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Acute Toxicity of Copper, Zinc, Iron, and Manganese and of the Mixtures Copper–Zinc and Iron–Manganese to Whiteleg Shrimp *Litopenaeus vannamei* Postlarvae

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Cu, Zn, Fe and Mn are physiologically essential for the normal development and growth of aquatic organisms; however, they may act as enzyme inhibitors if their concentrations are different from the actual physiological requirements, which may lead to either a toxic effect or to inhibition of growth (Bambang et al. 1995). Elevated concentrations of these metals in coastal regions result from the natural and anthropogenic processes; and in Mexico, despite national policies regarding the use and handling of coastal resources, metal pollution problems are increasing (Villanueva and Botello 1998).

In the past 17 years, shrimp aquaculture has rapidly increased in Mexico and the most important species is the whiteleg shrimp *Litopenaeus vannamei*. Studies carried out in Mexico for more that 25 years have demonstrated an increase of metal concentrations in lagunar and estuarine ecosystems (Villanueva and Botello 1998) and these systems are essential environments for shrimp production. However, information about metal toxicity for *L. vannamei* is not available, in spite of their ecological and commercial importance. The purpose of this study was to assess under laboratory conditions the acute toxicity of individual concentrations of Cu, Zn, Fe and Mn on *L. vannamei* postlarvae. Additionally, due similar physiological/toxic effects (Cu-Zn), and by adsorption and co-precipitation reactions (Fe-Mn); the interaction (1:1) of these metals is examined.

MATERIALS AND METHODS

Experiments were carried out at the Faculty of Marine Sciences, UAS. 500 postlarvae (PL12) of *L. vannamei* (1.2±0.2 cm) were obtained from a commercial laboratory located close to Mazatlan (Mexico), and acclimated for three days under laboratory conditions in a 50 l container (46x41x44 cm). During acclimation the postlarvae were fed with *Artemia* sp nauplii, and seawater was renewed every 24 h (Buikema et al. 1982). The seawater used in the bioassays was pumped from the Mazatlan Bay and was filtered through a sand and gravel bed, one cartridge system (10 to 1 μm) and finally treated with activated charcoal. Chemical characteristics of seawater have been specified by Frías-Espericueta et al. (2001).

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Metal test solutions were prepared every day with $CuCl_2$, $ZnCl_2$, $FeCl_3 \bullet 6H_2O$ and $MnCl_2 \bullet 4H_2O$ (Baker GR grade) diluted in seawater. Since metals in solution occur in many forms, all values are reported as total concentrations (nominals), which ranged from 12 to 128, 1 to 30, 30 to 80, and 50 to 350 mg L^{-1} for Cu, Zn, Fe, and Mn, respectively. Throughout the bioassays temperature varied from 26.8 to 27.6°C, salinity was 34 ppt, and pH and dissolved oxygen were kept between 8.0-8.1 and 5.05-5.69 mg L^{-1}, respectively.

In individual bioassays, short-term (96 hr) median lethal concentration (LC_{50}) toxicity tests were conducted following the methods described in APHA-AWWA-WPCF (1992). Postlarvae were sampled randomly from the 50 l container and placed in triplicate 600 ml glass beakers for each of the treatments, where they were kept for three days before the bioassays, with 12:12 hr (light:dark) diurnal cycle. All glassware was acid-washed before use. Each flask containing 300 ml of test solution and 10 test postlarvae, was aerated by an airstone. The test solutions and the control were renewed daily in accordance with the static renewal method for toxicity tests (Buikema et al. 1982). All observations on survival and culture conditions were carried out at 12-hr intervals. The absence of response to a gentle mechanical stimulus was the criterion for death.

LC_{50} values and their 95% confidence limits for any 24 hr interval were calculated with the standard method of probit analysis described by Finney (1971). Once the estimated probit line and results of a chi-square test for goodness of fit were determined, a z-test for the comparison of two LC_{50} values at the 5% level of significance (APHA-AWWA-WPCF 1992) was carried out between each LC_{50} value.

After the 96-hr LC_{50} value for each metal was obtained, Cu-Zn and Fe-Mn bioassays (168 hr) were carried out at a toxicity ratio (TR) of 1:1 at different toxic units (TU). These were calculated according to Sprague and Ramsay (1965) as:

$$TR = M_1\text{-}m/LC_{50}\ M_1 : M_2\text{-}m/LC_{50}\ M_2 \ ; \ TU = M_1\text{-}m/LC_{50}\ M_1 + M_2\text{-}m/LC_{50}\ M_2$$

Where M_1-m and M_2-m refer to metal concentrations (mg L^{-1}) in the mixture, and $LC_{50}\ M_1$ and $LC_{50}\ M_2$ refer to their 96-hr LC_{50} (mg L^{-1}).

In addition, to determine the effect of any given metal mixture, the sum of toxic contributions (S) was calculated as:

$$S = ([A]/\ LC_{50}\ M_1) + ([B]/LC_{50}\ M_2)$$

Where [A] and [B] are the concentrations of each metal in the binary mixture causing 50% mortality. When the calculated S value was <1,=1, or >1, this was taken as proof of synergism, simple additivity or antagonism, respectively (Vermeulen 1995).

In order to obtain a more representative value for the species under study, the postlarvae came from different hatches, from different broodstocks, and were obtained at different times.

RESULTS AND DISCUSSION

No shrimp died in the controls run in parallel with the bioassays of postlarvae exposed to different concentrations of total Cu, Zn, Fe and Mn. In 12 mg L^{-1} of Cu, no shrimp died during the 96 hr of exposure. In the 16, 32, 48, 64 and 80 mg L^{-1} total Cu solutions, mortalities of 20, 43.3, 56.6, 70, and 86.6% were recorded, respectively, and a 100% mortality occurred with 96, 112, and 128 mg L^{-1} after 72, 48, and 24 hr of exposure. According to the existing literature on the toxicity of Cu, death was probably due to excess mucous covering gills as a result of irritation, linked to a disruption of the gill function; besides, uptake of Cu is known to cause acidosis reinforced by hypercapnea and accumulation of lactic acid, since it restricts respiratory exchanges with the internal tissues hypoxia, being the lethal mechanism (Boitel and Truchot 1989).

Final mortalities in 1, 1.5, 2.5, and 5 mg L^{-1} total Zn were 0, 33.3, 76.6, and 90%, respectively, while with 10, 15, 20, 25, and 30 mg L^{-1}, 100% mortality occurred at 96, 96, 72, 60, and 24 hr, respectively. Lethal effect of Zn is to induce cytological damage to gills and the physiological cause of death is related to the breakdown of respiratory and osmoregulatory processes (Crespo 1984).

Mortalities of 0, 23.3, 40, and 93.3% were noted in 30, 40, 45, and 50 mg Fe L^{-1}, respectively; with 100% mortality after 84, 60, 48, 36 and 12 hr of exposure to 55, 60, 65, 70, and 80 mg L^{-1}, respectively. Jansen and Groman (1993) pointed out that, in decapods, Fe precipitates are deposited on the carapace and the gills, occluding the respiratory lamellae.

For total Mn, 100% mortality was found only with 250, 300, and 350 mg L^{-1} at 72, 48 and 24 hr, respectively; for 50 100, 150 and 200 mg L^{-1}, mortality ranged from 0 to 86.6 % at 96 hr of exposure. Baden and Neil (1998) found that the toxic effect of Mn is the disruption of physiological processes related to the neuromuscular system.

According to Bambang et al. (1995), the physiology of shrimps is linked with the molt cycle, and their susceptibility to toxicants varies with molt stages, since during ecdysis, the water influx favours metal uptake with an increase of the toxic effect. For this, crustaceans are supposed to be more sensitive to metals at the time of molting, as observed in *Carcinus maenas* by Boitel and Truchot (1989), and some authors reported that postlarvae die in the act of molting.

In the present study, no relation was observed between mortality and ecdysis in the Fe and Mn bioassays. However, in the experiments with 1.25 mg Zn L^{-1}, all postlarvae found dead were in the ecdysis phase. The highest numbers of exuvies

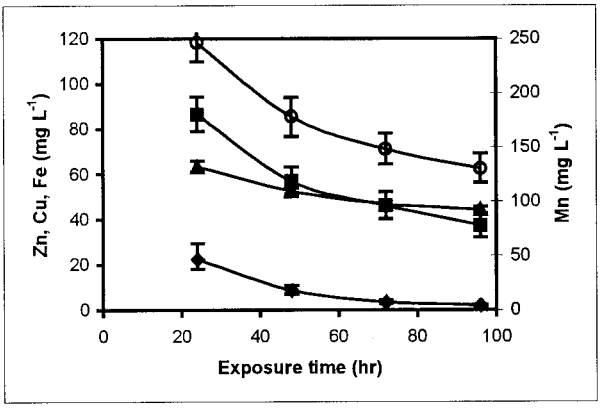

Figure 1. LC_{50} values and 95% confidence limits (bars) for *L. vannamei* postlarvae (PL18) exposed from 24 to 96 hr to total Zn (◆), Cu (■), Fe (▲) and Mn (O)

(32) was observed in the Cu bioassay, at 12 mg L^{-1}, while in the control only 19 organisms reached the ecdysis stage. Bjerregaard and Vislie (1986) pointed out that Cu increases Ca levels in hemolymph and affects the production or function of the molt-inhibiting hormone and this is associated with the initiation of molting processes.

Statistical analysis showed a satisfactory fit ($P<0.05$) to the probit model and that, with the exception of the comparison between 72-hr and 96-hr in the case of Mn, all LC_{50} calculated for each metal at different exposure times (Fig 1) were significantly different ($P<0.05$).

During the Cu and Fe assays, We observed the presence of particulates at the bottom of the experimental vessels. The dissolved fractions of Cu and Fe determined by atomic absorption spectrophotometry; and the LC_{50} was calculated with the dissolved concentrations, and both dissolved and total LC_{50} are reported in the table 1. Evidently, LC_{50} calculated with dissolved values, was significantly lower than total value.

Dissolved metals are supposed to be more toxic, because they are more easily absorbed by aquatic organisms than the particulate fraction. However we also decided to report our results as a function of total Cu and Fe, because to their benthic behavior, our test organisms were always in contact with the particulate (as well as with the dissolved Cu and Fe), and this precipitates could cause occlusion of the gills affecting some physiological processes. In addition, Borgmann and Ralph (1983) found that Cu toxicity is not only a function of free Cu ion concentrations, especially in the presence of others ligands/complexes, and that it is also dependent on the organisms tested.

In table 1, a selection of toxicity studies carried out with crustaceans in larval/postlarvae stage is showed. Total Zn was more toxic than total Cu (even dissolved LC_{50}) and this metal than Fe and Mn. Postlarvae of *L. vannamei* were more tolerant than most of larval crustaceans, our 96-hr LC_{50} value for total Zn was 2.08 mg L^{-1}, within the interval reported by EPA (0.09-58.1 mg L^{-1}).

Regarding dissolved Cu, *L. vannamei* showed a tolerance higher than those enlisted in the table 1, with the exception of *Metapenaeus ensis*, which showed a LC_{50} of 4.76 mg L^{-1}. Cu is commonly used in Mexican commercial hatcheries to improve growth and to control fungi population, which could increase the tolerance to Cu by postlarvae. However chemical speciation varies with salinity and the effect of free ion at different salinities must be investigated in *L. vannamei*

The Mn tolerance of *L. vannamei* is high, because they accumulate high concentrations without lethal effect. Limited research has been reported establishing the toxicity of Mn to aquatic organisms (Lasier et al. 2000), and only few comparisons were carried out (Table 1).

Table 1. 96-hr LC_{50} values (mg L^{-1}) of total Cu, Zn and Mn for larval crustaceans

Species	Cu	Zn	Mn	Author
Ceriodaphnia dubia			15*	Lasier et al (2000)
Hyalella azteca			3*	Lasier et al. (2000)
Maja squinado	0.05**			Mariño-Balsa et al. (2000)
Penaeus japonicus	1.45			Bambang et al. (1995)
Metapenaeus ensis	4.76*			Wong et al. (1993)
Scylla serrata	0.08			Ramachandran et al. (1997)
Portunus pelagicus		0.56*		Greenwood and Fielder (1983)
Portunus sanguinolentus		0.77*		Greenwood and Fielder (1983)
Paragrapsus quadridentatus	0.17	1.23		Ahsanullah and Arnott (1978)
Cancer magister	0.05	0.45		Martin et al. (1981)
Palaemon serratus	3.3**			Mariño-Balsa et al. (2000)
L. vannamei	[d]4.2/37	2.08	130	This study

* 48-hr, ** 72-hr LC_{50} values. [d]Dissolved/total value

Metal interactions may alter their toxicity, as shown in table 2 for Cu-Zn and Fe-Mn. In both bioassays the sum of toxic contributions (S) and the TU_{50} were higher than 1 at 168-hr of toxic exposure (S= 3.3 and 1.87, respectively), indicating an antagonistic effect similar to the results of Negilski et al. (1981) in the shrimp *Callianassa australiensis*, who attributed the toxic effect to substitution and competition between available sites during protein synthesis. No studies about interaction of Fe-Mn were found, thus the comparison was not possible.

Maximum permissible concentrations (MPC), were calculated according to Mariño-Balsa et al. (2000): MPC= LC_{50}/100. In this case, the MPC for rearing of *L. vannamei* in seawater would be 1300, 20.8 and 42/373, 9.5/443 μg L^{-1}, for

total Mn, Zn, and dissolved/total Cu and Fe, respectively. In order to validate the MPC calculated from these bioassays, the first physiological response of organisms to sublethal concentrations might occur at molecular level and this must be investigated in *L. vannamei*.

$CuSO_4$ is commonly applied to shrimp ponds to control the abundance of algae, and its effect on growth of this species should be the object of further studies. In this context, this MPC is of great practical utility to provide biological criteria to establish quality standards that protect resources of the coastal environment, especially in the shrimp farms of the Northwest coast of Mexico.

Table 2. TU_{50} values and confidential limits (p=0.05) (between parenthesis) of *L. vannamei* postlarvae exposed to Cu-Zn and Fe-Mn mixtures.

Exposure time (hr)	TU_{50} of mixtures			Individual LC_{50} (mg L^{-1})	
	TU_{50}	Equivalent concentration			
		Cu	Zn	Cu	Zn
48	6.09 (5.2, 7.2)	140	6.33	56.8	8.67
96	2.98 (2.5, 3.5)	68.5	3.09	37.3	2.08
168	1.44 (1.2, 1.7)	33.1	1.49		
		Fe	Mn	Fe	Mn
48	2.52 (2.41,2.63)	55.4	163	52.5	178
96	1.88 (1.74, 2.02)	41.6	122	44.2	130
168	1.43 (1.33, 1.53)	31.6	92.9		

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