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Reversibility of trace metals effects on sea urchin embryonic development

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

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

*Corresponding author.
E-mail address: l.morroni@univpm.it (L. Morroni); annalisa.pinsino@ibim.cnr.it (A. Pinsino)

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. Overall, this study confirmed the enhanced capability of ITI in assessing interferences of pollutants on sea urchin development, supporting a more accurate use of this embryo-toxicity assay in various practical applications.

Keywords: *Paracentrotus lividus*, Toxicity index, Trace metals, Reversibility, Developmental anomalies

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 evaluate 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 widespread choice, which allowed to demonstrate 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 2016; Morroni et al. 2016).

Many authors have characterized dose–response relationships exposing sea urchin embryos to increasing concentrations of individual metals or mixtures (Warnau et al. 1996; Fernàndez and Beiras 2001; Radenac et al. 2001; Arizzi Novelli et al. 2003; Xu et al. 2011): 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 were investigated since several decades (Pagano et al. 1982; Graillet et al. 1993).

Although these studies extensively describes alterations of embryo morphology, such effects have rarely been taken into consideration to develop a standardized scale of toxicity assigning a different weight to various embryonic malformations depending on their severity and reversibility during the embryonic development. While Carballeira et al. (2012) proposed to calculate the percentage of abnormal larvae classifying embryos based on their observed skeletal malformations, 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 more sensitive and realistic integrative toxicity index (ITI), moving from the simple observation of general effects to the classification of their severity.

With the aim of further testing the potentiality of the new integrative toxicity index, improving the ecological relevance of the embryo toxicity bioassay, this study focussed on 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 hours), were compared with treatments in which each metal was removed after 24 hours, and embryos monitored in clean seawater during the following 48 hours of recovery phase. Results were evaluated both in terms of ITI and in terms 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 on 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 sea urchin 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 2004) 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 FSW collected at the same site as the sea urchins. 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, embryos were exposed for 72 h to increasing concentrations of Cd, Cu, Pb and Zn (Table 1). Control embryos were exposed to FSW only. To evaluate the reversibility of induced effects, additional experiments were performed in which metals were removed 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 following 48 h of recovery phase (from 24 to 72 h after fertilization). 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 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. Three replicates for each sample 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 (negative control) was ≥80%. Reference toxicant results (continuously Cu-exposed embryos) were accepted if they fell within

97 the laboratory acceptability ranges (between 34.598 and 68.344 µg/l) and literature data (Beiras and
98 Fernàndez 2001) at 48 h of development/exposure.
99
100 *2.2 Toxicity criteria*
101 The degree of metal toxicity was calculated using the integrative toxicity index (ITI) (Morroni et al.
102 2016) and the standard criteria of evaluation based on the calculation of the percentage of normal versus
103 abnormal embryos.
104 Groups of 100 embryos were analysed at 24, 48 and 72 hours (h) by optical microscopy (Leica
105 DMI3000B) and photographed using a digital camera (Leica DCF450C).
106 Embryos were classified as normal only when they satisfied all the following morphological criteria: (1)
107 correct schedule in reaching the developmental endpoint, (2) left/right and dorso/ventral embryonic axis
108 symmetry, (3) differentiation of oral/aboral ectoderm and endoderm. ITI was calculated by assigning a
109 different weight to various embryonic malformations depending on both their severity and the stage at
110 which malformations (delayed and/or abnormal embryos morphologies) appeared, quantified based on a
111 ranking of severity from 0 (none) to 10 (high). Lower toxicity values were given to delayed embryos
112 (embryos with delay in development and absence of malformations) and higher scores were attributed to
113 abnormal embryos (embryos with delay in development and malformations) with no chance to recover
114 development. The toxicity categories are shown in Table 2S and ITI is calculated as follows:
115 $$ITI = \sum^{n}_{i=10}(S_i * F_i)/100$$
116 Where S_i is the score associated to each abnormality and F_i is the frequency observed for that abnormality
117 (i=10).
118
119 *2.3 Chemical test and analysis of metals*
120 The concentrations of Cd, Cu and Zn were analysed by inductively coupled plasma optical emission
121 spectrometry (ICP-OES) and the concentration of Pb by Atomic Absorption Spectrometry (AAS). The
122 results showed that measured concentrations generally varied less than 15% from the nominal
123 concentrations (see Table 3S). Thus, all calculations were based on nominal concentrations.
124
125 *2.4 Data analysis*
126 Variations in embryo development related to metal exposure, incubation time and washing treatment
127 were examined through a permutational multivariate analysis of variance (PERMANOVA, Anderson
128 2001). The analyses were computed on a resemblance matrix, obtained by applying the Euclidean

distance index, and all observed morphotypes were considered as variables. Metal concentration, incubation time and washing were considered as fixed factors. When significant differences in embryo development were detected, additional pair-wise tests were performed. Primer v6 statistical package in conjunction with the Windows PERMANOVA + module (Anderson et al. 2008) were used to perform the statistical tests. The percentages of abnormal larvae and size inhibition were also considered to evaluate the toxic effects estimated as EC50 values. The EC values with 95% confidence limits were calculated by the Trimmed Spearman–Karber statistical method. Responses in each experimental condition were corrected for effects in control tests by applying Abbott's formula (Hamilton et al. 1978).

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}

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).

3. Results and discussion

The effects observed in embryos at various experimental conditions are reported in Fig. 1, the median effective concentration (EC50) and the values of ITI are given in Table 2 and Table 3, respectively. In general, embryos perturbed for 24 h and then cultured in FSW for the following 48 h recovered development but never completely resumed their normal patterns. Negative control embryos washed with FSW did not show any harmful effects related to the washing treatment ($t = 0.47$, $p = 0.65$).

A good concentration-dependent relationship between the four trace metals and the toxicity index was generally observed both during continuous exposures to Cd, Cu, Pb and Zn, and following the recovery period. After 48 h, washed embryos displayed toxicity index values higher than after 24 and 72 h development/exposure, with the exception of Zn-exposed embryos where the highest toxicity levels were observed at 72 h (Fig. 1, Table 2 and Table 3). Considering the multivariate PERMANOVA performed on all morphotypes used to estimate the index, the results showed a significant effect of various factors (metal, concentration, time of incubation, washing) and their interactions. The pair-wise comparison performed for the factor washing at each time level did not show significant differences at 48 h between washed and non-washed embryos ($t=0.538$, $p=0.621$), while after 72 h washed embryos showed values of normally developed embryos significantly higher than those measured in non-washed embryos ($t= 23.354$, $p=0.001$). As expected, an increase in recovery time typically raised the capability to restore

normal development (see Table 4). Specific effects depend on mechanisms by which trace metals interact with cellular components and molecular targets. In this respect, for example, an interference with calcium homeostasis and consequently Ca-dependent signalling cascades could be responsible for such metal-embryonic malformations as already demonstrated in *P. lividus* Mn-exposed embryos (Pinsino et al. 2011, 2014) and in embryos of *Strongylocentrotus purpuratus* exposed to Cu, Pb and Zn (Tellis et al. 2014a, 2014b). Cadmium (Cd) is well-known to accumulate within cells/tissues during *P. lividus* development, causing teratogenesis and stimulating apoptotic processes (Agnello et al. 2006, 2007; Filosto et al. 2008). A certain delay between exposure time and the onset of visible alterations during development may at least partly depend on the simultaneous effects of growth and surface adsorption of the embryos. In addition, the sea urchin fertilization envelope establishes a physical and biochemical barrier that protects the zygote from supernumerary sperm, as well as environmental and microbial agents until hatching. In this respect, Cd, Cu, Pb and Zn apparently provoked limited effects after 24 h of exposure although a different uptake mechanism causing delayed damages can not be excluded. A number of published studies demonstrated that exposure of sea urchin embryos to several chemical and physical stressors (including metals) induce redox anomalies, oxidative stress, DNA damage, mitotic aberrations to early life stages (Pagano et al. 1996; 2016). However, embryos are prepared to counteract environmental fluctuations by having high levels of cellular defences, buffer stress and alternative pathways that can be independent of developmental programs (Hamdoun and Epel 2007). The recovery capability has important implications for the adult populations, being larval dispersal crucial for the maintenance and durability of a sea urchin population. Several studies demonstrated that metal exposure in *P. lividus* embryos induced the synthesis of HSPs and of metallothioneins (MT) genes (Pinsino et al. 2011), two detoxification mechanisms which can contribute to the decrease the onset of anomalies, as observed in the present study.

3.1 Cd-exposed embryos

Regarding the onset and reversibility of effects by specific metals, after 24 h more than 85% of embryos continuously exposed to different concentrations of Cd (from 1000 to 2500 µg/L) displayed a normal development (EC50 value of 4183.40), with ITI values almost comparable to controls (Fig.1, Table 2 and Table 3). Conversely, at 48 h, Cd exposure was effective in producing a concentration-dependent increase of both teratogenesis (EC50 value of 1724.40) and ITI values (from 1.24 to 3.05). These effects appeared mostly related to the higher percentage of delayed (prism/early pluteus) and abnormal embryos with crossed skeletal rods (42 and 50% at 2000 and 2500 µg/l respectively), in agreement with abnormal

embryos previously observed by Arizzi Novelli *et al.* (2003). Nevertheless, at 72 h of development, these authors found an EC50 value ten times lower than those reported in the present study (230 µg/l versus 2115.90 µg/l). Conversely, our results are more closely related to those obtained by Warnau et al. (1996) (3372 µg/l <EC50<11241 µg/l), and lower than those reported by_Fernandez and Beiras (2001) (9240 µg/l). Many factors, such as the physiological state of the organisms, the moment at which embryos first encounter toxicant, the water physicochemical characteristics, metal compounds used for the exposure, may influence the sensitivity of the sea urchin embryos in a different manner: these differences can also be due to methodological variations for testing, as well as the sea urchin pool of gametes utilized for the experiments. Interestingly, Pagano et al. (1982) observed that embryos exposed to Cd from hatching to pluteus stage were affected to the same extent as if treatment had stared at fertilization for the same Cd levels, meaning that the toxic effects start from hatching. Roccheri et al (2004) demonstrated that Cd removal from culture medium, after incubation for more than 15 hours (with 1 mM $CdCl_2$) did not allow rescuing of normal embryo development, suggesting that prolonged treatments induced irreversible damage. Authors observed a plateau stage in the synthesis of stress proteins (HSPs) after 24 hours of Cd-exposure, suggesting the existence of a threshold for this cell defence mechanism.

Table 2 – Toxicity results in non-washed and washed embryos, considering as endpoint the percentage of normal versus abnormal embryos. Effect Concentration 50% (EC50) are given in bold and expressed in µg/l. The lower values represent the respective 95% confidence limits, and the straight-line slopes (in italics).

Treatment	NON-WASHED EMBRYOS			WASHED EMBRYOS		
Incubation time	24h	48h	72h	24h	48h	72h
Cd	**4183.40** (n.c.) *5.90*	**1724.40** (1624.30-1807) *12.15*	**2115.90** (1980.50-2258) *6.87*	**4183.40** (n.c.) *5.90*	**1862.10** (1425-1859) *11.74*	**4484.90** (n.c) *5.33*
Cu	**72.82** (68.24 - 82.83) *7.96*	**45.78** (42.36 -47.89) *15.50*	**40.65** (36.38 -43) *6.64*	**72.82** (68.24 - 82.83) *7.96*	**47.46** (42.03-50.65) *9.78*	**212.13** (111.8 – 868.5) *1.50*
Pb	**310.68** (228.70-613.50) *1.73*	**107.88** (102.75- 112.04) *14.40*	**164.45** (150.75 - 178.61) *7.10*	**310.68** (228.70-613.50) *1.73*	**113.38** (100.7- 144.04) *6.66*	**216.70** (177.50-235.35) *7.88*
Zn	**86.87**	**72.65**	**79.49**	**86.87**	**83.13**	**105.30**

	(59.9- 107.96)	(40.55- 91.17)	(39.62 - 106.59)	(59.90- 108)	(46.47-99.69)	(100.97-108.70)
	2.34	*9.34*	*7.82*	*2.34*	*10.61*	*15.88*

Table 3- – .Values of ITI obtained for washed and non-washed embryos at different trace metal concentrations and time of exposure/recovery.

		NON-WASHED EMBRYOS			WASHED EMBRYOS		
		Time					
Metal	Concentration (µg/L)	24 h	48 h	72 h	24 h	48 h	72 h
CTR	-	0.23	0.79	0.34	0.23	0.88	0.43
Cd	1000	0.13	1.24	0.65	0.13	1.22	0.47
	1500	0.22	1.88	1.12	0.22	1.78	0.51
	2000	0.13	2.79	1.49	0.13	1.88	0.62
	2500	0.45	3.05	3.83	0.45	2.76	0.98
Cu	20	0.35	1.36	0.75	0.35	1.36	0.75
	50	0.95	3.5	2.96	0.95	3.62	0.89
	60	1.36	4.38	3.36	1.36	4.53	1.05
	70	3.33	5.38	3.24	3.33	5.51	1.71
Pb	80	0.72	1.2	0.35	0.72	0.97	0.28
	100	0.78	2.48	0.59	0.78	0.85	0.25
	120	0.73	2.05	0.78	0.73	1.45	0.45
	250	1.09	4.48	2.71	1.09	4.64	1.04
Zn	60	0.84	1.05	1.23	0.84	1.60	0.78
	70	1.64	1.41	2.89	1.64	1.05	0.92
	100	1.78	2.48	4.22	1.78	2.73	2.74
	120	1.95	2.75	6.60	1.95	3.01	5.07

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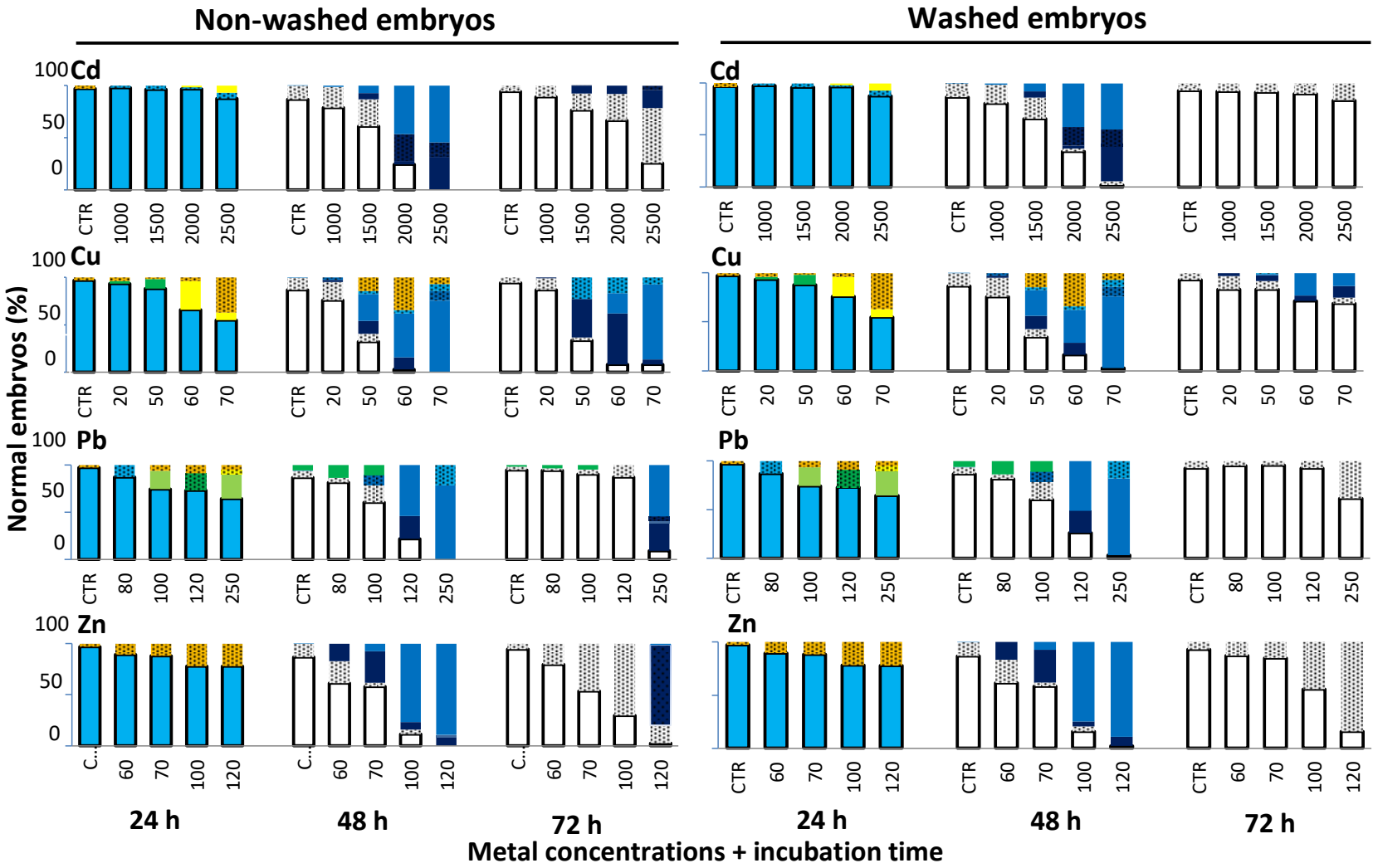

Fig. 1 –Effects of Cd, Cu, Pb and Zn on the sea urchin embryo development during continuous (non-washed) and after washing (washed embryos). Data are referred to effects after 24, 48 and 72 h of development. On X axis metal concentrations are reported in µg/l (CTR: control). Each stage of development reached by embryos is represented by bars in a colour scale from red to white. Normal embryos are rimmed in black and are late gastrula at 24 h (light blue bars) and pluteus stage at 48-72 h (white bars). Malformed embryos are represented with black spots at each stage of development. Pl: Pluteus; ePl: early Pluteus; Pr: Prism. lG: late Gastrula; mG: mid Gastrula; eG: early Gastrula; mBl: mesenchyme Blastula; Bl: Blastula; M: Morula.

221

Table 4 – Effects of washing treatment on embryo development of *P. lividus*. Results reported on the table were obtained with PERMANOVA pair-wise tests performed for the factor washing at each level of time incubation and for factor time of incubation at each level of washing treatment. W48: washed embryos observed at 48 h of development; NW48: non-washed embryos at 48 h of development; W72: washed embryos at 72 h of development; NW72: washed embryos at 72 h of development. Significant p-value are reported in bold.

Groups	Cd		Cu		Pb		Zn	
	t	P (perm)	t	P (perm)	T	P (perm)	T	P (perm)
W48 vs NW48	0.229	0.931	0.215	0.961	0.240	0.898	0.072	0.998
W48 vs W72	9.580	**0.001**	1.136	0.258	6.359	**0.001**	5.178	**0.001**
NW48 vs NW72	2.136	**0.026**	1.080	0.292	1.733	0.103	2.512	**0.001**
W72 vs NW72	13.171	**0.001**	6.031	**0.001**	8.054	**0.001**	5.375	**0.001**

If Cd insult is not too strong, embryos restore normal development (Roccheri et al 2004), probably because only a few cells are damaged and removed through apoptosis, allowing the restoring of the physiological morphology. In agreement, in this work we found that embryos exposed to Cd for 24 h and then cultured in FSW for the following 24 h (48 h development) exhibited increased malformations with developmental effects comparable to those of continuously exposed embryos (EC50 value of 1862.10, ITI values range from 1.22 to 2.76). On the contrary, embryos exposed to Cd for 24 h and then cultured in FSW for the following 48 h (72 h development) appear almost totally recovered (EC50 2671.08, ITI values range from 0.47 to 0.98).

3.2 Cu-exposed embryos

Cu-exposed embryos (from 20 to 70 µg/L) displayed a moderate number of abnormalities after 24 h (EC50 values of 72.82), with a dose-dependent increase of ITI values (from 0.35 to 3.33) mostly associated to a high number of abnormal mesenchyme blastula (Fig.1, Table 2 and Table 3). At 48 h of development/exposure, Cu induced a great number of malformed prisms with a severe impairment of the skeletal architecture of the embryos (e.g. crossed and separated tip at the hood apex arms, fused arms, as well as incomplete or absent skeletal rods) (EC50 45.78, ITI values range from 1.36 to 5.38). Embryos exposed to Cu for 24 h and then cultured in FSW for a following rescue period of 24 h (48 h development) showed a marked increase of malformations, with embryo toxicity values fully comparable to those of 48 h continuously exposed embryos (EC50 47.46, ITI values range from 1.36 to 5.51).

In agreement with data from Warnau *et al.* (1996) (48 µg/l <EC50<64 µg/l), Radenac et al. (2001) (50 µg/l <EC50< 100 µg/l), Arizzi Novelli *et al.* (2003) (62 µg/l) and Fernandèz and Beiras (2001) (66.76 µg/l), at 72 h of development, the majority of Cu-exposed embryos showed a prismoid shape, with

slightly increased values of developmental anomalies (EC50 40.65, ITI values range from 0.75 to 3.24). The reversibility of effects was more evident in embryos exposed to Cu for 24 h and then cultured in FSW for a following rescue period of 48 h, showing an elevated capability to recover normal development (EC50 212.13, ITI values range from 0.75 to 1.71). To the best of our knowledge, this is the first study underling the sea urchin embryonic recovery capability after Cu treatment.

3.3 Pb-exposed embryos

The 24 h exposure to Pb (from 80 to 250 µg/L) determined moderate teratogenic effects at the blastula and gastrula stage including delayed embryos (EC50 310.68, ITI values range from 0.72 to 1.09). At the pluteus stage (48 h), continuously Pb-exposed embryos displayed a high number of abnormalities with a dose-dependent trend, exhibiting crossed and separated tip at the hood apex arms, as well as a delay in the developmental schedule (EC50 107.88, ITI values range from 1.2 to 4.48). Also for lead, 24 h depuration after 24 h exposure (48 h development) was associated with an increase of developmental effects, which did not exhibit significant differences compared to those observed in continuously exposed embryos (EC50 113.38, ITI values range from 0.97 to 4.64).

At 72 h, the percentage of abnormal embryos was lower than that measured at 48 h of development/Pb-exposure (EC50 164.45, ITI values range from 0.35 to 2.71). These values appears slightly higher than those obtained by Radenac et al. 2001 (between 10 and 100 µg/l) and Arizzi Novelli et al. (2003) (68 µg/l) but lower than the results of Fernandez and Beiras (2001) (509.5 µg/l). Embryos exposed to Pb for 24 h and then cultured in FSW for the following 48 h (72 h development) showed an elevated capability to recover normal development (EC50 216.70, ITI values range from 0.28 to 1.04). However, at the highest Pb concentration (250 µg/L), more than 70% of washed embryos reached the pluteus stage but exhibited a severe inhibition of skeleton formation, confirming that such kind of malformations are more difficult to rescue even when development is recovered (Pinsino et al. 2011).

3.4 Zn-exposed embryos

Exposure to Zn (from 60 to 120 µg/L) caused a moderate number of abnormalities after 24 h (EC50 values of 86.87), with ITI values higher than those observed for Cd and Pb (from 0.84 to 1.95, Fig.1, Table 2 and Table 3). At the pluteus stage (48 h), continuously exposed embryos showed a high number of delayed embryos (prismoid shape), and a moderate number of skeletal malformed embryos (EC50 72.65, ITI values from 1.05 to 2.75). Embryos exposed to Zn for 24 h and then cultured in FSW for a following rescue period of 24 h (48 h development) showed an evident incapability to recover a normal

development with effects comparable to those of non-washed embryos (EC50 83.13, ITI values range from 1.60 to 3.31).

At 72 h of development, Zn-exposed embryos showed abnormalities represented by radialized arms causing, in some cases, a body shape deformation (EC50 79.49, ITI values range from 1.23 to 6.60). The EC50 values appears comparable with the results of Radenac (2001) (between 50 and 250 µg/l) and those estimated from Arizzi Novelli et al. (2003) (49 µg/l). These abnormalities influenced the ITI values more than standard EC50, and similar results were obtained in embryos exposed for 24 h and then cultured in FSW for 48 h (EC50 105.30, ITI values range from 0.78 to 5.07). The reversibility of effects caused by Zn-exposure was the lowest among tested metals, confirming a particularly high percentage of plutei with radialized arms previously observed also in the sea urchin *Anthocidaris crassispina* (Kobayashi and Okamura, 2004).

Interestingly, Tellis et al. (2014) demonstrated that during the development of the sea urchin *Strongylocentrotus purpuratus*, Zn inhibits the physiological oscillations of the intracellular calcium, essential to the correct functioning of the cell machinery, causing irreversible effects after gastrulation. In agreement, a competition with calcium uptake and internalization could be responsible for our results on embryos of *P. lividus* exposed to Zn.

4. Conclusions

Overall, results highlighted an astounding level of developmental plasticity of the sea urchin embryos exposed to Cd, Cu, Pb and Zn, determined by the ability to restore the underlying developmental pattern after a 48-hour recovery period. Nevertheless, even if recovery may occur, prolonging time in the plankton due to increased development time can have a range of adverse effects on larvae, such as increased predation risk, dispersal away from suitable habitat, lower settlement success and juvenile growth rate (Pechenik 1999). All trace metals caused developmental toxicity in a concentration- and time-dependent manner, being responsible for malformations and developmental delays with Zn and Cu more toxic than Pb, and Cd.

The specific differences in reversibility and intensity caused by different metals are highlighted by the use of the ITI, which recognizes and weights the delay and degree of various abnormalities and allow a better discriminatory ability than standard criteria. In particular, when embryos present malformations, ITI values appear more sensitive than standard criteria, which underestimate this form of toxicity. In fact, only ITI attributes higher toxicity values at severely malformed morphotypes that, in spite of low recover ability, are not considered by the standard criteria. An example of this difference, is represented by Zn-

exposed embryos, where the numerous morphotypes with body deformation and radialized arms (72 h) influence ITI much more than standard EC50. These results reinforce the notion that the ITI enhances the capability to discern interferences on sea urchin development in an accurate manner, appearing particularly relevant to validate the sea urchin embryo toxicity assay, and supporting its usefulness in various practical applications.

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