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(Article begins on next page)

1 **Towards sea cucumbers as a new model in embryo bioassays:**

2 ***Holothuria tubulosa* as test species for the assessment of marine pollution**

3
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12
13
14 **Abstract**

15 Sea cucumbers are widely distributed deposit-feeders that represent an important component of
16 benthic communities. These echinoderms were recently proposed as candidates in embryo bioassays
17 to provide a new tool in the toxicity assessment of pollutants in marine water and sediments. The aim
18 of this study is therefore to evaluate the usefulness of a new species, *Holothuria tubulosa* (Gmelin,
19 1788), as a model organism for sensitive embryo bioassays, defining the acceptability of controls,
20 minimum sample size, embryo density and salinity range. Cadmium (Cd), copper (Cu), lead (Pb),
21 mercury (Hg), sodium dodecyl sulphate (SDS) and 4-n-nonylphenol (NP) were used as reference
22 toxicants to assess specific embryotoxicity endpoints. Sea cucumber and sea urchin embryos were
23 subsequently incubated with elutriates of harbour marine sediments to compare the responsiveness
24 of these two species. The results showed an acceptability threshold of 10% (abnormal embryos), a
25 minimum sample size of 200 embryos, an embryo density of 200 embryos/ml and an optimal salinity
26 range of 36-37‰. The sensitivity to the environmental pollutants and matrices tested revealed values
27 (expressed as EC₅₀) comparable with those of embryos belonging to other marine invertebrates
28 commonly used in bioassays, indicating that this species has a good level of responsiveness. A
29 specific integrative toxicity index (ITI) was applied, combining the frequency of developmental
30 anomalies and weighting their severity. Data elaborated with ITI demonstrated good discrimination
31 of sample toxicity, with a dose-dependent increase of teratogenic effects for all the tested substances,
32 indicating *H. tubulosa* as a promising species for future assessments of marine pollution.

34 **1. Introduction**

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

59 bioassays are not expected to detect the full range of potential effects of pollutants, the use of a battery
60 of bioassays is strongly recommended for the assessment and monitoring of the marine environment
61 (Rodríguez-Romero 2016, Regoli et al. 2019). Additionally, the use of a single species can present
62 some practical limitations, such as a seasonal-dependent spawning period, difficulties in finding
63 organisms in specific areas due to local fishing and overexploitation - as in the case of sea urchins
64 (Guidetti et al. 2004). In order to provide a wider choice among suitable species, sea cucumbers have
65 recently been proposed as candidates for the development of embryo toxicity bioassays (Morrone et
66 al. 2020b). Sea cucumbers represent an important component of benthic communities since they are
67 widely distributed ecosystem engineers that play a key role in sea floor dynamics by processing and
68 bioturbating the sediment (Purcell et al. 2016; Rakaj et al. 2015; 2019), as well as in the creation of
69 seagrass detrital pathways for Mediterranean coastal ecosystems (Boncagni et al. 2019). The
70 sensitivity of echinoderms to pollutants, together with their ecological importance, has prompted
71 suggestions for their use as model organisms in the testing of the effects of marine pollution (ASTM
72 2012, Pagano et al. 2017, Morrone et al. 2016, 2018, 2020b). *Holothuria tubulosa* (Gmelin, 1791) is
73 one of the most common and widely distributed sea cucumbers in the Mediterranean basin, it is
74 relatively simple to maintain in a laboratory conditions, and spawning can be easily induced through
75 a recently developed protocol for aquaculture (Grosso et al., 2020; Rakaj et al. 2018). The
76 reproductive period of *H. tubulosa* occurs between June and October, reaching full maturity at the
77 same time as the sea urchin *Paracentrotus lividus* enters the recovery gonadal stage, limiting its
78 availability for embryo bioassays. Due to the reproductive cycle of *H. tubulosa*, its high fecundity,
79 widespread distribution and important ecological role, this species can be considered as an additional
80 candidate for testing the embryotoxicity of environmental samples in ecotoxicological studies.

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

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

102 **2. Material and Methods**

103 *2.1 Sampling and acclimatization*

104 Adult sea cucumbers (*H. tubulosa*) were collected through snorkelling (1-5 m) at Santa Marinella in
105 the central Tyrrhenian Sea, Italy (42°3'0"N, 11°49'9"E) from June to October 2019. The organisms
106 were transported to the Laboratory of Experimental Ecology and Aquaculture (University of Rome
107 Tor Vergata) inside 30 L tanks equipped with aerators and ice to maintain the temperature below
108 28°C, and then acclimatized in a recirculating aquaculture system (RAS) at 24 °C for 7 days.

109

110 *2.2 Sea cucumber embryo tests with H. tubulosa*

111 A sea-cucumber embryo test was performed following the protocol developed by Morrioni et al.
112 (2020b). Specifically, before spawning was induced, adults were kept in substrate-free aquaria for 48
113 h in order to void their gut contents. After this period spawning was induced by thermal shock. To
114 perform this operation the sea cucumbers were transferred individually to 5 L spawning tanks with
115 0.45 μm filtered and UV-sterilized seawater (FSW) and the water temperature was increased rapidly
116 by 3–5°C (from 24°C to 27–29°C). This temperature was maintained for 1.5 h, after which the water
117 was cooled to the initial temperature (Rakaj et al. 2019). Eggs were collected from each female from
118 the bottom of spawning tanks and were re-suspended in separate beakers with FSW. Before pooling
119 the eggs, their quality was assessed under a microscope: females releasing non-round eggs, immature
120 forms or debris were discarded. Males were identified after the first spawn and removed from the
121 spawning tanks. Dry sperm was collected from at least three males and stored at 4°C until use. Sperm
122 motility was checked under a microscope and diluted sperm suspension was added to an aliquot of
123 egg suspension to check the formation of the fertilization envelope. A total of 100 eggs were checked
124 in order to calculate the percentage of fertilization success. Males fertilizing fewer than 95% of the
125 eggs were discarded (Morrioni et al 2020b).

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

136

137 2.3 *Endpoints and toxicity criteria*

138 100 embryos were analyzed by optical microscopy (Zeiss Axio Vert A1) and photographed using a
139 digital camera (Zeiss Axiocam 105 color) with a 25x zoom. The mid-auricularia stage was selected
140 as the developmental endpoint. The degree of toxicity was calculated using the Integrative Toxicity
141 Index (ITI), based on the percentage and severity of embryo malformations (Morrone et al., 2020b).
142 More specifically, embryos were classified as normal if they satisfied the following morphological
143 criteria (Morrone et al. 2016): (1) correct schedule in reaching the developmental endpoint, (2)
144 left/right and dorso/ventral embryonic axis symmetry, (3) correct differentiation of the digestive
145 system (mouth, stomach, and anus). The toxicity was quantified by counting the frequency of delayed
146 and/or abnormal embryo morphology and by ranking the severity of effects quantitatively from 0
147 (none) to 5 (high). Anomalies were classified as delays in development with or without further
148 morphological alterations, and different stages weighted depending on the severity of anomalies
149 observed. Lower toxicity values were given to embryos with delays in development without
150 anomalous defects, while higher scores were attributed to embryos that displayed both delayed
151 development and malformations. Details of the scores, with four photographic examples per
152 morphotype are given in Table 1. The toxicity score is 0 when normal mid-auricularia (mAu) is
153 observed, and 1 for well-developed early-auricularia (eAu) larvae, i.e. with only development delays
154 but without malformations. Toxicity values increase for malformed embryos and larvae, depending
155 on the stage of development: higher toxicity values are attributed to malformed mAu (score 3), still
156 higher for delayed and malformed early-auricularia larvae (score 4) and, finally, the highest score at
157 Gastrula/Blastula/Morula stage (G/Bl/M) (score 5).

158

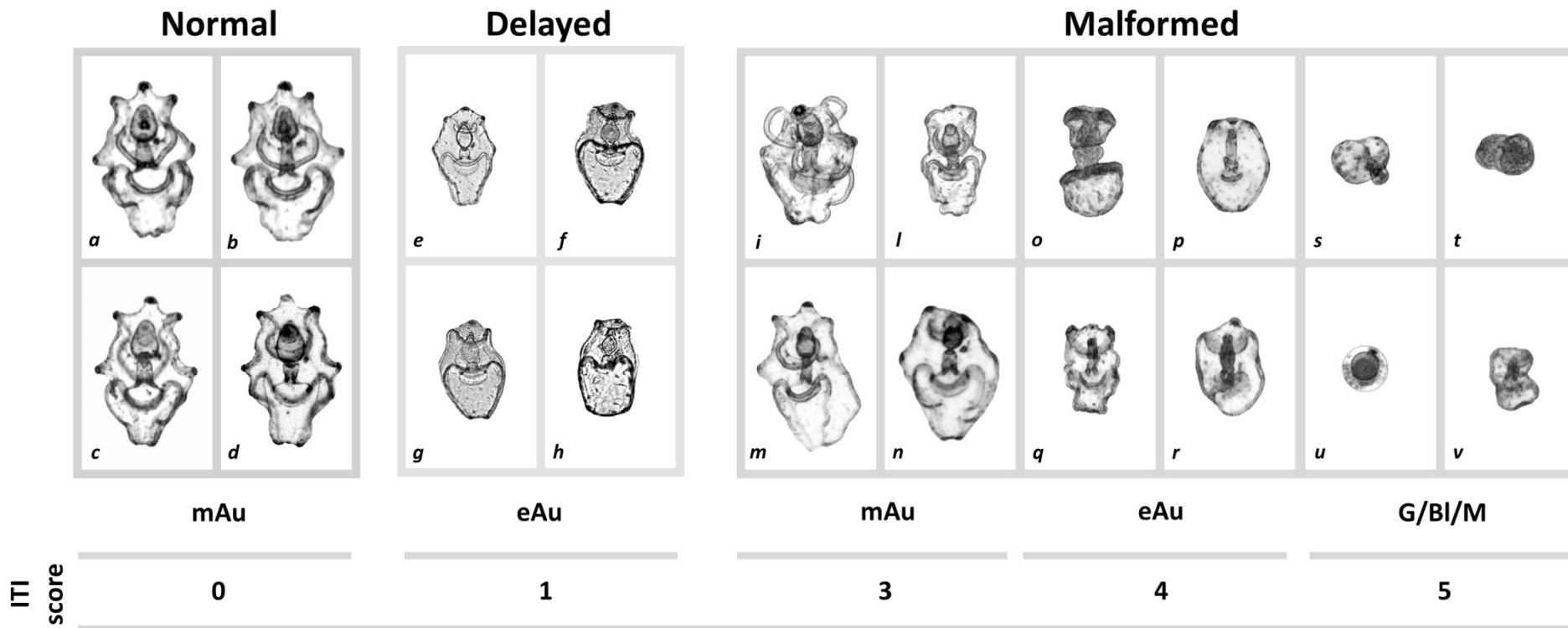
159 The integrative toxicity index (ITI) was calculated as follows:

160
$$ITI = \sum_{i=1}^{10} (S_i * F_i) / 100$$

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

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166

167 **Table 1** - Classification of developmental anomalies in *Holothuria tubulosa* according to the Integrative Toxicity Index (ITI) based on developmental delays
 168 (delayed embryos) and malformations (malformed embryos). mAu: mid-auricularia; eAu: eraly-auricularia; G: Gastrula; Bl: Blastula; M: Morula. *a, b, c, d*: normal
 169 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.
 170



171

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 finally placed in each six-well microplate). All glassware
196 was sterilized before the experiments with washes of HNO₃ (10% vol.), acetone and ultra-pure water,

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

199

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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 |

207

208 2.7 Elutriate samples

209 The responsiveness of *H. tubulosa* embryos was compared with that of *P. lividus* testing these species
210 toward the elutriates of environmental matrices. The sea cucumber and sea urchin embryos were
211 exposed in parallel to elutriate samples from harbour sediments, using 3 dilutions in FSW (90%, 50%,
212 25%), following the methodology reported by Morroni et al. (2018).

213 Sediments were collected from 4 stations in Piombino harbour, in the north Tyrrhenian Sea (Italy).

214 Elutriates were prepared within 10 days from the sampling, with a storage temperature of 4°C, in
215 accordance with USEPA (1991) guidelines and literature studies (Volpi Ghirardini et al., 2005).

216 Sediment samples were mixed in a 1:4 (v/v) ratio of sediment to FSW and placed on a rotary shaker
217 table for 1 h, at a speed of 300 rpm, at room temperature. The dilutions were made up with FSW
218 collected in a long-term monitored reference site located far from human activities. After mixing, the
219 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 (Cruceu 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

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

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

263

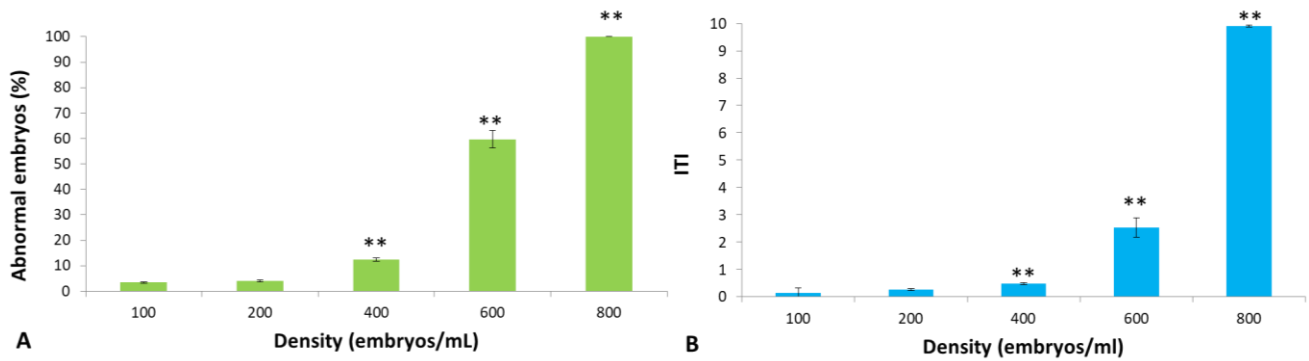
264 3. Results

265 3.1 *Optimal embryo density*

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

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

273



274

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

280

281 3.2 Test acceptability and sample size

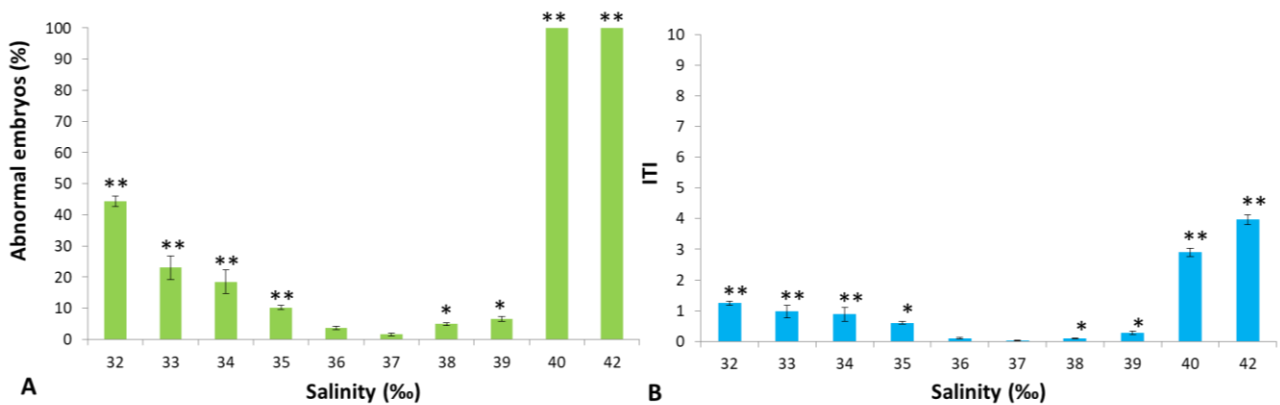
282 Control data showed a normal distribution expressed both as a percentage of abnormal embryos
283 (Shapiro-Wilk W: 0.95, p<0.01) and as ITI (Shapiro-Wilk W: 0.92, p<0.01). The 10th percentile of
284 the distribution corresponds to 6.91% (abnormal embryos) and 0.04 (ITI); thus, thresholds of
285 acceptability were fixed at 10% and 0.05, respectively. Concerning minimum sample size, setting an
286 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,
287 0.40, 0.25, 0.10 and 0 were, respectively, 58, 200, 334, 400, 400, 400, 334, 200 and 58. After applying
288 the correction for finite populations, a minimum sample size of 200 embryos allows a 95% confidence
289 in the estimate with an error of 5% and hence was considered as ideal for the test.

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

292

293 3.3 Optimal salinity range

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



300
 301 **Figure 2** - The effects of salinity on *Holothuria tubulosa* embryo development. The X axis reports the
 302 increasing salinities used in the experiments, while the Y axis shows the endpoint of embryo development.
 303 Data are expressed as a mean percentage of abnormal embryos (A, green bars) and ITI (B, blue bars) \pm standard
 304 error. Asterisks indicate significant differences between each treatment and the optimum value of 36 ‰ (*:
 305 $p < 0.05$, **: $p < 0.01$).

307 **3.3 Reference toxicants**

308 The results of the chemical analysis showed that measured concentrations generally varied less than
 309 13% from the nominal concentrations. Thus, all calculations reported above were based on nominal
 310 concentrations.

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

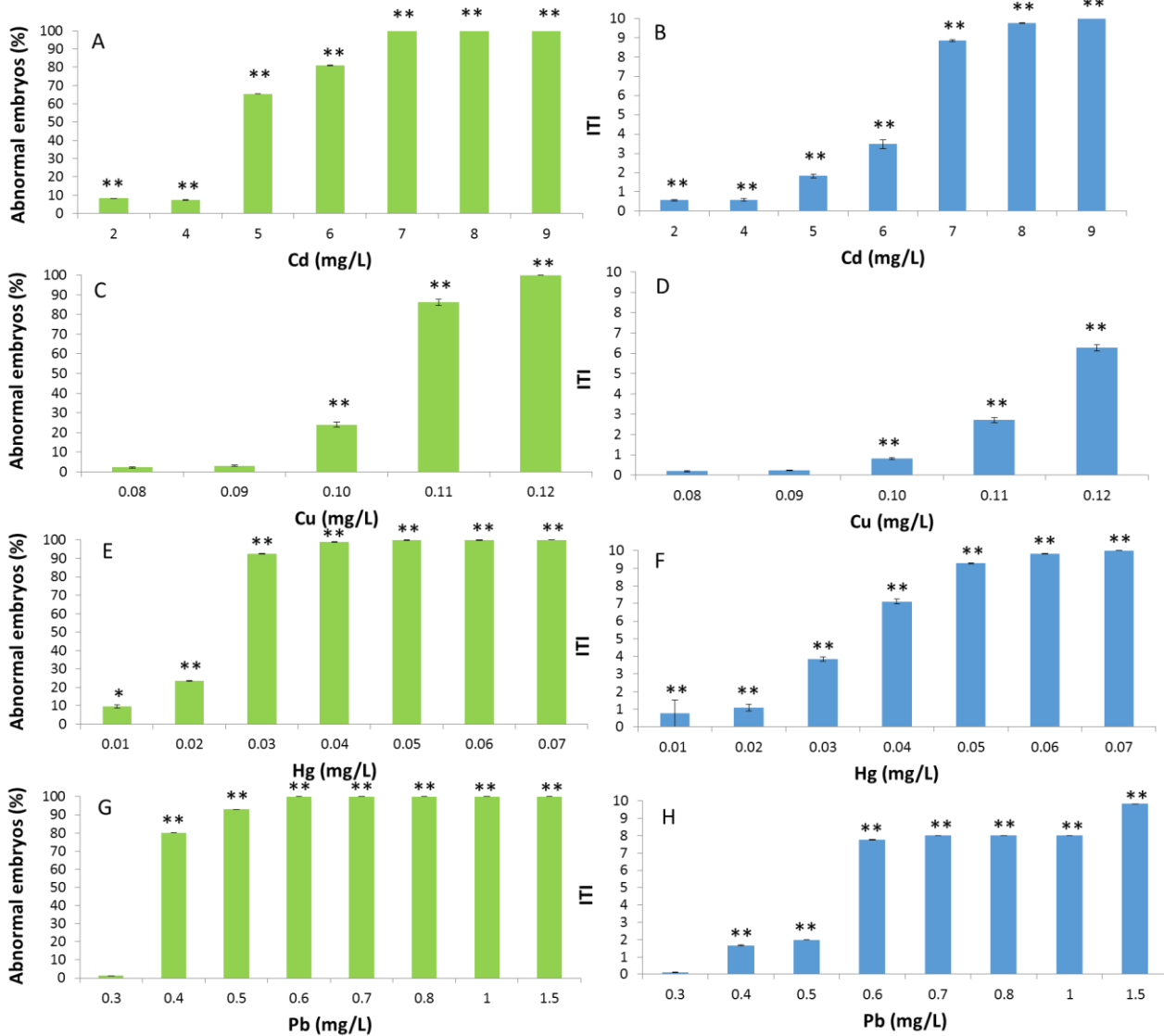
314 The effects of Cd^{2+} on embryo development resulted in an EC_{50} ($\pm 95\%$ confidence limit) of 4.978
 315 (3.884-5.628, Table 3). Specifically, Cd showed a percentage of abnormal embryos of 7, 65, 81, 100%
 316 at 4, 5, 6, 7 mg/L, respectively (Fig. 3A), and ITI more gradually increased with 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} ($\pm 95\%$ 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} ($\pm 95\%$ 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



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339

340 **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 (*: $p < 0.05$, **: $p < 0.01$).

345
346 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.

349 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).
351 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,
352

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 (EC₅₀) values of *Holothuria tubulosa* embryos exposed to reference
 360 toxicants. Values are reported with respective 95% confidential limits. n.c.= not calculated.

| | 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 | <i>H. tubulosa</i> : 60.056% (56.040-64.930%) <i>P. lividus</i> : >100% | | |
| | S2 | <i>H. tubulosa</i> : 32.878% (29.689-35.987%) <i>P. lividus</i> : 68.762% (58.211 - 78.11%) | | |
| | S3 | <i>H. tubulosa</i> : 44.324% (27.738-55.943%) <i>P. lividus</i> : >100% | | |
| | S4 | <i>H. tubulosa</i> : >100% <i>P. lividus</i> : >100% | | |

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363

364 3.4 Elutriate samples of marine sediments

365 Table 3 reports the mean EC₅₀ 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* (EC₅₀ = 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%.

372 4 Discussion

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

398 2020b), thus facilitating the embryo abnormality count. The difference between the two species is
399 likely to be related to the size of the fertilized eggs and the following developmental stages: *H. polii*
400 fertilized eggs range from a diameter of $247.46 \pm 15.48 \mu\text{m}$ to $623.42 \pm 49.04 \mu\text{m}$ in length at mid
401 auricularia stage (Rakaj et al 2019), whereas *H. tubulosa* has a diameter of $172.1 \pm 1.7 \mu\text{m}$ in fertilized
402 eggs and $464.1 \pm 4.9 \mu\text{m}$ length at mid auricularia, with lower disturbance in respiration and available
403 space during embryogenesis at the same density of *H. polii*.

404 Fluctuations in salinity occur naturally in shallow waters, and global variations are expected due to
405 anthropogenic climate change (Delorme and Sewell 2014, IPCC 2014). Echinoderms are generally
406 sensitive to changes in salinity, with well-known consequences on embryo/larval development
407 (Carballeira et al. 2011, Russel 2013, Mak and Chan 2018). In the present study a salinity range of
408 36-37 ‰ was found to be optimal for embryo development in *H. tubulosa*, values that are slightly
409 higher than those obtained for *H. polii* (between 34 and 36%, Morroni et al., 2020b). From the
410 applicative point of view, the measured optimum in salinity is appropriate for testing environmental
411 matrices in seawater, avoiding the salinity corrections necessary for *H. polii*.

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

423 2018). The mussel *Mytilus galloprovincialis* exhibits lower values than *H. tubulosa*, with an EC50
424 for Cu of 0.007 – 0.018 mg/L (Prato and Biandolino 1997; Beiras and Albentosa 2004, Arnold et al.
425 2010; Boukadida et al. 2016) and a Cd effect concentration of 1.93 mg/L. This value, that was
426 identified by Beiras and Albentosa (2004), appears higher than those of- 0.037-0.212, 0.180 and 0.790
427 obtained in embryos of *Crassostrea gigas*, *Hydroides elegans* and *Ficopomatus enigmaticus*,
428 respectively (Mai et al, 2012;; His et al. 1999; Gopalakrishnan et al. 2008; Oliva et al. 2018), that
429 were found to be more sensitive to Cd than *H. tubulosa*. Concerning the embryotoxicity of Hg (EC₅₀=
430 0.023 mg/L), the results for the sea cucumber, *H. tubulosa* fit with the 0.008-0.046 mg/L range
431 obtained in *P. lividus* by His et al. (1999), Arizzi Novelli (2003), Fernandez and Beiras (2001), and
432 are comparable to the other embryo-larval models (Table 4). The median effect of Pb concentrations
433 found in this study (0.372 mg/L) falls within the range of 0.068 - 1.250 found in *P. lividus* embryos
434 (Fernandez and Beiras 2001; Arizzi Novelli 2003; Manzo et al. 2010; Morroni et al. 2018) and is
435 lower than values reported for *H. elegans* (1.130 mg/L) and *C. gigas* (0.660 mg/L) (Gopalakrishnan
436 et al. 2008; Xie et al 2016). In this study, the EC50 values for Cd and Cu in *H. tubulosa* were 4.98
437 and 0.10, thus revealing a lower sensitivity in comparison to another species of sea cucumbers, *H.*
438 *polii*, with an EC50 of 2.26 and 0.026 mg/L for Cd and Cu, respectively (Morroni et al., 2020b).
439 Despite the different embryotoxicity of Cu, this metal seems to cause a delay in development in both
440 sea cucumber species at lower concentrations, in line with the effects observed on sea urchin embryo
441 development (Arizzi Novelli et al. 2003, Morroni et al. 2018). Moreover, in sea cucumbers this metal
442 can cause a significant size reduction in the mid auricularia development stage and, at higher
443 concentrations, a malformation in the early auricularia stage, causing an a-typical body shape (Table
444 1o) typically associated with exposure to Cu and Hg. This peculiar malformation, which was also
445 seen in *H. polii*, highlights a developmental anomaly that is typical of sea cucumber larvae in specific
446 stress conditions.

447 With regards to the organic compounds, the EC₅₀ of NP in *H. tubulosa* embryos (0.710 mg/L) is
448 almost ten times lower in respect to that of *F. enigmaticus* (6.81 mg/L) (Oliva et al 2018), but higher

449 than those found in sea urchin *P. lividus* and in the bivalve *M. galloprovincialis*, with 0.085 and 0.140
 450 mg/L, respectively (Tato et al. 2018). The EC₅₀ of SDS (8,501 mg/L) is very close to *F. enigmaticus*
 451 EC₅₀ (8.68 mg/L) and in the same order of magnitude than those of *P. lividus*, *M. galloprovincialis*
 452 and *Ciona intestinalis*, with EC₅₀ of 4.100, 2.253 and 5.145 mg/L, respectively (Bellas 2005; Beiras
 453 and Bellas 2008).

454

455

456 **Table 4** – EC₅₀ values (expressed in mg/L with 95% confidence limits) of *Holothuria tubulosa* compared
 457 with other groups from literature: *Holothuria polii*, *Ciona intestinalis*, *Crassostrea gigas*, *Ficopomatus*
 458 *enigmaticus*, *Hydroides elegans*, *Mytilus galloprovincialis*, *Paracentrotus lividus* for Cd²⁺, Cu²⁺, Hg²⁺, Pb²⁺,
 459 SDS and NP in **embryo toxicity assays**.

460

| Test specie | Cd ²⁺ | Cu ²⁺ | Hg ²⁺ | Pb ²⁺ | SDS | NP |
|----------------------------------|--------------------------------------|---------------------------------------|-------------------------------------|-------------------------------------|--|---------------------------------------|
| <i>Holothuria tubulosa</i> | 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) |
| <i>Holothuria polii</i> | 2.26 ^a (1.69- 3.31) | 0.026 ^a (0.017-0.029) | - | - | - | - |
| <i>Ciona intestinalis</i> | 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) | - |
| <i>Crassostrea gigas</i> | 0.037-0.212 ^{ef} | 0.013 ^e (0.011 – 0.014) | 0.012 ^f | 0.660 ^g (0.453~1.062) | - | - |
| <i>Ficopomatus enigmaticus</i> | 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) |
| <i>Hydroides elegans</i> | 0.180 ⁱ (0.140-0.220) | - | 0.054 ⁱ (0.042-0.068) | 1.130 ⁱ (0.817-1.782) | - | - |
| <i>Mytilus galloprovincialis</i> | 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) |
| <i>Paracentrotus lividus</i> | 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)

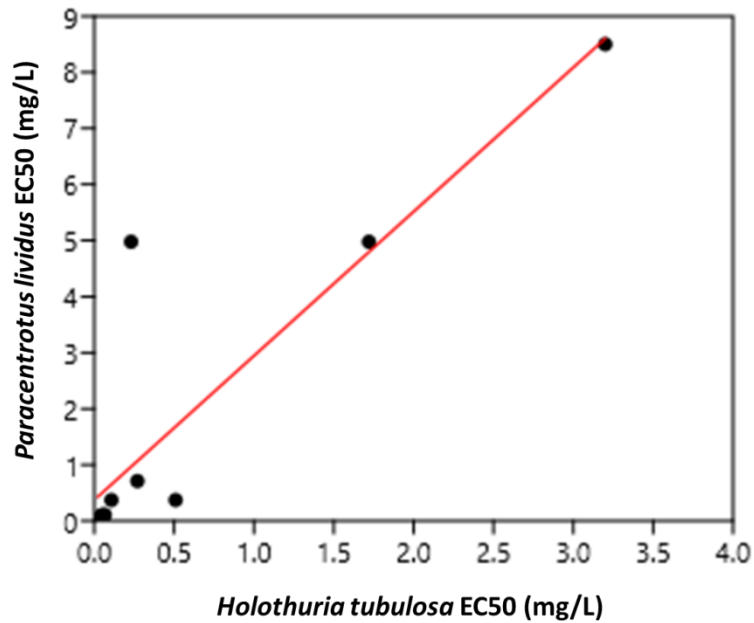
h : Oliva et al. (2018)
i: Gopalakrishnan et al (2008)
l:: Beiras and Albentosa (2004)
m: Boukadida et al. (2016)
n: Prato and Bianolino (1997)
o: Arnold et al. (2010):
p: Beiras and Bellas (2008)
q: Tato et al. (2018)
r: Morroni et al. (2018)
s; Waranu et al. 1996
t :Arizzi Novelli (2003)
u :Fernandez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

461

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

482 Concerning the elutriate sample, a higher sensitivity of *H. tubulosa* embryos with respect to sea urchin
483 *P. lividus* was observed. In two samples in particular, (S1, S3), toxic effects on sea cucumbers were
484 observed, whereas these were not seen in sea urchins. In S2 samples, *H. tubulosa* demonstrate twice
485 as high a sensitivity than that of *P. lividus*, which also exhibits toxic effects. These three samples
486 were characterized by the presence of Cd (0.9-5.8 mg/kg), organic pollutants such as PAHs (5400-
487 30000 µg/kg) and organotin compounds (Table S1). These concentrations were positively correlated
488 with developmental defects ($p < 0.001$) and were higher than the maximum limits established in the
489 Italian law decree determining quality class and management options for dredged marine sediments
490 (DM 173/2016). Ammonia was also found in the elutriate sample, with a concentration range between
491 0.560 – 1.240 mg/L. These values are higher than the effect concentrations in sea urchins found by
492 Losso et al (2007) and Saco-Alvarez et al. (2010) and could potentially affect correct sea cucumber
493 embryo development.

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



508

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

513

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

517

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

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

560

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