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Reversibility of trace metals effects on sea urchin embryonic development

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ABSTRACT

The sea urchin embryo-toxicity test is widely used to assess the toxicity of contaminants and environmental matrices. In standard guideline and literature studies, the classical toxicity criteria are based on distinguishing between normal and abnormal embryos at pluteus stage. The aim of this research was to further expand the potentiality of the recently developed Integrative Toxicity Index (ITI), investigating the reversibility of the effects induced by various trace metals (cadmium, copper, lead and zinc) on sea urchin development. For this purpose, embryos were observed after different periods of exposure and recovery to metals. Results were analysed comparing ITI with standard criteria, thus moving from the simple observation of general effects to the classification of their severity. The onset and reversibility of effects by trace metals were more efficiently discriminated by the use of the ITI, which recognized and weighted the delay and degree of various abnormalities. Above, this study was expected to provide new insights into the capability of each metal to induce anomalies leading to a block or delay in embryogenesis of the embryos to recover normal development after metal exposure, thus adding further ecological value to the sea urchin bioassay.

1. Introduction

Urban and industrial activities in coastal areas introduce significant amounts of pollutants and metals certainly represent one of the classes of major concern for their toxicological potential toward marine organisms. Many biomonitoring studies and normative guidelines integrate the analyses on chemical characterization of abiotic matrices with the assessment of their effects at different levels of biological organization, from molecular changes up to community disturbance. The use of ecotoxicological bioassays is a widely recognized approach to evaluating toxicological endpoints at organism level, using a wide selection of biological endpoints and species of different trophic levels in standardized conditions. Bioassays with embryos of marine invertebrates are routinely used to assess the ecotoxicological quality of environmental matrices, and the sea urchin is one of the most sensitive and widely used choices, highlighting the teratogenic effects of trace metals from the elutriate of marine sediments or seawater (His et al., 1999; Beiras et al., 2003; Kobayashi and Okamura, 2004, 2005; Khosrovyan et al., 2015; Rodríguez-Romero et al., 2016; Morroni et al., 2016).

Many authors have characterized dose–response relationships by exposing sea urchin embryos to increasing concentrations of individual metals (Warnau et al., 1996; Fernàndez and Beiras, 2001; Radenac et al., 2001; Arizzi Novelli et al., 2003; Xu et al., 2011), metal oxide nanoparticles (Manzo et al., 2013; Buric et al., 2015), mixtures of metals (Fernàndez and Beiras, 2001; Manzo et al., 2010; Xu et al., 2011) and mixtures of metals with biocides (Manzo et al., 2008). In these studies, embryos were typically classified at pluteus stage as normal/abnormal, calculating their relative percentages to estimate metal toxicity. More detailed toxic effects of xenobiotics on gametes and specific developmental stages have been investigated over several decades (Pagano et al., 1982; Graillet et al., 1993). Although these studies extensively describe alterations of embryo morphology using different experimental approaches, data coming from recovery experiments are not reported. As a consequence, the reversibility of the observed anomalies has not been established, without distinguishing between block and delay of embryogenesis. In a few old studies of perturbation experiments it was found that embryos perturbed for a short period eventually recovered skeleton development but never completely resumed their normal patterns (Hardin et al., 1992; Zito et al., 1998; Pinsino et al., 2011). Nevertheless, information on the capability of sea urchins to recover from trace metals remains scant. Moreover, the toxic effects have rarely been taken into consideration to develop a standardized scale of toxicity assigning a different weight to

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Table 1
Metal concentrations used in the experiments. Values are expressed in μg/L (left part of the panel) and using molar notations (right part of the panel).

Metal	μg/L	M
Cd	1000, 1500, 2000, 2500	8.89×10^{-6}, 1.33×10^{-5}, 1.78×10^{-5}, 2.22×10^{-5}
Cu	20, 50, 60, 70	3.15×10^{-7}, 7.87×10^{-7}, 9.44×10^{-7}, 1.10×10^{-6}
Pb	80, 100, 120, 250	3.86×10^{-7}, 4.8×10^{-7}, 5.79×10^{-7}, 1.21×10^{-6}
Zn	60, 70, 100, 120	9.47×10^{-7}, 1.10×10^{-6}, 1.58×10^{-6}, 1.89×10^{-6}

various embryonic malformations depending on their severity and reversibility during the embryonic development. While authors have proposed calculating the percentage of abnormal larvae classifying embryos based on their observed skeletal malformations or larval size (Beiras et al., 2012; Carballeira et al., 2012; Saco-Álvarez et al., 2010), a more recent study proposed a new toxicity scale associating different values to each morphotype in relation to specific malformations and developmental stage of sea urchin embryos (Morroni et al., 2016). This approach allows for a better categorization of the teratogenic potential of environmental matrices and chemicals through a sensitive and realistic integrative toxicity index (ITI), moving from the simple observation of general effects to the classification of their severity. The weighed criteria have already been validated in several environmental conditions and proposed for the revision of current guidelines (Piva et al., 2011; Benedetti et al., 2012, 2014; Regoli et al., 2014; Bebianno et al., 2015).

With the aim of further testing the potentiality of the new integrative toxicity index this study focussed on an unclear issue in literature studies, i.e. the possible reversibility of teratogenic effects described for common trace metals such as cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn). In this respect, experiments performed with embryos continuously exposed to various metals from fertilization up to the pluteus stage (72 h), were compared with treatments in which each metal was removed after 24 h, and embryos were monitored in clean seawater during the subsequent 48 h of recovery phase. Results were evaluated in terms both of ITI and of adopting the standard endpoints of normal/abnormal embryos. The overall comparison of the evaluation procedures and of results obtained from different experimental treatments was expected to provide new insights into the capability of each metal to induce anomalies leading to a block or delay in embryogenesis of the embryos to recover normal development after metal exposure, thus adding further ecological value to the sea urchin bioassay. The possibility to understand and weigh the reversibility of toxic effects could open up a new perspective to consider the impact of persistent contaminants in the marine environment, improving the ecological relevance of the embryo toxicity bioassay.

2. Materials and methods

2.1. Sea urchin fertilization and embryo toxicity experiments

Adult sea urchins (*P. lividus)* were collected during the breeding season by free divers along the southern coast of Livorno, Italy (43° 25.602′ N – 10° 23.780′ E). After collection, the sea urchins were transported in an insulated container to the laboratory and acclimatized for up to one week in flowing seawater at a temperature of 15 °C ± 1, salinity 38 and natural photoperiod. Embryotoxicity tests were performed in accordance with standard procedure (ASTM, 2012) and literature data (Volpi Ghirardini et al., 2005). Three males and three females were induced to spawn by injecting 1 ml of 0.5 M KCl into the sea urchin body cavity through the peristomial membrane surrounding the mouth. Eggs were collected by placing spawning females on 100 ml beakers with 0.45 μm filtered seawater (FSW) collected at the same site as the sea urchins. Egg quality was checked by observing the eggs under a microscope. Females with eggs not round, immature forms or debris were discarded. Sperm was collected “dry” from the gonopores of male sea urchins using a pipette and was kept on melting ice until use (< 1 h). Sperm motility was checked under the microscope and diluted sperm suspension was also added to an aliquot of egg suspension (sperm/egg ratio 50:1) in order to verify the success of fertilization (formation of the fertilization membrane with a fertilization rate > 95%). Once mobility was checked, 5 μl of sperms were diluted in 50 ml of FSW and added to 350 ml of egg suspension (1000 eggs/ml), sperm/egg ratio 50:1. After fertilization, a period of 20 min was allowed to pass before to starting the incubation with test solution. Embryos were exposed for 72 h to increasing concentrations of Cd, Cu, Pb and Zn (Table 1) and maintained in 10 ml sterile capped polystyrene six-well micro-plates (1 ml per well, corresponding to a final density about 100 embryos/ml) at a temperature of 20 °C in a dark room. Control embryos were exposed to FSW only. To evaluate the reversibility of induced effects, additional experiments were performed in which embryos were removed from metal solution after 24 h of development/exposure (gastrula stage). The same treatment was applied to control embryos in order to verify the absence of any embryo alteration due to the washing procedure. Specifically, embryos were filtered using a 55 μm nylon mesh in order to remove them from the metal solution, and then cultured in clean FSW during the subsequent 48 h of recovery phase (from 24 to 72 h after fertilization). The filter with embryos was first washed by immersion in FSW and embryos were then transferred to the new well with gentle washing in FSW. Washed embryos were compared with embryos continuously exposed from fertilization to the pluteus stage (72 h post-fertilization) (non-washed embryos). Metal-induced malformations were analysed at 24 h, 48 h and 72 h development/exposure (Table 1S). Embryos exposed to Cd, Cu, Pb, Zn were maintained in sterile capped polystyrene six-well micro-plates (1 ml per well, corresponding to about 1000 embryos) at a temperature of 20 °C in a dark room. Three replicates for each sample (100 embryos/ml) were carried out. At the end of the experiment samples were preserved by adding a few drops of 40% buffered formalin and morphological evaluation was performed.

Tests were accepted if the percentage of control embryos at 48 h of development at 20 °C (negative control) was ≥ 80% as performed in literature data Fernandez and Beiras, 2001) and recommended by standard procedure (ASTM, 2012). Reference toxicant results (continuously Cu-exposed embryos) were accepted if they fell within the laboratory acceptability ranges (between 34.6 and 68.3 μg/l) and literature data (Fernàndez and Beiras, 2001; Manzo et al., 2010) at 48 h of development/exposure.

2.2. Toxicity criteria

The degree of metal toxicity was calculated using the integrative toxicity index (ITI) (Morroni et al., 2016) and the standard criteria of evaluation based on the calculation of the percentage of normal versus abnormal embryos.

Groups of 100 embryos were analysed at 24, 48 and 72 h (h) by optical microscopy (Leica DMI3000B) and photographed using a digital camera (Leica DCF450C).

Embryos were classified as normal only when they satisfied all the following morphological criteria: (1) correct schedule in reaching the developmental endpoint, (2) left/right and dorso/ventral embryonic axis symmetry, (3) differentiation of oral/aboral ectoderm and