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Research Article

Evaluation of potential confounding factors in sediment toxicity tests with *Hyalella azteca* (Saussure, 1858)

Anelise Destefani[1,2], Juliana Cristina Friedrichsen[1] & Charrid Resgalla Jr.[1]
[1]Universidade do Vale do Itajaí, Centro Itajaí, SC, Brazil
[2]Instituto Federal Catarinense, Araquari, SC, Brazil
Corresponding author: Anelise Destefani (anelise.destefani@ifc.edu.br)

ABSTRACT. Ecotoxicological tests performed in waters with low salinities, typical of estuarine environments, are limited regarding the availability of test organisms. The amphipod *Hyalella azteca,* an organism protocoled for trials with freshwater sediments, presents a potential for use in saltwater tests in which the confounding factors, such as salinity and sediment grain size, should be evaluated to avoid errors in the results in relation to the sensitivity of the organism. Thus, amphipod cultivations in salinities of 0, 5, 10 and 20 were conducted, to perform tests of tolerance to salinity, and sensitivity to a reference toxicant. The tolerance of the species to different sediment grain sizes and organic matter contents was evaluated in tests with reconstituted sediments in five different compositions, varying the content of organic matter (5 to 20%), clay and sand (0 to 95%). The results of the cultivations showed that animals maintained present the best reproduction and survival rates at salinity 5, and a tolerance limit under chronic conditions, at salinity above 10. The salinity can be considered a confounding factor since the tests carried out with the reference toxicant showed the higher resistance of the species under conditions of higher salinities. The species present tolerance to different sediment grain sizes and organic matter contents; therefore, this factor does not interfere in the survival of *H. azteca* during the ecotoxicological tests.

Keywords: *Hyalella azteca***,** salinity, sediment, grain size, organic matter content, acclimation.

INTRODUCTION

Toxicity tests in estuarine sediments have been used since the 1980s, especially in locations with waste effluents or dredging activities (USEPA, 2000, Environment Canada, 2013;). In these assays, different species are proposed as test organisms, with advantages and disadvantages as well as various applications such as availability, sensitivity, and tolerance to environmental conditions. In the particular case of Brazil, the amphipods *Leptocheirus plumulosus*, *Tiburonella viscana* (Melo & Abessa, 2002; Bertoletti, 2011), *Grandidierella bonnieroides* (Molisani *et al*., 2012) and the Tanaid *Monokaliapseudes shubartii* (Resgalla Jr. & Laitano, 2002; Zamboni & Costa, 2002; Prósperi & Nascimento, 2008) have been used for this type of study, but each of them has its particular tolerance limit under test conditions. For example, *M. schubartii* shows high tolerance to contaminants (Mottola *et al*., 2009), while *T. viscana* presents sensitivity in sediments dominated by clay or sand (Bertoletti, 2011).

The amphipod *Hyalella azteca* can be used as an alternative freshwater organism to expand the number of species for ecotoxicological evaluation in estuarine environments and conditions of low salinity. Besides its use for evaluating dredged material in inland and coastal waters (salinity of up to 15), as recommended by the USEPA (2000), there have been no studies on the influence of salinity on species sensitivity in brackish water trials; the only studies conducted to date have focused on tolerance to survival and reproduction at low salinities culturing (Nebeker & Miller, 1988).

A second limitation of tests with sediments is the responses that the test organisms can present concerning different sediment grain sizes and organic matter contents. Sediment particle size is a parameter that often defines whether a substrate is a suitable habitat for determined species (Simpson *et al*., 2005), and it can be considered an additional stress factor and

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a confounding factor in ecotoxicological studies. According to Spies (1989), if the physical and chemical characteristics of the test sediment are less suitable for the test organism than the control sediment, this can lead to a false-negative result. According to USEPA (2000) *H. azteca* presents a broad tolerance to grain size variation of the sediment; however, there had no specific simulated tests on this interference factor (Ankley *et al*., 1994) or simulations of the characteristics of the sediments in Babitonga Bay, which are characterized by high organic matter content according to Destefani (2017).

On the other hand, due to the variability in sediment grain size and the high levels of organic matter in estuarine environments, it is difficult to find places free of contaminants, since there is a direct relationship between the percentage of fines and organic matter of the sediments, and the presence of organic and inorganic contaminants (Eggleton & Thomas, 2004). As a result, obtaining reference sediments (of the same texture but without contaminants) for use in environmental impact studies may be limited to the use of reconstituted sediments (González, 2012). These sediments are prepared using sand, clay and non-toxic organic constituents obtained from commercial sources, with natural or reconstituted water (OECD, 2004), but tests need to be conducted for their application in ecotoxicological assays.

This study aims to determine if salinity influences on the amphipod *H. azteca* sensitivity to contaminants, and to confirm if these organisms are tolerant to reconstituted sediments with high organic matter contents. This investigation intends to contribute to the application of whole sediment toxicity test with the amphipod *H. azteca*.

MATERIALS AND METHODS

Cultivation of the amphipod *Hyalella azteca* and acclimation tests

The cultivations of *H. azteca* were performed at the Ecotoxicology Laboratory of Univali. The organisms were cultivated in 2.5 L plastic boxes with lids, in a static system with mild aeration and 100 initial organisms, as recommended by the ABNT (2013). Freshwater was obtained from a natural source and filtered through a 45 µm filter, presenting hardness of between 36 to 45 mg L^{-1} $CaCO_3$ and pH of 7.6 ± 0.2. The photoperiod was 16L:8D and the temperature was kept at 23 ± 1ºC. Compound RL (a fish food mixed with yeast and primrose oil), prepared according to Araújo (2005), was offered daily at a ratio of 2.5 mL to each batch of 100 organisms. The substrates used in the cultivations consisted of banana leaves (Family Musaceae) previously autoclaved and left in immersion and aeration in water for seven days to remove the lignin. This substrate was provided in sufficient quantities to cover the bottom of the plastic boxes. The cultivation was maintained every 7 days, observing the general condition of the organisms, the number of pairs, and the presence of juveniles and intruders. The juveniles used in the tests were obtained by separating them from the adults by filtering through a 145 µm filter.

For the acclimation tests, four cultivations were performed on a smaller scale (1.5 L and 50 organisms) and executed in salinities of 0 (control), 5, 10 and 20, and under the same conditions as described previously, and maintained for 10 weeks. Freshwater mixed with natural seawater provided the different cultivation salinities and confirmed with a refractometer. For acclimatization of the organisms, the salinity was increased by two units for each week of cultivation, until the desired final concentration reached. The maintenance of 50 adults per treatment was performed by the cultivation of production itself and started in the seventh week. Mortality rates of the adults, the birth rate or the number of juveniles produced by the adults, and the number of new pairs present in the cultivation were determined weekly, by counting, and transferred to new culture using a wide mouth dropper.

Tests with the reference substance

Tests were performed with the reference substance, to evaluate the sensitivity of the organisms cultivated, and to determine the influence of salinity in the tests. For this purpose, zinc sulfate ($ZnSO_4$ $7H_2O$) was used as the reference toxicant, in a 96 h test without sediment. The tests were performed in 200 mL beakers of the test solution, at six different concentrations (from 0.15 to 10 mg L^{-1}) of zinc sulfate and four replicates of each concentration, plus controls. For each replicate, 10 juveniles of *H. azteca* aged between 7 and 14 days were transferred, and the number of dead organisms was recorded every 24 h. The tests were considered valid when the mortality in the control flasks was lower than 20%. Based on the mortality rates, the values of LC_{50} (Median Lethal Concentration), together with their respective 95% confidence intervals, were calculated by the Trimmed Spearman-Karber Method (USEPA, 2002).

Ten tests were performed with the reference toxicant according to the ABNT (2013), under static conditions, with organisms at 7 to 14 days of age, temperature from 22 to 26°C and a photoperiod of 12 h of light.

Salinity tolerance tests

For the salinity tests, the juveniles from the cultivations at salinities of 0 and 5 were used, as these cultivations presented higher birth rates, and consequently, more test organisms for use in the tests.

An initial evaluation was carried out to determine the tolerance of the organisms to variations in salinity (based on the mortality rate). In this case, juveniles obtained in the cultivations in salinities of 0 and 5, and exposed to different salinities (from 0 to 25) in acute conditions, were used. The experiments were the same as those described for the tests with the reference substance.

In a second assessment, the sensitivity of organisms cultivated in salinities of 0 and 5 to the reference toxicant zinc sulfate was determined, for which solutions were prepared in salinities of 0.5 and 10. The test conditions were the same as those described for the tests with the reference substance.

Tests with reconstituted sediment

Two tests were performed to evaluate the reconstituted sediments and the influence of particle size of the sediment, simulating the characteristics of the sediments in Brazilian estuaries, on the survival of *H. azteca*. Five different grain sizes and organic matter content compositions were used, with organic matter content ranging from 5 to 20%, and clay and sand content of between 0% and 95%, including the composition recommended by the OECD (2004) (Table 1).

For this evaluation, the sediments were created from the contaminant-free material, with organic matter consisting of earthworm humus, sieved through a 1 mm filter, washed sand (obtained from the continental platform of the south Brazil at a depth of 70 m) and clay composed of caulite (commercial type used in porcelain production). For the reconstitution of the sediments, 11.5 times the dry weight of the organic matter was added to deionized water, to obtain a moisture content of 30% (Arrate *et al.*, 2004). The organic matter was gently stirred (in a mechanical stirrer) for 48 h, according to the recommendation of the OECD (2004). After this period, the pH was measured (between 6.5 and 7.5), and the mixture containing all the constituents of the sediment was stored in a dark place for 7 days, to mature.

The sediment toxicity tests were conducted according to the ABNT (2013) protocol; 100 g of reconstituted sediment was distributed in 600 mL beakers, and a further 200 mL of cultivation water (salinity 0) was carefully added, with four replicates of each prepared formulation. Before the start of the tests, the pH and dissolved oxygen of the overlying water were measured, and 10 juveniles of *H. azteca* (cultivated in salinity 0) were transferred to each test beaker. Every two days, two-thirds of the test water was renewed, and the food was added to the test bottles (2 mL of compound RL). The test was maintained at a temperature of 23 ± 1ºC, with a photoperiod of 16L:8D and diffuse lighting. After 10 days, the test was terminated, and the numbers of living (retained amphipods) and dead (disappeared) organisms were counted by sieving the sediments.

Table 1. Percentage of reconstituted sediment components used in the *H. azteca* assays.

Components	S1	OECD	S2	S3	S4	S5
Sand	95	75-76	60	30	10	0
Clay	5	20	35	60	75	80
Organic matter	0	4-5	5	10	15	20

Data treatment

The data were analyzed as percentages of mortality and survival for the acclimation tests, salinity tolerance and grain size and organic matter content of the sediments. Comparisons between treatments and control were performed by analysis of variance (ANOVA) with *a posteriori* Dunnett's test. Lethal concentration (LC_{50}) values for the zinc sulfate assays were estimated using the TSK Program (Montana State University).

RESULTS

The results of the acclimatization tests showed mortality rates of less than 20% for the control (salinity 0) and the salinities of 5 and 10 during 10 weeks of cultivation. The amphipods did not survive to the transition process from salinity 0 to 20, as they all died when the salinity reached 16, in the 8^{th} week of cultivation (Fig. 1).

The reproduction rates were higher at salinities 5 and 10 (averages of 5.9 and 3.6 juveniles/adults respectively) in reference to the control (salinity 0) with an average of 1.4 juveniles/adults (Fig. 1). Differences were observed between the reproductive periods in different cultivations, with peaks being observed later in the culture for salinities 5 and 10. For the cultivation at salinity 20, a decrease in the reproduction rate was observed after the 6^{th} week of acclimation, when the salinity was above 10.

Concerning tolerance, the organisms cultivated in salinities 0 and 5 presented low mortality rates, up to salinity 10 (Fig. 2). The organisms cultivated in salinity 5 showed higher resistance to the salinities of 15 and 20, but still showed mortality rates above 20% in these conditions (ANOVA, F = 53,972 salinity 0 and F = 35,004 salinity 5).

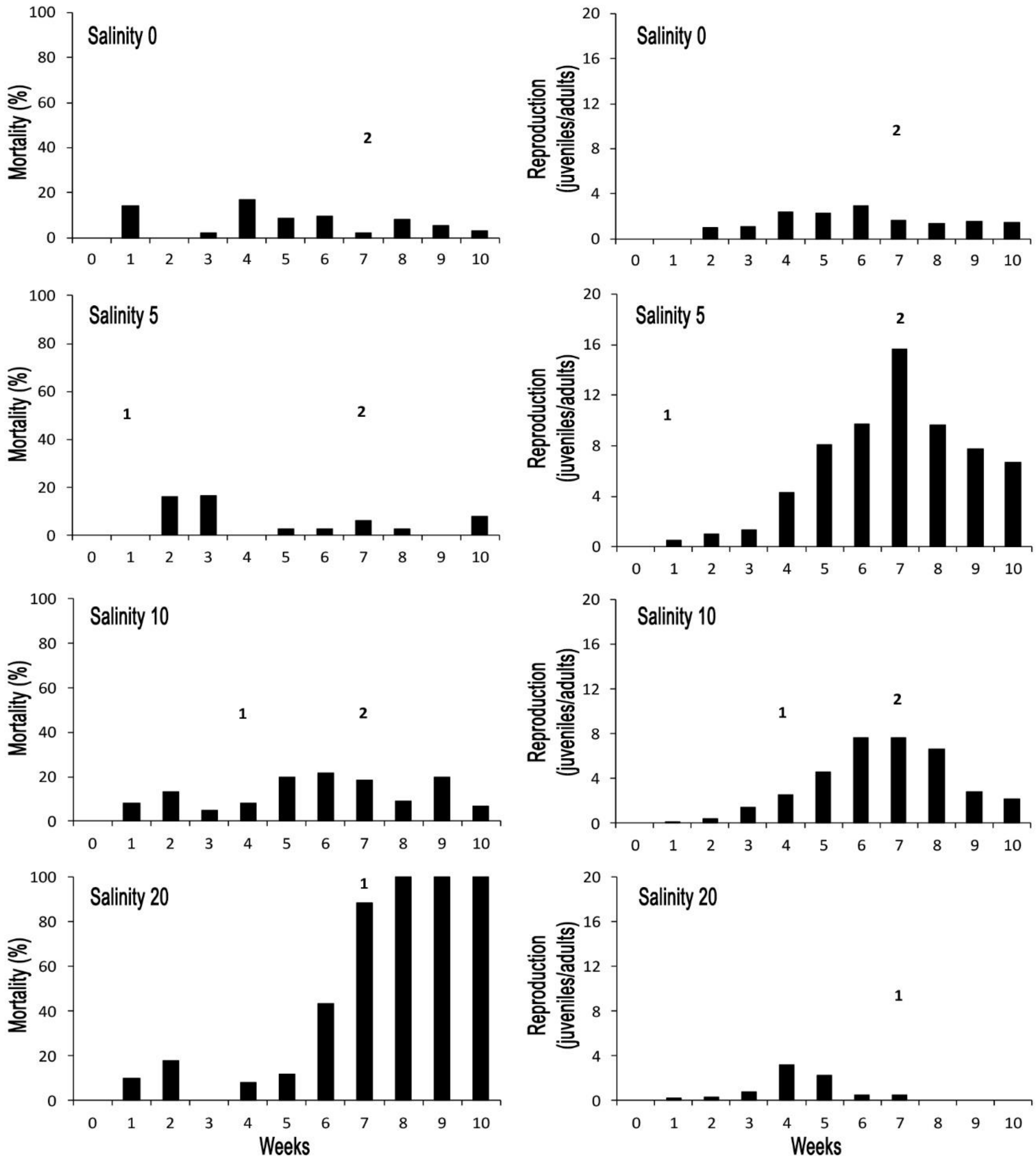

Figure 1. Mortality rates (%) and production of juveniles per adult per week, and at different cultivation salinities. 1) Corresponds to the week in which the desired salinity was reached, except for the cultivation at salinity 20, in which salinity reached 16 in week seven, 2) corresponds to the week in which the adults were replaced in the cultivations.

Concerning the sensitivity of *H. azteca* to the reference substance ($ZnSO_4.7H_2O$), it was observed that for the tests in fresh water, the average LC_{50} was 0.682 mg L^{-1} with a coefficient of variation (CV) of 25.36% (N of 13 tests) (Table 2). This LC_{50} value was similar to those observed in the tests performed in fresh water with acclimated organisms in salinity of 0 and 5 (Fig. 3).

These results suggest that the amphipod presents an increase in resistance to the toxicant as the salinity of the dilution water increases (Fig. 3). This trend was observed for organisms cultivated at salinity 0 but was more pronounced for organisms cultivated at salinity 5.

For the tests at different sediment grain size and organic matter content, the results demonstrated that variations in levels of organic matter, sand and clay sho-

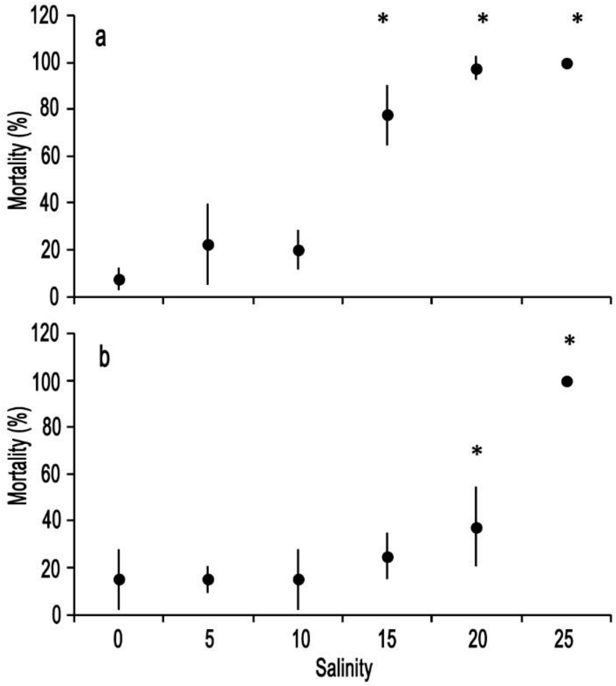

Figure 2. Mean (point) and standard deviation (line) of mortality (%) of *H. azteca* in acute tests in different salinities under two acclimation conditions: a) in fresh-water and b) in water at salinity 5. *Refers to treatment significantly different from control (salinity 0).

wed no influence on the survival rates of the amphipod. All the formulations showed average survival rates of over 80% (Fig. 4), and there was no clear trend regarding grain size preference.

DISCUSSION

The cultivations conducted at different salinities were evaluated relative to survival of organisms and within the mortality acceptability rate of 20%, according to the ABNT of the recommendations (2013). In this aspect only, the cultivation at salinity 20 presented mortality rates that made cultivation impossible, confirming that the species has a salinity survival limit of 15, as suggested by Ingersoll *et al*. (1992), McGee *et al*. (1993) and USEPA (2000). The cultivation at salinity 5 presented higher survival rates compared to the control, which may suggest that this species adapts very well to salinity 5.

This tolerance was confirmed by the production of juveniles, which were initially stimulated at salinity 5 and decreasing at salinities 10 and above. This result is in agreement with Nebeker & Miller (1988) who observed reproductive inhibition from salinity 10.4.

Although the results suggest that salinity does not promote any delay in the start of reproduction, compared

Table 2. Values of LC_{50} (mg L^{-1}) and lower (LL) and upper limits (UL) of the 95% confidence interval for zinc sulfate in *Hyalella azteca* assays in freshwater. SD: standard deviation.

Assay	LC_{50}	LL	UL
1	0.95	0.78	1.16
2	0.69	0.53	0.90
3	0.52	0.45	0.59
4	0.67	0.56	0.80
5	0.95	0.78	1.16
6	0.92	1.04	1.57
7	0.70	0.51	0.96
8	0.60	0.45	0.80
9	0.78	0.67	0.90
10	0.56	0.41	0.77
11	0.47	0.32	0.69
12	0.58	0.46	0.73
13	0.47	0.33	0.67
Average = 0.682		SD = 0.173	

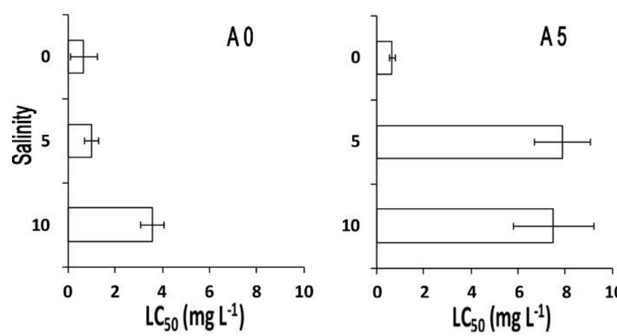

Figure 3. Values of LC_{50} (bar) and confidence interval of 95% (line) for zinc sulfate in tests at different salinities and using organisms acclimatized to salinities of 0 (A0) and 5 (A5).

with cultivation in freshwater, the reproductive peaks in weeks 6 and 7 of cultivation were more premature than those presented by Araújo (2005), who observed reproductive peaks between weeks 5 and 6, and the highest rate from week 8. These differences can be attributed to cultivation conditions such as temperature and water hardness than salinity itself.

In the acclimatization test at different salinities, in physiology, it is known that organisms acclimatized under abnormal conditions respond by changing their tolerance limits to the physicochemical parameters in which they are maintained (Schmidt-Nielsen, 1997). Borgmann (2002) emphasizes the need for *H. azteca* lots used in brackish water tests to be evaluated using an additional salinity control, resulting in more realistic information on environmental conditions.

In terms of sensitivity, the values of CL_{50} obtained for zinc sulfate demonstrate the higher sensitivity of *H.*

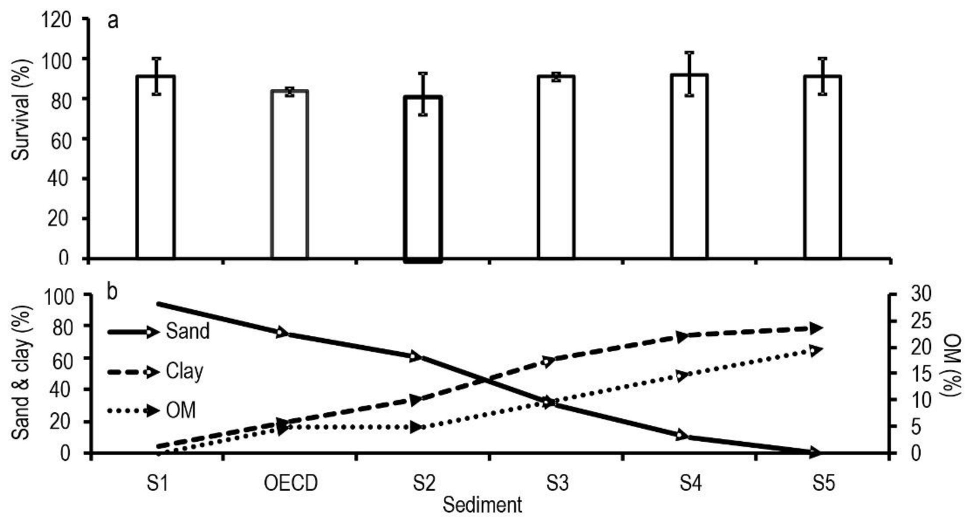

Figure 4. a) Average values (bar) and standard deviation (line) for survival rates of *H. azteca* in tests with reconstituted sediment with different grain size and organic matter contents, b) percentage of silt, clay and organic matter content of the tested sediment.

azteca compared to the marine amphipod *Tiburonella viscana* and the tanid *Monokaliapseudes Schubartii* (Mottola *et al*., 2009). However, *H. azteca* shows a loss of sensitivity when the test is performed above salinity 5, or when organisms acclimated to salt water are used. This loss of sensitivity appears to be associated with both the physiology of the organism and the bioavailability of metals in water with higher salinity (McLuski & Hagerman, 1987), as the sensitivity of *H. azteca* was also altered by its acclimation to salinity 5. Despite the behavior of metals, combined with physiology, it is still not a clear process and presents variability depending on the metals involved (Dutton & Fisher, 2011). The effect of salinity on the presence of zinc can also result in a decrease in its toxicity (Rainbow & Black, 2002; Loro *et al*., 2012; Yung *et al*., 2015).

According to Grosell *et al*. (2007) aquatic organisms, including crustaceans, are more sensitive to metal stress when hyperosmoregulating than when they are closer to their isosmotic point. This fact may be associated with osmoregulation physiology and Na^+ gradients between blood and water in the medium. In a more recent study, Loro *et al*. (2014) point out that Zn exposure causes pathological changes in both Ca^{2+} and Na^+ homeostasis, and that increasing salinity exerts protective effects (with Ca^{2+} competing with Zn for binding sites in the gills) against both sublethal and lethal Zn toxicities, as well as acting in the regulation of Na^+. These ions may compete directly with the mechanism of zinc absorption by the organism, reducing the absorption rate of this metal (Jones, 1975; Bambang *et al*., 1995; Pinho *et al*., 2007).

Likewise, zinc in high salinities forms a complexation by chlorides, decreasing the presence of free metal ions, particularly in the form of Zn^{2+}, which is the most bioavailable and reactive form (Loro *et al.*, 2012; Park *et al*., 2014), leading to lower toxicity of the metal (Paquin *et al*., 2000).

The high tolerance of *H. azteca* concerning the diversity of sediment grain size and organic matter content gives it salient methodological advantages for environmental quality evaluation tests. These results corroborate the studies of Ingersoll & Nelson (1990) and Suedel & Rodgers (1994), who observed that this species has broad tolerance to sediment particle size, with no changes observed in survival or growth rates of the species. Even for variations in the organic content of the sediment, Ingersoll & Nelson (1990), Ankley *et al*. (1994) and did not observe any changes in the survival rates of *H. azteca*.

On the other hand, a study developed by Hindarti *et al*. (2015) demonstrated that the amphipod *Grandidierella bonnieroides* presents survival above 80% in sediments consisting predominantly of fine sediments (silt and clay), while in sediments consisting predominantly of sand, the survival rates were significantly lower (44%). Similarly, studies conducted by Melo & Nipper (2006) show that the survival of *Tiburonella viscana* was not affected by particle size in sediments ranging 60 to 95% fine sand, collected in the field, but the survival was significantly reduced in sediments containing only one or two particle sizes, suggesting that a heterogeneity of particle sizes is necessary for the survival of this amphipod.

CONCLUSIONS

The results of this study indicate that salinity may be considered a confounding factor for the tests using the freshwater amphipod *H. azteca*. At salinities of 5 to 10, the test organism may present a higher resistance to toxic effects of contaminants. On the other hand, the cultivation of this species in saltwater may be advantageous in the production of juveniles for use in ecotoxicological tests for sediments from brackish waters.

The species shows tolerance to several variations in sediment particle size, with sand contents ranging from 0 to 95%, clay contents from 80 to 5% and organic matter contents from 0 to 20%, therefore it is not considered a confounding factor in ecotoxicological tests.

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