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Toxicity Testing with Embryos of Marine Mussels: Protocol Standardization for *Perna perna* (Linnaeus, 1758)

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Toxicity tests using bivalve embryos have been widely employed since the 1960's for the evaluation of biological effects of contaminants to marine organisms (Davis 1961; Calabrese 1972, Granmo 1972; Armstrong and Milleman 1974). The American Society for Testing and Materials published standard testing procedures for bivalve embryo toxicity tests using two species of oysters and two species of mussels occurring in the temperate region of North America (ASTM 1992). Those standardized tests provide sensitive, rapid and low cost toxicity assessment, and therefore are widely used in water quality assessment of effluents and other environmental samples, as well as for toxicity characterization of chemical pollutants (Chapman et al. 1992; Fichet et al. 1998; His et al. 2000).

Marine ecotoxicology studies are relatively recent in Brazil (Abessa et al. 1998); therefore only a few methods for toxicity testing using native species have been published (Nipper 1998). Standardized procedures for water-column toxicity testing are available for sea-urchin embryos (CETESB 1999), mysids (CETESB 1992), mangrove oysters (Nascimento 2002), and calanoid copepods (Nipper et al. 2002) as test species. The Brazilian shore is about 8,000 km long, and comprises a wide diversity of coastal habitats, most presently facing acute or chronic chemical contamination stress. Adequate assessments of contaminated coastal sites in Brazil require the development of additional standardized toxicity tests to provide more options to researchers and decision-makers.

The objective of the present study was to adapt the marine bivalve embryo standard testing methods developed for temperate species (ASTM 1992) for use with the tropical mytilid *Perna perna*, and to provide preliminary data of test performance using select contaminants.

The brown mussel *P. perna* occurs on rocky reefs, forming dense colonies at the low- and inter-tidal levels, especially in high hydrodynamic sites. This organism is economically important in the Southern coastal region of Brazil, representing an increasingly relevant food resource for coastal populations. Its exploration supports several economic activities, such as harvesting, culturing, processing and trading. *Perna perna* are similar to other mytilids (His et al. 2000) in several aspects of their biology and ecology (Lunetta 1969). However, detailed knowledge on the biology of *P. perna*, such as seasonal changes in fecundity, sensitivity of embryos to physical-chemical factors such as salinity, temperature

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and chemical pollutants, must be investigated for the proper use of this species in ecotoxicological studies.

MATERIALS AND METHODS

About 100 adult individuals were collected from rocky reefs in Ponta Grossa, Ubatuba, SP, Brazil (45°06'20"W, 23°29'40"S) and transported to the laboratory. Freshly collected organisms were rinsed and freed of incrustations. Gamete release was by thermal induction or biological stimulation.

Uncontaminated natural seawater was collected off Ilha Anchieta, Ubatuba, filtered through a 0.22 μm Millipore membrane, and left under aeration for at least 4 hours for use as control and dilution water. All the assays were conducted in glass test tubes containing 10 mL of test water or contaminant solution. Five replicates were used for each treatment. The experiments lasted about 48h, the time necessary for the embryos to develop from egg to D-larvae. At the end of the exposure period, embryos were fixed by the addition of 0.5 mL 4% neutralised formaldehyde. Larvae were observed under a stereomicroscope using a Sedgewick-Rafter chamber. Larvae were classified as normal or abnormal according to ASTM (1992) guidelines. Normal larvae are D-shaped with discernable internal visceral content and closed shelves. The minimum acceptable rate of normal development for the controls was 70% (ASTM 1992).

In vitro fertilization success depends on the proportion between sperm cells and ovules. Adequate sperm-to-ovules ratios should provide fertilization of all the eggs while minimizing the incidence of poly-spermatic eggs. To evaluate the influence of sperm cell densities on fertilization success of *P. perna*, the density of ovules was kept constant at 35 cells mL^{-1}, which was considered proper to allow fertilization in other mytilids (Cherr et al 1990). Sperm cells were at 3 different densities and added to the test tubes containing the ovules.

The density of sperm cells in the sperm solution was estimated by diluting the full strength sperm water in filtered seawater (1:99). Target sperm densities were 10^2; 10^3; 10^5 sperm cells per ovule, according to recommendations of ASTM (1992) and Cherr et al (1990). Actual densities were determined using a Fucus-Rosental haemocyte meter. Fertilization was determined by the presence of any cell division or embryological development after 24 h, at 25°C.

The density of eggs in the test tube has potential to influence larval development under laboratory conditions, especially due to dissolved oxygen consumption and metabolite excretion (Bayne 1976). Thus, an experiment was conducted in order to evaluate this influence and establish the most adequate density which allows a normal development rate greater than 70%. This assay consisted exposing new fertilized eggs to clean seawater in the following densities: 10, 20, 30, 40, 50 and 60 eggs/mL^{-1}. This experiment was conducted at 25 ± 2°C and had 48h duration.

Physical variables, such as temperature and salinity, may influence larval development. Optimal water quality conditions should be maintained when conducting toxicity tests to avoid confounding factors on the test outcome. Two experiments were performed to assess the effects of different combinations of salinity and temperature on egg development. In the first experiment, eggs were

exposed to different salinities (15, 20, 25, 30, 35 and 40‰) at 20, 25 and 30°C. An additional assay was then conducted using a smaller range of salinities (25, 30, 35 and 40‰) and range of temperatures (15, 20 and 25°C).

The sensitivity of the *P. perna* embryo assay relative to other water-column toxicity tests was evaluated using select chemicals widely used as reference toxicants, the detergent sodium dodecil sulphonate (SDS) and potassium dichromate ($K_2Cr_2O_7$). Five tests were conducted for each substance to allow the establishment of performance charts. Five SDS concentrations were prepared (0.32; 0.63; 1.25; 2.50; 5.00 mg/L^{-1}) by diluting a 100 mg/L^{-1} stock solution in filtered seawater. For the $K_2Cr_2O_7$, a range-finding test was conducted using a wide range of dilutions (0.1, 0.5, 1.0, 10.0, 50 mg/L^{-1}). In the definitive experiments six concentrations were used (1.50, 3.12, 6.25, 12.50, 25.00, 50.00 mg/L^{-1}).

The results of the experiments with sperm cells and egg densities were analysed by one-way analysis of variance followed by Tukey's pair-wise comparison tests. The results of the salinity and temperature experiments were analysed using the Student Newman Keuls multiple amplitude test at each individual temperature among salinities. For the tests with contaminants, the 48h-effects concentration (48h-EC50) for SDS and $K_2Cr_2O_7$ were estimated using the Trimmed Spearman Karber method (Hamilton et al 1977) after Abbott's correction. For both contaminants, the mean and standard deviation of the individual EC50 values were calculated, as well the coefficient of variation (CV). Then, the preliminary sensitivity zone for this species was estimated by using the mean EC50 ± 2CV.

RESULTS AND DISCUSSION

Chemical, physical or biological methods have been employed to induce spawning in marine bivalves (ASTM 1992). For *P. perna*, the thermal induction method was successful as it produced spawning in more than 80% of the mussels. The combination of thermal and biological methods has also proved adequate for inducing spawning in *P. perna*, whereas the biological stimulation alone did not produce effective spawning.

All the sperm cell densities tested produced fertilization in 100% of the exposed ovules. Cherr et al (1990) recommend the use of more than 100 sperm cells per ovule to reach fast fertilization and thus ensure age homogeneity among embryos.

No significant differences in larval development success were observed among different egg densities, (Fig 1). However, larval counting is more laborious at low densities; of the sizes between 100 and 113μm, therefore the use of densities greater than 30 $eggs/mL^{-1}$ is encouraged. In the first experiment, normal larval development rate greater than 70% occurred only at 35‰ salinity and 20-25°C. Larval development did not occur at 30°C (Table 1) and at salinities 15 and 20‰. The second experiment produced a similar result (Table 2), and acceptable larval of development occurred only at 35 and 25°C. Larval development at low temperatures (15 and 20°C) was significantly lower than at 25°C.

Table 1. Percent of normal larvae at various combinations of salinity and temperature Experiment 1 (standard deviations in parenthesis). The same symbols represent no significant estatistical difference (α= 0,05)

	Percent of Normal Larvae		
Salinity (‰)	**20 °C**	**25 °C**	**30 °C**
15	0	0	0
20	0	0	0
25	8.6# (6.3)	20.6* (5.0)	0
30	65.0* (9.8)	49.3# (9.8)	0
35	77.4* (5.5)	80.4* (7.8)	0
40	39.3* (3.4)	29.5* (5.9)	0

Table 2. Percent of normal larvae at various combinations of salinity and temperature (α= 0,05) Experiment 2 (standard deviations in parenthesis).The same symbols represent no significant estatistical difference (α= 0,05)

	Mean Percent of Normal Larvae		
Salinity (‰)	**15 °C**	**20 °C**	**25 °C**
25	0#	5.0 #/*(3.2)	10.8*(10.0)
30	0.4#(0.9)	60.4* (11.0)	68.6* (9.0)
35	3.0§ (5.7)	63.2# (6.4)	72.0* (3.8)
40	0§	41.6#(11.2)	63.3* (13.1)

According to Bayne (1976), lamellibranches embryos require a narrow range of salinity for normal development compared to later life-stages and larval development rate is temperature-dependant. Tolerance to varying salinities is suspected to be genetically influenced. Embryos from adults living in salinity areas of varying salinity tend to be more tolerant to salinity changes than embryos from animals inhabiting less variable areas.

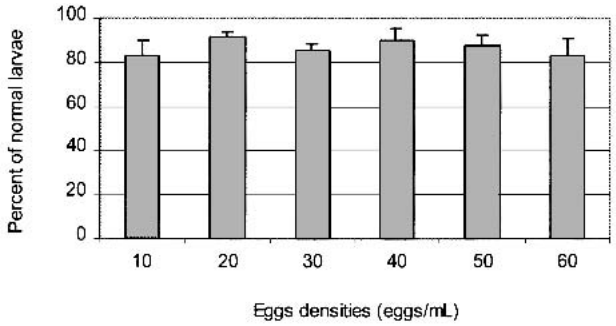

Figure 1. Percent of normal larval of *P. perna* at different eggs densities.

The mussels used in this study were collected from a site with low salinity variation (i.e., 33 to 36‰), therefore explaining the low tolerance of the embryos to low salinities. Our results indicate that the optimum conditions for larval development, and consequently the conditions recommended for conducting toxicity testing, are 25°C and 35‰ salinity.

Potassium dichromate and SDS are frequently used as reference substances in toxicity testing using aquatic invertebrates (Rand 1995). The 48h-EC50 obtained for both contaminants are shown in Table 3. *Perna perna* embryos were more sensitive to SDS than to potassium dichromate, and the respective mean 48h-EC50 values were 1.72 ± 1.07 mg/L^{-1} and 16.45 ± 3.24 mg/L^{-1}.

According to Rand (1995), some desirable characteristics for references substances are low variability among tests and the production of detectable effects in short-term exposures. In the present investigation, potassium dichromate produced less variable results, whereas SDS produced substantially more variable results.

Table 3. Individual and mean 48h-EC50 values obtained for SDS and potassium dichromate (mg/L^{-1}).

	SDS	**$K_2Cr_2O_7$**
Test 1	1.30	20.89
Test 2	3.57	12.70
Test 3	1.70	17.15
Test 4	1.09	17.59
Test 5	0.96	13.93
Mean ± Standard deviation	1.72 ± 1.07	16.45 ± 3.24
Coefficient of variation (%)	62.03	19.68

The mean EC50 value for *P. perna* embryos exposed to SDS was similar to the values reported for sea urchin (*Lytechinus variegatus)* embryos (EC50-24h = 1.35 – 2.5 mg/L^{-1}) (Abessa et al. 2002) and the range of sensitivity of *P. perna* embryos to potassium dichromate was within the range estimated for the amphipod *Tiburonella viscana* (Abessa and Sousa 2003).

Using the EC50 mean values and their respective standard deviations, it was possible to establish initial levels of sensitivity to the selected reference substances, as shown in Table 4. The 48h-EC50 values outside those intervals may indicate unusual embryo sensitivity and therefore non-recommended use in toxicity tests. The interval calculated for SDS was too broad and included a negative value, thus it should not be used for further comparisons. Therefore, only potassium dichromate is recommended as a reference substance when conducting toxicity tests with *P. perna* embryos.

Table 4 – Sensitivity intervals calculated to *Perna perna* embryos.

Sensitivity level	Concentration (mg*L^{-1})	
	SDS	**Potassium dichromate**
Lower level	-0.41 (0.00)	9.98
Upper level	3.86	22.93

Overall, the ASTM (1992) method for toxicity testing using bivalve embryos was considered adequate for use with *P. perna.* However, a few recommended modifications in the ASTM recommendations are proposed in Table 5 as a result of this study.

The embryo toxicity testing using *P. perna* proposed in this study is rapid, inexpensive, simple and apparently has similarly sensitive to other tests currently used in Brazil. In addition, brood stocks are easily collected along the coast. Therefore, the routine use of this test in ecotoxicological evaluations conducted along the southern coast of Brazil in recommended.

Table 5. Toxicity test procedure recommendations for using *Perna perna* and for North American mussels according to ASTM (1992).

	Test using *Perna perna*	**ASTM (1992)**
Method for spawning induction	Physical or physical + biological	Chemical, physical and/or biological
Volume of sample	10 mL	From 1-2 to 10-30 mL
Sperm cell density per ovule	$>10^3$ (1-2 mL sperm solution)	10^5 to 10^7
Eggs density (eggs mL^{-1})	30 to 60	15 to 100
Temperature (°C)	25 ± 2	16 to 25
Salinity	35	18 to 32 ± 1

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