Science of the Total Environment 758 (2021) 144025

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Acute mercury toxicity and bioconcentration in shrimp *Litopenaeus vannamei* juveniles: Effect of low salinity and chemical speciation

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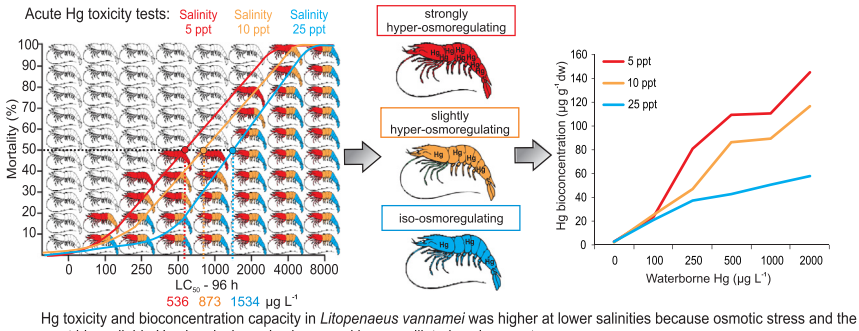
HIGHLIGHTS

- Hg toxicity and bioconcentration in shrimp were tested at 5, 10 and 25 ppt salinity.
- Hg LC_{50}–96 h values were 536, 873 and 1534 μg L^{-1} for 5, 10 and 25 ppt salinity.
- Acute Hg toxicity in *Litopenaeus vannamei* juveniles was higher at lower salinities.
- Hg bioconcentration in whole-body of shrimp was greater at lower salinities.
- Osmotic stress and most bioavailable Hg chemical species increased at low salinity.

GRAPHICAL ABSTRACT



Hg toxicity and bioconcentration capacity in *Litopenaeus vannamei* was higher at lower salinities because osmotic stress and the most bioavailable Hg chemical species increased in more diluted environments

ARTICLE INFO

Article history:
Received 28 August 2020
Received in revised form 6 November 2020
Accepted 17 November 2020
Available online 08 December 2020

Editor: Mae Sexauer Gustin

Keywords:
Bioaccumulation
Bioavailability
Hypo-osmotic stress
Safe-level
Hg speciation

ABSTRACT

This study evaluated low salinity effect on acute Hg toxicity and bioconcentration capacity in *L. vannamei* juveniles (8.4 ± 0.7 g), because this species is frequently exposed to hypo-osmotic environments in its natural habitat, and in farming ponds. Hg LC_{50} values were 1723, 1272, 983 and 536 μg L^{-1} at salinity of 5 ppt (parts per thousand); 2203, 1740, 1340 and 873 μg L^{-1} at 10 ppt; and 7013, 5693, 1759 and 1534 μg L^{-1} at 25 ppt for 24, 48, 72 and 96 h, respectively. After 96 h, acute Hg toxicity in shrimp was significantly higher in salinity of 5 ppt than in 25 ppt; likewise, Hg bioconcentration in shrimp exposed to different Hg treatments was statistically greater in salinity of 5 ppt than in 25 ppt. The chemical speciation calculated in experimental waters suggested that neutral chemical Hg species ($HgCl_2$ and HgClOH) were the most bioavailable because their fractions (51–62%) increased when salinity decreased. Therefore, the inverse relationship between Hg toxicity and salinity was due to osmotic stress and the neutral chemical Hg species fractions that increased at lower salinities. Hg bioconcentration factors indicated that the higher Hg waterborne concentrations were the most saturated uptake and storage mechanisms in shrimp. Thus, Hg concentrations in organisms did not increase in proportion to waterborne Hg concentrations in the three salinities. These results support the hypothesis of an effect of low salinity on Hg toxicity and bioconcentration capacity in *L. vannamei*. The safe Hg concentrations 5.4, 8.7 and 15.3 μg L^{-1} were proposed for shrimp exposed to salinity of 5, 10 and 25 ppt, respectively. This information allows recognizing risky environments for both wild and cultured healthy growth of these shrimp, which can help decision makers on coastal management and shrimp pond managers to have better water quality.

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https://doi.org/10.1016/j.scitotenv.2020.144025
0048-9697/

1. Introduction

Mercury (Hg) is a highly teratogenic and carcinogenic non-essential metal, classified as a priority pollutant (EPA, 2020). Since the late 19th century Hg concentrations in the atmosphere and in surface ocean waters have increased 300–500 and 200%, respectively due to intensification of anthropogenic activities (Mason et al., 1994; Outridge et al., 2018). Aquatic organisms are susceptible to experiencing the toxic Hg effects, because most of the mobilized metals by anthropogenic sources are transported via atmosphere and runoff to coastal ecosystems where they may accumulate at high concentrations (Luoma and Rainbow, 2008). For example, Hg concentrations of up to 4.0–10.2 μg L^{-1} have been found in waters of estuaries and coastal lagoons located in the northern Gulf of California, Mexico (Páez-Osuna et al., 2017). Such values exceed that of the Continuous Hg Concentration Criterion (CCC) (0.9 μg L^{-1}) for marine aquatic life (EPA, 2020). Therefore, these ecosystems could be potentially risky for developing aquatic organisms vulnerable to this metal.

Crustaceans have demonstrated to be more sensitive to Hg (Eisler and Hennekey, 1977) than other metals (Connor, 1972; Mariño-Balsa et al., 2000; Verslycke et al., 2003). *Litopenaeus vannamei* is a crustacean with ecological and economic importance for countries that make up the Pacific coast from the Gulf of California (Mexico) to Tumbes (Peru) where this species is naturally distributed (Dugassa and Gaetan, 2018). It represents 35% of the total production of wild shrimp caught in estuaries and bays of Mexico, and 99% of the total shrimp production by aquaculture (CONAPESCA, 2020). Likewise, this species is relevant to different countries in North and South America, and Asia where shrimp farming is practiced, which makes it the main shrimp culture species globally (FAO, 2020).

During the post-larval and juvenile stages, wild shrimp *L. vannamei* inhabit in estuaries and coastal lagoons where they can experience a wide range of salinities (ranging from 1 to 45 parts per thousand (ppt); Mair et al., 1982; Dugassa and Gaetan, 2018) and be impacted by high Hg concentrations (Páez-Osuna et al., 2017). Likewise, these ecosystems have been used as water sources to fill shrimp farming ponds, frequently with low salinity (i.e., 2–5 ppt mainly; Esparza-Leal et al., 2009; Roy et al., 2010). This factor is relevant in toxicological terms since the passive uptake of metals generally increases when euryhaline organisms are exposed to hypo-osmotic stress (Paquin et al., 2002; Grosell et al., 2007). Furthermore, the most bioavailable metal fractions of inorganic chemical species of metals (e.g., Hg^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+}) for biota are known to increase at lower salinities (Blewett et al., 2015; Bielmyer-Fraser et al., 2018). For these reasons, metal toxicity can be inversely related to water salinity.

The inverse relationship between low salinity and toxicity of some metals, such as Cd (Verslycke et al., 2003; Barbieri and Tavares-Paes, 2011), Zn (Verslycke et al., 2003; Barbieri and Doi, 2011), Pb (Verslycke et al., 2003; Santos et al., 2014), Cu (Grosell et al., 2007; Martins et al., 2011) and Ni (Leonard et al., 2011; Blewett et al., 2015) has been demonstrated in different crustaceans. In *Eriocheir sinensis* adult crabs, Hg toxicity increased when they were exposed to freshwater compared to water with 35 ppt salinity. A similar tendency was determined in *Carcinus maenas* adult crabs exposed to 12 ppt salinity compared with water with 35 ppt of salinity (Bianchini and Gilles, 1996). However, the inverse relationship between salinity and Hg toxicity has not been proved in shrimp with high osmoregulatory capacity (Das and Sahu, 2002; Verslycke et al., 2003).

Das and Sahu (2002) reported that acute Hg toxicity in *Penaeus monodon* and *Penaeus setiferus* juveniles with large size (55–75 mm in length) was affected by low salinity. However, in both species, juveniles with smaller sizes (35–55 mm), Hg toxicity was not affected by low salinity, because their hyper-osmoregulation mechanisms were more efficient in younger organisms. On the other hand, Verslycke et al. (2003) observed that Hg sensitivity of *Neomysis integer* juveniles exposed to low salinity (5 ppt) was statistically comparable to that determined in the control salinity (25 ppt), because of their adaptation to hypo-osmotic environments.

Shrimp *L. vannamei* has a high hyper-osmoregulation capacity capable of acclimating to salinity of 0.5 ppt even though its isosmotic point is close to 25 ppt (Roy et al., 2010); nevertheless, as *P. monodon* and *P. setiferus*, the ability of *L. vannamei* to adapt to low salinity environments decreases in larger organisms (Charmantier et al., 1994; Péqueux, 1995; Roy et al., 2010). Therefore, this study implicates the hypothesis that *L. vannamei* juveniles exposed to lower salinities show higher Hg accumulation due to hypo-osmotic stress compared with those exposed to isosmotic salinity, where osmotic stress is not present; thus, acute Hg toxicity in shrimp is greater at lower salinities.

According to the previous information and considering that no study was available where low salinity effect on Hg acute toxicity and bioconcentration capacity in penaeids had been investigated, this study established two objectives (1) contrast acute Hg toxicity in *L. vannamei* juveniles exposed to low salinity environments (5 and 10 ppt) and an isosmotic environment (25 ppt) and (2) determine the low salinity effect on Hg bioconcentration capacity in shrimp exposed to different waterborne Hg concentrations after 96 h.

2. Material and methods

2.1. Testing organisms and acclimation process

Litopenaeus vannamei (7–9 g wet weight) juveniles (n = 1500) were collected from a shrimp farm located in northwest Mexico (State of Sinaloa) and transported to Yum Kaax experimental module in the city of Mazatlán, Sinaloa. Shrimp were distributed in nine high-density 500-L polyethylene tanks containing diluted seawater at salinity of 27 ppt (same salinity level in the culture pond); the tanks remained without change in salinity for two days although 50% of the water in each one was exchanged daily. After that, the tanks were divided into three groups and subsequently acclimated for eleven days (salinity changes of 2 ppt/day with an acclimation rate of 0.5 ppt/h) until they reached the required salinities (5, 10 and 25 ppt). Then, the shrimp remained two days without changes in salinity before starting the experiments (Li et al., 2008; Ramírez-Rochín et al., 2019).

Shrimp were fed with a commercial diet (30% protein, 5.8% fat, 5% crude fiber, 13% ash and 12% moisture) (CamaronEX 30, Nutrimentos Purina S.A., de C.V., Ciudad Obregón, SON, MX) three times daily during the acclimation process. Seawater utilized for bioassays was pumped from Mazatlán Bay and filtered through a system with activated carbon and silica sand followed by 10, 5 and 1 μm cartridge filters coupled to ultra-violet (UV) light filter. Municipal tap water (salinity of 0.2 ppt) filtered through a deep bed system with sand, gravel, activated carbon and zeolite (WaterTec®, Tucson, AZ, U.S.A.) was used during acclimation process and toxicity test. The experimental procedures were developed considering the guidelines for accommodation and care of animals used for experimental and other scientific purposes established by the EC (2007) (notified under document number C (2007) 2525).

2.2. Acute toxicity test

Mercury toxicity tests with *L. vannamei* male and female juveniles (8.4 ± 0.7 g wet weight and 83 ± 12 mm in length) acclimated to salinities of 5, 10, and 25 ppt were carried out in triplicate by the static renewal method for toxicity testing (APHA-AWWA-WPCF, 1992; Roos-Muñoz et al., 2019). The nominal Hg concentrations (treatments) used as test solutions were 0, 100, 250, 500, 1000, 2000, 4000, and 8000 μg L^{-1} for three salinity levels. A stock Hg solution with 500 mg L^{-1} was made dissolving 676.8 mg of $HgCl_2$ (Baker GR grade, DE) in 1 L of Milli-Q water. The test solutions were prepared by placing adequate volumes of the stock solution in 10 L of diluted seawater to the required salinity, which were contained in 26-L polyethylene tanks (washed and rinsed with a 2 M HNO_3).

Ten shrimp randomly selected in the intermolt stage (de Oliveira-Cesar et al., 2006) were placed in each test tank; 100% of test solutions were renewed every 24 h for 96 h of test duration, time in which the organisms were not fed (Ramírez-Rochín et al., 2019). Water samples (100 mL) filtered throughout a membrane of 0.45 μm (MF-Millipore, Darmstadt, DE) were collected from each renewed test solutions to determine the analytical initial concentrations of dissolved Hg and major seawater ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}), as well as total alkalinity (as $CaCO_3$).

Dead organisms were removed at 12-h intervals up to 96 h. Shrimp death was assumed when an organism remained immobile after being touched with a glass rod. Cumulative mortality was recorded each 24 h, the same periods in which the LC_{50} analyses (24, 48, 72, and 96 h) were performed. A dissolved oxygen meter (model DO200, YSI, OH, U.S.A.) was used and temperature of the test solutions were measured each 12 h; while pH was determined with a pH meter (model HI98121, Hanna Instruments, TX, U.S.A.) and salinity with a refractometer (model STX-3, VEE GEE, WA, U.S.A.). The light-dark cycle was 12–12 h.

The range of Hg concentrations chosen to determine acute toxicity was based on previous studies with *L. vannamei* (Frías-Espericueta et al., 2001; Roos-Muñoz et al., 2019), as well as in preliminary results obtained from this study; these Hg concentrations were adequate to determine the acute toxicity under the different salinities evaluated. Conversely, these Hg values could hardly be present in marine and surface low salinity waters, though it is more common to find concentrations of this magnitude in contaminated groundwater (e.g., ~800 μg L^{-1}; Richard et al., 2016). The wide tolerance to low salinity and availability of *L. vannamei* postlarvae throughout the year make this shrimp a popular species for cultivation in inland areas, where sometimes groundwater is used, which could reach Hg values comparable to those used in this study.

2.3. Analytical procedures in shrimp and water samples

Shrimp that survived after 96 h of exposure to different Hg test solutions were rinsed with Milli-Q water and the whole-body of the organisms were collected from each test tank in the same polyethylene container (washed with 2 M HNO_3) and frozen at −20 °C (see Supplementary material). The samples were freeze-dried for 72 h (−49 °C and 133×10^{-3} mbar), and consequently pulverized and homogenized in an agate mortar (one pool per replicate). Aliquots (samples 254 ± 2 mg of dry tissue) were digested in Teflon vials (Savillex, EdenPraire, U.S.A.) with 5 mL of HNO_3 (Ultrapure 69%, J.T. Baker, Radnor, U.S.A.) at 120 °C for three h (Ramírez-Rochín et al., 2019). Digested samples were adjusted to a final volume of 15 mL with purified Milli-Q water.

The accuracy and precision of the analytical method were determined by digesting blank samples and reference materials Dorm-4 (fish protein; NRC-CNRC, 2012) and Dolt-5 (Dogfish liver; NRC-CNRC, 2014) together with the shrimp samples following the same procedure (i.e., one of each in every batch of 24 shrimp samples). Concentrations of Hg in shrimp (i.e. Hg bioconcentration, which is defined as metal concentration accumulated in aquatic organisms considering water as the only exposure route; Newman and Unger, 2003; DeForest et al., 2007) and water samples were analyzed by cold vapor Atomic Absorption Spectrometry (AAS) (SpectraAA 220, Varian VGA-110) with a detection limit of 0.2 μg L^{-1} and a precision estimated as coefficient of variation of 2.3%. The average recovery rate of Hg from Dorm-4 (certified Hg value 0.412 ± 0.036 μg g^{-1}) and Dolt-5 (certified Hg value 0.440 ± 0.180 μg g^{-1}) were 106.4 ± 3.4 (n = 3 replicates) and 102.7 ± 4.6% (n = 3), respectively. The Hg concentrations were expressed as means ± standard deviation μg g^{-1} of dry weight (dw). Major seawater cations Na^+, K^+, Ca^{2+} and Mg^{2+} were analyzed using flame AAS (SpectraAA 220, Varian VGA-110), and major anions SO_4^{2-} and Cl^- were determined spectrophotometrically (Perkin Elmer UV/VIS Lambda 10) and by $AgNO_3$ titration (Metrohm system), respectively. Total alkalinity was analyzed by HCl titration (Metrohm system). These analytical techniques were developed following standard procedures outlined by APHA-AWWA-WPCF (1992).

2.4. Calculation of chemical speciation, lethal median concentrations, bioconcentration factors and safe mercury concentration

Inorganic Hg speciation was performed with Visual MINTEQ version 3.1 metal speciation program, considering the physicochemical composition of each salinity level and maximum and minimum Hg concentrations tested in toxicity (Blewett et al., 2015). Computer software (GraphPad Prism Version 7.04) fed with the Hg concentrations tested and mortality percentage recorded at each exposure time (24, 48, 72, and 96) was used to determine the LC_{50} and their 95% confidence intervals (Ramírez-Rochín et al., 2019). Bioconcentration factors (BCFs) were calculated considering the difference between Hg concentrations in shrimp exposed to Hg treatments and those in shrimp (as μg g^{-1} dw) exposed to the control treatments divided by the Hg concentrations in water (Hg treatment as mg L^{-1}) (McGeer et al., 2003; Leonard et al., 2011). The Hg safe concentrations for shrimp (i.e., the maximum Hg concentration that theoretically should not cause any adverse effect to shrimp physiological process (growth, respiration, reproduction and susceptibility to diseases) Mariño-Balsa et al., 2000; Newman and Unger, 2003) were estimated by multiplying the LC_{50}–96 h, corresponding to each salinity level and applying factor 0.01 (Mariño-Balsa et al., 2000; Frías-Espericueta et al., 2001; Boudet et al., 2013; Roos-Muñoz et al., 2019).

2.5. Statistical analyses

The *Z*-test of comparison of means was used to compare the LC_{50} values calculated for different salinities according to APHA-AWWA-WPCF (1992), Frías-Espericueta et al. (2001) and Ramírez-Rochín et al. (2019). The Hg bioconcentration in shrimp (Hg bioconcentration) and BCFs data were not normal (Kolmogorov-Smirnov test) and/or homoscedastic (Bartlett's test). Therefore, they were transformed to arcsine square root (Zar, 1984; Ramírez-Rochín et al., 2019); consequently, data were compared using two-way ANOVA and post hoc Tukey's test using STATISTICA software version 10.0 (2010) (Tulsa, OK, U.S.A). All statistical analyses were performed with a significance level of 0.05.

3. Results

3.1. Chemical composition, mercury concentrations and speciation in test solutions

During the acute Hg toxicity test developed at salinities of 5, 10 and 25 ppt, relatively stable average values of temperature (27.6 ± 0.6 °C), dissolved oxygen (5.4 ± 0.3 mg L^{-1}) and pH (7.6 ± 0.3) were determined (see Supplementary material). The major ions and total alkalinity analyses of testing waters with different salinities showed that their concentrations decreased proportionally according to salinity, as expected (see supplementary material). In regards to the analytical concentrations of waterborne Hg determined in the bioassay test solutions with different salinities, they showed a variation lower than 10% when compared with the nominal concentrations (see supplementary material).

The Hg chemical speciation calculated showed that the waterborne Hg concentration did not affect the distribution of chemical metal species for the same salinity, but salinity changes clearly affected the Hg species distribution for the same waterborne Hg concentration (Fig. 1). The Hg fraction as a free ion (Hg^{2+}) was extremely low (<0.3%) in all cases when compared with the total dissolved Hg concentration. The chemical Hg species with the highest metal proportions were $HgCl_2$ (43.3%), $HgCl_4^{2-}$ (36.4%) and $HgCl_4^{2-}$ (67.8%) shown at salinities of 5, 10 and 25 ppt, respectively (see Supplementary material).

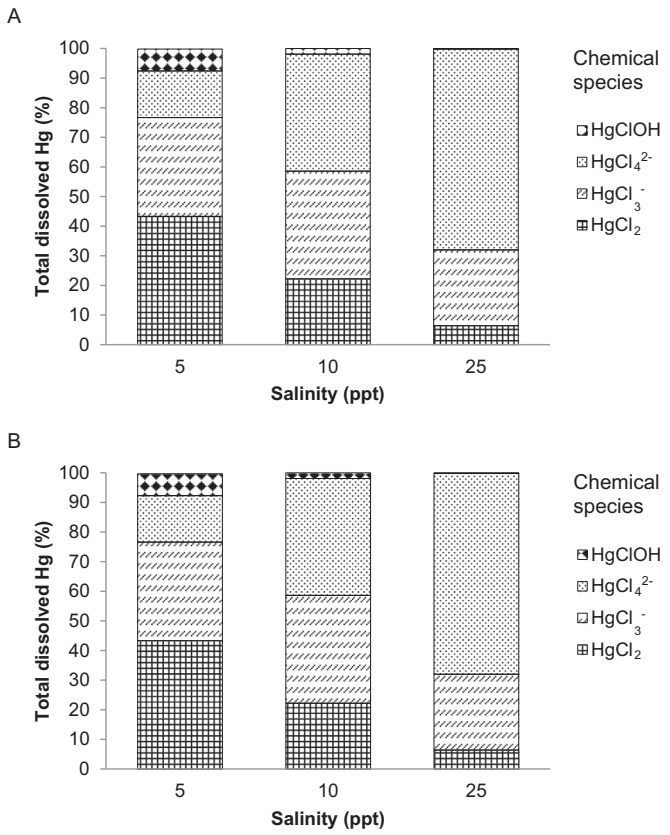

Fig. 1. Chemical species distribution of inorganic mercury (% of total dissolved mercury) in the test solutions with the extreme mercury levels used in the acute toxicity tests performed with *Litopenaeus vannamei* exposed to different salinities. The chemical species Hg^{2+} and $Hg(OH)_2$ showed distributions < 0.3% in relation to total dissolved mercury.

3.2. Mortality, LC_{50} values and safe mercury concentrations

The cumulative mortality of *L. vannamei* observed at different Hg treatments in toxicity tests with distinct salinity each 24 h after 96 h of exposure is shown in Fig. 2. No dead organisms were recorded in the control treatments during the experiments. In contrast, a clear positive dose-mortality relationship was observed in the bioassays, which was more acute at lower salinity (see supplementary material).

Mean LC_{50} values (and their 95% confidence intervals) of Hg were 1723 (1200−2300), 1272 (900–1700), 983 (650–1199) and 536 (300–800) μg L^{-1} for salinities of 5 ppt; 2203 (1808–2600), 1740 (1293–2300), 1340 (886–1953) and 873 (536–1360) μg L^{-1} for 10 ppt; and 7013 (6100–7700), 5693 (4721–6200), 1759 (1300−2200) and 1534 (1000−2000) μg L^{-1} for 25 ppt at 24, 48, 72, and 96 h of exposure, respectively (Fig. 3).

No significant differences ($p > 0.05$) were observed between LC_{50} values of Hg estimated for salinities of 5 and 10 ppt during the four days of exposure. Nevertheless, the LC_{50} values of Hg calculated for 25 ppt at 24 and 48 h of exposure were significantly higher ($p < 0.05$) than those determined for salinities of 5 and 10 ppt at the same exposure times although the LC_{50} values calculated for 25 ppt were only statistically higher than those estimated for 5 ppt for 72 and 96 h of exposure. The safe waterborne Hg concentration determined for *L. vannamei* juveniles exposed to salinities of 5, 10, and 25 ppt were 5.4, 8.7, and 15.3 μg L^{-1}, respectively.

3.3. Mercury bioconcentration in Litopenaeus vannamei juveniles

The Hg bioconcentration in *L. vannamei* increased at higher waterborne Hg concentrations in the three salinities examined (Fig. 4 and

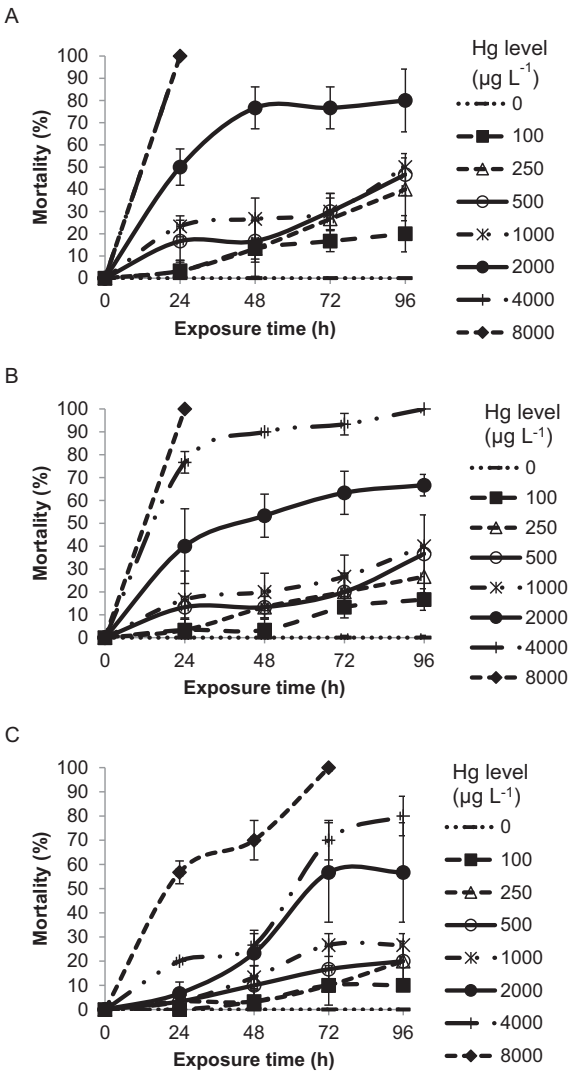

Fig. 2. Mean cumulative mortality (%) ± standard deviation (n = 3) of *Litopenaeus vannamei* juveniles exposed to different concentrations of waterborne mercury and exposure time in salinities of 5 (A), 10 (B), and 25 (C) ppt.

supplementary material). These increases were significant ($p < 0.05$) between the control group and rest of the treatments in all salinities and between treatments of 100, 250, 500, and 2000 μg L^{-1} of Hg in salinities of 5 and 10 ppt, as well as for 100 and 500 μg L^{-1} of Hg in 25 ppt.

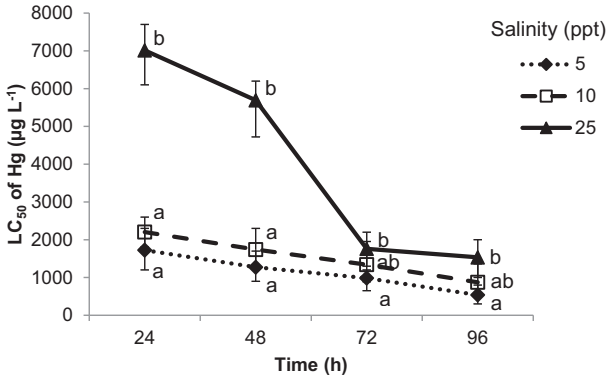

Fig. 3. Mercury LC_{50} and 95% confidence interval (error bars) (n = 3) means for *Litopenaeus vannamei* juveniles exposed to different salinity and time exposure. Different letters indicate significant differences between values calculated for same time exposure and different salinities ($p < 0.05$).

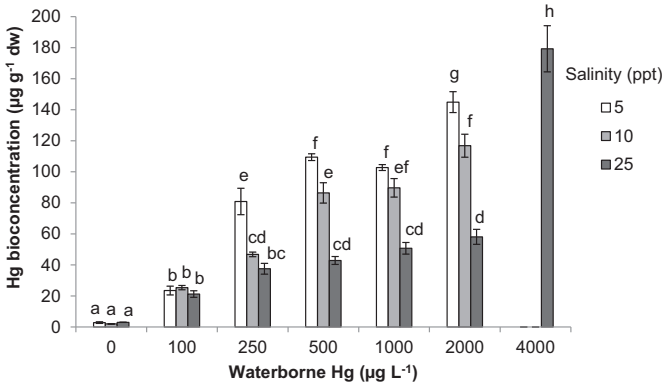

Fig. 4. Mercury bioconcentration ± standard deviations (n = 3) means in *Litopenaeus vannamei* juveniles after 96 h of exposure to different waterborne mercury concentrations and salinities. Different letters between treatments indicate significant differences ($p < 0.05$). Hg bioconcentration values corresponding to 4000 µg L^{-1} of waterborne Hg with 5 and 10 ppt of salinity are not shown because all shrimp died before 96 h of exposure.

Similarly, negative relationships between salinity and Hg bioconcentration in shrimp were observed in all the treatments. However, they were significant between salinities of 5 ppt when compared with 10 and 25 ppt in the treatment with 250 µg L^{-1} of Hg, among the three salinities for 500 and 2000 µg L^{-1} of Hg and between salinities of 5 and 10 ppt concerning 25 ppt in the treatment with 1000 µg L^{-1} of Hg. In the case of the 4000 µg L^{-1} of Hg treatment, only were surviving organisms in the group exposed to a salinity of 25 ppt, which was statistically ($p < 0.05$) the highest level of Hg bioconcentration. The statistical analysis (two-way ANOVA) demonstrated that a significant salinity and waterborne Hg concentration interaction occurred on Hg bioconcentration in *L. vannamei*.

3.4. Mercury bioconcentration factors in Litopenaeus vannamei juveniles

Mercury BCFs determined in *L. vannamei* exposed at different salinities and waterborne Hg concentrations after 96 h oscillated from 29.1 ± 1.2 to 312.3 ± 36.2, which were calculated for 2000 µg L^{-1} of Hg at salinity of 25 ppt and for 250 µg L^{-1} of Hg at 5 ppt, respectively (Fig. 5 and supplementary material). BCFs of Hg decreased when waterborne Hg concentrations increased in shrimp exposed to salinities of 10 and 25 ppt. A similar tendency was observed in organisms exposed to 5 ppt in all the cases except for BCF calculated for 250 µg L^{-1} of Hg

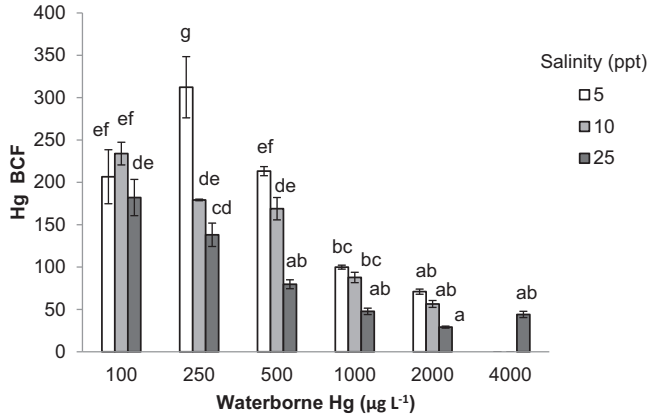

Fig. 5. Mercury bioconcentration factor ± standard deviation (n = 3) means in *Litopenaeus vannamei* juveniles after 96 h exposure to different waterborne mercury concentrations and salinities. Different letters between treatments indicate significant differences ($p < 0.05$). Hg bioconcentration values corresponding to 4000 µg L^{-1} of waterborne Hg with 5 and 10 ppt of salinity are not shown because all shrimp died before 96 h of exposure.

because it increased significantly ($p < 0.05$) compared with the BCF estimated for 100 µg L^{-1} of Hg. A significant decrease was observed between the BCF determined for salinities 250, 500, and 1000 µg L^{-1} of Hg at 5 ppt; 100 and 1000 µg L^{-1} of Hg at 10 ppt and between 100 and 500 µg L^{-1} of Hg at 25 ppt. Significant differences ($p < 0.05$) between salinities were found in BCFs determined in treatment with 250 µg L^{-1} of Hg where BCF corresponding to a salinity of 5 ppt was higher than those given for 10 and 25 ppt. Likewise, in the treatment with 500 µg L^{-1} of Hg, the BCFs calculated for salinities 5 and 10 ppt were higher than that determined for 25 ppt. A significant interaction of salinity and waterborne Hg concentration on BCFs of Hg was observed in *L. vannamei* (Fig. 5).

4. Discussion

4.1. Low salinity effect on acute mercury toxicity and safe concentrations in Litopenaeus vannamei juveniles

Although acute Hg toxicity has been studied in numerous crustacean species, such as *C. maenas*, *Crangon crangon*, *Homarus gamarus* (Connor, 1972), *P. setiferus* (Green et al., 1976), *Metapenaeus dobsoni* (Sivadasan et al., 1986), *Pagurus longicarpus* (Eisler and Hennekey, 1977), *Palaemon serratus*, *Maja squinado* (Mariño-Balsa et al., 2000), *L. vannamei* (Frías-Espericueta et al., 2001; Roos-Muñoz et al., 2019), *Farfantepenaeus brasiliensis* (Barbieri et al., 2005), and *Macrobrachium rosenbergii* (Kaoud et al., 2011), the relationship between low salinity and this metal has been poorly examined. Thus, it becomes relevant considering the high toxicity of Hg and the fact that euryhaline decapods are often found in environments with salinities lower than their isosmotic point.

To our knowledge, the effect of low salinity on the acute Hg toxicity in crustaceans has been evaluated only in three studies (Bianchini and Gilles, 1996; Das and Sahu, 2002; Verslycke et al., 2003). In the first one, the authors showed that acute Hg toxicity was significantly higher in *E. sinensis* (strong hyper-osmoregulator) and *C. maenas* (weak hyper-osmoregulator) adults exposed to 0 and 12 ppt of salinity, respectively, in relation to 35 ppt (Bianchini and Gilles, 1996). In the second study, acute Hg toxicity was observed in *P. monodon* and *P. indicus* juveniles with two sizes (35–55 and 55–75 mm in length for each species, respectively; weight not reported) at salinities of 5, 15, and 25 ppt. A significant effect of low salinity was not found on acute Hg toxicity in small size juveniles of *P. monodon* and *P. setiferus* while it was statistically higher in large size shrimp of both species exposed to the lowest salinity (5 ppt) compared to those exposed to an isosmotic environment (25 ppt) (Das and Sahu, 2002). The third study was developed in *N. integer* juveniles (weight and size not reported) exposed to salinities of 5 and 25 ppt, where low salinity did not significantly affect Hg toxicity (Verslycke et al., 2003).

In this study, an increase of acute Hg toxicity was observed in *L. vannamei* juveniles exposed to lower salinity (Fig. 3), which was consistent with the results reported for adults of *E. sinensis*, *C. maenas* (Bianchini and Gilles, 1996) and large size *P. monodon* and *P. setiferus* juveniles (Das and Sahu, 2002). The inverse relationship between low salinity and acute Hg toxicity has been directly related to the osmoregulatory capacity of each species, because environmental Hg uptake in organisms increases when osmotic stress caused by exposure to diluted medium is higher (Bianchini and Gilles, 1996; Das and Sahu, 2002). *E. sinensis*, *P. monodon*, *P. setiferus* and *L. vannamei* possess a strong hyper-osmoregulatory capacity that decreases as organisms are exposed to more hypo-osmotic environments (Charmantier et al., 1994; Péqueux, 1995). This result explains why the acute Hg toxicity in *L. vannamei* juveniles examined in this study was statistically higher at the lowest salinity evaluated (5 ppt) with respect to salinity (25 ppt, isosmotic point) in the control group. On the contrary in intermediate salinity (10 ppt), metal toxicity was not significantly different from that determined in the control group salinity – a tendency that was also observed in *E. sinensis* adults (Bianchini and Gilles, 1996) and

large size shrimp (55–75 mm in length) of *P. monodon* and *P. setiferus* (Das and Sahu, 2002) – although in distinct salinity levels.

On the other hand, the results of this study (Fig. 3) differed from those determined in *P. monodon* and *P. setiferus* small size juveniles (Das and Sahu, 2002), as well as *N. integer* juveniles (Verslycke et al., 2003), because the sensitivity of these organisms to Hg was not significantly affected by exposure to low salinity environments. These results indicated that *P. monodon* and *P. setiferus* -small size juveniles (35–55 mm in length)- demonstrated to have greater hyper-osmoregulation capacity at a salinity of 5 ppt than *L. vannamei* juveniles (71–96 mm in length) examined in this study. This result can be explained because the osmoregulation mechanisms of euryhaline penaeids are better adapted to low salinities when they are in early stages of their life cycle (Péqueux, 1995; Roy et al., 2010). In the case of *N. integer* juveniles, their high adaptability to low salinity was demonstrated, considering that acute Hg toxicity was similar at salinities of 5 and 25 ppt. This situation was not observed in *L. vannamei* juveniles, because this shrimp only inhabits in estuarine ecosystems during its larval and juvenile stages (Dugassa and Gaetan, 2018), while *N. integer* completes its entire life cycle in estuarine ecosystems (Verslycke et al., 2003).

The acute Hg toxicity in *L. vannamei* juveniles was previously investigated by Roos-Muñoz et al. (2019) but in a hyper-osmotic environment (34 ppt salinity) where 500 μg L^{-1} was reported as LC_{50}–96 h for shrimp with 8.6 g of mean weight. The *L. vannamei* juveniles (8.4 g of mean weight) tested in this study at salinities of 5, 10 and 25 ppt were 7.2, 74.6, and 206.8% less sensitive than those examined by Roos-Muñoz et al. (2019) at salinity of 34 ppt. These results suggested that Hg toxicity in *L. vannamei* was lower in hypo- and isosmotic environments than in hyper-osmotic. Nevertheless, future studies investigating Hg toxicity in *L. vannamei* at low and high salinities are needed to support this conclusion with stronger evidence.

The results of the acute toxicity tests can also be used to estimate the toxicity threshold levels in aquatic species to know their environmental risks and estimate safe concentrations (Newman and Unger, 2003; Barbieri et al., 2013). An appropriate criterion for estimating safe metal concentrations is to multiply the LC_{50}-96 h and a factor of 0.01 (Mariño-Balsa et al., 2000; Frías-Espericueta et al., 2001; Boudet et al., 2013; Roos-Muñoz et al., 2019). However, it is important to note that this "safe concentration" should be tested during chronic exposure to ensure that it does not cause adverse effects (e.g., decrease in growth rate, respiration, reproduction and increase in susceptibility to diseases) in a particular species (Mariño-Balsa et al., 2000). Based on the above, the provisional safe waterborne Hg concentrations for *L. vannamei* juveniles exposed to different salinities proposed in this study (i.e., 5.3, 8.7 and 15.3 μg L^{-1} for 5, 10 and 25 ppt, respectively) were supported. The CCC of Hg for the marine aquatic life established by the EPA is 0.9 μg L^{-1} (EPA, 2020). The maximum permissible Hg limit established by the Mexican Official Standard for coastal waters dedicated to fishing exploitation is 10.0 μg L^{-1} (DOF, 1997). Therefore, the maximum permissible limit followed by EPA (2020) is safe for *L. vannamei* juveniles; however, the Mexican Official Standard is safe for *L. vannamei* juveniles exposed a salinity of 25 ppt but not for shrimp exposed to 5 and 10 ppt.

Hg concentrations as high as 98, 138, and 1384 μg L^{-1} associated with shallow hydrothermal activity (5 m deep) have been determined in waters of Gulf of California (Concepción Bay, Baja California Sur) (Prol-Ledesma et al., 2004), which exceed the safety Hg levels proposed in this study. Similarly, in the Upper Gulf of California and Colorado River Delta where low salinity levels in rainy season have been recorded, moderate or high Hg concentrations have also been specifically determined in the Ciénega de Santa Clara wetlands (mean concentration 4.0 μg L^{-1}), Hardy River north (10.2 μg L^{-1}), and Hardy River south (4.3 μg L^{-1}) (Páez-Osuna et al., 2017) that are comparable to the safe Hg values proposed. These results are relevant considering that such region of the Gulf of California is characterized by housing wild and cultivated populations of *L. vannamei*. Thus, it can be specified that environments in Mexico, especially those with low salinity, may be risky for the development of wild and cultivated populations of this shrimp species.

4.2. Low salinity effect on mercury bioconcentration in Litopenaeus vannamei juveniles

Bioconcentration capacity of metals in crustaceans has also been shown to increase in low salinities that was observed in the case of Zn in *Palaemon elegans* (Nugegoda and Rainbow, 1989), Cd in *Palaemon longirostris* (Pierron et al., 2007) and Ni in *C. maenas* (Blewett et al., 2015). In the case of Hg, this phenomenon was reported in *E. sinensis* adults exposed to 1000 μg L^{-1} in salinities of 18 ppt and 35 ppt; in *C. maenas* adults exposed to 1000 μg L^{-1} in 12 ppt and 35 ppt (Bianchini and Gilles, 1996); and in *C. maenas* adult exposed to 1 μg L^{-1} in 8 and 33 ppt (Laporte et al., 1997). A similar tendency was observed in this study. However, in *L. vannamei* juveniles exposed to 250, 500, 1000, and 2000 μg L^{-1} of Hg in salinities of 5 and 25 ppt although in shrimp exposed to 100 μg L^{-1}, a significant effect of low salinity on Hg bioconcentration was not observed (Fig. 4).

The increase in metal bioconcentration capacity caused by low salinity in euryhaline crustaceans has been related to higher passive water uptake produced by the difference in osmotic pressure of the organisms' internal fluids (hyper-osmotic) and exterior environment (hypo-osmotic) (i.e., osmotic stress). This situation promotes dissolved metals to enter into the cells through diffusion (Bianchini and Gilles, 1996; Grosell et al., 2007; Blewett et al., 2015). Furthermore, the exposure to hypo-osmotic medium contributes to the fact that the main environmental cations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+} - which are essential - can be replaced in the cellular ligands (i.e., channels, carriers and pumps) by analogous toxic cations (e.g., Cd^{2+}, Pb^{2+} and Hg^{2+}) dissolved in the environment. This situation causes disruption in osmoregulation mechanisms and increases osmotic stress (Péqueux, 1995; Paquin et al., 2002; Veltman et al., 2008).

Regarding Hg, higher environmental metal concentrations have been observed to cause more severe disruptions in osmoregulation mechanisms in euryhaline crustaceans (Bjerregaard and Vislie, 1985; Barradas and Péqueux, 1996), with the effect being more acute at lower salinity (Péqueux et al., 1996; Laporte et al., 1997). This result explains why in this study (Fig. 4), a significant effect of low salinity on Hg bioconcentration capacity was not observed in organisms exposed to the lowest waterborne Hg concentration (100 μg L^{-1}), and a significant effect of low salinity on this variable was observed in higher waterborne Hg concentrations (250, 500, 1000 and 2000 μg L^{-1}).

Low salinity is known to affect the chemical metal speciation, such as Hg (Laporte et al., 1997), Pb, Zn (Verslycke et al., 2003), Ni (Leonard et al., 2011), Cu (Martins et al., 2011) and Cd (Bielmyer-Fraser et al., 2018), which is relevant in toxicology, because within its inorganic chemical species, free-ionized forms (Hg^{2+}, Pb^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+}) are the most bioavailable and toxic to aquatic organisms (Newman and Unger, 2003). The metal fraction in their free-ionized form is lower at higher salinities due to the formation of seawater metal-major anion (i.e., Cl^-, SO_4^{2-}, F^-, HCO_3^-, CO_3^{2-}) complexes; thus, metal bioavailability and toxicity are lower at higher salinity (Paquin et al., 2002; Bielmyer-Fraser et al., 2018). In this study, negative relationships were observed between salinity and the Hg^{2+} fraction determined in the test solutions; however, the Hg^{2+} fractions were very low (<0.3%) in the three salinities evaluated (Fig. 1). For this reason, the lethal effects recorded in the toxicity tests were unlikely to have been caused by Hg^{2+}, which coincided with that concluded by Laporte et al. (1997) in a study developed in salinities comparable (8 and 23 ppt with *Callinectes sapidus*) to those examined in this study (5, 10, and 25 ppt).

The bioavailability of different chemical species of inorganic Hg in crustaceans has been studied by various authors (Péqueux et al., 1996; Laporte et al., 1997; Andres et al., 2002; Laporte et al., 2002); however,

the results in this subject have been inconsistent. For example, Péqueux et al. (1996) found evidence suggesting that $HgCl_3^-$ and $HgCl_4^{2-}$ can be captured by the Cl^- channels and branchial epithelium transporters in *E. sinensis*, assuming an active environmental Hg uptake. In contrast, Laporte et al. (1997) showed that Hg bioaccumulation in *C. maenas* gills was positively correlated with $HgCl_2$ and not with $HgCl_4^{2-}$, suggesting that Hg bioavailability was related to a neutral chemical species, assuming a passive Hg uptake. Subsequently, Andres et al. (2002) and Laporte et al. (2002) concluded that the environmental Hg was captured by *C. sapidus* gills and intestines through active and passive mechanisms that involved the channels designed for amino acid transport. The results of this study suggested that neutral chemical Hg species ($HgCl_2$ and HgClOH) were the most bioavailable because their fractions increased when salinity decreased (Fig. 1), which contributed to explain the negative relationship between acute Hg toxicity and bioconcentration capacity concerning salinity. In addition, they were consistent with that concluded by Laporte et al. (1997), Andres et al. (2002) and Laporte et al. (2002) since toxic effects determined in *L. vannamei* juveniles examined in this study were mainly caused by $HgCl_2$ because it was the most abundant neutral chemical species in the three salinity levels evaluated.

4.3. Low salinity effect on mercury bioconcentration factors in Litopenaeus vannamei juveniles

In aquatic organisms, the BCF indicates the resulting ratio of the concentration of the metal accumulated in the organisms and the concentration of the dissolved metal in water. In other words, it indicates the number of times that the metal accumulates in an organism in proportion to the dissolved metal concentration in water (Newman and Unger, 2003). This information has been frequently used to generally determine the ability of a species to regulate and store the metal accumulated based on the concentrations of the dissolved metal in water and other environmental variables (e.g., different metal concentrations, salinity, pH and exposure periods) (McGeer et al., 2003; DeForest et al., 2007; Williams et al., 2006; Leonard et al., 2011). For example, in a study carried out by Leonard et al. (2011) in *L. vannamei* juveniles exposed 96 h to different Zn concentrations at salinities of 5, 10 and 25 ppt, they observed that the metal uptake rate (BCF) in shrimp decreased when the waterborne Zn concentrations increased for both salinities. These result suggested that the shrimp showed a higher rate of Zn uptake, regulation and storage at lower environmental Zn concentrations, because it was required to satisfy the metabolic needs of the organisms. However, at higher Zn concentrations, its storage and regulatory mechanisms became saturated, inhibiting the rate of metal uptake in body.

A similar trend to that observed by Leonard et al. (2011) in relation to Zn was determined in this study with respect to Hg in the same shrimp species but at salinities of 5, 10 and 25 ppt, because the Hg accumulation rate (BCF) in *L. vannamei* juveniles was higher at lower waterborne Hg concentrations (Fig. 5). These results suggest that even though Hg is not an essential metal for shrimp metabolism (Delgado-Alvarez et al., 2015), the toxicodynamic mechanisms of Hg were more efficient when the environmental metal concentrations were lower. On the other hand, Andres et al. (2002) concluded that the high Hg affinity to bind with thiol groups found in cell membranes of *C. sapidus* caused them to saturate, decreasing the rate of environmental Hg uptake (BCF) at higher concentrations of waterborne Hg. This conclusion explains the decrease in the Hg accumulation rate observed in this study in *L. vannamei* juveniles exposed to higher waterborne Hg concentrations at salinities of 5, 10 and 25 ppt (Fig. 5), which also coincides with that concluded by Leonard et al. (2011) in the case of Zn. This inverse relationship between BCFs and the environmental metal concentrations has been widely demonstrated in various aquatic species (McGeer et al., 2003; Williams et al., 2006; DeForest et al., 2007) and shown to be characteristic of all metals.

5. Conclusions

This study demonstrated that the acute Hg toxicity and bioconcentration capacity in the whole-body of *L. vannamei* juveniles exposed to low salinity environment (5 ppt) was significantly higher than in those exposed to an isosmotic environment (25 ppt). This result may be explained by hypo-osmotic stress, as well as higher fractions of Hg neutral chemical species ($Hg(OH)_2$ and $HgCl_2$) found at lower salinities. Therefore, the hypothesis that establishes an increase in acute Hg toxicity and bioconcentration capacity in *L. vannamei* juveniles caused by exposure to low salinity environments is accepted. The analyses of Hg bioconcentration factors demonstrated that Hg accumulation in whole-body of shrimp did not increase in proportion to waterborne Hg concentrations because the metal uptake and storage mechanisms in organisms were more saturated at higher Hg environmental concentrations in the three salinities tested (5, 10 and 25 ppt). Provisional Hg safe concentrations for *L. vannamei* juveniles are provided in this study (i.e., 5.4, 8.7 and 15.3 $\mu g\ L^{-1}$ for salinities of 5, 10 and 25 ppt). This information allows knowing threshold toxicity values of this metal and recognizing potentially risky environments for wild and reared shrimp populations exposed to brackish and low salinity waters, which have ecological and economic implications. Finally, future research should focus on demonstrating shrimp ability to accumulate inorganic and organic Hg (MeHg) using the environment (water and diet) as exposure route at different salinities. This information contributes to a better understanding of the accumulation mechanisms of this metal in shrimp, as well as the toxicological impact of Hg and MeHg.

CRediT authorship contribution statement

Javier Ramírez-Rochín: Formal Analysis, Methodology, Writing – Original Draft Preparation, Investigation, Conceptualization, Visualization

Ángel I. Campa-Córdova: Conceptualization, Writing-Review & Editing

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Marcela G. Fregoso-López: Investigation, Methodology, Writing & Editing

Irasema E. Luis-Villaseñor: Methodology, Resources, Writing & Editing

Federico Páez-Osuna: Conceptualization, Visualization, Writing – Review & Editing, Resources, Funding Acquisition, Supervision, Project Administration

Declaration of competing interest

The authors Javier Ramírez-Rochín, Ángel I. Campa-Córdova, Martín G. Frías-Espericueta, Marcela G. Fregoso-López, Irasema E. Luis-Villaseñor and Federico Páez-Osuna of the manuscript titled "Acute mercury toxicity and bioconcentration capacity in shrimp *Litopenaeus vannamei* juveniles: effect of low salinity and chemical speciation" submitted to Science of the Total Environment declare the have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Melissa Macias, Milton Chávez and Humberto Bojórquez Leyva for their important collaboration during the experimental work and chemical analysis; Germán Ramírez Reséndiz and Carlos Suárez Gutiérrez for the statistical support provided; Diana Fischer for editorial English services. This work was supported by the Dirección General de Asuntos del Personal Académico, Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica and the Universidad Nacional Autónoma de México (DGAPA-PAPIIT-UNAM)

Project IN200619 titled “Metales y metaloides en ríos y presas de cuencas mineras de Sinaloa: identificando contaminación y riesgos para la salud”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144025.

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