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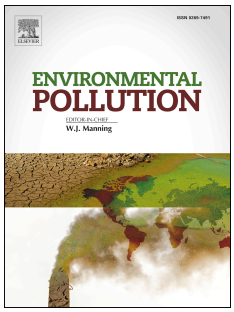

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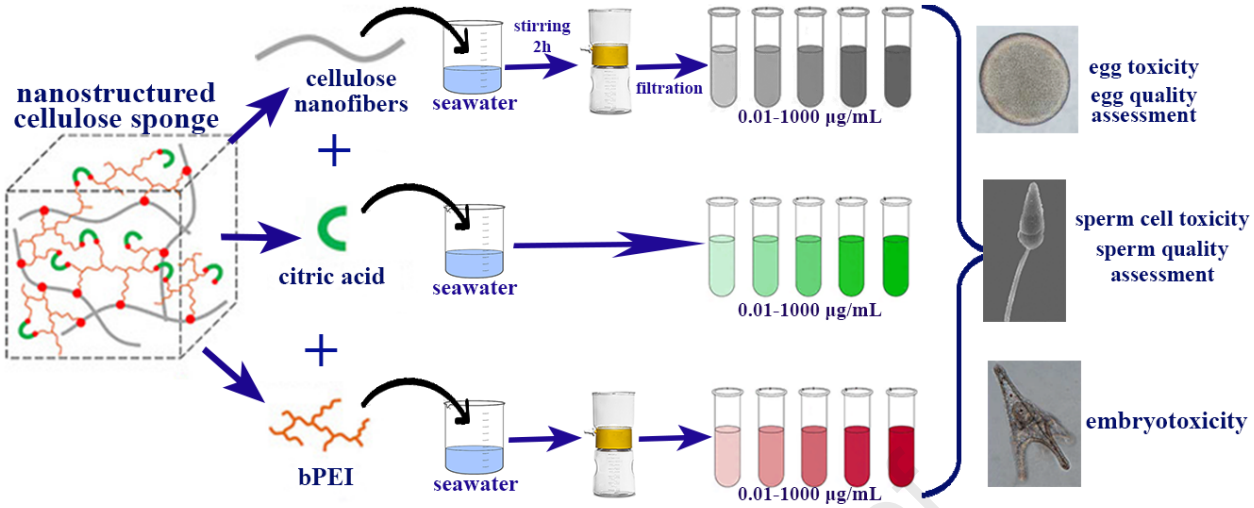

Reproductive toxicity assessment of cellulose nanofibers, citric acid, and branched polyethylenimine in sea urchins: eco-design of nanostructured cellulose sponge framework (Part B)

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

Materials and methods

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

Animal and gamete collection

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

Gamete spawning was induced by injecting 1 mL of 0.5 M KCl through the peristomal membrane. Eggs were collected in filtered natural seawater (FNSW) and preserved at 18±1°C until use. Spermatozoa were collected dry directly from the gonopore and stored at 4°C. Finally, gametes were checked for preliminary quality assessment and counted.

Test solutions

Branched polyethyleneimine

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

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

Citric acid

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

TEMPO-oxidized cellulose nanofibers

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

Ecotoxicological bioassays with sea urchins

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

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

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

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

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

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

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

Assessment of gamete quality

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

Male gametes

The MMP and pH_i were evaluated by staining 1 x 10^6 spermatozoa/mL respectively with 5 μM of the mitochondrial dye JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) and 5 μM of the cell-permeant dye BCECF-AM ((2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-carboxyfluoresce in acetoxymethyl ester). The oxidative status was assessed by analysing the intracellular ROS levels staining 5 x 10^6 spermatozoa/mL with 10 μM H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) and the intracellular content of superoxide anions (O_2^-) by staining 60 x 10^6 spermatozoa/mL with 2 μM DHE (dihydroethidium).

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

For male gametes, motility was also evaluated by an expert operator via a visual estimation, which was carried out by loading an aliquot of sperm suspension (80 x 10^6 spermatozoa/mL) on a sperm counting chamber, employing a microscope equipped with an objective 40X and analysing at least 5 visual fields. The percentage of motile spermatozoa was determined as a ratio between motile and total sperm number.

Female gametes

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

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

Fluorescence Spectroscopic analysis

Each fluorochrome was detected by exciting it at a specific wavelength and recording its fluorescence emission spectra in a selected range. In particular, for JC-1 the excitation wavelength was at 488 nm and the emission spectrum recorded between 500 and 620 nm, the ratio between the fluorescence peak values at ~595 nm and ~525 nm indicates the MMP value; for H_2DCFDA and DHE, whose fluorescence intensity is proportional to the intracellular ROS levels, the excitation wavelength was respectively set to 488 nm 350 nm and fluorescence emission spectra recorded in a range of 500-560 nm and 500-620 nm, respectively. BCECF-AM exhibits a primary excitation peak at 440 nm and a secondary peak at 490 nm with an emission peak that remains constant at 535 nm. The ratio between these two peaks was converted into pH_i values based on a linear regression analysis.

CNS leachate characterization

The CNS leachate, obtained by Esposito et al. (2023) by allowing CNS to leach in FNSW while simulating the remediation process condition, was characterized to identify the presence of chemical additives possibly released in solution by the adsorbent material. Twenty mL of the leachate were freeze-dried by an SP Scientific BenchTop Pro Lyophilizer, providing a solid mainly consisting of inorganic salts. The solid was washed with methanol (3 x 10 mL) to extract the organic residue (4 mg). The organic phases collected together were concentrated under vacuum and characterized by ^{1}H NMR in D_2O (NMR spectrometer Brüker 400 MHz, 1024 scans) and elemental analysis after dehydration of the sample by freeze-drying process (Costech ECS 4010 analyser based on the Dumas method for the simultaneous determination of CHNS elements).

Statistical analysis

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

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

Results

Ecotoxicological bioassays

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

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

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

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

percentage starting from 100 μg/mL in *A. lixula* and from 1000 μg/mL in *P. lividus* (Table 2S). Otherwise, the pre-exposure of both gametes to TOCNF did not affect the sperm fertilizing ability and the egg fertilizing competence in both sea urchin species (Tables 2S).

Gamete quality assessment

Mitochondrial membrane potential (MMP)

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

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

Oxidative status

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

In *A. lixula*, the intracellular H_2O_2 levels significantly increased after sperm exposure to all the tested bPEI concentrations; whereas the O_2^- intracellular levels significantly increased only after sperm exposure to 10 μg/mL bPEI (Fig. 3; Table 3S). On the other hand, LPO was not significantly affected 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 ^{1}H-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

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

Up to now, the impact of CNF has been barely investigated and mostly in freshwater species with negligible effects (Harper et al., 2016; Ogonowski et al., 2018; Ong et al., 2017; Pengiran et al., 2022). CNF reduces growth, cell viability, and intracellular ATP levels as well as induces ROS generation in freshwater green microalgae at concentrations far higher than those predicted to reach the aquatic environment (1 μg/mL) (Pereira et al., 2014). Otherwise, CNF did not affect vitality, morphology and swimming behaviour in fish and crustaceans (Ogonowski et al., 2018; Pengiran et al., 2022). The only study so far on marine species revealed that neither oxidative stress nor biotransformation were affected in the digestive glands and gills of the marine mussel *M. galloprovincialis*, although a CNF uptake and disruption of gill functionality and immune cells by mechanical interaction was observed (Rusconi et al., 2024). Starting from these findings, the need for more in-depth investigations emerged to promote CNF eco-safe applications as in remediation. As far as our ecotoxicity results on single CNS components, TOCNF did not affect gamete quality and fertilization competence in sea urchins; however, it altered embryo development but only at concentrations (100 and 1000 μg/mL) much higher than those predicted to reach the natural environment (Stoudmann et al., 2019).

Nowadays, CA is classified as low acute toxicity based on the scarce ecotoxicity data available for marine organisms, even if the subacute toxic limit concentration is given as a wide range between 1 and 300 mg/L (Development, 2001). Herein, consistent EC_{50} values for CA, i.e., 107.2 μg/mL in *P. lividus* and 5.7 μg/mL in *A. lixula*, were measured. Additionally, CA did not impair the quality and fertilization competence of sea urchin female gametes; but, negatively affected the fertilizing ability of spermatozoa and embryo development. The earliest stages of sea urchin embryo development are fuelled by maternal RNAs and proteins deposited into the unfertilized egg and activated after fertilization. At the 64-cell stage embryo, the depletion of maternal mRNAs occurs and from this time

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

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

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

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

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

Conclusion

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

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

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566 **Fig. captions**

567 **Fig. 1.** Concentration-response curves for bPEI on embryotoxicity, spermiotoxicity and egg toxicity
568 tests with the sea urchin *P. lividus* and *A. lixula*. In the graphs, the dashed lines indicate the EC_{50}.
569 Values represented the mean of triplicate experiments with error bars indicating the standard error.

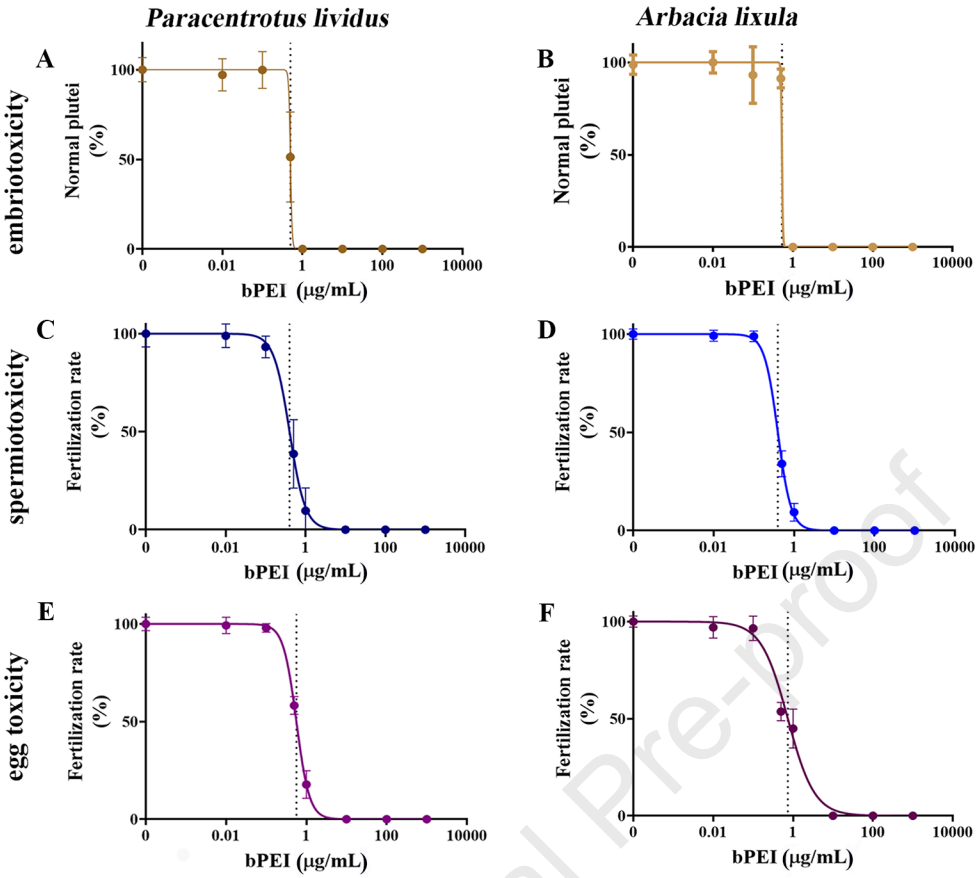
570

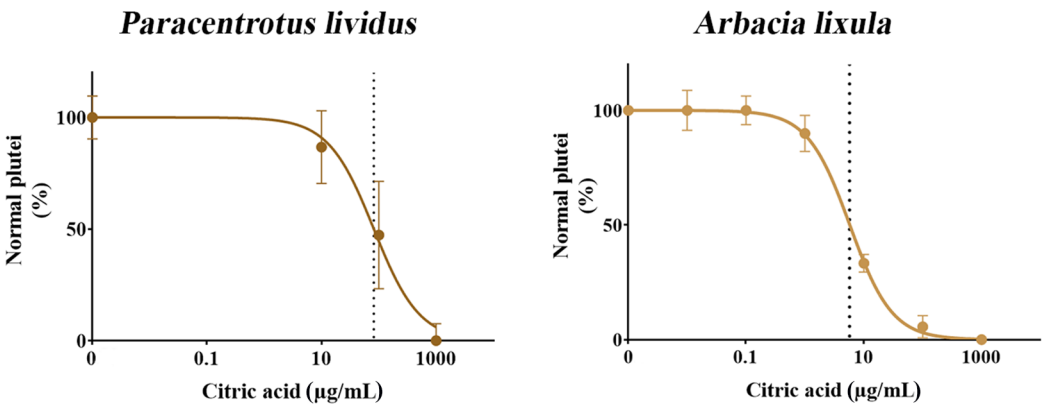
Fig. 2. Concentration-response curves for citric acid on embryotoxicity test with the sea urchin *P. lividus* and *A. lixula*. In the graphs, the EC_{50} is depicted by the dashed line. Values represented the mean of triplicate experiments with error bars indicating the standard error.

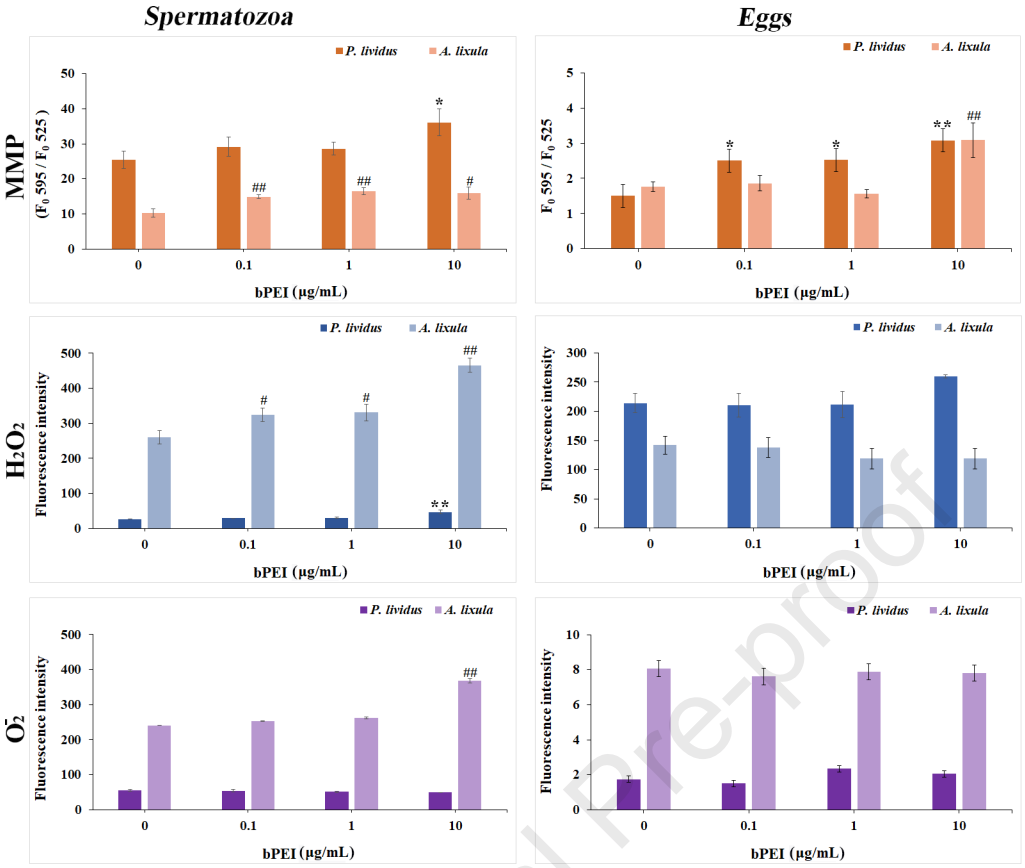
Fig. 3. Gamete quality assessment upon exposure to bPEI in *P. lividus* and *A. lixula.* Values (as the mean of triplicate experiments with error bars indicating standard error) of mitochondrial membrane potential (MMP) evaluated by JC-1; intracellular levels of hydrogen peroxide (H_2O_2) estimated with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA); intracellular content of superoxide anions (O_2^-) assessed with dihydroethidium (DHE). * or # indicate a significant difference ($p < 0.05$) from control gametes; ** or ## indicate a significance level lower than 0.01 ($p < 0.01$).

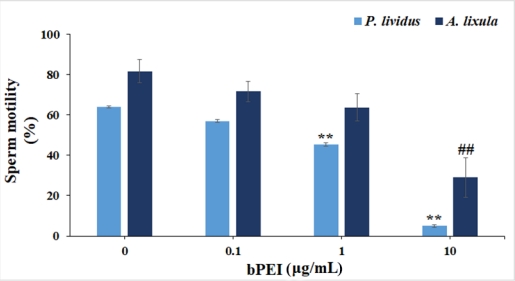
Fig. 4. Sperm motility assessment upon exposure to bPEI in *P. lividus* and *A. lixula.* Percentage of motile spermatozoa determined through visual estimation after 1 h exposure to different concentrations of bPEI. ** or ## indicate a significance level lower than 0.01 ($p < 0.01$).

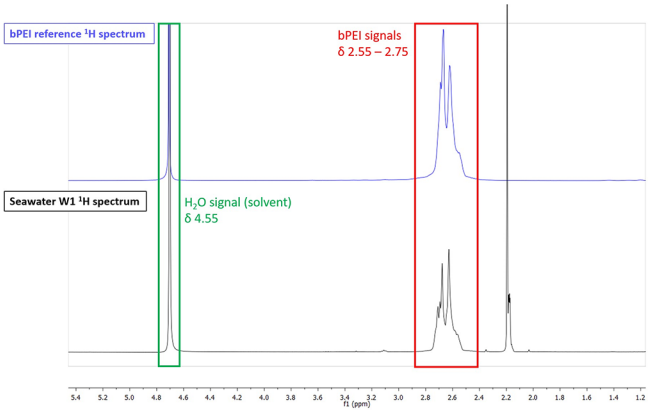
Fig. 5. Superimposition of **a**) ^{1}H NMR spectrum of reference bPEI and **b**) ^{1}H NMR spectrum of seawater sample after extraction process. It is possible to identify by comparison of spectrum (**b**) with reference spectrum (**a**) the characteristic signals of bPEI, confirming its presence in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











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: