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Evaluation of the viability of a microscale method for the short-term chronic toxicity test using *Lytechinus variegatus* embryos

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Abstract: The aim of the present work was to propose some modifications to the standardized method of toxicity test with embryos of sea urchins, such as: i) use of 24-well tissue culture plates instead of glass tubes; ii) reduction of sample volume in each replicate from 10 mL to 2.5 mL, and iii) increase in number of exposed embryos in each replicate from 300 to 500 The experiments evaluated the effects of widely used reference toxicants - zinc and sodium dodecyl sulfate (SDS), using the standard protocol and the new proposed method. The mean EC_{50} values for SDS were 3.35 ± 0.78 and 2.89 ± 0.58 mg L^{-1} for microscale and standard methods, respectively, whereas, for zinc, the EC_{50} was 0.07 ± 0.02 mg L^{-1} regardless of the method. The modifications in the toxicity test using *Lytechinus variegatus* embryos proposed were acceptable, at the least, for the reference substances tested. Such modifications may mean improvements to this method, for it represents costs reduction, reduced water consumption and minimization of contamination due to poor handling of glassware. However, much effort on the validation of the microscale method should be done especially to access its applicability considering hydrophobic chemicals and environmental samples.

Keywords: Sea urchin, zinc, sodium dodecyl sulfate, 24-well microplates, embryo density, quality assurance.

Resumo. Avaliação da viabilidade de um método em microescala para o teste de toxicidade crônica de curta duração com embriões de *Lytechinus variegatus*. O objetivo deste trabalho é propor as seguintes modificações no método padronizado para o bioensaio com embriões de ouriço do mar: i) uso de placas 24-multicavidades ao invés de tubos de vidro; ii) redução do volume do teste em cada réplica de 10 a 2,5 mL e iii) aumento no número de embriões expostos de 300 para 500, em cada réplica. Experimentos foram executados seguindo os procedimentos padronizado e modificado, comparando os efeitos do zinco e do dodecil sulfato de sódio (DSS). Os valores médios de CE_{50} para o DSS foram 3,35 ± 0,78 e 2,89 ± 0,58 mg L^{-1}, para o método padrão e o micrométodo respectivamente, e 0,07 ± 0,02 mg L^{-1} para o zinco, indiferentemente do método. As modificações no teste de toxicidade utilizando os embriões de *Lytechinus variegatus* propostos podem ser consideradas aceitáveis, pelo menos para as substâncias referência testadas. Essas modificações podem ser consideradas melhorias no método padronizado, já que levam à redução nos custos, a um consumo reduzido de água e amostras e minimizam a contaminação pela manipulação inadequada da vidraria. Entretanto, esforços devem ser investidos na validação desse método especialmente para verificar sua aplicabilidade para avaliação de compostos hidrofóbicos e amostras ambientais.

Palavras-chave: ouriço do mar, zinco, dodecil sulfato de sódio, placa 24-multicavidades, densidade de embriões, controle de qualidade.

Introduction

Acute and chronic toxicity assays with different life stages of aquatic organisms (fishes and invertebrates) have been extensively used in environmental monitoring programs to evaluate the effects of aquatic pollution. Among the myriad of effects that can be measured, gametogenesis and embryonic development are categories that deserve special attention due to their potential to produce alterations up to the population and community levels (Cherr et al. 1992, 1993).

In this context, embryos from different species of sea urchins have been widely used to evaluate the quality of marine and estuarine environments (Environmental Canada 1992, USEPA 1995). For several decades, sea urchin bioassays have been used with different purposes, including the quality assessment of marine waters and the evaluation of some properties of natural products, such as cytotoxicity, genotoxicity, teratogenicity and antimitotic activity (Kobayashi 1973, Fusetani 1987, Zúñiga *et al*. 1995, Saotome *et al.* 1999, Costa-Lotufo *et al*. 2002, Hansen *et al.* 2003, Cummings & Kavlock 2004). Several factors contribute to increase the use of sea urchin embryos as test-organisms: their worldwide distribution, the easy collection of adults at field, the possibility to obtain large amounts of ovules and sperm cells for *in vitro* fertilization and the rapid, clear and highly successful embryonic development throughout larvae formation.

In Brazil, the use of toxicity tests in environmental quality analyses started in the beginning of the 1970s with the standardization, by the environmental agency from São Paulo State (CETESB), of some tests using freshwater organisms (Resgalla & Laitano 2002, Zagatto 2006a). But only ten years later were the test protocols for marine organisms published (CETESB 1987, 1992, 1999, Resgalla & Laitano 2002, Zagatto 2006a). Since then, toxicity tests with sea urchin embryos have been extensively used in environmental pollution monitoring (Mastroti 1997, Prósperi 1993, 2002, Abessa *et al.* 2002, Nilin *et al*. 2007). *Echinometra lucunter* and *Lytechinus variegatus* are among the most common Echinoidea species from the Brazilian coast, being them both commonly used in ecotoxicological assays (Nascimento *et al*. 2000, Prósperi & Araújo 2002, Mastroti 1997, Prósperi 1993, 2002, Abessa *et al.* 2002, Nilin *et al*. 2007). Resgalla & Laitano (2002) compiled data from 60 toxicity tests using 21 species of marine organisms under effects of four reference substances (copper, chrome, zinc and sodium dodecyl sulfate) and concluded that sea urchin embryos (*E. lucunter* and *L. variegatus*) exhibited a high sensitivity, but it was specific to the type of tested contaminant. Furthermore, Araújo & Nascimento (1999) and Nascimento *et al*. (2000) compared the sensitivity of the sea urchin *E. lucunter* with the mangrove oyster *Crassostrea rhizophorae* eggs during fertilization and early embryogenesis using water-soluble extracts of crude or refined oils and sodium dodecyl sulfate (SDS). They concluded that the oyster eggs were more sensitive than sea urchins eggs.

Nowadays, the short-term chronic toxicity test with sea urchins *L. variegatus* or *E. lucunter* embryos is standardized in Brazil by CETESB (1999) and more recently by Associação Brasileira de Normas Técnicas (ABNT) (2006). The aim of the present work is to propose some modifications in the standardized method, such as: i) use of 24-well tissue culture plates instead of glass tubes; ii) reduction of sample volume from 10 mL to 2.5 mL, and iii) increase in the number of exposed embryos from 300 to 500. To access the viability of these modifications, experiments were performed comparing the responses of embryos to zinc and SDS when submitted to the standard protocol and the modified conditions (microscale method).

Materials and Methods

Test organisms

Adult *L. variegatus* sea urchin individuals were collected at Lagoinha beach, Paraipaba, Ceará. The animals were maintained in 100-L aerated glass tanks containing natural sea water, each holding about 15 urchins. The animals were checked daily and the unhealthy ones were discarded. The dilution water was collected at the same place as the sea urchins and filtered (0.45µm membrane) before the experiments. The water was checked for pH, salinity, temperature and ammonia and presented values within the desirable range for use in toxicity testing.

Test solutions

Two reference toxicants (zinc and SDS) were used to validate the applicability of the microscale method considering previously defined acceptability ranges for the tests and the results obtained with standardized tests. Stock solutions were prepared in distilled water at 50 mg L^{-1} for zinc sulfate and 200 mg L^{-1} for SDS. These solutions were diluted to get final concentrations of 1.0; 0.5; 0.25; 0.12 and 0.062 mg L^{-1} for zinc sulfate (or 0.227; 0.113; 0.056; 0.028 and 0.014 mg of Zn L^{-1}) and 5.1; 4.2; 3.2; 2.4; 2.2; 1.8 and 1.3 mg L^{-1} for SDS.

Collection of Gametes and fertilization

Gamete elimination was induced by injecting 3.0 mL of 0.5 M potassium chloride (KCl) solution into the perivisceral cavity of one male and one female for each experiment. Gender recognition was attempted based on the criteria described by Abessa et al. (2001). The ovules were allowed to settle to the bottom of a graduate cylinder filled with filtered sea water. This process was repeated twice to wash off the gelatinous coat from the ovules. Concentrated sperm was collected with a Pasteur pipette. Fertilization was performed by adding 1 mL of a sperm suspension (0.05 mL of concentrated sperm plus 2.45 mL of filtered sea water) to 100 mL of filtered sea water containing the ovules. Fecundation was confirmed by visualizing of the fertilization membrane. The eggs were counted in a chamber using a dissecting microscope at 40X, and the concentration was adjusted to 10000 eggs mL^{-1}.

Test procedures – Microscale method

In the new proposed method, non-treated sterile polystyrene 24-well tissue culture plates with lids (TPP, Switzerland) were used as test chambers. After the fertilization, as described above, 500 eggs (in a maximum volume of 50 µL) were added to each well. The final volume per well of the tested sample was 2.5 mL. The plates were then were kept under a 12:12 h light–dark cycle (lights on at 06:00 h) at 25 ± 2ºC. After 24 hours, a 10 µL aliquot was taken to verify the embryos development. When the control showed at least 80% of well-developed pluteus larvae (26 ± 2 hours), 125 µL of formaldehyde were added to each well for fixation of the embryos.

Test procedures – standardized method (CETESB)

In the standardized method, 15 mL glass tubes were used as test chambers carrying 10 mL of each sample. After the fertilization, 300 eggs (in a maximum value of 100 µL) were added. The test tubes were kept under a 12:12 h light–dark cycle (lights on at 06:00 h) at 25 ± 2ºC during 24 hours. After this interval, aliquots were taken to verify the embryos development. When the control showed at least 80% of well-developed pluteus larvae (26 ± 2 hours), the content of each test tube was transferred to a glass tube containing 0.5mL of formaldehyde for fixation of the embryos.

Statistical analysis

According to USEPA (2002), three experimental replicates were used for each dilution and for control tests. Seven and six experiments were executed simultaneously using both methods for zinc and SDS, respectively, in order to use the same pool of gametes.

One hundred eggs or embryos were counted for each concentration of test substance to obtain the percentage of normal larvae. Data were analyzed as means ± standard deviation (SD). The Effective Concentration to affect 50% of tested organisms (EC_{50}) and their confidence intervals were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). The EC_{50} values obtained for tested substances in the present study using both methods were compared using Student's T test with 5% significance level. Further, EC_{50} values obtained in the present study were compared to those described in the literature for the same species and substances using one-way ANOVA followed by Student Newman Keuls, also with 5% significance level.

Results and discussion

In the present study, some modifications in the short-term chronic toxicity test with *L. variegatus* embryos standardized by Brazilian environmental authorities (CETESB 1999, ABNT 2006) were proposed to accommodate the test in a microscale using non-treated sterile 24-well microplates.

Firstly, the development of normal pluteus larvae using 24-well microplates was compared to that obtained by the standardized method, and the Figure 1 shows control data obtained using both methods. No significant differences were observed between the mean normal embryonic development rates ($p > 0.05$); the mean value of normal pluteus was 88.5 ± 0.9 % (coefficient of variation = 8.7%, n=75) in the 24-well assays, and 90.3 ± 0.9 % (coefficient of variation = 6.3%, n= 39) in the standardized method. The mean normal development rates of both methods were above 80%, which is one of the acceptability criteria for the sea-urchin embryos test (CETESB 1999, Prósperi & Araújo 2002, ABNT 2006), demonstrating the data reliability. Moreover, the low coefficient of variation indicated a good reproducibility of the 24-well assay.

Dinnel *et al*. (1987) and Lera *et al.* (2006) argued that plastic dishes might have a toxic effect due to the leaching of chemicals from newly manufactured plastic on the sea urchin spermatozoa, leading to a low fertilization success. However, further studies conducted by Vaschenko *et al*. (1999) demonstrated that there was no significant difference in the fertilization rates of experiments running in

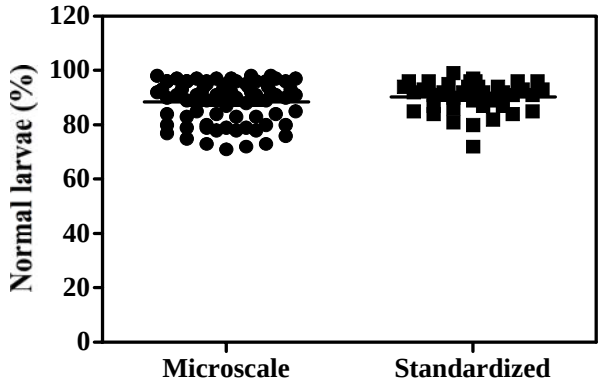

Figure 1. Development of normal pluteus larvae using the microscale (24-well microplates) and the standardized method (glass tubes) under control conditions.

parallel in both plastic plates and small glass beakers. It is worthwhile mentioning that, in the present study, the fertilization was performed in a glass beaker, and only after confirmed fertilization that the eggs were transferred to the 24-wells microplates.

The sensitivity of exposed embryos was assessed by comparative studies on the toxicity of SDS and zinc using both microscale and standardized methods. The results are presented as the obtained EC_{50} (mean ± 2SD) in Figure 2. The mean EC_{50} values for SDS were 3.35 ± 0.78 and 2.89 ± 0.58 mg L^{-1} using the microscale and standardized methods, respectively, whereas, for zinc, it was 0.07 ± 0.02 mg L^{-1} regardless of the method used (Figure 2). It is worthwhile mentioning that there was no difference in the statistical analysis when only the experiments conducted in parallel were considered.

Data obtained in the present study were in good agreement with those obtained from literature using the standardized method with the same species, as shown in Table I. PhD. Denis Abessa (personal communication) compared the sensibility of three *L. variegatus* populations collected at different beaches, Ubatuba, Santos and São Sebastião, all located on the coast of the state of São Paulo, and demonstrated that the EC_{50} values ranged from 0.04 to 0.06 mg L^{-1} for zinc, and 1.35 to 2.50 mg L^{-1} for SDS. Mastroti (1997) obtained values for SDS toxicity in the range of 1.52 to 2.70 mg L^{-1}, with a mean value of 2.06 ± 0.38 mg L^{-1}. On the other hand, Prósperi (2002) obtained EC_{50} values for zinc in the range of 0.05 to 0.12 mg L^{-1}, with a mean value of 0.07 ± 0.02 mg L^{-1}. Statistical comparison among EC_{50} values revealed significant differences within values obtained for SDS using the standardized method, considering these from the present work and those obtained in literature, and

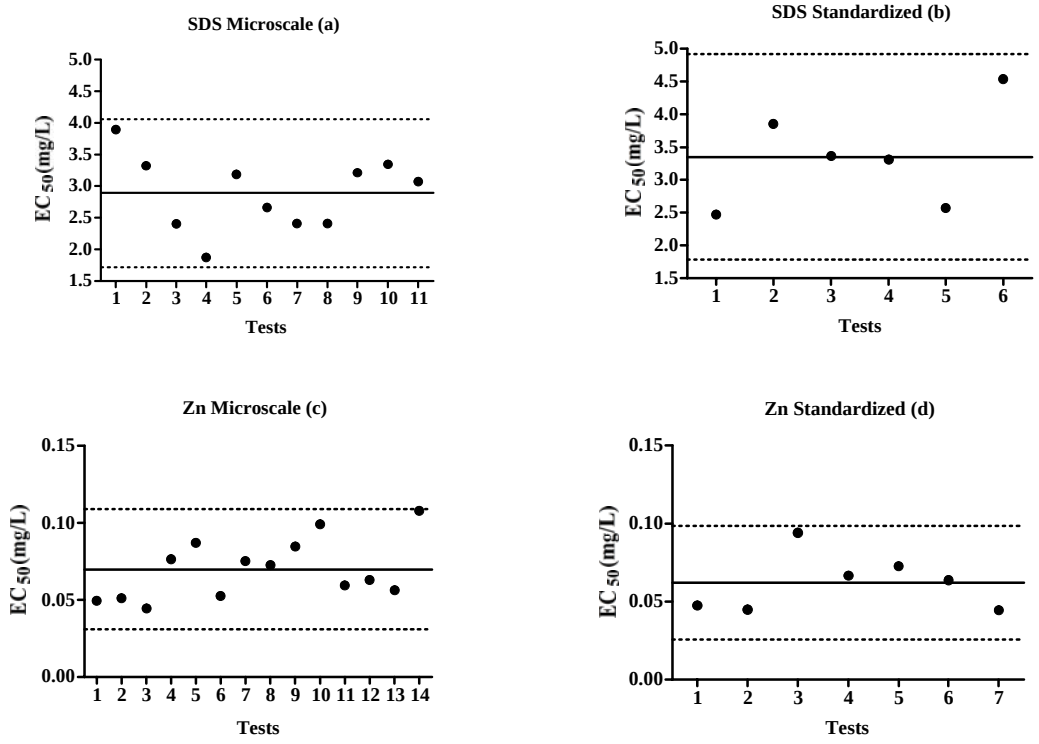

Figure 2. Control-charts for two reference substances (SDS and Zn), showing the mean EC_{50} values and the upper and lower intervals obtained from the short-term chronic toxicity test with *Lytechinus variegatus* embryos using microscale and standardized methods. Figures 2a and 2c: data for SDS and Zn, obtained from 11 and 14 microscale tests, respectively. Figures 2b and 2d: data for SDS and Zn, from 6 and 7 standardized tests, respectively.

Table I. Mean EC_{50} values ± SD obtained for the reference toxicants sodium dodecyl sulfate (SDS) and Zinc (Zn) in the short-term chronic toxicity test with embryos of *Lytechinus variegatus*. Number of tests (n) performed are shown in parentheses.

EC $_{50}$ value mg L^{-1} (n)		Chamber	Reference
SDS	Zn		
2.06 ± 0.38 (6)	-	Tube (10 mL)	Mastroti 1997
1.91 ± 0.82 (4)	0.04 ± 0.01 (4)*	Tube (10 mL)	[a] PhD. Denis Abessa (pers. comm.)
1.35 ± 0.69 (4)	0.06 ± 0.02 (4)	Tube (10 mL)	[b] PhD. Denis Abessa (pers. comm.)
2.50 ± 0.96 (3)	0.05 (1)	Tube (10 mL)	[c] PhD. Denis Abessa (pers. comm.)
-	0.07 ± 0.02 (9)	Tube (10 mL)	Prósperi 2002a
3.35 ± 0.78* (6)	0.07 ± 0.02 (7)	Tube (10 mL)	This study
2.89 ± 0.58 (11)	0.07 ± 0.02 (14)	Microscale (2.5 mL)	This study

[a] Animals collected in Santos, São Paulo.
[b] Animals collected in Ubatuba, São Paulo.
[c] Animals collected in São Sebastião, São Paulo.
* $p < 0.05$, ANOVA followed by Student Newman Keuls.

the EC_{50} obtained by PhD. Denis Abessa (personal communication) for zinc using animals collected at Santos and all other EC_{50} values, including these from the present work. Despite the statistical significance, the biological relevance of these differences should be looked very carefully since all values are in the same range.

Another important point that should be addressed in the design of an ecotoxicological method is biomass availability (embryo densities), especially in the evaluation of environmental samples and hydrophobic organic contaminants (Evans & Nipper 2007, 2008). In fact, the number of exposed embryos is a controversial issue, and it is far to be homogeneous considering different standardized protocols. Table II shows the number of exposed embryos of different sea urchin species used by several authors. This number may range from 17.5 embryos mL^{-1} (Roepke *et al*. 2005) to 400 embryos mL^{-1} (Carr *et al*. 2000). The experiments for the proposed microscale method were performed with 200 embryos mL^{-1} in each well, instead of 30 embryos mL^{-1}, as recommended in the Brazilian standardized method (CETESB 1999, ABNT 2006). According to Evans & Nipper (2008), the biomass interfered with the identification of the toxicity of chemicals with higher hydrophobicity, and, moreover, the lower biomass may increase the uptake of chemicals, allowing a critical body residue capable of causing toxic effects. It is worthwhile mentioning that the microscale method was only evaluated for SDS and zinc, toxicants that present a good solubility in water. To accurately evaluate the effects of biomass increase, assays with serial dilutions of biomass using chemicals with variable hydrophobicity, and also environmental samples, should be performed to establish the critical conditions to increase the sensitivity of the proposed method and its environmental significance.

Despite the intrinsic variation in the sensitivity of tested organisms from different populations, variability in toxicity test can be described in terms of two types of precision: intralaboratory and interlaboratory precision (Rand *et al*. 1995, Zagatto 2006b). Intralaboratory precision is a reflection of (1) the ability of trained personnel to obtain consistent results repeatedly when performing the same test on the same species using the same chemical, (2) test organism condition and sensitivity, (3) dilution water quality and (4) temperature control (USEPA 1995). In the present work, the obtained coefficients of variation ranged from 20.22% for SDS using the microscale method to 29.40% for zinc using the standardized method. According to USEPA (1995), the coefficient of variation for the EC_{50} values were 22% and 39% to indicate an acceptable intralaboratory and interlaboratory precisions, respectively. These values were calculated using cooper as a reference toxicant and *Strongylocentrotus purpuratus* as the test species. Considering data for the short-term chronic toxicity test using *L. variegatus*, the coefficients of variation ranged from 25.5% (for animals collected in Santos (PhD. Denis Abessa, personal communication) to 31.0% (Prósperi 1993) using zinc as a reference toxicant, and from 18.8% (Mastroti 1997) to 51.4% (for animals collected in Ubatuba,) using SDS. Thus, the variations observed in the present study are in accordance with the literature.

In conclusion, the modifications in the short-term chronic toxicity test using *Lytechinus variegatus* embryos proposed in the present paper were considered acceptable for the types of chemical

Table II. Number of sea urchin embryos from different species used in the short-term chronic toxicity test.

Species	Number of embryos mL^{-1}	Reference
Paracentrotus lividus	30	Bellas *et al*. 2005
	300	Lera & Pellegrini 2006
Arbacia punctulata	400	Carr *et al.* 2000
	50	Evans and Nipper 2007
	50, 100, 200 and 400	Evans and Nipper 2008
Lytechinus variegatus	30	CETESB 1999
	200	This study
Strongylocentrotus purpuratus	17.5	Roepke *et al*. 2005

use in the experiments, since the normal viability of embryos were not altered and the sensitivity for reference toxicants, SDS and zinc, were in agreement with data obtained using the standardized method. We believe that such modifications improved this test, since it has a reduced cost (plastic microplates are generally less expensive than glass tubes), a reduced water consumption and minimization of contamination problems due to poor handling of glassware. However, much effort on the validation of the microscale method should be done, especially to access its applicability considering hydrophobic chemicals and environmental samples.

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