, Roesijadi G (1987) Metallothioneins: a monitoring tool. In: Vernberg FJ (eds) Pollution physiology of estuarine organisms. University of South Carolina Press, USA, pp 421–438

Foster CA, Howse HD (1978) A morphological study on gills of the brown shrimp, *Penaeus aztecus*. Tissue Cell 10:77–92

Gibson R, Barker PL (1979) The decapod hepatopancreas. Oceanogr Mar Biol Ann Rev 17:285–346

Gilles R, Pequeux A (1983) Interactions of chemical and osmotic regulation with the environment. In: Vernberg FJ, Vernberg WB (eds) The biology of Crustacea. Environmental adaptations, vol. 8. Academic, New York, pp 109–177

Grey DL, Dall W, Baker A (1983) A guide to the Australian Penaeid prawns. Northern Territory Government Printing Office, Darwin, Australia. 140 pp

Hirsch GC, Jacobs W (1930) Der Arbeitsrhythmus der Mitteldarmdruse von Astacus leptodactyus. II. Teil: Wachstum als primarer Faktor des Rhythmus eines polyphasischen organigen Sekretionssystems. Z Vergl Physiol 12:524–557

Holthuis LB (1980) FAO Species Catalogue. Vol. 1. Shrimps and prawns of the world. An annotated catalogue of species of interest to fisheries. Food and Agriculture Organization of the United Nations, Rome, 271 pp

Hopkin SP (1989) Ecophysiology of metals in terrestrial invertebrates. Elsevier Applied Science, London, 366 pp

Hopkin SP, Nott JA (1980) Studies on the digestive cycle of the shore crab *Carcinus meanes* (L.) with special reference to the B-cells in the hepatopancreas. J Mar Biol Assoc UK 60:891–907

Icely JD, Nott JA (1992) Digestion and absorption: digestive system and associated organs. In: Harrison FW, Humes AG (eds) Microscopic anatomy of invertebrates, Decapod Crustacea, vol. 10. Wiley-Liss Inc, New York, pp 147–201

Jacobs W (1928) Untersuchungen uber die Cytologie der Sekretbildung in der Mitteldarmdruse von *Aztecus leptodactylus*. Z Zellforsch Mikrosk Anat 8:1–62

Johnston W, Barber AA (1969) Reconstitution of functional hemocyanin from apohemocyanin: the hepatopancreas as copper donor. Comp Biochem Physiol 28:1259–1273

Joseph KO, Srivastava JP, Kadir PMA (1992) Acute toxicity of five heavy metals to the prawn, *Penaeus indicus* H. Milne Edwards in brackishwater medium. J Inland Fish Soc India 24:82–84

Law RJ, Waldock MJ, Allchin CR, Laslett RE, Bailey KJ (1994) Contaminants in seawater around England and Wales: results from monitoring surveys, 1990–1992. Mar Pollut Bull 28:668–675

Legras S, Mouneyrac C, Amiard JC, Amiard-Trichet C, Rainbow PS (2000) Changes in metallothionein concentrations in response to variation in natural factors (salinity, sex, weight)

and metal contamination in crabs from a rich-metal estuary. J Exp Mar Biol Ecol 246:259–279

Le Reste L (1978) Biologie d'une population de crevettes *Penaeus indicus* H. Milne Edwards sur la cote nord-ouest de Madagascar. O.R.S.T.O.M., Paris. 291 pp

Marigomez I, Soto M, Carajaville MP, Angulo E, Giamberini L (2002) Cellular and subcellular distribution of metals in molluscs. Microsc Res Technol 56:358–392

Martin GG, Hose E (1992) Vascular elements and blood (Hemolymph). In: Harrison FW, Humes AG (eds) Microscopic anatomy of invertebrates, Decapod Crustacea, vol. 10. Wiley-Liss Inc, New York, pp 117–149

Mason AZ, Jenkins KD (1995) Metal detoxification in aquatic organisms. In: Tessier A, Turner RA (eds) Metal speciation and bioavailability in aquatic systems, vol. 3. Wiley, Chichester, pp 479–578

McClurg TP (1984) Effects of fluoride, cadmium and mercury on the estuarine prawn, *Penaeus indicus*. Water SA 10:40–45

Moksnes PO, Lindahl U, Haux C (1995) Metallothionein as a bioindicator of heavy metal exposure in the tropical shrimp, *Penaeus vannamei*: a study of dose-dependant induction. Mar Environ Res 39:143–146

Mouneyrac C, Amiard-Trichet C, Amiard JC, Rainbow PS (2001) Comparison of metallothionein concentrations and tissue distribution of trace metals in crabs (*Pachygrapsus mamoratus*) from a metal-rich estuary, in and out the reproductive season. Comp Biochem Physiol C 129:193–209

Nassiri Y, Rainbow PS, Amiard-Triquet C, Rainglet F, Smith BD (2000) Trace-metal detoxification in the ventral caeca of *Orchestia gammarellus* (Crustacea: Amphipoda). Mar Biol 136:477–484

Nimmo DWR, Lightner DV, Bahner LH (1977) Effects of cadmium on the shrimps, *Penaeus duorarum, Palaemonetes pugio* and *Palaemonetes vulgaris*. In: Vernberg FJ (Ed) Physiological responses of marine biota to pollutants. Academic, New York, pp 131–183

Nunez-Nogueira G, Rainbow PS (2005a) Kinetics of zinc uptake from solution, accumulation and excretion by the decapod crustacean *Penaeus indicus*. Mar Biol 147:93–103

Nunez-Nogueira G, Rainbow PS (2005b) Cadmium uptake and accumulation by the decapod crustacean *Penaeus indicus*. Mar Environ Res 60:339–354

Nunez-Nogueira G, Smith BD, Rainbow PS (2006) Assimilation efficiency of zinc and cadmium in the decapod crustacean *Penaeus indicus*. J Exp Mar Biol Ecol 332:75–83

Olafson RW, Olsson PE (1991) Electrochemical detection of metallothionein. Method Enzymol 205:205–213

Papathanassiou E, King PE (1984) Effects of starvation on the fine structure of the hepatopancreas in the common prawn *Palaemon Serratus* (Pennant). Comp Biochem Physiol 77A:243–249

Pérez-Farfante I, Kensley B (1997) Penaeoid and sergestoid shrimps and prawns of the world. Mémoires du Museum National d'Histoire Naturelle, Paris 175, pp 1–233

Pourang N, Dennis JH, Ghourchian H (2004) Tissue distribution and redistribution of trace elements in shrimp species with emphasis on the roles of metallothionein. Ecotox 13:519–533

Rainbow PS (1997) Ecophysiology of trace metal uptake in crustaceans. Estuar Coast Shelf Sci 44:169–175

Rainbow PS (1998) Phylogeny of trace metal accumulation in crustaceans. In: Langston WJ, Bebianno M (eds) Metal metabolism in aquatic environments. Chapman and Hall, London pp 285–319

Rainbow PS, Scott AG (1979) Two heavy metal-binding proteins in the midgut gland of the crab *Carcinus maenas*. Mar Biol 55:143–150

Roesijadi G (1981) The significance of low molecular weight, metallothionein-like proteins in marine invertebrates: current status. Mar Environ Res 4:167–179

Roesijadi G (1993). Metallothioneins in metal regulation and toxicity in aquatic animals. Aquat Toxicol 22:81–114

Soegianto A, Charmantier-Daures M, Trilles JP, Charmantier G (1999a) Impact of copper on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Decapoda). J Crust Biol 19:209–223

Soegianto A, Charmantier-Duares M, Trilles JP, Charmantier G (1999b) Impact of cadmium on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). Aquat Living Resour 12:57–70

Taylor HM, Taylor EW (1992) Gills and lungs: exchange of gases and ions. In: Hamson FW, Humes AG (eds) Microscopic anatomy of invertebrates, Decapod Crustacea, vol. 10. Wiley-Liss Inc, New York, pp 203–293

Thompson JAJ, Cosson RP (1984) An improved electrochemical method for the quantification of metallothioneins in marine organisms. Mar Environ Res 11:137–152

Viarengo A, Burlando B, Dondero F, Marro A, Fabbri R (1999) Metallothionein as a tool in biomonitoring programmes. Biomarkers 4:455–466

Vogt G, Quinitio ET (1994) Accumulation and excretion of metal granules in the prawn, *Penaeus monodon*, exposed to water-borne copper, lead, iron and calcium. Aquat Toxicol 28:223–241

Williams B (2000) Biostatistics. Chapman and Hall/CRC. Boca Raton, 201pp

Wong VWT, Rainbow PS (1986) Two metallothioneins in the shore crab *Carcinus maenas*. Comp Biochem Physiol A 83(1):149–156