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Histopathological Alterations in Gills of White Shrimp, *Litopenaeus vannamei* (Boone) After Acute Exposure to Cadmium and Zinc

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Abstract White shrimp, *Litopenaeus vannamei*, a globally important cultured prawn species, is an ideal animal for studying the impairment caused by the effects of heavy metals that are often detected in coastal areas. In this present study, we detected the acute toxicity of cadmium (Cd) and zinc (Zn) to *L. vannamei*. Medium lethal time (LT50) values of Cd and Zn on *L. vannamei* were estimated. Furthermore, we also demonstrated that acute exposure to high concentrations of Cd and Zn resulted in morphological alterations in gills of *L. vannamei* in this present study.

Keywords *Litopenaeus vannamei* · Cadmium · Zinc · Gills · Histopathology

Shrimp culture farms are often located near the coast and use seawater directly from coastal areas. However, coastal seawater is easily contaminated by heavy metals due to human activities. Thus, the impact of heavy metals should be considered on cultured shrimp individuals, culture industries, and human health. For marine animals, gills are crucial for respiration, excretion, acid–base balance, and osmotic and ionic regulation (Soegianto et al. 1999a). Compared to other parts of the body covered by exoskeleton providing the first protection in crustaceans, gills are immediately exposed to the environment external to the body and are the first organs exposed to pollutants, such as heavy metals. Direct damage to gill structures and functions caused by exposure to higher levels of heavy metals was rarely studied in prawns. It is only in *Penaeus japonicus* that the effects of heavy metals on gill structures and functions have been well studied (Bambang et al. 1995; Soegianto et al. 1999a, b), and relatively less so in other prawn species such as *P. indicus* (Chinni et al. 2002). Even though *L. vannamei* is a very important cultured prawn species, surprisingly few related investigations have been conducted on it to date. Cd is biologically non-essential but an important metal with industrial applications, while Zn is an ubiquitous and nutritionally essential metals playing important roles on physiological functions. Both metals are commonly apparent in coastal seawater. The objective of this present study was, therefore, to estimate the acute toxicity of Cd and Zn on *L. vannamei*, and to examine histological alterations in gills of *L. vannamei* after exposure to high levels of Cd and Zn.

Materials and Methods

Postlarvae *L. vannamei* were obtained from a commercial shrimp hatchery in Pingtung, southern Taiwan and maintained in the laboratory for acclimation. Water conditions during shrimp rearing and the experimental period were: temperature 25°C, salinity 15 p.s.u., DO 5.8–6.5 mg/L, pH 7.15–7.87, and Eh 32.152 μS/cm, under a 12:12-h light–dark regime with continuous aeration and filtration.

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Acute toxicities of Cd and Zn were studied as previously described (Wu and Chen 2004) with water renewal methods based on the *Standard Guide for Conducting Acute Tests with Fishes* (EPA/ROC 1998). According to the results of mortality and medium lethal concentration (LC_{50}) from probit analysis (Finney 1971; Wu and Chen 2004), medium lethal time (LT_{50}) values were estimated based on the regression equations calculated by the software Microcal Origin vers. 6.0.

Juveniles of *L. vannamei* (2.15 ± 0.18 g in mass; 9.83 ± 0.59 cm in length) in the intermolt stage were subjected to acute exposure to Cd or Zn and then sacrificed for the histological study on the gills. Animals were divided into three groups with one group exposed to 3 mg/L Cd, one exposed to 3 mg/L Zn, and another exposed to no metal as a control group. Samples were taken after 24 and 48 h of exposure. Gill samples were very carefully dissected out and fixed in 4% buffered formalin, embedded in paraffin, sectioned at 8-μm thickness on a microtone (Microm, HM330, Heidelberg, Germany), stained with hematoxylin and eosin (H&E), and examined with a Olympus microscope (Tokyo, Japan).

Results and Discussion

According to reported information, the average concentration of Cd is about 0.05 μg Cd/L in unpolluted water, while in coastal waters it tends to increase to over 0.1 μg Cd/L and even over 10 μg Cd/L in some areas due to anthropogenic input, local geological conditions, and human activities, according to reported data (Soegianto et al. 1999a). Likewise, Zn concentrations in coast water had also been detected as 4–800 μg Zn/L (Bryan 1976). Thus, the impact of heavy metals Cd and Zn should be seriously considered when coastal seawater is directly used for shrimp culture and the human health is concerned.

Estimation of LT_{50} values of *L. vannamei* was based on the results of mortality and the determined LC_{50} values. Regression curves were drawn from LC_{50} values and exposure times as shown in Table 1 and Fig. 1. LT_{50} values were calculated based on the regression equations as follows: $Y = 23.80323 + 71.54851 \times (e^{((X - 0.02938)/0.07509)})$ ($R^2 = 0.999$) for the Cd set; and $Y = 3.68762 + 93.3528 \times (e^{(-(X - 0.13033)/0.30121)})$ ($R^2 = 0.989$) for the Zn set, where Y is the exposure time (h) and X is the LC_{50} metal concentration (mg/L) for either Cd or Zn. Standard checks of both regression equations were between −10% and 10% as shown in Table 1. The results of the estimation of LT_{50} values after calculations based on LC_{50} data sets are shown in Table 2. According to our results, LT_{50} values of *L. vannamei* for 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg/L concentrations were estimated as shown in Table 2.

Table 1 Values of the 24-, 48-, 72-, and 96-h medium lethal concentrations of *Litopenaeus vannamei* to Cd and Zn

	Time (h)	LC_{50} (mg/L)[a]	Log (LC_{50})	Time′ (h)	Standard check[b] (%)
Cd	24	2.58	0.41	24.24	1.00
	48	1.30	0.11	46.99	−2.15
	72	1.14	0.06	73.38	1.88
	96	1.07	0.03	95.35	−0.68
Zn	24	3.98	0.60	23.31	−2.97
	48	2.14	0.33	51.71	7.18
	72	1.75	0.24	67.89	−6.06
	96	1.35	0.13	97.04	1.07

Time′ is the estimated time calculated with the regression equations $Y = 23.80323 + 71.54851 \times (e^{(-(X - 0.02938)/0.07509)})$ for the Cd set and $Y = 3.68762 + 93.3528 \times (e^{(-(X - 0.13033)/0.30121)})$ for the Zn set, where Y is the time (h) and X is the metal concentration (mg/L), when using LC_{50} values to represent metal concentrations

[a] (Wu and Chen 2004)

[b] Standard check = (1 − (Time′ − Time)) × 100%

Gills of *L. vannamei* are dendrobranchiate, with an axis that supports numerous secondary laminae giving rise, at right angles, to filaments divided into two branches near their termini (Fig. 2). Further branching was also observed in terminal areas of some filaments. The central axis is attached to the cephalothoracic wall via a tubular structure. Primary filaments branch from the central axis; each primary filament further divides into secondary filaments. Filaments are elongated and have a delicate shape. A longitudinal septum divides the lumen of each axis, branch, and filament into afferent and efferent vessels. The surface of each lamina or filament is covered by a thin cuticle underlain by an epithelial monolayer. Each filament tip has a hemolymphatic lacuna. Connective tissues are present in the septum of filaments and in the axis of gills. Epithelial pillar cell processes span the distance between opposite cuticular walls. Interspersed between the pillar cells are lacunae, through which the hemolymph actually moves.

Blackened gills appeared in several shrimp treated with 3 mg/L Cd and could be observed with the naked eye (Fig. 3a). After exposure for 24 h, gill filaments were obviously thickened and dilated when examined with a light microscope (Fig. 3b). Filaments were heavily hypertrophic after 48 h of exposure to Cd, which resulted in a decrease in the space between filaments (Fig. 3c). Epithelial edema and cell proliferation were observed in gill filaments when further examined under higher magnification, which caused an increase in the distance between the central hemolymphatic lacunae and the ambient water (Fig. 3d). A high density of vacuoles appeared within cells of secondary filaments. In addition, structural impairments of gill filaments also presented, since we observed that the

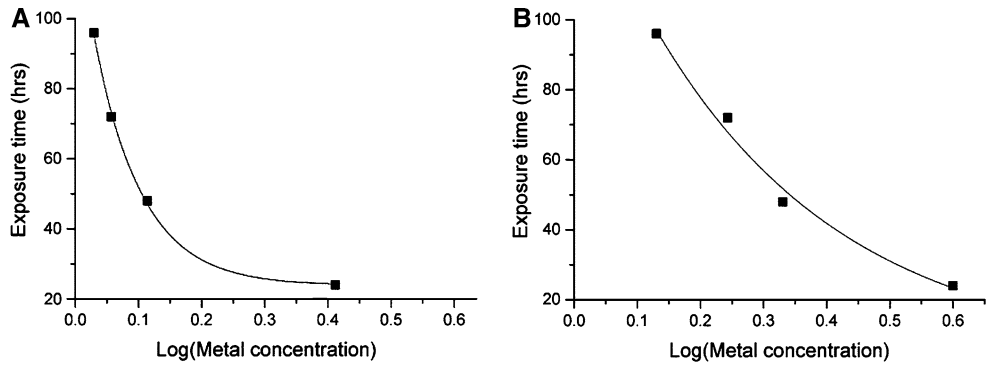

Fig. 1 Correlation between exposure time (h) and logarithmic values of metal concentrations from the results of acute toxicity assays when *Litopenaeus vannamei* were exposed to **a** Cd, with regression equation $Y = 23.80323 + 71.54851 \times (e^{(-(X - 0.02938)/0.07509)})$, or **b** Zn, with the equation $Y = 3.68762 + 93.3528 \times (e^{(-(X - 0.13033)/0.30121)})$

Table 2 Values of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/L LT_{50} of *Litopenaeus vannamei* to Cd and Zn calculated according to the regression curves shown in Fig. 1

Metal concentration (mg/L)	Cd LT_{50} (h)	Zn LT_{50} (h)
1.0	129.64	147.62
1.5	33.93	83.87
2.0	25.72	56.64
2.5	24.33	42.06
3.0	23.99	33.19
3.5	23.88	27.30
4.0	23.84	23.17

epithelium covering the secondary filaments had sloughed off and formed a detached layer of tissue lying above and between the desquamated filaments. The gill alterations we observed resulted in narrowed or obstructed hemolymphatic vessels. Furthermore, hyperemia seemed to have occurred, since we observed that hemocytes were heavily distributed within the hemolymphatic vessels of gill filaments of shrimp exposed for 48 h, as shown in Fig. 3d.

Previous studies conducted on some crustacean species have shown that Cd can alter gill structures (Bubel 1976; Couch 1977; Nimmo et al. 1977; Papathannnasiou and King 1983; Papathannnasiou 1985; Darmono et al. 1990; Soegianto et al. 1999a). In these species studied, black-

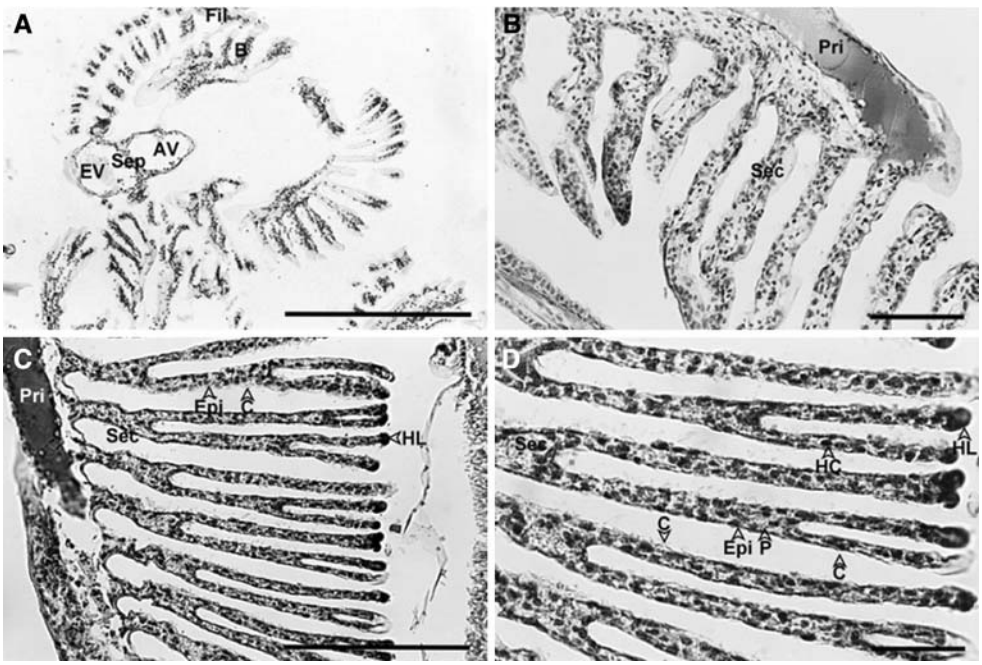

Fig. 2 Gills from control *Litopenaeus vannamei*. **a** Transverse section of a gill, bar = 200 μm. **b** Primary gill filament, bar = 50 μm. **c** Secondary filaments, bar = 200 μm. **d** Secondary branching gill filaments, bar = 50 μm. *AV* afferent vessel, *B* branch, *C* cuticle, *Epi* epithelium, *EV* efferent vessel, *Fil* filament, *HC* hemocyte, *HL* hemolymphatic lacuna, *P* pillar cell system, *Pri* primary filaments, *Sec* secondary filaments, *Sep* septum

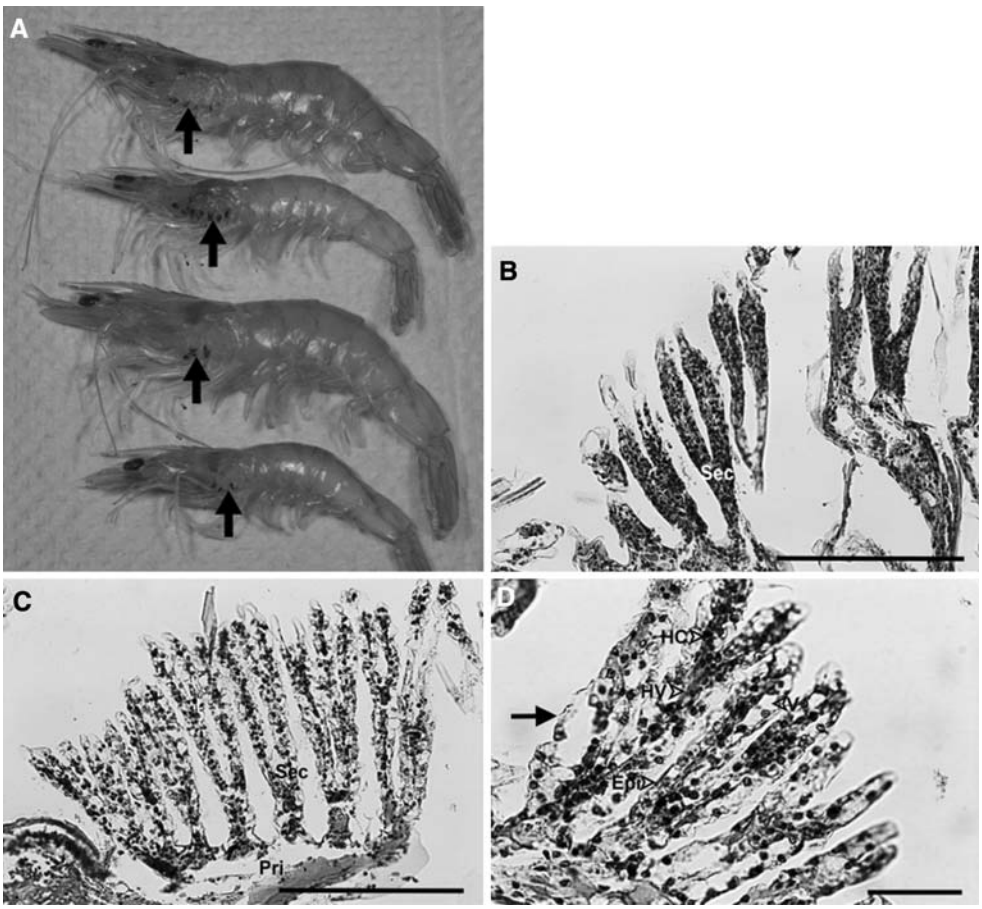

Fig. 3 Gills from *Litopenaeus vannamei* treated with 3 mg/L Cd. **a** Cd exposed shrimps after removal of the left branchiostegite. Note that the blackened regions appeared in gills as indicated by *arrows*. **b** Secondary gill filaments of shrimp exposed to Cd for 24 h, bar = 200 μm. **c** Secondary gill filaments of shrimp exposed to Cd for 48 h, bar = 200 μm. **d** Further examination of gill filaments from shrimp exposed to Cd for 48 h show that epithelium has sloughed off as indicated by the *arrow*, and rich amounts of hemocytes appear within the hemolymphatic vessels, bar = 50 μm. *C* cuticle, *Epi* epithelium, *HC* hemocyte, *HV* hemolymphatic vessel, *Pri* primary filaments, *Sec* secondary filaments, *V* vacuole

pigmented lesions, complete loss of cell structural integrity and organization, and bacterial and fungal invasion were observed after exposure to cadmium. The black-pigmented lesions consisted of lamellar hemocyte aggregation and melanization of branchial lamellae, accompanied by necrosis, especially in distal gill filaments (Darmono et al. 1990; Soegianto et al. 1999a). Soegianto et al. (1999a) examined ultrastructural changes in the gills of *P. japonicus* exposed to Cd. Results showed that gill cells of *P. japonicus* exposed to 2 and 200 μg/L Cd for 15 days displayed no important structural changes. However, a profound alteration of the gills occurred after exposure to 2,000 and 4,000 μg/L Cd for 4 days. At those concentrations, the number of nephrocytes increased in the gill filaments; necrosis of gill cells appeared; spaces opened up between the cuticle and epithelial cells into which numerous bacteria infiltrated; black electron-dense material was also observed within the spaces between cuticle and the epithelial; and in certain gill filaments, the nucleus was fragmented. These results are similar to our observations of *L. vannamei* exposed to 3 mg/L Cd for 24 or 48 h, including necrosis of gill cells and structural impairments resulting in loss of gill structural integrity (Fig. 3). It is believed that at least two types of phagocytic cells exist in gill tissues: hemocytes and nephrocytes (Soegianto et al. 1999a). One of the most important functions of hemocytes is to remove foreign particles that may gain access to the hemocoel, achieved by a combination of phagocytosis, nodule formation, and an encapsulation reaction, depending on the dimensions of the foreign body (Tsing et al. 1989; Battistella et al. 1996; Soegianto et al. 1999a). Hyperemia, in which hemocytes are heavily distributed in hemolymphatic vessels of gill filaments, was observed, and might have been related to the removal reaction of foreign materials, Cd in this case, by hemocytes. Nephrocytes, although not very clearly observed in our results, are believed to contribute to the ultrafiltration, regulation, and detoxification of hemolymph components (Doughtie and

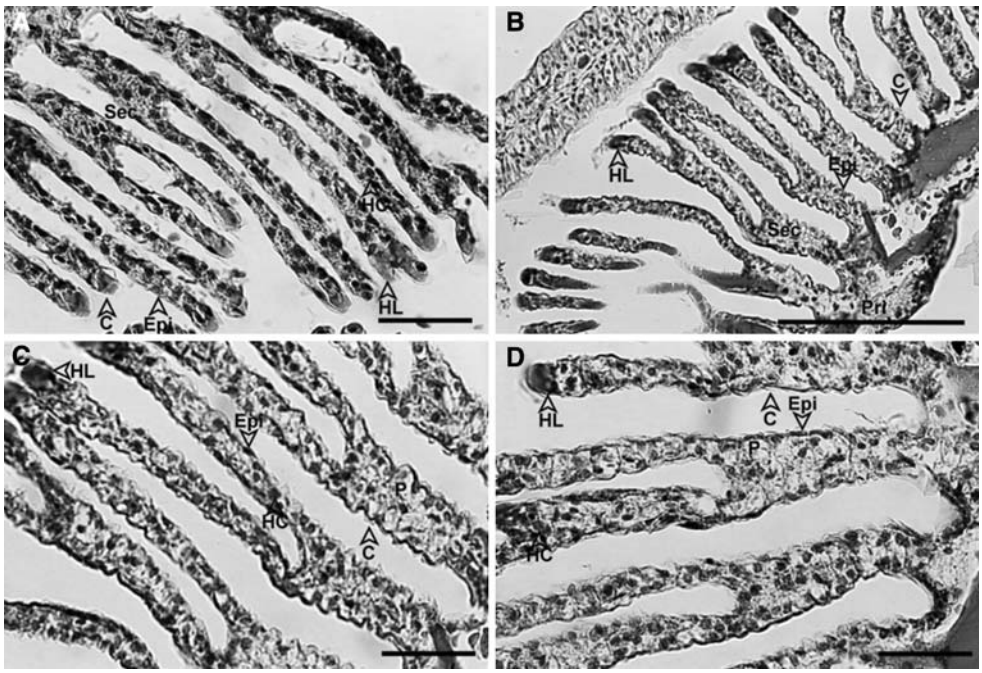

Fig. 4 Gills from *Litopenaeus vannamei* treated with 3 mg/L Zn. **a** Secondary filaments of shrimp exposed to Zn for 24 h, bar = 50 μm. **b** Filaments of shrimp exposed to Zn for 48 h are dilated, bar = 200 μm. **c** and **d** Epithelial edema and cell proliferation apparent on gill filaments, resulting in a roughening of the surfaces of the gill filaments, bar = 50 μm. *C* cuticle, *Epi* epithelium, *HC* hemocyte, *HL* hemolymphatic lacuna, *P* pillar cell system, *Pri* primary filaments, *Sec* secondary filaments

Rao 1981; Maina 1990; Lawson et al. 1994; Soegianto et al. 1999a). The appearance of blackened gills was observed by Soegianto et al. (1999a) when they worked on *P. japonicus* exposed to Cd. They proposed two hypotheses regarding the presence of black gills in Cd-exposed shrimp. First, the invading metal can be removed by phagocytosis, and this foreign material becomes entrapped in several layers of hemocytes forming a nodule, which normally becomes heavily melanized due to the phenoloxidase activity of host. 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.

Compared to Cd, there have been comparatively fewer studies on the histological effects of Zn on the gill structures of shrimp. Unlike Cd, a non-essential and xenobiotic element, Zn plays a role as an essential element in most organisms, and most organisms possess the ability to regulate the level of Zn entering into and being excreted from the body to maintain a stable level within the body when they are exposed to ambient Zn. For example, a prawn species, *Macrobrachium malcolmsonii*, shows the ability to regulate its tissue concentration of the essential metal Zn to an approximately constant level when exposed to a wide range of dissolved zinc concentrations (26–653 μg Zn/L), until a threshold dissolved concentration (373 μg Zn/L) is reached when regulation collapses, and the net accumulation of Zn begins (Vijayram and Geraldine 1996). Therefore, injuries caused by Zn are usually slighter than those caused by Cd when the same organism is exposed to these metals at the same concentration. This was also confirmed by this study, since serious structural destruction and necrosis were only observed in gills of shrimp treated with 3 mg/L Cd but not in individuals treated with Zn at the same concentration. Even so, alterations of gills of white shrimp treated with 3 mg/L Zn were observed in this study, especially those exposed for 48 h. Similar to Cd, Zn has also been reported to affect gill structures of certain aquatic organisms, including detachment and sloughing of epithelial cells, coalescence of adjacent secondary lamellar epithelia, and cytoplasmic abnormalities such as extensive vacuolation, swelling of nuclei and mitochondria, and cellular disintegration (Matthiessen and Brafield 1973). Vacuolation resulting in filament edema was also observed here (Fig. 4).

In conclusion, we have estimated the acute toxicities of Cd and Zn on *L. vannamei*, and demonstrated the histopathological alterations in the gills after acute exposure to Cd and Zn.

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