

Sensitivities of two tropical epibenthic amphipods to physical chemical variables and reference toxicants

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Abstract

This study sought to evaluate the sensitivity of two epibenthic amphipods (*Hyalenigra* and *Hyaleyoungi*) to reference substances in order to evaluate their potential as test-organisms in ecotoxicological studies. First, the optimal ranges of salinity, temperature, and pH were estimated for both species. Then, toxicity tests with different reference toxicants were conducted. The selected contaminants were cadmium chloride (CdCl₂), sodium dodecyl sulfate detergent (C₁₂H₂₅NaO₄S), ammonium chloride (NH₄Cl), potassium dichromate (K₂Cr₂O₇), and zinc sulfate (ZnSO₄). The mortalities were recorded and the effective concentrations to 50% organisms (LC50_{96h}) were estimated. Sensitivities of both species were similar to those reported in studies on other amphipods, which is evidence of the potential of these species to serve as test organisms in ecotoxicological studies.

Keywords: Reference substances, *Hyalenigra*; *Hyaleyoungi*; Toxicity tests.

INTRODUCTION

Over the last few decades, aquatic ecosystems have been degraded due to human activities. Pollution is considered the main threat to aquatic environments, and it demands attention from stakeholders and actions from the government. Environmental agencies have focused on the development and employment of environmental monitoring techniques, among which chemical and ecotoxicological approaches stand out (Environmental Canada 1992; USEPA 1996; 2002). Environmental analyses have placed emphasis on sediment quality assessment (Bohrer 1995), since this area of environmental studies consists of one substrate for many organisms; however, sediments can accumulate chemical substances in concentrations much higher than those found in the water column (Adams *et al.* 1985; Petrovic and Barceló 2004). Moreover, contaminated sediments represent a secondary source of pollutants for the benthic and epibenthic biota and the water column, and they are capable of negatively

impacting the quality of waters and biota even when the primary source of pollution has ceased (Araújo *et al.* 2006).

When one also considers the increasing number of potentially toxic substances that are released into marine and coastal environments, the development of new methods to properly evaluate the risks and effects in different environments and organisms is more clearly understood as an urgent need that has not been met. In this case, toxicity tests are cost-effective tools that may provide direct, quantifiable evidence of the biological consequences of contamination, and can be used to measure the interactive toxic effects of complex contaminant mixtures in water and sediment (Cesar *et al.* 2002).

Toxicity testing has been widely used to assess the quality of sediments around the world, and most studies have employed amphipods as test-organisms (Nipper *et al.* 1989; Cesar *et al.* 2002; Molisani *et al.* 2013; Basallote *et al.* 2014), since they are highly responsive to many kinds of contaminants, and have been used to evaluate the quality of whole sediments (Environment Canada 1992; ASTM 2014;

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Podlesinska&Dabrowska 2019). However, in the tropical and subtropical regions of the Southwest Atlantic, there are only two native amphipod species (*Tiburonellaviscana* and *Grandidierellabonnieroides*) with standardized protocols for their use in toxicity tests (ABNT NBR 15638/2008; Abessa *et al.* 1998; Melo 1993; Melo & Abessa 2002; Molisani *et al.* 2013). Considering the diversity of coastal and marine environments from the Southwest Atlantic, this number is clearly insufficient, thus, there is a need to expand the number of possible test species and protocols in order to better represent the marine/estuarine biota in ecotoxicological studies, especially when sediments are considered.

According to the USEPA (1994), potential test organisms must fulfill some characteristics (for example, they must be sensitive to contaminants but tolerant to laboratory conditions and handling). Additionally, the organism's distribution, biology and physiology, living habits, and level of sensitivity must be known in order to guarantee that the species will produce reliable results on the toxic effects of a substance or an environmental sample.

This study sought to assess the sensitivity of two Brazilian epibenthic marine amphipods (*Hyalenigra* and *Hyaleyongi*) to reference contaminants in order to determine their potential as test-organisms in toxicity tests. Passarelli *et al.* (2016) reported the potential of sensitivity of the Brazilian amphipod *Hyalenigrato* be used as test organisms in sediment toxicity tests and another study showed the sensitivity of *Hyaleyongi* to enrichment of CO₂ in seawater (Goulding *et al.* 2017). However, this is the first attempt evaluating the sensitivity of these species to toxic substances in water that is useful as reference assay, and considering the environmental parameters.

The ultimate goal of this study was to provide new ecotoxicological tools for evaluating the quality of tropical and subtropical marine ecosystems that include not only whole sediment testing, but also elutriate and sediment-water interface since the *H. youngi* and *H. nigra* species have epibenthic habits and are exposed to the contaminants by different routes.

MATERIALS AND METHODS

Collection, Selection and Acclimation of Test Organisms

Individuals of *Hyalenigra* (Fig. 1a) and *Hyaleyongi* (Fig. 1b) were collected from stalks of the macroalgae *Ulva* sp which were

obtained from rocky reefs from the beaches of Santos and Guarujá, particularly from Palmas Island (24°0.511'S – 46°19.448'W), which is located in the Santos Bay, São Paulo, Brazil.

The algae stalks were collected in the intertidal zone, conditioned in cool boxes with seawater and aeration, and then transported to the laboratory (ABNT NBR 15638/2008). The collected material was then screened, and the organisms were separated by species; juveniles and egg-bearing females were excluded. Species identification was conducted with the aid of specialized bibliography and identification keys (Barnard & Karaman 1991; Serejo 2001). The selected organisms were acclimated to the test conditions in glass tanks with 1L of filtered natural seawater. During the acclimatization period, the organisms received daily feedings of *Ulva lactuca* and 1.5ml of concentrated fish food (2g of TetraMin® fish food in 80 ml of pond seawater that were stirred until small flakes formed). Small pieces of nylon-type mesh were used as an artificial substrate; these mesh pieces were added to each test chamber using the procedure established by the Brazilian protocol for toxicity testing with the freshwater amphipod *Hyaella* spp (ABNT NBR 15470/2007). Table 1 shows the conditions and feasibility criteria of the toxicity tests with *H. nigra* and *H. youngi* specimens found during the preliminary tests performed to establish the limiting parameters.

Characterization of Limiting Parameters

Hyalenigra and *H. youngi* specimens were exposed to different salinities (5, 10, 15, 20, 25, 30, 35 and 40) at three temperatures (15°C, 20°C and 25°C). The test solutions were prepared by diluting filtered clean seawater with distilled water or using reconstituted water obtained by the dilution of commercial sea salts (RedSea®) in distilled water; 3 replicates were used for each treatment. Then, the test-organisms were transferred to 1L-polyethylene chambers containing 400ml of test solution with continuous gentle aeration and daily feed. After 10 days, the surviving amphipods in each replicate were counted. Subsequently, and using a similar test procedure, the organisms were exposed to three pH conditions (7.5; 8.0; 8.5) for 10 days using the optimal values of salinity and temperature estimated in the previous tests. The seawater used in this step presented an initial pH of 8.67, which required

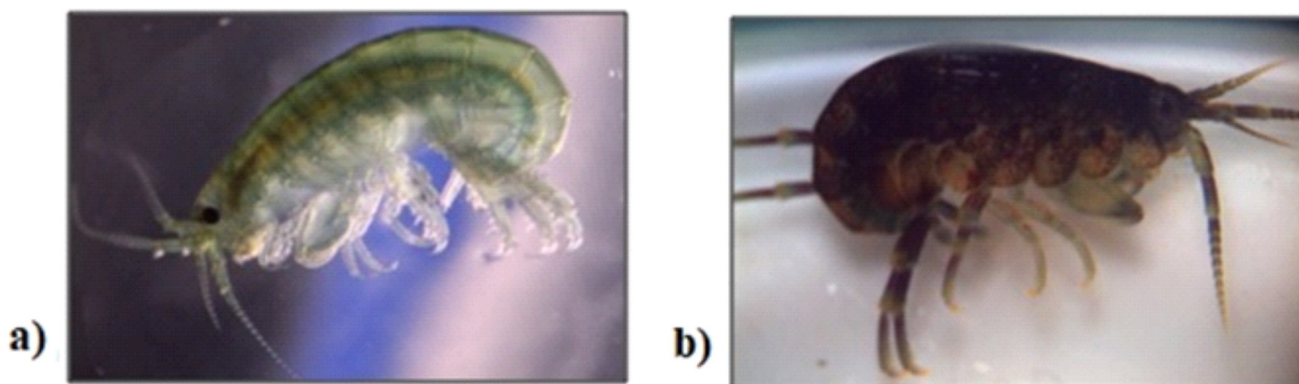


Figure 1. Species employed in the study: 1a. *Hyalenigra* and 1b. *Hyaleyongi*. (scale: 0 to 2 cm)

Table 1. Summary of conditions and feasibility criteria of the toxicity tests (aqueous fraction) with *Hyaleyoungi* and *Hyalenigra*.

Parameters	Terms
Type of essay	Static
Exposure period	96 hours
Temperature	20°C - 25°C
Salinity	35±2 (30-40)
pH	7.5 – 8.5
Photoperiod	Continuous light
Test chamber	1L poly-ethylene chambers
Volume of test solution	400 ml
Number of organisms per replication	10 organisms
Number of replicates per treatment	≥ 3 replicates
Aeration	Continuous and gentle
Dilution water	Natural filtered seawater (0.45 µm)
Age of organisms tested	Adults (no ovigerous females)
Observed effect	Lethality (LC50)
Test validity	Minimum survival in control = 85%

adjustments: 10% HCl was added the water in order to reduce the pH to the proposed values used in the experiment.

Sensitivity of Test Organisms

The sensitivity of *H. nigra* and *H. youngi* was evaluated through toxicity tests, which were performed using the following reference toxicants: cadmium (as cadmium chloride - CdCl₂); sodium dodecyl sulfate detergent (SDS - C₁₂H₂₅NaO₄S), ammonia (as ammonium chloride - NH₄Cl), chromium (as potassium dichromate - K₂Cr₂O₇), and zinc (as zinc sulfate - ZnSO₄). The selected toxicants have been recommended worldwide as reference substances (ASTM 1992; Environment Canada 1995; Schipper *et al.* 1999; Swartz *et al.* 1985; USEPA 1994; ABNT NBR 15638/2008).

The definitive concentrations were determined from preliminary tests with dilution factors of 1.5 to 2.0. Dilutions were performed with natural filtered seawater (0.45 µm) and chemical agents; all reagents used in the assays were of analytical grade. Five tests were performed with each substance and *H. nigra*; in the tests with *H. youngi*, the number of experiments changed according to the substance: three tests were performed with CdCl₂, SDS and K₂Cr₂O₇, two tests were conducted with NH₄Cl, and one test was performed with ZnSO₄. Five concentrations plus control seawater were used; the final concentrations were determined from preliminary tests. For all tested substances, 3 replicates were prepared for each concentration. They were prepared in 1L polyethylene bottles containing 400ml of the test solution and ten organisms. Small pieces of nylon mesh were introduced into each replicate as an artificial substrate. All tests lasted 96h and were set up in a temperature-controlled room (25±2°C) with continuous light. At the beginning and the end of each experiment, the overlying water physical-chemical parameters (temperature, salinity, dissolved oxygen and pH)

were measured in order to ensure the acceptability of the tests, and measurements followed standard methods (APHA 1998).

Data Analyses

The results of the characterization parameters were analyzed statistically using TOXSTAT Software, version 3.5. The data were checked for normality using the chisquare method and for homogeneity of variances using Bartlett's test and then their percentage were measured.

As an estimation of the lethal concentrations for the amphipods, the LC50_{96h} values and their respective 95% confidence limits were calculated for all substances using the Trimmed Spearman-Kärber method (Hamilton *et al.* 1977) with Abbott's formula. The precision of the laboratory data was calculated for each species/substance through the calculation of the coefficients of variation (CVs), using the mean (LC50) and relative standard deviation with the following equation:

$$CV\% = \frac{\text{Standard deviation of LC50}}{\text{Mean LC50}} \times 100 \quad (1)$$

RESULTS

The results obtained in the experiments combining different salinities and temperatures are shown in Figure 2. The results showed optimal survival rates of *H. nigra* at a salinity of 35 and at 20°C, conditions under which 95% of exposed organisms survived (Fig. 2a). For *H. youngi*, the best survival rates were observed at salinity of 35 and at both 20°C and 25°C; under these conditions, survival rates were ≥ 86% (Fig. 2b). However, the minimum survival rate acceptable by ABNT NBR 15638 is 85%. In this sense, it was also observed great survival at salinity 30 and at 20°C and 25°C, using the *H. youngi* species and from salinity 30 to 40 and under all temperatures employed for the *H. nigra* species.

The best results obtained in the tests with different pH levels occurred at 8.5 in the tests with both species. Survival rates ≥ 80% were also observed under other conditions, including salinities between 30 and 40, temperatures between 20°C and 25°C, and pH levels between 7.5 and 8.5 (Table 2).

The results of the tests with reference substances are shown in Table 3. The mean LC50_{96h} values in the tests with NH₄Cl₂ were 139.73±18.5 mgL⁻¹ for *H. nigra* and 214.54±23.84 mg L⁻¹ for *H. youngi*. In the tests with SDS, the mean LC50_{96h} values were 4.63±0.59 mgL⁻¹ and 16.67±8.20 mgL⁻¹, respectively. In the tests with potassium dichromate, mean LC50_{96h} values were relatively similar, with respective values of 36.37±8.25 mgL⁻¹ and 28.83±2.44 mgL⁻¹; both species exhibited similar a mean LC50_{96h} value in the tests with CdCl₂ (0.53 ± 0.11 mg L⁻¹ and 0.44 ± 0.08 mgL⁻¹) and ZnSO₄ (0.60 ± 0.18 mgL⁻¹ and 0.37 mgL⁻¹) for *H. nigra* and *H. youngi*, respectively. The sensitivity of both species to the reference toxicants tended to vary little among experiments, and CVs lower than 30% were observed in some substances. Sodium dodecyl sulfate produced the lowest CV among the studies on *H. nigra* (17%), while in the studies on *H. youngi*, the lowest CV was observed with potassium dichromate (10%).

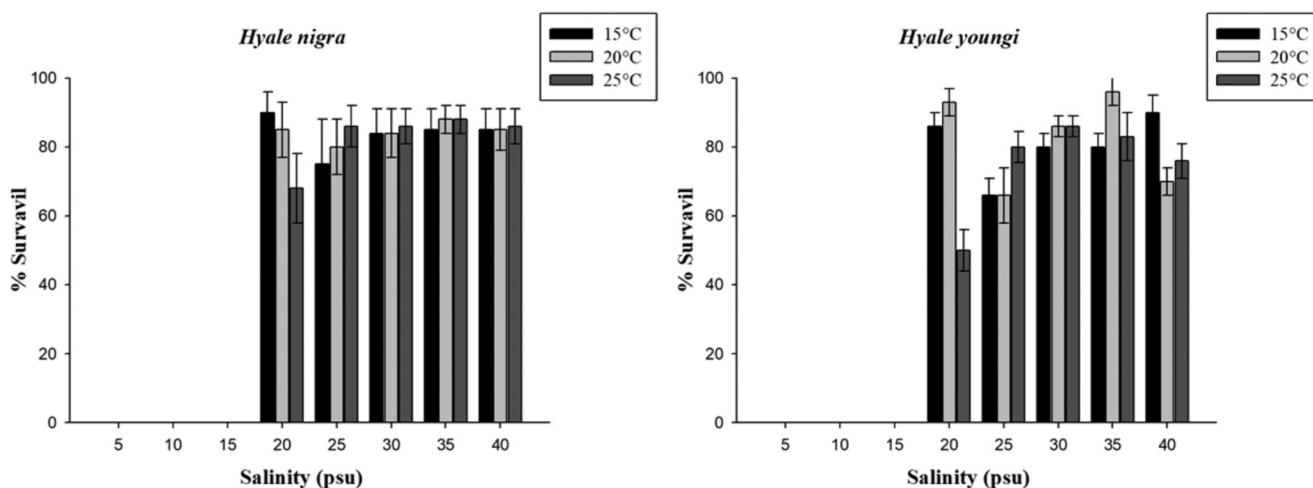


Figure 2. Mean values of different salinity and temperature to the amphipods *Hyale nigra* (2a) and *Hyale youngi* (2b). (± standard deviation)

Table 2. Results of the tests with different pH levels using the amphipods *Hyale youngi* and *Hyale nigra*.

pH	% Survival	% Survival
	(<i>Hyale youngi</i>)	(<i>Hyale nigra</i>)
7.5	85%	83%
8.0	85%	85%
8.5	90%	86%

Table 3. Mean LC50_{96h} values obtained in toxicity tests with *Hyale nigra* and *Hyale youngi* exposed to reference substances. Concentrations are expressed as mg L⁻¹. Results are expressed as means and respective standard deviations (SD) and coefficients of variation (CV).

Substance	Species			
	<i>H. nigra</i>		<i>H. youngi</i>	
	Mean ± SD	CV	Mean ± SD	CV
NH ₄ Cl ₂	139.73 ± 18.5	18%	214.54 ± 2 3.84	14%
C ₁₂ H ₂₅ NaO ₄ S	4.63 ± 0.59	17%	16.67 ± 8.20	46%
K ₂ Cr ₂ O ₇	36.37 ± 8.25	41%	28.83 ± 2.44	10%
CdCl ₂	0.53 ± 0.11	34%	0.44 ± 0.08	29%
ZnSO ₄	0.60 ± 0.18	75%	0.37	-

When both species were compared, *H. nigra* was found to be more sensitive to NH₄Cl₂ and C₁₂H₂₅NaO₄S; in the studies using the other substances, sensitivities can be considered comparable.

DISCUSSION

Amphipods are considered suitable indicators of environmental quality because they are sensitive to different types of contaminants in aquatic environments.

An important aspect that must be known regarding any species' potential to be a biological model in ecotoxicological evaluations is the definition of its tolerance limits to physical-chemical parameters. These parameters may interfere on the interpretation of the data, and they could be considered confounding factors that can sometimes cause false-negative and/or false-positive results. According to Serejo (2001) *H. nigra* and *H. youngi* specimens occur along the coast of the Brazilian states of Bahia, Rio de Janeiro and São Paulo, and they are generally associated with macroalgae found in rocky shores. Temperature is a factor that strongly influences these species, and it must be monitored and controlled during the conduction of laboratory tests (Kinne 1961; Fish & Preece 1970; Barnett 1971).

The tolerance range for *H. nigra* and *H. youngi* (highest survival at salinities between 30 and 40 and temperatures between 20°C and 25°C) were expected as they are typical of marine habitats in tropical and subtropical areas. Although the *H. youngi* amphipod has showed a lower survival rates at salinity 20 under 25°C, its application is still recommended and acceptable when the experiments conditions are controlled following the criteria mentioned in the table 1 (optimal conditions for the application of these species). Moreover, a previous study has reported a significant mortality of *H. youngi* species at pH ≤ 6.5 (Goulding *et al.* 2017) while the results obtained in this study showed higher survival at the highest pH level tested (pH 8.5) which confirm the sensitivity of this species to acidified conditions (CO₂ enrichment).

Another pre-requisite of a test-species candidate is the sensitivity to a range of contaminants. This can be measured through experiments with reference substances. These experiments are performed with determined toxicants (USEPA 1994; ABNT NBR 15638/2008) which toxicities are known for many species, in order to allow further comparisons (Lee 1980). The LC50_{96h} values found in this study to both species (*Hyale youngi* and *H. nigra*) were similar to those reported in the literature for other species. Both species present similar habits and aspects with specific difference in their taxonomy. In the case of *H. nigra*, the taxonomic differentiation is found in pereopods from 5 to 7 with 3 single spines present and in

the branch of uropods 1 and 2 with marginal spines. On the other hand, *H. youngi* presents large and striated arrows in the dyspods of pereopods from 3 to 7, and the peduncle of uropode 1 with lateral spine (Serejo 2001).

Table 4 shows a comparison of the LC50_{96h} values obtained in tests with several reference toxicants, including the species used in the current study. Responses to the different substances tended to vary little among experiments, with exception of *H. nigra* response of to ZnSO₄, for which the CV was 75%.

The literature recommends CVs of 30% or lower for control charts produced with at least 20 tests (Environment Canada 1992); however, the higher values observed in this study may have been due to the low number of experiments and also because the organisms were collected in the field, being subject to environmental fluctuations that could have influenced on their physiology and conditions. Further studies with these species and reference toxicants could provide more reliable data on their sensitivity and the refinement of the sensitivity ranges; they would also subsidize the preparation of control charts, which consist of an important tool to assess the sensitivity of test-organisms.

The mean values of LC₅₀ found for cadmium (CdCl₂) were 0.32 mg L⁻¹ for *H. nigra* and 0.27 mg L⁻¹ for *H. youngi*, similar to those reported for the amphipods *Corophium multisetosum*, *Corophium insidiosum* and *Gammarus aequicauda* that were: 0.31 mg L⁻¹, 0.35 mg L⁻¹ and 0.26 mg L⁻¹, respectively (Ré et al. 2009; Prato et al. 2006). However, higher values were found for the amphipods *Corophium insidiosum* (1.68 mg L⁻¹) and *Corophium orientale* (3.3 mg L⁻¹) (Picone et al. 2008; Prato & Biandolino 2006).

The results obtained in the present study produced a LC50_{96h} of 0.26 mg L⁻¹ and 0.37 mg L⁻¹ of ZnSO₄ for *H. nigra* and *H. youngi*, respectively, which were relatively close to the value of the LC₅₀ reported in the literature for *T. viscana* (0.79 mg L⁻¹) (Melo & Nipper 2007).

King et al. (2006) evaluate the sensitivity to zinc in seven species of amphipods (*Chaetocorophium lucasi*,

Corophiumcolo, *Grandidirella japonica*, *Hyalelongicornis*, *Melita awa*, *Melita matilda* e *Melita plumulosa*) in adult (10 days) and juvenile (96 hours) phases. Their results showed that the highest sensitivity was exhibited by *H. longicornis* at juvenile stage (LC50 of 0.05 mg L⁻¹), and adult individuals (LC50 of 0.19 mg L⁻¹). The most tolerant species reported by King et al. (2006) was *C. color* (LC50 of 0.45 mg L⁻¹), and its sensitivity was comparable with those presented in this study by *H. nigra* and *H. youngi*.

In addition, both species (*H. nigra* and *H. youngi*) of amphipods investigated in this study were relatively tolerant to ammonia. This should be considered advantageous for the evaluation of the toxicity of sediments since ammonia is considered a non-persistent confounding factor rather than a contaminant of concern; besides, it confirms the potential of these species as bioindicators of aquatic environmental health.

On the other hand, it is highlighted that the majority of standardized species have burrowing habits however the use of epibenthic species may be very versatile since these organisms are exposed to the contaminant in the sediment from different ways. Although the species used in this study have epibenthic habitats, individuals of both species (*H. nigra* and *H. youngi*) were observed buried in the sand near the algae during collection, indicating that this behavior may occur depending on the situation in which the organism is found.

Furthermore, there are other species with distinct habits that are standardized, such as *Gammarus aequicauda* and *Ampelisca abdita* (Cesar et al. 2004; USEPA 1994). Also, the Brazilian standard ABNT NBR 15470/2007 establishes a protocol to sediment toxicity tests with epibenthic species of sweet habits (*Hyalella* sp.), which is basically the same applied in the present study, except for some adaptations regarding the amount of sample used and age of the test organisms.

CONCLUSION

The present study provides information on the sensitivity of epibenthic marine amphipods *Hyalella nigra* and *Hyalella youngi*

Table 4. Comparison of LC50 values obtained from tests with reference substances and different species of marine amphipods. a) LC50_{96h}; b) LC50_{48h}; c) LC50_{72h}.

Species	Reference Toxicant (mg L ⁻¹)					Author(s)
	NH Cl	SDS	CdCl	K Cr O	ZnSO	
<i>Hyalella nigra</i> ^{a)}	103.42 ²	3.45	0.32 ²	19.88 ⁷	0.26 ⁴	This study
<i>Hyalella youngi</i> ^{a)}	166.85	8.47	0.27	23.95	0.37	This study
<i>Tiburonellaviscana</i> ^{b)}	-	3.41	-	9.8	0.79	Melo & Nipper 2007
<i>Tiburonellaviscana</i> ^{b)}	-	-	-	11.22	-	Abessa & Sousa 2003
<i>Tiburonellaviscana</i> ^{c)}	28.74	-	-	-	-	Abessa 2002
<i>Tiburonellaviscana</i> ^{b)}	-	5.87	-	9.8	-	Abessa 2002
<i>Gammarus aequicauda</i> ^{b)}	-	5.56	0.26	9.52	-	Cesar et al. 2004
<i>Microdeutopus gryllotalpa</i> ^{b)}	-	2.98	-	6.06	-	Cesar et al. 2004
<i>Corophium multisetosum</i> ^{a)}	-	-	0.31	-	-	Ré et al. 2009
<i>Eohaustorius estuarius</i> ^{a)}	125.5	-	-	-	-	Kohn et al. 1994
<i>Grandidirella japonica</i> ^{a)}	148.3	-	-	-	-	Kohn et al. 1994
<i>Corophium insidiosum</i> ^{a)}	-	-	0.35	-	-	Prato et al. 2006
<i>Corophium orientale</i> ^{a)}	-	-	3.3	-	-	Picone et al. 2008

to physical-chemical water variables and reference substances. The results showed that these species present typical marine and tropical habits with excellent survival between salinities at 30 - 40, 20 °C - 25 °C of temperature and pH levels of 8.5. In the tests with the toxic reference substances, the results indicated that the amphipods used in this study are sensitive to the different compounds tested, and were similarly responsive compared to other invertebrate species used in toxicity testing. The amphipod *H. nigra* showed higher sensitivity than *H. youngi*, with exception of cadmium chloride, which was more toxic to the latter.

Therefore, the results obtained in this study showed the good sensitivity of the species *Hyalellognathus nigra* and *Hyalellognathus youngi* to all the reference substances tested, and consequently, prove their potential of application in toxicity tests providing new options of species to be used to evaluate quality of sediment when demanded by the Brazilian legislation. Further studies on the biology of these species and their sensitivity to other environmental parameters (such as sediment textures and organic enrichment) and environmental samples should be conducted, in order to confirm the results of this study and the usefulness of both species in toxicity testing with sediments.

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