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Impact of cadmium on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Crustacea: Decapoda)

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Abstract – The objective of this study was to investigate the impact of cadmium on the structure of gills and epipodites in late juvenile *Penaeus japonicus*. Cadmium titrations were performed by atomic absorption flame spectrophotometry, and structural observations were performed through light- and electron-microscopy. The cadmium concentration increased in different tissues (mainly gills, epipodites and hepatopancreas) according to the external cadmium concentration and the exposure time. The structural changes which occurred in the gill and epipodite cells appeared to be a function of cadmium accumulation in these tissues. Gill cells of shrimps exposed to 2 and 200 µg $Cd{\cdot}L^{-1}$ for 15 d did not display discernible structural changes. An increased number of nephrocytes was noted in gill filaments of shrimps exposed to 200 µg $Cd{\cdot}L^{-1}$. Exposure to 2 000 and 4 000 µg $Cd{\cdot}L^{-1}$ during 4 d resulted in profound structural changes. The epithelial cells were necrosed, disorganized and vacuolized. Exposure to 2 µg $Cd{\cdot}L^{-1}$ for 15 d did not result in discernible structural changes of epipodite cells. Exposure to 200 µg $Cd{\cdot}L^{-1}$ for 15 d, 2 000 and 4 000 µg $Cd{\cdot}L^{-1}$ during 4 d induced profound alterations of the epipodites: increased thickness, decreased number of apical microvilli, basal infoldings and mitochondria, occurrence of pseudomyelinic structures. These alterations are discussed in relation to the respiratory and osmoregulatory functions of gills and epipodites. © Ifremer/Elsevier, Paris

Cadmium / gill / epipodite / histology / ultrastructure / *Penaeus japonicus*

Résumé – Impact du cadmium sur la structure des branchies et des épipodites de la crevette *Penaeus japonicus* (Crustacea : Decapoda). Cette étude a pour objectif d'étudier l'impact du cadmium sur la structure des branchies et des épipodites de juvéniles de *Penaeus japonicus*. Les dosages de cadmium ont été réalisés par spectrophotométrie d'absorption atomique avec flamme et les observations structurales par microscopie photonique et électronique. La concentration en cadmium dans les différents tissus (en particulier branchies, épipodites et hépatopancreas) augmente avec la concentration en métal du milieu extérieur et la durée de l'exposition. Les changements structuraux au niveau des cellules épithéliales des branchies et des épipodites sont fonction de la concentration en métal dans ces organes. Les cellules branchiales des crevettes exposées à 2 et 200 µg $Cd{\cdot}L^{-1}$ durant 15 j n'ont pas présenté de changements structuraux importants. Une augmentation du nombre des néphrocytes a été notée dans les filaments branchiaux des crevettes exposées à 200 µg $Cd{\cdot}L^{-1}$. L'exposition à 2 000 et 4 000 µg $Cd{\cdot}L^{-1}$ durant 4 j a entraîné des changements structuraux profonds. Les cellules épithéliales branchiales étaient nécrosées, désorganisées et vacuolisées. L'exposition à 2 µg $Cd{\cdot}L^{-1}$ durant 15 j n'a pas entraîné de changements structuraux importants des épipodites. L'exposition à 200 µg $Cd{\cdot}L^{-1}$ durant 15 j, et à 2 000 et 4 000 µg $Cd{\cdot}L^{-1}$ durant 4 j a provoqué des altérations profondes des épipodites : augmentation d'épaisseur, diminution du nombres des microvillosités apicales, invaginations basales et mitochondries, apparition de structures pseudomyéliniques. Ces altérations sont discutées en relation avec la fonction respiratoire et osmorégulatrice des branchies et des épipodites. © Ifremer/Elsevier, Paris

Cadmium / branchie / épipodite / histologie / ultrastructure / *Penaeus japonicus*

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1. INTRODUCTION

Cadmium exists in trace quantities in the marine environment, largely complexed with ions such as Cl [63], and only a small proportion, the free hydrated metal ion form Cd^{2+}, is bioavailable to organisms [20]. Its average concentration in unpolluted waters is about 0.05 µg $Cd{\cdot}L^{-1}$ [67]. Due to anthropogenic input and local geological conditions, concentrations of cadmium in coastal waters tend to increase. For instance, higher cadmium concentrations have been reported in the following areas: 0.1–0.4 µg $Cd{\cdot}L^{-1}$ in the Gironde estuary [14] and 0.4 µg $Cd{\cdot}L^{-1}$ in Marseille coastal waters, France [42], 0.45 µg $Cd{\cdot}L^{-1}$ in the Severn estuary, England [5], 0.14–9 µg $Cd{\cdot}L^{-1}$ in the coastal waters of Patras, Greece [40], 4.24–5.22 µg $Cd{\cdot}L^{-1}$ in the Ebro river delta, Spain [69]. In the heavily polluted estuary of Reghaia, Algeria, cadmium concentration is up to 200–1 000 µg $Cd{\cdot}L^{-1}$ [9].

Cadmium is a very toxic metal [36, 66], without apparent physiological function [82, 84]. It can accumulate in crustacean tissues [54, 65, 82, 84] according to the external metal concentration. There is no evidence that crustaceans can regulate internal cadmium concentrations [17, 29, 79, 81, 84].

Several studies have shown that high concentrations of cadmium were lethal for crustaceans, such as *Penaeus duorarum* [54], *P. merguiensis* [28], *P. japonicus* [6], *Palaemonetes vulgaris* [54], *Callianassa australiensis* [1].

Several adverse effects of cadmium have been reported on crustaceans physiology, especially on respiration and osmoregulation. The impact of cadmium on respiration has been studied in the crabs *Carcinus maenas*, *Cancer pagurus* [75], *Uca pugilator* [79], in the lobster *Homarus americanus* [74] and in the mysid *Leptomysis lingvura* [33]. Adverse effects of cadmium on osmoregulation have been reported in the crab *C. maenas* [11, 75], in the isopods *Idotea neglecta*, *Jaera albifrons* [39] and in the shrimp *P. japonicus* [6].

In *P. japonicus*, the 96-h LC_{50} in 37 PSU (per salinity unit) was 5 500 µg $Cd{\cdot}L^{-1}$ in late juveniles [6]. These authors have also demonstrated that exposure to sublethal concentrations of 2 000 and 4 000 µg $Cd{\cdot}L^{-1}$ in 37 PSU seawater significantly reduced the hypo-osmoregulatory capacity of exposed individuals by 28 and 53 %, respectively.

The gills of marine animals are crucial for respiration, acid-base balance, osmotic and ionic regulation [19, 51, 62]. Since the branchial epithelium is a tissue where both active and passive exchanges occur between the organism and its environment [68], it is likely to be a site of action of metals. A number of investigations have described morphological damage to gills in different crustaceans chronically exposed to cadmium [26, 27, 57, 58], that may result in disruption of respiratory and osmoregulatory functions.

In *P. japonicus*, ultrastructural and physiological studies have demonstrated that the location of osmoregulatory tissues in the branchial chamber changes during the early post-embryonic development [12, 13]. In late juveniles, these tissues were mainly found in epipodites. In contrast, the gill epithelium was very thin and slightly differentiated, leading to the hypothesis that these organs were probably mainly involved in respiration.

Following the demonstration of the adverse effect of cadmium on osmoregulation [6], the objective of this study was to measure the concentration of cadmium in different tissues and to investigate the effect of cadmium on gills and epipodites of juveniles of the peneid shrimp *P. japonicus* (Bate, 1888).

2. MATERIALS AND METHODS

2.1. Animals

Late juveniles of *Penaeus japonicus* approximately 6-month-old (13 ± 1 g) were obtained from France-Étang S.A., a Mediterrannean shrimp farm located in Port-Leucate, Aude, France. After transport to the laboratory, the shrimps were maintained for 1 week prior to the experiments in recirculated natural seawater (Eheim filters and pumps) obtained from Palavas, Hérault, at a salinity of 37 ± 1 PSU and a temperature of 25 ± 1 °C, with a 12-h light and 12-h dark cycle. They were fed with mussels during the preacclimatory period.

Since crustacean physiology, and particularly the tegument structure and haemolymph osmolality, fluctuates as a function of the intermolt stages [22], these were a prime consideration in the selection of experimental animals. Only shrimps in stage C or D_0 were considered for the experimental study. The molt stages were determined by microscopical examination of antennal scales according to Drach and Tchernigovtzeff's method [32].

2.2. Exposure of shrimps to cadmium

A stock solution of cadmium (1 000 mg $Cd{\cdot}L^{-1}$) was prepared in deionized water from $CdCl_2.2H_2O$ (Fluka). Selected experimental concentrations were made by addition of adequate volumes of the stock solution to seawater.

In a previous work, Bambang et al. [6] reported that the 96-h LC_{50} value for cadmium was 5 500 µg$\cdot L^{-1}$ (95 % confidence interval: 4 567–6 869 µg $Cd{\cdot}L^{-1}$) in late juvenile *P. japonicus*, and that their hypo-osmoregulatory capacity was significantly reduced after a 4-d exposure to 2 000 and 4 000 µg $Cd{\cdot}L^{-1}$ in seawater at 37 PSU. Based on these values, different sublethal concentrations and times of exposure were used and are indicated below. Temperature was maintained at 25 ± 1 °C. Seawater was aerated but not filtered. Cadmium concentration was checked every two or three days in each medium just before and after renewal; measurements were performed on a Varian

AA-1275 atomic absorption flame spectrophotometer to follow the concentration of cadmium during the time of exposure.

2.2.1. Exposure to different cadmium concentrations during 4 d

Three groups of five late juveniles were exposed for 4 d in 20-L plastic tanks to seawater containing cadmium at the following concentrations: C (control medium, natural seawater: < 2 µg $Cd \cdot L^{-1}$), 2 000 and 4 000 µg $Cd \cdot L^{-1}$. Each exposure was duplicated and the media were renewed every 2 d. The shrimps were not fed during the exposure period. The intermolt stages were determined at the end of the experiment.

2.2.2. Exposure to different cadmium concentrations during 15 d

Three groups of five late juveniles were exposed for 15 d in 20-L plastic tanks to seawater containing cadmium at the following concentrations: C (control medium, natural seawater), 2 and 200 µg $Cd \cdot L^{-1}$. Each exposure was duplicated and test media were renewed every 3 d. Cadmium concentration was checked in each medium every 3 d. The shrimps were fed with mussels. Excess food and faeces were removed daily during the exposure period. The intermolt stages were determined at the end of the experiment.

2.3. Measurement of cadmium in different tissues

Four shrimps were dissected and the following organs were removed from each individual of each experimental group: gills and epipodites (together because of small size of epipodites), hepatopancreas, abdominal muscle and carapace. The tissue samples were dried at 60 °C for 48 h, homogenized and weighed then transferred to 3 mL concentrated nitric acid at 90 °C for 4 h. After filtration and dilution to 50 mL with deionized water, cadmium concentrations were mesured on a Varian AA-1275 atomic absorption flame spectrophotometer [3]. ANOVA and Dunnett's test were used for statistical analysis [53].

2.4. Histology

At the end of the period of cadmium exposure, four shrimps in intermolt stages C and D_0 from control and treated groups were sacrificed. The third to eighth gills and the second to fifth epipodites were dissected and fixed in Halmi's fluid. Samples were embedded in paraffin, sectionned at 5-µm thickness on a Minot Leitz Wetzlar microtome, stained with Masson's trichrome (variant Goldner) [50] and examined with a Leica-microscope.

For electron-microscopy, gills and epipodites were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer in saline. The osmolality of the fixative was adjusted through the addition of sodium chloride to the osmolality of shrimp haemolymph. Osmolalities of the haemolymph and of the media were measured with a Roebling osmometer. Tissue samples were embedded in LR-White resin. Semithin sections (2 µm) were cut with a glass knife on a Reichert ultramicrotome, and stained with a methylene blue solution. Ultrathin sections (0.1 µm) cut with a Diatome diamant knife on the same ultramicrotome, collected on copper grids, were stained with an aqueous saturated solution of uranyl acetate and lead citrate. Sections from at least four shrimps per treatment were examined with a Jeol 200CX transmission electron-microscope at 100 kV.

3. RESULTS

3.1. Cadmium concentration in seawater and in the test media

Cadmium concentrations in control seawater and in the different test media are given in *table I*. After 48 h of exposure time, cadmium concentrations in experimental media decreased by 12.5 % at 2 000 µg $Cd \cdot L^{-1}$ and by 7.0 % at 4 000 µg $Cd \cdot L^{-1}$; after 72 h, the cadmium concentration decreased by 16.3 % at 200 µg $Cd \cdot L^{-1}$.

Table I. Concentration of cadmium in control seawater (C) and in the different test media (mean ± SD). A, Freshly prepared medium; B, after 48 h (2 000, 4 000 µg $Cd \cdot L^{-1}$) or 72 h (0, 2, 200 µg $Cd \cdot L^{-1}$) exposure. Number of measurements per condition: 4 (2 000, 4 000 µg $Cd \cdot L^{-1}$) and 10 (0, 2, 200 µg $Cd \cdot L^{-1}$).

Expected concentration (µg $Cd \cdot L^{-1}$)	Measured concentration (µg $Cd \cdot L^{-1}$)	
	A	B
0 (C)	< 2	< 2
2	< 2	< 2
200	209 ± 16	175 ± 16
2 000	2 022 ± 31	1 770 ± 25
4 000	4 013 ± 34	3 732 ± 30

3.2. Cadmium concentration in different tissues

The concentrations of cadmium in shrimp tissues exposed to different treatments are given in *table II*. In control shrimps, the concentration of cadmium in the carapace, hepatopancreas and abdominal muscles was low (from 0.8 to 3.8 µg $Cd \cdot g^{-1}$ dw), and it was slightly higher in gills and epipodites (9–10 µg $Cd \cdot g^{-1}$ dw). Compared to controls, the cadmium concentration in the carapace increased significantly by 1 000 % in shrimps exposed to 200 µg $Cd \cdot L^{-1}$ for 15 d and by 1 475 and 2 700 % in shrimps exposed to 2 000 and 4 000 µg $Cd \cdot L^{-1}$, respectively, for 4 d, respectively. The concentration of cadmium in the abdominal muscle increased by 1 770 % in shrimps exposed to 4 000 µg $Cd \cdot L^{-1}$ for 4 d. In the hepatopancreas, the cadmium concentration increased by 2 530 % in shrimps exposed to 200 µg $Cd \cdot L^{-1}$ for 15 d and by 2 460 and 8 500 % in shrimps exposed to 2 000 and

Table II. Concentration of cadmium in different tissues of *Penaeus japonicus* exposed to different media. *, Significant difference with controls ($P < 0.05$). Number of measurements for each condition: 4.

Cadmium concentrations in media (μg Cd·L^{-1})	Cadmium concentrations in tissue (μg Cd·g^{-1} dw) (mean ± SD)			
	Gills and epipodites	Hepatopancreas	Abdominal muscle	Carapace
0, 15 d	9.0 ± 6.4	3.8 ± 2.3	1.1 ± 1.1	1.2 ± 0.9
2, 15 d	10.0 ± 4.1	2.7 ± 2.2	0.8 ± 0.4	1.7 ± 1.1
200, 15 d	399.0 ± 66.5 *	99.8 ± 18.5 *	4.5 ± 1.6	13.2 ± 6.2 *
0, 4 d	10.2 ± 6.1	3.1 ± 3.1	1.0 ± 0.6	1.3 ± 1.2
2 000, 4 d	386.2 ± 51.8 *	97.2 ± 16.3 *	4.8 ± 3.5	18.9 ± 8.8 *
4 000, 4 d	1 017.0 ± 257.9 *	326.5 ± 93.0 *	20.6 ± 8.8 *	33.8 ± 10.1 *

4 000 μg Cd·L^{-1} for 4 d, respectively. The cadmium concentration in gills and epipodites increased significantly by 4 300 % in shrimps exposed to 200 μg Cd·L^{-1} for 15 d and by 4 200 and 11 200 % respectively after exposure to 2 000 and 4 000 μg Cd·L^{-1} for 4 d.

3.3. Gills in control shrimp

Penaeus japonicus possess 18 gills in each branchial chamber. In late juveniles, the gills were 3.5–7 mm long. The gills of *P. japonicus* are dendrobranchiate, with an axis that supports numerous secondary laminae giving rise, at right angles, to filaments divided into two branches near their termini (*figure 1*a). A longitudinal septum divides the lumen of each axis, branch, and filament into afferent and efferent vessels (*figure 1*a).

In late juveniles, the surface of each lamina or filament was covered by a thin (0.7–1 μm) cuticle underlain by a monolayer epithelium. Connective tissues were present in the septum of filaments and in the axis of the gill. Nephrocytes (15–30 μm in diameter) were observed near the septum, close to the loose connective tissue bordering the haemolymphatic lacuna and they were surrounded by a basal lamina (*figure 1*b). These cells were limited by an interdigitating cell membrane forming pedicels; they contained one or more nuclei, one or more large and dense vacuoles, and other small vacuoles and organelles in the cytoplasm.

The epithelium of the branches and filaments was generally thin, about 0.7–2 μm in transverse section; it was thicker near the nuclei (5–7 μm) and at the tip of the filaments (1–2 μm) which were widened to form a distal lacuna. The cytoplasm of the epithelial cells contained few organelles, and these included the nucleus and a few spherical or elongated mitochondria (*figure 1*c). Apical microvilli and basal infoldings were generally very limited or absent.

Similar morphology was noted between different filaments along the length of the gill axis, or between the anterior and posterior gills.

3.4. Epipodites in control shrimp

The epipodites, or mastigobranches, are elongated, thin, biramous structures, attached to coxopodites of the first to the sixth thoracic appendages. Six of them are present in each branchial chamber. In juveniles, they were 4–6 mm long and about 20–30 μm thick.

Each side of the epipodite was limited by a cuticle and an underlying monolayer epithelium. The two facing epithelia were separated by a central connective tissue. The epithelium was made of high columnar cells (9–13 μm), with their nucleus located in the apical part of the cell under a thin cuticle (1–2 μm) (*figure 1*d). Ultrastructurally, the epithelial cells featured both apical microvilli and basal infoldings (*figure 1*e, f). The apical microvilli, about 2 μm deep, were oriented at right angles to the cuticle (*figure 1*e). A thick basal lamina separated the epithelium from the central connective tissue (*figure 1*f). The basal infoldings were organized as a compact network penetrating deeply, up to 12 μm, into the cytoplasm. Abundant elongated mitochondria (1–2 μm) were inserted between the infoldings (*figure 1*f).

3.5. Effect of cadmium on gills

After cutting out a branchiostegite, the gills of control shrimp and of those exposed to 2 and 200 μg Cd·L^{-1} presented a white or pale yellow colour. In contrast, the gills were blackened in shrimps exposed to 2 000 and 4 000 μg Cd·L^{-1}. The blackening intensity was higher in shrimp exposed to 4 000 μg Cd·L^{-1} than in those exposed to 2 000 μg Cd·L^{-1} (*figure 2*a).

Exposure to 2 μg Cd·L^{-1} for 15 d did not result in any discernible structural changes of the gill cells compared to those of control shrimps.

After 15 d of exposure to 200 μg Cd·L^{-1}, an increased number of nephrocytes was observed in the filaments (*figure 2*b). The epithelial layer of the filaments was still very thin (0.8–2 μm) and contained few intracellular organelles. Its structure was comparable with that of control shrimps.

Exposure to 2 000 μg Cd·L^{-1} during 4 d resulted in important alterations of the gill filaments such as necrosis and a thickening of the epithelia of the distal filament and of the septum, resulting in narrower or obstructed haemolymph vessels (*figure 2*c). Some distal filaments were completely disorganized, with autolysis and loss of cytoplasmic organelles (*figure 2*d). Numerous vacuoles and nuclear pycnosis were noted in their epithelial cells. In some necrosed gill filaments, the epithelial cells were separated from the

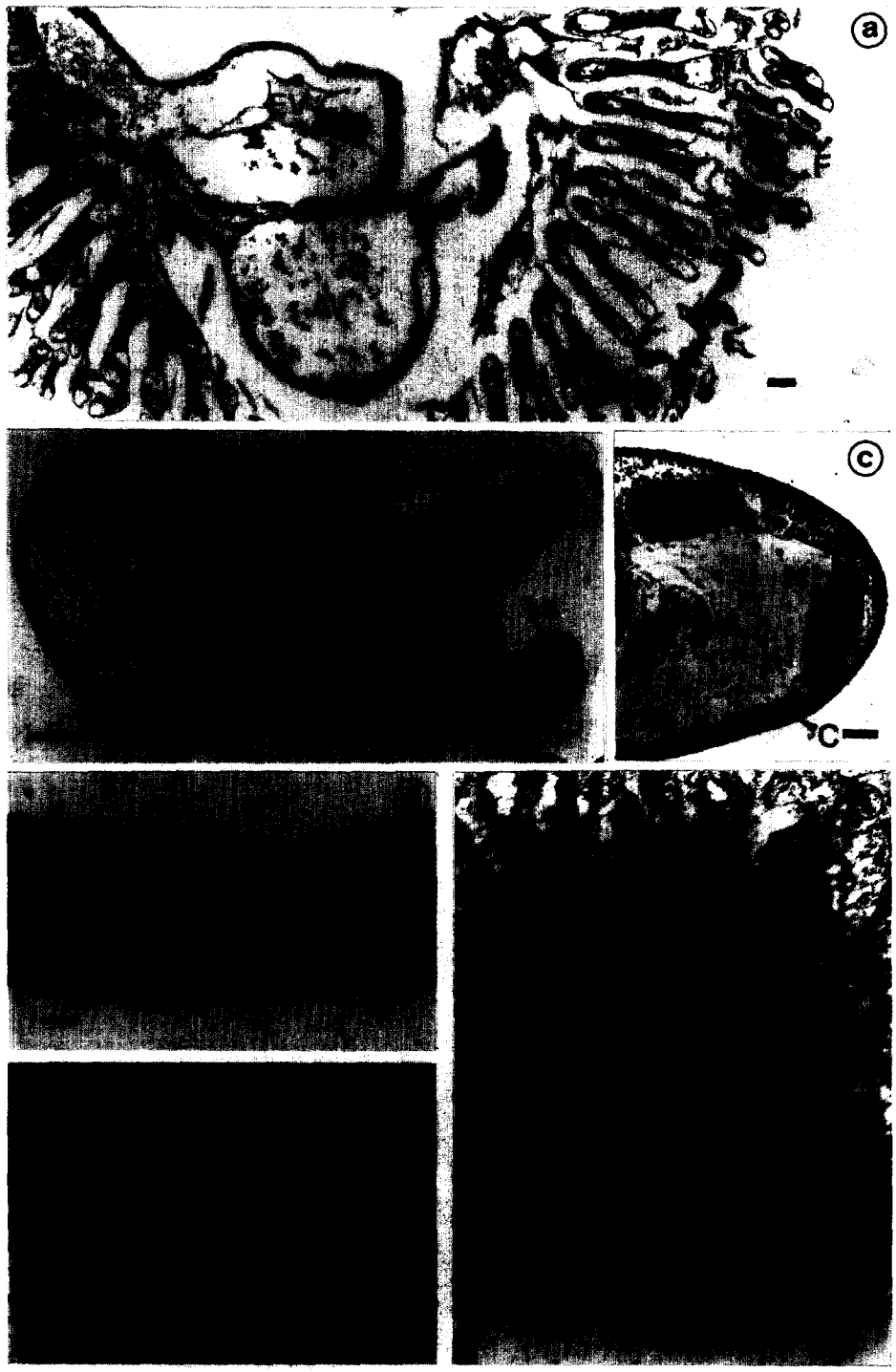

Figure 1. Gills and epipodites from control *Penaeus japonicus*. (a) Transverse section of a gill: the axis is divided into afferent and efferent vessels, and supports branches and filaments (bar size 10 μm). (b) Semithin section of a gill filament: the thin monolayer epithelium is covered by a thin cuticle. Each tip of the filament is occupied by a haemolymphatic lacuna. Note the presence of a nephrocyte (bar size 4 μm). (c) Ultrastructure of the tip of a gill filament: the cuticle covers the epithelium. The cytoplasm contains few mitochondria (bar size 2.5 μm). (d) Transverse section of an epipodite: two simple columnar epithelia are separated by a central connective tissue. The nuclei are located in the apical part of the cell under a thin cuticle (bar size 5 μm). (e) Ultrastructure of the apical part of an epipodite: numerous microvilli are located under the cuticle (bar size 0.5 μm). (f) Ultrastructure of the basal part of an epipodite with numerous basal infoldings associated with mitochondria (bar size 0.5 μm). AV: afferent vessel; B: branch; BL: basal lamina; C: cuticle; CT: connective tissue; E: epithelium; EV: efferent vessel; F: filament; H: haemolymphatic lacuna; Ht: haemocyte; IN: basal infolding; M: mitochondria; MV: apical microvilli; N: nucleus; NP: nephrocyte; S: septum.

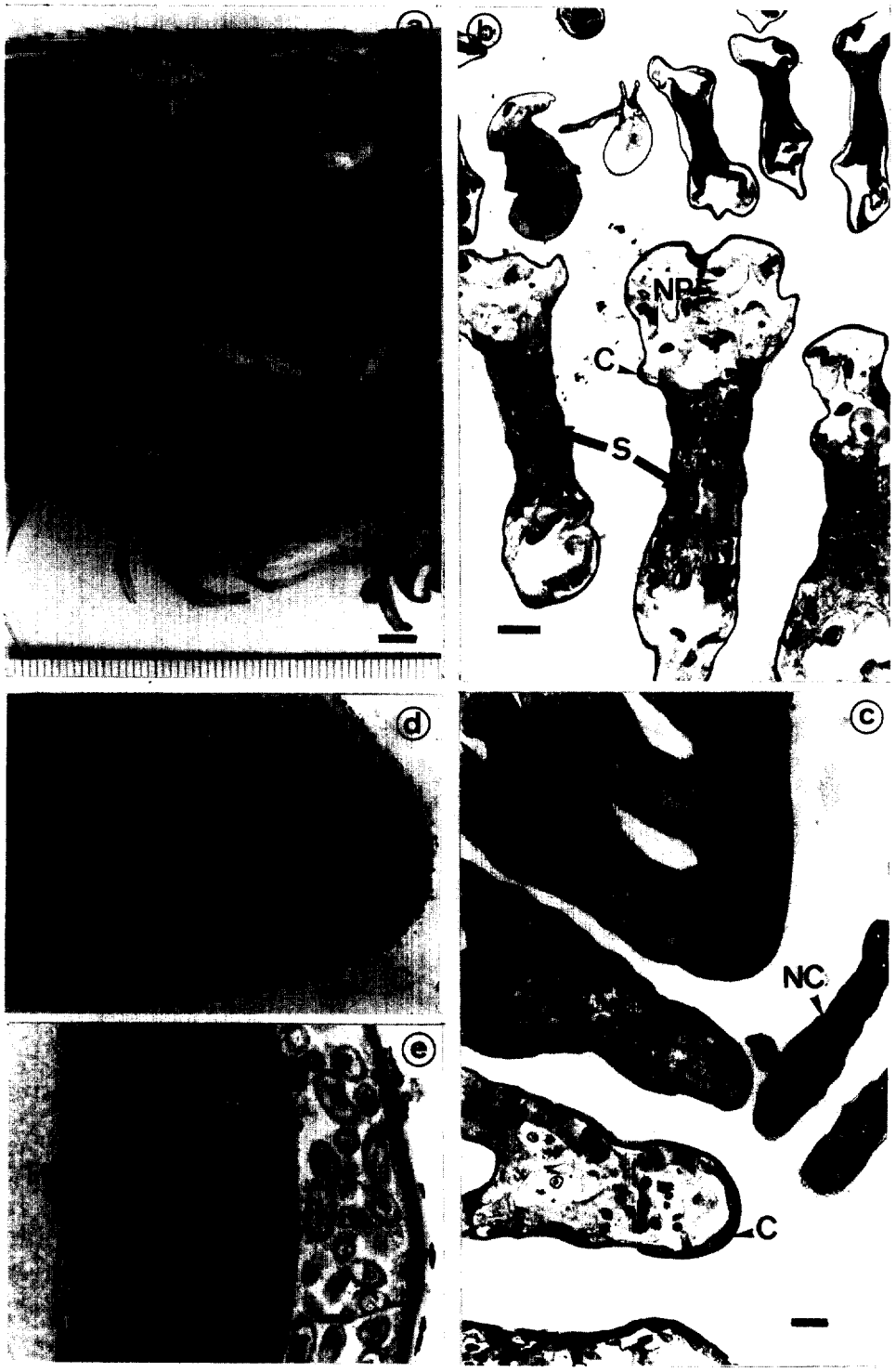

Figure 2. Gills from *Penaeus japonicus* exposed to cadmium. (a) Control and cadmium-exposed shrimps after removal of the left branchiostegite. a: Control shrimp, b: shrimp exposed to 2 000 µg $Cd{\cdot}L^{-1}$, c: shrimp exposed to 4 000 µg $Cd{\cdot}L^{-1}$ (bar size 3 mm). (b) Exposure to 200 µg $Cd{\cdot}L^{-1}$ for 15 d, semithin section of gill filaments. Nephrocytes are abundant (bar size 20 µm). (c) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, semithin section of gill filaments. Note the numerous nephrocytes and the necrosis of filaments (bar size 20 µm). (d) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, ultrastructure of a gill filament. Epithelial cells are completely disorganized, with autolysis and loss of cytoplasmic organelles (bar size 2 µm). (e) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, ultrastructure of a gill filament. A space containing bacteria is present between the cuticle and the epihelium (bar size 0.5 µm). Bt: bacteria; C: cuticle; E: epithelium; Ep: epipodite; G: gills; H: haemolymphatic lacuna; N: nucleus; NC: necrosis; NP: nephrocyte; S: septum; V: vacuole.

cuticle by a space where a large number of bacteria were present (*figure* 2e).

Exposure to 4 000 µg $Cd \cdot L^{-1}$ during 4 d resulted in the presence of necrosed areas in almost all filaments. The epithelial cells were deeply swollen, resulting in narrowed haemolymph vessels (*figure* 3a). In some necrosed filaments, large spaces with no cytoplasm were observed beneath the cuticle, which separated the epithelial cells from the cuticle (*figure* 3b). In other necrosed terminal filaments, both spaces present beneath and along the cuticle and the haemolymphatic lacuna were infiltrated by a black electron-dense material (*figure* 3c). In some gill filaments, nuclei were fragmented (*figure* 3d).

3.6. Effect of cadmium on epipodites

The epipodites of control shrimp and of those exposed to 2 and 200 µg $Cd \cdot L^{-1}$ during 15 d displayed a whitish or yellowish colour. In contrast, the epipodites from shrimp exposed to 2 000 and 4 000 µg $Cd \cdot L^{-1}$ presented a black colour. The intensity of blackening was higher in shrimp exposed to 4 000 µg $Cd \cdot L^{-1}$ than in those exposed to 2 000 µg $Cd \cdot L^{-1}$ (*figure* 2a).

Exposure to 2 µg $Cd \cdot L^{-1}$ during 15 d did not result in discernible structural changes of the epipodite cells, compared to those of control shrimps.

The epipodites from shrimp exposed to 200 µg $Cd \cdot L^{-1}$ for 15 d showed slight structural changes. They were slightly swollen (23–45 µm thick compared to 20–30 µm in controls). In some regions, the two facing epithelia were in contact (*figure* 3e). Apical microvilli expanded to lacunae at their basis. Numerous vacuoles were observed beneath the apical microvilli (*figure* 3f). Basal infoldings developed into multilayer membranes with a high number of swollen hypertrophied mitochondria (1.3–4 µm) and vacuoles were located between them (*figure* 3g).

The epipodites from shrimp exposed to 2 000 µg $Cd \cdot L^{-1}$ for 4 d showed deeper structural changes. They were largely swollen (55–77 µm thick). The epithelial cells were profoundly disorganized with large patches of necrotic area, and the differentiation between the two epithelia and the central connective tissue disappeared (*figure* 4a). Ultrastructurally, most cells had lost their apical microvilli. Mitochondria decreased in number and size, and their structures were condensed. Large vacuoles containing electron-dense materials were observed in some necrosed epipodites (*figure* 4b). Most nuclear matrices were condensed with vacuoles around the nucleus. Basal membrane infoldings were disorganized, transformed into an irregular network interspersed with numerous vacuoles, or they were totally absent (*figure* 4c). Pseudomyelinic structures were also noted in some epithelial cells of the epipodites (*figure* 4d).

After exposure to 4 000 µg $Cd \cdot L^{-1}$, the epipodites were largely swollen (64–105 µm thick) (*figure* 4e). The epithelial layers were deeply disorganized: the cell structure was lost with heavy autolysis and necrosis. All organelle structures were completely deformed and undistinguishable (*figure* 4f).

4. DISCUSSION

In *Penaeus japonicus*, the cadmium concentrations in gills and epipodites, hepatopancreas, carapace and abdominal muscle of control shrimps were low, below 10 µg $Cd \cdot g^{-1}$ dw. Similar levels (0.45–4.70 µg $Cd \cdot g^{-1}$ dw) have been found in the same tissues of the carid shrimp *Palaemon elegans* [82]. After exposure to higher concentrations of cadmium in the medium (> 2 µg $Cd \cdot L^{-1}$), our results showed that the cadmium concentration increased in different tissues according to the external cadmium concentration and the exposure time. The pattern of accumulation of cadmium in different tissues of juvenile *P. japonicus* is generally in agreement with similar findings in other decapod crustaceans [38, 54, 65]. Other experimental studies have also shown that the concentration of cadmium in tissues was directly proportional to the concentration in the surrounding seawater [38, 79, 82, 84]. In *P. japonicus*, the increase in metal level varied with the tissue. The highest cadmium accumulation was observed in gills and epipodites, followed by the hepatopancreas, the carapace and the abdominal muscle. A similar hierarchy of accumulation was observed in *Palaemon elegans* after exposure to 100 µg $Cd \cdot L^{-1}$ for 5 d, but it changed after exposure to the same concentration for 15 d: hepatopancreas, gills, cuticle and muscle [82]. In *P. japonicus* and *P. elegans*, the highest cadmium concentration in gills after exposure to cadmium for 4 or 5 d might be related to the important quantity of this metal in the haemolymph and/or in the necrosed tissues, or these organs might constitute the entry sites of the metal and act as a transient store for accumulated cadmium [49]. The lower cadmium concentration in the hepatopancreas could originate from a progressive transfer of cadmium from the gills to the hepatopancreas via the haemolymph [10], and/or from a process of differentiation of the hepatopancreas epithelium (as observed by Alikhan [2] in the isopod *Porcellio spinicornis*) leading to a transfer of the metal into the intestinal lumen and from this site to the exterior (as observed by Brown [16] in crayfish). However, the higher cadmium concentration in the hepatopancreas of *P. elegans* after exposure to cadmium for 15 d also suggested that this organ plays a role in metal storage and/or in detoxification by a metal-binding component [83].

In the shrimp *Penaeus japonicus*, gills and epipodites are present in each branchial chamber and appear concurrently during post-embryonic development [13]. In late juveniles, gills and epipodites attach at the same location on the coxopodite of maxillipeds and pereopods. The epithelium of the gills is very thin. The cytoplasm of epithelial cells contains few

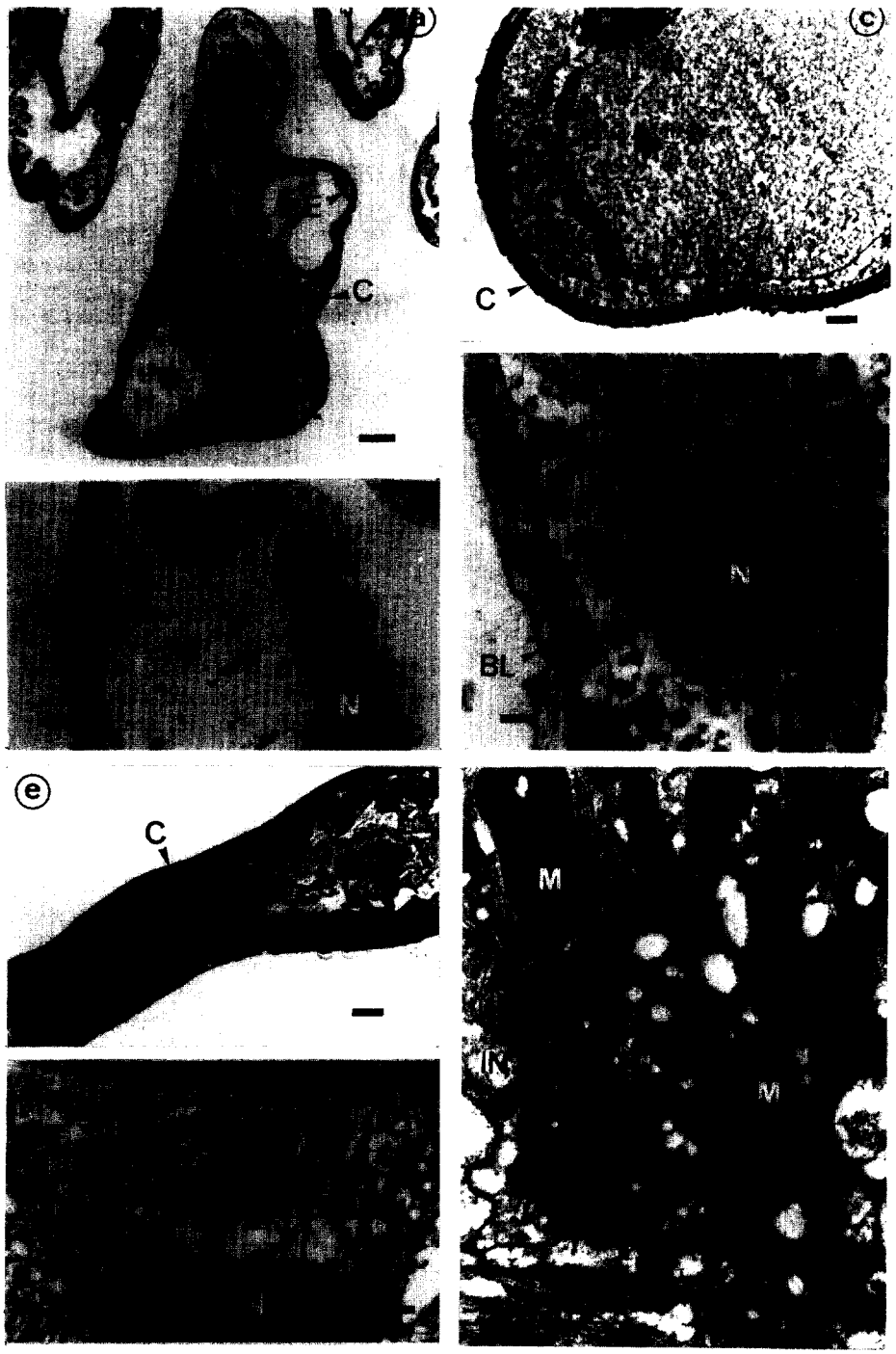

Figure 3. Gills and epipodites from *Penaeus japonicus* exposed to cadmium. (a) Exposure to 4 000 µg $Cd \cdot L^{-1}$ for 4 d, semithin section of gill filaments. Numerous nephrocytes and necrosed areas are visible (bar size 20 µm). (b) Exposure to 4 000 µg $Cd \cdot L^{-1}$ for 4 d, ultrastructure of a gill filament. A large space (*) separates the cuticle from the epithelium (bar size 1 µm). (c) Exposure to 4 000 µg $Cd \cdot L^{-1}$ for 4 d, ultrastructure of a gill terminal filament. A space (*) separates the epithelium from the cuticle, and is infiltrated by the cytoplasm rich in electron-dense materials (bar size 0.5 µm). (d) Exposure to 4 000 µg $Cd \cdot L^{-1}$ for 4 d, ultrastructure of a gill filament. The nucleus is fragmented (bar size 0.5 µm). (e) Exposure to 200 µg $Cd \cdot L^{-1}$ for 15 d, semithin section of an epipodite. The epithelial cells are slightly necrosed. The two facing epithelia are in contact (bar size 5 µm). (f) Exposure to 200 µg $Cd \cdot L^{-1}$ for 15 d, ultrastructure of the apical part of epithelial cells of an epipodite. Note numerous vacuoles; the microvilli expand into lacunae (bar size 1 µm). (g) Exposure to 200 µg $Cd \cdot L^{-1}$ for 15 d, ultrastructure of the basal part of epithelial cells of an epipodite. Basal infoldings develop into multilayer membranes with numerous swollen mitochondria and vacuoles between them (bar size 0.5 µm). BL: basal lamina; C: cuticle; CT: connective tissue; E: epithelium; H: haemolymphatic lacuna; IN: basal infolding; M: mitochondria; MV: apical microvilli; N: nucleus; NC: necrosis; NP: nephrocyte; V: vacuole.

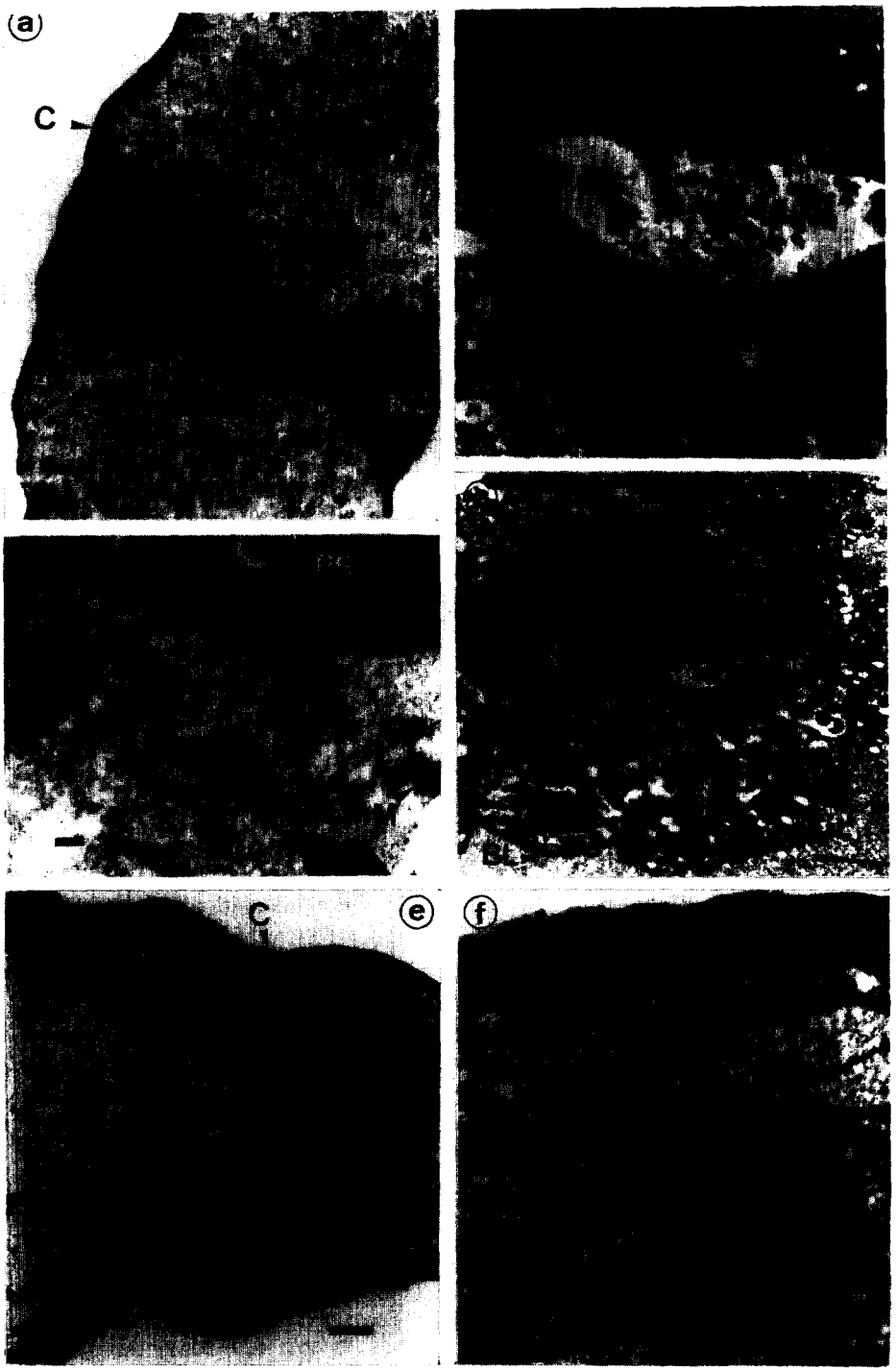

Figure 4. Epipodites from *Penaeus japonicus* exposed to cadmium. (a) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, semithin section of an epipodite. The epithelial cells are necrosed and disorganized (bar size 7 µm). (b) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, ultrastructure of the apical part of the epithelium of an epipodite. The epithelial cells have no detectable apical microvilli but they contain numerous vacuoles. Note a large vacuole containing electron-dense materials (bar size 1 µm). (c) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, ultrastructure of the basal part of the epithelium of an epipodite. The basal infoldings host numerous vacuoles and few transformed mitochondria containing condensed cristeae (bar size 0.5 µm). (d) Exposure to 2 000 µg $Cd{\cdot}L^{-1}$ for 4 d, ultrastructure of an epipodite. The epithelial cells contain pseudomyelinic structures in necrosed regions (bar size 0.5 µm). (e) Exposure to 4 000 µg $Cd{\cdot}L^{-1}$ for 4 d, semithin section of a swollen epipodite. The epithelial cells are necrosed and disorganized (bar size 7 µm). (f) Exposure to 4 000 µg $Cd{\cdot}L^{-1}$ for 4 d, ultrastructure of an epipodite. The epihelial cells are deeply disorganized. Their cellular structure is lost and all organelle structures are deformed and undistinguishable (bar size 1 µm). BL: basal lamina; C: cuticle; E: epithelium; M: mitochondria; N: nucleus; NC: necrosis; PS: pseudomyelinic structure; V: vacuole.

organelles, including the nucleus and a few mitochondria. Some cells present apical microvilli, but basal infoldings and other organelles are scarce. Based on widely admitted anatomo-functional relations [61], these observations lead to the hypothesis already proposed by Bouaricha et al. [13] that the gills of *P. japonicus* are implicated mainly in respiration. Compared to gills, the epithelium of epipodites is thicker and more differentiated. The structure of the epipodite cells is very similar to the thick cells, or ionocytes, observed in the posterior gills of crabs [7, 25, 61, 73], characterized by numerous microvilli, basal infoldings and associated mitochondria. This typical aspect of ion-transporting tissues suggests that the epipodites are most probably involved in osmoregulation. The high level of Na^+-K^+-ATPase activity measured in epipodites of late juvenile *P. japonicus* supports this hypothesis [13].

The gill cells of shrimps exposed to 2 and 200 µg $Cd \cdot L^{-1}$ during 15 d did not display important structural changes. However, the number of nephrocytes increased in gill filaments exposed to 200 µg $Cd \cdot L^{-1}$. A profound alteration of gills occurred after exposure to 2 000 and 4 000 µg $Cd \cdot L^{-1}$ for 4 d. At these concentrations, gills tended to blacken. In shrimp exposed to 2 000 µg $Cd \cdot L^{-1}$, the necrosis of gill cells resulted in narrowed or obstructed haemolymphatic vessels. In certain necrosed filaments, a space was present between the cuticle and the epithelial cells and was infiltrated by numerous bacteria. After exposure to 4 000 µg $Cd \cdot L^{-1}$, a large space separating the epithelial cells from the cuticle and containing black electron-dense material was observed in the necrosed filaments. In certain gill filaments, the nucleus was fragmented. These structural alterations seem to be in direct relation to the external cadmium concentration and to the time of exposure.

Previous studies conducted in a few species of crustaceans have shown that cadmium can alter the gill structure. Similar findings, such as black-pigmented lesions occurred in the gills of the shrimps *Penaeus duorarum* [26, 54] and *P. merguiensis* [27] after exposure to cadmium. After exposure to 763 µg $Cd \cdot L^{-1}$ for 15 d, Couch [26] found that black lesions occurred in distal gill filaments of *P. duorarum*. Blackening of gills was followed by a complete loss of cell structural integrity and organization, and by bacterial and fungal invasion. In the same species, exposure to 5 000 µg $Cd \cdot L^{-1}$ for 5 to 7 d also induced gill blackening [54]. Severe black-pigmented lesions, which occurred in the gills of *P. merguiensis* after 15 d exposure to 500 µg $Cd \cdot L^{-1}$, consisted of lamellar haemocyte aggregation and melanization of branchial lamellae, accompanied by necrosis, especially in the distal gill filament [27]. Papathannasiou and King [58] reported that the fine structure of gill cells of *Palaemon serratus* was affected by exposure to cadmium at 50 $mg \cdot L^{-1}$ during 44 h; the mitochondria and the membrane system supposedly involved in osmoregulation were the most affected organelles. A similar alteration in gill structure was reported in the shrimp *Crangon crangon* after exposure to the same concentration for 22 h [57]. In addition, Bubel [18] reported that after exposure to 20 mg $Cd \cdot L^{-1}$ for 24 h, the structure of pleopodial gills of the isopod *Jaera nordmanni* was completely disorganized. Similar effects such as necrosis, cell proliferation, epithelial lifting and dilated lamellae were observed in gills of fish exposed to metals, including cadmium [48].

In crustaceans, two types of phagocytic cells have been observed in gill tissues, mobile haemocytes [77] and nephrocytes [31, 43, 77]. The most important function of haemocytes is the removal of any foreign particles that may gain access to the haemocoele. This function is achieved by a combination of phagocytosis, nodule formation and encapsulation reaction, depending on the dimension of the foreign body [8, 77]. From our results, two hypothesis can be proposed regarding the presence of black gills in cadmium-exposed shrimp. First, the invading metal can be removed by phagocytosis. This foreign material would become entrapped in several layers of haemocytes forming a nodule. Normally the nodule becomes heavily melanized because of the host's phenoloxidase activity [8]. Or second gill blackening might result from non-specific autolysis and necrosis leading to the deposition of black electron-dense material in the necrosed tissues, such as a metallic sulphite or even cadmium [26, 52]. The effects of metal on the haemocyte number could therefore lead to a reduction in immunocompetence in crustacea [76].

Nephrocytes are believed to contribute to the ultrafiltration, regulation and detoxification of haemolymph components [31, 41, 43, 47, 73]. The increased number of nephrocytes that we observed in exposed gills may indicate the contribution of these phagocytic cells to the response to the presence of cadmium. Nephrocytes might play a prominent role in the uptake of cadmium from the haemolymph and they might then store this metal in their central vacuole, as has been previously observed for inorganic mercury by Andersen and Baatrup [4] and Laporte [41]. A nephrocytic hyperplasia was also observed in gill laminae of juvenile *P. japonicus* exposed to 300 µg TBTO (tributyltin oxide)$\cdot L^{-1}$ [45].

In our study, we observed the presence of bacteria in gill filaments of shrimps exposed to 2 000 µg $Cd \cdot L^{-1}$. The sensibility of exposed gills to bacterial infection has been previously described in other shrimps exposed to cadmium [26, 27]. We, thus, suppose that bacteria contribute to the final phase of tissue alteration. Their presence has also been observed in gills of *P. japonicus* [70] and of the crayfish *Astacus leptodactylus* [46] infected by fungi.

According to our observations, epipodites were more susceptible to cadmium than gills. The exposure to three cadmium concentrations (200 µg$\cdot L^{-1}$ during 15 d, 2 000 and 4 000 µg$\cdot L^{-1}$ during 4 d) resulted in profound alterations of epipodites, and the degree of

alteration increased as a function of metal concentration. The apical microvilli were transformed into lacunae and even disappeared. Mitochondria decreased both in number and in size, and their structure was disorganized. In some regions, the nuclear matrices were condensed and vacuoles were present around the nucleus. The shape of basal membrane infoldings was transformed, and they decreased in number or were totally absent. Numerous vacuoles were observed between them. Histological studies showed that the epipodites of juvenile *P. japonicus* also displayed profound alterations after exposure to the pesticide fenitrothion [45] and to tributyltin oxide [44].

After exposure to 2 000 µg $Cd \cdot L^{-1}$, numerous pseudomyelinic structures were also observed in certain epithelial cells of epipodites. The presence of these structures has been reported in cells of other species exposed to pollutants [18, 37, 59, 72]. The reorganization of the endoplasmic reticulum into concentric membrane arrays is believed to play a role in detoxification by active metabolic processes [15, 24, 35, 55]. They would provide a greatly increased membrane surface area to ensure maximal contact of the toxin with the detoxifying enzymes [34, 56, 71, 72]. However, this type of cellular structure was also found during degenerating processes, e.g. during the degeneration of the androgenic glands in the crab *Ocypode quadrata* [60], of the Y organs in the isopod *Sphaeroma serratum* [23] and of the pavement and chloride cells in gills of the teleost *Oreochromis mossambicus* [80]. Thus, we suggest that both interpretations might be retained in *P. japonicus* for these structures, with a detoxifying function at the lower doses of cadmium, and as a sign of degeneration at higher concentration, the latter being confirmed by the generalized alteration of all other cellular structures.

Since high cadmium concentrations result in serious damage to gills and epipodites of *P. japonicus*, the metal may consequently inhibit the physiological functions of these organs. Since the gills of this shrimp are probably involved in gas exchange, we suppose that their alteration results in the disruption of respiration. This is supported by results showing that cadmium (120 to 8 000 µg $Cd \cdot L^{-1}$) depressed gill tissue oxygen consumption in two crab species, *Carcinus maenas* and *Cancer irroratus* [75]. However, an increase in gill tissue oxygen consumption was noted in cadmium-exposed lobsters *Homarus americanus* [74]. This contrast may be due to specific differences or to the different exposure regimes used. The crabs were exposed for 48 h to high cadmium concentrations (120 to 8 000 µg $Cd \cdot L^{-1}$), whereas the lobsters were exposed for 30 d to lower levels (3 and 6 µg $Cd \cdot L^{-1}$) [74, 75]. Another study reported that 1 µg $Cd \cdot L^{-1}$ greatly depressed the respiration rate in stage V zoeae of the fiddler crab *Uca pugilator*, but increased respiration in stage I and III zoeae [79]. At 18 °C, the respiration rate of mysid *Leptomysis lingvura* decreased rapidly at concentrations between 50 and 100 µg $Cd \cdot L^{-1}$; at a higher concentration (up to 250 µg $Cd \cdot L^{-1}$), respiration continued to decrease but more steadily [33].

Epithelial cells of epipodites of *P. japonicus* are characterized by the abundance of apical microvilli, basal infoldings and mitochondria. The lower number of these features in shrimps exposed to cadmium suggests that the osmoregulatory function of these cells is disturbed. In this species, an impairment of osmoregulation by cadmium has been reported: after 4 d exposure to 2 000 and 4 000 µg $Cd \cdot L^{-1}$, the hypo-osmoregulatory capacity in seawater decreased by 28 and 53 %, respectively, and the hyper-osmoregulatory capacity in dilute seawater decreased by 24 % after 2 d and by 47 % after 4 d exposure to 2 000 µg $Cd \cdot L^{-1}$ [6]. A clear relation can thus be established between the structural damages to epipodites reported in the present study and the impairment of osmoregulation. In other species, exposure to cadmium also had adverse effects on the ability to osmoregulate. Thurberg et al. [75] reported that osmoregulation was disrupted in *C. maenas* after 48 h exposure to high levels of cadmium (500 to 8 000 $\mu g \cdot L^{-1}$). In the same species, the haemolymph ion content (Na^+, K^+ and Cl^-) was unaffected after exposure of the crabs to 1 000 µg $Cd \cdot L^{-1}$, and it was slightly affected at 10 000 µg $Cd \cdot L^{-1}$ [11]. In the isopods *Idotea negleta* and *Jaera albifrons*, osmoregulation was impaired after exposure to 10 000 µg $Cd \cdot L^{-1}$ in seawater at 27 and 17 PSU salinity, respectively [39]. Other studies have also shown that cadmium exposure negatively affects the gill Na^+-K^+-ATPase activity, in *Scylla serrata* [30], *C. irroratus* [78], *Nephrops norvegicus* [21] and *C. maenas* [64]. Thus, there are very few demonstrations of the adverse effects of cadmium on both osmoregulation and osmoregulatory organs, since physiological studies and structural observations have been conducted in different species, excepting the case of the isopod genus *Jaera* [18, 39]. We report here in the species *Penaeus japonicus* that a decrease in osmoregulatory ability following exposure to cadmium [6] most probably originates from a structural degradation of epipodites by the metal, a fact that indirectly confirms the prominent function of these organs in the osmoregulation of peneid shrimps.

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