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Development and validation of new analytical methods using sea urchin embryo bioassay to evaluate dredged marine sediments

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Abstract

Management of dredged materials disposal is regulated in accordance to several environmental normative requirements, and it is often supported by integration of chemical data with ecotoxicological characterization. The reliability of a bioassay to assess the potential toxicity of dredged sediments requires the selection of quality criteria that should be based on simple analytical methods and easily understandable hazard for politicians and environmental managers. The sea urchin embryo-toxicity bioassay is considered an essential component for evaluating the quality of sediments in harbour areas but its use, when based exclusively on the observation of normal vs. abnormal embryos, may alter the interpretation of the results, overestimating the risk assessment. To improve the reliability of this assay in establishing causative relationship between quality of sediments and sea urchin embryonic development, here we developed and validated three Integrative Toxicity Indexes (ITI 2.0, ITI 3.0, ITI 4.0), modifying the already-known ITI (here ITI 1.0). Based on this aim, we used Taranto harbour as a model pilot-study to compare results to those obtained from standard criteria. Among the tested indexes, the ITI 4.0, discriminating strictly developmental delay and morphological defects from gastrula to fertilized eggs, resulted the most promising.

Keyword list: *Paracentrotus lividus*; teratogenicity; delay; elutriates; contaminants

1. Introduction

To evaluate the impact of chemical pollutants in the environment, nowadays it is widely recognized the importance to assess the biological effects of contaminants, using an integrated approach with the chemical data. In fact, the chemical approach by itself does not provide information on real bioavailability and biological risk of measured pollutants. Ecotoxicological batteries of bioassays have progressively been applied to quantify the potential biological hazard caused by bioavailable multi-factorial contamination, thus providing a more relevant response not restricted by a predetermined list of contaminants (Morroni et al., 2020). For these reasons these tools are often included in legislative requirements. The last Italian Decree on management of dredged sediments (DM 173/2016) foresees a list of key species to be used in a battery of bioassays to assess the sediment toxicity. This ecological risk assessment is based on a multidisciplinary weight of evidence (WOE) approach, considering chemical analyses and ecotoxicological bioassays as different lines of evidence (LOEs) through a quantitative integration. As result of weighted elaboration, a quality classification of marine sediments and different management options were then suggested according to dumping legislation. In recent years WOE approach was validated in several case studies for environmental risk assessment associated with polluted sediments, harbour areas, or complex natural and anthropic impacts on the marine environment (Piva et al., 2011; Benedetti et al., 2014; Pittura et al., 2018; Regoli et al., 2019). Based on this aim, the ecotoxicological bioassays have a crucial role to evaluate the overall environmental quality status, and to suggest appropriate management decisions. Data are typically obtained from different species, strains, exposure times and different end-points including survival, reproduction, and growth (Picone et al., 2016). Among target species, sea urchins are worldwide considered an ideal choice for marine eco-toxicological tests as their embryos are enough sensitive to detect adverse effects related to a huge range of pollutants and natural matrices, including metals and metals mixtures (Kobayashi and Okamura 2004; Morroni et al., 2018; Bonaventura et al., 2018), micro- and nano-plastics (Pinsino et al., 2017; Oliviero et al., 2019), UV radiation (Lister et al., 2010; Russo et al., 2014), ocean acidification (Passarelli et al 2017; Dorey et al., 2018), sediments (Khosrovyan et al., 2013; Pagano et al., 2017). Sea urchin embryos can be easily obtained in laboratory conditions, and the development to pluteus stage is completed in 24-48 hrs, depending on

the species. In the DM 173/2016, the embryo-toxicity test on the Mediterranean species, *Paracentrotus lividus*, is measured after 48 hours of development (ASTM, 1995; USEPA, 1995; Environment Canada, 2011), and embryos are conventionally classified in "normal" or "abnormal", reporting the percentage of abnormally developed embryos (standard toxicity criteria). The general limit of such standard toxicity criteria, is that developmental analysis does not distinguish among different malformations, block, and delay of embryogenesis. In order to overcome this limitation, some recent studies developed new analytical indexes to weigh the teratogenic effects in the sea urchin embryos, by integrating the frequency of abnormal embryos with the severity of such abnormalities (Morroni et al., 2016), or by using a selective criterion such as detailed skeleton malformation (Carballeira et al., 2012). Although these analytic methods result highly performant, they are less rapid and simple than traditional toxicological testing strategy based on the observation of normal *vs.* abnormal embryos.

To further improve the use of promising analytical methods to establish causative relationships between contaminants and sea urchin embryonic teratogenicity or delay, here we developed and tested three additional new Integrative Toxicity Indexes (ITIs) modifying the pioneer ITI published by Morroni et al (2016), and comparing results to those obtained from standard toxicity approach. Based on this aim, we used sea urchin embryo-toxicity data (48 hours of development as end-point) generated by 43 elutriates obtained from representative sediments samples of Taranto harbour, which was chosen as a model case-study. Notably, Taranto harbour was of interest because environmental and epidemiological investigations in the area have provided evidence of environmental contamination (e.g., particulate matter, heavy metals, polycyclic aromatic hydrocarbons, and organ-halogenated compounds) (Pirastu et al 2013). The new ITIs were based on the frequency of delayed and/or abnormal embryonic morphologies calculated using a simplified scale from 0 (absence of toxicity) to 5 (maximum toxicity). To achieve the intended goals in terms of reliable harbour-sediment hazard assessment and related risk evaluation, studies on the development of fast and reliable methods become mandatory.

2. Material and Methods

2.1 Sediment sampling and elutriate preparation

Sediments were collected during a large characterization and monitoring project in the Taranto harbour (from September 2016 to February 2017). Elutriates from 43 representative sediment samples collected at different depth levels (from 0 to 150 cm) were prepared according to the guidelines (USEPA 1991; APAT-ICRAM, 2007) and literature studies (Morroni et al., 2016).

2.2 Sea urchin harvesting, embryonic cultures and exposure

Specimens of the sea urchin *Paracentrotus lividus* were collected along the unpolluted coast of Sicily (Italy), and were brought back to the laboratory. Toxicity tests were performed following the method reported by Morroni et al. (2016) with slight modifications, as described. At least three males and three females were induced to spawn by injecting 0.5 M KCl into the sea urchin body cavity through the peristomal membrane around the teeth. Eggs were collected by placing spawning females on 100 ml beakers with 0.45 µm filtered artificial seawater (ASW), while sperms were collected dry (directly from the surface of the sea urchin) using a micropipette with the end of the tip cut off, maintained in a sealed container at room temperature, and used in 30 minutes. Egg quality and sperm motility were inspected by observing the gametes under an optical microscope (OLYMPUS CKX31). Sperms were diluted in 10 ml of ASW and added to the egg suspension (10,000 eggs mL^{-1} dilution). After fertilization, embryos were maintained in a 24-well plate at the final concentration of 500 embryos/ml, at a temperature of 18°C. Embryos were then exposed to elutriates (1:4 ratio of sediment to water), from fertilization (0 h post-fertilization) to the pluteus stage (48 h post-fertilization).

Three replicates were performed for each elutriate sample as well as for controls. At 48 hours after fertilization (h), live embryos were observed and photographed using an optical microscope equipped with a digital camera (OLYMPUS CKX3). Formaldehyde (10% in ASW) was added to each well at the final concentration of 0.015% just prior to count and categorize embryos.

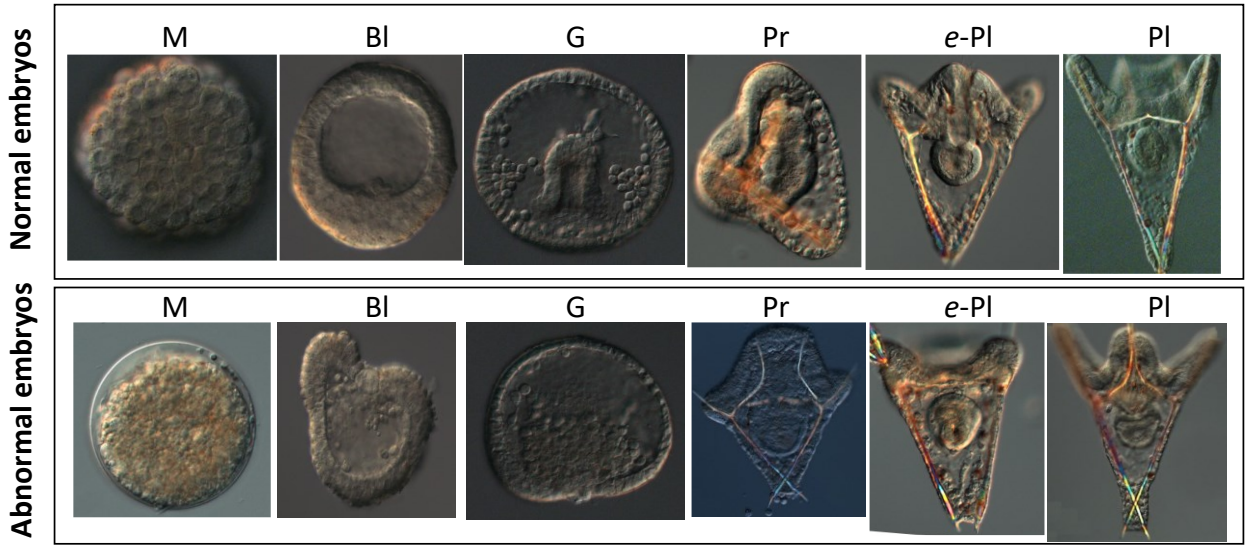
2.3 Toxicity criteria

The toxicity of elutriate samples from Taranto harbour was estimated by calculating the percentage of abnormal embryos at pluteus stage (48 h of development), according to the standard criteria, the Integrative Toxicity Index (ITI) published by Morroni et al (2016) (here called ITI 1.0), and three additional new ITIs developed from ITI 1.0, respectively called ITI 2.0, ITI 3.0 and ITI 4.0. The standard criteria calculate the percentage of normal and abnormal embryos without considering different typologies of malformations or the phase in which they appear. Conversely, all the ITI methodologies count the frequency of delayed and/or abnormal embryonic morphologies and quantitatively rank the severity of effects.

Embryos were classified as normal if they satisfied the morphological criteria as follows: i) suitable schedule in reaching the developmental endpoint (pluteus at 48 h); ii) correct skeleton development and patterning; iii) right ectoderm, mesoderm and endoderm germ layer differentiation; iv) conform left/right or dorso/ventral axis symmetry. On the other hand, embryos displaying impairment of the axis symmetry, as well as germ layer defects were marked as abnormal (see **Table 1** showing

representative images of normal and abnormal *P. lividus* embryos at different embryonic developmental stages).

Table 1 –Developmental stage and abnormalities of *P. lividus* observed in this study



M, Morula; Bl, Blastula; G, Gastrula; Pr, Prism; ePl, early Pluteus; Pl, Pluteus

The ITI 1.0 from previous study used a toxicity scale from 0 (absence of toxicity) to 10 (maximum toxicity), which was here implemented with a second generation of ITIs, using more simplified criteria, grouping embryos on a toxicity scale from 0 (absence of toxicity) to 5 (maximum toxicity) as shown in **Table 2**. The lowest level (0) assigned to each ITIs was associated with a "no effect" in development, including only normal embryos reaching the 48-h endpoint (Pluteus). The only exception was the ITI 4.0 where the zero-effect level was extended also to those embryos displaying a slight delay, considered as a negligible effect (pluteus and early pluteus). A score ranging from 1 to 3 was assigned to the ITI 2.0 and ITI 3.0 as follows: 1 for delayed embryos at the pluteus stage (*e*-Pl); 2 for malformed embryos at the pluteus stage (*m*-Pl); and 3 for delayed embryos at the pluteus stage displaying malformations (*em*-Pl). For ITI 2.0, the level 4 was associated with the delayed embryos from prism to fertilized egg (Pr-F) and the level 5 with the malformed Pr-F (*m*-Pr-F); in ITI 3.0 the levels 4 and 5 were assigned to include delayed and/or malformed Pr (Pr and/or *m*-Pr), and delayed and/or malformed embryos from gastrula stage to fertilized egg (G-F and/or *m*-G-F), respectively. On the other hand, the score 1 assigned to the ITI 4.0 was associated with the Pl and *e*-Pl displaying malformations (*m*-Pl and *em*-Pl), the score 2 was associated with the stage of prism (Pr), and the score 3 with the Pr displaying malformations (*m*-Pr). The highest levels of toxicity (4 and 5) assigned to the ITI 4.0 were associated with the delayed and/or malformed embryos at the gastrula stage (G and/or *m*-G), and with the delayed and/or malformed embryos from blastula stage to fertilized egg (Bl-F and/or *m*-Bl-F). Therefore, the lower degree of toxicity was assigned to Pl with

absence of abnormalities, while the higher degree was attributed to embryos displaying severe delay, and/or delay *plus* abnormalities simultaneously.

The ITIs applied in this study are calculated as follows:

$ITI = \sum^{n}_{i=10}(S_i * F_i)/100$

Where Si is the score associated to each abnormal embryonic morphology and Fi is the frequency observed for that morphotypes (i=10).

Table 2 – Integrative Toxicity Indexes (ITIs) tested in this study

	Toxicity categories												
ITI 1.0	**Normal**	**Delayed**						**Malformed**					
	Pl	*e*-Pl	Pr	G	mBl	Bl	M	Pl	Pr	G	mBl	Bl	M
Score	**0**	**2**	**3**	**4**	**4.5**	**5**	**5.5**	**6**	**7**	**7.5**	**8**	**9**	**10**
ITI 2.0	**Normal**	**Delayed**						**Delayed and Malformed**					
	Pl	*e*-Pl			Pr-F			Pl	*e*-Pl		Pr-F		
Score	**0**	**1**			**4**			**2**	**3**		**5**		
ITI 3.0	**Normal**	**Delayed**			**Malformed**			**Delayed and/or Malformed**					
	Pl	*e*-Pl			Pl	*e*-Pl		Pr			G-F		
Score	**0**	**1**			**2**	**3**		**4**			**5**		
ITI 4.0	**Normal**	**Delayed**			**Malformed**			**Delayed and/or Malformed**					
	Pl/*e*-Pl	Pr			Pl/*e*-Pl	Pr		G			Bl-F		
Score	**0**	**2**			**1**	**3**		**4**			**5**		

F: fertilized egg; M, Morula; mBl, mesenchyme Blastula; Bl, Blastula; G, Gastrula; Pr, Prism; *e*-Pl, early Pluteus; Pl, Pluteus. Bl-F, from Blastula to fertilized egg; G-F: from Gastrula to fertilized egg; Pr-F: from Prism to fertilized egg.

3. Results and Discussion

The evaluation based on the standard criteria and the thoughtful ITIs (from ITI 1.0 to ITI 4.0) of the impact on the sea urchin embryonic development is shown in Figure 1. Based on the standard criteria, the majority of tested elutriates (46.5%) presented an extremely severe or severe toxicity with a percentage of abnormal embryos higher than 75% (20 of 43 samples; Figure 1A, red bars); a moderate number (28%) displayed from severe to moderate toxicity with a percentage of abnormal embryos ranging from 75% to 20% (12 of 43; Figure 1A, blue bars); the remaining 25.5% did not show any significant impact compared to the controls (11 of 43; figure 1A, compare green bars with those grey) being under the threshold of 20%.

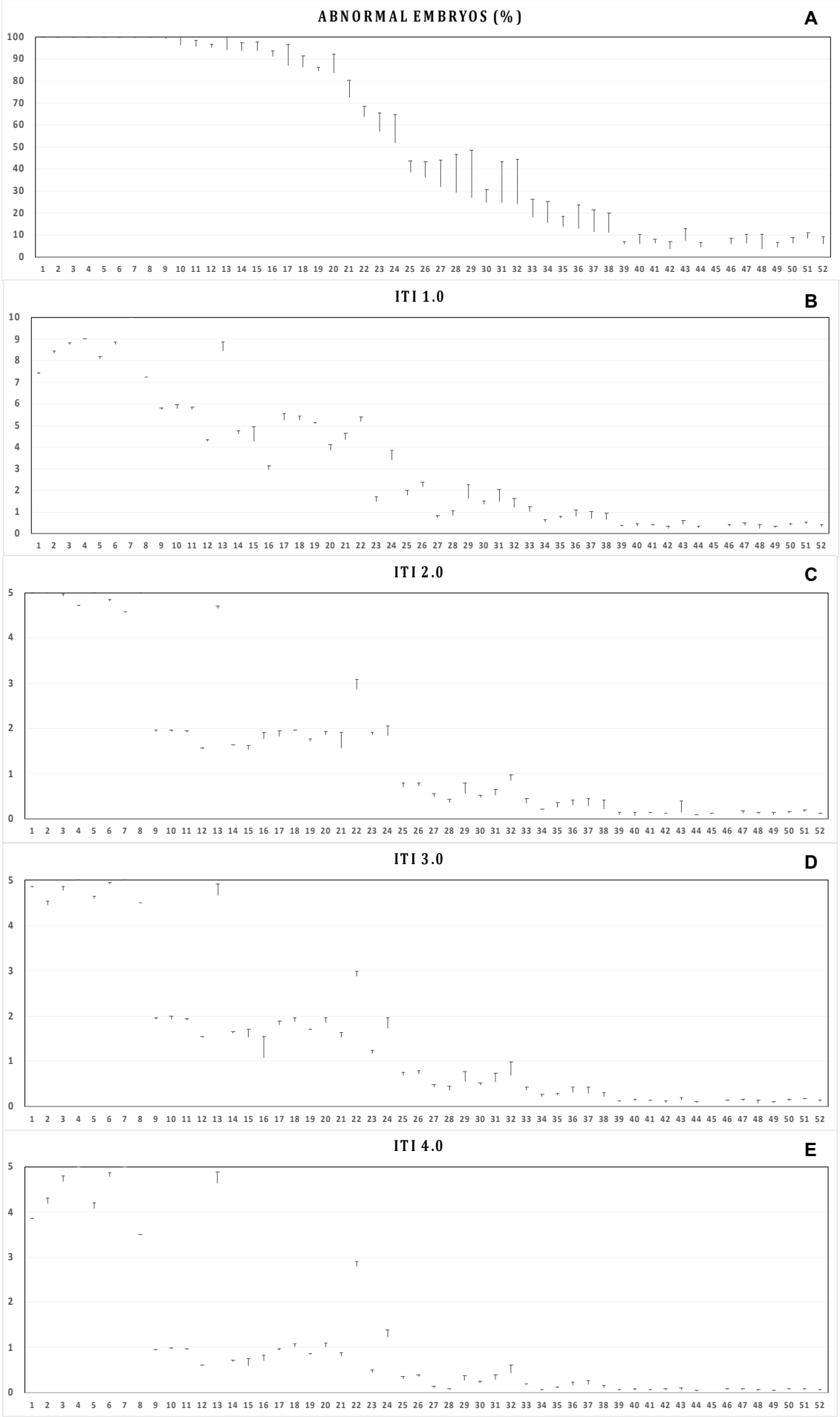

ABNORMAL EMBRYOS (%)
A
ITI 1.0
B
ITI 2.0
C
ITI 3.0
D
ITI 4.0
E

Figure 1. Sea urchin embryonic development evaluated according to standard criteria and thoughtful ITIs. Histograms represent the results expressed as mean percentage (%) of abnormal embryos±SD (A), and values of the ITI 1.0 (B), ITI 2.0 (C), ITI 3.0 (D), ITI 4.0 (E) ± SD. Data are referred to each elutriate sample of Taranto harbour, reported on X- axis. Controls (CTR) are reported in the right part of the panels for a total of 9.

The evaluation of embryo-toxicity based on the ITI 1.0 method, which discriminate the frequency of delayed and/or abnormal embryonic morphologies and quantitatively rank the severity of effects on a pondered scale from 0 (absence of toxicity) to 10 (maximum toxicity), leads to an attenuate scenario of morbidity (Figure 1B). The difference appeared more accentuated for those samples classified as extremely toxic by standard criteria (Figure 1B, red bars): only one sample (number 7) confirmed the maximum level of toxicity (10), while eight samples presented a level ranging from 7 to 9 (number 1-6, 8, and 13), six samples ranged from 5 to 6 (moderate toxicity, number 9-11, 17-19), three samples from 4 to 5 (low toxicity, number 12, 14-15), and two samples were below the value 4 (very low toxicity, number 16, and 20). Therefore, the extremely toxic effects assessed by standard method for 20 sediment samples were confirmed by ITI 1.0 only for 9 of them (from level 7 to level 10), corresponding to about 50% of the cases. In agreement with our recent report, this result provides the evidence that the ITI 1.0 allows to better separate the samples according to the frequency and severity of delayed and/or abnormal morphologies (Morroni et al., 2016). ITI 1.0 is more sensitive than traditional toxicological testing strategy but presents the disadvantage that the rigorous morphological analysis may be applied by trained personnel on the sea urchin embryonic development: in this respect, for unspecialized operators, it may result less rapid, simple and direct than conventional methods based on observation of normal *vs.* abnormal embryos only. To simplify the promising approach of ITI for determining a more realistic toxicological evaluation of dredged sediments, we tested a second generation of indices still based on the frequency of delayed and/or abnormal embryonic morphologies but calculated using a simplified scale, in which embryos are grouped on a toxicity scale from 0 (absence of toxicity) to 5 (maximum toxicity) (see Table 2, Material and Methods). Based on this aim, scores assigned by ITI 2.0 range from 1 to 5 as follows: 1 for delayed plutei (*e*-Pl); 2 for malformed plutei (*m*-Pl); 3 for delayed and malformed plutei (*em*-Pl); 4 for delayed embryos from prism to fertilized eggs (Pr-F); 5 for malformed embryos from prism to fertilized eggs (*m*-Pr-F). The evaluation of embryo-toxicity of Taranto elutriates based on the ITI 2.0 is reported in Figure 1C. In agreement with the ITI 1.0 results, extremely toxic effects evaluated by standard criteria for the 20 samples were confirmed only for 9 of them (from level 4 to 5); the remaining 11 samples displayed a low toxicity with levels below 2. Even if the ITI 2.0 appear less discriminating than ITI 1.0, this index displayed a similar level of performance in samples classified as extremely toxic (compare Figure 1B and 1C).

The ITI 3.0 showed a trend comparable to ITI 2.0, with the difference that levels below 2 were much less compliant (Figure 1D). The ITI 3.0 assign the score from 1 to 3 as for ITI 2.0 (1 for *e*-Pl; 2 for *m*-Pl; 3 for *em*-Pl), whereas different criteria have been used for attributing values of 4 and 5: 4 for delayed and/or malformed prisms (*em*-Pr), and 5 for delayed and/or malformed embryos from gastrula to fertilized eggs (*em*-G-F). These differences increased the ability of ITI 3.0 to discriminate among groups compared to the ITI 2.0.

Considering the results of the first six samples, the toxic levels were, on average, 5% lower than those obtained from ITI 2.0. This slight increase in sensitivity was mostly observed in the sample number 2 and 5, with 13% and 5% of the embryos at the prism stage, respectively. This stage was not well discriminated in ITI 2.0 as the score 4 is assigned to embryos at the stages from prism (Pr) to fertilized egg (F).

Finally, when we used the ITI 4.0, discriminating strictly developmental delay and morphological defects from gastrula to fertilized eggs (1 for *em*-Pl; 2 for *e*-Pr; 3 for *m*-Pr; 4 for *em*-G; 5 for *em*-Bl-F), we still increased the ability to discriminate among groups (see Figure 1E). Notably, several samples showed a lower and more distributed values of toxicity, such as samples number 1, 2, 5 and 8 (ITI 4.0 values ranging from 3.5 to 4.2). Other samples, as the number 7, maintained the maximum level of toxicity (5), confirming the good discriminatory ability of this index, which consider the different degree of severity assigned at early stages, discriminating between gastrula and pre-gastrula stages (from blastula to fertilized eggs) (see Table 2).

The sea urchin embryo is a simple model to monitor the developmental stages from fertilization to pluteus stage; *Paracentrotus lividus*, under controlled conditions of temperature (18°C) reaches the pluteus stage after 48 h. The embryonic development requires a prompt and synchronised combination of cell proliferation, fate specification and movement, controlled by gene regulatory networks (Erkenbrack et al 2018). Cell fate is specified at the appropriate space and time (blastula-early gastrula stage of development) when cells become able to express a set of differentiated germ layer-exclusive genes (Davidson et al 1998**)**. Elevated metabolic rates decrease capability for growth, and promote developmental delay of the sea urchin embryos; for example, this happens under acidified seawater conditions (Stumpp et al 2011).

The most documented explanation on sea urchin embryonic delay as an effect of toxicity, is the reduction in the ability to uptake calcium and, in turn, to maintain intracellular homeostasis related to a low extracellular pH; calcium-contaminant trafficking competition also affects the normal gene regulatory network controlling development (Stumpp et al 2011; Pinsino et al 2011). A number of developmental steps such as fertilization, cleavage, neuronal development, skeletogenesis, cell death and body modelling are known to be dependent on calcium ion trafficking (Webb and Miller 2003).

On the other hand, regulatory studies reveal that the embryos present an early sequence of encoded "fail-safe" regulatory devices (Smith and Davidson 2009). Based on this evidence, we speculate that in the early embryonic stages (from gastrula to fertilized egg), when the cell fate has not been specified yet, the probability for embryos to recover from the delay and continue the development is really scant, thereby justifying the assignment of the higher degree of toxicity to embryos displaying severe delay, abnormalities, or delay plus abnormalities occurring simultaneously. On the contrary, at the late embryonic phases (from prism to early pluteus), when the cell fate is already specified, the embryos have high probability to continue the development, thus explaining the assignment of the lower degree of toxicity. The increasing grading of mild, moderate and severe effects assigned on the severity of delay and teratogenicity, was progressively emphasized from the ITI 2.0 to the ITI 4.0. All these indexes can be considered valid tools to better evaluate the embryo toxicity effects on sea urchin based on objective and solid scientific criteria, with clearly important applicative consequences when assessing the quality of dredged marine sediments: among the various indexes, ITI 4.0 which stress mainly the severity of delay, offers the higher sensitivity and discriminatory efficiency.

4. Conclusions

The use of the WOE integration, which combine and weight different typologies of data and analyses, allows to better discriminate the presence of contaminants and their short or long-term consequences, especially when apparently contrasting results are provided by various LOEs. The possibility to convert complex scientific information into simple hazard indexes, easily understandable from policy makers and environmental managers, can facilitate and orientate the more appropriate and site-specific decisions on environmental sediment management (Morroni et al., 2020). In this context, the sea urchin embryo-toxicity bioassay is considered an essential component for evaluating the quality of sediments in harbour areas, with important environmental and economic implications. Classifications based on the worst result are still in use and significant consequences may arise depending on the choice of the ecotoxicological assays within a battery. In particular, this study demonstrated that care should be taken in the evaluation of embryo-toxicity results suggesting to weight developmental delay and morphological defects in a balanced way.

The development of such sensitive method is of great utility to properly achieve a reliable harbour-sediment hazard assessment and related risk evaluation. The results obtained in the present study indicate that ITI 4.0 is a promising approach for dredged sediments, better discriminating samples with intermediate toxicity from those highly toxic.

References

APAT-ICRAM, 2007. Manuale per la movimentazione di sedimenti marini, pp. 1–67. Available at 〈 www.apat.gov.it〉 and 〈www.icram.org.

ASTM E1563, 1995. Standard guide for conducting static acute toxicity tests with echinoid embyos. E 1563-95 In Annual Book of ASTM Standards, Philadelphia, PA, vol. 11(5), pp. 999–1017.

Benedetti, M., Gorbi, S., Fattorini, D., D'Errico, G., Piva, F., Pacitti, D., Regoli, F., 2014. Environmental hazards from natural hydrocarbons seepage: Integrated classification of risk from sediment chemistry, bioavailability and biomarkers responses in sentinel species. Environ. Pollut. 185, 116–126.

Bonaventura R, Zito F, Chiaramonte M, Costa C, Russo R (2018). Nickel toxicity in P. lividus embryos: Dose dependent effects and gene expression analysis. Mar Environ Res. 2018, 139:113-121.

Carballeira, C., Ramos-Gómez, J., Martín-Díaz, L., DelValls, T.A., 2012. Identification of specific malformations of sea urchin larvae for toxicity assessment: application to marine pisciculture effluents. Mar Environ Res. 77, 12-22.

Davidson, E. H., Cameron, R. A., and Ransick, A. (1998). Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. Development. 125, 3269–3290.

DM 173/2016. Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Supplemento ordinario alla Gazzetta Ufficiale, n. 208 del 6 settembre 2016-Serie generale. Regolamento recante modalità e criteri tecnici per l′autorizzazione all'immersione in mare dei materiali di escavo di fondali marini.

Dorey, N., Martin, S., Oberhänsli, F., Teyssié, J.L., Jeffree, R., Lacoue-Labarthe, T., 2018. Ocean acidification modulates the incorporation of radio-labeled heavy metals in the larvae of the Mediterranean sea urchin Paracentrotus lividus. J Environ Radioact. 190-191, 20-30.

ECHA, 2016. Guidance on Information Requirements and Chemical Safety Assessment. Chapter R.7b: Endpoint specific guidance.

Environment Canada, 2011. Biological Test Methods: Fertilization Assay using Echinoids (Sea Urchins and Sand Dollars). EPS 1/RM/27.

Erkenbrack EM1, Davidson EH2, Peter IS1. Conserved regulatory state expression controlled by divergent developmental gene regulatory networks in echinoids. Development. 2018 Dec 18;145(24). pii: dev167288.

Khosrovyan, A., Rodríguez-Romero, A., Salamanca, M.J., DelValls, T.A., Riba,I., Serrano, F., 2013. Comparative performances of eggs and embryos of sea urchin (Paracentrotus lividus) in toxicity bioassays used for assessment of marine sediment quality. Mar. Pollut. Bull. 70(1-2), 204-209.

Kobayashi, N., Okamura, H., 2004. Effects of heavy metals on sea urchin embryo development. 1.Tracing the cause by the effects. Chemosphere 55(10),1403–1412.

Leogrande, S., Alessandrini, E.R., Stafoggia, M., Morabito, A, Nocioni, A., Ancona, C., Bisceglia, L., Mataloni, F., Giua, R., Mincuzzi, A., Minerba, S., Spagnolo, S., Pastore, T., Tanzarella, A., Assennato, G., Forastiere, F., 2019. Industrial air pollution and mortality in the Taranto area, Southern Italy: A difference-in-differences approach. Environ Int. 132, 105030.

Lister, K.N., Lamare, M.D., Burritt, D.J., 2010. Oxidative damage in response to natural levels of UV-B radiation in larvae of the tropical sea urchin Tripneustes gratilla Photochem. Photobiol. 86, 1091-1098.

Morroni, L., Pinsino, A., Pellegrini, D., Regoli, F., Matranga, V., 2016. Development of a new integrative toxicity index based on an improvement of the sea urchin embryo toxicity test. Ecotoxicol. Environ. Saf. 123, 2–7.

Morroni, L., Pinsino, A., Pellegrini, D., Regoli, F., 2018. Reversibility of metal induced malformations in sea urchin embryos. Ecotox. Environ. Saf. 148, 923-929.

Morroni, L., d'Errico, G., Sacchi, M., Molisso, F., Armiento, G., Chiavarini, S., Rimauro, J., Guida, M., Siciliano, A., Ceparano, M., Carraturo, F., Tosti, E, Gallo, A., Libralato, G., Patti, F.P., Gorbi, S., Fattorini, D., Nardi, A., Di Carlo, M., Mezzelani, M., Benedetti, M., Pellegrini, D., Musco, L., Danovaro, R., Dell'Anno, A., Regoli, F., 2020. Integrated characterization and risk management of marine sediments: the case study of the industrialized Bagnoli area (Naples, Italy). Mar. Environ. Res. In press.

Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S., Beiras, R., 2019. Leachates of micronized plastic toys provoke embryotoxic effects upon sea urchin Paracentrotus lividus. Environ. Pollut. 247, 706–715.

Pagano, G., Thomas, P.J., Guida, M., Palumbo, A., Romano, G., Trifuoggi, M. Oral, R., Trifuoggi, M., 2017. Sea Urchin Bioassays in Toxicity Testing: II. Sediment Evaluation. Expert Opin. Environ. Biol. 6, 1.

Pinsino A1, Roccheri MC, Costa C, Matranga V. Manganese interferes with calcium, perturbs ERK signaling, and produces embryos with no skeleton. Toxicol Sci. 2011 Sep;123(1):217-30.

Pinsino, A., Bergami, E., Della Torre, C., Vannuccini, M.L., Addis, P., Secci, M., Dawson, K.A., Matranga, V., Corsi, I., 2017. Amino-modified polystyrene nanoparticles affect signaling pathways of the sea urchin (*Paracentrotus lividus*) embryos. Nanotoxicology, 11, 201-209.

Passarelli, M.C., Cesar, A., Riba, I., DelValls, T.A. 2017. Comparative evaluation of sea-urchin larval stage sensitivity to ocean acidification. Chemosphere 184, 224–234.

Picone, M., Bergamin, M., Losso, C., Delaney, E., Arizzi Novelli, A., Ghirardini, A.V., 2016. Assessment of sediment toxicity in the Lagoon of Venice (Italy) using a multi-species set of bioassays. Ecotoxicol. Environ. Saf. 123, 32-44.

Pirastu, R., Comba, P., Iavarone, I., Zona, A., Conti, S., Minelli, G., Manno, V., Mincuzzi, A., Minerba, S., Forastiere, F., Mataloni, F., Biggeri A., 2013. Environment and Health in Contaminated Sites: The Case of Taranto, Italy. Int J Environ Res Public Health 3, 753719.

Pittura, L., Avio, C.G., Giuliani, M.E., D'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regol, F., 2018. Microplastics as vehicles of environmental PAHs to marine organisms: Combined chemical and physical hazards to the mediterranean mussels, Mytilus galloprovincialis. Front. Mar. Sci. 5(103).

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., Regoli, F., 2011. Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: A practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. Chemosphere 83, 475–485.

Regoli, F., D'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., Di Carlo, M., Pellegrini, D., Gorbi, S., 2019. Application of a Weight of Evidence Approach for Monitoring Complex Environmental Scenarios: the Case-Study of Off-Shore Platforms. Front. Mar. Sci. 6, 1–15.

Russo, R., Bonaventura, R., Matranga, V., 2014. Time-and dose-dependent gene expression in sea urchin embryos exposed to UVB. Mar. Environ. Res. 93, 85–92.

Smith J1, Davidson EH. Regulative recovery in the sea urchin embryo and the stabilizing role of fail-safe gene network wiring. Proc Natl Acad Sci U S A. 2009 Oct 27;106(43):18291-6.

Stumpp M1, Wren J, Melzner F, Thorndyke MC, Dupont ST; CO2 induced seawater acidification impacts sea urchin larval development I: elevated metabolic rates decrease scope for growth and induce developmental delay. Comp Biochem Physiol A Mol Integr Physiol. 2011 Nov;160(3):331-40

USEPA, 1991. Evaluation of dredged material proposed for ocean disposal testing manual. EPA 503/8-91/001.

USEPA, 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. EPA/600/ R-95/136.

Webb SE1, Miller AL. Calcium signalling during embryonic development. Nat Rev Mol Cell Biol. 2003 Jul;4(7):539-51.