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Dates: Received: 05 July, 2016; Accepted: 30 December, 2016; Published: 31 December, 2016

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Keywords: Sediment; Amphipod; Hyale nigra; Spiked sediment.

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Research Article

Using the Amphipod *Hyale Nigra* to Assess the Quality of Marine Sediments

Abstract

Few species are standardized and have been used as test organisms around the world in ecotoxicological assays. In the case of sediment assessment, there are only two amphipod species (*Tiburonella viscana* and *Grandierella bonnieroides*) standardized protocols for toxicity test in South Atlantic region. In order to evaluate the possibility of using the epibenthic marine amphipod *Hyale nigra* in toxicity tests, a sensitivity test using spiked sediment method was applied, with cadmium chloride as a reference toxicant (CdCl₂). Tests with environmental samples were performed to validate the test organisms. The samples were taken from Santos Estuary and Bay in state of Sao Paulo, Brazil. The historical toxicity and contamination are mentioned in the literature. The results obtained in the spiked sediment tests showed little variation in the sensitivity of *Hyale nigra* amphipod with a mean LC₅₀ value of 5.73 mg.L⁻¹ (1.51 mg.L⁻¹ – 8.75 mg.L⁻¹). In the tests with environmental samples, the results obtained were similar to those found in the literature regarding this sampling area. This study demonstrates the potential of *Hyale nigra* amphipod as a test organism in toxicity tests, once that the specie is sensitive and its results were similar to those reported in the literature on other species of amphipods that are already used in ecotoxicological studies.

Introduction

Toxicity tests have been used worldwide with different objectives, such as to determine environmental health, establish maximum limits for the discharge of chemicals and effluents, define critical areas, and provide a biological understanding of the chemicals found in the environment and among others goals [1-4]. Most of the purposes cited above require environmental studies (monitoring and assessment) or the establishment of maximum limits for chemical discharge into the environment. Thus, in order to provide proper estimations of the environmental effects or risks resulting from contamination, toxicity tests, preferably, with local species must be conducted.

The use of native species is important, because these species represent the local biota that may be directly exposed to the environmental contamination, and their use thus allows for the extrapolation of the results to the field with more reliability than non-indigenous organisms can provide [5]. However, despite the fact that ecotoxicological tests have been incorporated into environmental studies since the 1990s, few species have been used as test organisms around the world [6-8]. The limited number of species that present standardized protocols for toxicity tests is especially worrisome in Brazil,

since the country has an extensive coastline that comprises many different ecosystems and environments, from the equatorial Amazon areas in the North to the temperate and subtropical regions in the South. Therefore, there is a crucial need for new local species to be found — ones that can serve as test organisms in ecotoxicological studies and thus provide reliable information for the protection of marine and coastal ecosystems. The search of new species for ecotoxicological purposes requires knowledge for varied aspects of the organisms, including their biology, reproductive cycles, growth patterns, feeding habits, seasonality, interactions with the environment, and sensitivity to contaminants or mixtures. According to [9], the sensitivity to toxicants plays a key role, since the use of extremely sensitive or resistant species may influence the results and reliability of toxicity tests.

In the case of sediments, the sensitivity of the test organisms can be estimated either through spiked sediment toxicity tests that use known concentrations of reference substances or by exposing the organisms to field-collected sediments that contain a range of single or multiple contaminants [10].

Spiked sediment toxicity tests involve the addition of one or more chemicals to clean sediments at different concentrations, followed by the evaluation of the effect that the concentrations

have on the exposed organisms [11-13]. This approach has been used to evaluate the sensitivity of test organisms to contaminants [5,14,15], particularly their sensitivity to metals such as cadmium, copper, and zinc [16-18].

In Brazil, the National Environment Council (CONAMA) has requested that ecotoxicological assays be part of studies in order to evaluate the quality of waters, sediments and effluents [19], and also to provide information on the management and disposal of dredged sediments [20]. However, when it comes to sediments, there are ecotoxicological protocols available for only two species of amphipods, two species of benthic copepods, one species of shrimp and one species of tanaid, and certainly this reduced amount is not nearly enough to cover the country's coastline or its diversity of environments. [21] highlights the need for tests that are ecologically more realistic and which rely on native species. Results obtained from the use of local species may be also important for the development of local/regional sediment quality guidelines (SQGs) [13].

This way, the objective of the current study was to evaluate the sensitivity of the native amphipod *Hyale nigra* to contaminated sediments, as a first step in determining its suitability as a standard test species. The tests considered the species' exposure to sediments spiked with reference toxicants, and also to field-collected samples with varied levels of contamination.

Materials and Methods

Test-organism

The *Hyale nigra* specie is a crustacea, amphipod from Hyalidae Family Bulycheva, (1957). These crustaceans are representatives of vagile epifauna and stand out from the other groups for its abundance and wealth specie in macrophyte communities around the world [22-24]. Moreover, these organisms are important source feed to some fish and crustaceans species [25, 26], thus enabling the transfer to contaminants throughout the food chain, for instance, by the biomagnification process.

Collection, selection and acclimation of test organisms

Hyale nigra specimens were collected from stalks of the macroalgae *Ulva* sp obtained from rocky reefs in Palmas Island (24°0.511'S - 46°19.448'W) Santos Bay - São Paulo, Brazil. The algae stalks were collected at the intertidal zone, conditioned in cool boxes filled with seawater, and transported to the laboratory. The organisms collected were sorted and separated by species. Juveniles and egg-bearing females were excluded. *H. nigra* specimens were identified using an identification keys [27-28], transferred to aquariums of 1L with seawater, and acclimated for two days to the test conditions (salinity of 35, > 80% O.D and 25°C). Small pieces of nylon mesh (600µm) were used as artificial substrate, following the recommendations set by the Brazilian protocol for toxicity testing with *Hyalella* spp [29]. During the acclimatization period, the organisms were fed both *Ulva lactuca* and 1.5ml of concentrated fish food daily

(the concentrated fish food was 2g of fish food Tetra-Min® diluted into 80 ml of pond seawater, and stirred until small flakes formed).

Experiment set-up

The sensitivity of *H. nigra* amphipod was tested on different steps. At first, its sensitivity to different sediment textures (very coarse sand, coarse sand, medium sand, fine sand and very fine sand) was assessed. For it, the control sample was washed, sieved and separated according to the Wentworth scale. The experiments were based on preliminary studies and followed the recommendations established by the ABNT NBR 15470/2007 standard [29], which describes the test conditions for freshwater species. The tests were conducted in 1-L chambers containing 2 cm layer (~150g) of sediment and 600 ml of seawater. Next, the pieces of nylon mesh were added to all replicates. Three replicates with 10 organisms were used. The experiment lasted 10 days with renewal of the overlying water (2/3) in each 3 days, and gentle aeration and continuous light.

Secondly, the control sediment was spiked with cadmium [30-31]. The sediment spiking was prepared via the suspension method, in which 600 ml of saturated solutions of CdCl₂ were mixed with 150 g of control sediment; the mixtures were stirred for 4 hours at 130 rpm (rotations per minute) with the help of a magnetic stirrer. After 24 h, the liquid solution was withdrawn from the test bottles. Then the sediments were left to rest for 14 days in order to allow for the stabilization between the sediment and the added metal. Aliquots of the sediments were then separated and sent to a commercial analytical laboratory (Ecolabor - São Paulo; ISO/IEC 17025 accredited) in order to measure the concentrations of Cd in the sediments. Five tests were performed by exposing *H. nigra* to spiked sediment using 4 concentrations of spiked sediment (1 mg, 2 mg, 4 mg and 8 mg Cd/Kg of wet sediment) and four replicates. The tests were carried out with the same conditions described above in the first step experiment. At the beginning and the end of each experiment, the overlying physical-chemical parameters of the water (temperature, salinity, dissolved oxygen, and pH) were measured in order to ensure the acceptability of the tests; the measurements followed standard methods (APHA 1998).

The third experiment step was performed with environmental samples from Santos Estuary and Bay. The establishment of the sampling sites (Figure 1) was based on previous studies [32-36], which showed contamination gradients from the inner portions of the estuary to the external areas. Four sampling sites were collected following [11] recommendations. Two kind of experiment were performed with the environmental samples. The whole sediment toxicity tests were conducted following the international and Brazilian standards for sediment testing [28-30]. The basic description of the tests was previously presented in the description. The sediment samples were also evaluated using liquid phase assays employing sediment elutriate. For it, the sediment samples and seawater (ratio 1:4 v/v) were mixed for 30 minutes. The samples remained stabilized for 1 hour, and the supernatant was separated and used in the ecotoxicological

tests [37]. The tests were conducted using 4 replicates per treatment. Each replicated containing 400ml of elutriates. The elutriate tests lasted for 96h. At the end of all experiment step, the organisms were transferred to Petri dishes and examined under a stereomicroscope. The numbers of dead and living organisms were counted. When the final sum did not reach 10, the organisms that were not found were considered dead.

Data Analyses

The results of the toxicity tests were analyzed statistically using TOXSTAT Software, version 3.5. The results of both sediment elutriation and whole sediment were analyzed using ANOVA, followed by Dunnett's test in order to compare the survival rates to that reference sample (P4). Student's t-test for independent samples was used to compare the survival rates of the specimens in each sample to their specific control ($p=0.05$). Prior to the statistical analysis, the data were checked for normality using the chisquare method and for homogeneity of variances using Bartlett's test. For the tests with cadmium-spiked sediments, the lethal concentrations after 10 days of exposure (10-d LC₅₀) and their respective 95% confidence limits were calculated using the Trimmed Spearman-Kärber method [38].

Results and Discussion

The tests with different sediment textures showed survival rates of *H. nigra* in the sediment control above 80%. The control sample presented a predominance of medium sands. There are no significant differences in the results with different sediment textures when compared to the control ($p < 0.05$) to *H. nigra* amphipod. According to [39], sediment grain size distribution has been found to directly influence to test results with amphipods and it may influence their survival rates. In the current study, *H. nigra* was not presented a survival significantly different from the control (>60%) when exposed to different particle sizes tested being the best result from medium sand.

In relation to cadmium-spiked sediment tests the amphipod *H. nigra* showed little variation in the results (CV % = 26) and the mean LC₅₀ estimated after 10 days was 5.73 mg Cd/kg (1.51 – 8.75 mg Cd/kg). The data are shown in table 1.

The results showed that *H. nigra* is highly sensitive to Cd in sediments. This value is close to the LC₅₀ value (5.6 mg Cd/kg) obtained by [16] in their study on the freshwater amphipod *Hyalella curvispina*. When the LC₅₀ values (LC₅₀ 10-d value of 5.73 mg Cd/kg, $n=5$) are compared to the Brazilian sediment quality guidelines for dredged sediments [20], which are 1.2 and 7.2 mg Cd/kg for the threshold and probable effect levels, respectively, it becomes clear that these levels may not completely protect the native biota [40]. Reported that these sediment guidelines did not provide effective protection for marine and estuarine ecosystems, since many toxic samples exhibited concentrations of chemicals below the theoretical threshold levels. These authors also stated these SQGs were established by adopting the values proposed by the North American and Canadian protocols [41, 42] and without performing studies with native species

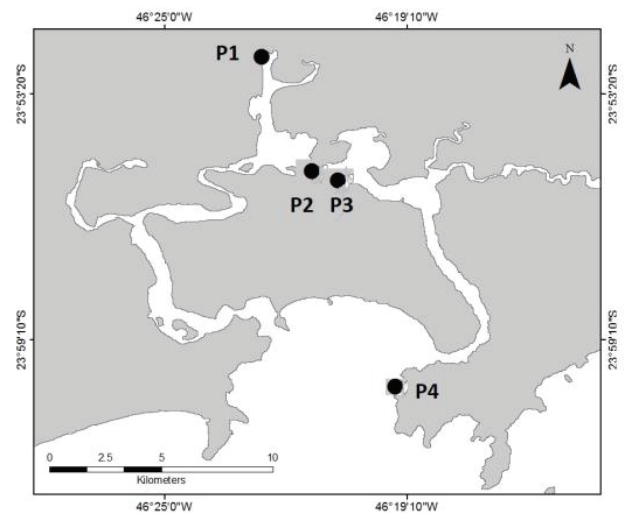


Figure 1: Study area and sampling sites used in the toxicity test with *Hyale nigra* amphipod.

Table 1: Results of medium lethal concentration (LC₅₀) using spiked sediment tests with *Hyale nigra* amphipod (mg.Cd/kg). * Means lower and upper limits.

Test	Cd - Spiked sediment
	LC ₅₀ - 10 days
01	6.46 (5.19 – 8.09)*
02	4.90 (3.39 – 7.07)*
03	6.17 (4.34 – 8.77)*
04	7.51 (5.62 – 10.0)*
05	3.59 (2.16 – 5.97)*
Mean	5.73 (1.51 – 8.75)*

and at the conditions expected to be found in the Brazilian sediments [43]. Showed that the interim sediment quality guidelines (ISQGs) were conservative, since the amphipod *Melita plumulosa* was not affected in concentrations below the ISQGs. In this sense, both cadmium-spiked sediment tests and tests using other chemicals may provide information that can improve the federal legislation and achieve the effective protection of native biota. Further tests with other aquatic species should be run in order to provide information on the Cd sensitivity of a broad range of organisms.

The results obtained in whole sediment and elutriate toxicity tests with *H. nigra* are shown in figure 2. The sediment grain size distribution presented higher values of silt at P1, P2, and P3; at P4, the highest values were found in medium sand. Out of all of the samples tested, the total concentration of ammonia was highest at P2 (1.47 mgL⁻¹). Organisms exposed to the sediment from P4 (control) presented the highest survival rate, while sediments from P2 and P3 displayed significantly lower survival relative to the reference control.

The Santos Estuarine System is considered a strategic region in Brazil, since it comprises the biggest port of Latin America, an industrial complex, and a large urban area. The SES is also of high ecological importance because it is surrounded by mangroves and a set of protected areas. The region constitutes a refuge and shelter for many species of vertebrates and invertebrates alike.

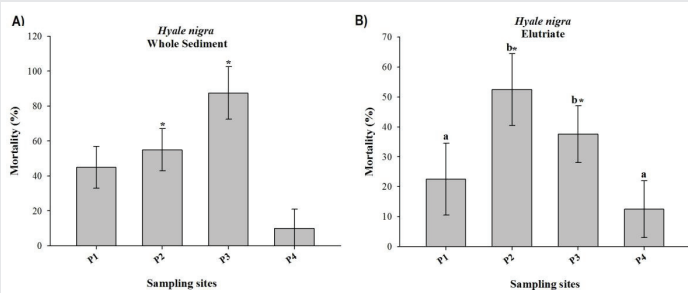


Figure 2: Mean results of tests with environmental samples. A) Whole sediment toxicity test. B) Elutriate test (*= significant reference; $p = 0.05$; Different letters correspond to significant differences ($p < 0.01$)).

Although in this study the concentrations of contaminant did not have been quantified, a number of past and present studies have shown that the SES region is ecologically damaged due to the presence of multiple contaminants in the area, some of which have increased in quantity [3,31–35,44]. As previously mentioned in this article, many contamination sources are located within the estuarine portion or in its surroundings. Thus, sediments from the region have been considered contaminated and toxic and present moderate to high levels of chlorine, detergent, ammonia, sulfur, and mercury, among other pollutants.

Sediments from P1, P2 and P3 were considered contaminated and toxic to marine invertebrates (results which were consistent with [32,45], and especially [44]), whereas sediment from P4 was considered only slightly degraded. The aforementioned authors attributed toxicities to metals, PAHs, and detergents. In the current study, sediments from P2 and P3 were toxic relative to the reference sediment (P4). These results are in accordance with these previous studies since the sediment sample from P4 is usually used as a reference site (control site) in the environment studies in this area. Additionally, it is important to highlight that the sediment sample from P4 is located in Santos Bay and receives less influence from the estuary degradation than the others. Moreover, the absence of toxicity in the sediment from P1 may be explained by the fact that the region was dredged a few months before the sampling.

Conclusion

In conclusion, the amphipod *Hyale nigra* presents a good tolerance to different grain sizes of sediment and high sensitivity to contaminated sediments. Our result proves that *H. nigra* is a suitable potential test-species in ecotoxicological studies with sediment. Furthermore, because *H. nigra* presents epibenthic habits, it can also be used to evaluate different means of exposure, as demonstrated in the elutriate tests and whole-sediment tests.

Therefore, *H. nigra* represents a suitable species for use in toxicity tests with sediments. This species is also advantageous in that it can be used to evaluate different means of exposure. In studies on local issues, this species represents a new option to be considered when toxicity tests are demanded by the Brazilian legislation.

Acknowledgements

This study was funded by the São Paulo Research Foundation – FAPESP (grant #2011/159248). The first author would like to thank the Erasmus Mundus Program for the doctoral fellowship (2014–0693/001–EMJD). A. Cesar and D. Abessa thank the National Council known as CNPq (*Conselho Nacional de Desenvolvimento Científico e Tecnológico*, MEC – Brazil) for their respective research productivity fellowships (PQ#305869/2013–2 and PQ#308649/2011–7) and also to the Research Project ECO₂Mar (CAPES 126/2012–6).

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