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Histological changes and survival of *Litopenaeus vannamei* juveniles with different copper concentrations

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ABSTRACT

Survival of juvenile *Litopenaeus vannamei* exposed to five Cu concentrations ranging from 10 to 0.003% the 96 h median lethal concentration, which were supposed to be safe in the long term, was 0% after 1, 5 and 6 weeks for Cu concentrations of 3.512, 1.756 and 0.877 mg L^{-1} (10, 5 and 2.5% the 96 h LC50). With these three concentrations there were severe time- and dose-dependent structural damages, such as necrosis, loss of regular structure and infiltration of hemocytes in the gill tissues, as well as atrophy, necrosis and irregular tubular structure in the hepatopancreas. After 9 weeks of exposure, only minor histological changes were observed with the two lower concentrations.

1. Introduction

The estimated 2005 total yields of the 43,500 ha dedicated to shrimp cultures in NW Mexico was close to 95,000 ton of the whiteleg shrimp *Litopenaeus vannamei*, most of which were grown in semi-intensive farms in the NW states of Sinaloa and Sonora (Gutierrez-Venegas, 2006).

Shrimp farm practices include the use of a wide range of bioactive compounds (disinfectants, therapeutics, feed additives, algaecides and fertilizers), which may be a source of biological damage to the surrounding biota and even to the species in culture (Dias-Bainy, 2000). Among these, copper sulfate is used to control macroalgae and bivalve growth in some Mexican semi-intensive shrimp farms.

Copper is toxic to shrimp (Manisseri and Menon, 1995; Frías-Espericueta et al., 2003) and the low water exchange rates used in most semi-intensive shrimp farms may cause sub-optimal environmental conditions and induce adverse shrimp responses, due to copper accumulation in the pond sediments (Páez-Osuna et al., 2003; Liao et al., 2006). However, previous exposure to sublethal Cu concentrations may induce physiological and metabolic adjustments (Hashemi et al., 2008), leading to acclimation and survival at otherwise toxic levels of environmental pollutants. The purpose of this study was to determine under laboratory conditions the survival and the possible histological alterations in the gills and hepatopancreas of *L. vannamei* during 9 weeks of exposure to copper sulfate concentrations which, according to Sprague (1971), are supposed to be "safe" even in the long term for aquatic organisms.

2. Materials and methods

Juveniles of *L. vannamei* (1.5–2 g) were obtained from a shrimp farm located close to the laboratory, and acclimated during 3 days to the experimental conditions in a common container. Throughout this stage and the rest of the experiments the organisms were fed "ad libitum" twice daily with pelleted 35% protein shrimp food, and kept with a 12:12 h light-dark photoperiod. Uneaten food was collected after 1 h and water exchanges were 100% every 24 h, using natural seawater filtered through a sand and gravel bed, one multiple cartridge system (10, 5 and 1 μm and 0.5 μm-mesh activated charcoal). The background dissolved Cu concentration determined by atomic absorption spectrophotometry (Spectra AA, Varian) was 2.1 μg L^{-1} and throughout the experiments the mean water temperature, pH, DO and total ammonia concentrations were 28±1 °C, 8.15±0.1, 6.3±0.25 mg L^{-1} and 4.1±0.8 μg L^{-1}, respectively.

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Table 1
LC50 values and 95% confidence limits for Cu exposure of *Litopenaeus vannamei* juveniles

Time (h)	LC50 (mg/L)	95% Confidence limits
24	47.23a	43.03–51.20
48	39.15b	36.61–41.59
72	39.15b	36.61–41.59
96	35.12c	30.12–36.77

Different letters indicate significant differences (one-way ANOVA, $\alpha = 0.05$; a > b > c).

The Cu content of the pelleted food was not determined directly (according to the label, 0.011% $CuSO_4$). However, since the organisms of all treatments were fed the same food and the same daily ration, any histological alteration in comparison to the control group was taken as indicative of the effect of waterborne Cu used in the challenge experiment (Odendaal and Reinecke, 2007).

After this first stage, salinity was decreased with dechlorinated tap water at a rate of 0.5‰ h^{-1} until 25‰, which is the isosmotic point of *L. vannamei* (Castille and Lawrence, 1981). The shrimp were kept at this salinity for 1 week prior to the first experiment, which served to determine the short-term (96 h) median lethal concentration (LC50) as in Frías-Espericueta et al. (2003), using triplicate cultures of 30 specimens and 12 Cu concentrations ranging from 0 (control) to 210 mg L^{-1}.

Uneaten food was removed after 1 h and the test solutions were renewed every 24 h; observations on survival and removal of dead specimens and exuviae were every 12 h, assuming death when non-motile shrimp showed no response to stimulation with a glass rod (Chen and Lin, 2001). At the end of this experiment, the LC50 values and their 95% confidence limits for every 24 h interval were calculated by probit analysis (Finney, 1971) and compared at the 5% level of significance (APHA-AWWA-WPCF, 1992).

In the following experiment shrimp were exposed in duplicate containers to five Cu concentrations which were supposed to have no lethal or sublethal effect, and were calculated multiplying the LC50 96 h by progressively decreasing application factors (AF) starting with AF = 0.1, tentatively suggested as safe by Sprague (1971).

The five AFs tested were: 0.1, 0.05, 0.025, 0.01 and the copper sulfate concentration used by Mexican farmers (4 kg ha^{-1} of $CuSO_4 \cdot 5H_2O$), which, assuming 1–1.2 m-deep ponds, would give a Cu concentration within the range 0.102–0.085 mg L^{-1}. Two additional containers without $CuSO_4$ addition served as control cultures.

For each AF, two organisms of each container in the intermolt stage were selected at random every week; the hepatopancreas and the anterior and posterior abdominal regions were injected with Bouin's solution (Li et al., 2007) and the organisms were left in the same solution to ensure tissue fixation. Shrimp were later transferred to 50% ethanol, the hepatopancreas and gill tissues were dissected, dehydrated in increasing ethanol concentrations, embedded in paraffin and 5 μm sections were obtained with a conventional microtome. The sections were re-hydrated, stained with hematoxylin-eosin (Yang et al., 2007), examined under a microscope and photographs were obtained with a digital camera.

3. Results

The 24h LC50 value (47.23 mg L^{-1}) was significantly higher than those obtained for 48 and 72 h (39.15 mg L^{-1} in both cases) and the lowest value was that obtained after 96 h (35.12 mg L^{-1}; Table 1). Thus, the Cu concentrations used in the second experiment were: AF 0.1 = 3.512 mg L^{-1}; AF 0.05 = 1.756 mg L^{-1}; AF 0.025 = 0.877 mg L^{-1}; AF 0.01 = 0.351 mg L^{-1} and AF "farm" = 0.101 mg L^{-1}.

At the end of the 9 weeks, survival was 100% in the controls and with AF 0.01 and "farm". Mortality was 100% before the end of the first week with AF 0.1 and after 5 and 6 weeks with AF 0.05 and 0.025, respectively.

Throughout the experiment, no histological alterations were observed in the tissues of the controls, whereas in the treatments they were dependent on the Cu concentration and on the time of exposure.

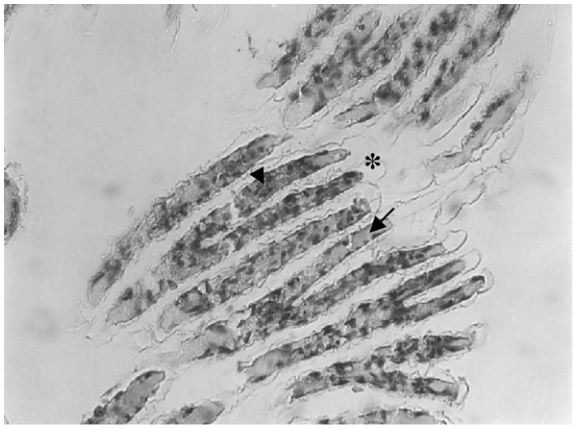

Fig. 1. Gills of shrimp exposed to 0.05 AF for 3 weeks. Absence of pillar cells (arrow). Loss of regular structure of cuticular epithelium (*). Multifocal necrosis (arrow head). H&E 400X.

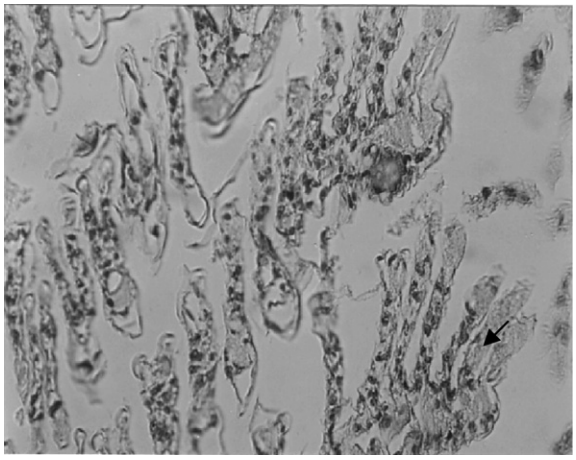

Fig. 2. Gills of shrimp exposed to 0.025 AF for 6 weeks. Loss of regular structure and absence of pillar cells of the secondary filaments (arrow). H&E 400X.

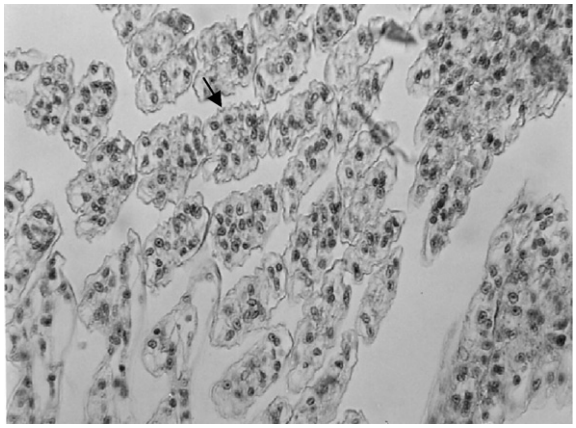

Fig. 3. Gills of shrimp exposed to 0.01 AF for 9 weeks. Loss of regular structure of cuticular epithelium (arrow). H&E 400X.