Towards sea cucumbers as a new model in embryo-larval bioassays: Holothuria tubulosa as test species for the assessment of marine pollution

Towards sea cucumbers as a new model in embryo bioassays: *Holothuria tubulosa* as test species for the assessment of marine pollution

Arnold Rakaj [ǂ1], Lorenzo Morroni [ǂ2*], Luca Grosso [1], Alessandra Fianchini [1], David Pellegrini [2], Francesco Regoli [3]

[1]: Laboratorio di Ecologia Sperimentale ed Acquacoltura, Dipartimento di Biologia, Università di Roma "Tor Vergata", Roma, Italy.
[2]: Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Livorno, Italy.
[3]: Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche, Ancona, Italy.
ǂ: contributed equally.
*: Corresponding author

Abstract

Sea cucumbers are widely distributed deposit-feeders that represent an important component of benthic communities. These echinoderms were recently proposed as candidates in embryo bioassays to provide a new tool in the toxicity assessment of pollutants in marine water and sediments. The aim of this study is therefore to evaluate the usefulness of a new species, *Holothuria tubulosa* (Gmelin, 1788), as a model organism for sensitive embryo bioassays, defining the acceptability of controls, minimum sample size, embryo density and salinity range. Cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), sodium dodecyl sulphate (SDS) and 4-n-nonylphenol (NP) were used as reference toxicants to assess specific embryotoxicity endpoints. Sea cucumber and sea urchin embryos were subsequently incubated with elutriates of harbour marine sediments to compare the responsiveness of these two species. The results showed an acceptability threshold of 10% (abnormal embryos), a minimum sample size of 200 embryos, an embryo density of 200 embryos/ml and an optimal salinity range of 36-37‰. The sensitivity to the environmental pollutants and matrices tested revealed values (expressed as EC_{50}) comparable with those of embryos belonging to other marine invertebrates commonly used in bioassays, indicating that this species has a good level of responsiveness. A specific integrative toxicity index (ITI) was applied, combining the frequency of developmental anomalies and weighting their severity. Data elaborated with ITI demonstrated good discrimination of sample toxicity, with a dose-dependent increase of teratogenic effects for all the tested substances, indicating *H. tubulosa* as a promising species for future assessments of marine pollution.

1. Introduction

Marine ecosystems are being subjected to increasing pressure due to a wide range of anthropogenic activities that result in the release of a large variety of chemicals into coastal waters, leading to the accumulation of a multiplicity of substances in marine sediments (Reguera et al. 2018). Among these are trace metals (Morroni et al. 2018), hydrocarbons (Bellas et al. 2008), Persistent Organic Pollutants (POPs) (Bocquené and Abarnou 2013) and other emerging pollutants such as nanomaterials (Corsi et al. 2014, Oliviero et al 2019), pharmaceuticals (Aguirre-Martínez et al. 2015), microplastics and new bioplastics (Avio et al. 2017; Campani et al. 2020). Nowadays, it is widely recognized that the impact of these compounds should be evaluated by placing increasing importance on the assessment of the biological effects these have on marine organisms following an integrated approach incorporating chemical data. Within this framework, ecotoxicological bioassays have progressively been applied to quantify the potential biological hazard caused by bioavailable multi-factorial contamination, taking into consideration also synergic effects, and thus providing a more significant response that is not restricted to a predetermined list of contaminants (Morroni et al. 2020a). Embryo bioassays with marine invertebrates are considered sensitive ecotoxicological tests that can be employed to assess the impact of marine pollution since the early life stage of many marine invertebrates, including benthic invertebrates, is often that of planktonic larva. For these larva, the embryogenesis and the subsequent larval metamorphosis are particularly delicate events which can be easily altered by environmental contaminates present in the water column. Embryos and larvae are directly exposed since they have no protection and lack the ability to escape from chemically polluted waters (Saco-Alvarez 2008), making them more vulnerable to environmental contaminants than adults. The transparent embryo is also suitable for the observation of malformations, making it possible to detect sub-lethal effects of pollutants on multicellular body formation at an early stage in development. For this reason, various test species of marine invertebrates have been proposed in the last few decades for embryo-larval bioassays (His et al. 1999; Bellas et al. 2005; Perez et al. 2019). As single species

bioassays are not expected to detect the full range of potential effects of pollutants, the use of a battery of bioassays is strongly recommended for the assessment and monitoring of the marine environment (Rodríguez-Romero 2016, Regoli et al. 2019). Additionally, the use of a single species can present some practical limitations, such as a seasonal-dependent spawning period, difficulties in finding organisms in specific areas due to local fishing and overexploitation - as in the case of sea urchins (Guidetti et al. 2004). In order to provide a wider choice among suitable species, sea cucumbers have recently been proposed as candidates for the development of embryo toxicity bioassays (Morroni et al. 2020b). Sea cucumbers represent an important component of benthic communities since they are widely distributed ecosystem engineers that play a key role in sea floor dynamics by processing and bioturbating the sediment (Purcell et al. 2016; Rakaj et al. 2015; 2019), as well as in the creation of seagrass detrital pathways for Mediterranean costal ecosystems (Boncagni et al. 2019). The sensitivity of echinoderms to pollutants, together with their ecological importance, has prompted suggestions for their use as model organisms in the testing of the effects of marine pollution (ASTM 2012, Pagano et al. 2017, Morroni et al. 2016, 2018, 2020b). *Holothuria tubulosa* (Gmelin, 1791) is one of the most common and widely distributed sea cucumbers in the Mediterranean basin, it is relatively simple to maintain in a laboratory conditions, and spawning can be easily induced through a recently developed protocol for aquaculture (Grosso et al., 2020; Rakaj et al. 2018). The reproductive period of *H. tubulosa* occurs between June and October, reaching full maturity at the same time as the sea urchin *Paracentrotus lividus* enters the recovery gonadal stage, limiting its availability for embryo bioassays. Due to the reproductive cycle of *H. tubulosa*, its high fecundity, widespread distribution and important ecological role, this species can be considered as an additional candidate for testing the embryotoxicity of environmental samples in ecotoxicological studies.

The aim of this paper is therefore to assess the suitability of *H. tubulosa* as a model species for embryo bioassays, developing a harmonized methodology in order to integrate this new bioassay with those already standardised for other marine invertebrates, in particular with the sea urchin sister-group. Following the protocol for sea cucumber embryo bioassays developed by Morroni et al (2020b), a

series of experiments was set up to measure the responsiveness of embryos to salinity, and to define the reproducibility of the test, establishing the minimum sample size and the optimal larval density. The embryos were also exposed to organic and inorganic pollutants, cadmium (Cd^{2+}), copper (Cu^{2+}), mercury (Hg^{2+}), lead (Pb^{2+}), sodium dodecyl sulphate (SDS) and 4-n-nonhyphenol (NP), selected as reference toxic compounds for embryos of several marine species (Beiras and Albentosa 2004; Oliva et al. 2018; Morroni et al 2018, 2020b,). The *H. tubulosa* embryo bioassay was further validated with exposure to elutriates from marine sediments, comparing the teratogenic effects on larval development with those obtained from sea urchin embryos. The percentage of abnormal embryos was chosen as the first measured endpoint. A second endpoint, consisting of a specific Integrative Toxicity Index (ITI) (Morroni et al 2016) was developed and applied. More specifically, a new toxicity scale was developed in this study by linking different values at each morphotype with germ layer specific malformations and developmental stage, associating different scores to various typologies of developmental anomalies. This information was then summarised to form an integrative index. Implementing this particular approach, it is possible to discriminate between various adverse effects and give different scores depending on developmental stage and the severity of the malformations: this aspect is particularly important for moderately toxic sediments, enhancing the sensitivity of the bioassay (Morroni et al. 2016).

2. Material and Methods

2.1 Sampling and acclimatization

Adult sea cucumbers (*H. tubulosa*) were collected through snorkelling (1-5 m) at Santa Marinella in the central Tyrrhenian Sea, Italy (42°3′0″N, 11°49′9″E) from June to October 2019. The organisms were transported to the Laboratory of Experimental Ecology and Aquaculture (University of Rome Tor Vergata) inside 30 L tanks equipped with aerators and ice to maintain the temperature below 28°C, and then acclimatized in a recirculating aquaculture system (RAS) at 24 °C for 7 days.

2.2 Sea cucumber embryo tests with H. tubulosa

A sea-cucumber embryo test was performed following the protocol developed by Morroni et al. (2020b). Specifically, before spawning was induced, adults were kept in substrate-free aquaria for 48 h in order to void their gut contents. After this period spawning was induced by thermal shock. To perform this operation the sea cucumbers were transferred individually to 5 L spawning tanks with 0.45 µm filtered and UV-sterilized seawater (FSW) and the water temperature was increased rapidly by 3–5°C (from 24°C to 27–29°C). This temperature was maintained for 1.5 h, after which the water was cooled to the initial temperature (Rakaj et al. 2019). Eggs were collected from each female from the bottom of spawning tanks and were re-suspended in separate beakers with FSW. Before pooling the eggs, their quality was assessed under a microscope: females releasing non-round eggs, immature forms or debris were discarded. Males were identified after the first spawn and removed from the spawning tanks. Dry sperm was collected from at least three males and stored at 4°C until use. Sperm motility was checked under a microscope and diluted sperm suspension was added to an aliquot of egg suspension to check the formation of the fertilization envelope. A total of 100 eggs were checked in order to calculate the percentage of fertilization success. Males fertilizing fewer than 95% of the eggs were discarded (Morroni et al 2020b).

To perform fertilization, a suspension of 1×10^4 spermatozoa/mL was diluted in 500 mL of egg suspension (1000 eggs/mL). After the formation of the fertilization envelope the embryos were washed three times by decantation, removing the sperm in the supernatant and adding FSW. After 20 minutes, 1 mL of fertilized eggs suspension was added to 9 mL of test solution, thus reaching a final density of 100 embryos/mL (Morroni et al. 2020). Experiments were set up using 10 mL sterile capped polystyrene six-well microplates, with 6 replicates being carried out per experimental condition. Embryos were incubated in dark conditions at a temperature of 26°C for 72h, as recommended by Morroni et al (2020). At the end of the incubation period samples were fixed and preserved by adding one drop of Lugol's iodine solution. Embryo morphology was evaluated under an inverted microscope.

2.3 Endpoints and toxicity criteria

100 embryos were analyzed by optical microscopy (Zeiss Axio Vert A1) and photographed using a digital camera (Zeiss Axiocam 105 color) with a 25x zoom. The mid-auricularia stage was selected as the developmental endpoint. The degree of toxicity was calculated using the Integrative Toxicity Index (ITI), based on the percentage and severity of embryo malformations (Morroni et al., 2020b). More specifically, embryos were classified as normal if they satisfied the following morphological criteria (Morroni et al. 2016): (1) correct schedule in reaching the developmental endpoint, (2) left/right and dorso/ventral embryonic axis symmetry, (3) correct differentiation of the digestive system (mouth, stomach, and anus). The toxicity was quantified by counting the frequency of delayed and/or abnormal embryo morphology and by ranking the severity of effects quantitatively from 0 (none) to 5 (high). Anomalies were classified as delays in development with or without further morphological alterations, and different stages weighted depending on the severity of anomalies observed. Lower toxicity values were given to embryos with delays in development without anomalous defects, while higher scores were attributed to embryos that displayed both delayed development and malformations. Details of the scores, with four photographic examples per morphotype are given in Table 1. The toxicity score is 0 when normal mid-auricularia (mAu) is observed, and 1 for well-developed early-auricularia (eAu) larvae, i.e. with only development delays but without malformations. Toxicity values increase for malformed embryos and larvae, depending on the stage of development: higher toxicity values are attributed to malformed mAu (score 3), still higher for delayed and malformed early-auricularia larvae (score 4) and, finally, the highest score at Gastrula/Blastula/Morula stage (G/Bl/M) (score 5).

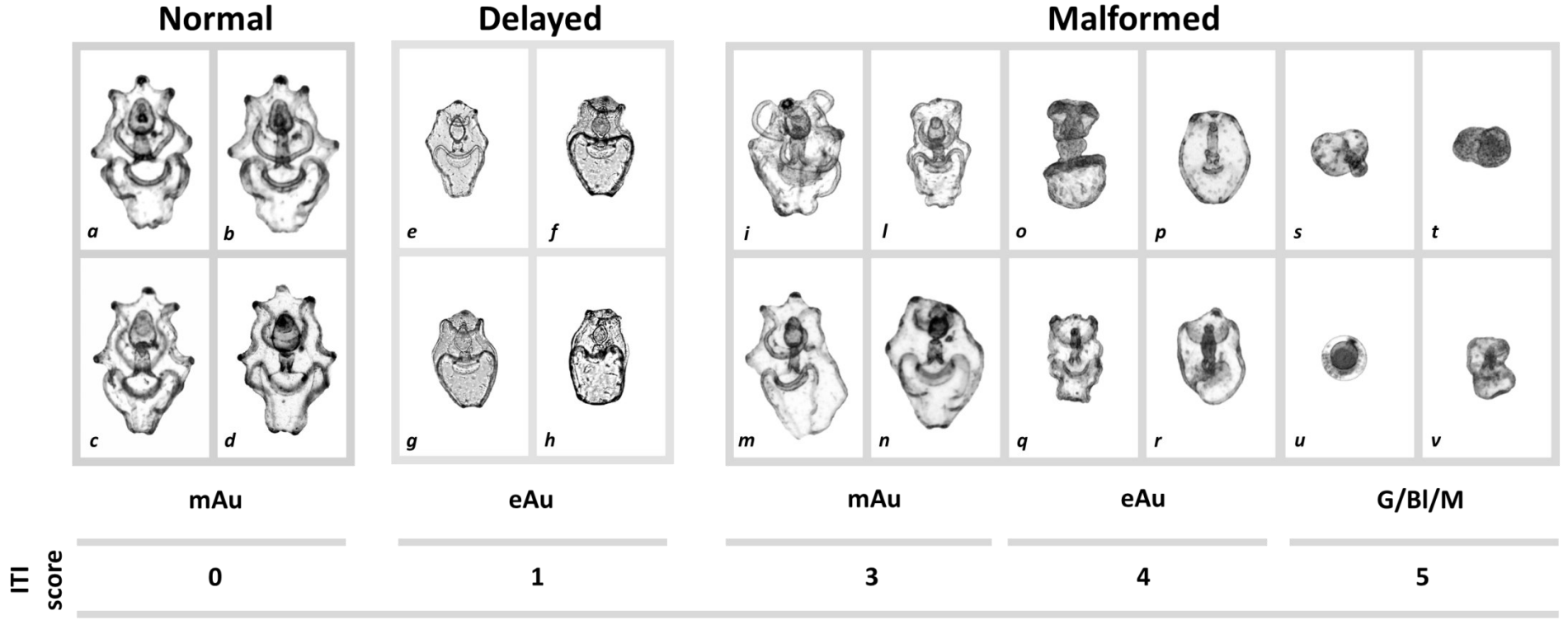
The integrative toxicity index (ITI) was calculated as follows:

$$ITI = \sum_{ni}=10(S_i*F_i)/100$$

Where S_i is the score associated at each abnormality and F_i is the frequency observed for that abnormality (i=10).

163
164
165
166

Table 1 - Classification of developmental anomalies in *Holothuria tubulosa* according to the Integrative Toxicity Index (ITI) based on developmental delays (delayed embryos) and malformations (malformed embryos). mAu: mid-auricularia; eAu: eraly-auricularia; G: Gastrula; Bl: Blastula; M: Morula. *a, b, c, d*: normal mAu; *e, f, g, h*: normal eAu; *i, l, m, n*: malformed mAu; *o, p, q, r*: malformed eAu; s, t, u, v: malformed G/Bl/M.



172 *2.4 Assessment of embryo density*

173 Tests were carried out using different egg suspensions (100, 200, 400, 600, 800 fertilized eggs/ml), to
174 find the optimal egg density for morphological observations without negative effects on embryo/larval
175 development.

176

177 *2.5 Assessment of salinity effects*

178 To investigate the optimal conditions for embryogenesis and larval development, *H. tubulosa*
179 embryos were exposed to ASW with different degrees of salinity: tests were performed by incubating
180 fertilized eggs at 32, 33, 34, 35, 36, 37, 38, 39, 40, 42 ‰ up to 72 hours. The ASW was prepared
181 according to the formulation from Lorenzo et al. (2002) to obtain 42 ‰ salinity, after which it was
182 diluted with ultra pure water (Milli Q) to obtain 5 L of reference sea water for each experimental
183 salinity. Before the experiment, the accuracy of the reference sea water salinity was measured using
184 a salinity probe (HI98319).

185

186 *2.6 Reference toxicants*

187 Cadmium chloride, copper nitrate, lead nitrate, mercury chloride, Sodium Dodecyl Sulphate (SDS)
188 and 4-nonylphenol (NP) were used as reference toxicants. Stock solutions were prepared by
189 dissolving reagent grade (Sigma Aldrich srl, Milan, Italy) in 1000 ml glass flasks with bidistilled
190 water (BDW) to obtain a concentration of 1000 mg/L. Test solutions were obtained by diluting 10 ml
191 of the stock solution in 100 or 1000 mL glass flasks with FSW. Finally, the stock solutions in FSW
192 were placed in the six-well microplates, with 6 replicates being carried out per experimental condition
193 to obtain the concentrations of toxicants reported in Table 2, taking into account also the 10% dilution
194 of the nominal concentrations caused by the addition of the egg suspension to the experimental
195 solution(1 ml of sea cucumber eggs were finaly placed in each six-well microplate).All glassware
196 was sterilized before the experiments with washes of HNO_3 (10% vol.), acetone and ultra-pure water,

as indicated by Bellas et al. (2004), and chemical analyses were performed for the tested solutions (See).

Table 2 – Exposure concentrations of reference toxicants.

Reference toxicant	Exposure concentrations (mg/L)
Cd	2,4,5,6,7,8,9
Cu	0.08; 0.09; 0.10; 0.11; 0.12
Hg	0.01; 0.02; 0.03; 0.04; 0.05; 0.06; 0.07
Pb	0.30; 0.40; 0.50; 0.60; 0.70; 0.80; 1,00; 1.50
NP	0.30; 0.40; 0.50; 0.60; 0.80; 1
SDS	6;7;8;10;12

2.7 *Elutriate samples*

The responsiveness of *H. tubulosa* embryos was compared with that of *P. lividus* testing these species toward the elutriates of environmental matrices. The sea cucumber and sea urchin embryos were exposed in parallel to elutriate samples from harbour sediments, using 3 dilutions in FSW (90%, 50%, 25%), following the methodology reported by Morroni et al. (2018).

Sediments were collected from 4 stations in Piombino harbour, in the north Tyrrhenian Sea (Italy). Elutriates were prepared within 10 days from the sampling, with a storage temperature of 4°C, in accordance with USEPA (1991) guidelines and literature studies (Volpi Ghirardini et al., 2005). Sediment samples were mixed in a 1:4 (v/v) ratio of sediment to FSW and placed on a rotary shaker table for 1 h, at a speed of 300 rpm, at room temperature. The dilutions were made up with FSW collected in a long-term monitored reference site located far from human activities. After mixing, the samples were centrifuged (Thermo Scientific SL 16R, Rodano, Italy) for 20 min at 3000 rpm (4 °C)

220 and the aqueous fractions (elutriate samples) were poured off and stored for 30 days at -20 °C until
221 use in embryo incubation.
222
223 2.8 *Chemical analysis*
224 Concentrations of trace metals (Al, As, Cd, Cu, Cr, Hg, Ni, Pb, Zn), Polycyclic Aromatic
225 Hydrocarbons (PAHs), Organochlorine Pesticides (OPs), Organotin Compounds were determined in
226 marine sediments used for elutriates. In particular, high performance liquid chromatography (HPLC)
227 with diode array (DAD) and fluorimetric detection were used for PAHs, atomic absorption
228 spectrophotometry (AAS) for trace metals (Benedetti et al., 2014). PCBs and OPs were analysed
229 using the EPA 3545a (extraction), EPA 3630 (clean-up) and EPA 8270D methods for analytical
230 determination. Organotin compounds (TBT) were analysed according to ICRAM (2001). Total
231 concentrations / levels of ammonia and nitrite were determined by spectrophotometer (HACH
232 LANGE GmbH DR 2800 using kit 304 and 340 HACH LANGE GMBH LCK) on elutriate samples.
233 Trace metals in test solutions were, determined through inductively coupled plasma optical emission
234 spectrometry (ICP-OE, Agilent Technologies 7900, Santa Clara, CA, USA). Concentration of NP
235 was measured via an HPLC-fluorescence detection method (Cruceru et al. 2012), while SDS was
236 measured as a methylene blue active substance (MBAS) using a Perkin Elmer Lambda 45
237 spectrophotometer (George and White 1999).
238
239 *2.8 Data analysis*
240 The results of negative controls with FSW obtained in the experiments were used to assess the quality
241 of the biological material. Minimum acceptable percentages of abnormal embryos and ITI values
242 were established in order to define test acceptability. Minimum sample size (n) was also estimated
243 on the basis of tables provided by Burnstein (1971), calculating the proportion of abnormal embryos
244 in the whole population (p) at a given confidence level, assuming sampling without replacement. The

obtained sample is a non-negligible fraction of the total population N (i.e. n >5% N), hence n is over-estimated, and thus the obtained value was corrected for a finite population (Bellas et al. 2003).

To evaluate the toxic effect of reference toxicants and elutriate samples, the percentages of abnormal embryos were considered, estimating EC_{50} values which were then compared with literature data available for other test species. EC values with 95% confidence limits were calculated following the Trimmed Spearman-Karber statistical method. Responses in each experimental condition were corrected for effects in control tests by applying Abbott's formula (Hamilton et al., 1978). Variations in embryo development related to salinity and embryo density were analysed using one-way ANOVA. The homogeneity of variances was tested through Cochran's C-tests; Student–Newman–Keuls (SNK) tests were performed to check for *a posteriori* comparisons of mean values after significant effects in ANOVA (Underwood 1997). Significant differences between the percentage of abnormal larvae in FSW (control) and in the reference substance solutions were determined by one-way ANOVA followed by a Dunnett's test for multiple comparison (Carballeira et al. 2012; Murado and Prieto 2013). Two levels of significance were established: $p < 0.05$ and $p < 0.01$. The effect of each toxicant was determined by a parametric Pearson correlation test. Significance was established at 95% ($p < 0.05$). A correlation test to compare the sensitivity of *P. lividus* and *H. tubulosa* to different pollutants was also performed. Both ANOVA and Pearson correlation tests were performed using PAST statistical software (Hammer et al., 2001).

3. Results

3.1 Optimal embryo density

The density of fertilized eggs in the initial suspension significantly affected the embryo/larval development during the bioassay ($p<0.01$), as reported in Figure 1. No significant difference was observed between the densities of 100 and 200 embryos/ml, considering both the percentage of normal embryos (Fig. 1A) and ITI (Fig. 1B). On the contrary, higher densities caused a significant increase in developmental anomalies. Consequently, 200 embryos/ml was chosen as optimal density,

since this level combines the absence of negative effects on embryogenesis with a good number of embryos, allowing for a minimum sample size and easy counting of the embryos.

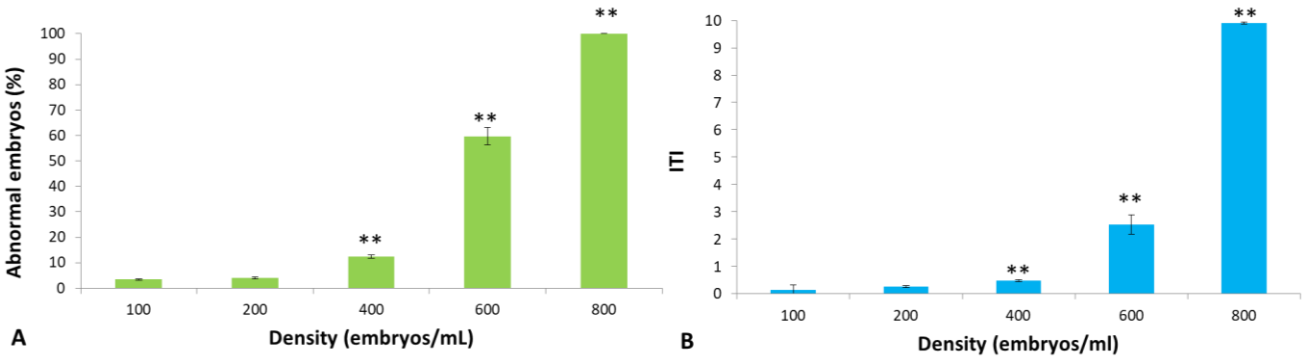


Figure 1 - The effects of embryo density on *Holothuria tubulosa* embryo development. The X axis represents the increasing densities of embryos, while the Y axis shows the endpoint of embryo development. Data are expressed as a mean percentage of abnormal embryos (A, green bars) and ITI (B, blue bars) ± standard error. Asterisks indicate significant differences between each treatment and the optimum value of 36 ‰ (*: $p<0.05$, **: $p<0.01$).

3.2 Test acceptability and sample size

Control data showed a normal distribution expressed both as a percentage of abnormal embryos (Shapiro-Wilk W: 0.95, $p<0.01$) and as ITI (Shapiro-Wilk W: 0.92, $p<0.01$). The 10th percentile of the distribution corresponds to 6.91% (abnormal embryos) and 0.04 (ITI); thus, thresholds of acceptability were fixed at 10% and 0.05, respectively. Concerning minimum sample size, setting an error of 0.05 (confidence level of 95%), values of n for expected p values of 1, 0.90, 0.75, 0.60, 0,5, 0.40, 0.25, 0.10 and 0 were, respectively, 58, 200, 334, 400, 400, 400, 334, 200 and 58. After applying the correction for finite populations, a minimum sample size of 200 embryos allows a 95% confidence in the estimate with an error of 5% and hence was considered as ideal for the test.

Having a final density of 200 eggs/mL 1, a minimum of 1mL in each vial treatment therefore needed to be examined. Each experimental condition was replicated in 6 wells.

3.3 Optimal salinity range

Salinity significantly affected embryo development ($p<0.01$) and detailed effects are reported in Figure 2. The optimal range was found to be between 36 and 37‰, without significant differences between these two levels. This salinity range is the same for both the endpoints established: i.e. the percentage of normal embryos (Fig. 2A) and the ITI (Fig. 2B). The effects are between 2% and 3%, expressed as a percentage of normal embryos (Fig. 2A) and between 0.01 and 0.03 (Fig. 2B) using the integrative toxicity index, with the absence of significant differences.

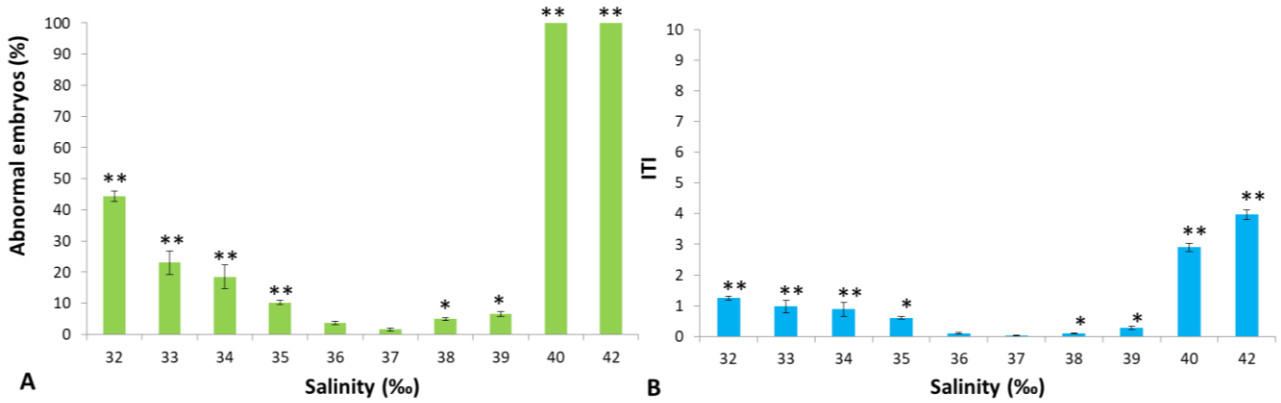


Figure 2 - The effects of salinity on *Holothuria tubulosa* embryo development. The X axis reports the increasing salinities used in the experiments, while the Y axis shows the endpoint of embryo development. Data are expressed as a mean percentage of abnormal embryos (A, green bars) and ITI (B, blue bars) ± standard error. Asterisks indicate significant differences between each treatment and the optimum value of 36 ‰ (*: $p<0.05$, **: $p<0.01$).

3.3 Reference toxicants

The results of the chemical analysis showed that measured concentrations generally varied less than 13% from the nominal concentrations. Thus, all calculations reported above were based on nominal concentrations.

The incubation of *H. tubulosa* embryos in water containing any of the types of trace metals or organic compounds induced significant abnormalities ($p<0.01$) in a dose-dependent-manner, as reported in Figure 3.

The effects of Cd^{2+} on embryo development resulted in an EC_{50} (±95% confidence limit) of 4.978 (3.884-5.628, Table 3). Specifically, Cd showed a percentage of abnormal embryos of 7, 65, 81, 100% at 4, 5, 6, 7 mg/L, respectively (Fig. 3A), and ITI more gradually increased with values of 0.59, 1.84,

317 3.47, 8.85, 9,74, 10 at 4, 5, 6, 7, 8, 9 mg/L, respectively (Fig. 3B). The capability of ITI to discriminate
318 toxicity alongside a greater range of metal concentrations, reflects a slight developmental delay at 5
319 mg/L, with a gradual shift from early auricularia to malformed auricularia at 6 mg/L and an increase
320 in malformations in gastrula/blastula/morula embryos at higher concentrations.
321 The percentages of abnormal embryos caused by Cu were 3, 23, 86, 100 % at 0.09, 0.10, 0.11, 0.12
322 mg/L (Fig. 3C), with an estimated EC_{50} (±95% confidence limits) of 0.104 (0.103-0.105, Table 3).
323 The ITI increased from 0.22 at 0.09 mg/l to 6.26 at 0.12 mg/L (Fig. 3D), appearing mainly related to
324 a developmental delay of up to 0.11 mg/L (71% of early auricularia) with a shift to malformed early
325 auricularia at 0.12 mg/L (63%, data not shown).
326 Hg was the most toxic among the tested metals, showing an EC_{50} (±95% confidence limits) of 0.023
327 (0.022-0.025, Table 3). Embryos incubated with Hg exhibited 10, 23, 92, 99, 100 % of abnormalities
328 at 0.01, 0.02, 0.03, 0.04, 0.05 mg/L, respectively (Fig. 3E), and ITI values of 0.78, 1.10, 3.84, 7.10,
329 9.28, 9.82, 10 at 0.01, 0.02, 0.03, 0.04, 0.05, 0.06 mg/L, respectively (Fig 3F). These values are due
330 to a developmental delay of up to 0.03 mg/L (63% of early auricularia) and a gradual shift from
331 malformed auricularia to gastrula stage at higher concentrations, reaching 100% of gastrula embryos
332 at 0.06 mg/L (data not shown).
333 The effects of Pb on embryo development were evident at 0.4 mg/L with 80% of abnormal embryos,
334 showing an increase to 93% and 100% at 0.5 and 0.6 respectively (Fig. 3G) (EC_{50}= 0.372 mg/L, Table
335 3). The increase in ITI is slightly more gradual than that of the percentages of abnormalities, ranging
336 from 1.66 at 0.4 mg/l to 9.83 only at 1.5 mg/L (Fig 3H). This difference is a result of the various
337 effects on embryo development depending on Pb exposure and detected by the

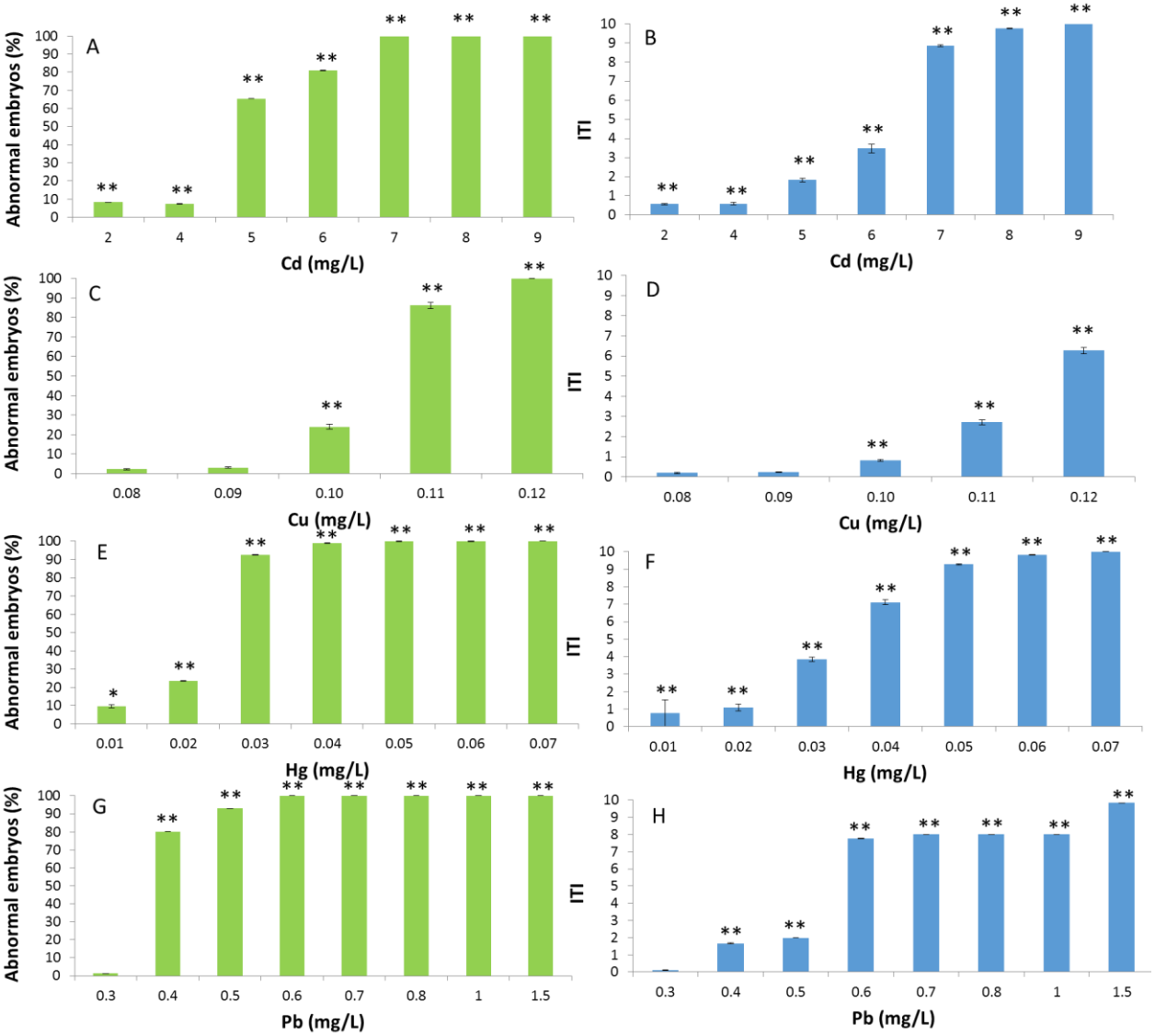


Figure. 3 – Dose-response relationships for Cd (A, B), Cu (C,D), Hg (E, F), Pb (G, H), SDS (I, L) and NP (M, N) on *Holothuria tubulosa* embryo development expressed as mean percentage of abnormal embryos (green bars) and ITI (blue bars) ± standard error. The X axis reports the increasing concentrations of reference toxicants, while the Y axis shows the endpoint of embryo development. Significant differences from controls are shown (*: $p<0.05$, **: $p<0.01$).

toxicity index. In particular, a developmental delay only was noted at 0.4 and 0.5 mg/L (90% of early auricularia), malformations at early auricularia appeared between 0.6 and 1 mg/L for almost the 100% of larvae and, finally, a shift to gastrula stage was seen at 1.5 mg/L.

Concerning the organic compounds used in this study, NP exhibited an EC_{50} (±95% confidence limit) of 8,501 (8,302 – 8,514), while for SDS the EC_{50} (±95% confidence limit) was 0.710 (0.683 – 0.734). The percentage of malformed NP-exposed embryos was 4, 21, 74, 100 % at 0.5, 0.6, 0.8, 1 mg/L, respectively (Fig 3I); SDS exhibited percentages of abnormal embryos of 3, 36, 91, 100 at 7, 8, 10,

353 12 mg/L, respectively (Fig. 3M). Concerning ITI values, 3.84, 7.10, 9.28, 9,82 were estimated at 0.5,
354 0.6, 0.8, 1 mg/L of NP (Fig. 3L), while values of 0.25, 0.88, 2.13, 7.57 were calculated at 7, 8, 10, 12
355 mg/L of SDS (Fig. 3N), respectively. For both toxicants, the variations in ITI values were due to an
356 initial shift from early auricularia without malformation (up to 0.8 mg/L of NP and 10 mg/L of SDS)
357 to the same developmental stage with severe malformation. This morphotype was observed at higher
358 concentrations for almost 90% of larvae, with only 5% of larvae blocked at the gastrula stage.

359 **Table 3** – Median effective concentration (EC_{50}) values of *Holothuria tubuosa* embryos exposed to reference
360 toxicants. Values are reported with respective 95% confidential limits. n.c.= not calculated.
361

	Test solution	EC_{50} (± 95% confidence limits)	NOEC (mg/L)	LOEC (mg/L)
Trace metals	**Cd**	4.978 mg/ (3.884-5.628 mg/L)	< 2	< 2
	Cu	0.104 mg/L (0.103-0.105 mg/L)	0.090	0.1000
	Hg	0.023 mg/L (0.022-0.025 mg/L)	< 0.010	< 0.01
	Pb	0.372 mg/L (n.c.)	0.300	< 0.372 - > 0.300
Organic compounds	**NP**	0.710 mg/L (0.683 – 0.734 mg/L)	0.500	0.600
	SDS	8,501 mg/L (8,302 – 8,514 mg/L)	6	7
Elutriate of marine sediments	**S1**	*H. tubulosa*: 60.056% (56.040-64.930%) *P. lividus*: >100%		
	S2	*H. tubulosa*: 32.878% (29.689-35.987%) *P. lividus:* 68.762% (58.211 - 78.11%)		
	S3	*H. tubulosa*: 44.324% (27.738-55.943%) *P. lividus*: >100%		
	S4	*H. tubulosa*: >100% *P. lividus*: >100%		

362
363
364 *3.4 Elutriate samples of marine sediments*
365 Table 3 reports the mean EC_{50} values calculated in *H. tubulosa* and sea urchin *P. lividus* embryo
366 bioassays for each elutriate sample. The comparison of the results showed higher toxic effects of S1
367 for *H. tubulosa* (EC_{50} = 60.056%), than for *P. lividus* (EC50 >100%). Similar differences between
368 species sensitivity were observed for S2 sample with higher EC50 in *H. tubulosa* than in *P. lividus*
369 (32.878 vs 68.762%), and for S3, with 44.324% of *H. tubulosa* EC50 and absence of evident
370 toxicity in *P.lividus*. Finally, reference site S4 showed an absence of toxic effects in both sea
371 cucumber and sea urchin embryo bioassays, with EC50 values higher than 100%.

4 Discussion

Many sea cucumbers belong to widely distributed species of great ecological importance, in addition to which they are easy to handle, meaning that embryo bioassays can be carried out without sophisticated equipment. A reproducible test methodology was developed for the first time in sea cucumbers using *H. polii* s (Morroni et al 2020b), demonstrating the suitability of this bioassay, and the possibility for this species to be included in ecotoxicological batteries. Our study was aimed at assessing the usefulness of *H. tubulosa* as a new species to be employed in sea cucumber embryo bioassays. To verify the applicability of this new species in ecotoxicological tests, the control acceptability, minimum sample size, embryo density effects, salinity effects, toxicity of organic and inorganic pollutants and elutriate of marine sediments were assessed, developing and validating specific endpoints. The results of our study allowed for a successful estimation of an acceptability limit for the negative control as less than 10% of abnormal embryos, corresponding to 0.05 of ITI value. Higher percentages of embryos with developmental anomalies could reflect the scarce quality of biological material, which could interfere with the responsiveness of the test and the correct sensitivity of embryos to environmental pollutants. The threshold of the acceptability of controls found in this study for *H. tubulosa* appears to be the same as those found with the embryo test performed using sea urchin (Saco-Alvarez et al. 2010) and bivalve embryos, such as oysters and mussels (His et al. 1997). In contrast, the embryo bioassays using limpets (Perez et al 2016, 2019) and ascidian (Bellas et al. 2003) show higher values, which range between 50 and 60% of abnormal larvae. Interestingly, also the bioassay with *H. polii* shows higher values (40%, Morroni et al 2020b) than those obtained for *H. tubulosa* in this study (10%). These differences may depend on the reproductive traits of the species, including polyspermy and oocyte immaturity, and their natural variability. With regard to minimum sample size, we suggest performing the bioassays by carrying out 6 replicates per experimental condition with a final density of 60 eggs/ml, thus exceeding the estimated minimum value of 200 embryos. The ideal density appeared to be 200 embryos/ml, more than three folds higher than the optimal value assessed for *H. polii* (60 embryos/ml, Morroni et al

2020b), thus facilitating the embryo abnormality count. The difference between the two species is likely to be related to the size of the fertilized eggs and the following developmental stages: *H. polii* fertilized eggs range from a diameter of 247.46 ± 15.48 µm to 623.42 ± 49.04 µm in length at mid auricularia stage (Rakaj et al 2019), whereas *H. tubulosa* has a diameter of 172.1 ±1.7 µm in fertilized eggs and 464.1 ± 4.9 µm length at mid auricularia, with lower disturbance in respiration and available space during embryogenesis at the same density of *H. polii.*

Fluctuations in salinity occur naturally in shallow waters, and global variations are expected due to anthropogenic climate change (Delorme and Sewell 2014, IPCC 2014). Echinoderms are generally sensitive to changes in salinity, with well-known consequences on embryo/larval development (Carballeira et al. 2011, Russel 2013, Mak and Chan 2018). In the present study a salinity range of 36-37 ‰ was found to be optimal for embryo development in *H. tubulosa*, values that are slightly higher than those obtained for *H. polii* (between 34 and 36%, Morroni et al., 2020~~b)~~ From the applicative point of view, the measured optimum in salinity is appropriate for testing environmental matrices in seawater, avoiding the salinity corrections necessary for *H. polii.*

The applicability of the tests with *H. tubulosa* towards toxic compounds was assessed by exposing the embryos to four trace metals and two organic pollutants. Our results showed a good responsiveness for this species, with an increase in teratogenic effects in a dose dependent manner for all the substances tested. Trace metals are ranked in the following order from the highest to the lowest toxicity in the early life stage of *H. tubulosa* embryo development: Hg>Cu>Pb>Cd. The EC_{50} of Cd was one order of magnitude higher than Cu and Pb, and two orders higher than Hg. These data are, on average, consistent with previous findings on the embryos and larvae of other marine invertebrates commonly used in bioassays (Table 4). In particular, the results for Cd and Cu (4.978 and 0.104 mg/L) are comparable with those of the sea urchin *Paracentrotus lividus,* which shows EC_{50} values between 0.230 – 9.24 mg/L for Cd, and between 0.045 – 0.068 ml/L for *Cu* (Warnau et al. 1996; Fernandez and Beiras 2001; Arizzi Novelli et al. 2003; Manzo et al. 2010; Morroni et al.

2018). The mussel *Mytilus galloprovincialis* exhibits lower values than *H, tubulosa,* with an EC50 for *Cu* of 0.007 – 0.018 mg/L (Prato and Biandolino 1997; Beiras and Albentosa 2004, Arnold et al. 2010; Boukadida et al. 2016) and a Cd effect concentration of 1.93 mg/L. This value, that was identified by Beiras and Albentosa (2004), appears higher than those of- 0.037-0.212, 0.180 and 0.790 obtained in embryos of *Crassostrea gigas, Hydroides elegans* and *Ficopomatus enigmaticus,* respectively (Mai et al, 2012;; His et al. 1999; Gopalakrishnan et al. 2008; Oliva et al. 2018), that were found to be more sensitive to Cd than *H. tubulosa*. Concerning the embryotoxicity of Hg (EC_{50}= 0.023 mg/L), the results for the sea cucumber, *H. tubulosa* fit with the 0.008-0.046 mg/L range obtained in *P. lividus* by His et al. (1999), Arizzi Novelli (2003), Fernandez and Beiras (2001), and are comparable to the other embryo-larval models (Table 4). The median effect of Pb concentrations found in this study (0.372 mg/L) falls within the range of 0.068 - 1.250 found in *P. lividus* embryos (Fernandez and Beiras 2001; Arizzi Novelli 2003; Manzo et al. 2010; Morroni et al. 2018) and is lower than values reported for *H. elegans* (1.130 mg/L) and *C. gigas* (0.660 mg/L) (Gopalakrishnan et al. 2008; Xie et al 2016). In this study, the EC50 values for Cd and Cu in *H. tubulosa* were 4.98 and 0.10, thus revealing a lower sensitivity in comparison to another species of sea cucumbers, *H. polii,* with an EC50 of 2.26 and 0.026 mg/L for Cd and Cu, respectively (Morroni et al., 2020b). Despite the different embryotoxicity of Cu, this metal seems to cause a delay in development in both sea cucumber species at lower concentrations, in line with the effects observed on sea urchin embryo development (Arizzi Novelli et al. 2003, Morroni et al. 2018). Moreover, in sea cucumbers this metal can cause a significant size reduction in the mid auricularia development stage and, at higher concentrations, a malformation in the early auricularia stage, causing an a-typical body shape (Table 1*o*) typically associated with exposure to Cu and Hg. This peculiar malformation, which was also seen in *H. polii,* highlights a developmental anomaly that is typical of sea cucumber larvae in specific stress conditions.

With regards to the organic compounds, the EC_{50} of NP in *H. tubulosa* embryos (0.710 mg/L) is almost ten times lower in respect to that of *F. enigmaticus* (6.81 mg/L) (Oliva et al 2018), but higher

than those found in sea urchin *P. lividus* and in the bivalve *M, galloprovincialis*, with 0.085 and 0.140 mg/L, respectively (Tato et al. 2018). The EC_{50} of SDS (8,501 mg/L) is very close to *F. enigmaticus* EC_{50} (8.68 mg/L) and in the same order of magnitude than those of *P. lividus, M. galloprovincialis* and *Ciona intestinalis*, with EC_{50} of 4.100, 2.253 and 5.145 mg/L, respectively (Bellas 2005: Beiras and Bellas 2008).

Table 4 – EC_{50} values (expressed in mg/L with 95% confidence limits) of *Holothuria tubulosa* compared with other groups from literature: *Holothuria polii, Ciona intestinalis, Crassostrea gigas, Ficopomatus enigmaticus, Hydroides elegans, Mytilus galloprovincialis, Paracentrotus lividus* for Cd^{2+}, Cu^{2+} Hg^{2+}, Pb^{2+}, SDS and NP in embryo toxicity assays.

Test specie	Cd^{2+}	Cu^{2+}	Hg^{2+}	Pb^{2+}	SDS	NP
Holothuria tubulosa	4.97 (3.884-5.628)	0.104 (0.103-0.105)	0.023 (0.022-0.025)	0.372 (n.c.)	0.710 (0.683 – 0.734)	8,501 (8,302 – 8,514)
Holothuria polii	2.26 [a] (1.69- 3.31)	0.026 [a] (0.017-0.029)	-	-	-	-
Ciona intestinalis	0.721 [b] (0.691-0.751)	0.036-0.054 [bc]	0.044 [b] (0.041-0.050)	-	5.145 [d] (4.939 – 5.367)	-
Crassostrea gigas	0.037-0.212 [ef]	0.013 [e] (0.011 – 0.014)	0.012 [f]	0.660 [g] (0.453~1.062	-	-
Ficopomatus enigmaticus	0.79 [h] (0.590 – 0.990)	0.2 [h] (0.150 – 0.240)	-	-	8.680 [h] (7.170 – 10.190)	6.810 [h] (6.560 – 7.160)
Hydroides elegans	0.180 [i] (0.140-0.220)	-	0.054 [i] (0.042-0.068)	1.130 [i] (0.817-1.782)	-	-
Mytilus galloprovincialis	1.925 [l] (1.690–2.223)	0.007-0.018 [lmno]	0.005 [l] (0.005-0.006)	0.221 [l] (0.059–0.346)	2.253 [p] (1.566 – 7.733)	0.140 [q] (0.127-0.152)
Paracentrotus lividus	0.230-9.240 [rstuv]	0.045-0.068 [rstuv]	0.08-0-046 [tuf]	0.068-1.250 [ruv]	4.100 [d] (3.750–4-580)	0.085 [q] (0.063- 0.112)

a: Morroni et al. (2020b)
b: Bellas et al. (2004)
c : Bellas et al. (2003)
d :Bellas et al. (2005)
e : Mai et al. (2012)
f: His et al. (1999)
g: Xie et al (2017)

h : Oliva et al. (2018)
i: Gopalakrishnan et al (2008)
l:: Beiras and Albentosa (2004)
m: Boukadida et al. (2016)
n: Prato and Bianolino (1997)
o: Arnold et al. (2010):
p: Beiras and Bellas (2008)
q: Tato et al. (2018)
r: Morroni et al. (2018)
s; Waranu et al. 1996
t :Arizzi Novelli (2003)
u :Fernandez and Beiras (2001)
v: Manzo et al (2010)

The detailed effects on *H. tubulosa* embryo development were considered and weighted using the ITI. With this endpoint, the alterations were scored according to the severity of effects, thus discriminating between larvae in which development was simply delayed and abnormal embryos/larvae. The results of this study confirm that the differences between ITI and the standard criteria are particularly evident when the experimental conditions cause 100% of abnormal embryos. For example, in embryos exposed to 7, 8, 9 mg/L, 100% of embryos were abnormal while ITI discriminated values of 8.85, 9.7 and 10, respectively (Fig. 2A, B). Also embryos incubated with Pb concentrations between 0.6 and 1.5 mg/L showed severe anomalies in 100% of cases, while the ITI value ranged from 7.77 to 9.83 (Fig 2E, F), highlighting a gradual increase in toxic effects not detected using the standard criteria. These data are in line with those obtained using the same approach with different species, such as *H. polli* and the sea urchin *P. lividus* (Morroni et al. 2016, 2020). Although the assessment of a long list of delayed stages and morphotypes could make endpoint assessment more time-consuming compared to traditional approach, sea cucumber larvae present a single symmetric observation plane and the morphotypes, including larval protrusions, correspond to precise proportions of the body shape without overlapping arms, thus making these organisms particularly suitable for the development of automatized classification methods. In addition, the great advantage in the use of this integrated toxicity index is the better discrimination between the samples according to the severity and developmental stage at which an effect is observed, considering delays in development as being less severe than delays combined with embryo morphological malformations (Morroni et al. 2020b).

Concerning the elutriate sample, a higher sensitivity of *H. tubulosa* embryos with respect to sea urchin *P. lividus* was observed. In two samples in particular, (S1, S3), toxic effects on sea cucumbers were observed, whereas these were not seen in sea urchins. In S2 samples, *H. tubulosa* demonstrate twice as high a sensitivity than that of *P. lividus*, which also exhibits toxic effects. These three samples were characterized by the presence of Cd (0.9-5.8 mg/kg), organic pollutants such as PAHs (5400-30000 μg/kg) and organotin compounds (Table S1). These concentrations were positively correlated with developmental defects ($p<0.001$) and were higher than the maximum limits established in the Italian law decree determining quality class and management options for dredged marine sediments (DM 173/2016). Ammonia was also found in the elutriate sample, with a concentration range between 0.560 – 1.240 mg/L. These values are higher than the effect concentrations in sea urchins found by Losso et al (2007) and Saco-Alvarez et al. (2010) and could potentially affect correct sea cucumber embryo development.

Data obtained in the present study indicate that *H. tubulosa* is a useful and highly promising species for use in embryo bioassays, also considering the fact that it has a longer spawning period and higher percentage of well-developed larvae in negative control than *H. polii* (Morroni et al 2020b) . The responsiveness of the endpoints, their sensitivity to salinity, various pollutants and elutriate samples indicates that *H. tubulosa* is a highly responsive model organism for ecotoxicological investigations.. Bivalve and sea urchins are the most commonly used marine invertebrates in embryo-larval bioassays. As demonstrated by Beiras and Bellas (2008), the comparison of the sensitivity between these two groups indicated that they are two complementary tools. In particular, bioassays using the sea urchin *P. lividus* embryos have been extensively and routinely used in environmental quality assessments (Pagano et al 2017). The present study shows, on average, a similar sensitivity to the tested environmental contaminates and matrices between *P. lividus* and sea cucumbers, as shown above. A statistical comparison of the responsiveness to pollutants of *P. lividus* and *H. tubulosa* yields a correlation $r^2= 0.88$ ($p<0.001$) and a slope of 2.57 (Fig. 4). Generally speaking, this means that the two groups produce comparable results.

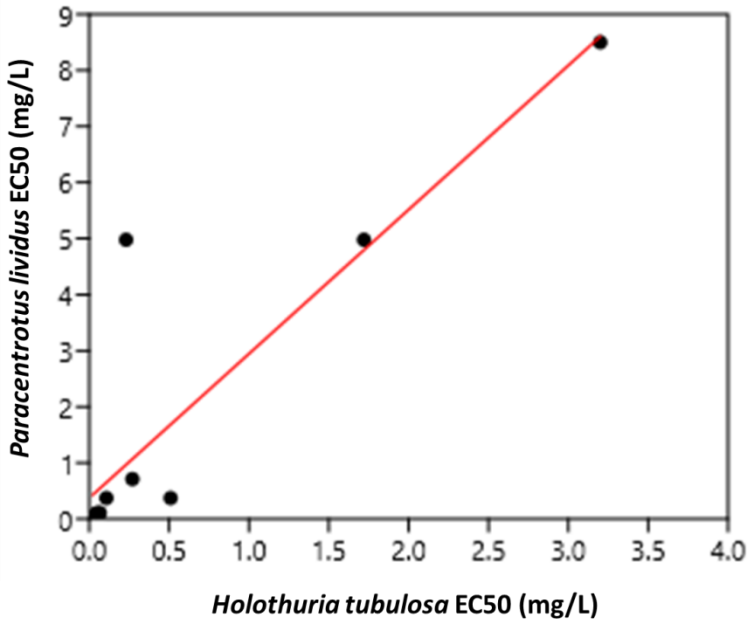


Figure 4 – Correlation between sea cucumber *Holothuria tubulosa* EC50 (X axis) and sea urchin *Paracentrontrotus lividus* EC50 (Y axis) for trace metals (Cd, Cu, Hg, Pb) and organic compounds (SDS, NP). Data for *P. lividus* are taken from literature stuidies (His et al. 1999; Fernandez and Beiras 2001; Arizzi Novelli et al. 2003; Bellas et al. 2005; Manzo et al. 2010; Morroni et al. 2018; Tato et al 2018).

Thus the choice of sea urchin or sea cucumber bioassays can be made depending on the availability of the test species and the biological material, which greatly facilitates the routine use of these biological tools in pollution studies all year round.

Notably, the use of *P. lividus* has some limitations, since samples can be difficult to find given the fact that they are found only in rocky bottoms and are subject to overfishing. Echinoid populations have in fact been increasingly over-exploited due to high market demand and, as consequence, numerous sea urchin fisheries have collapsed in several locations around the world (Casal et al. 2020). Moreover, the spawning period is generally limited. For example, in European seas *P. lividus* spawn in winter and spring when the photoperiod is short and water temperature is between 13.5°C - 18°C (Byrne et al., 1990). Interestingly, sea cucumbers spawn in summer, with a distinct annual reproductive cycle (Aydin and Erkan 2015), in a period that is complementary to that of sea urchins. Given these characteristics and the comparable sensitivity observed towards toxicants and environmental matrices, *H. tubulosa* could represent a complementary species to *P. lividus*, allowing

528 researchers to perform embryo bioassays also during the summer season, when gametes of sea urchins
529 are not naturally available. In addition, the organism can be found easily in both soft and rocky
530 bottoms, increasing the possibilities of collection in various marine areas. These factors add further
531 importance to the *H. tubulosa* embryo bioassay, which should be considered not as a substitute for
532 the existing embryo-larval bioassays, but as a new and promising tool that could be integrated into
533 existing ecotoxicological batteries, guaranteeing an all-year-round availability of sexually mature
534 species with differentiated responses.
535 These results are thus encouraging and could have important consequences when applied. Currently,
536 the use of bioassays in ecological risk assessment is becoming of fundamental importance. In recent
537 years a multidisciplinary weight of evidence (WOE) approach, which considers chemical analyses
538 and ecotoxicological bioassays as different lines of evidence (LOEs) through quantitative integration,
539 has been validated in several case studies associated with the natural and anthropic impacts on the
540 marine environment (Piva et al. 2011, Bebianno 2015, Pittura et al. 2018, Regoli et al. 2019, Morroni
541 et al. 2020a). Additionally, as part of a decision-making process, various WOE methodologies have
542 recently been formalized in different fields, for instance by US-EPA (Linkov et al., 2015) and with
543 the latest Italian law on the management of dredged sediments (DM 173/2016). As a consequence,
544 batteries of ecotoxicological bioassays have taken on a crucial role in the evaluation of the ecological
545 status for decisions regarding sediment management. This study presents a good alternative embryo
546 test and a new candidate that could expand the list of test species, paving the way for the use of sea
547 cucumbers in ecotoxicological bioassays. Additional studies in the future addressing the sensitivity
548 of *H. tubulosa* to a mixture of chemicals and different environmental matrices could further increase
549 the potential of this form of bioassay, reinforcing the applicability of this species in a WOE integration
550 for the assessment of the environmental quality of the marine environment.
551
552 **5. Conclusions**

This experimental study allowed us to standardize fundamental test parameters, confirming the suitability of *H. tubulosa* as a test species in sea cucumber embryo bioassays. This new model organism is widely distributed in the wild, is easy to collect and is of high ecological value. Our results show a sensitivity to different environmental pollutants that is comparable to other marine invertebrates, together with an integrative toxicity index that can be applied to further discriminate between developmental anomalies. This bioassay represents a promising tool that could be adopted in future ecotoxicological studies in order to assess the toxicity of sea water and sediments.

Acknowledgements

We are grateful to Dr. Stefano Ferrari for his precious support in the chemical analysis.

References

Aguirre-Martínez, G.V., Owuor, M.A., Garrido-Perez, C., Salamanca, M.J., Del Valls, T.A., Martín-Díaz, M.L. (2015). Are standard tests sensitive enough to evaluate effects of human pharmaceuticals in aquatic biota? Facing changes in research approaches when performing risk assessment of drugs. Chemosphere 120:75-85.

Aydin, M., Erkan, S., (2015). Identification and some biological characteristics of commercial sea cucumber in the Turkey coast waters. Int. J. Fish. Aquat. Stud. 3:260–265.

Arizzi Novelli, A., Losso, C., Ghetti, P.F., Volpi Ghirardini, A. (2003). Toxicity of heavy metals using sperm and embryo toxicity bioassay with Paracentrotus lividus (Echinodermata: Echinodea): comparison with exposure concentrations in the lagoon of Venice, Italy. Environ Toxicol Chem 22(6):1295-1301.

Arnold, W.R., Cotsifas, J.S., Ogle, R.S., DePalma, S.G.S., Smith, D.S. (2010). A comparison of the copper sensitivity of six invertebrate species in ambient saltwater of varying dissolved organic matter concentrations. Environ Toxicol Chem 29:311–319.

ASTM E1563, 98 (2012). Standard guide for conducting static acute toxicity tests with echinoid embryos. E1563-95. In Annual Book of ASTM Standards, Philadelphia, PA,vol.11(5), pp.999–1017.

Avio, C.G., Gorbi, S., Regoli, F., (2017). Plastics and microplastics in the oceans: from emerging pollutants to emerged threat. Mar. Environ. Res. 128:2–11.

Bebianno MJ, Pereira CG, Rey F, Cravo A, Duarte D, D'Errico G, Regoli F. (2015). Integrated approach to assess ecosystem health in harbor areas. Sci Total Environ 514:92–107.

584 Bellas, J., Beiras, R., Vázquez, E. (2003). A standardization of *Ciona intestinalis* (Chordata,
585 Ascidiacea) embryo-larval bioassay for ecotoxicological studies. Water Res. 37: 4613–4622.
586 Bellas, J., Beiras, R., & Vázquez, E. (2004). Sublethal effects of trace metals (Cd, Cr, Cu, Hg) on
587 embryogenesis and larval settlement of the ascidian Ciona intestinalis. Archives of
588 environmental contamination and toxicology, 46(1):61-66.
589 Bellas, J., Beiras, R., Marino-Balsa, J.C., Fernandez, N. (2005). Toxicity of organic compounds to
590 marine invertebrate embryos and larvae: a comparison between the sea urchin embryogenesis
591 bioassay and alternative test species. Ecotoxicology 14:337–353.
592 Beiras, R. and Albentosa, M. (2004). Inhibition of embryo development of the commercial bivalves
593 *Ruditapes decussatus* and *Mytilus galloprovincialis* by trace metals; implications for the
594 implementation of seawater quality criteria. Aquaculture 230:205–213.
595 Beiras, R. and Bellas, J. (2008). Inhibition of embryo development of the *Mytilus galloprovincialis*
596 marine mussel by organic pollutants; assessment of risk for its extensive culture in the Galician
597 Rias. Aquaculture, 277: 208-212.
598 Benedetti, M., Gorbi, S., Fattorini, D., D'Errico, G., Piva, F., Pacitti, D., & Regoli, F. (2014).
599 Environmental hazards from natural hydrocarbons see page: integrated classification of risk
600 from sediment chemistry, bioavailability and biomarkers responses in sentinel species.
601 Environmental pollution, 185:116-126.
602 Bocquené, G. and Abarnou, A. (2013). Organochlorinated pesticides, PCBs, dioxins, and PBDEs in
603 grey mullets (*Liza ramada*) and allis shads (*Alosa alosa*) from the Vilaine estuary (France).
604 Environ Sci Pollut Res. 20:667–675.
605 Boncagni, P., Rakaj, A., Fianchini, A., Vizzini, S. (2019). Preferential assimilation of seagrass
606 detritus by two coexisting Mediterranean sea cucumbers: *Holothuria polii* and *Holothuria*
607 *tubulosa*. Estuarine, Coastal and Shelf Science 231, 106464.
608 Boukadida, K., Banni, M., Gourves, P.-Y., Cachot, J. (2016). High sensitivity of embryo- larval stage
609 of the Mediterranean mussel, *Mytilus galloprovincialis* to metal pollution in combination with
610 temperature increase. Mar. Environ. Res. 122 :59–66.
611 Burnstein, H. (1971). Attribute sampling. McGraw-Hill, New York, p.464.
612 Byrne M. (1990). Annual reproductive cycles of the commercial sea urchin *Paracentrotus lividus*
613 from an exposed intertidal and a sheltered subtidal habitat on the west coast of Ireland. Mar
614 Biol 104: 275-289..

615 Campani T., Casini, S., Caliani, I, Pretti, C., Fossi M.C. (2020). Ecotoxicological Investigation in
616 Three Model Species Exposed to Elutriates of Marine Sediments Inoculated With Bioplastics.
617 Frontiers in Marine Science 7:229. doi: 10.3389/fmars.2020.00229.
618 Carballeira, C., Martin-Diaz, L., DelValls, T.A. (2011). Influence of salinity on fertilization and larval
619 development toxicity tests with two species of sea urchin. Mar. Environ. Res. 72:196–203.
620 Carballeira, C., Ramos-Gómez, J., Martín-Díaz, L., DelValls, T. A. (2012). Identification of specific
621 malformations of sea urchin larvae for toxicity assessment: application to marine pisciculture
622 effluents. Marine environmental research 77 : 12-22.
623 Casal, G., Fernández-Boán, M., Fernández, N., Freire, J., Fernández, L. (2020). Spatial structure and
624 abundance of the sea urchin *Paracentrotus lividus* in subtidal fishing grounds of the Galician
625 coast (NW- Spain). Estuar Coast Shelf Sci 239:106753
626 Corsi, I., Cherr, G.N., Lenihan, H.S., Labille, J., Hassellov, M., Canesi, L., et al. (2014). Common
627 strategies and technologies for the ecosafety assessment and design of nanomaterials entering
628 the marine environment. ACS Nano. 8:9694–9709.
629 Cruceru, I., Iancu, V., Petre, J., Badea, I.A., Vladescu, L. (2012). HPLC-FLD determination of 4-
630 nonylphenol and 4-tert-octylphenol in surface water samples. Environ. Monit. Assess. 184,
631 2783–2795.
632 Delorme, N.J. and Sewell, M.A. (2014). Temperature and salinity: Two climate change stressors
633 affecting early development of the New Zealand sea urchin *Evechinus chloroticus*. Mar. Biol.
634 161:1999.
635 DM 173/2016. Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Supplemento
636 ordinario alla Gazzetta Ufficiale, n. 208 del 6 settembre 2016-Serie generale. Regolamento
637 recante modalità e criteri tecnici per l′autorizzazione all'immersione in mare dei materiali di
638 escavo di fondali marini.
639 George, A.L., and White, G.F. (1999). Optimization of the methylene blue assay for anionic
640 surfactants added to estuarine and marine water. Environ. Toxicol. Chem. 18 (10):2232–2236.
641 Gopalakrishnan, S., Thilagam, H., Raja, P.V. (2008). Comparison of heavy metal toxicity in life
642 stages (spermiotoxicity, egg toxicity, embryotoxicity and larval toxicity) of *Hydroides elegans*.
643 Chemosphere 71:515–528.
644 Grosso, L., Rakaj, A., Fianchini, A., Morroni, L., Cataudella, S., Scardi, M. (2020). Integrated Multi-
645 Trophic Aquaculture (IMTA) system combining the sea urchin *Paracentrotus lividus*, as
646 primary species, and the sea cucumber *Holothuria tubulosa* as extractive species. Aquaculture,
647 736268.

Guidetti, P., Terlizzi, A., Boero, F. (2004). Effects of the edible sea urchin, *Paracentrotus lividus*, fishery along the Apulian rocky coast (SE Italy, Mediterranean Sea). Fisheries Research 66(2–3): 287-297.

Fernàndez, N. and Beiras, R. (2001). Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. Ecotoxicology 10:263–271.

His, E., Seaman, M.N.L., Beiras, R. (1997) . A simplification of the bivalve embryogenesis and larval development bioassay method for water quality assessment. Water Res 31:351–355.

His, E., Heyvang, I., Geffard, O., De Montaudouin, X. (1999). A comparison between oyster (*Crassostrea gigas*) and sea urchin (*Paracentrotus lividus*) larval bioassays for toxicological studies. Water Res. 33:1706–1718.

Hammer, O., Harper, D.A.T., Ryan, P.D. (2001). PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 1–9

ICRAM. Metodologie analitiche di riferimento. (2001). App. 1: Metodo per la determinazione di composti organostannici in sedimenti e matrici biologiche tramite GC-MS e GC-FPD. Ed. Italian Ministry for Environment. pp. 122.

IPCC (2014). Climate change 2014: Impact, adaptation and vulnerability. Working Group II contribution to the IPCC 5th Assessment Report.

Linkov, I., Massey, O., Keisler, J., Rusyn, I., Hartung, T. (2015). From "weight of evidence to quantitative data integration using multicriteria decision analysis and Bayesian methods". Altex 32:3–8.

Lorenzo, J. I., Nieto, O., Beiras, R. (2002). Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. Aquatic Toxicology, 58(1-2): 27-41.

Losso, C., Arizzi Novelli, A., Picone, M., Marchetto, D., Pantani, C., Ghetti, P.F., Volpi Ghirardini, A. (2007). Potential role of sulfide and ammonia as confounding factors in elutriate toxicity bioassays with early life stages of sea urchins and bivalves. Ecotox. Environ. Saf. 66 :252–257.

Mai, H., Cachot, J., Brune, J., Geffard, O., Belles, A., Budzinski, H., Morin, B. (2012). Embryotoxic and genotoxic effects of heavy metals and pesticides on early life stages of Pacific oyster (*Crassostrea gigas*). Mar. Poll. Bull. 64:2663-2670.

Mak, K.K.Y., Chan, K.Y.K. (2018) Interactive effects of temperature and salinity on early life stages of the sea urchin Heliocidaris crassispina. Mar Biol 165: 57.

Mezali, K., Zupo, V., and Francour, P. (2006). Population dynamics of Holothuria (*Holothuria tubulosa*) and *Holothuria (Lessonothuria) polii* of an Algerian *Posidonia oceanica* meadow. Biologia Marina Mediterranea, 496 13: 158–161.

682 Manzo, S., Buono, S., Cremisini, C. (2010). Cadmium, lead and their mixtures with copper:
683 *Paracentrotus lividus* embryotoxicity assessment, prediction, and offspring quality evaluation
684 Ecotoxicology, 19:1209-1223..
685 Morroni, L., Pinsino, A., Pellegrini, D., Regoli, F., Matranga, V. (2016). Development of a new
686 integrative toxicity index based on an improvement of the sea urchin embryo toxicity test.
687 Ecotoxicol. Environ. Saf. 123: 2-7.
688 Morroni, L., Pinsino, A., Pellegrini, D., Regoli, F. (2018). Reversibility of metal induced
689 malformations in sea urchin embryos. Ecotoxicol Environ Saf 148:923-929.
690 Morroni L., d'Errico G., Sacchi M., Molisso F., Armiento G., Chiavarini, S, Rimauro, J., Guida M.,
691 Siciliano A., Ceparano M.T., Aliberti F., Gallo A., Libralato G., Patti F.P, Gorbi S., Fattorini,
692 D., Nardi A., Di Carlo M., Mezzelani M., Benedetti M, Pellegrini D., Musco L., Danovaro R.,
693 Dell'Anno A., Regoli F., (2020a). Integrated characterization and risk management of marine
694 sediments: the case study of the industrialized Bagnoli area (Naples, Italy). Mar Environ Res.
695 160:104984.
696 Morroni, L., Rakaj, A., Grosso, L., Fianchini, A., Pellegrini, D., Regoli, F. (2020b). Sea cucumber
697 *Holothuria polii* (Delle Chiaje, 1823) as new model for embryo bioassays in ecotoxicological
698 studies. Chemosphere 240: 124819.
699 Murado, M. A., and Prieto, M. A. (2013). NOEC and LOEC as merely concessive expedients: Two
700 unambiguous alternatives and some criteria to maximize the efficiency of dose–response
701 experimental designs. Science of the Total Environment 461:576-586.
702 Oliva, M., Mennillo, E., Barbaglia, M., Monni, G., Tardelli, F., Casu, V., Pretti, C. (2018). The
703 serpulid *Ficopomatus enigmaticus* (Fauvel, 1923) as candidate organisms for ecotoxicological
704 assays in brackish and marine waters. Ecotoxicol. Environ. Saf. 148:1096–1103.7
705 Oliviero, M., Tato, T., Schiavo, S., Fernández, V., Manzo, S., Beiras, R. (2019). Leachates of
706 micronized plastic toys provoke embryotoxic effects upon sea urchin Paracentrotus lividus.
707 Environ. Pollut. 247: 706–715.
708 Pagano, G., Thomas, P.J., Guida, M., Palumbo, A., Romano, G., Trifuoggi, M. Oral, R., Trifuoggi,
709 M. (2017). Sea Urchin Bioassays in Toxicity Testing: II. Sediment Evaluation. Expert Opin.
710 Environ. Biol. 6, 1. DOI: 10.4172/2325-9655.1000141.
711 Pérez, S., Fernández, N., Ribeiro, P.A. (2016). Standardization of a *Patella* spp. (Mollusca,
712 Gastropoda) embryo–larval bioassay and advantages of its use in marine ecotoxicology.
713 Ecotoxicol. Environ. Saf. 127: 175–186.

714 Pérez, S., Sánchez Marín, P., Bellas, J., Viñas, L., Besada,V., Fernández, N. 2019. Limpets (*Patella*
715 spp .Mollusca, Gastropoda) as model organisms for biomonitoring environ-mental quality.
716 Ecol. Ind.10:150–162.
717 Pittura, L., Avio, C.G., Giuliani, M.E., D'Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regol, F.
718 (2018). Microplastics as vehicles of environmental PAHs to marine organisms: Combined
719 chemical and physical hazards to the Mediterranean mussels, *Mytilus galloprovincialis*. Front
720 Mar Sci 5(103).
721 Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., Regoli, F. (2011). Assessing
722 sediment hazard through a weight of evidence approach with bioindicator organisms: A
723 practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and
724 ecotoxicological bioassays. Chemosphere 83:475–485.
725 Prato, E. and Biandolino F. (1997). Combined toxicity of mercury, copper and cadmium on
726 embryogenesis and early larval stages of the *Mytilus galloprovincialis.* Environ. Technol.,
727 28:915-920.
728 Purcell, S. W., Conand, C., Uthicke, S., & Byrne, M. (2016). Ecological roles of exploited sea
729 cucumbers. Oceanography and Marine Biology: An Annual Review, 54:367–386.
730 Rakaj, A., Boncagni, P., Fianchini, A., Gravina, M. F. (2015). RISULTATI PRELIMINARI SULLA
731 RIPRODUZIONE ARTIFICIALE DI HOLOTHURIA TUBULOSA (HOLOTHUROIDEA,
732 ECHINODERMATA)/PRELIMINARY RESULTS ON THE ARTIFICIAL
733 REPRODUCTION OF HOLOTHURIA TUBULOSA (HOLOTHUROIDEA,
734 ECHINODERMATA). Bio. Mar. Med. 22:64-65.
735 Rakaj, A., Fianchini, A., Boncagni, P., Lovatelli, A., Scardi, M., Cataudella, S. (2018). Spawning and
736 rearing of *Holothuria tubulosa*: a new candidate for aquaculture in the Mediterranean region.
737 Aquac. Res. 49:557–568.
738 Rakaj, A., Fianchini, A., Boncagni, P., Scardi, M., Cataudella, S. (2019). Artificial reproduction of
739 *Holothuria polii*: A new candidate for aquaculture. Aquaculture 498:444–453.
740 Regoli, F., D'Errico, G., Nardi, A., Mezzelani, M., Fattorini, D., Benedetti, M., Di Carlo, M.,
741 Pellegrini, D., Gorbi, S. (2019). Application of a Weight of Evidence Approach for Monitoring
742 Complex Environmental Scenarios: the Case-Study of Off-Shore Platforms. Front Mar Sci 6:1–
743 15.
744 Reguera, P., Couceiro, L., Fernández, N. (2018). A review of the empirical literature on the use of
745 limpets *Patella* spp. (Mollusca: Gastropoda) as bioindicators of environmental quality.
746 Ecotoxicol. Environ. Safe. 148:593–600.
747 Russell, M.P. (2013). Echinoderm responses to variation in salinity. Adv Mar Biol 66:171–212.

748 Saco-Álvarez, L. Beiras, R. Durán, I. Lorenzo, J.I. (2010). Methodological basis for the optimization
749 of marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality.
750 Ecotox. Environ. Saf. 73:491–499.
751 Tato, T., Salgueiro-González, N., León, V. M., González, S., & Beiras, R. (2018). Ecotoxicological
752 evaluation of the risk posed by bisphenol A, triclosan, and 4-nonylphenol in coastal waters
753 using early life stages of marine organisms (*Isochrysis galbana*, *Mytilus galloprovincialis*,
754 *Paracentrotus lividus*, and *Acartia clausi*). Environmental Pollution 232:173-182.
755 Underwood A.J. (1997). Experiments in Ecology. Their Logical Design and Interpretation Using
756 Analysis of Variance. Cambridge University Press, Cambridge.
757 Warnau, M., Iaccarino, M., De Biase, A., Temara, A., Jangoux, M., Dubois, F., Pagano G. 1996.
758 Spermiotoxicity and embryotoxicity of heavy metals in the echinoid *Paracentrotus lividus*.
759 Environmental Toxicology and Chemistry 15(11):1931-1936.
760 Xie, J., Yang, D., Sun, X., Cao, R., Chen, L., Wang, Q., ... & Zhao, J (2017). Combined toxicity of
761 cadmium and lead on early life stages of the Pacific oyster, *Crassostrea gigas*. Invertebrate
762 Survival Journal, 14(1), 210-220.