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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:	<p style="margin-right: 0cm; margin-left: 0cm; font-size: medium; font-family: "Times New Roman", serif; caret-color: rgb(0, 0, 0); color: rgb(0, 0, 0); text-align: justify; line-height: 24px;">Management of dredged materials disposal is regulated by several environmental normative requirements, and it is often supported by the 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 a 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 fertilized egg to gastrula stage, resulted in the most promising. <o:p></o:p></p>

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

Rosa Bonaventura[a], Francesca Zito[a], Lorenzo Morroni[b], David Pellegrini[b], Francesco Regoli[c], and Annalisa Pinsino[a*]

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

Cover Letter

Dear Dr. Paolo Roccaro,
Please find here the recent resubmission of our manuscript entitled "Development and validation of new analytical methods using sea urchin embryo bioassay to evaluate dredged marine sediments" Ms. Ref.No. JEMA-D-20-08004. The manuscript has been revised according to the reviewer's comments. We appreciated their work on this manuscript as both made a constructive review and appeared familiar with the topic. We did follow all of their recommendations. We hope that you will find this revised manuscript suitable for publication in the Journal of Environmental Management.
Below, point by point responses:

Reviewers' comments:

Reviewer #1:
The manuscript JEMA-D-20-08004, supplies an important tool to evaluate marine matrices in the light of environmental safety and management. The authors present an improvement of their previous work by use of sea urchin early developmental stages, by supplying three Integrative Toxicity Indexes (ITI) which can put together chemical data with ecotoxicological characterization. This work reports a modification of the already known ITI, developed previously by the same authors. The case of Taranto harbor is examined as a pilot study, aimed at demonstrating the efficiency and possible predictivity of the methods. The developmental aspects of the biological system are described and shown, so that the method is easy to understand and reproducible in other laboratories.
The work is carefully explained, the results are convincing, the references are up-to date and appropriate, the English language is fluent and easy to understand, the figures clearly show the results. Thus, n my opinion the paper deserves publication in the Journal of Environmental management.
I have just one observation:

Q1. Page 14, line 265: from gastrula to fertilized eggs: I would write "from fertilized egg to gastrula stage". At these stages, the possibility of recovery is rather higher than expected, cause of the possibility demonstrated by Giudice (1971) to reaggregate dispersed cell as soon as the environmental conditions are improved. Moreover, the plasticity of sea urchin larvae (Fenaux et al) may cause a morphogenetic damage milder than expected, also at later stages.

R1. The point raised by the reviewer is very important. As for his suggestion, the words "from gastrula to fertilized eggs" have been replaced by "from fertilized egg to gastrula stage" throughout the text (see text).

Reviewer #2: This is a very interesting work describing new analytical methods using the sea urchin embryo bioassay to evaluate dredged marine sediments. Authors evaluated three new Integrative Toxicity Indexes (ITI) in addition to a previous one reported in a study published by the same group in 2016. The method selected among the three (ITI 4.0) represents a powerful tool to assess embryotoxicity of dredged marine sediments and for other ecotoxicological assessments. This method improves the reliability of the sea urchin embryo bioassay, since it weights developmental delay and morphological defects in a balanced way.
Although the paper is well conceived and clearly written, the Authors should address some minor point before the manuscript could be published.

Q1. Par. 2.2 Information about control conditions chosen for this experiment should be given. Please add it.
R1. As suggested, we added some information about control conditions as follows (see highlighted text):

Three replicates were performed for each elutriate sample as well as for controls. **Control embryos were exposed to ASW only, for a total of nine control cultures (each in triplicates).** At 48 hours after fertilization (h), live embryos were observed and photographed using an optical microscope equipped with a digital camera (OLYMPUS CKX3). **The results were considered valid and acceptable only if each set of control presented at least 80% of normal embryos.** Formaldehyde (10% in ASW) was added to each well at the final concentration of 0.015% just prior to count and categorize embryos.

Q2. Line 166-168. It is not clear to me why in the summation notation i=10. Written this way it should mean that 10 is the lower bound of summation, and n is the upper bound of summation. This is the standard mathematical notation for the summation. If the meaning is here different, please specify.

R2. The point raised by the reviewer is correct. We acknowledge that the lower bound of summation notation i=10 was incorrect and we thank this reviewer to highlighted the mistake. Following your question, the text has been changed as follows:

"The ITIs applied in this study are calculated as follows:
ITI = $\sum^{n}i=1(Si*Fi)/100$
Where Si is the score associated with each abnormal embryonic morphology and Fi is the frequency observed for that abnormality expressed as a percentage (n=13 for ITI 1.0 and n=6 for ITI 2.0, ITI 3.0, and ITI 4.0)."

Q3. Line 182-183. Please indicate a reference for the threshold of 20% to be considered as significant.
R3. The reference has been added as suggested (see text)
Morroni, L., Sartori, D., Costantini, M., Genovesi, L., Magliocco, T., Ruocco, N., Buttino, I. 2019. First molecular evidence of the toxicogenetic effects of copper on sea urchin Paracentrotus lividus embryo development. Water Res. 160: 415-423. doi: 10.1016/j.watres.2019.05.062

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R4. As for your suggestion, the colour code has been added to the figure legend (see text).

Q5. Line 233: "…… 13% and 5% of the embryos at the prism stage, respectively..": are these data reported in a supplementary file?
R5. These data have been not reported in a supplementary file because they are out of the scope of this short communication. Thank you for your comment, to avoid any misunderstanding a "not shown" has been added at the end of this sentence.

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Highlights

- *Paracentrotus lividus* embryo bioassay was used to evaluate dredged marine sediments
- The harbour of Taranto was chosen as a model pilot-study for sediment evaluation
- New Integrative Toxicity Indices was developed and validated to improve the reliability of the sea urchin embryo assay
- ITI 4.0 weighted developmental delay and malformations in the most balanced way
- ITI 4.0 resulted in a promising tool in the quality assessment of dredged sediments

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

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Abstract

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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. 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 the 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 a 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 (Morroni et al., 2016, 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. 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 a 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).

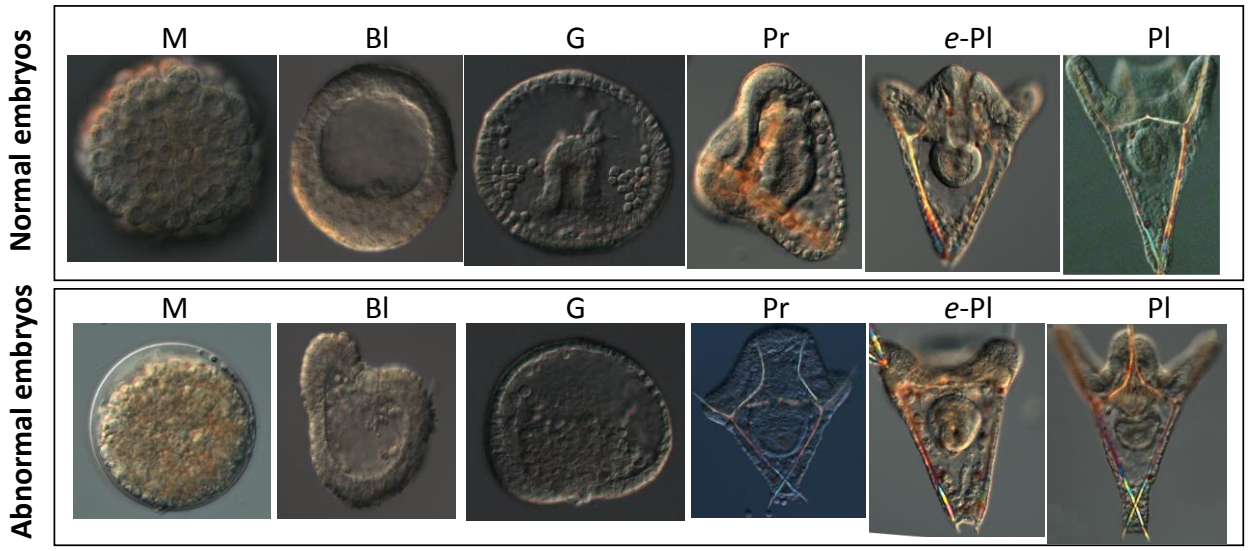
Three replicates were performed for each elutriate sample as well as for controls. Control embryos were exposed to ASW only, for a total of nine control cultures (each in triplicates). At 48 hours after fertilization (h), live embryos were observed and photographed using an optical microscope equipped with a digital camera (OLYMPUS CKX3). The results were considered valid and acceptable only if each set of control presented at least 80% of normal embryos. 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; *e*Pl, early Pluteus; Pl, Pluteus

The ITI 1.0 from the 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, level 4 was associated with the delayed embryos from fertilized egg to prism (F-Pr) and level 5 with the malformed F-Pr (F-*m*Pr); 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 fertilized egg to gastrula stage (F-G and/or F-*m*G), 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 the 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 fertilized egg to blastula stage (F-Bl and/or F-*m*Bl). Therefore, the lower degree of toxicity was assigned to Pl with the 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:

$ITI = \sum^{n}_{i=1}(S_i * F_i)/100$

Where Si is the score associated with each abnormal embryonic morphology and Fi is the frequency observed for that abnormality expressed as a percentage (n=13 for ITI 1.0 and n=6 for ITI 2.0, ITI 3.0, and ITI 4.0).

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			F-Pr			Pl		*e*Pl		F-Pr	
Score	**0**	**1**			**4**			**2**		**3**		**5**	
ITI 3.0	**Normal**	**Delayed**			**Malformed**			**Delayed and/or Malformed**					
	Pl	*e*Pl			Pl		*e*Pl	Pr			F-G		
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			F-Bl		
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. F-Bl, from fertilized egg to Blastula; F-G: from fertilized egg to Gastrula; F-Pr: from fertilized egg to Prism.

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 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% (Morroni et al., 2019).

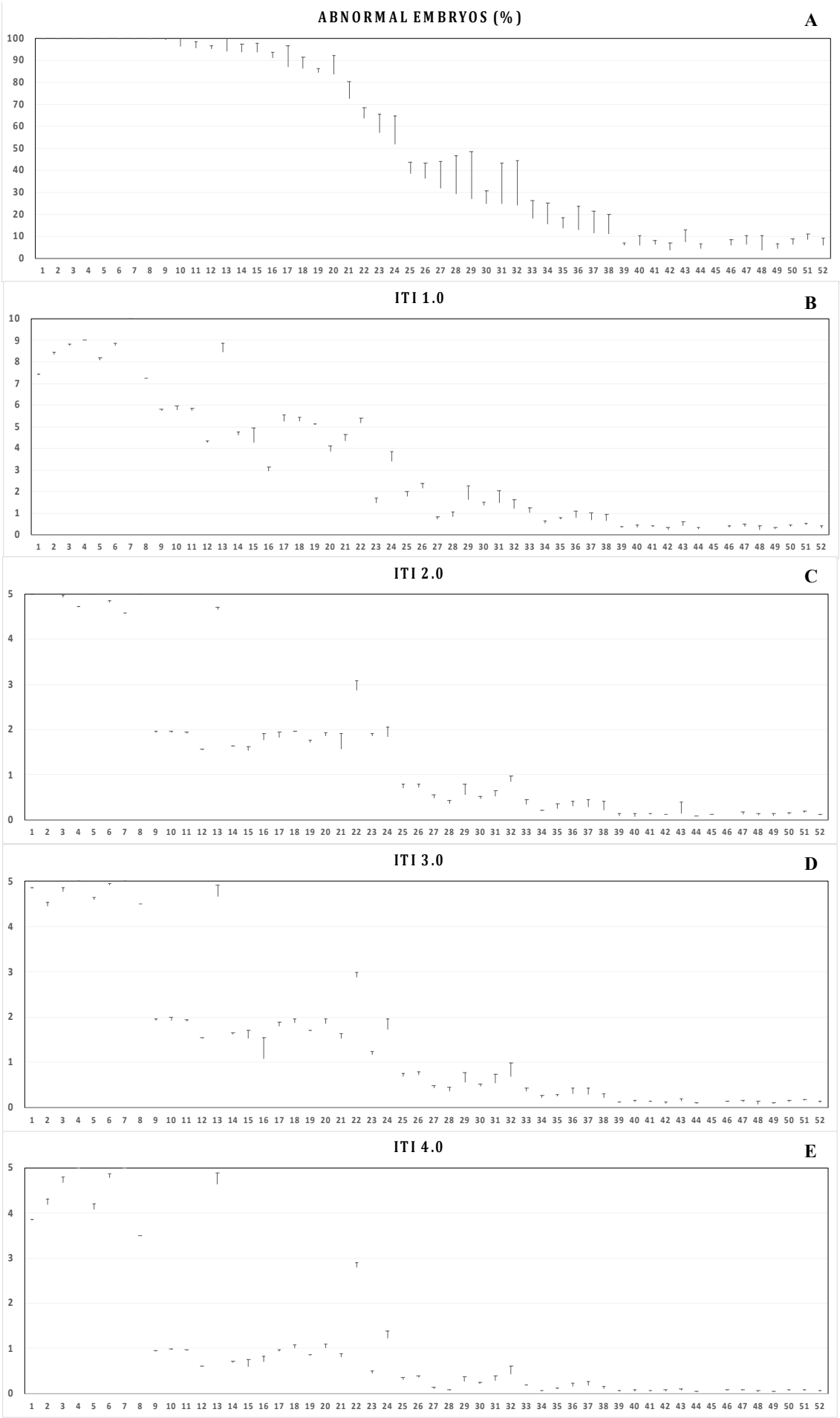


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Figure 1. Sea urchin embryonic development evaluated according to standard criteria and thoughtful ITIs. Histograms represent the results expressed as a mean percentage (%) of abnormal embryos±SD (A), and values of the ITI 1.0 (B), ITI 2.0 (C), ITI 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. Red bars: percentage of abnormal embryos higher than 75%; Blue bars: percentage of abnormal embryos ranging from 75% to 20%; Green bars: percentage of abnormal embryos lower than 20%; Grey bars: controls.

The evaluation of embryo-toxicity based on the ITI 1.0 method, which discriminates the frequency of delayed and/or abnormal embryonic morphologies and quantitatively ranks 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 the 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 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 the 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 fertilized egg to prism (F-Pr); 5 for malformed embryos from fertilized egg to prism (F-*m*Pr). 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 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 flattened (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 fertilized egg to gastrula (F-*em*G). These differences increased the ability of ITI 3.0 to discriminate among groups compared to 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 sample number 2 and 5, with 13% and 5% of the embryos at the prism stage, respectively (not shown). This stage was not well discriminated in ITI 2.0 as score 4 is assigned to embryos at the stages from fertilized egg (F) to prism (Pr).

Finally, when we used the ITI 4.0, discriminating strictly developmental delay and morphological defects from fertilized egg to gastrula (1 for *em*Pl; 2 for *e*Pr; 3 for *m*Pr; 4 for *em*G; 5 for F-*em*Bl), we still increased the ability to discriminate among groups (see Figure 1E). Notably, several samples showed 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 fertilized egg to blastula) (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). Several

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 fertilized egg to gastrula), when the cell fate has not been specified yet, the probability for embryos to recover from the delay and continue the development is 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 a 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 to 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 embryotoxicity 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 combines 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 by policymakers 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 weight developmental delay and morphological defects in a balanced way.

The development of such a 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.

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CRediT authorship contribution statement

Rosa Bonaventura: Investigation, Data Curation; Writing-Original Draft, Project administration; **Francesca Zito:** Investigation; **Lorenzo Morroni:** Software, Formal analysis, Writing-Review & Editing; **David Pellegrini:** Writing-Review & Editing; **Francesco Regoli:** Writing-Review & Editing, Validation, Software; **Annalisa Pinsino:** Conceptualization, Investigation, Data Curation, Writing-Original Draft, Writing-Review & Editing; Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interest.

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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 ~~(200 words)~~

Management of dredged materials disposal is regulated by~~in accordance to~~ several environmental normative requirements, and it is often supported by the 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 a 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 fertilized egg to gastrula stage~~gastrula to fertilized eggs~~, resulted in 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, t~~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 the 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 a 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 (Morroni et al., 2016, 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. To~~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 a 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

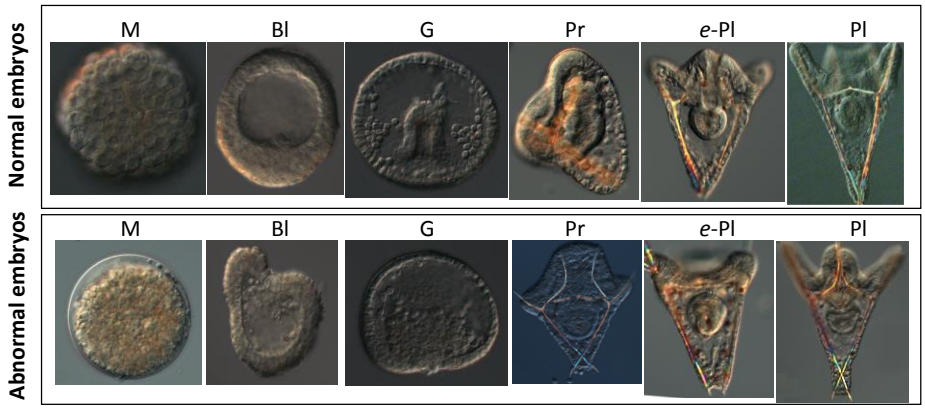
ml beakers with 0.45 µm filtered artificial seawater (ASW), while sperms were collected dry (directly from the surface of the sea urchin) using a micropipette with the end of the tip cut off, maintained in a sealed container at room temperature, and used in 30 minutes. Egg quality and sperm motility 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. Control embryos were exposed to ASW only, for a total of nine control cultures (each in triplicates). At 48 hours after fertilization (h), live embryos were observed and photographed using an optical microscope equipped with a digital camera (OLYMPUS CKX3). The results were considered valid and acceptable only if each set of control presented at least 80% of normal embryos. 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; *e*Pl, early Pluteus; Pl, Pluteus

The ITI 1.0 from the 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 fertilized egg to prism ~~to fertilized egg~~ (F-Pr-~~F~~) and ~~the~~ level 5 with the malformed F-Pr-~~F~~ (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 fertilized egg to gastrula stage ~~to~~

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fertilized egg (F-G -F and/or F-*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 the 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 fertilized egg to blastula stage to fertilized egg (F-Bl-F and/or F-*m*-Bl-F). Therefore, the lower degree of toxicity was assigned to Pl with the 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:

$ITI = \sum_{i=1}^{n} (S_i * F_i)/100$

Where Si is the score associated with to each abnormal embryonic morphology and Fi is the frequency observed for that abnormality expressed as a percentage that morphotypes (ni=10 13 for ITI 1.0 and n=6 for ITI 2.0, ITI 3.0, and ITI 4.0).

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			F-Pr-F			Pl		*e*-Pl		F-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		F-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		F-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. F-Bl-F, from fertilized egg to Blastula to fertilized egg; F-G-F: from fertilized egg to Gastrula to fertilized egg; F-Pr-F: from fertilized egg to 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% (Morroni et al., 2019).

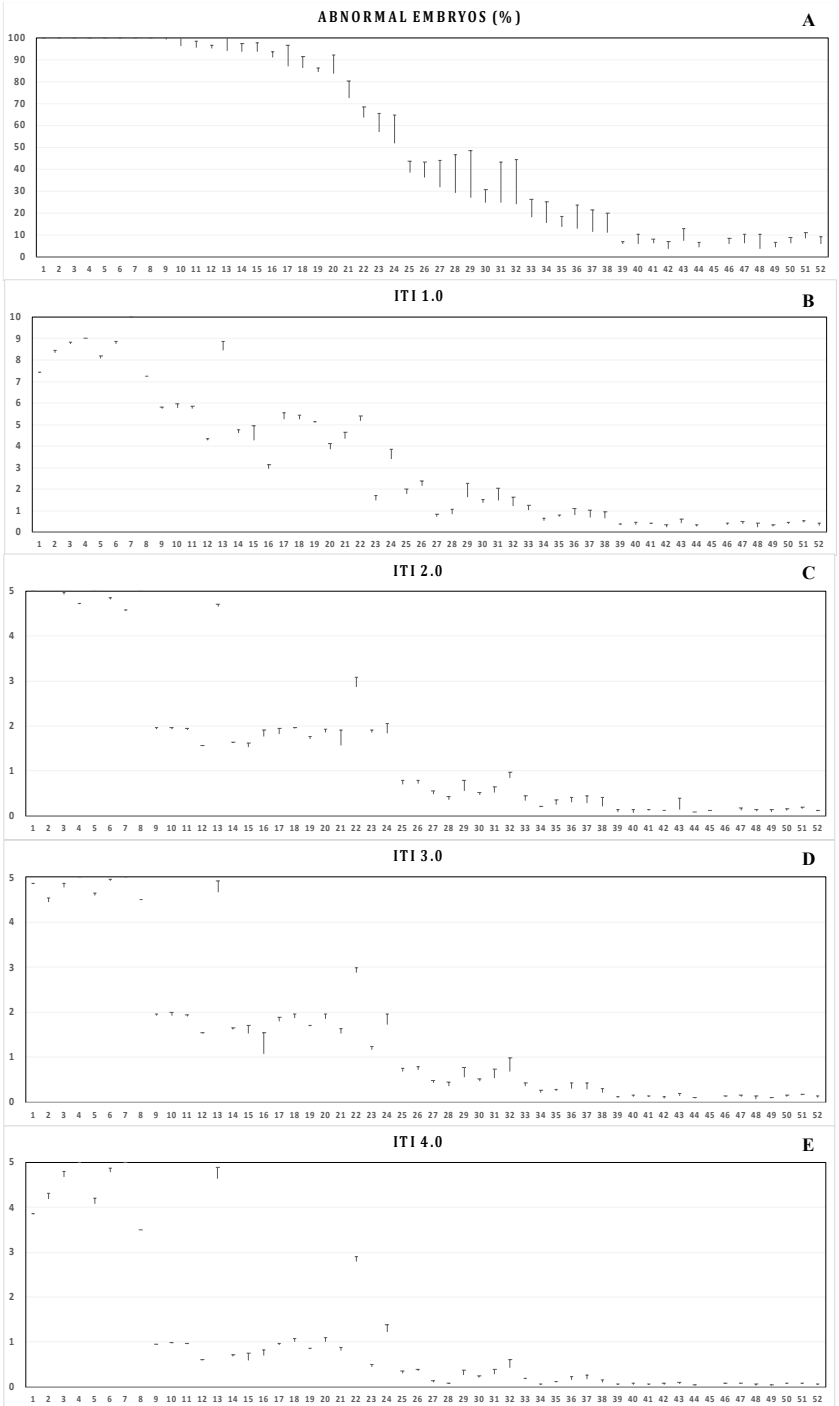

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Figure 1. Sea urchin embryonic development evaluated according to standard criteria and thoughtful ITIs. Histograms represent the results expressed as a mean percentage (%) of abnormal embryos±SD (A), and values of the ITI 1.0 (B), ITI 2.0 (C), ITI 3.0 (D), ITI 4.0 (E) ± SD. Data are referred to each elutriate sample of Taranto harbour, reported on X-axis~~X- axis~~. Controls (CTR) are reported in the right part of the panels for a total of 9. Red bars: percentage of abnormal embryos higher than 75%; Blue bars: percentage of abnormal embryos ranging from 75% to 20%; Green bars: percentage of abnormal embryos lower than 20%; Grey bars: controls.

The evaluation of embryo-toxicity based on the ITI 1.0 method, which discriminates~~discriminate~~ the frequency of delayed and/or abnormal embryonic morphologies and quantitatively ranks~~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 the 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 the~~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 fertilized egg to prism ~~to fertilized eggs~~ (F-Pr-~~F~~); 5 for malformed embryos from fertilized egg to prism ~~to fertilized eggs~~ (F-*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 flattened (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 fertilized egg to gastrula ~~to fertilized eggs~~ (F-*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 (not shown). This stage was not well discriminated in ITI 2.0 as ~~the~~ score 4 is assigned to embryos at the stages from fertilized egg (F) to prism (Pr) ~~to fertilized egg (F)~~.

Finally, when we used the ITI 4.0, discriminating strictly developmental delay and morphological defects from fertilized egg to gastrula ~~to fertilized eggs~~ (1 for *em*-Pl; 2 for *e*-Pr; 3 for *m*-Pr; 4 for *em*-G; 5 for F-*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 fertilized egg to 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). Several~~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 fertilized egg to 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 a 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 to~~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 embryotoxicity~~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 combines~~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 by~~from~~ policymakers~~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 a 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.

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CRediT authorship contribution statement

Rosa Bonaventura: Investigation, Data Curation; Writing-Original Draft, Project administration; **Francesca Zito:** Investigation; **Lorenzo Morroni:** Software, Formal analysis, Writing-Review & Editing; **David Pellegrini:** Writing-Review & Editing; **Francesco Regoli:** Writing-Review & Editing, Validation, Software; **Annalisa Pinsino:** Conceptualization, Investigation, Data Curation, Writing-Original Draft, Writing-Review & Editing; Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Figure 1

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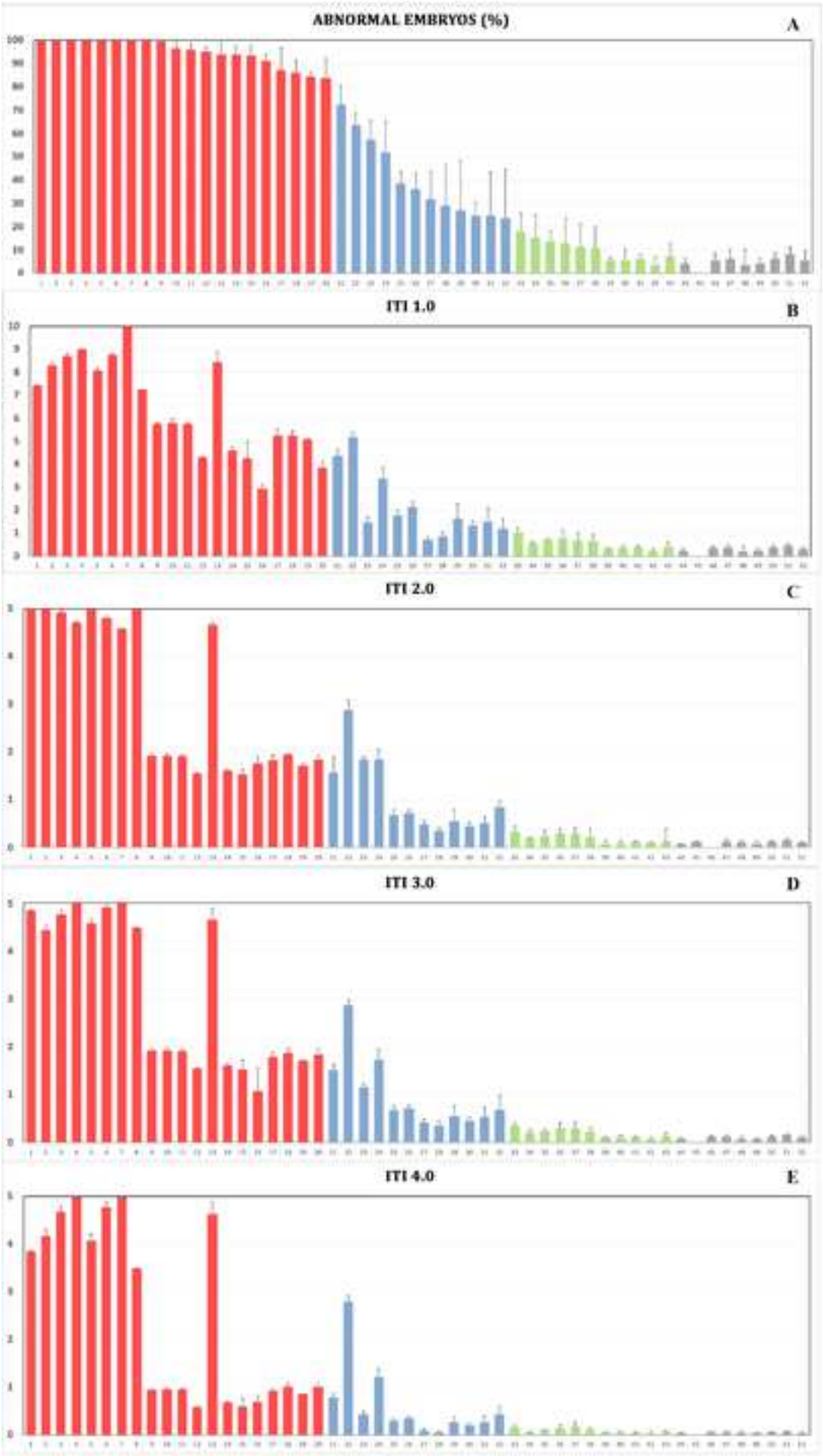

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Rosa Bonaventura: Investigation, Data Curation; Writing-Original Draft, Project administration; **Francesca Zito:** Investigation; **Lorenzo Morroni:** Software, Formal analysis, Writing-Review & Editing; **David Pellegrini:** Writing-Review & Editing; **Francesco Regoli:** Writing-Review & Editing, Validation, Software; **Annalisa Pinsino:** Conceptualization, Investigation, Data Curation, Writing-Original Draft, Writing-Review & Editing; Project administration, Funding acquisition.

Declaration of interests

☐ X **The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.**

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: