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Development of a new integrative toxicity index based on an improvement of the sea urchin embryo toxicity test

L. Morroni [a,b,1], A. Pinsino [c,1], D. Pellegrini [b], F. Regoli [a], V. Matranga [c,*]

[a] Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy
[b] Istituto Superiore per la Protezione e la Ricerca Ambientale, Livorno, Italy
[c] Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy", Consiglio Nazionale delle Ricerche, Palermo, Italy

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ABSTRACT

The sea urchin embryo toxicity test is classically used to assess the noxious effects of contaminated marine waters and sediments. In Italian guidelines on quality of dredged sediments, the standard toxicity criteria used for this assay are based on a single endpoint at 48 hours of development, corresponding to the pluteus stage. Different typologies of abnormalities, including those which occur at earlier stages, are not categorized, thus preventing the evaluation of the actual teratogenic hazards. A new integrative toxicity index has been developed in this study based on the analysis of two developmental stages, at 24 and 48 h post-fertilization, and the differentiation between development delays and germ layers impairments: the new toxicity index is calculated by integrating the frequency of abnormal embryos with the severity of such abnormalities. When tested on dredged sediments, the evaluation of increasing levels of toxicity affecting embryonic outcomes enhanced the capability to discriminate different samples, appearing particularly relevant to validate the sea urchin embryo toxicity assay, and supporting its utility in practical applications such as the sediments classification in harbor areas.

1. Introduction

The sea urchin embryo-toxicity test is worldwide used as a reliable, sensible and inexpensive tool to assess the toxicity of a large range of pollutants and natural matrices. From the initial studies on sensitivity of various developmental stages of sea urchins to chemicals (Kobayashi, 1980), most recent investigations still confirmed the responsiveness of this embryo development to metals (Arizzi Novelli et al., 2003; Russo et al., 2003; Pinsino et al., 2010, 2011a, 2014; Moureaux et al., 2011), pharmaceuticals (Graillet and Girard, 1994; Aguirre-Martínez et al., 2015), biocides (Pesando et al., 2003; Marín et al., 2007), X-ray and UV radiation (Matranga et al., 2010, Bonaventura et al., 2006, Bonaventura et al., 2011, Russo et al., 2014), and also complex environmental matrices such as marine waters and sediments (Kobayashi and Okamura, 2004; Beiras et al., 2003a, 2003b; Volpi Ghirardini et al., 2005; Cesar et al., 2009; Khosrovyan et al., 2013). Most of these studies apply protocols for embryo-toxicity testing based on standardized methods (ASTM, 1995; USEPA, 1995; Carr, 1998; Environment Canada, 2011). Slight differences in the characteristics and duration of the assay may depend on the exposed sea urchin species and laboratory conditions, including the incubation temperature. For the Mediterranean species, *Paracentrotus lividus*, the protocol typically consists in the exposure of sea urchin eggs (about 300 eggs/mL) to a contaminated solution and in the morphological observation of 100 randomly chosen embryos at a developmental endpoint of 72hours (at 18°C), corresponding to the pluteus stage (Arizzi Novelli et al., 2006). Embryos are conventionally classified in two major categories of biological quality, normal and abnormal, and the percentage of abnormal embryos in exposed groups is compared to the control frequency.

The most common effects observed in the development of sea urchin embryos exposed to classical and emerging toxicants are a general delay in the developmental schedule and/or the impairment of the correct differentiation of the so called germ layers, namely ectoderm, mesoderm, endoderm, and/or their derivatives (Hardin et al., 1992; Livingston and Wilt, 1989; Timourian, 1968; Russo et al., 2003; Bonaventura et al., 2005, 2006, 2015; Aluigi et al., 2012; Kiyomoto et al., 2010; Matranga et al., 2010, 2011; Pinsino et al., 2011b)

A general limit of embryo-toxicity test on sea urchin is that developmental analyses are carried out only at the pluteus stage, without distinguishing between different morphotypes occurring at stages before pluteus, thus preventing to understand if embryos exhibits malformation, block or delay of embryogenesis. Although

* Corresponding author.
E-mail address: Matranga@ibim.cnr.it (V. Matranga).
[1] Equally contributed to this study.

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alteration on embryo morphology have been extensively described, these effects have been rarely considered to develop a standardized scale of toxicity assigning a different weight to various embryonic malformations depending on their severity. Only Carballeira et al. (2012) proposed to calculate the percentage of abnormal larvae at 48 h (at 20° C) of exposure, classifying embryos in 4 classes with increasing toxicity on the basis of their observed skeletal malformation. The evaluation and quantification of the different adverse effects on embryo morphology and development could greatly increase the sensitivity of the assay and its capability of discriminate from slight to severe levels of embryo-toxicity.

The embryo-toxicity test with *Paracentrotus lividus* is frequently included in the batteries of ecotoxicological tests used to evaluate the sediments quality in Italian harbor areas (APAT-ICRAM, 2007) and, according to well established and validated protocols (Bay et al., 1993; Beiras et al., 2003a, 2003b; Khosrovyan et al., 2013), the assay is applied to sediment elutriates. These batteries should include at least 3 bioassays measuring different biological endpoints in species representative of various trophic levels (APAT-ICRAM, 2007). Although weighted criteria have been developed to integrate the results of the whole battery considering specific thresholds, biological endpoints and exposure conditions of different assays, regulatory guidelines on dredged sediments still rely on the "worst" result to classify the ecotoxicological potential of a sample. In this respect, the elevated responsiveness of the sea urchin embryo-toxicity test to factors other than environmental pollutants (i.e. elevated content of organic matter or ammonia) might negatively affect the classification of sediments quality.

With the aim to enhance the capability of the sea urchin embryo-toxicity test to discriminate the ecotoxicity of sediments, a new toxicity scale has been developed in this study associating different values at each morphotype in relation with germ layer specific malformations and developmental stage. Tests were performed on elutriates obtained from representative sediments samples of the Trapani harbor which, in the framework of a larger project, was chosen as a model case-study for the application of an integrated, multidisciplinary Weight Of Evidence (WOE) approach to improve criteria of sediment quality characterization, and resulting management options (Piva et al., 2011; Benedetti et al., 2012).

The overall results were expected to improve the capability of the *Paracentrotus lividus* embryo-toxicity test to discriminate different samples, moving from the simple observation of general effects to the classification of their severity; this approach should allow to better categorize the teratogenic potential of marine sediments and their quality classification through a more sensitive and realistic toxicity index.

2. Materials and methods

2.1. Sediment sampling and elutriate preparation

Sediments were collected in June/July 2012 during a large characterization and monitoring projects in the Trapani harbor. Elutriates from 24 representative sediment samples were prepared in accordance with USEPA (1991) guidelines and literature studies (Volpi Ghirardini et al., 2005). The sediment samples were mixed in a 1:4 (v/v) ratio of sediment to water and placed on a rotary shaker table for 1 h, at a speed of 300 rpm, at room temperature. The dilutions were made up with 0.22 μm filtered sea water (FSW) collected in a long-term monitored reference site located far from human activities. After mixing, the samples were centrifuged for 20 min at 3000 rpm (4 °C) and the aqueous fractions (elutriate samples) were poured off and stored for 30 days at −20 °C until use for the toxicity tests.

2.2. Sea urchin embryo toxicity test

Adult sea urchins (*Paracentrotus lividus*) were collected during the breeding season by SCUBA divers along the North-Western coast of Sicily (Italy). After collection sea urchins were transported in an insulated plastic container to the laboratory and immediately used to obtain eggs and sperms. Toxicity tests were performed in accordance with adapted U.S. Environmental Protection Agency guideline (USEPA, 1995). Gametes from at least three females and three males were collected from gonads and pooled prior to fertilization as reported by Bonaventura et al. (2003) and Russo et al. (2014). The sperm was collected dry (directly from the surface of the sea urchin) using a micropipette with the end of the tip cut off, and maintained in a sealed container at 4 °C. The eggs were placed in FSW, washed three times and used for bioassays within 30 min of collection. 10 μl of dry sperm were diluted in 50 ml of FSW and added to 300 ml of egg suspension diluted to 20,000 eggs/ml (sperm/egg ratio 50:1). After fertilization, embryos were maintained in 60 mm Petri dishes (10 ml per dish, corresponding to about 2×10^4 embryos per dish) at a temperature of 18 ± 2 °C. Embryo cultures were exposed from fertilization to the pluteus stage (48 h post-fertilization) to 24 different elutriate samples prepared as described above. According to guidelines on dredged materials (APAT-ICRAM, 2007), three replicates were performed for each elutriate sample. Morphological evaluation was performed on live embryos: to this purpose, groups of 20 embryos were collected and immediately inspected at the 24 h and 48 h endpoints by optical microscopy (Zeiss Axioskop 2 Plus microscope) and photographed by a digital camera.

2.3. Toxicity criteria

The toxicity of elutriate samples from Trapani harbor was determined by calculating the percentage of normal embryos at gastrula (24 h) and pluteus (48 h) stage, according to both standard criteria and a new integrative index of toxicity. The standard criteria evaluate the percentage of normal and abnormal embryos without considering different typologies of malformations or the phase in which they appear. Embryos are classified as normal if they satisfy the following morphological criteria: (1) correct schedule in reaching the developmental endpoint (gastrula at 24 h and pluteus at 48 h), (2) left/right and dorso/ventral embryonic axis symmetry, (3) differentiation of oral/aboral ectoderm and endoderm territories, (4) skeleton development and patterning (for a detailed description see Henry, 1998; Pinsino et al., 2010). The new integrative toxicity index (ITI) is based on a detailed analysis of development delays and germ layer impairments, i.e. the inability to properly differentiate ectoderm, mesoderm and endoderm territories. The toxicity is quantified by counting the frequency of delayed and/or abnormal embryos morphologies and by quantitatively ranking the severity of effects from 0 (none) to 10 (high). Embryos displaying impairment of the left/right or dorso/ventral axis symmetry, as well as oral/aboral ectoderm, mesoderm and endoderm territories have been scored in this study as abnormal, without subcategories distinctions. Anomalies are classified as delays in development with or without further morphological alterations, and assigned with an increasing weight depending on the severity and the stage at which they appear after 24 or 48 h from fertilization. Lower toxicity values were given to embryos with delay in development and absence of malformations; higher scores were attributed to embryos showing contemporarily delayed and malformed morphotypes. Different scales and scores were tested by comparing with expert judgment the calculated indices with morphologic observations. Details about selected scores and stages of development are given in Table 1 that illustrates delayed and abnormal morphotypes, as compared to