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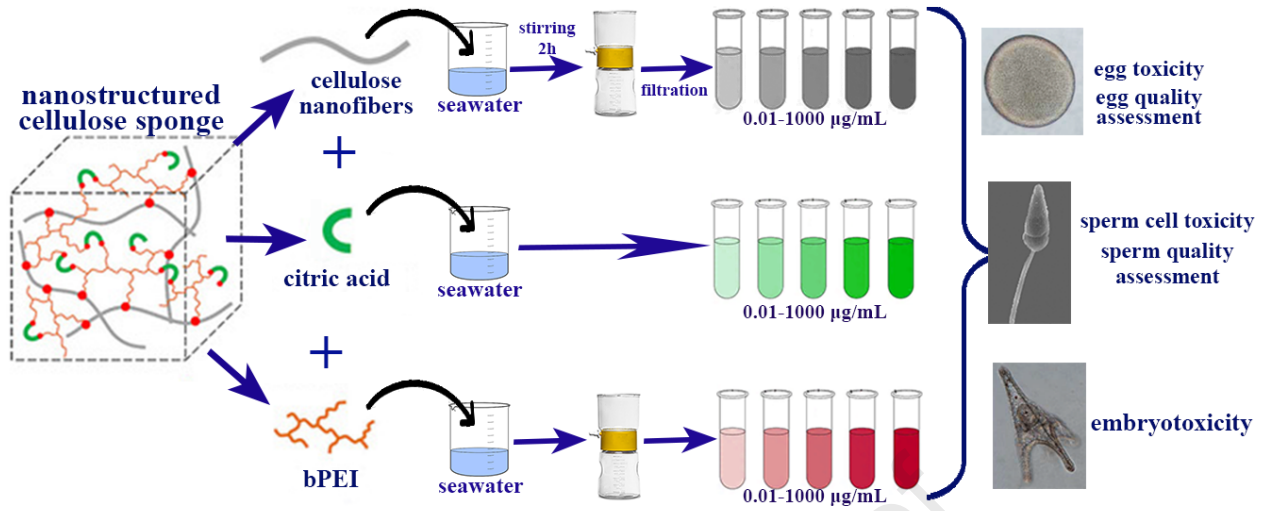
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1 **Reproductive toxicity assessment of cellulose nanofibers, citric acid, and branched**
2 **polyethylenimine in sea urchins: eco-design of nanostructured cellulose sponge framework**
3 **(Part B)**

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28 **Abstract**

29 In the framework of a safe-by-design approach, we previously assessed the eco-safety of
30 nanostructured cellulose sponge (CNS) leachate on sea urchin reproduction. It impaired gamete
31 quality, gamete fertilization competence, and embryo development possibly due to the leaching of
32 chemical additives deriving from their chemical synthesis. To extend this observation and identify
33 the component(s) that contribute to CNS ecotoxicity, in the present study, we individually screened
34 the cytotoxic effects on sea urchin *Arbacia lixula* and *Paracentrotus lividus* gametes and embryos of
35 the three main constituents of CNS, namely cellulose nanofibers, citric acid, and branched
36 polyethylenimine. The study aimed to minimize any potential safety risk of these components and to
37 obtain an eco-safe CNS. Among the three CNS constituents, branched polyethylenimine resulted in
38 the most toxic agent. Indeed, it affected the physiology and fertilization competence of male and
39 female gametes as well as embryo development in both sea urchin species. These results are
40 consistent with those previously reported for CNS leachate. Moreover, the characterisation of CNS
41 leachate confirmed the presence of detectable branched polyethylenimine in the conditioned seawater
42 even though in a very limited amount. Altogether, these data indicate that the presence of branched
43 polyethylenimine is a cause-effect associated with a significant risk in CNS formulations due to its
44 leaching upon contact with seawater. Nevertheless, the suggested safety protocol consisting of
45 consecutive leaching treatments and conditioning of CNS in seawater can successfully ameliorate the
46 CNS ecotoxicity while maintaining the efficacy of its sorbent properties supporting potential
47 environmental applications.

48

49 **Keywords:** eco-safety; embryotoxicity test; engineered nanomaterial; gamete quality assessment; sea
50 urchin; sperm cell toxicity test.

51

52 **Introduction**

53 Nanostructured cellulose sponges (CNS) are engineered nanomaterials (ENMs) developed for marine
54 environmental remediation following a safer-by-design approach (Fiorati et al., 2020), which
55 introduces the assessment of ENM ecotoxicity along with their performance and efficacy at the design
56 stage before their launch into the market (Corsi et al., 2018; Corsi et al., 2023). The eco-safety of
57 CNS was previously assessed on the sea urchin reproduction demonstrating gamete quality, gamete
58 fertilization competence, and embryo development alteration probably associated with the presence
59 and consequent release of unreacted chemical additives used during the CNS synthesis process
60 (Esposito et al., 2023). This hypothesis was confirmed by overcoming the eco-toxicity by consecutive
61 leaching treatments and conditioning of CNS in seawater. At the same time, the new safety protocol
62 did not affect the CNS-sorbent properties (Esposito et al., 2023). CNS was produced following a two-
63 step protocol, consisting first of the production of TEMPO-oxidized cellulose nanofibers (TOCNF),
64 followed by their cross-linking in the presence of branched polyethyleneimine (bPEI) and citric acid
65 (CA) (Fiorati et al., 2020). The core of the safer-by-design approach is ensuring the safety and
66 minimizing (eco)toxicity of ENMs. To achieve this goal, it is fundamental to understand the factors
67 that contribute to (eco)toxicity and design out them during the synthesis or manufacturing processes
68 of ENMs (Corsi et al., 2023; Lin et al., 2018). The present study aims to fill the knowledge gap on
69 the absence of eco-toxicity data in sea urchin reproduction of the building block components of CNS,
70 namely cellulose nanofibers (TOCNF), bPEI and CA, by testing them individually to ultimately
71 reduce and/or eliminate any potential safety risk associated with the use of CNS and improve their
72 eco-safety.

73 Cellulose nanofibers (CNFs) are natural nanoscale fibres made purely from cellulose. Due to their
74 appealing physicochemical and mechanical properties, CNFs are drawing increasing attention as
75 promising new bio-based nanomaterials for use in many applications including biomedical, food
76 packaging to environmental remediation (Fen et al., 2022). This inevitably will lead to large-scale
77 production of CNFs and, thereby, an increasing release of them into the environment. Due to its

78 natural origin, CNFs are assumed to be non-toxic; however, being in the nanoscale dimension any
79 potential biological risk must be disclosed for their safety application (Stoudmann et al., 2019). To
80 date, the aquatic environmental toxicity of CNFs has been poorly investigated (Fen et al., 2022). In
81 particular, few ecotoxicity data are available for freshwater organisms demonstrating that CNFs cause
82 acute toxicity in freshwater algae (Munk et al., 2015), crustaceans and fish (Wang et al., 2020). On
83 the other hand, low toxic effects of CNFs was recently documented in the marine mussel *Mytilus*
84 *galloprovincialis* (Rusconi et al., 2024).

85 The bPEI is a synthetic cationic polymer characterised by repeating units of amine groups (-[CH₂-
86 CH₂-NH₂]-) spaced by two carbon atoms with a backbone chain characterized by primary, secondary
87 and tertiary amine groups (Kunath et al., 2003). bPEI possesses several potential applications due to
88 its chemical functionality arising from the high density of amines. In the environmental remediation
89 application, bPEI is considered an ideal candidate for the synthesis of highly efficient adsorbent
90 materials to employ for the capture and removal of multiple compounds, including heavy metals due
91 to its ability to form complexes with metal ions (Finny et al., 2022; Melone et al., 2015). Moreover,
92 bPEI applications have been suggested as an effective technology for harmful algal bloom control
93 (Kim et al., 2021). However, safety concerns regarding its toxicity remain unresolved and claim for
94 more investigation for its safe use. The limited studies on the eco-toxicity of bPEI in freshwater
95 organisms reveal high toxicity for microalgae (Yoshitomi et al., 2021) and negligible for crustaceans
96 (Kim et al., 2021). Similarly in marine species, bPEI has been documented to cause limited effects
97 on bacteria and microalgae (Fiorati et al., 2020; Rychter et al., 2019).

98 CA is a natural metabolite of energy metabolism in all animal and plant cells. It is the most widely
99 employed organic acid in food, beverage, pharmaceutical, nutraceutical and cosmetic products,
100 agriculture, and other industrial applications (Singh Dhillon et al., 2011). Furthermore, other
101 promising biomedical and industrial applications of CA have been found as a crosslinking agent in
102 the synthesis of several bio-based nanomaterials and environmental remediation (Ciriminna et al.,
103 2017; Salihu et al., 2021). According to the Organisation for Economic Co-operation and

104 Development, CA is classified as a chemical compound of low concern to the environment, since it
105 exhibits low acute toxicity to freshwater and marine species (algae, protozoan, decapod crustacean)
106 (Development, 2001). Nevertheless, CA eco-toxicity at the early life stages of marine species has
107 been overlooked.

108 The Mediterranean echinoids *Paracentrotus lividus* and *Arbacia lixula* are considered excellent
109 model organisms for ecotoxicological studies and represent ecologically important sea urchin species
110 inhabiting the northeast Atlantic and the Mediterranean Sea where they play a key role in structuring
111 benthic communities being dominant grazers (Boudouresque and Verlaque, 2013). Any distress
112 caused by anthropogenic activities including pollution remediation on these species may have
113 repercussions for the whole ecosystem and associated services. The preservation of the species strictly
114 depends on gamete quality and the ability they have to reach and overcome the critical stage of
115 embryo development. The male and female gametes, spermatozoa and eggs respectively, are
116 specialized cells, which, during fertilization, fuse producing a diploid fertilized egg cell, named
117 zygote, that undergoes numerous cycles of mitosis giving rise to a new genetically distinct organism
118 (Tosti and Ménézo, 2016). The quality of gametes is a determining factor in fertilization and embryo
119 development success and its evaluation is commonly based on different parameters, mainly
120 morphology, vitality, mitochondrial activity, intracellular reactive oxygen species (ROS) level,
121 intracellular pH, and motility for spermatozoa. The evaluation also included the assessment of
122 fertilization and developmental competence (Gallo et al., 2018; 2020; Gallo et al., 2022; Gallo et al.,
123 2021). In broadcast spawning marine invertebrates, gametes are released into seawater where
124 fertilization and embryo development occur; thereby, the quality of gametes, fertilization and embryo
125 development may be influenced by chemical agents introduced into seawater with severe
126 repercussions on the persistence of marine species (Gallo et al., 2020; Gallo and Tosti, 2019).
127 Although for embryo development to be successful in the production of viable offspring, good quality
128 gametes are required, ecotoxicological studies with sea urchins traditionally focused on one life-
129 history stage, commonly embryo and larvae stages starting from fertilized eggs, omitting that

130 environmental stressors leading to a disturbance in gamete quality can carry over into the following
131 embryo stage (Podolsky and Moran, 2006). In the present study, the ecotoxicity of single components
132 used in CNS formulation, such as TOCNF, bPEI and CA have been tested on sea urchin reproductive
133 processes. A multi-responses integrated approach was adopted, which combines standardized
134 ecotoxicity tests, such as sperm cell toxicity and embryotoxicity, with innovative bioassays along
135 with gamete quality assessment. Overall, the present study addresses, for the first time, the current
136 concerns related to the safety of CNS for environmental application by disclosing the safety of single
137 components and chemicals used during the synthetic process.

138 **Materials and methods**

139 All the fluorochromes used for gamete quality assessment were purchased from Thermo Fisher
140 Scientific (Milan, Italy).

141 **Animal and gamete collection**

142 Adult sea urchins were collected from the Gulf of Naples by the personnel of the Material Collection
143 and Diving service of the Stazione Zoologica Anton Dohrn and transported in a cool box to the Marine
144 Biological Resources service. Herein, sea urchins were maintained in tanks (1 animal/5 L) with
145 running filtered natural seawater at the temperature of $18 \pm 2^\circ\text{C}$, pH 8.1 ± 0.1 , salinity 39 ± 0.5 ppm,
146 a photoperiod of 10 h L: 14 h D and fed with fresh green algae *Ulva sp.*

147 Gamete spawning was induced by injecting 1 mL of 0.5 M KCl through the peristomal membrane.
148 Eggs were collected in filtered natural seawater (FNSW) and preserved at $18 \pm 1^\circ\text{C}$ until use.
149 Spermatozoa were collected dry directly from the gonopore and stored at 4°C . Finally, gametes were
150 checked for preliminary quality assessment and counted.

151 **Test solutions**

152 *Branched polyethyleneimine*

153 The bPEI (25 kDa; CAS: 9002-98-6) was purchased from Merck Life Science (Milan, Italy) and
154 dissolved in double distilled water to obtain a stock solution of 5000 $\mu\text{g/mL}$, which was magnetically
155 stirred for 20 min at room temperature (RT). Then, the bPEI stock solution was filtered with a 0.22

156 μm filter and diluted in FNSW to obtain the tested concentrations (0.01, 0.1, 0.5, 1, 10, 100, 1000
157 $\mu\text{g}/\text{mL}$), which were chosen based on the bPEI EC_{50} values reported in the data sheet (1 - 10 mg/L
158 and 10 - 100 mg/L , respectively for *Danio rerio* and *Daphnia magna*) and based on previous findings
159 (data not shown). Before use, the pH of each test solution was checked by using a bench pH meter
160 and, if necessary, adjusted to the pH of 8.1 (FNSW).

161

162 *Citric acid*

163 The citric acid (Merck Life Science) was dissolved into double distilled water to prepare a stock
164 solution of 5000 $\mu\text{g}/\text{mL}$, which was then diluted in FNSW to obtain the final tested concentrations of
165 0.01, 0.1, 1, 10, 100, 1000 $\mu\text{g}/\text{mL}$, selected on acute toxicity data for aquatic invertebrate and
166 preliminary experiments (data now shown).

167

168 *TEMPO-oxidized cellulose nanofibers*

169 The TEMPO-oxidized cellulose nanofibers (TOCNF) were obtained from the oxidation of the cotton
170 fibres through the 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO)/NaClO/NaBr system (Pierre et al.,
171 2017). A stock solution of 1000 $\mu\text{g}/\text{mL}$ TOCNF in FNSW was prepared, stirred at RT for 2 h, and
172 then filtered through a 0.45 μm filter. The stock solution was diluted in FNSW to obtain the test
173 concentrations of 0.01, 0.1, 1, 10, 100, and 1000 $\mu\text{g}/\text{mL}$ TOCNF based on acute and chronic toxicity
174 data (Ogonowski et al., 2018; Wang et al., 2020).

175

176 **Ecotoxicological bioassays with sea urchins**

177 Three ecotoxicological bioassays, embryotoxicity, spermotoxicity and egg toxicity, with *P. lividus*
178 and *A. lixula* were performed to investigate the toxicity of bPEI, TOCNF and CA in different life
179 stages. Differently from *P. lividus* for which traditional and standardized protocols for embryotoxicity
180 and spermotoxicity bioassays are widespread, for *A. lixula*, despite these bioassays have been already
181 carried out by different research groups, standardized procedures are not yet available since the

182 experimental conditions significantly differ from each other. Regarding the egg toxicity bioassay, it
183 has been not performed before with the sea urchin *P. liviuds* and *A. lixula*. Thereby, a set of
184 preliminary tests using the reference toxicant has been carried out to set up the suitable experimental
185 conditions used herein and in our previous study (Esposito et al., 2023).

186 Briefly, for each bioassay, male and female gametes of three species were selected, mixed and used;
187 furthermore, a positive control test with copper as a reference toxicant was conducted. The bioassays
188 were accepted if they met the previously defined requirements of acceptability in the negative control
189 and reference toxicant tests (Ghirardini et al., 2005).

190 In the embryotoxicity test, the eggs were fertilized with spermatozoa according to a sperm: egg ratio
191 of 50:1 in *P. lividus* and 1000:1 in *A. lixula*. After 20 min, 1000 fertilized eggs/mL were transferred
192 into a test chamber containing 9 mL of the test solution and incubated in a culture chamber for 48 h
193 at 18°C for *P. lividus* and 20 °C for *A. lixula*. 48 h after fertilization, embryos were fixed by adding
194 4% glutaraldehyde in FNSW and the percentage of plutei with normal development in each test
195 solution was determined by observing 100 larvae.

196 In the spermotoxicity bioassay, spermatozoa were exposed to test solutions for 1 h at 18°C for *P.*
197 *lividus* and 20°C for *A. lixula*. After exposure, eggs were added to the test chambers in a sperm/egg
198 ratio of 15.000:1 in *P. lividus* and 10000:1 in *A. lixula*.

199 In the egg toxicity bioassay, eggs were exposed to test solutions for 1 h at 18°C for *P. lividus* and
200 20°C for *A. lixula*. After exposure, spermatozoa were added to the test chambers according to a sperm:
201 egg ratio of 100:1 in *P. lividus* and of 1000:1 in *A. lixula*.

202 The spermotoxicity and egg toxicity bioassays were arrested by adding 4% glutaraldehyde 20 min
203 after fertilization (i.e., at the zygote stage) for *P. lividus* and 90 min post fertilization (i.e., at the 2-
204 cell stage embryo) for *A. lixula* and the percentage of fertilized eggs, i.e., fertilization rate (FR), was
205 determinate on a random sample of 200 eggs.

206

207 **Assessment of gamete quality**

208 Gamete quality assessment was performed as previously reported (Esposito et al., 2023). Briefly,
209 after male and female gamete exposure for 1 h to test solutions, different physiological parameters,
210 such as mitochondrial membrane potential (MMP), oxidative status, and intracellular pH (pH_i), were
211 evaluated by employing fluorescent staining coupled with fluorescence spectroscopy (Boni et al.,
212 2022; Gallo et al., 2018; Gallo et al., 2022).

213 *Male gametes*

214 The MMP and pH_i were evaluated by staining 1 x 10⁶ spermatozoa/mL respectively with 5 μM of the
215 mitochondrial dye JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide)
216 and 5 μM of the cell-permeant dye BCECF-AM ((2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-
217 carboxyfluorescein acetoxymethyl ester). The oxidative status was assessed by analysing the
218 intracellular ROS levels staining 5 x 10⁶ spermatozoa/mL with 10 μM H₂DCFDA (2',7'-
219 dichlorodihydrofluorescein diacetate) and the intracellular content of superoxide anions (O₂⁻) by
220 staining 60 x 10⁶ spermatozoa/mL with 2 μM DHE (dihydroethidium).

221 Briefly, aliquots of spermatozoa were incubated with each fluorochrome for 30 min at 18°C, in the
222 dark. Then, samples were centrifuged at 900g at 4°C for 10 min and the pellet re-suspended in FNSW.
223 After 30 min, spermatozoa were centrifuged again, excepting those staining with DHE, the pellets
224 were re-suspended in FNSW, and samples were analysed with the spectrofluorometer (Shimadzu RF-
225 5301, Tokyo, Japan).

226 For male gametes, motility was also evaluated by an expert operator via a visual estimation, which
227 was carried out by loading an aliquot of sperm suspension (80 x 10⁶ spermatozoa/mL) on a sperm
228 counting chamber, employing a microscope equipped with an objective 40X and analysing at least 5
229 visual fields. The percentage of motile spermatozoa was determined as a ratio between motile and
230 total sperm number.

231 *Female gametes*

232 Eggs were stained as previously described (Gallo et al., 2022). Briefly, 1000 eggs/mL were incubated
233 with each fluorochrome for 30 min in the dark at 18°C; then, eggs were washed and incubated for 30

234 min in FNSW. Afterwards, the eggs were washed again, except the DHE stained eggs, re-suspended
235 in FNSW and analysed to the microplate reader (Tecan Infinite® m1000 pro).

236 *Fluorescence Spectroscopic analysis*

237 Each fluorochrome was detected by exciting it at a specific wavelength and recording its fluorescence
238 emission spectra in a selected range. In particular, for JC-1 the excitation wavelength was at 488 nm
239 and the emission spectrum recorded between 500 and 620 nm, the ratio between the fluorescence
240 peak values at ~595 nm and ~525 nm indicates the MMP value; for H₂DCFDA and DHE, whose
241 fluorescence intensity is proportional to the intracellular ROS levels, the excitation wavelength was
242 respectively set to 488 nm 350 nm and fluorescence emission spectra recorded in a range of 500-560
243 nm and 500-620 nm, respectively. BCECF-AM exhibits a primary excitation peak at 440 nm and a
244 secondary peak at 490 nm with an emission peak that remains constant at 535 nm. The ratio between
245 these two peaks was converted into pHi values based on a linear regression analysis.

246 *CNS leachate characterization*

247 The CNS leachate, obtained by Esposito et al. (2023) by allowing CNS to leach in FNSW while
248 simulating the remediation process condition, was characterized to identify the presence of chemical
249 additives possibly released in solution by the adsorbent material. Twenty mL of the leachate were
250 freeze-dried by an SP Scientific BenchTop Pro Lyophilizer, providing a solid mainly consisting of
251 inorganic salts. The solid was washed with methanol (3 x 10 mL) to extract the organic residue (4
252 mg). The organic phases collected together were concentrated under vacuum and characterized by ¹H
253 NMR in D₂O (NMR spectrometer Brüker 400 MHz, 1024 scans) and elemental analysis after
254 dehydration of the sample by freeze-drying process (Costech ECS 4010 analyser based on the Dumas
255 method for the simultaneous determination of CHNS elements).

256

257 **Statistical analysis**

258 Each bioassay was conducted in triplicate and replied three times. Statistical comparisons were
259 conducted by performing the one-way variance analysis (ANOVA) followed by a parametric test

260 using the software Systat 11.0 (Systat Software Inc.). The minimum level of significance was fixed
261 as $p < 0.05$. The data are stated as mean \pm standard error (SE). GraphPad Prism version 8.0 (software
262 package, San Diego, CA, USA) was used to calculate the 50% effective concentration values (EC_{50})
263 i.e., the concentration that gives half-maximal response.

264

265 **Results**

266 **Ecotoxicological bioassays**

267 The performed bioassays revealed that bPEI affects fertilization success and embryo development in
268 both sea urchin species in the range of the tested concentrations (Fig. 1). In particular, the increase in
269 bPEI concentration resulted in the reduction of the percentage of normal larvae up to the total absence.
270 The EC_{50} values calculated were 0.50 $\mu\text{g/mL}$ and 0.53 $\mu\text{g/mL}$ bPEI for *P. lividus* and *A. lixula*,
271 respectively (Fig. 1A and B). Sperm as well as egg pre-exposure to bPEI significantly decreased
272 fertilization rate (FR) in a concentration-dependent manner (Fig. 1C, D, E and F). The EC_{50} value of
273 0.4 $\mu\text{g/mL}$ was determined for both *P. lividus* and *A. lixula* for spermiotoxicity bioassays (Fig. 1C
274 and D). Slight different EC_{50} values were calculated in *P. lividus* (0.56 $\mu\text{g/mL}$) and *A. lixula* (0.74
275 $\mu\text{g/mL}$) for the egg toxicity test (Fig. 1E and F).

276 The CA also affects sea urchin embryo development. In particular, the exposure of fertilized eggs
277 leads to a significant decrease in the percentage of normal embryo at pluteus stage with a calculated
278 EC_{50} of 107.2 $\mu\text{g/mL}$ in *P. lividus* and 5.7 $\mu\text{g/mL}$ in *A. lixula* (Fig. 2).

279 Sperm fertilizing ability was affected by CA only in *P. lividus*. Indeed, a significant reduction of FR
280 was observed after sperm pre-exposure to 1000 $\mu\text{g/mL}$ CA (Table 1S). The EC_{50} was not calculated
281 for this bioassay because of the absence of at least two concentrations whose response was less than
282 50%. Differently, the egg fertilization competence was not impaired by CA pre-exposure in both sea
283 urchin species (Table 1S).

284 The TOCNF influences sea urchin embryo development only at the highest tested concentrations. In
285 particular, the exposure of fertilized eggs to TOCNF induced a significant decrease of normal larvae

286 percentage starting from 100 $\mu\text{g}/\text{mL}$ in *A. lixula* and from 1000 $\mu\text{g}/\text{mL}$ in *P. lividus* (Table 2S).
287 Otherwise, the pre-exposure of both gametes to TOCNF did not affect the sperm fertilizing ability
288 and the egg fertilizing competence in both sea urchin species (Tables 2S).

289 **Gamete quality assessment**

290 *Mitochondrial membrane potential (MMP)*

291 The MMP was significantly impaired by bPEI in sea urchin female and male gametes. In particular,
292 in *P. lividus* spermatozoa exposed to 0.1 and 1 $\mu\text{g}/\text{mL}$ bPEI, the MMP value did not differ from that
293 measured in unexposed spermatozoa; nevertheless, it significantly increased after exposure to 10
294 $\mu\text{g}/\text{mL}$ bPEI (Fig. 3; Table 3S). Otherwise, in *A. lixula* spermatozoa, after exposure to all tested bPEI
295 concentrations a significant rise of MMP was detected (Fig. 3; Table 3S).

296 In *P. lividus* eggs, MMP significantly increased after exposure to all tested bPEI concentrations in
297 comparison to the control (Fig. 3; Table 4S). Differently, in *A. lixula* eggs, the MMP values
298 significantly increased only after exposure to the highest tested bPEI concentration (Fig. 3; Table 4S).
299 The CA as well as TOCNF exposure did not affect MMP in spermatozoa and eggs of two sea urchin
300 species (Table 3S and 4S).

301

302 *Oxidative status*

303 The oxidative status of sea urchin gametes was assessed directly by analysing the intracellular level
304 of two ROS species, H_2O_2 and O_2^- , and indirectly by evaluating LPO. In *P. lividus* spermatozoa, the
305 intracellular H_2O_2 levels significantly increased after exposure to the highest tested bPEI
306 concentration. Differently, the intracellular O_2^- levels in spermatozoa were not significantly affected
307 by sperm exposure to bPEI (Fig. 3; Table 3S).

308 In *A. lixula*, the intracellular H_2O_2 levels significantly increased after sperm exposure to all the tested
309 bPEI concentrations; whereas the O_2^- intracellular levels significantly increased only after sperm
310 exposure to 10 $\mu\text{g}/\text{mL}$ bPEI (Fig. 3; Table 3S). On the other hand, LPO was not significantly affected
311 at all tested concentrations in both sea urchin spermatozoa (Table 3S). In both sea urchin species, egg

312 exposure to bPEI, CA and TOCNF did not significantly modify intracellular ROS levels and LPO
313 (Tables 3S and 4S).

314

315 ***Intracellular pH***

316 The pH_i of spermatozoa and eggs of *P. lividus* as well as *A. lixula* was not significantly affected after
317 exposure to bPEI, CA, and TOCNF (Table 3S and 4S).

318 ***Sperm motility***

319 In *P. lividus*, exposure to 0.1 µg/mL bPEI did not alter sperm motility; whereas, a significant decrease
320 in the percentage of motile spermatozoa was measured compared to the control after exposure to 1
321 and 10 µg/mL bPEI. Similarly, in *A. lixula*, the percentage of motile spermatozoa was significantly
322 reduced only after exposure to the highest tested bPEI concentration (Fig. 4; Table 3S).

323 The percentage of motile spermatozoa was not significantly affected after exposure to CA as well as
324 TOCNF in both sea urchin species (Table 3S).

325 ***Leachate characterization***

326 The elemental analysis of the residual organic matter extracted from leachate provided the following
327 mass distribution: 29.01 % in N (Nitrogen), 50.49 % in C (Carbon) and 10.50 % in H (Hydrogen),
328 consistent with that measured for bPEI, with a percentage of O (Oxygen, 10 %, calculated by
329 difference) which, can be ascribed to the high hygroscopicity of the polymer.

330 The ¹H-NMR analysis provided further confirmation: the spectrum of the extracted organic residue
331 resulted very similar to that of bPEI used for the original formulation of CNS, with a broad signal
332 attributed to the methylene hydrogens of the polymer in the range between 2.5 and 2.8 ppm (Fig. 5).

333

334 **Discussion**

335 In our recently published article (Esposito et al., 2023), the ecotoxicity of CNS leachate on sea urchin
336 reproductive processes has been proved suggesting a potential leaching of the chemicals embedded

337 in the CNS formulation upon dispersion in seawater. Starting from this hypothesis, in the present
338 study, the three constituents of CNS, such as bPEI, CA and TOCNF, were, here, individually tested
339 on gamete quality, fertilization competence and embryo development of the sea urchins *P. lividus*
340 and *A. lixula* to disclose their eco-toxicity for a safer CNS design.

341 Up to now, the impact of CNF has been barely investigated and mostly in freshwater species with
342 negligible effects (Harper et al., 2016; Ogonowski et al., 2018; Ong et al., 2017; Pengiran et al., 2022).

343 CNF reduces growth, cell viability, and intracellular ATP levels as well as induces ROS generation
344 in freshwater green microalgae at concentrations far higher than those predicted to reach the aquatic
345 environment (1 µg/mL) (Pereira et al., 2014). Otherwise, CNF did not affect vitality, morphology and
346 swimming behaviour in fish and crustaceans (Ogonowski et al., 2018; Pengiran et al., 2022). The
347 only study so far on marine species revealed that neither oxidative stress nor biotransformation were
348 affected in the digestive glands and gills of the marine mussel *M. galloprovincialis*, although a CNF
349 uptake and disruption of gill functionality and immune cells by mechanical interaction was observed
350 (Rusconi et al., 2024). Starting from these findings, the need for more in-depth investigations emerged
351 to promote CNF eco-safe applications as in remediation. As far as our ecotoxicity results on single
352 CNS components, TOCNF did not affect gamete quality and fertilization competence in sea urchins;
353 however, it altered embryo development but only at concentrations (100 and 1000 µg/mL) much
354 higher than those predicted to reach the natural environment (Stoudmann et al., 2019).

355 Nowadays, CA is classified as low acute toxicity based on the scarce ecotoxicity data available for
356 marine organisms, even if the subacute toxic limit concentration is given as a wide range between 1
357 and 300 mg/L (Development, 2001). Herein, consistent EC₅₀ values for CA, i.e., 107.2 µg/mL in *P.*
358 *lividus* and 5.7 µg/mL in *A. lixula*, were measured. Additionally, CA did not impair the quality and
359 fertilization competence of sea urchin female gametes; but, negatively affected the fertilizing ability
360 of spermatozoa and embryo development. The earliest stages of sea urchin embryo development are
361 fuelled by maternal RNAs and proteins deposited into the unfertilized egg and activated after
362 fertilization. At the 64-cell stage embryo, the depletion of maternal mRNAs occurs and from this time

363 embryo development largely depends on the expression of the zygotic genes, which have to be
364 activated at specific times and in specific territories for an embryo to develop properly (Adonin et al.,
365 2021). It is well known that CA forms stable chelate complexes with metal ions, such as calcium and
366 magnesium, which may play an essential role in sea urchin embryo development and their deprivation
367 has been proved to impair gastrulation, skeletogenesis and animal-vegetal axis development (Martino
368 et al., 2019). Therefore, the embryotoxic effects herein observed for CA may be linked to a possible
369 reduction of these ions within the fertilized eggs. Given the widespread current and future
370 applications, i.e., as a cross-linker, the CA toxicity toward other marine environmentally relevant
371 species needs to be further investigated.

372 Concerning bPEI, it severely affects the quality and the fertilization competence of sea urchins' male
373 and female gametes, as well as embryo development. It has been widely documented that bPEI
374 induces cytotoxicity in different cell lines but the mechanism has not been elucidated yet (Hunter,
375 2006). In particular, the exposure of spermatozoa to bPEI resulted in a motility decrease as well as an
376 increase of mitochondrial activity and intracellular ROS levels, consistent with the known positive
377 correlation between MMP and ROS production in spermatozoa of different species (Gallo et al., 2021;
378 Turrens, 2003). Inside the cell, the MMP increase may be caused either by the closure of the
379 mitochondrial permeability transition pore or the inhibition of ATP synthase (Suski et al., 2018). The
380 decrease in sperm motility herein observed can be due to ATP depletion, which serves as an energy-
381 carrying molecule, suggesting that the mechanism of toxic action of bPEI in sea urchin spermatozoa
382 relies on ATP synthase inhibition, and subsequently, oxidative stress promotion because at high
383 membrane potential, mitochondria produce more ROS.

384 Otherwise, in sea urchin eggs, bPEI exposure affects mitochondrial activity promoting an MMP rise
385 that is not associated with an increase in intracellular ROS levels, probably because, differently from
386 spermatozoa that are deficient in antioxidant defence, the eggs are characterized by an efficient
387 antioxidant system that counterbalances the generation of ROS (Dowling and Simmons, 2009).
388 Additionally, bPEI impairs the fertilization competence of female gametes as previously

389 demonstrated for CNS leachate (Esposito et al., 2023). Fertilization is a cell-cell membrane fusion
390 event involving two steps consisting in the attachment of two membranes through cell-surface
391 molecules and followed by the physical merger of the plasma membrane lipids. Since in our previous
392 study we also revealed that after exposure to CNS leachate egg surface was characterized by the
393 presence of several aggregates (Esposito et al., 2023), it is possible to hypothesize that the aggregates
394 observed on the egg surface are made up to bPEI molecules, which inducing membrane damage and
395 phospholipids reshuffling, as already reported in other cell types (Hunter, 2006), may prevent
396 spermatozoa binding hindering the fertilization process.

397 Several studies indicated that bPEI exhibits high cytotoxicity and induces apoptosis, but the
398 mechanism(s) triggering cell death induction is poorly understood (Fischer et al., 2003;
399 Khansarizadeh et al., 2016). Apoptosis is a physiological process, which occurs during sea urchin
400 embryo development playing a key role in shaping and sculpting the developing embryos and
401 eliminating damaged or unnecessary cells (Agnello et al., 2015). Changes in the level of apoptosis
402 upon exposure to physical and chemical contaminants have been reported in different sea urchin
403 species representing a defence strategy to remove damaged cells (Di Tuccio et al., 2023). Thereby, it
404 is possible to hypothesise that the exposure of sea urchin embryos to bPEI causes irreversible cell
405 damage and apoptosis activation, which, in turn, can result in an altered developmental program with
406 consequent embryo abnormalities. Future works will be devoted to investigating the biochemical
407 pathways involved in apoptotic activation by bPEI and, possibly, to identifying its specific molecular
408 target(s) with the support of computational approaches.

409

410 **Conclusion**

411 This study represents the first report on the bPEI cytotoxicity in sea urchin gametes. Overall, the
412 ecotoxicological data obtained in the present study indicate that, among the constituents of CNS, the
413 bPEI is the most toxic and, thereby, could pose a higher risk in the final CNS formulation due to its
414 leaching during aging and/or contact with seawater. The analysis of CNS leachate, indeed, confirmed

415 the presence of bPEI in the conditioned seawater even though in a very limited amount. Considering
416 that each gram of CNS contains 440 mg of bPEI, which falls within the range of concentrations tested,
417 in a hypothetical sequence of events in which the total amount of bPEI per gram of CNS was released
418 into seawater due to prolonged CNS use, an ecological risk for sea urchins may occur. However, this
419 worrying scenario can be overcome as shown in our previous study (Esposito et al., 2023), in which
420 we demonstrated that multi-leaching treatment and conditioning of CNS in seawater significantly
421 reduce their toxicity. This protocol can help in removing the excess of bPEI down to levels not
422 hazardous for marine life and supporting the eco-safety of CNS without affecting the adsorbent
423 efficiency and the mechanical integrity of the sponge. Such evidence promotes a safe environmental
424 application of CNS including in marine pollution remediation.

425

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437

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566 **Fig. captions**

567 **Fig. 1.** Concentration-response curves for bPEI on embryotoxicity, spermotoxicity and egg toxicity
568 tests with the sea urchin *P. lividus* and *A. lixula*. In the graphs, the dashed lines indicate the EC₅₀.
569 Values represented the mean of triplicate experiments with error bars indicating the standard error.

570

571 **Fig. 2.** Concentration-response curves for citric acid on embryotoxicity test with the sea urchin *P.*
572 *lividus* and *A. lixula*. In the graphs, the EC₅₀ is depicted by the dashed line. Values represented the
573 mean of triplicate experiments with error bars indicating the standard error.

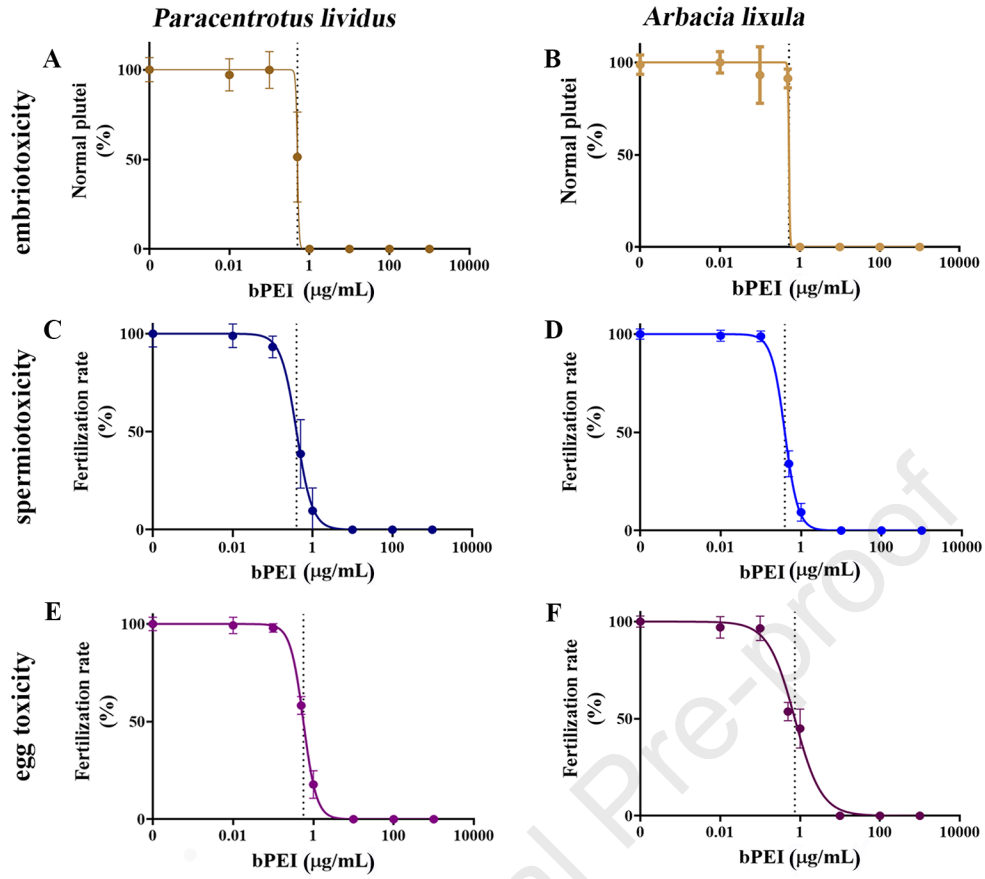
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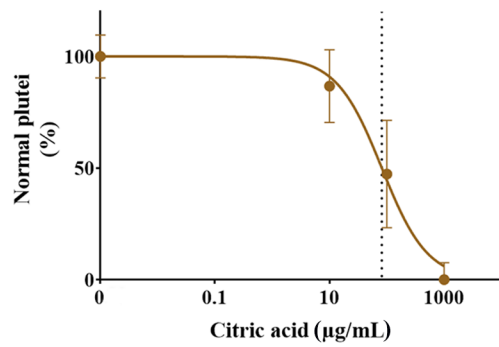
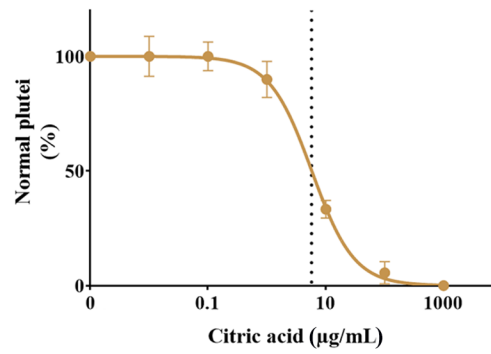
575 **Fig. 3.** Gamete quality assessment upon exposure to bPEI in *P. lividus* and *A. lixula*. Values (as the
576 mean of triplicate experiments with error bars indicating standard error) of mitochondrial membrane
577 potential (MMP) evaluated by JC-1; intracellular levels of hydrogen peroxide (H₂O₂) estimated with
578 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA); intracellular content of superoxide anions
579 (O₂⁻) assessed with dihydroethidium (DHE). * or # indicate a significant difference (p < 0.05) from
580 control gametes; ** or ## indicate a significance level lower than 0.01 (p < 0.01).

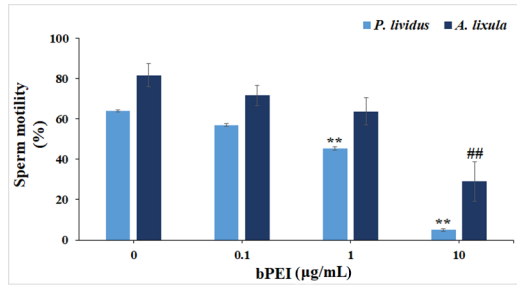
581

582 **Fig. 4.** Sperm motility assessment upon exposure to bPEI in *P. lividus* and *A. lixula*. Percentage of
583 motile spermatozoa determined through visual estimation after 1 h exposure to different
584 concentrations of bPEI. ** or ## indicate a significance level lower than 0.01 (p < 0.01).

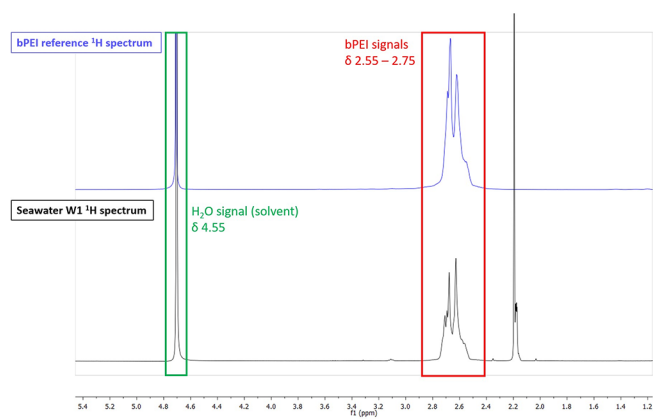
585 **Fig. 5.** Superimposition of **a)** ¹H NMR spectrum of reference bPEI and **b)** ¹H NMR spectrum of
586 seawater sample after extraction process. It is possible to identify by comparison of spectrum (**b**) with
587 reference spectrum (**a**) the characteristic signals of bPEI, confirming its presence in the extract from
588 the CNS leachate.



Paracentrotus lividus*Arbacia lixula*



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Highlights

Cellulose nanofibers, citric acid, branched polyethylenimine were assessed one by one

Spermiotoxicity, embryotoxicity, egg toxicity, and gamete quality were evaluated

Branched polyethylenimine results the most toxic compound

Branched polyethylenimine poses a threat to the reproductive success of sea urchins

Declaration of interests

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

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

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