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Cadmium and copper mixture effects on immunological response and susceptibility to *Vibrio harveyi* in white shrimp *Litopenaeus vannamei*

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ABSTRACT

Cadmium (Cd2+) and copper (Cu2+) are considered immunotoxic metals and their presence in combination in the aquatic environment may cause effects on shrimp species as *Litopenaeus vannamei*. Thus, this research evaluates the combined effects of Cd2+ and Cu2+ on shrimp inoculated with *Vibrio harveyi* bacteria. The experiments were performed at 96-h of exposure to sublethal concentrations of both metals. No mortality was observed in organisms exposed to the sum of Criterion of Continuous Concentration (ΣCCC) in Cd + Cu mixture and those inoculated with *V. harveyi*. Higher clotting times were recorded in Cd + Cu + *V. harveyi* treatment at higher metal concentrations. No significant differences ($P > 0.05$) were recorded in hemocyanin content between shrimp exposed to metals and those experimentally infected. Significantly higher ($P < 0.05$) total hemocyte count (THC) was recorded at 96 h exposure in the ΣCCC and 10% treatments of Cd + Cu + *V. harveyi* experiment. Regarding Cd + Cu + *V. harveyi* bioassay, the highest phenoloxidase (PO) activity was recorded in shrimp inoculated with *V. harveyi* (0.326 ± 0.031 PO units/mg protein) at 96-h exposure. The lowest PO activity was observed in organisms exposed to Cd + Cu + *V. harveyi*. Regarding superoxide dismutase (SOD) activity, shrimp exposed to higher metal concentrations at 96 h showed the lowest hemolymph activity (6.03 ± 0.62 SOD units/mL). Protein decrease was observed in organisms exposed to metal mixture. The results showed that *L. vannamei* could be more susceptible to *V. harveyi* when exposed to Cd + Cu.

1. Introduction

Sinaloa is one of the most important agricultural states of Mexico with 1 067 526 ha of several crops [1], and this activity includes a high use of agrochemicals (*i.e.*, fertilizers and pesticides) to control and improve crops. Moreover, northwest Mexico (states of Sonora and Sinaloa) is the main shrimp farming area in Mexico. Most of the shrimp farms are located on the margins of the coastal lagoon ecosystems surrounded by intensive agricultural fields. Therefore, shrimp farms are potentially exposed to contaminated waters with a myriad of agrochemical residues, including heavy metals, such as cadmium (Cd^{2+}) and copper (Cu^{2+}); in fact, the presence of Cd and Cu has been reported in farmed shrimp hepatopancreas with values of 1.27 ± 1.33 and 229 ± 113.6 μg/g, respectively [2]. Both metals represent a potential risk for the growing shrimp farming and consumers.

The environmental stress related to variations in temperature and salinity, hypoxia, and presence of contaminants increase shrimp disease susceptibility because total hemocyte count (THC), Prophenoloxidase (proPO)-activating system, and phagocytic activity decrease, while reactive oxygen species (ROS) increase [3]. In this context, Liao et al. [4] pointed out the relationship between metal stress and disease susceptibility in aquaculture species. Shrimp diseases have a major impact on shrimp aquaculture industry worldwide with high economical losses annually [5]. Major challenges to shrimp farm success in Mexico also

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include proper disease management.

Litopenaeus vannamei is the main shrimp species farmed in Mexico and worldwide, but viral (*i.e.*, Taura, WSSV) and bacterial (*i.e.*, *Vibrio alginolyticus*, *V. parahaemolyticus, V. harveyi*) diseases have limited their production [6]. *Vibrio* spp. are abundant in shrimp ponds [7], thus, knowledge of shrimp cellular and humoral defense mechanisms is required when shrimp are exposed to pollutants.

Circulating hemocytes are very important in shrimp immune defense: (1) hyaline cells are responsible for phagocytosis, encapsulation, and nodulation of foreign particles; (2) semi granular and (3) granular cells are responsible for encapsulation, coagulation, and the proPO system [5]. When proPO is activated and converted to phenoloxidase (PO), it catalyzes several reactions to produce quinones, and subsequently, the microbicide melanin [8].

The phagocytosis process releases several ROS, mainly superoxide anion (O_2^-), which has a microbicide activity [9]. To avoid biomolecule damage (*i.e.,* DNA, cellular membrane), the antioxidant enzyme superoxide dismutase (SOD) catalyzes O_2^- dismutation to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [10]. When oxidative stress occurs, ROS could bind to biological molecules affecting normal cellular functions [11,12].

The presence of heavy metals in the environment causes immunosuppression on aquatic organisms [11,13]. Total hemocyte count (THC) reduction was reported by Lorenzon et al. [14] on shrimp *Palaemon elegans* when exposed to mercury, cadmium, lead, and copper. Exposure to Cd^{2+} caused THC reduction and PO increase in the crab *Sinopotamon henanense* [15]. Moreover, several studies have evaluated metal toxicity on *L. vannamei* [16] and freshwater crayfish *Cherax quadricarinatus* [17] inoculated with bacterium and virus, respectively. However, the effects on the immune system of aquatic organisms when exposed to sublethal concentrations of essential and non-essential metals and their interaction with pathogenic vibrio have been little investigated. Therefore, the objectives of this contribution are to evaluate survival and immune response (cellular and humoral) of the white shrimp *L. vannamei* exposed to Cd + Cu and inoculated with a *Vibrio harveyi* pathogenic strain. Therefore, the hypothesis of this study is that *L. vannamei* immunological response and susceptibility to *V. harveyi* depends on Cd^{2+} and Cu^{2+} levels in the mixture and exposure time.

2. Material and methods

2.1. Sampling and acclimation

Two samplings were carried out at different dates, collecting 200 shrimp in each sampling (total length of 10.55–13.28 cm) from a disease-free commercial shrimp farm (certified by the state of Sinaloa: Commission for Disease Control in Aquaculture) and transported to the aquarium of the experimental facilities in the ICMyL-UNAM (Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México).

Shrimp specimens from each sampling were maintained in tanks with aeration for three days and fed *ad libitum* with 35% protein of commercial shrimp feed (Vimifos, S.A., MX). Environmental conditions were salinity 35 ± 0 practical salinity units (PSU), temperature 22 ± 1 °C, oxygen 5 ± 1 mg L^{-1}, total ammonia-N 5.8 μg/L, 12:12 h light-dark photoperiod and 80% daily water exchange with ultraviolet (UV)-treated and 10-μm filtered seawater. After this acclimation period, groups of 16 shrimp (physical integrity without injury, good viability, and natural color) were placed in individual 40-L glass aquaria and acclimated for three additional days under similar conditions previously described [18,19]. All the experiments in this study complied with the Care and Use of Test Animal guidelines.

2.2. Exposure to sublethal concentrations of metals and inoculation with Vibrio harveyi

The experimental design consisted of two stages. For experimental stage 1 (Cd + Cu exposure), four treatments (each in duplicate) were used: (1) shrimp not exposed to metal mixture and non-inoculated (+control); (2) shrimp exposed to Cd + Cu mixture at their respective Criterion of Continuous Concentrations (CCC for Cd^{2+} 8.8 and Cu^{2+} 3.1 μgL^{-1}) proposed by the United States Environmental Protection Agency [20]; (3) at 1%, and (4) at 10% of the median lethal concentration (LC_{50} 96 h) levels of each metal. LC_{50}-96 h for Cd^{2+} and Cu^{2+} are 2 489 and 37 300 μg/L, respectively [21,22]. The Cd^{2+} and Cu^{2+}mixture concentrations for inoculated and non-inoculated shrimp were 1% (Cd^{2+}: 24.89 μg/L: Cu^{2+}: 373 μg/L) and 10% (Cd^{2+}: 248.9 μg/L: Cu^{2+}: 3730 μg/L) of their respective LC_{50}-96 h. The third metal mixture concentrations were the sum of CCC for Cd^{2+} and Cu^{2+}.

At the end of experimental stage 1, all materials were acid cleaned and disinfected to start the next stage. For experimental stage 2 (Cd + Cu + bacteria), five treatments (each in duplicate) were used: (1) shrimp non-exposed to metal mixture and non-inoculated (+control); (2) shrimp non-exposed to metal mixture but inoculated with *V. harveyi* (–control); (3, 4 and 5) shrimp exposed to metal mixture (CCC, 1 and 10% of their respective LC_{50}-96 h) and inoculated with *V. harveyi*.

The strain *V. harveyi* CAIM 1792 was previously isolated from hemolymph from shrimp affected by the Bright Red Syndrome [23]. The bacterial inoculum was obtained according to the methodology of Soto-Rodriguez et al. [24]. Briefly, the strain was recovered from the cryovials and inoculated in 10 mL of tryptic soy broth (TSB) supplemented with 2.0% NaCl and incubated overnight at 30 °C. Colonies were suspended in sterile 2.5% NaCl and centrifuged at 5 724×*g* for 10 min at 15 °C. The bacterial suspension was adjusted to an optical density of 1.0 at 610 nm. These suspensions were plated onto thiosulfate-citrate-bile salts (TCBS) (Bioxon Laboratory, Poland), after serially diluted to determine the real bacterial density inoculated in the challenge. Shrimp were removed from the aquaria, disinfected and injected in the third abdominal segment with 100 μL of *V. harveyi* CAIM 1792 at 1 x 10^4 colony forming units (CFU) mL^{-1} or injected with 100 μL of sterile 2.5% NaCl for the control group [24].

The duration of the exposure experiment to the metal mixture (Cd + Cu) was 96 h after the shrimp specimens were inoculated with bacteria. Hemolymph taken from the ventral sinus of five organisms/aquarium were sampled with a 1 mL sterile syringe at four sampling times 0, 5, 48 and 96 h post-infection (hpi). A total of 10 hemolymph samples/treatment at each sampling time were collected. To avoid differences in immunological variables, only shrimp in intermolt (stage C) were used [12–20] and identified according to Robertson et al. [25]. Feeding and water renovation were suspended after metal mixture addition [26,27]. Shrimp survival percentages were daily recorded, and dead organisms were immediately removed from the aquaria.

2.3. Metal concentrations

The solutions of both metals were prepared with $CdCl_2$ and $CuCl_2$ (Baker, reagent grade) dissolved in distilled water to obtain a stock of 1 mg L^{-1} of each metal solution. The experimental metal mixture solutions were obtained by adding the appropriate volume of each stock solution to the aquarium water to obtain equitoxic concentrations [28]. The seawater used in the experiments had metal background levels for Cu^{2+} and Cd^{2+} (0.5–0.7 and 0.35–0.42 μg/L, respectively) and was subsequently analyzed by atomic absorption spectrophotometry.

2.4. Hemolymph clotting time and hemocyanin concentration

According to Jussila et al. [29], the hemolymph clotting time was determined at a room temperature of 22 ± 1 °C; a 1.15 mm internal diameter capillary tube with 20 μL of hemolymph was inverted

continuously until hemolymph stopped flowing. The coagulation time was counted from the moment the needle was inserted in the shrimp ventral sinus [30].

Hemocyanin was quantified with the technique of Pascual et al. [31]: 10 μL of the hemolymph (without anticoagulant) was taken in duplicate, quickly placed in 1.5 mL cuvettes and diluted with 990 μL of Milli-Q water. Absorbance readings were made in an ultraviolet visible (UV-VIS) spectrophotometer (GENESYSTM), at 335 nm wavelength [27].

2.5. Total hemocyte count

Hemocyte counts were performed with a hemocytometer, recording the cells in four quadrants (corners) at an additional quadrant (at random) for each hemolymph sample [32].

2.6. Phenoloxidase (PO) activity

The PO activity determination was made following the technique proposed by Hernández-López et al. [33] and Ji et al. [34]. Hemocytes and cell-free plasma were separated by centrifugation at 800*g* at 4 °C for 3 min. A second centrifugation was performed at 16 800 g, at 4 °C for 10 min. A volume of 30 μL of hemocyte lysate (in triplicate/shrimp) was suspended in equal volumes of cacodylate buffer and trypsin solution and allowed to stand at 25 °C for 10 min. Then, 170 μL of L-DOPA (L, 3, 4-dihydroxyphenylalanine) were added to the mixture. The absorbance of this mixture was read in a microplate reader at 492 nm [27] and transformed to relative PO units (U/min/mg protein), using the protein concentration of the lysate determined with Bradford's method [35].

2.7. Superoxide dismutase activity

Superoxide dismutase activity was quantified with a RANSOD Kit (Randox, Crumlin, UK, Cat. No. SD 125). The hemocyte lysate of each sample was diluted with phosphate buffer (0.01 M, pH 7) in a 1:49 ratio (v/v), and 20 μL of the dilutions were placed in a 96-well microplate. Then, 130 μL of the substrate (xanthine 0.05 mmol/L and 0.025 mmol/L of I.N.T.) were added, mixed for 30 s, and finally, 20 μL of xanthine oxidase were added. Absorbance readings were taken at 492 nm on a microplate reader (Labsystems) every 30 s, until the final reading was recorded at 3 min. A reference standard was used, and the activity was expressed as SOD units/mL [30].

2.8. Protein concentration

The protein concentration in the hemocyte lysate was determined by Bradford's method (1976), adapted to microplate [36], using Bio-Rad reagent as a reaction solution in a 1:4 ratio with Milli-Q water. For calibration, a standard solution of 1.0 mg mL^{-1} of serum albumin from bovine (BSA-SIGMA) was used. The calibration was carried out according to the instructions of the Bio-Rad kit; the protein concentration was determined using 10 μL of hemocyte lysate from each sample in triplicate, to which 200 μL of the reaction solution was added. Absorbance readings at 595 nm were taken after 12 min.

2.9. Statistical analyses

Data were analyzed for normality and homoscedasticity (Smirnov-Kolmogorov and Bartlett tests). When data were not homoscedastic, the two-way analysis of variance (ANOVA) tests were run after rank transformation [37]. Finally, the Holm-Sidak and Student– Newman–Keuls (SNK) tests were carried out to isolate significant differences; all tests were performed at $P < 0.05$ as significant level.

3. Results

3.1. Mortality

Mortality was not observed in the organisms exposed to the sum of CCC in the Cd + Cu mixture and those inoculated with *V. harveyi*. Regarding shrimp exposed to 1 and 10% of the LC_{50}-96 h in the metal mixture experiment, 3.3 and 6.7% mortalities were recorded respectively, but no significant differences ($P > 0.05$) were observed. In the metal mixture + bacteria experiment, only 3.1% of mortality was recorded in the 1% treatment (Table 1).

3.2. Hemolymph clotting time

In Cd + Cu mixture, hemolymph clotting time did not show a dose-dependent trend as a function of exposure time. The highest value was recorded in shrimp exposed to 10% LC_{50}, which was significantly ($P < 0.05$) different from those of ΣCCC and 1% LC_{50} treatments. While, in Cd + Cu + *Vibrio*, higher clotting times were recorded in higher metal concentrations (Table 2).

3.3. Hemocyanin concentration

In Cd + Cu, no significant differences ($P > 0.05$) were observed between the control and those in metal treatments (Table 3). In metals + bacteria bioassay, no significant differences ($P > 0.05$) were observed, except for a trend to lower hemocyanin content with higher metal concentration (Table 3).

3.4. Total hemocyte count (THC)

No significant differences ($P > 0.05$) were observed in shrimp THC from all Cd + Cu treatments at 5 h exposure. However, the hemocytes number decreased ($P < 0.05$) in shrimp exposed to metal mixture at 48 and 96 h (Table 4).

No significant differences were recorded between negative (−) and positive (+) controls in the metal + *Vibrio* experiment. Nevertheless, significantly higher ($P < 0.05$) THC were recorded at all exposure times in those shrimp exposed to 10% LC_{50} treatment (Table 4).

3.5. Phenoloxidase (PO) activity

No direct effect was observed with the Cd + Cu mixture on PO activity at 96 h of exposure ($P > 0.05$); however, PO activity was lower ($P < 0.05$) in shrimp exposed to 1 and 10% LC_{50} than those of the control (Table 5).

Regarding the bioassay metals + bacteria, the highest PO activity

Table 1
Mean values of mortality percentages (±SD) of *Litopenaeus vannamei* juveniles exposed to Cd + Cu mixture and Cd + Cu + *Vibrio harveyi* (*Vh*) combinations, equivalent to 1 and 10% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control (+)	Control (−)	ΣCCC	1%	10%
Cd + Cu					
5 h	0.0[a]	nt	0.0[a]	0.0[a]	0.0[a]
48 h	0.0[a]	nt	0.0[a]	0.0[a]	0.0[a]
96 h	0.0[a]	nt	0.0[a]	3.3 ± 2.4[a]	6.7 ± 4.7[a]
Cd + Cu + *Vh*					
5 hpi	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]
48 hpi	0.0[a]	0.0[a]	0.0[a]	3.1 ± 0.0[a]	0.0[a]
96 hpi	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]

nt = no treatment; standard deviation = SD; LC_{50} = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA and Holm-Sidak test).

Table 2
Mean values (minutes) (±SD) of hemolymph clotting time of *Litopenaeus vannamei* juveniles exposed to Cd + Cu and Cd + Cu + *Vibrio harveyi* (*Vh*) combinations equivalent to 1 and 10% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control +	Control -	ΣCCC	1%	10%
Cd + Cu					
5 h	1.87 ± 0.41^{ab}	nt	1.95 ± 0.29^{ab}	2.29 ± 1.13^{ab}	2.35 ± 0.83^{ab}
48 h	2.23 ± 1.00^{ab}	nt	2.70 ± 0.93^{b}	1.78 ± 0.27^{ab}	1.72 ± 0.43^{ab}
96 h	1.99 ± 0.43^{ab}	nt	1.61 ± 0.17^{a}	1.46 ± 0.04^{ab}	2.83 ± 1.66^{ab}
Cd + Cu + *Vh*					
0 hpi	1.63 ± 0.20^{a}	2.23 ± 1.08^{ab}	1.38 ± 0.25^{a}	1.65 ± 0.20^{ab}	1.41 ± 0.07^{a}
5 hpi	1.92 ± 0.48^{ab}	2.03 ± 0.62^{ab}	1.95 ± 0.50^{ab}	1.68 ± 0.17^{ab}	1.88 ± 0.64^{a}
48 hpi	1.77 ± 0.17^{a}	2.21 ± 0.57^{a}	1.85 ± 0.26^{a}	1.76 ± 0.31^{a}	2.78 ± 0.37^{b}
96 hpi	1.86 ± 0.23^{ab}	2.15 ± 0.43^{ab}	2.25 ± 0.50^{ab}	2.30 ± 0.67^{ab}	2.10 ± 0.25^{ab}

nt = no treatment; standard deviation = SD; LC_{50} = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA and Student-Newman-Keuls test).

Table 3
Mean values (±SD) of hemocyanin (mmol/L) in the hemolymph of *Litopenaeus vannamei* juveniles exposed to Cd + Cu and Cd + Cu + *Vibrio harveyi* (*Vh*) combinations equivalent to 1 and 10% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control (+)	Control (−)	ΣCCC	1%	10%
Cd + Cu					
5 h	2.32 ± 0.35^{c}	nt	2.18 ± 0.48^{bc}	2.08 ± 0.40^{abc}	2.07 ± 0.33^{abc}
48 h	2.15 ± 0.19^{bc}	nt	1.94 ± 0.29^{ab}	2.15 ± 0.33^{b}	2.04 ± 0.12^{ab}
96 h	1.91 ± 0.35^{ab}	nt	2.16 ± 0.26^{b}	2.39 ± 0.24^{bc}	2.06 ± 0.25^{ab}
Cd + Cu + *Vh*					
0 hpi	0.13 ± 0.10^{a}	0.66 ± 0.18^{ab}	0.44 ± 0.13^{a}	0.51 ± 0.30^{ab}	0.14 ± 0.07^{ab}
5 hpi	0.85 ± 0.26^{c}	0.73 ± 0.18^{bc}	0.66 ± 0.14^{bc}	0.67 ± 0.18^{bc}	0.51 ± 0.07^{bc}
48 hpi	0.71 ± 0.13^{bc}	0.66 ± 0.11^{bc}	0.78 ± 0.17^{c}	0.60 ± 0.21^{bc}	0.56 ± 0.07^{b}
96 hpi	0.56 ± 0.14^{ab}	0.69 ± 0.29^{ab}	0.54 ± 0.14^{b}	0.59 ± 0.34^{ab}	0.58 ± 0.12^{ab}

nt = no treatment; standard deviation = SD; LC50 = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA and Student-Newman-Keuls test).

Table 4
Mean values (±SD) of total hemocytes (10^6 cells/mL) of *Litopenaeus vannamei* juveniles exposed to Cd + Cu and Cd + Cu + *Vibrio harveyi* combinations equivalent to 1 and 10% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control (+)	Control (−)	ΣCCC	1%	10%
Cd + Cu					
5 h	4.61 ± 2.80^{bc}	nt	4.11 ± 3.01^{bc}	3.86 ± 2.63^{ab}	3.09 ± 2.60^{b}
48 h	9.74 ± 4.88^{d}	nt	5.84 ± 4.74^{c}	3.84 ± 1.70^{bc}	4.21 ± 3.09^{bc}
96 h	7.68 ± 2.02^{d}	nt	2.64 ± 1.67^{ab}	3.69 ± 0.87^{b}	1.13 ± 0.62^{a}
Cd + Cu + *Vh*					
0 hpi	1.24 ± 0.66^{ab}	3.86 ± 0.99^{e}	1.12 ± 0.41^{a}	2.86 ± 0.11^{d}	4.47 ± 1.59^{f}
5 hpi	1.23 ± 1.01^{ab}	1.03 ± 0.64^{a}	1.02 ± 0.63^{a}	1.11 ± 0.75^{a}	3.18 ± 1.48^{d}
48 hpi	0.98 ± 0.86^{a}	0.85 ± 0.41^{a}	1.08 ± 0.64^{a}	1.53 ± 1.05^{b}	2.36 ± 1.23^{c}
96 hpi	1.11 ± 0.98^{ab}	1.46 ± 1.08^{b}	3.35 ± 1.08^{d}	1.55 ± 0.58^{bc}	3.79 ± 0.80^{e}

nt = no treatment; standard deviation = SD; LC_{50} = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA Student-Newman-Keuls test).

Table 5
Mean values (±SD) of PO activity (PO units/mg protein) in *Litopenaeus vannamei* juveniles exposed to Cd + Cu and Cd + Cu + *Vibrio harveyi* (*Vh*) combinations equivalent to 1, 10, and 50% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control (+)	Control (−)	ΣCCC	1%	10%
Cd + Cu					
5 h	0.188 ± 0.103^{c}	nt	0.311 ± 0.135^{d}	0.286 ± 0.267^{d}	0.185 ± 0.103^{c}
48 h	0.214 ± 0.150^{bc}	nt	0.140 ± 0.053^{ab}	0.107 ± 0.030^{a}	0.068 ± 0.013^{a}
96 h	0.155 ± 0.071^{bc}	nt	0.094 ± 0.024^{ab}	0.157 ± 0.062^{ab}	0.155 ± 0.032^{bc}
Cd + Cu + *Vh*					
0 hpi	0.144 ± 0.005^{a}	0.147 ± 0.004^{a}	0.164 ± 0.011^{b}	0.153 ± 0.008^{ab}	0.148 ± 0.002^{a}
5 hpi	0.123 ± 0.008^{b}	0.326 ± 0.012^{e}	0.111 ± 0.011^{d}	0.194 ± 0.007^{f}	0.160 ± 0.012^{c}
48 hpi	0.132 ± 0.007^{c}	0.173 ± 0.016^{b}	0.135 ± 0.010^{c}	0.132 ± 0.007^{c}	0.197 ± 0.010^{d}
96 hpi	0.232 ± 0.022^{d}	0.326 ± 0.031^{e}	0.223 ± 0.051^{a}	0.187 ± 0.011^{df}	0.280 ± 0.029^{b}

nt = no treatment; standard deviation = SD; LC50 = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA and Student-Newman-Keuls test).

value was recorded in shrimp inoculated with *V. harveyi* (0.326 ± 0.031 PO units/mg protein) at 5 and 96 hpi ($P < 0.05$) (Table 5).

3.6. Superoxide dismutase (SOD) activity

The lowest mean value of SOD activity (6.03 ± 0.62 SOD units/mL of hemolymph) was determined in shrimp from the metal mixture experiment at 96 h exposure in the 10% LC_{50} 96 h treatment (Table 6). Regarding Cd + Cu + bacteria experiment, no significant differences ($P > 0.05$) were observed among positive (+) control with the other treatments (Table 6).

3.7. Protein concentration

Only protein content in shrimp exposed at 48 h of exposure and 10% LC_{50} was higher ($P < 0.05$) than those of control and CCC and 1% LC_{50} treatments (Table 7). Regarding metals + bacteria, specimens of negative (−) control showed lower protein content at 5 and 96 h exposure than those of Cd + Cu + bacteria treatments, without significant differences (Table 7).

4. Discussion

In Mexico, some shrimp farmers use copper sulfate to eradicate micro and macroalgae and gastropod mollusks [28] while the most common Cd^{2+} source to shrimp ponds is the fertilizer runoff discharged to coastal lagoons [19]. Both metals are entering shrimp farms, causing costly molecular/biochemical events to detoxify and maintain cellular homeostasis, weakening shellfish immunological defense [5].

Table 6
Mean values (±SD) of superoxide dismutase activity (SOD units/mL of hemolymph) in *Litopenaeus vannamei* juveniles exposed to Cd + Cu and Cd + Cu + *Vibrio harveyi* (*Vh*) combinations equivalent to 1 and 10% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control (+)	Control (−)	ΣCCC	1%	10%
Cd + Cu					
5 h	11.29 ± 0.16^{c}	nt	13.48 ± 0.43^{d}	12.38 ± 1.54^{cd}	12.28 ± 1.15^{cd}
48 h	8.31 ± 1.65^{ab}	nt	8.00 ± 1.04^{bc}	7.71 ± 1.01^{ab}	7.34 ± 0.80^{b}
96 h	7.43 ± 1.17^{b}	nt	9.01 ± 0.057^{c}	7.34 ± 1.75^{b}	6.03 ± 0.62^{a}
Cd + Cu + *Vh*					
0 hpi	15.65 ± 0.24^{d}	16.11 ± 0.40^{d}	16.45 ± 0.24^{d}	16.16 ± 0.32^{d}	15.65 ± 0.24^{d}
5 hpi	11.28 ± 3.08^{abe}	10.23 ± 1.87^{a}	12.52 ± 1.50^{bc}	12.00 ± 1.58^{be}	12.43 ± 1.13^{bef}
48 hpi	13.48 ± 1.21^{cf}	12.12 ± 1.36^{bf}	13.97 ± 0.73^{c}	12.79 ± 1.62^{bc}	14.25 ± 1.03^{cd}
96 hpi	12.67 ± 1.73^{bcf}	12.61 ± 1.57^{bf}	13.89 ± 2.19^{c}	11.14 ± 3.57^{ab}	13.16 ± 1.33^{cf}

nt = no treatment; standard deviation = SD; LC50 = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA and Student-Newman-Keuls test).

Table 7
Mean values (±SD) of total protein concentration (mg/mL) in *Litopenaeus vannamei* juveniles exposed to Cd + Cu and Cd + Cu + *Vibrio harveyi* (*Vh*) combinations equivalent to 1 and 10% of the respective LC_{50} 96 h, and of the two values of CCC.

Time	Control (+)	Control (−)	ΣCCC	1%	10%
Cd + Cu					
5 h	12.54 ± 4.10^{a}	nt	9.75 ± 5.68^{a}	14.34 ± 5.90^{a}	12.56 ± 4.21^{a}
48 h	14.74 ± 8.06^{ab}	nt	17.78 ± 4.70^{b}	17.97 ± 9.69^{ab}	22.71 ± 11.06^{d}
96 h	20.41 ± 7.13^{c}	nt	21.59 ± 7.39^{c}	21.13 ± 7.18^{bc}	17.70 ± 5.36^{c}
Cd + Cu + *Vh*					
0 hpi	8.66 ± 1.50^{bc}	17.22 ± 10.17^{cd}	11.02 ± 8.45^{cd}	13.35 ± 5.84b^{bc}	12.35 ± 6.60^{bc}
5 hpi	15.54 ± 7.38^{cd}	5.65 ± 3.80^{a}	17.39 ± 11.22^{d}	9.46 ± 3.86^{ab}	11.84 ± 7.39^{bc}
48 hpi	13.17 ± 5.46^{cef}	9.85 ± 6.99^{bf}	13.08 ± 6.87^{ce}	16.62 ± 16.13^{ce}	8.55 ± 4.60^{b}
96 hpi	9.13 ± 6.05^{ab}	6.69 ± 4.12^{ab}	10.72 ± 5.04^{bc}	11.14 ± 6.64^{b}	8.20 ± 5.49^{ab}

nt = no treatment; standard deviation = SD; LC_{50} = median lethal concentration; CCC = Criterion of Continuous Concentrations; hpi = hours post infection. Different letters indicate statistical differences (two-way ANOVA and Student-Newman-Keuls test).

4.1. Shrimp mortality

In this experimental research, low mortality was observed at higher exposure times and metal concentrations, including organisms inoculated with *V. harveyi* and exposed to Cd + Cu mixture (Table 1). This low mortality observed in the present study could be by the natural immune response of some shrimp species (as *L. vannamei*) to *Vibrio harveyi* [8]. This result agrees with Pan et al. [27], who reported no mortality in *L. vannamei* inoculated at 1×10^5 CFU mL^{-1} of the same bacteria. However, *L. vannamei* inoculated with *V. alginolyticus* (1×10^5 CFU mL^{-1}) had 50% mortality after 72 h [38], indicating different lethal effects of *Vibrio* species to *L. vannamei*.

4.2. Hemolymph clotting time

Hemolymph coagulation is an excellent sign of an efficient response of the crustacean immune system, and clotting time increase may be used as an indicator of crustacean stress [29]. In this study, no significant effect was observed in clotting time, which agrees with the study of Bautista-Covarrubias et al. [39] who reported non-Cd^{2+} effect in *L. vannamei* clotting time. However, Cu^{2+} exposure (3 730 μg/L) increased hemolymph clotting time in *L. vannamei* [26], indicating a possible antagonism effect.

Sarathi et al. [40] pointed out that a hemolymph clotting time increase occurs when organisms are inoculated with bacteria and viruses. Yoganandhan et al. [41] reported an increase in clotting time on *Penaeus indicus* infected with white spot syndrome virus (WSSV). Regarding the Cd + Cu + *Vibrio* experiment in this study, higher hemolymph clotting times were observed at the 10% treatment, indicating a synergism effect of these metals with this *Vibrio* species.

4.3. Hemocyanin content

Hemocyanin is the main hemolymph Cu^{2+}-based protein that transports oxygen to all tissues, which acts as metal carrier and protein storage and also plays an important role in resistance to microorganism infections [27]. Its content in aquatic organisms is affected by stressors, including metals, and its decrease was reported in red swamp crayfish *Procambarus clarkii* exposed to Cu^{2+} [42].

Hemocyanin plays an important role in shrimp immune response [43]. Its increase in *L. vannamei* exposed to 200 μg Cu^{2+}/L was reported by Qian et al. [44]. Bautista-Covarrubias et al. [39] also reported an increase in *L. vannamei* exposed to Cd^{2+} (8.8 μg/L), but hemocyanin decreased when shrimp was exposed to 1 245 μg/L. These authors concluded that its increase at low Cd^{2+} concentration occurs as a compensatory mechanism response, but its decrease is due to a deleterious immune effect. The tendency to lower the hemocyanin content reported in this study could affect several physiological processes when shrimp are exposed to Cd + Cu + bacteria because less oxygen is available for metabolic and immune shrimp functions.

4.4. Total hemocyte count

Metals are immunotoxic in decapods because they decrease THC. Experimental studies with shrimp *P. elegans* exposed individually to Pb^{2+}, Hg^{2+}, Zn^{2+}, Cr^{2+}, Cu^{2+}, Cd^{2+} [14], and with shrimp *L. vannamei* exposed (individually) to Cd^{2+} and Cu^{2+} [27,40] have evidenced the effect on THC decrease. The results in this study also confirm that THC decreases in white shrimp when exposed to Cd + Cu. The deleterious THC effect in decapods exposed to metals is caused by oxidative stress affecting hemocytes, as explained by Zhou et al. [45] and Guo et al. [46] after their experiments with the crab *S. henanense* and shrimp *L. vannamei*, respectively. These findings are highly relevant ecologically because low THC causes an increase in shrimp susceptibility to diseases [3].

Regarding studies on shrimp inoculated with pathogen microorganisms and exposed to metals, Yeh et al. [16] and Abad-Rosales et al. [18] reported a significant THC reduction in *L. vannamei* inoculated with *V. alginolyticus* and WSSV and exposed to Cu^{2+} and a metal mixture (Cd^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}), respectively. However, in this study, shrimp exposed to sublethal Cd + Cu concentrations + *V. harveyi*, showed a THC increase at higher exposure times, which could be a defense mechanism of white shrimp [45], detonated by *V. harveyi* presence.

4.5. Phenoloxidase activity

The Prophenoloxidase (proPO)-activating system is an essential mechanism of the crustacean immune system [8], which included PO, a copper-containing enzyme that catalyzes reactions to melanin synthesis [27]. PO activity reduction has been associated with environmental stress [11]. In this study, no direct effect of Cd + Cu exposure on PO

activity was observed. However, Wei and Yang [11] observed a PO activity reduction in *P. clarkii* when exposed to low Cu^{2+} levels, but its activity increased at higher Cu^{2+} levels. Bautista-Covarrubias et al. [39] reported a PO activity decrease in *L. vannamei* after 96 h to Cd^{2+} exposure (249 μg/L). This behavior indicates a PO time-dose-dependent activity when shrimp are exposed to metals.

Abad-Rosales et al. [18] reported that PO activity increased in shrimp exposed to metals and challenged with pathogenic microorganisms. Chen and Wang [47] and Yeh et al. [16] exposed giant freshwater prawn *Macrobrachium rosenbergii* and *L. vannamei* at sublethal concentrations of copper sulfate and challenged with bacteria *Lactococcus garvieae* and *V. harveyi*, respectively; reporting that PO activity decreased in both crustaceans. These results indicate a wide variety of responses, which involve decapod, stressors, and pathogen microorganisms.

4.6. Superoxide dismutase activity

Copper is an element recognized as essential to organisms, a cofactor of numerous enzymes like cytochrome *c* oxidase and SOD [11]. However, an excess of Cu^{2+} produces ROS through Haber-Weiss reaction [10]. According to Yeh et al. [16], when pathogen microorganisms enter shrimp body, NADPH-oxidase is activated to reduce oxygen molecules and produce ROS as O_2^- (respiratory burst), which has microbicide activity.

Both Cd^{2+} (non-redox-active) and Cu^{2+} (redox-active) cause oxidation stress affecting SOD activity as observed in hemocytes of crab *S. henanense* [13] and *L. vannamei* [47], respectively. In this study, significant differences were observed between shrimp exposed to higher metal mixture concentration. Cu^{2+} exposure causes SOD increases as a function of time and concentration in *L. vannamei* [46], but Wei and Yang [11] reported a SOD activity reduction on *P. clarkii*. Whereas in Cd^{2+} exposure, SOD activity decreases as concentration and exposure time increases, such as in the decapod *Austinogebia edulis* [48]. Thus, a synergistic effect occurred during Cd + Cd exposure on *L. vannamei*.

Duan et al. [49] reported that in shrimp *Penaeus monodon* inoculated with *Vibrio parahaemolyticus*, ROS production increases during the first inoculation time. ROS are produced as a defense mechanism. SOD increases at first inoculation time (3 hpi) but decreases at higher time (12 hpi). Hsieh et al. [50] reported that SOD activity decreased 50% at 36 h after *V. alginolyticus* inoculation, and no SOD activity recovery was observed at the end of 72 h. Yeh et al. [16] determined an increase in the superoxide anion in *L. vannamei* exposed to Cu^{2+} and inoculated with *V. alginolyticus*. In this research, no increase in SOD activity was observed in *L. vannamei*. The ROS production (O_2^- and H_2O_2 by respiratory burst) induced by Cd + Cu exposure and bacteria inoculation decreased SOD activity [10], indicating an antioxidant failure during vibriosis disease [49] and Cd + Cu exposure.

4.7. Protein content

Exposure to metal mixtures induces ROS production and cause damage to proteins, lipids, and cellular apoptosis [46]. Moreover, protein carbonylation could produce peptide cleavage, cross-linking and modification of their molecular structure [11]. Moreover, Cu^{2+} exposure caused a protein carbonyl induction after 24 h in *P. clarkii* [42].

Exposure to Cd^{2+} and Cu^{2+} decreased protein content in this study, which agrees with Qin et al. [13] and Bautista-Covarrubias et al. [39], who reported that protein content decreased after Cd^{2+} exposure in the crab *S. henanense* and shrimp *L. vannamei*, respectively. Copper exposure also decreased protein content in *L. vannamei* [26]. These authors pointed out that this low protein content could indicate a higher energy demand by shrimp due to induced stress by metals. Moreover, Cd + Cu exposure caused a synergistic effect in the shrimp of this study. These alterations in proteins may alter the metabolic functions affecting protein/enzyme synthesis of *L. vannamei* as an immune response.

5. Conclusion

Litopenaeus vannamei inoculated with *V. harveyi* and exposed to Cd + Cu showed an increase on THC and a reduction on PO activity, which could indicate a higher susceptibility to *V. harveyi*. Regarding shrimp exposed to Cd + Cu in the CCC values and *V. harveyi* inoculated, THC increased while PO activity decreased. The results evidence that metal stress caused a higher disease susceptibility in exposed shrimp. Therefore, CCC values are not sufficient to protect health of aquatic organisms. Further studies should consider an adjustment to lower CCC values.

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CRediT authorship contribution statement

Juan Carlos Bautista-Covarrubias: Conceptualization, Writing – review & editing. **Iriana Edith Valdez-Soto:** Methodology. **Marisela Aguilar-Juárez:** Writing – review & editing. **Jonathan Omar Arreola-Hernández:** Methodology. **Martín Federico Soto-Jiménez:** Writing – review & editing. **Sonia Araceli Soto-Rodríguez:** Writing – review & editing. **José Armando López-Sánchez:** Investigation. **Carmen Cristina Osuna-Martínez:** Writing – review & editing. **Martín Gabriel Frías-Espericueta:** Project administration, Conceptualization, Writing – review & editing.

Declaration of competing interest

Authors declare no competing interest.

Data availability

No data was used for the research described in the article.

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