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REGULAR ARTICLE

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Cellular responses to increasing Cd concentrations in the freshwater crab, *Potamonautes warreni*, harbouring microbial gill infestations

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Abstract We investigated the uptake, transport, storage and defence mechanisms in the freshwater crab, *Potamonautes warreni*, harbouring microbial gill infestations and exposed to increasing chronic (0.2, 0.5, 1.0 mg l^{-1}) and acute (2.0 mg l^{-1}) cadmium (Cd) concentrations under controlled laboratory conditions over a period of 21 days. Transmission electron microscopy and X-ray microanalysis revealed that the microbial gill fauna was eliminated on exposure to 0.2 mg Cd^{2+} l^{-1} and that Cd became increasingly adsorbed and incorporated into lamellar crystal deposits and permeated the cuticle of the gills of *P. warreni*. Degeneration of the apical membrane infoldings and vacuolation of epithelial cells occurred concurrently with pinocytosis, endocytosis and pronounced phagocytotic activity in the epithelia and haemal canal of the gills. Elevated Cd exposures (0.5 or 1.0 mg l^{-1}) resulted in the swelling and dissociation of mitochondrial outer membranes together with an increase in transport of Cu, Cl and S by haemocytes in the haemal canal to epithelial tissues depleted in these elements. Cd also accumulated in tightly coiled concentric membrane whorls in the haemal canal, whereas the highest concentrations of Cd were found within aggregates of lysosome-like bodies in cuticulin-secreting cells of the gill stem. Chronic exposure to Cd induced increased fatigue and mild uncoordinated motor activity. In contrast, at an acute exposure of 2.0 mg l^{-1} over 48 h, *P. warreni* showed a time-specific rapid loss of motor function, although only mild cellular lesions occurred in the gill tissues. The significance of cellular changes in the gill epithelia and altered motor activity of *P. warreni* with increased waterborne Cd are discussed as potential biomarker responses in monitoring aquatic pollution.

Keywords Microbial gill infestation · Increasing Cd pollution · Cellular defence and behavioural responses · TEM · Semi-quantitative X-ray microanalysis · Freshwater crab, *Potamonautes warreni* · Crustacea

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Introduction

Comparisons of inter- and intra-specific responses of aquatic species to non-essential metals, such as cadmuim (Cd), are often difficult to interpret as the uptake, accumulation and excretion of these non-essential metals in individual organs vary. Acclimation and the physiological state of the organism together with the availability of Cd in its ionic state in the aqueous environment are also variable factors (Bryan 1979; Depledge and Rainbow 1990; Bryan and Langston 1992; Shugart 2000). Moreover, little is known about the evolution of tolerance or acclimation of aquatic species to synergistic effects of pollutants and disease, as few ecosystems have been continuously monitored and the majority of studies address the effects of individual pollutants, often with single parameters (Pascoe and Cram 1977; Pascoe and Woodworth 1980; Collins et al. 1999; Hollis et al. 1999; Schuwerack et al. 2001c). This therefore contributes to the variability observed in the levels of the uptake of Cd and the mechanisms of detoxification in aquatic organisms.

The uptake of essential and non-essential metals in marine and freshwater crustacean and fish species may occur from solution or via food but little is known about the importance of these uptake routes (Jennings and Rainbow 1979; Depledge and Rainbow 1990; Chan and Rainbow 2000). Gills are major uptake sites of metals in solution and also targets of sessile microbes and parasitic invaders. These may cause cellular lesions in gill lamellae and disturb functional processes such as oxygen uptake, CO_2 removal, osmotic, ion and acid-base regulation,

nitrogenous excretion and defence mechanisms (Jennings et al. 1979; Johnson 1980; Burggren and McMahon 1988; Schuwerack et al. 2001a; Galloway and Depledge 2001).

The uptake of metals across biological membranes is dependent on metal speciation and bioavailibility, which are in turn influenced by the chemical composition of water, its ionic strength, pH and hardness and the concentration of dissolved organic and inorganic matter in complex or particulate form (Depledge et al. 1994; Mason and Jenkins 1995). Thus, metal toxicity in aquatic biota is not only determined by the concentration of metals in solution, but also by their speciation, which is influenced by specific environmental factors and causes enhanced or reduced metal uptake via biological membranes (Simkiss and Taylor 1995). When permeating across cell membranes, metals become rapidly sequestered by intracellular ligands. Under healthy conditions, homeostatic processes regulate metal speciation and thus prevent increased binding to ligands involved in important physiological processes, which may otherwise become impaired (Mason and Jenkins 1995). A disturbance in either the intracellular levels of the metal or the concentrations or types of ligand will alter steady-state conditions with respect to transport, storage, excretion and result in the formation of abnormal physiologically essential proteins (Mason and Jenkins 1995).

On a cellular level, Cd causes cumulative cell breakdown in *Jaera nordmanni* (Bubel 1976), black gill syndrome in the pink shrimp *Penaeus duorarum* (Couch 1978; Sparks 1985) and lamellar aneurisms in the teleost *Gnathonemus petersii* (Alazemi et al. 1996). Furthermore, Cd has been shown to induce non-specific immune responses, such as phagocytotic activity and changes in the density of haemocytes in decapod crustaceans (Bubel 1976; Johnson 1980, 1987; Victor 1993; Cheng and Chen 2001; Galloway and Depledge 2001). Cd can also interfere with olfactory primary neurons and synaptic neurotransmitters in the brain of rats (Hastings and Evans 1991; Minami et al. 2001) and may damage the structure of DNA in invertebrates and vertebrates (Shugart 2000). Knowledge of immuno- and neurotoxic responses in invertebrates and their links to altered behaviour is however scarce and occasionally controversial. Even less is known about the induction of neuro-immune responses and the simultaneous change in behaviour in diseased aquatic invertebrates encountering waterborne pollutants (Chisholm and Smith 1992; Galloway and Depledge 2001) or about the interaction of these responses at different levels of biological organisation within individuals and whether they trigger, or are subordinate to, other responses at increasing pollutant levels (Ader et al. 1995).

In 1995, the South African freshwater crab, *P. warreni*, collected from the Mooi River at Noordbrug, was acclimated to multiple stressors, including faecal contamination, elevated NH_3 levels, microbial gill infestations and traces of Cd^{2+} (Schuwerack et al. 2001a, 2001b). This work showed that gill infestations in *P. warreni* alter the respiratory tissues, which in turn induce changes in growth, oxygen consumption and heart rate in rested crabs, resulting in reduced metabolic and motory activities. Schuwerack et al. (2001a) showed that, in crabs that were acclimated and chronically exposed to 0.2 mg Cd^{2+} l^{-1} for 21 days in the laboratory, the highest concentrations of Cd were found in the gills, followed by the digestive gland, although Cd was regulated in the haemolymph. This implies that *P. warreni* possesses mechanisms of detoxification and defence that enable it to tolerate high chronic levels of Cd. The present study therefore addresses the following questions: (1) how does Cd exposure affect microbial gill infestation, (2) where is Cd accumulated in acclimated infested *P. warreni,* (3) how does Cd uptake, transport and accumulation occur in gill tissues of chronically and acutely exposed infested *P. warreni*, (4) what defence mechanisms are shown in these crabs with exposure to elevated levels of waterborne Cd, and (5) can the degree of modulation of gill infestation and cellular responses and the link between these responses be identified in chronically and acutely exposed *P. warreni*?

Materials and methods

Collection and maintenance of P. warreni

Wild indigenous populations of *P. warreni* (female and male; *n*=40; mass: 80–91 g; carapace width: 53–61 mm), were collected from the Mooi River, at Noordbrug (26°40′S/27°05′E), approximately 60 km downstream from an unpolluted site, a spring named Bovenste Oog (Schuwerack et al. 2001a) and 1 km south and north of Potchefstroom Dam and of Potchefstroom Town, Northwest Province (NWP), South Africa, respectively (Schuwerack et al. 2001a).

Crab burrows were submerged in muddy substrates at the riverbanks at Noordbrug and had a maximal depth of 0.5 m and a restricted water flow (0.0025 m^3/s). Biological analyses of water quality revealed faecal contamination of *Escherichia coli* (58 counts/100 ml; Schuwerack et al. 2001a). Physico-chemical analyses of water samples in the field and laboratory, respectively, included: conductivity (670 and 500 μS cm^{-1}); dissolved oxygen (8.3 and 4.73 mg l^{-1}); Cl (21.0 and 11.0 mg l^{-1}); Na (23.0 and 11.3 mg l^{-1}); SO_4 (1.3 and 0.1 mg l^{-1}); PO_4 (0.01 and 0.0 mg l^{-1}); total hardness as $CaCO_3$ (322 and 310 mg l^{-1}); NH_4^+ (0.12 mg l^{-1} and 0.067 mg l^{-1}); and the following metals: Ca (45 and 41 mg l^{-1}), Zn (0.037 and 0.030 mg l^{-1}), Mg (35.4 and 31.3 mg l^{-1}), Cu (0.032 and 0.01 mg l^{-1}), Fe (0.03 and 0.01 mg l^{-1}) and K (3.8 and 4.4 mg l^{-1}). Cd concentrations were 0.009 mg l^{-1} in the field and 0.006 mg l^{-1} in laboratory water and, as analysed by scanning electron microscopy and X-ray microanalyses, the levels of Cd were higher in crab burrow sediments (0.89% by weight) compared with riverbed surface sediments (0.33% by weight; Schuwerack et al. 2001b).

Cd pollution scenarios in the laboratory

Following transfer to the laboratory, individual crabs were kept fully submerged and acclimated in 900 ml aerated de-chlorinated water in a static system for 7 days. To investigate cellular and behavioural changes in response to chronic and acute Cd exposure, each of six crabs were exposed chronically to 0.2, 0.5 or 1.0 mg l^{-1} Cd^{2+} ($CdCl_2$; Merck) and acutely to 2.0 mg Cd^{2+} l^{-1} for up to 21 days. Control animals were kept in fresh water without added Cd. Each crab was fed two cat food pellets (Brand Epot) in freshly changed water prior to the addition of $CdCl_2$ to promote metal uptake through the water, which was changed every 72 h. Aqueous

Cd concentrations in the glass containers decreased by 50% during the first 12 h, with a further 50% reduction occurring after 36 h until the concentration remained constant (Schuwerack et al. 2001b).

Histopathological assays

Transmission electron microscopy

Unexposed and Cd-exposed gill tissues of sacrificed crabs were dissected out and transverse sections of the apex, mid region and gill stem of the pleurobranch on thoracopod V, the posterior arthrobranch and the podobranch on maxilliped III of the gills were fixed in Todd's fixative (Aldrich and Todd 1986) . Gill tissues were preserved in Todd's fixative for 12 h, washed in 0.1 M sodium cacodylate buffer (pH 7.2; 2×20 min), postfixed in 1% osmium tetroxide for 1 h and washed in distilled water (3×20 min). For transmission electron microscopy (TEM), gills were *en-bloc* stained with 2% uranyl acetate for 30 min, washed in distilled water (3×20 min), dehydrated in an acetone series (50%, 70%, 90%, 100%, 100%) for 15 min each and embedded in Spurr's resin (Spurr 1969) at 60°C for 8 h. Sections (0.5 µm thick) were stained in 0.5% toluidine blue and viewed in the first instance under a light microscope (Optiphot, Nikon, Japan). Additionally, 100 nm sections were stained with 2% uranyl acetate and lead citrate (Reynolds 1963) and examined under a Philips CM10 transmission electron microscope. Cadmium uptake, transport, storage and immunological responses, such as phagocytosis and changes in haemocyte morphology, in the cuticle, underlying epithelia and haemal canal in the apex and mid region of the lamellae and in the central axial canal of the gill stem, were assessed by image analyses, using a light microscope (Optiphot; Camera model: FM2, Nikon, Japan) or a Philips CM10 transmission electron microscope. Specific cellular responses were recorded within three randomly selected areas (550 μm^2) in each of five ultrathin sections.

X-ray microanalysis

Semi-quantitative X-ray microanalysis on unstained sections (100–120 nm) was performed with a Philips CM120 BioTwin transmission electron microscope, equipped with an energy-dispersive X-ray microanalysis system (EDS) and a detector fitted with a berillium window. The accelerating voltage was set at 80 KeV and spot analyses were carried out in the TEM bright field mode with X-ray count rates ranging between 200 to 1.500 counts per second (cps). X-ray spectra and maps were collected for 30 s or 2.5 h, respectively, and expressed as a net intensity (NI) and a peak to background ratio (P/B). Cd was identified from the L-alpha X-ray intensities at 3.133 KeV or in the case of other elements from the K-alpha or M-alpha X-ray intensities and analysed by using a window width of 8 µm and an absorbance of 1041 eV for an acquisition time of 30 s and a spot size of 140 nm^2. X-ray spectra and element maps were generated for the various cellular types in order to investigate Cd uptake, bioaccumulation and transport, together with ion concentrations in the cuticle and tissues at the apex and mid region of gill lamellae and the gill stem. As the X-ray microanalysis system lacked a frame grabber, the images included various magnifications of video prints and maps. Elemental concentrations were measured and expressed in NI and P/B with a detectable signal being designated as having a ratio greater than 3:1.

Results

Behaviour

Following initial attempts to escape from their environment, unexposed infested crabs settled down and responded with co-ordinated motory activity including territorial display. In crabs exposed to chronic waterborne Cd, attempts to escape became increasingly intense and prolonged with increasing chronic Cd concentrations. Following chronic exposure to 0.5 and 1.0 mg Cd^{2+} l^{-1} crabs showed fatigue and uncoordinated motor activity, including impaired grasping behaviour of food particles. At an acute exposure of 2.0 mg Cd^{2+} l^{-1}, *P. warreni* responded with attempts to escape (0–3 h), vigorous opening and closing of the third maxilliped and movements of the antennae (3–8 h), locking of chelae (8–48 h) and loss of balance, followed by paralysis and a coma ending in death (<66 h).

Histopathological analysis of gills of *P. warreni*

Unexposed infested gill tissues

Individual gill lamellae of *P. warreni* from Noordbrug were infested with peritrichous ciliates, such as *Zoothamnium, Epistylis, Lagenophrys* spp., and motile species of protozoans embedded in dense clumps of bacteria when compared with uninfested gills from crabs collected at an unpolluted source of the river as observed during a previous study (Fig. 1a, b; Schuwerack et al. 2001a). Gill epithelia of *P. warreni* showed lesions induced by the microbial gill fauna. These included dilation of the cuticle and large randomly distributed subcuticular spaces at apical membrane infoldings with the stalked peritrichous ciliate, *Zoothamnium* sp. Attached *Lagenophrys* sp. caused resorption of membrane infoldings, as shown by Schuwerack et al. (2001a). The lesions protruded deeply into the epithelia resulting in indentations of the epicuticle in the presence of bacterial colony attachments and microvilli were resorbed or unfolded at the apical membrane. Furthermore, in heavily infested crabs, more mitochondria were found near the apical membrane infoldings when compared with uninfested tissues (Fig. 1a, b). Occasionally, single circulating haemocytes with a central nucleus and eccentrically arranged granules of irregular shapes were found together with single fixed phagocytes containing ingested debris within the artery of the haemal canal (see Fig. 1b) and separated from the epithelial cells by a basolateral membrane. No differences in the degree of infestation or effects were observed in the pleurobranchs compared with posterior arthrobranchs, but the smaller podobranchs were predominantly infested with bacteria and occasionally *Lagenophrys* sp. The main elemental constituents of the cuticle and epithelia of unexposed infested crabs identified in the X-ray spectra included Cu, with NI peaks at 139.84 cps, followed by Cl (8.15 cps), S (3.57 cps) and Fe (3.29 cps). Cd was not detected in these tissues by X-ray microanalysis (Table 1).

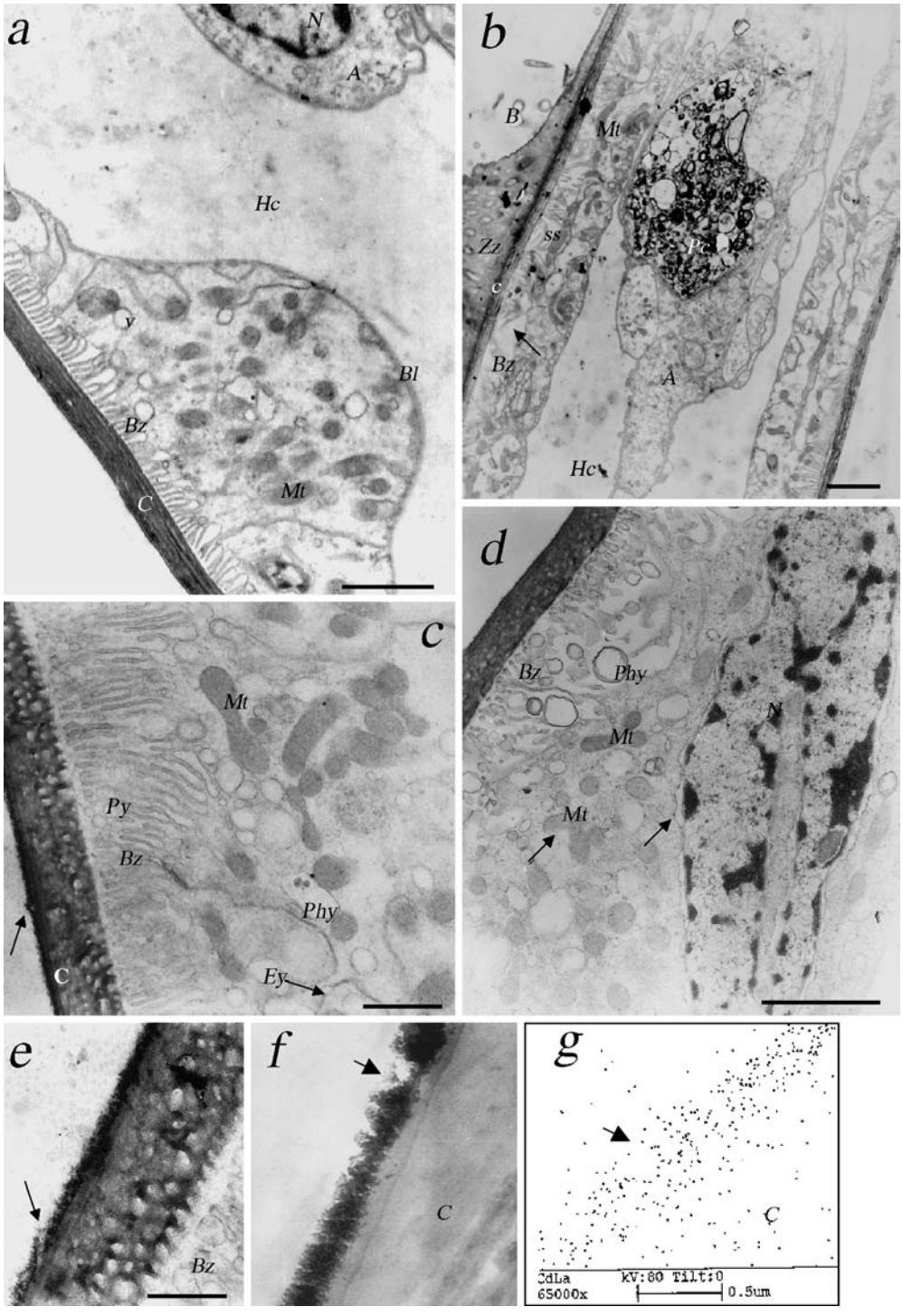

Fig. 1a–g Gill lamellae of (**a**) unexposed uninfested and (**b**) naturally exposed infested *P. warreni*, of (**c**) infested *P. warreni* exposed to 0.2 mg Cd^{2+} l^{-1} and of (**d–g**) infested *P. warreni* exposed to 0.5 mg Cd^{2+} l^{-1} for 21 days. **a** Uninfested unexposed gill lamellae of *P. warreni* collected at an unpolluted source of the river showing the clean cuticle (*c*), the underlying epithelia with apical membrane infoldings (*Bz*), vesicles (*v*), the mitochondria-rich (*Mt*) cytoplasm and the central haemal canal (*Hc*) of posterior arthrobranch (*N* nucleus, *Bl* basolateral membrane). **b** Infested gill lamellae with bacteria (*B*) and the stalked peritrichous ciliate, *Zoothamnium* sp. (*Zz*) showing loss of apical membrane infoldings (*Bz*) under the stalk, elongation of mitochondria (*Mt)* migrating to the apical membrane and fixed phagocyte (*Pc*) in the artery (*A*) of the haemal canal (*Hc*). Note the dilation of the lamella cuticle (*c*) under the stalk attachment, the subcuticlar spaces (*ss*) at apical membrane infoldings (*Bz*) and the elongated mitochondria (*Mt*) in the epithelia of pleurobranch. **c** Crystal deposition on cuticle (*c*, *arrow*) with dissociating infoldings of the apical membrane (*Bz*) and pinocytotic activity (*Py*), endocytosis (*Ey*) and phagocytotic (*Phy*) activity in the epithelium of pleurobranch. **d** Severe dissociation of the apical membrane infoldings (*Bz*) and pinocytosis (*Py*), increased phagocytosis (*Phy*), swelling (*arrow*) of mitochondria (*Mt*) and membrane blebs on nuclei (*N*, *arrow*) of posterior arthrobranch. **e** Fine granular extracellular crystal deposition on the cuticle of pleurobranch; which occasionally lifts off (*arrow*). **f** Video print of **g**. **g** X-ray mapped Cd adsorption to crystal deposition (*arrow*) and of the permeation of Cd across the epi-, exo- and endocuticles (*c*, *arrow*). *Bars* 0.5 μm (**a**), 2 μm (**b**), 0.5 μm (**c, d**), 1.0 μm (**e**)

Table 1 TEM X-ray microanalysis of Cd uptake and bioaccumulation in the crystal deposits associated with the cuticle and lamellar epithelia of *P. warreni;* concentrations of Cd are expressed in net intensity (*NI*) and peak to background ratio (*P/B*)

Element	Cd^{2+} (mg l^{-1})									
	0.0		0.2		0.5		1.0		2.0	
	NI	P/B	NI	P/B	NI	P/B	NI	P/B	NI	P/B
Cd L[a]	0.0	0.0	0.74	0.78	–	–	0.93	0.96	0.69	0.63
Cl K[a]	8.15	5.25	3.06	1.23	2.89	1.35	1.81	2.03	3.16	2.06
Cu K	139.84	49.60	87.19	62.97	121.43	106.25	39.89	46.12	116.16	1.31
S K	3.57	0.69	1.37	1.58	2.11	1.07	0.95	2.09	–	–
Al K	–	–	13.73	4.96	2.50	2.80	0.70	0.65	1.54	0.94
Fe K	3.29	0.94	21.50	10.96	32.29	20.09	0.00	0.00	17.47	1.81
Si K	–	–	3.45	3.80	5.93	4.37	2.53	8.60	–	–

[a] K, L represent specific emission peaks

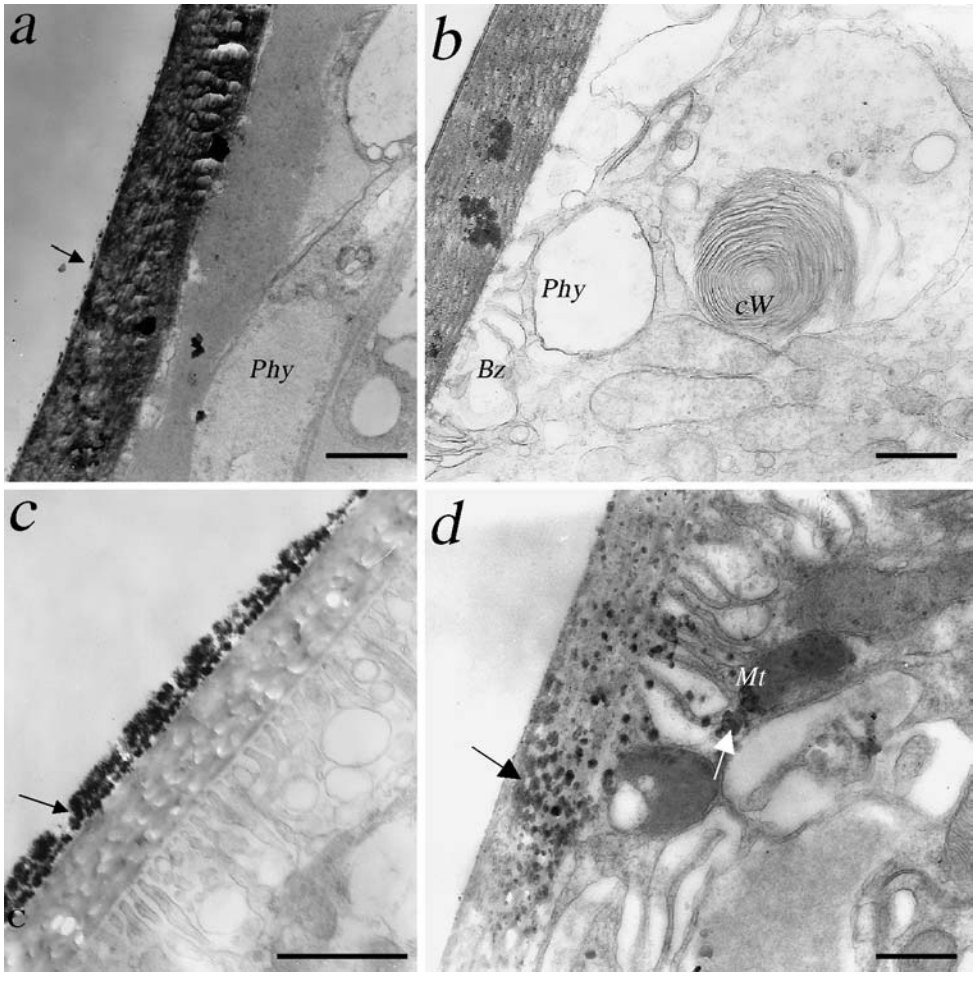

Fig. 2a–d Gill tissue of infested *P. warreni* (*a, b*) chronically exposed to 1.0 mg Cd^{2+} l^{-1} for 21 days and (**c, d**) acutely exposed to 2.0 mg Cd^{2+} l^{-1} for 48 h. **a** Cuticle with increasingly coarse granular crystal deposition (*arrow*), loss of apical membrane infoldings and mitochondria in the majority of tissues and the formation of large phagocytic vesicles (*Phy*) with dense cytoplasm in lamellae epithelial cells of pleurobranch. **b** Tightly coiled membrane whorls (*cW*) in epithelia in posterior arthrobranch after 1.0 mg Cd^{2+} l^{-1} exposure for 21 days (Bz loss of apical foldings). **c** Thick film of coarse granular matrix (*arrow*) on the cuticle of posterior arthrobranch after 2.0 mg Cd^{2+} l^{-1} exposure for 48 h. **d** Localised permeation of granular substance (*black arrow*) across the epi-, exo- and endocuticles into the apical membrane infoldings and adsorption to mitochondrial membranes (*Mt*, *white arrow*) at apex of posterior arthrobranch. *Bars* 2.0 μm (**a**) 1.0 μm (**b, c**) 0.5 μm (**d**)

Gill tissues chronically (0.2, 0.5, 1.0 mg Cd^{2+} l^{-1}) and acutely (2.0 mg Cd^{2+} l^{-1}) exposed for up to 21 days

The apical and mid regions of the cuticular lamellar surfaces and the gill stem of *P. warreni* exposed to 0.2 mg Cd^{2+} l^{-1} were free from infestation (Fig. 1c–g). However, gill lamellae were exceedingly blackened in colour and covered with a deposit having a crystalline matrix, which was coarser than the polysaccharide-like film induced by infestations identified in previous studies (Fig. 1b–f; see Schuwerack et al. 2001a). The crystal deposits on the cuticle occasionally detached and, as demonstrated by TEM X-ray spectra and mapping (Fig. 1e–g, Table 1), the Cd signal increased within the crystal deposition, coinciding with a decrease of Cu and S and an increase of Fe and Si. This was accompanied by a dose-dependant decrease in Cl and Al at 0.2, 0.5 and 1.0 mg Cd^{2+} l^{-1} exposures (Table 1).

With an increase in chronic exposure to Cd, microvilli at the apical membrane of lamellar epithelia were withdrawn from the cuticular membrane, and pinocytosis or endocytosis respectively occurred within the extensive

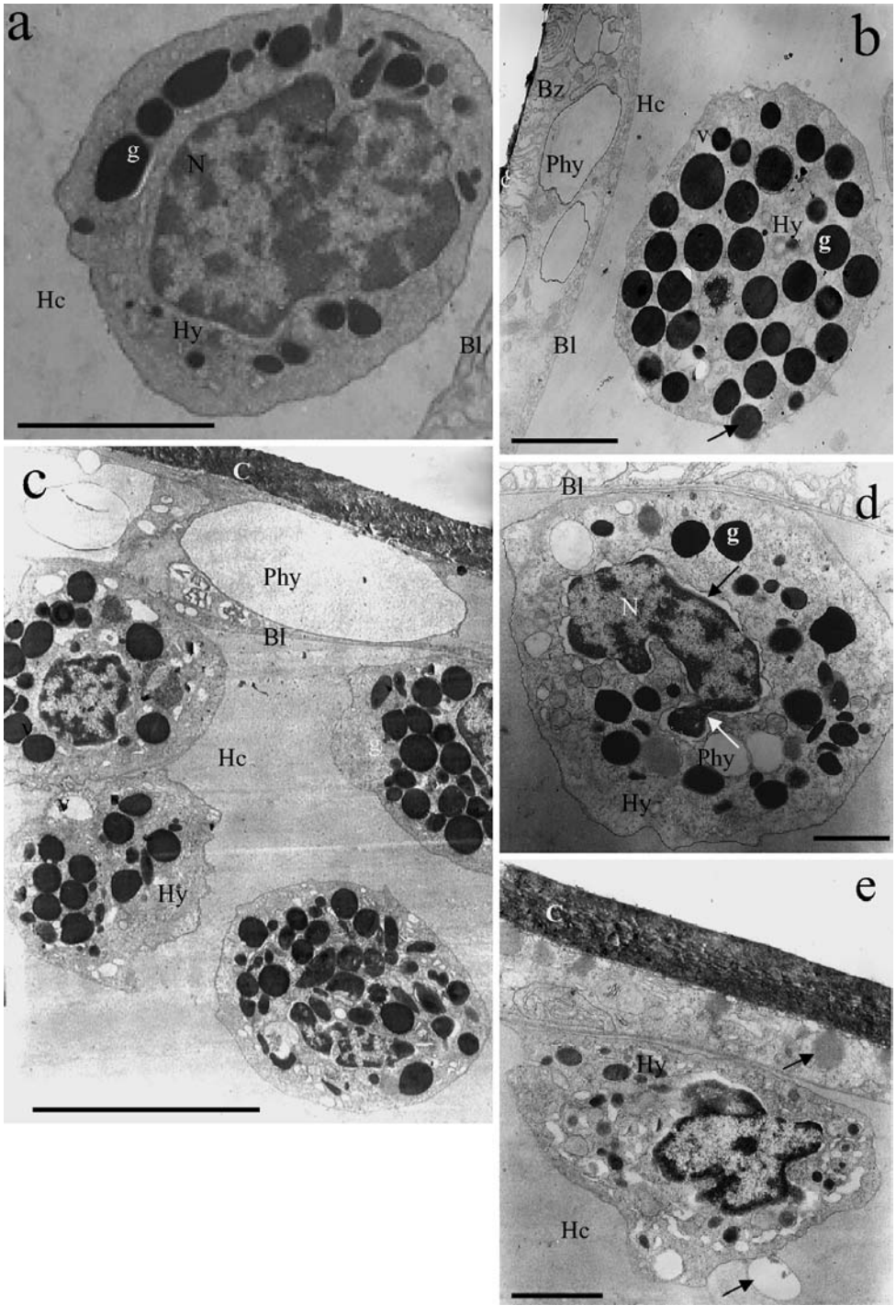

Fig. 3a–e Haemocyte activity in (**a**) naturally exposed infested *P. warreni*, (**b**) crabs exposed to 0.5 mg Cd^{2+} l^{-1}, (**c–e**) crabs exposed to 1.0 mg Cd^{2+} l^{-1} for 21 days. **a** Typical circulating haemocyte in haemal canal (*Hc*) of pleurobranch lamella with a central nucleus and eccentrically distributed granules (*g*) with electron-dense material. **b** Circulating haemocyte with increasing numbers of granules containing highly electron-dense material and showing the onset of granular exocytosis (*arrow*) at the basolateral membrane (*Bl*) in the haemal canal (*Hc*) of posterior arthrobranch at 0.5 mg Cd^{2+} l^{-1}. **c** Aggregates of four settled haemocytes at the basolateral membrane (*Bl*) in the haemal canal (*Hc*) of a pleurobranch lamella. **d** Membrane blebs (*black arrow*) and exocytosis of membrane-bound chromatin from nucleoli (*white arrow*) with signs of pycnosis in posterior arthrobranch (*N* nucleus, *Phy* phagocytotic activity, *g* granule). **e** Exocytosis of membrane-bound small phagocytes from the haemocyte (*Hy*) into the haemal canal (*Hc*) of posterior arthrobranch (*arrows*). *Bars* 0.1 μm (**a, c**) 2 μm (**b**), 1 μm (**d, e**)

apical membrane infoldings or along loose plasmalemma strands. Phagocytosis, fragmented nuclear membranes and swelling of mitochondria were commonly found in the underlying epithelia (Fig. 1c, d). A small number of Cd-enriched particles were recorded in the cytoplasm (Fig. 1g). With an increase in chronic exposure to 0.5 mg Cd^{2+} l^{-1} these lesions became progressively severe (Fig. 1c–g) and a concentration of 1.0 mg Cd^{2+} l^{-1} led to a loss of mitochondria and apical membrane infoldings in the majority of the gill epithelia examined (Fig. 2a, b). The cytoplasm of epithelial cells became dense with tightly coiled membranous concentric whorls and large phagocytotic vesicles (Fig. 2a, b). In gills exposed to acute concentrations of 2.0 mg Cd^{2+} l^{-1} for 48 h, the crystal deposits were exceedingly coarse in texture (Fig. 2c) and a localised increased influx of Cd particles occurred across parts of the epi-, exo- and endocuticle of lamellar epithelia and permeated the membranes of mitochondria near the apical membrane infoldings at the apex of gill lamellae (Fig. 2d, Table 1).

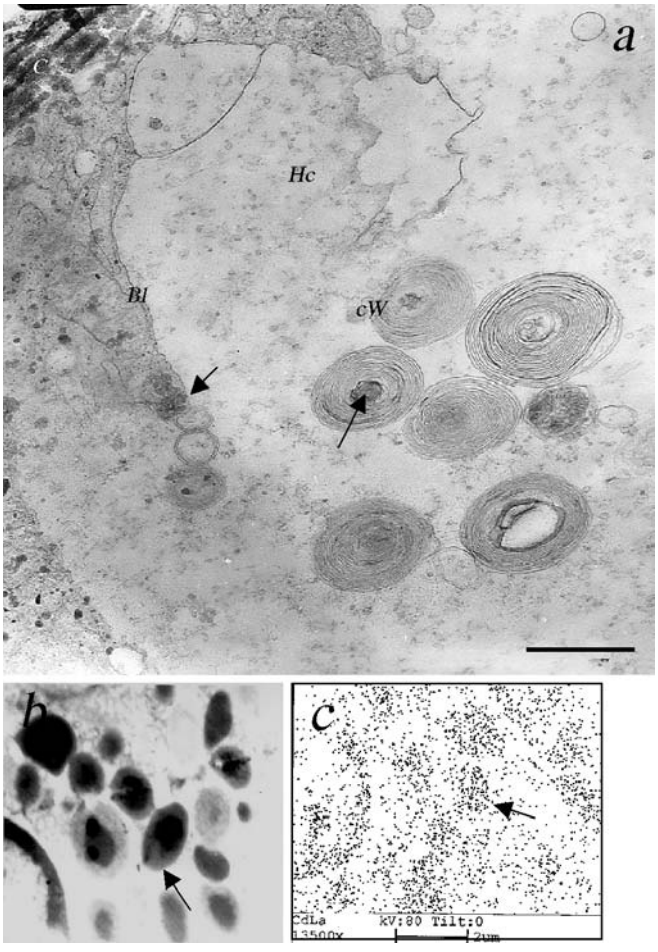

Fig. 4a–c Transport mechanisms in the haemal canal (*Hc*) of pleurobranch lamella of *P. warreni* exposed to 1.0 mg Cd^{2+} l^{-1}. **a** Formation and aggregation of tightly coiled concentric membrane whorls (*cW*, *arrow*) at the basolateral membrane (*Bl*) with Cd inclusions (*long arrow*) in the haemal canal (*Hc*). **b** Video print of **c**. **c** X-ray mapped, tightly coiled concentric whorls (*arrows*) with incorporated Cd. *Bar* 1.0 μm (**a**)

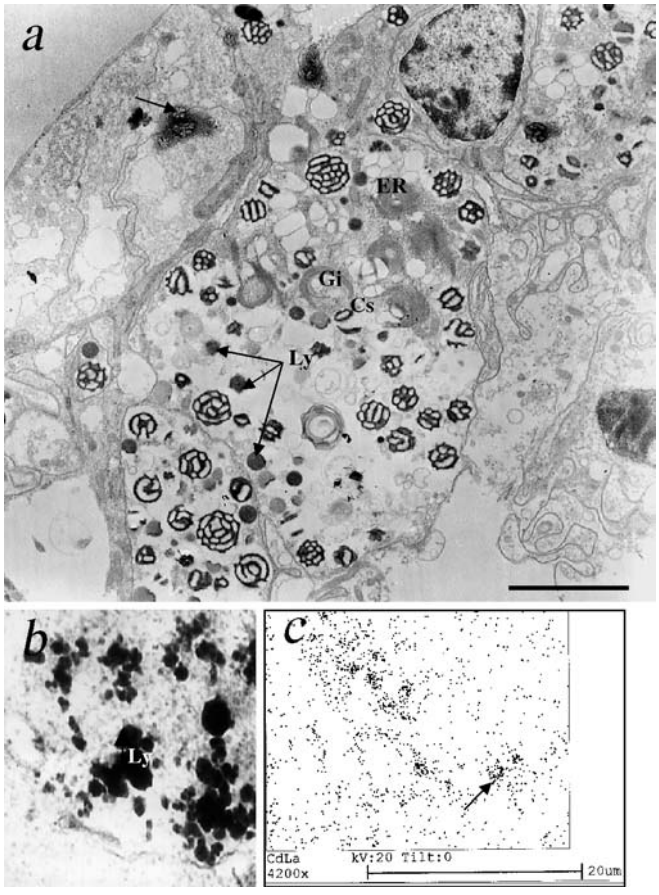

Fig. 5a–c Storage site for Cd granules in lysosome-like bodies of cuticulin-secreting cell of the gill stem of crabs exposed to 1.0 mg Cd^{2+} l^{-1}. **a** Lysosome-like bodies (*Ly*) (*arrows*) and increase in Golgi (*Gi*) with the formation of cytoarchitectural structures (*arrow*), endoplasmic reticulum (*ER*), and cisternae (*Cs*). **b** Video print of **c**. **c** X-ray mapped Cd stored in lysosomal aggregates (*arrow*) in the gill stem. *Bar* 1 μm (**a**)

Cu and Cl levels were reduced and the X-ray count rates for Fe increased 5.3-fold (Table 1).

At a concentration of 0.5 mg Cd^{2+} l^{-1}, profound morphological changes occurred in circulating haemocytes in the haemal canal when compared with those in the lamellae of unexposed gills (Fig. 3a, b). The number and density of granules increased (Fig. 3b) when compared with acclimated infested crabs (Fig. 3a) and those exposed to 0.2 mg Cd^{2+} l^{-1} (not shown). At a concentration of 1.0 mg l^{-1} waterborne Cd^{2+}, aggregates of haemocytes (Fig. 3c) contained more phagocytotic vesicles that were exocytosed into the haemal canal (Fig. 3c–e). In addition nuclear membrane blebs were evident and electron-dense material was exocytosed as membrane-bound granules from the nucleoli, which also showed signs of pycnosis (Fig. 3d, e). Haemocyte granules were particularly rich in Cu and S and also contained Si and Cl (Table 2). Furthermore, tightly coiled concentric whorls with inclusions of Cd, Cu, Si and Cl aggregated in the haemal canal near the basolateral membrane from which they originated (Fig. 4a–c, Table 2).

Within the gill tissues, the highest X-ray signals for Cd were in the cuticulin-secreting cells in the central axial canal of the gill stem. Within these cells, Cd particles became increasingly incorporated into lysosome-like bodies with increasing levels of waterborne Cd (4.12 NI; Fig. 5a–c, Table 2). Similarly, the greatest NI of Cu (508.27 cps), Cl (25.23 cps), Al (13.41 cps) and Fe (5.91 cps) were present in these lysosomes compared with all other tissues (Table 2). Moreover, cytoarchitectural structures derived from Golgi (Fig. 5a) rich in Cu, K, Si, Cl, S, P and Ca were observed to increase (Table 2) in size and number with increasing exposure to Cd in these cells (Fig. 5a).

Table 2 TEM X-ray microanalysis of Cd bioaccumulation in the hemal canal and gill stem of the gills of *P. warreni;* concentrations of Cd are expressed in net intensity (*NI*) and peak to background ratio (*P/B*)

Element	Hemocyte/granules (0.5 mg Cd^{2+} l^{-1})		Whorls in hemal canal (0.5 mg Cd^{2+} l^{-1})		Lysosome in gill stem (0.5 mg Cd^{2+} l^{-1})		Cytoarchitectural structures (1.0 mg Cd^{2+} l^{-1})	
	NI	P/B	NI	P/B	NI	P/B	NI	P/B
Cd L[a]	0.00	0.00	1.43	2.03	4.12	0.38	–	–
Cl K	1.87	1.13	3.50	5.40	25.23	2.29	1.52	3.68
Cu K	23.17	59.38	71.76	76.25	508.27	33.28	18.40	41.99
S K	1.90	2.36	–	–	–	–	0.95	2.01
Al K[a]	–	–	–	–	13.41	1.32	–	–
Fe K	–	–	–	–	5.91	0.62	–	–
Si K	1.95	3.64	2.53	8.60	–	–	1.60	2.66
P K	–	–	–	–	–	–	0.82	1.70
Ca K	–	–	–	–	–	–	0.62	1.15
K K	–	–	–	–	–	–	1.69	3.69

[a] K, L represent specific emission peaks

Discussion

P. warreni from a polluted site in the Mooi River at Noordbrug was subjected to a variety of contaminants, including elevated NH_4^+, Cd and faecal contamination, during the autumn of 1995 in contrast to those crabs collected from an unpolluted site during the summer period as previously described by Schuwerack et al. (2001a). Restricted water flow and high temperatures in the presence of these pollutants led to microbial gill infestations in *P. warreni* and caused cellular lesions in the gill epithelia, specific to the attachment of bacteria and stalkless and stalked peritrichous ciliates (Schuwerack et al. 2001a). Attached bacterial colonies caused indentations of the cuticle and the resorption and dissociation of the apical membrane infoldings of the gill epithelia. Peritrichous ciliates, namely, *Zoothamnium* and *Lagenophrys* spp., both caused large subcuticular spaces within the apical membrane infoldings. *Zoothamnium* also induced dilation of the cuticle by stalk attachment, whereas an unfolding of the apical membrane was evident in gill epithelia with *Lagenophrys*. Moreover, infested crabs showed a significant reduction in growth and heart rate and a significant increase in oxygen consumption (Schuwerack et al. 2001a). Chronic exposure to elevated concentrations of Cd led to progressive cellular lesions and immune and compensatory responses and mild impairment of locomotory function during this study, whereas the mild cellular responses did not reflect the acute neurotoxic time-specific events in *P. warreni* to an acute exposure of 2.0 mg Cd^{2+} l^{-1}.

Cd accumulation in infested gill tissues in *P. warreni*

Although Cd (0.009 mg l^{-1}) and NH_4^+ (0.12 mg l^{-1}) were elevated in unexposed infested *P. warreni,* lesions in gill lamellar tissues, associated with these pollutants, were not evident. An earlier study by Schuwerack et al. (2001b), using flame atomic absorption spectroscopy (FAAS), had shown that gills were the main target for Cd accumulation, compared with that in the digestive gland and haemolymph. Cadmium in infested gill tissues of *P. warreni* collected at Noordbrug in the present study was not detected by TEM X-ray microanalysis. It is therefore likely that the Cd detected by FAAS was associated with the microbial gill fauna, which in turn provided protection to the crab host. The microfaunal community, embedded in a polysaccharide-like film on the cuticle of *P. warreni*, acts as a physical barrier to O_2 uptake and ion exchange and therefore to metal uptake (Schuwerack et al. 2001a). Despite its acclimation, the gill microfauna show a greater sensitivity to chronic levels (0.05–0.2 mg l^{-1}) of Cd than in the crab host, *P. warreni* (Schuwerack et al. 2001b). Fluctuating pollutant levels compared with constant concentrations require higher metabolic costs and thus make adaptation by aquatic species more difficult (Linton et al. 1998). Little is known about the uptake of pollutants in these microbes attached to animate surfaces and this initiated a parallel investigation into the interaction of both stressors, i.e. Cd and the microbial gill fauna, and their possible synergistic effects on the histopathology and physiology of *P. warreni* (Schuwerack et al. unpublished). The effects of multiple stressors can vary with pollutant concentration and time of exposure, making it difficult to identify and co-ordinate specific responses to individual stress factors. In the present study, no histopathological lesions were observed in the gill tissues of *P. warreni* naturally exposed to Cd and NH_4^+, possibly because the microbial fauna afforded protection but, as soon as they were eliminated by exposure and an increase in Cd uptake, an increase in cellular alterations in the gills was observed. Studies on the impact of multiple stressors, on the uptake of Cd on unacclimated microbial gill fauna and their host, will further our understanding of the acclimation of these aquatic species to pollutants. Overall the sensitivity of the gill microbial fauna to fluctuating levels of Cd may make these communities valuable bioindicators and this merits further investigation.

Uptake, transport and bioaccumulation of Cd in *P. warreni* following chronic and acute exposure

The bioaccumulation of Cd in the gills of *P. warreni* following chronic exposure to Cd (0.2, 0.5, 1.0 mg l^{-1}) indicates a capacity to tolerate extremely high levels of this non-essential metal. Much of the Cd was adsorbed and incorporated into an extracellular film on the cuticle. This film became very granular as the concentration of Cd increased, probably as a result of a change in pH and other cuticular constituents (Handy 1989; Taylor et al. 1996; Tao et al. 2000). In penaid shrimps, crystal deposition on the cuticle is attributed to an overproduction of membrane proteins involved in crystal nucleation (Coblentz et al. 1998). Chitin, as a constituent of crustaceans, has proved to be an effective metal biosorbant in wastewater management, because of its beta (1–4)-linked *N*-acetyl glucosamine monomers, with considerable chain length, and its acidic polysaccharides with hydrophylic and polar properties, e.g. as a nitrogenous polysaccharide (McEldowney et al. 1993).

With the increased adsorption and obstruction of the epi-, exo- and endocuticles and the apical membrane infoldings of gill epithelia of *P. warreni*, restricted influx and efflux of ions is inevitable. This has been confirmed by the depletion of Cl in lamellar tissues and a restricted NH_4^+/NH_3 efflux recorded in these crabs (Schuwerack et al. unpublished) with rising Cd exposure. With the obstruction of ion exchange and oxygen uptake caused by the growing crystal depositions on the cuticle, increasing ammonia retention may contribute to a series of metabolic responses, including glutaminase inhibition. This, in turn, leads to a reduction of neurotransmitters (Korsgaard et al. 1995) and may cause the observed mild impaired motory performance at chronic pollutant levels and the rapid loss of neuromuscular performance with acute Cd exposure. This proposed cascade of events merits further investigation.

A reduction in Cl may reflect further an inhibitory effect of Cl^--ATPase by Cd and increased intracellular NH_4^+/NH_3 levels may lead to Na^+/K^+-ATPase inhibition. In the Brazilian estuarine crab, *Chasmagnathus granulata,* elevated waterborne ammonia inhibit the activity of Na^+/K^+-ATPase by as much as 80% (De Freitas Rebelo et al. 2000). Hanson et al. (1992) and Weeks et al. (1993) have also observed Na^+/K^+-ATPase inhibition upon Cu exposure in *Carcinus maenas*. In the present study, a 50% reduction of Na^+ and Cl^- in the laboratory water compared with concentrations in the Mooi River may have facilitated the uptake of these ions by *P. warreni*. The extracellular film on the cuticle of the gills was also particularly rich in Fe and Al in Cd-exposed crabs, compared with low concentrations of Fe and the absence of Al in unexposed infested crabs. Higher levels of Fe and Al in the laboratory water may have induced the adsorption to the cuticle and excretion of these ions by *P. warreni* (see also Potts and Parry 1964).

The Cu and S depletion in tissues and increased transport of these elements by haemocytes in the gills of exposed crabs suggest the involvement of metabolic processes in the haemocytes. Furthermore, the exocytotic activity in the nucleoli together with high S and Cu levels in the increasingly produced number of granules point to the haemocytes as a potential production site of haemocyanin and/or metallothionein. Furthermore, haemocytes are thought to originate in the digestive gland, which, in these crabs, shows induced concentrations of cytosolic microtubule-like proteins at 0.5 and 1.0 mg Cd^{2+} l^{-1} exposure (Schuwerack et al. unpublished). Moreover, the depression in Cu levels and thus in cytosolic hepatic Cu-bound ligands, such as haemocyanin, in the high molecular weight protein pool of exposed crabs may have been caused by a build-up of ammonia in these tissues, which occurs with restricted ion efflux (Schuwerack et al. unpublished). Cheng and Chen (1999) have for instance reported a decrease in haemocyanin in *Penaeus japonicus* exposed to ambient ammonia. Furthermore, the increase in haemolymph Cu levels in Cd-exposed *P. warreni* (Schuwerack et al. 2001b) and the coincident increased Cu transport by haemocytes may explain why Boone and Schoffeniels (1979) found elevated levels of Cu in the haemolymph but not in the body tissues of *C. maenas* (see Depledge and Bjerregaard 1989; Weeks et al. 1993).

The progressive interaction of Cd^{2+} in infested *P. warreni* resulted in a continuous degeneration of the microvilli at the apical border and intensive swelling of mitochondria with fragmentation and blebs in the nuclear membranes, compared with the milder histopathological lesions in naturally exposed infested crabs. Cytosolic Cd in the epithelial cells was probably detoxified by binding to diverse ligands, such as calmodulin, troponin or serine proteinases (Bellelli et al. 1985; Schuwerack et al. unpublished). The appearance of Cd particles across the epi-, exo- and endocuticlar membranes and the mitochondrial outer and inner membranes at an acute exposure of 2.0 mg Cd^{2+} l^{-1} after 48 h suggests a channel-mediated permeation of Cd rather than passive diffusion. However, this needs to be confirmed by a decrease in the Ca/Cd ratio on X-ray microanalysis during the 48-h exposure period. Similarly, Przelecka et al. (1991) have reported the irreversible replacement of Ca^{2+} by Cd^{2+} in deposits at cell membranes, mitochondria, vacuoles, cytoplasm and lysosomes in the cytosol of *Acanthamoeba* cells. Schuwerack et al. (in press) have demonstrated severe irreversible progressive dissociation of cristae and membranes of mitochondria, nuclei and Golgi in the thymic and pronephric tissues of Cd-exposed juvenile *Cyprinus carpio*, infected with the blood fluke, *Sangiunicola inermis*, after 48 h and 168 h exposure to 0.1 mg Cd^{2+} l^{-1}. The stereochemical properties of Cd, as a divalent soft metal, tending to bind covalently to soft electron-rich locations may explain these effects (Turner et al. 1985; Przelecka et al. 1991; Kiss and Osipenko 1994; Usai et al. 1999; Schuwerack et al. unpublished). In binding, Cd ions can perturb a structure to match its stereochemical requirements and may cause functional changes in essential macromolecules (Turner et al. 1985). The increased levels of Fe with elevated chronic and acute

Cd exposure indicate the interference of Cd with transferrin and the degradation of the protein.

The highest aggregation of Cd occurs in the lysosome-like granules within concentric membrane whorls of the haemal canal and in lysosome-like bodies in the cytosol of cuticulin-secreting cells of the central axial canal in the gill stem (see Maina 1990). These ultrastructural features may represent a possible detoxification mechanism, with Cd finally being incorporated into the exoskeleton and then lost in the process of moulting (Depledge and Rainbow 1990). An apparent increase in the Golgi and endoplasmic reticulum and cytoarchitectural structures in the gill stems of *P. warreni* on elevated exposures to chronic Cd indicate an interaction with protein synthesis, including haemocyanin, which also occurs in the digestive gland of these crabs (Schuwerack and Lewis 2003). Johnson (1980) has described similar structures in the digestive gland of the blue grab, *Callinectes sapidus*, and has suggested that they are composed of haemocyanin synthesised by the Golgi.

Defence mechanisms in the gill tissues of *P. warreni* with elevated Cd exposure

The detachment of crystal deposition and its absence on the lamellar cuticle in uninfested *P. warreni* taken from an unpolluted source in the Mooi River have provided evidence that the crabs possess cuticular defence mechanisms against toxic chemicals permeating their lamellar membranes (Schuwerack et al. 2001a). This merits further investigation as a first-line defence mechanism in teleosts (Handy 1989; Taylor et al. 1996).

Therefore *P. warreni* appears to respond to infestation by the ingestion of cell debris and foreign substances, by using fixed phagocytes in the haemal canal and with an increase in pinocytosis, endocytosis and phagocytotic activity as exposure to Cd increases from 0.2 to 0.5 and 1.0 mg l^{-1}. Phagocytotic activity, a functional attribute of haemocytes (Johnson 1987; Galloway and Depledge 2001), increases with rising waterborne Cd concentrations in the gill epithelia of *P. warreni* exposed to 1.0 mg Cd^{2+} l^{-1}. It also appears that the phagocytotic activities in the epithelia and the haemocytes are different processes. Furthermore, the functional involvement of haemocytes in the transport of Cu and S to depleted tissues suggests that the process of metabolic activity and immune responses in *P. warreni* are integrated. Such responses may have evolved with increasing levels of Cd derived from past mining activity, as indicated by the elevated levels of Cd in the sediments of the Mooi River at Noordbrug (Schuwerack et al. 2001b). The controversy concerning the origin, structure and functional role of haemocytes (Johnson 1987; Galloway and Depledge 2001) invites further investigations. These compensatory mechanisms found in *P. warreni*, with an increase in chronic Cd levels over 21 days, suggest further that the crab population is extremely tolerant and defence mechanisms may have evolved as part of an adaptation to high levels of Cd pollution (Schuwerack et al. 2001b).

Thus, gill infestation in *P. warreni* consistently leads to mild histopathological lesions, which are eliminated after exposure to 0.2 mg $Cd^{2+}l^{-1}$ for 21 days. This suggests that both stressors, i.e. infestation and Cd exposure, induce obstruction of oxygen uptake pathways and ion exchange. Species-specific lesions in the gill epithelial cells with microfaunal attachments are mild compared with those induced by rising chronic Cd exposure, which leads to pronounced concentration- and time-specific histopathological degeneration. However, the histo-pathological lesions in gill tissues, i.e. the mild changes at the microvilli profile, with exposure to 2.0 mg Cd^{2+} l^{-1} does not reflect the acute ambient Cd exposure. It would therefore be interesting to compare the uptake of Cd in storage organs, such as the digestive gland, in the crabs subjected to acute and chronic exposures at shorter time intervals and for prolonged periods.

Compared with lower vertebrates, such as fish, the immune system of the decapod crustacean, *P. warreni,* is relatively simple but nonetheless demonstrates a variety of transient, innate and cell-mediated immune responses. These responses coincided with metabolic requirements on the one hand and behavioural and impaired neuromuscular performances on the other, suggesting that these processes are integrated and subject to modulation by the brain. This merits further investigation.

Overall, the value of specific parameters, such as haemocyte density, as a biomarker is dependent on a number of variables, including pollutant concentrations, time of exposure, acclimation and tolerance within the organism. The optimal strength of biomarkers when assessed with other parameters at different levels of biological organisation should provide a holistic account of the effects of stressors on homeostasis.

P. warreni, as a model for the mode of action of Cd pollution, may have coped with chronic elevated concentrations of Cd at the time of investigation, partly because of acclimation and protection by the microbial gill fauna, which initially acted as a barrier to ion uptake. The neurotoxic effects exerted by the toxicant, and which reflected behavioural changes, may only become apparent though at a later stage in the life of the crab.

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