Ecosafety Assessment Of Cellulose-based Nanosponges Engineered For Marine Environmental Remediation In Sea Urchin Reproduction

Thesis

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Ecosafety assessment of Cellulose-based Nanosponges engineered for marine environmental remediation in sea urchin reproduction

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Abstract

A potential solution to marine pollution is the promising strategy of nanoremediation. Cellulose-based Nanosponges (CNS) were developed as eco-friendly and sustainable engineered materials for marine environmental remediation. This research aimed to assess the suitability of standardised bioassays, i.e. the spermiotoxicity and embryotoxicity assays, with the sea urchin *Paracentrotus lividus* in evaluating the safety of CNS. These tests were developed to screen the conventional contaminant toxicity and, thereby, may not be appropriate to evaluate the toxicity of nanomaterials due to their unique properties. Moreover, the eligibility of the sea urchin *Arbacia lixula* as a possible suitable alternative species in ecotoxicity testing has been investigated. For this species, appropriate bioassays have been developed since, to date, standardised procedures are not available. The obtained ecotoxicity data indicate that the two sea urchin species have a similar CNS sensitivity supporting the use of *A. lixula* in the ecotoxicity tests and that standardised bioassays with *P. lividus* are valuable tools for assessing the environmental impact of engineered material. In the framework of the reproductive risk assessment process that is beginning to expand, with new tests and endpoints, the traditional approach, based only on fertility and viable offspring as estimated endpoints, a new bioassay, named ovotoxicity test, has been developed to evaluate the potential CNS effects also on fertilisation competence of female gamete as well as a gamete quality assessment, in which gamete quality parameters underlying fertilisation and developmental competence were assessed as endpoints to screen the CNS impact on gamete quality. Overall, the ecotoxicity data indicate that CNS can affect gamete quality, gamete fertilisation competence, and embryo development due to a release of chemical additives from the manufacturing process. Hence, in the framework of the eco-design approach, these data suggest a re-design of CNS to obtain a safer device.

CHAPTER 1

General introduction

1.1. Marine environmental remediation

Marine environments are continuously threatened by a wide range of contaminants that may derive from anthropogenic or natural sources and pose risks to marine environmental health. Indeed, marine pollution may cause the loss of biodiversity and an ecological imbalance restricting the function of the ecosystem (Küpper & Kamenos, 2018). Thereby, there is an urgent need to protect and restore marine ecosystems by cleaning up contaminants from the environment by applying high-efficiency, but also eco-friendly and sustainable, remediation technologies.

Marine environmental remediation deals with the removal of contaminants from marine polluted matrices using several technologies such as coagulation, precipitation, filtration, *in situ* burning of the oil spill, sediment-capping and mechanical removal. Among these traditional technologies, thermal treatment, dredging, and capping are commonly applied to clean up marine sediments from hydrocarbons and metal (Akcil et al., 2015). Dredging is the process of removing contaminated sediment from a water body transporting and depositing it in another location far away from the contaminated site (Jain & Singh, 2003). Dredging harms the ecosystem destroying the ecology of the excavated areas as well as of the zones where the sands have been dumped. Moreover, during the dredging process, contaminants accumulated in sediments can spread into seawater (Martins et al. 2012). The capping method consists of placing an underwater cover or a layer of clean material on contaminated sediments to isolate pollutants from the surrounding aquatic environment; this action, however, can alter the composition of the sea floor. Moreover, over a long time, the erosive forces (tides, waves, bioturbation) can reduce the integrity of the capping layer leading to the release of contaminants from sediment (Zhang et al, 2016). The *in situ* burning is a technique used to remove the oil spilled into seawater by applying a controlled burning, which can cause the emission of particulates and toxic gases affecting air quality (Mullin & Champ, 2003).

It is evident that these traditional remediation techniques may seriously alter marine wildlife biodiversity and ecosystem. Despite the most recent achievements, traditional techniques of the remediation industry still present several deficiencies in efficiency and in the environmental footprint. Particularly, they require high energy and management costs and produce a high quantity of wastes difficult to recycle because of their complex composition.

Finally, for the reasons mentioned above, traditional methods can be employed to clean up a limited number of contaminants.

1.1.1. Nanoremediation

To overcome the several issues related to traditional remediation technologies, such as the high cost, time, energy, the elevated amount of chemical agents, and the production of toxic by-products or non-recyclable final wastes, more promising remediation technologies, i.e. nanoremediation, have been developed (Marcon et al., 2021). The shift from remediation to the emerging strategy of nanoremediation is an ongoing process. Nanoremediation relies on the use of engineered nanomaterials or nanoparticles (ENMs or NPs), designed to clean up polluted media. Compared to most conventional remediation technologies, nanoremediation has the potential advantage to be less costly and more effective. The intrinsic characteristics of nanomaterials used in the remediation process, such as the higher sensitivity to detect and remove/degrade lower concentrations of pollutants than traditional methods, but also the shorter remediation time and the reduced operation costs improve the efficiency of the entire process (Bardos et al., 2018; Blaise et al., 2008). Indeed, nanotechnology encompasses a sophisticated ability to manipulate matter at the nanoscale, resulting in new materials characterised by unique properties, which make them more efficient to face pollution as well as seawater pollution. Among these properties, the small size confers to nanosized material new chemo-physical characteristics compared to raw material, like a higher reactivity resulting from the larger reactive surface area that makes the access and the interaction with the target contaminant easier. Additionally, the specific ENM can be designed by changing the functional groups able to link or adsorb different molecules such as cations, anions, or organic compounds, increasing the affinity and the selectivity of the remediation process (Iravani, 2021). Consequently, the use of ENMs in marine environmental remediation minimises the addition of chemicals in the clean-up process and potentially enhances the range of contaminants to be treated and the efficacy of the *in situ* remediation technologies (Bardos et al., 2011; O'Carroll et al., 2013). Furthermore, the velocity of contaminant degradation or stabilisation by NPs can be increased, reducing the time frame and even the costs of the remediation process (Usepa, 2008; Carmalin Sophia et al., 2016; Mueller & Nowack, 2010). In the perspective of greener remediation, which aims at developing green, sustainable, efficient, and low-cost solutions for environmental remediation, the higher selectivity of ENMs offers the advantage to reduce the environmental impact of the clean-up process. In fact, only the specific contaminant is removed, while the chemical constitution of the media is preserved. Furthermore, unlike the mixture of wastes produced through traditional remediation approaches, contaminants removed by applying nanoremediation

techniques represent a recycling source, limiting the depletion of natural deposits (Prado-Audelo et al., 2021; Marcon et al., 2021). Until now, the main application of recovered contaminants is represented by the electronic industry. Indeed, recovered metals can be reused to produce LED lamps or energy storage devices (Bhattacharya & Fishlock, 2021).

ENMs employed to remediate marine environments are heterogeneous in size, shape, and chemical composition. To date, a unique and shared classification for ENMs still does not exist; commonly, they are classified based on their main component. Metals and their oxides constitute the most abundant class of ENMs due to their fast kinetics and high adsorption ability, which confer them a good capability to remove contaminants from water and seawater or to transform inorganic (As, Cu, Cr, Zn) and organic pollutants (solvents, pesticides, dyes) into less toxic compounds (Santhosh et al., 2016). ENMs of this group have long been studied as promising nanoadsorbent materials for aqueous system treatments (Coston et al., 1995; Agrawal & Sahu, 2006). More precisely, their adsorbent ability relies on the high surface area to volume ratio that allows them to have a high number of reaction sites for target chemicals in a small volume, improving the adsorption kinetics (Wang et al., 2006). However, more recently, it has been reported that the adsorption ability of metal-based nanomaterials can be influenced by extrinsic parameters such as pH, temperature, the concentration of the nanomaterial, stirring speed, contact time, and chemical species to adsorb. For instance, at the optimal conditions of pH 8 and 20 °C, nFe_3O_4/fly ash composite is able to remove 98.40% of the pesticide triphenyltin chloride (TPT) from seawater (Fatoki et al., 2014). While, the presence of higher temperature and acid pH negatively affect the adsorption ability nFe_3O_4/fly ash composite. The manganese ferrite NPs has been demonstrated to achieve the optimal arsenic adsorption rate when a concentration of 0.4 g/L of NPs, a stirring speed of 250 rpm, and a pH of 2 or 8 are applied. Particularly, the application of acid and basic pH allows to obtain the maximum adsorption rate of arsenate (As (III)) and arsenite, respectively (As(V)), which are the two oxidation states of arsenic present in surface water (Martinez-Vargas et al., 2018). Metal-based and carbon-based ENMs are extensively employed to produce nanofiltration membranes specifically designed for water and wastewater treatments (Chen et al., 2018; Bandehali et al., 2020). Nanofiltration membranes present a scaffold of carbon atoms that can assume various three-dimensional structures including spheres, cylinders, and sheets generating, respectively, fullerene, carbon nanotubes, graphene, and graphite. Over the ability to remediate seawater from metals, crude oil, and radioactive isotopes, carbon-based membranes exhibit an antifouling activity, which increases membrane lifetime and minimises the energy consumption of the remediation process (Brady-Estévez et al., 2008; Shen et al., 2019; Jiang et al., 2016; Xu et al., 2018). A different class of ENMs employed for environmental

remediation is represented by the magnetic-core nanocomposites, which consists of a superparamagnetic core, made of iron, nickel, cobalt, or their oxides, surrounded by a shell of inorganic components. The multilayer structure of the beads ensures a double functionality: the core increases the adsorption ability, whereas the functionalization of the shell confers the beads the ability to specifically adsorb inorganic contaminants, such as metals and radioactive isotopes, or organic contaminants like polycyclic aromatic hydrocarbons (PAHs) and oil mixtures (Yang et al., 2018; Yang et al., 2014; Hong et al., 2020; Yi et al., 2014; Dong et al., 2019). Unlike other nanomaterials, magnetic metallic adsorbents have the unique feature to react with an external magnetic field. This feature makes them particularly attractive for the *in situ* clean-up treatments, because they can be easily recovered from contaminated matrices by applying an external magnetic field (Juang et al., 2018).

Among ENMs, a growing interest is focused on the polysaccharides-based nanostructured materials since they are mainly based on natural carbohydrates (starch and cellulose) notoriously safe for the environment, wildlife and humans. Indeed, both starch and cellulose can be easily obtained from plants and transformed into the corresponding nanosized material by chemical, physical or enzymatic processes (Simsek et al., 2012). Nanosized carbohydrates are water-soluble and can be polymerized by using different cross linkers to produce gel and sponges with high surface area and good chemical reactivity to adsorb metals from water (Voisin et al., 2017). This class of ENMs is particularly sustainable because they are biodegradable, renewable, inexpensive, and energy efficient (Corsi et al., 2020; Gao et al., 2014; Ibrahim et al., 2020; Krishnani & Ayyappan, 2006; Gallo et al., 2021; Riva et al., 2021; Guidi et al., 2020; Riva et al., 2020; Liberatori et al., 2020; Bartolozzi et al., 2020). To implement the remediation process and extend the range of removable contaminants, the different classes of ENMs can be combined with other materials giving rise to hybrid nanocomposites that can benefit from the characteristics of each different constituent. For instance, the addition of magnetic beads of Prussian Blue (PB) to the graphene oxide-iron oxide (GO-Fe_3O_4) enhances the nanocomposite efficiency to remove radioactive cesium (Cs) from water, while the external sheets of graphene oxide (GO) increase the adsorption surface area and minimize the aggregation rate (Yang et al., 2014). The resulting product is an eco-friendly and highly efficient nanomaterial for Cs-decontamination in the marine environment.

Overall, the nanosized materials offer several advantages over the native materials by improving remediation efficiency and promoting the nanoremediation application, which, on the other hand, is limited by the scarce knowledge on the environmental effects of ENMs.

Therefore, the assessment of the potential risks that ENMs may pose for marine organisms is a top priority for the progress of nanoremediation.

1.1.2. Environmental risks associated with Engineered Nanomaterials for water remediation

Literature is full of lab-scale studies and reviews depicting the characteristics and the potential application of ENMs for water remediation, but studies that switch from lab-scale to pilot tests and finally to large-scale trials are scarce (Baby et al., 2019; Esposito et al., 2021; Lu & Astruc, 2020; Yu et al., 2017). Moreover, the most recent US EPA report "Table of Selected Sites Using or Testing Nanoparticles for Remediation " lists the pilot or large-scale remediation treatments employing ENMs applied to clean up environmental matrices. From this list, it has emerged that, up to date, ENMs have been employed only for the remediation of soil and groundwater, but not for seawater. Nevertheless, the large-scale studies on the use of nanoscale zero-valent iron (nZVI) for the treatment of groundwater and wastewater are an exception. Since the first pilot scale study carried out at the beginning of the 20th century in the USA, several large-scale studies on the nZVI have been performed also in Europe (Elliott & Zhang, 2001; Mueller et al., 2012; Su et al., 2012; Li et al., 2014). Nowadays, reports on the real applications of nZVI for remediation of groundwater and wastewater contaminated with chlorinated compounds include also the toxicity assessment with aquatic organisms and the cost-effectiveness analysis (Stefaniuk et al., 2016). On the contrary, strategies for nanoremediation of seawater are still at the laboratory scale and the street to real-world application is still far away. Indeed, beyond the benefits in terms of contaminant removal or degradation efficiency, the use of ENMs for marine environmental remediation raises concern for their environmental effects and impact on marine wildlife (Garner & Keller, 2014). Hence, once released into the marine environment, ENMs may undergo rapid transformations due to their intrinsic (size, chemical composition, and density) and extrinsic (agglomeration, surface charge, hydrophobicity, and solubility) properties, which may determine their ecotoxicity on the marine biota (Ahlbom et al., 2009). Considering the intrinsic properties that modulate the toxicity of ENMs, their nano size can be a threat because it facilitates mobility and, therefore, the incorporation of ENMs in natural biogeochemical cycles, making their identification and isolation more difficult (Bardos et al., 2018). ENM solubility is another important issue that is influenced by the physicochemical characteristics of seawater and it is essential to predict their environmental effects (Jiang et al., 2016). Seawater is considered a complex environmental matrix characterised by high ionic strength, an alkaline pH, and the presence of a wide variety of ionic species, colloids, and natural organic matter (NOM) (Handy et al., 2008; Blasco et al.,

2015). ENMs can enter the marine environment and dissolve into an ionic form driven by particle chemistry or they can aggregate among themselves (homoaggregation) or form complexes with non-homologous materials (heteroaggregation) making larger particles (Praetorius et al., 2020). Because of the increased volume and density, the mobility of ENM agglomerates is reduced promoting their sedimentation, which may influence ENMs fate and dispersion and, in turn, their bioavailability and toxicity. Indeed, sedimentation can limit the interactions of ENMs with pelagic species, but increase the bioavailability and the exposure risk for benthic species (Klaine et al., 2008; Rana & Kalaichelvan, 2013). Aggregation size and rate depend on the composition and shape of ENMs but also on the ionic strength and NOM content of the aqueous matrix (Adeleye et al., 2019). In seawater, the presence of a high concentration of salts (i.e. high ionic strength) promotes the aggregation of ENMs (Ciacci et al., 2019; Sillanpää et al., 2011). Otherwise, the concentration and the characteristics of NOM present in seawater can differently influence ENM aggregation and, in turn, their bioavailability to marine organisms. Immediately upon the release of ENMs in the aquatic environment, NOM is adsorbed on their surface inducing aggregation and stabilisation or dissolution of ENMs. Thereby, the interaction of ENMs with NOM can lead to two opposite ecotoxic effects. On one hand, it can lead to a reduction in the ENMs toxicity linked to their reduced bioavailability as well as complexation of metal components, or it can increase ENM stability enhancing the bioavailability and the risk for marine organisms. Temperature is an important factor that can influence the ENMs behaviour in aquatic systems. As an example, by increasing water temperature, the aggregation rate of metal oxide NPs decreases (Zhou et al., 2012). The environmental behaviour of ENMs also affects their functionalization, i.e. the charges of the substituents bound on their surface. According to the widely accepted Derjaguin-Landau-Verwey-Overbeek theory on the behaviour of charged ENMs, the presence of negative charges on the ENM surface promotes the rapid formation of micrometric agglomerates upon suspension in seawater (Cai et al., 2018; Tang et al., 2017). Differently, the positive charges, such as cationic amino-modified groups, prevent agglomeration enhancing their bioavailability to organisms (Wu et al., 2013).

To move towards sustainable and green environmental remediation, it is necessary to deepen the knowledge of the interactions between ENMs and marine environmental matrices (eco-interactions) and to understand how these interactions may affect the ENM toxicity to marine organisms (ecotoxicity). To date, the ecotoxicity of ENMs employed in marine environmental remediation on marine wildlife has been poorly assessed resulting in contrasting data on the absence of toxicity to sublethal effects, based on the species and the trophic level. The impact of metal-based nanomaterials has been recently reviewed highlighting the multiplicity of the toxic effects and the risk for marine biota at different

trophic levels such as algae, bacteria, molluscs, worms, arthropods, echinoderms, and chordate (Vasyukova et al., 2021). The ecosafety of AgNPs has been tested both on marine (*Phaeodactylum tricornutum)* and freshwater (*Raphidocelis subcapitata*) microalgae resulting in the absence of effects on algal growth (Prosposito et al., 2019). On the contrary, nickel NPs and titanium dioxide NPs ($nTiO_2$) have been demonstrated to cause growth inhibition, cell membrane damage and the increase of ROS levels in *Chlorella vulgaris*, *Karenia brevis*; *Skeletonema costatum*, and, *Nitzschia closterium* (Gong et al., 2019; Li et al., 2015; Xia et al., 2015).

Together with algae, molluscs are the most studied organisms to assess the ecotoxicological impact of ENM/NPs applied for marine remediation. In particular, bivalve molluscs are the main model used to assess the toxicity of ENM/NPs (Esposito et al., 2021). As filter feeders and sessile organisms, bivalves easily uptake contaminants as well as ENMs through the gills, but the main accumulation site is represented by the digestive gland (Tedesco et al., 2010). Metal-based NPs have been also demonstrated to induce oxidative stress, which causes tissue alterations in the gill and digestive gland, such as epithelial cells irregular in shape and necrosis (Xia et al., 2017). Oxidative stress seems to be one of the main toxic mechanisms of NPs. Studies on the risk of metal-based NPs for marine crustaceans highlighted that oxidative stress can be associated with the growth of ROS production and antioxidant enzymatic activity (Wong et al., 2020; Lai et al., 2020; Djebbi et al., 2021). Similarly, in fish, the presence of NPs in the gut and gill mucus has been reported and correlated with an increase in ROS levels and, thereby, the induction of oxidative stress (Baker, 2014; Federici et al.2007). Metal-based NPs are of concern also for their impact on reproduction. It has been observed that the exposure of adults and the neonate crustaceans to metal-based NPs leads to a decrease in the reproduction rate (number of neonates produced) and in the growth rate (rate of increase in juvenile body length) respectively (Fabrega et al., 2012). Furthermore, metal-based NPs have been proved to affect fish embryo development inducing morphological and functional alterations such as oedema, vacuolar degeneration of the enterocytes and the hepatocytes, spinal deformities, as well as inhibition of the hatching inducing mortality of embryos and larvae (Li et al., 2018).

Concerning the other classes of ENMs and NPs, literature on their impact on marine organisms is very scarce. Some studies on carbon-based nanomaterials toxicity highlight how the impact of ENMs depends on the sensitivity and the ability of the species to react to ENMs, but also on the exposure time, the preparation methods and the concentration. Indeed, it has been demonstrated that carbon-based nanomaterials at the relevant environmental concentrations (ng/L) are not toxic for marine organisms; whereas toxic effects have been observed in short-term experiments at high concentrations in the order of mg/L (Freixa et

al., 2018). Moreover, carbon-based nanomaterials have been demonstrated to induce oxidative stress causing physical damage to cellular membranes in oysters (Khan, 2019).
Together these studies underline the need for heterogeneous tools for the ecotoxicological assessment of ENMs. Indeed, the sublethal endpoints such as organism's behaviour, functional parameters and gene expression are increasingly being used along with the classic endpoints as mortality, growth and reproduction (Canesi & Corsi, 2016).
Despite the efforts that have been made up to date to assess the toxic effects induced by NMs, more studies are needed to strengthen the ecotoxicity data in living organisms in order to develop guidelines for the environmental risk assessment of NMs.

1.1.3. Cellulose-based Nanomaterials: facing the challenge of green nanoremediation in the framework of the eco-design approach

ENMs can enter the marine environment through accidental release during the production phase and the remediation process as well as during their end life, raising concern about the potential environmental effects of NMs throughout their life cycle. To overcome this issue, the holistic approach named life cycle assessment (LCA) has been applied to NMs. LCA allows the assessment of the environmental impact of a product throughout its entire life cycle from the extraction of raw material through material processing, manufacture, distribution, use, repair and maintenance up to its disposal or recycling (Muralikrishna & Manickam, 2017). Nevertheless, to date, the LCA of NMs presents many regulatory gaps and deficiencies. Weaknesses are represented by the limited knowledge of the environmental fate and behaviour of NMs, the absence of a catalogue of NMs employed for remediation, and the lack of standardised procedure for the ecotoxicological assessment of new NMs designed for remediation purposes (Hischier & Walser, 2012). Additionally, the parameters and the procedures traditionally used for the LCA of materials do not allow an accurate estimation of the human and environmental risk of NMs (Miseljic & Olsen, 2014; Hischier, 2014). Otherwise, in recent years, the most accredited international environmental institutions are giving indications to promote guidelines for the LCA of NMs. For instance, the U.S. Environmental Protection Agency (USEPA) published several documents supporting the “Green and Sustainable Remediation” (GSR) (United States Environmental Protection Agency (Usepa, 2008; Usepa, 2010). The green remediation strategy aims to reduce the environmental footprint of site remediation with a double approach: 1. minimising the total energy and water consumption and its impact on water resources; 2. maximising the use of renewable energy and reducing, reusing, and recycling material and waste (Favara et al., 2019). At the same time, a directive from the European Commission encouraged “the

integration of the environmental aspects into product design with the aim of improving the environmental performance of the product throughout its whole life cycle" (Directive, E.C., 2009). This directive drove the development of an innovative step-by-step process, named the "eco-design" approach (Figure 1) aimed to produce NMs that are at the same time highly efficient in the remediation process, sustainable and safe for the environment.

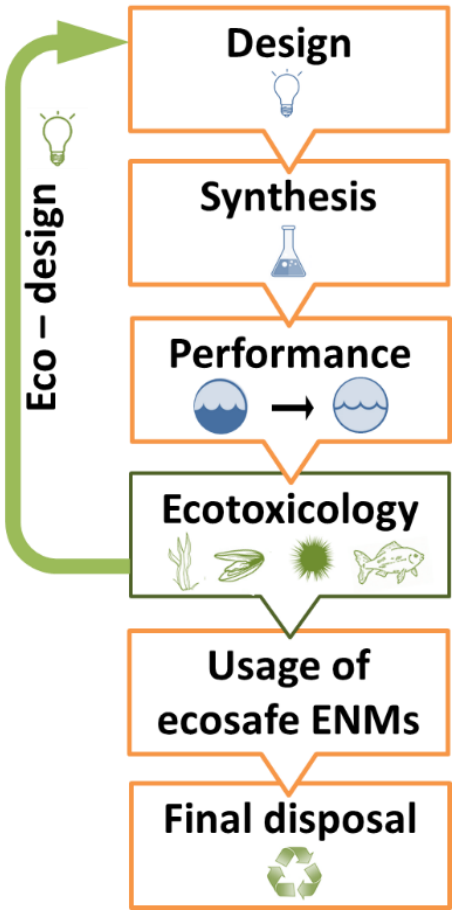


Figure 1. Schematic representation of the eco-design approach (modified by Esposito et al, 2021).

Following the "safety-by-design" strategy, each phase of the ENM developmental process, such as the initial design, the synthesis, and the performance, is carried out paying attention to reducing their costs in terms of time, money, and, particularly, energy and natural sources deployment but more important by limiting any risk for living beings (Corsi et al., 2018b). According to this approach, in the first step, a new NM is designed by trying to use a few components with the main component being natural, biodegradable, renewable, or deriving from recycled raw material. Furthermore, NM is synthesized limiting the use of chemicals in the production process and trying to use non-toxic reagents ("The Road to Green Nanotechnology," 2008). In the next step, the remediation efficacy of the new synthesized NM is carried out and is followed by the ecotoxicological assessment, which is the most stringent checkpoint of the entire process aimed at estimating the ecological risk of the new

NM before its application. If the ecotoxicological assessment highlights that the new synthesized NM can negatively affect marine biota, the NM returns to the starting phase to be re-designed modifying the chemical composition or the production protocol. After each modification, the ecotoxicological assessment is repeated until an eco-safe NM is developed. Therefore, ecotoxicology represents a key step of the eco-design approach for discrimination between toxic and safe NM before introduction into the market. By applying this strategy, also the last stage of the NM's life will have a low environmental impact because the NM can be recycled reducing the wastes generated by the final disposal.

As a successful example of eco-design strategy, cellulose-based nanosponges (CNS) represent the first designed ENM that followed the eco-design approach. Applying the safety-by-design strategy at the laboratory scale, CNS have been synthesised starting from the natural, biodegradable and renewable material, cellulose, using a simple, rapid, and low-cost protocol (Fiorati et al., 2020). Firstly, cellulose derived from recycled cotton was oxidized through a 2,2,6,6 tetramethylpiperidinyloxyl (TEMPO)-mediated oxidation to obtain nanofibers of cellulose (CNF), whose defibrillation was promoted by ultrasonication (Pierre et al., 2017; Riva et al 2021; Melone, et al., 2015a). Then, branched polyethyleneimine (bPEI) and citric acid (CA) were added to a 2% w/w water suspension of TEMPO-oxidized cellulose nanofibers (TOCNF), promoting the formation of the nanostructured network. Finally, thermal treatments were performed to increase the cross-linking density and promote the chemical and mechanical stability of the sponge-like nanoporous structure (Paladini et al., 2019). The resulting sponge-like material can be used as it is or can be ground to obtain a homogeneous powder (Figure 2).

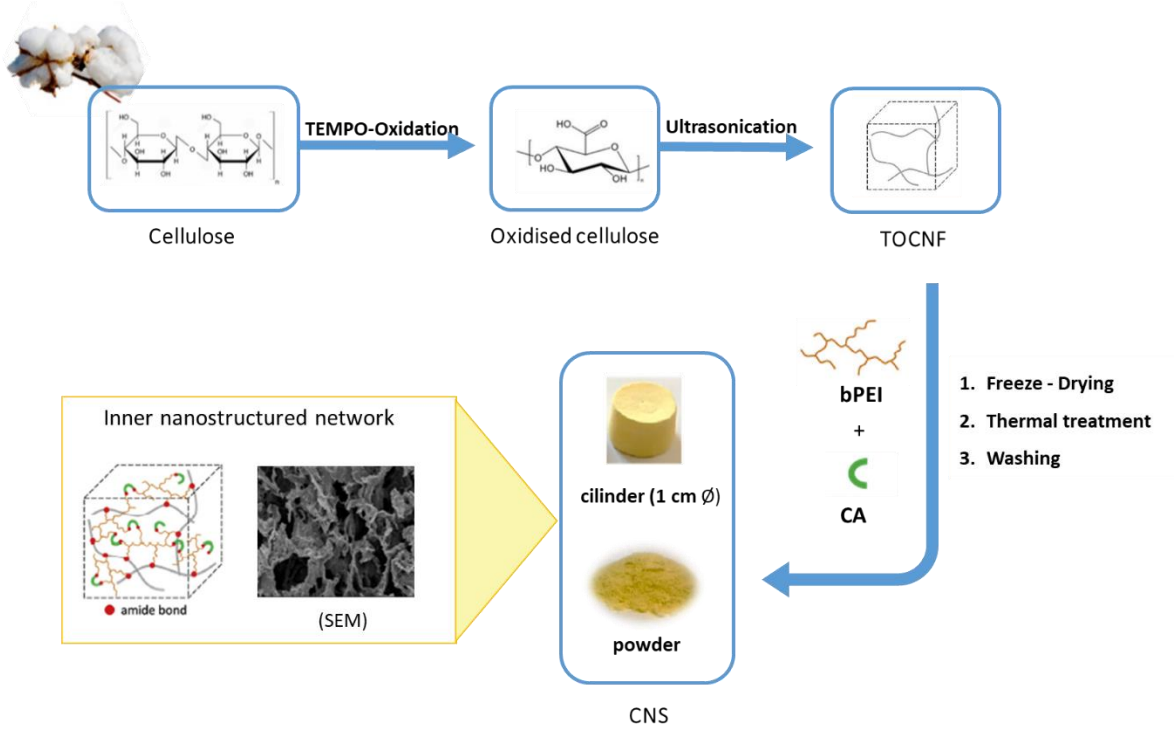


Figure 2. Main phases of the synthesis of Cellulose-based Nanosponges (CNS). The yellow box represents the inner structure and the micro-porosity of CNS analysed by Scanning Electron Microscopy (SEM). Abbreviations: TOCNF = TEMPO-oxidised cellulose nanofibers, bPEI = branched polyethyleneimine, CA = citric acid (Fiorati et al., 2017).

This formulation retains the remediation efficiency because the amino groups of bPEI and their chelating action towards metal ions are preserved. Moreover, since the nano-sized component (nanofibers of cellulose) is fixed into the reticular nanostructure of the sponge-like material, the potential risks related to the environmental behaviour of ENMs are limited (Corsi, et al., 2018b).

The CNS have been developed as an eco-friendly and sustainable material for marine environmental remediation, able to adsorb a wide range of metals (Cd^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, Cu^{2+}) and organic dyes (Corsi et al 2018a; Fiorati et al., 2020; Riva et al., 2020; Guidi et al., 2020; Guidi et al., 2021). However, despite their high remediation efficiency, the safety of CNS has been investigated only in two marine taxa as algae and mussels (Fiorati et al., 2020; Liberatori et al., 2020). Therefore, in order to support the application of the CNS in the marine environment and include more taxonomic levels as well as very sensitive life stages as embryos, sea urchin embryos are proposed as a new model for a more comprehensive ecosafety assessment.

1.2. Sea urchins in the ecological hazard assessment of marine pollutants

Sea urchins are a valid and versatile biological system employed in numerous research areas. Traditionally, they have been used as model organisms in developmental biology and, more recently, they acquired a relevant role in ecotoxicology due to their worldwide distribution, sensitivity to a wide range of contaminants, easy maintenance with low costs, production of a large number of gametes, rapid and synchrony embryo development and transparency of embryos (Chiarelli et al., 2019; Guillou & Michel, 1993; Quiniou et al., 1999; Kobayashi, 1971). To date, sea urchins are commonly used in biomonitoring programs for assessing the quality of coastal marine ecosystems and for predicting the biological effect of several contaminants at different levels of the organisation, from the single cell to tissues, to the entire organism and populations (Rhee et al., 2014; Savriama et al., 2015; Parra-Luna et al., 2020; Bošnjak et al., 2011; Privitera, et al., 2011a). Moreover, due to their ability to accumulate contaminants, like metals, the use of sea urchins in environmental studies allows to obtain information on contaminant bioavailability, bioaccumulation and biomagnification since they are prey for organisms at higher trophic levels (Martino et al., 2018; Ahn et al., 2009).

Ecotoxicity bioassays using sea urchins as test organisms have been developed worldwide and their reliability is well recognized. Sea urchins allow for studying a wide range of biological processes appropriate for the ecotoxicological assessment of marine contaminants (Morroni et al., 2018; Castellano et al., 2018). In particular, it is widely documented that the sea urchin's early developmental stages are more sensitive to contaminants than adults; therefore, gametes and embryos of sea urchins have been recognized as valuable tools in ecotoxicology (Bellas et al., 2022; Dinnel et al., 1989). Moreover, fertilisation and development are essential processes in fitness and survival; therefore, the modification of these processes may induce alterations up to the population and community levels (Savriama et al., 2015). To date, two standardised ecotoxicity tests with sea urchins, the sperm cell ecotoxicity test and the embryo-larval ecotoxicity test, are available (Usepa, 2002a; ASTM, 1995; ASTM, 2004). The species employed in these toxicity tests are selected based on the habitat of the study. *Paracentrotus lividus* is the ecological relevant species used in the multiple stressors studies in the Mediterranean Sea (Gambardella et al., 2021). For the Atlantic species, *Arbacia punctulata* is proposed in the standardised protocol to assess both the chronic and acute toxicity tests in salt water (Usepa 2002a), while, the guidelines for the acute toxicity test indicate *Strongylocentrotus droebachiensis* and *Strongylocentrotus purpuratus* as alternative species in cold water (Usepa, 2002b). The two standardised tests are used worldwide as a reliable, sensitive and inexpensive tool to assess the toxicity of

several marine pollutants and natural matrices. In the present thesis, the suitability of the spermiotoxicity and embryotoxicity tests for the ecosafety assessment of nanomaterials developed for marine environmental remediation has been evaluated using two different sea urchin species that coexist in the sublittoral zone of the Mediterranean coasts the *P. lividus* and *A. lixula*.

1.2.1. Anatomy and ecology of the sea urchin *Paracentrotus lividus* and *Arbacia lixula*

Sea urchins are intertidal and shallow subtidal invertebrates present on the east coast of the Atlantic and dominant in the Mediterranean Sea. Adult sea urchins present a round endoskeleton (test) containing the gut tube (digestive system), the pentaradial water vascular system, a primitive nervous system constituted by a network of nerves without any central brain, and five gonads (Figure 3). At the centre of the top side of the body is located the anus surrounded by five genital pores, while the mouth is at the opposite bottom side. The external surface of the globular body shows three innervate, sensitive and moveable structures. The hard spines, which cover the entire test, are used for defence but also for locomotion. The pedicellariae are claw-shaped appendages used to scare predators and, in some species, can emit venom. The third structure is represented by five double rows of tube feet, which are flexible and extensive structures with a suction cup-shape involved in locomotion, grabbing food, respiration, and allowing gas exchange. Furthermore, tube feet are considered the main sensory organ in sea urchins; indeed, they contain sensory receptors, which allow them to perceive variations in chemicals, touch and light intensity (Ullrich-Lüter et al., 2011).

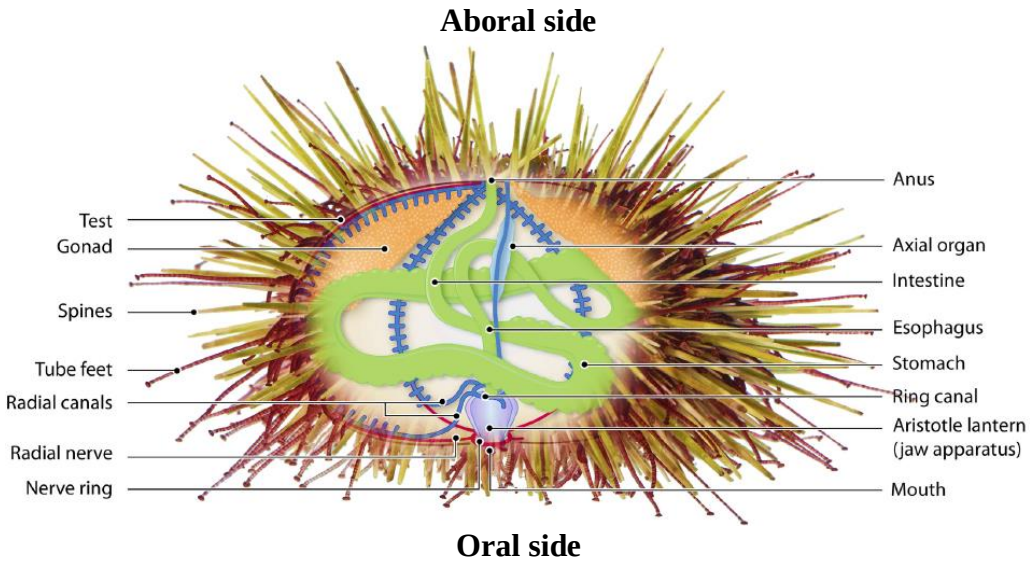


Figure 3. Anatomy of an adult sea urchin (modified from James., Siikavuopio., & Johansson, 2018).

The two sea urchin species employed in the present Doctorate projects split from a common ancestor and share the same evolutionary history until the superorder Echinacea that fork into six orders. Among these, from the order of Camarodonta and Arbacioida originate the species of *P. lividus* (Lamark, 1986), commonly called "purple sea urchin" and *A. lixula* (Linnaeus, 1758) the "black sea urchin", respectively (Table 1).

Table 1. Phylogenetic classification of *Paracentrotus lividus* and *Arbacia lixula.*

Phylum	Echinodermata	Echinodermata
Class	Echinoidea	Echinoidea
Subclass	Euechinoidea	Euechinoidea
Infraclass	Carinacea	Carinacea
Superorder	Echinacea	Echinacea
Order	Camarodonta	Arbacioida
Family	Parechinidae	Arbaciidae
Genus	Paracentrotus	Arbacia
Species	*Paracentrotus lividus (Lamark, 1986)*	*Arbacia lixula (Linnaeus, 1858)*

The sea urchins *A. lixula* and *P. lividus* (Figure 4) coexist and interact on the rocky shore of the Mediterranean Sea and on the northeast Atlantic coasts (Privitera et al., 2008). They are key species for coastal rocky reef ecosystem functions since their body covered by spines creates a safe microhabitat for both the juvenile urchins and small fishes, crustacean and invertebrates avoiding predation and protecting them from violent waves (Giglio et al., 2018; Stebbins, 1988).

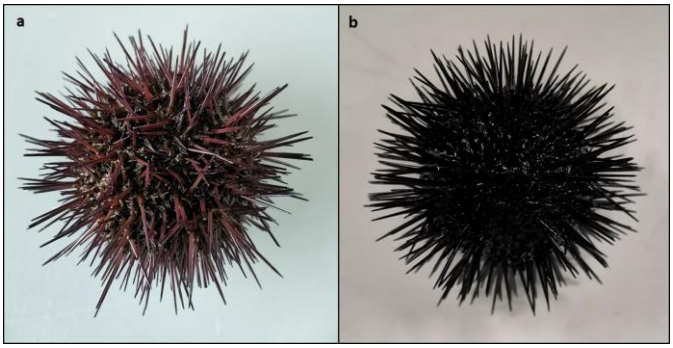


Figure 4. The two species of sea urchins investigated in this study view from the aboral side. *Paracentrotus lividus* (a) and *Arbacia lixula* (b).

As primary consumers, sea urchins mainly graze on macroalgae, even if some differences have been observed in the nutrition pattern of these species. *P. lividus* prefers the erect macroalgae, while *A. lixula* grazes encrusting coralline algae; however, in case of food scarcity, they also ingest sponges, mussels, barnacles and dead fish (Privitera et al., 2008). The grazing activity occurs through the Aristotle's Lantern, the particular mouth structure with five sharp teeth, which allows them to limit algal biomass modifying the seabed into desert-like barrens and consequently reducing the biodiversity of benthic communities in shallow marine ecosystems (Sala et al., 1998; Lawrence, 1975; Lawrence & Sammarco, 1982). Moreover, using Aristotle's Lantern, sea urchins are also able to shape the rocky reef by scraping and drilling holes in the rock to refuge from predators including humans. Differently from *A. lixula*, *P. lividus* is highly impacted by both fish and anthropogenic predation. In fact, fishes prefer to prey on *P. lividus* than *A. lixula* because of the length and/or robustness and disposition of spines and of the lower strength in the ability to remain attached to the substrate (Guidetti, 2004). Additionally, *P. lividus* is an edible species intensely harvested by humans since its gonads (roe) are the main appreciated echinoid delicacy consumed in Mediterranean and Atlantic European countries (Bertocci et al., 2018). Consequently, in many coastal habitats, overfishing has led to the total disappearance of this species. Trying to limit this phenomenon, over the last two decades, the interest in echinoculture has increased, but the cultivation of *P. Lividus* is not sufficient to cover the commercial supply because it is not economically sustainable (Ciriminna et al., 2020). Hence, depletion of natural populations persists (Sartori & Gaion, 2016). The combined action of natural predation, anthropogenic pressure and climate change are driving the reduction of the density of *P. lividus* population with respect to *A. lixula*, whose populations are growing up in some habitats to replace the *P. lividus* (Privitera, et al., 2011b). Compared to *P. lividus*, studies on the behaviour and biology of *A. lixula* are scarce. However, following the latest expansion of *A. lixula*, the scientific interest in the ecology of this species has increased. Recent studies attributed to *A. lixula* the same importance as *P. lividus*, as keystone species of the benthic coastal ecosystem, changing the traditional idea of *A. lixula* as a secondary species (Pérez-Portela et al., 2016). Nevertheless, more efforts are needed to reveal the interaction of these two species, to prevent their disappearance.

1.2.2. Sea urchin reproductive cycle

Sea urchins are dioecious and monomorphic organisms that reproduce sexually. This reproductive strategy ensures the survival of the species, enjoying the benefits of genetic

recombination such as rapid adaptability to novel ecological conditions and the transfer of the evolutionary changes through lineage.

Both male and female sea urchin gonads are composed of hundreds of acini, containing two types of cells: the nutritive phagocytes and the germinal cells (GCs). The former accumulate proteins, lipids and carbohydrates to supply energy for the development of the GCs. The latter derive from the embryonic precursors of the gametes, the primordial germ cells (PGCs) and undergo cell division and differentiation to form mature haploid gametes, through the biological process called gametogenesis. The nutrients stored in the nutritive phagocytes trigger gonad maturation from the recovery stage, through the growing stage, premature stage, and mature stage, until the spent stage which is characterised by empty gonads with thin acini lacking both gametes and phagocytes (Byrne, 1990). During gonad maturation, GCs size progressively increases up to the mature stage when they are predominant with respect to nutritive phagocytes. At this stage, functional and mature gametes, ready to be spawned, occupy the whole lumen of the acini.

In female sea urchins, oogenesis produces fully mature but quiescent eggs, where meiosis is completed and they are blocked at the G1-phase of the first mitotic division cycle (Figures 5a and 6a). Sea urchin eggs are spherical cells of 70-220 μm surrounded by a network of fibres forming the vitelline layer and an external colourless jelly layer of glycoproteins, which mediates the specie-specific interaction with spermatozoa (Deaker et al., 2019).

In male sea urchins, spermatogenesis originates mature spermatozoa, which are characterised by a cone-shaped head containing the nucleus and the acrosomal vesicle, a midpiece containing a ring of mitochondria, which supplies the energy for the flagellum movement, and a long flagellum. Spermatozoa are compact cells whose fundamental roles are to preserve and transport the genome to the egg for restoring the diploid genome, to biochemically activate the quiescent egg, and to allow the centrosomes to form the poles of the mitotic spindle that starts the segmentation of the zygote (Epel, 1990).

Sea urchins are broadcast spawners that release mature gametes into seawater, where fertilisation and embryo development occurs. Their breeding season can be characterised by one or more spawning events per year (Lozano et al., 1995). Generally, Mediterranean populations of *P. lividus* have a single spawning period, which occurs from October to June (Novelli et al., 2002); whereas, it has been long claimed that *A. lixula* is able to reproduce all the year (Lo Bianco, 1909; Tavares et al., 2004). However, more recently, it has been reported that the spawning of *A. lixula* occurs only between spring and summer (Elakkermi et al., 2021). Upon spawning, a large number of mature eggs and spermatozoa are released into seawater to ensure reproductive success. Before fertilisation, a precise sequence of events occurs. Firstly, eggs release species-specific chemoattractant peptides, which guide

the spermatozoa toward them (Hussain et al., 2017). Subsequently, the interaction between spermatozoa and the egg coat occurs and induces the sperm acrosome reaction, i.e. the exocytosis of the acrosomal vesicle that releases a lytic agent. These events enable spermatozoa to cross the extracellular matrix and reach the oocyte plasma membrane where the binding between the two cells occurs (Gallo & Costantini, 2012). This last event triggers the exocytosis of cortical granules, which in turn induces the formation of the fertilisation envelope, a hard barrier that protects the early embryo from chemical and mechanical injury (Figures 5b and 6b). At this phase, the structural block to polyspermy has been accomplished and it is followed by the fusion of the male and female nucleus leading to the activation of egg metabolism, mitosis and the beginning of the development (Vacquier, 2011). Sea urchin embryos exhibit a radial, holoblastic cleavage that culminates in the formation of a round ciliated blastula (Figures 5e and 6e), which rotates within the fertilisation envelope until hatching enzymes are secreted by the cells of the animal pole and digest the egg envelope (Lepage et al., 1992). Subsequently, the archenteron is formed by the cell invagination of the vegetative pole and the free-swimming hatched blastula moves to the gastrula stage (Figures 5f and 6f). During the last stages of gastrulation and coelom development, the embryo acquires a bilateral symmetry and afterwards becomes a planktotrophic larva. The feeding larvae need to accumulate nutrients to form the adult rudiment, containing some adult structures. Hence, it begins to feed immediately upon the development of the digestive system, which consists of the mouth, the tripartite gut (oesophagus, stomach, and intestine) and the anus. The feeding larva will be passively transported by the current and during this time it will develop through several stages from prism to *pluteus* (Figures 5g and 6g). The latter is characterised by arms, which help to canalise the food in the mouth and to glide through the water. All sea urchin species share the embryo development stages described above, but some characteristics, such as the colour of the egg/embryo, the presence of pigment cells (Figure 5h) or the skeletal “crown” (Figure 6h), are peculiar to each species.

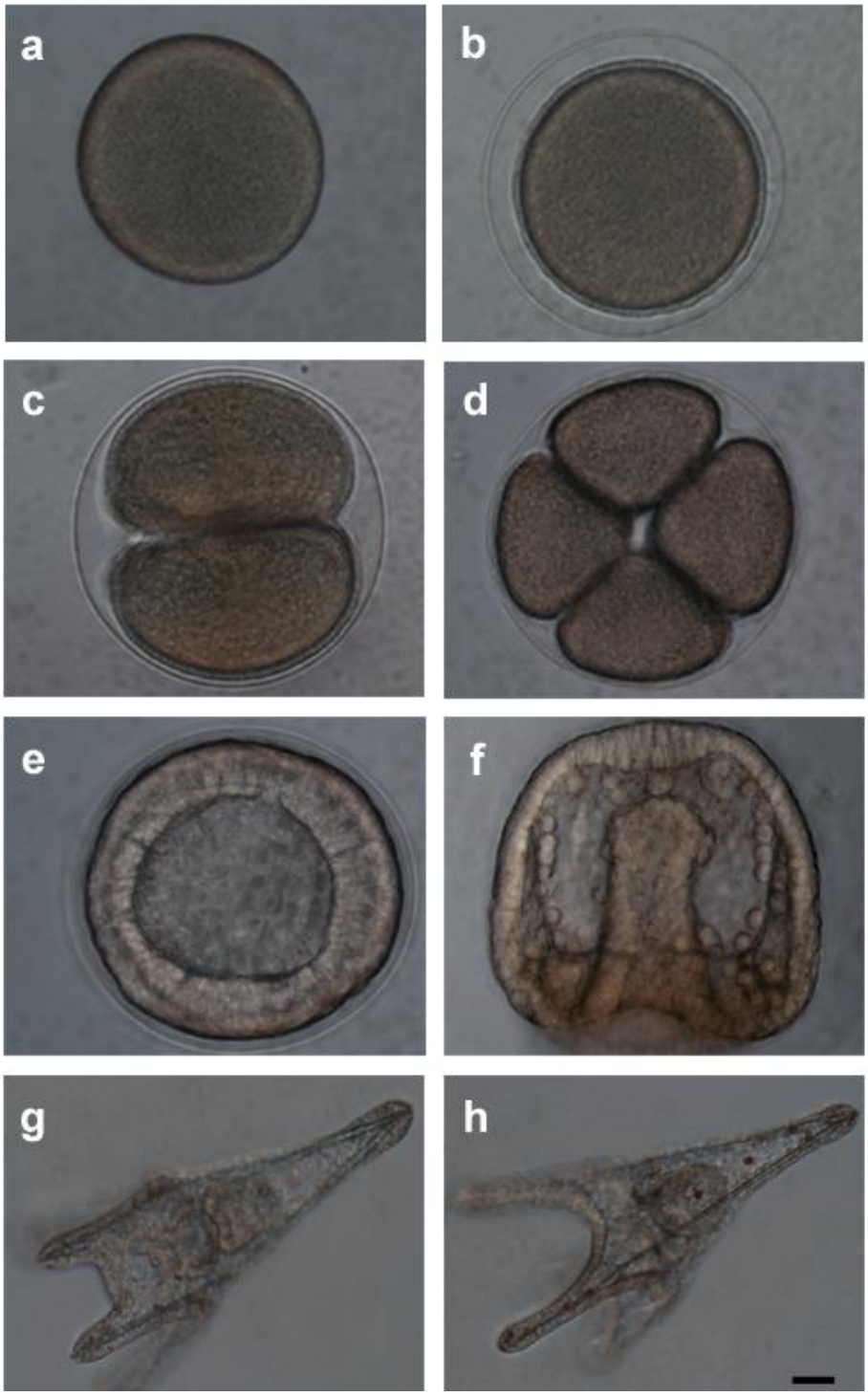


Figure 5. Embryo-larval development stages of *Paracentrotus lividus:* (a) unfertilised egg (Bar = 17 µm); (b) fertilised egg surrounded by the fertilization envelope (Bar = 17 µm); (c) two cell stage embryo (Bar = 17 µm); (d) four-cell stage embryo (Bar = 17 µm); (e) swimming blastula with the inner cavity (blastocoele) and the external monolayer of cells (trophoblast) (Bar = 17 µm); (f) late gastrula, lateral view; the round cells inside the embryo are the mesenchyme cells (Bar = 17 µm); (g) 4-arm pluteus with the pre-oral arms well visible (Bar = 45 µm); (h) 4-arm pluteus with the post-oral arms well visible, the dark spot are the pigment cells (Bar = 45 µm).

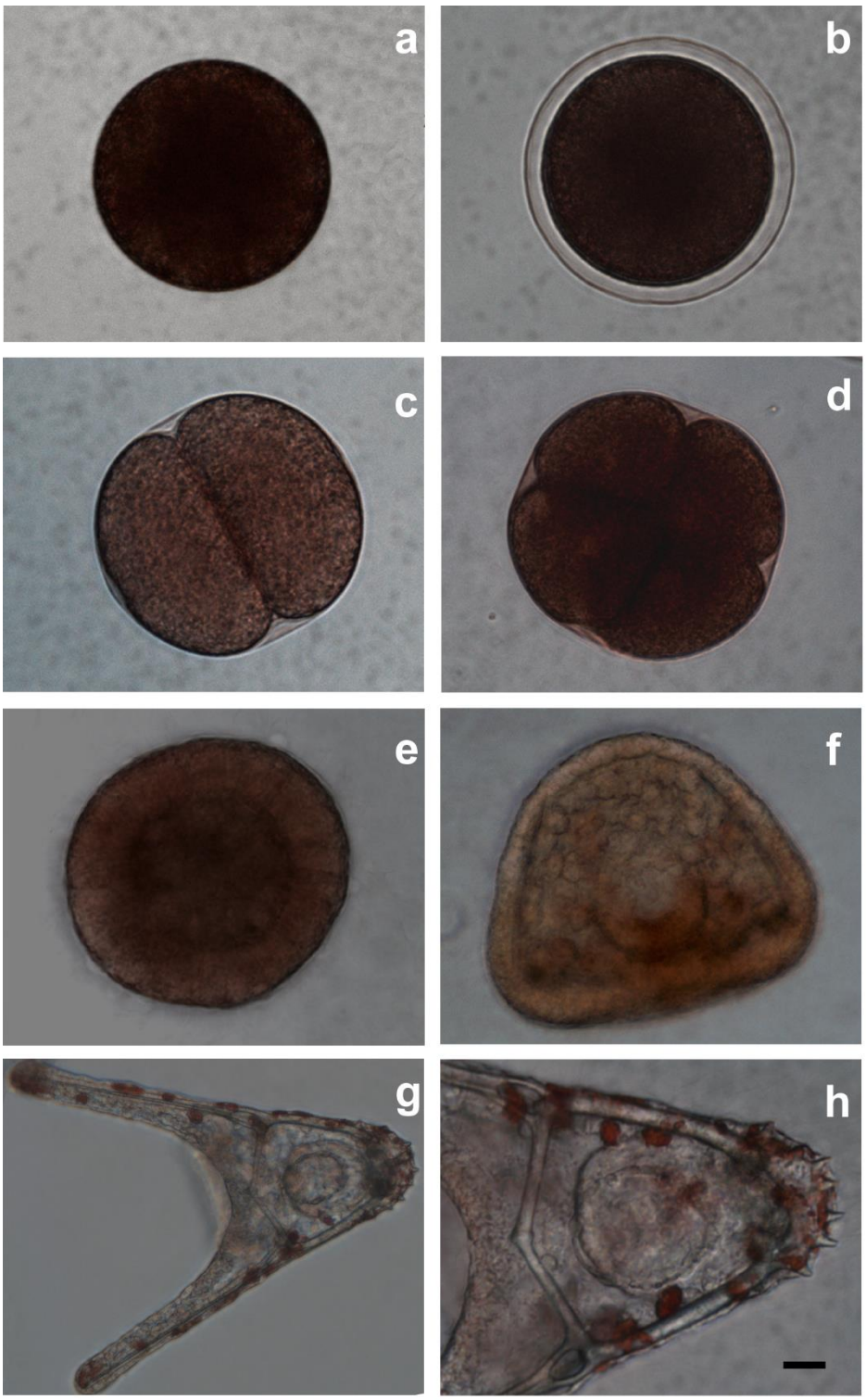


Figure 6. Embryo-larval development stages of *Arbacia lixula*: (a) unfertilised egg, the pigment granules confer the dark colour to the egg (Bar = 15 µm); (b) fertilised egg surrounded by the fertilization envelope (Bar = 15 µm); (c) two-cell stage embryo (Bar = 15 µm); (d) four-cell stage embryo (Bar = 15 µm); (e) swimming blastula (Bar = 15 µm); (f) late gastrula, ventral view (Bar = 15 µm); (g) 4-arm pluteus with the post-oral arms well visible (Bar = 30 µm); (h) magnification of the skeletal "crown" at the apical end, the pigment cells appear as red circles (Bar = 20 µm).

After approximately 30 days, when the adult rudiment on the left side of the larva is completely developed, all the pluteus structures, including the calcareous skeletal spicules, are lost. The echinopluteus rapidly metamorphoses in a penta-radial juvenile sea urchin no more than 1 mm in diameter (McClay, 2011). Hence, like most marine invertebrates, sea urchins exhibit an indirect development, i.e. the planktonic larvae with a bilateral symmetry undergo metamorphosis to generate a benthic adult with a distinct body plan (Peterson et al., 1997; Davidson & Erwin, 2006). Once the benthic juvenile sea urchin will reach sexual maturity, the spawning event will release the gametes into seawater, causing the shift from the benthic to the pelagic phase of the sea urchin life cycle. The sea urchin reproductive processes are influenced by different abiotic factors (Byrne, 1990; Gianguzza et al., 2013; Lozano et al., 1995). Temperature, pH, salinity, food availability and light have been demonstrated to affect gametogenesis, gonad growth and spawning. In particular, photoperiod and water temperature are the two main factors, which regulate gametogenesis and induce the spawning event in sea urchins (Siliani et al., 2016). Instead, the types of foods and their availability influence the gonad size and quality during gametogenesis (George et al., 2001).

1.3. Aim of the thesis

This PhD project aimed to assess the suitability of the sea urchin early life stages as *in vivo* model system for the ecotoxicological assessment of the cellulose-based nanosponges (CNS), developed as eco-friendly and sustainable engineered material for marine environmental remediation, employing two different species, *P. lividus* and *A. lixula*.
In particular, this project aimed to:

- investigate the suitability of standardised bioassays, i.e. the sperm cell toxicity and embryotoxicity assays, with the sea urchin *P. lividus* for the ecosafety assessment of CNS;
- assess the effects of CNS leachate and CNS components on the embryo development of sea urchins by carrying out the embryotoxicity assay;
- examine the impact of CNS leachate and CNS components on the sperm fertilising ability by performing the sperm cell toxicity test;
- develop a new bioassay, named ovotoxicity test, to evaluate the potential effects of CNS leachate and CNS components on the fertilisation competence of female gamete;
- develop a new approach to examine the impact of CNS leachate and CNS components on sperm and egg quality;
- evaluate the eligibility of the sea urchin *A. lixula* as a possible suitable alternative species for the environmental risk assessment of ENMs by comparing the sensitivity of the different early life stages;

In the contest of the eco-design approach, the obtained ecotoxicity data of CNS on sea urchin reproduction will contribute to the development of a safer device to employ in seawater remediation.
The sea urchin species employed in this project are not endangered or protected; furthermore, this project has been conducted according to the guidelines of the Declaration of Helsinki amended by the European Directive 2010/63 on the protection of animals used for scientific purposes, transposed into the Italian law by Legislative Decree 2014/26. Moreover, adult animals have been collected from a marine site in the Gulf of Naples, which is not privately owned or protected in any way, according to Italian legislation (DPR 1639/68, 19 September 1980, confirmed on 10 January 2000), by the personnel of the Material Collection and Diving service of the Stazione Zoologica Anton Dohrn. After collection, sea urchins were transported to the Marine Biological Resources service, where

animals were maintained in tanks (1 animal/ 5L) with running natural seawater at the following conditions: temperature of 18 ± 2°C, pH 8.1 ± 0.1, salinity 38 ± 0.5 ppm and a photoperiod of 10 h L: 14 h D. The animals were acclimated at least for 7 days before use and fed with fresh green algae *Ulva sp*.

CHAPTER 2

Ecosafety assessment of Cellulose-based Nanosponges and their components on sea urchin embryo development

2.1. Introduction

Sea urchin toxicity bioassays using embryos are internationally recognized as a rapid, sensitive, and cost-effective biological tool for biomonitoring marine environment and for estimating the toxicity of several contaminants (Chiarelli et al., 2019). The embryotoxicity test has been developed referring to standard procedures for different sea urchin species. It is considered a short-term chronic test and has been proved to be an effective, predictive and protective biological tool. The current standard procedure is based on the assessment as an endpoint of a qualitative response, the morphological normality of the larvae, which requires detailed microscopic observation of each individual and expertise in echinoderm embryology (ASTM, 1995). In Europe, the sea urchin *P. lividus* is recommended as test species due to its abundance and broad geographical distribution. Alternatively, other sea urchin species, which are locally or regionally abundant, can be used. To date, a standard method for embryotoxicity testing with regulatory applications is available for *P. lividus* (Sartori et al., 2017; Novelli et al., 2002), but not for *A. lixula*. Nevertheless, the new materials, as ENMs for marine remediation purposes, challenge the adequateness and fit-for-purpose of these standardised tests, being developed to assess hazards of chemical compounds. To date, the safety assessment of ENMs lacks regulatory standards, appropriate methods for monitoring their effectiveness, and protocols for the evaluation of potential environmental risks. Hence, there is a need to verify if the *P. lividus* embryotoxicity test can be successfully used to assess exposure and hazard of ENMs. It will also be of great support to develop an embryotoxicity assay based on *A. lixula* considering that this species is progressively replacing *P. lividus* (Carreras et al., 2021).

Accordingly, in this chapter, the *P. lividus* embryotoxicity test has been applied as well as the embryotoxicity assay with *A. lixula* has been set up to verify their suitability as *in vitro* tools to screen the CNS safety.

2.2. Materials and methods

2.2.1. Synthesis of Cellulose-based Nanosponges

Cellulose-based Nanosponges (CNS) have been supplied by Politecnico di Milano and synthesized following the protocol described in Fiorati et al (Fiorati et al., 2020). Briefly, cotton linter cellulose was oxidised via 2,2,6,6 tetramethylpiperidinyloxyl (TEMPO)-

mediated system and ultra-sonicated to promote the defibrillation and obtain a homogeneous solution of nanocellulose fibres (Melone, et al., 2015b). In the second phase, the cross-linking between cellulose nanofibers was promoted by adding 25 KDa branched polyethyleneimine (bPEI) and citric acid (CA) to a 2% w/w water suspension of TEMPO-oxidized cellulose nanofibers (TOCNF) according to the following ratio: 1g of bPEI, 1 g of TOUS-CNF (TEMPO-Oxidised and Ultra-Sonicated CNF) and 18 mol% of CA relative to primary amino groups in 25 kDa bPEI (Fiorati et al., 2020). The resulting xerogel was frozen (-80°C) and freeze-dried for 48h, in a 24-well plate. As a final step, CNS with nanoporous structure underwent thermal treatment in the oven (102°C for 16 h). The obtained sponge-like material with 1 cm diameter was ground to obtain the powder of CNS used in the next experiments.

2.2.2. Test solutions

All test solutions, schematically summarised in Table 2**,** were prepared fresh daily using natural seawater filtered through a 0.22 μm nitrocellulose filter (FNSW). The pH and salinity of all test solutions were checked with a benchtop pH meter (Mettler Toledo) and a manual refractometer, respectively, and adjusted, when necessary, taking as reference the values of FNSW (negative control).

Table 2. Experimental solutions and corresponding abbreviations were used in both sea urchin species.

NAME	**EXPERIMENTAL SOLUTIONS**
Negative control	FNSW
Positive control	Reference toxicants: $Cu(NO_3)_2$ *$3H_2O$ for *P. lividus* (20 - 150 μg/mL; 80 - 600 μM) $CuSO_4$ *$5H_2O$ for *A. lixula* (50 - 200 μg/mL; 200 - 800 μM)
W solution	Seawater exposed to CNS
T solution	Seawater exposed to TOCNF
CA solution	Citric acid solution (0.01 - 1000 μg/mL; 0.05 μM - 5 mM)
bPEI solution	bPEI solution (0.01 - 1000 μg/mL; 1 nM - 100 μM)

Reference toxicants (positive control) were used to validate the method. Indeed, they allow the estimation of the precision, accuracy, the intra- and inter-laboratory reproducibility of the assay and provide information on the sensitivity of the species used (Novelli et al., 2002). $Cu(NO_3)_2*3H_2O$ (Merck Life Science, Milan, Italy) was employed as a positive control in *P. lividus* and was prepared as a stock solution of l000 µg/L by dissolving copper in double distilled water. The copper stock solution was, then, diluted in FNSW to obtain the following test solutions: 20, 50, 70, 100, 150 µg/mL. Cu SO_4*5H_2O was used as a positive control in the embryotoxicity test with *A. lixula* (Giannetto et al., 2018). A stock solution of l000 µg/L Cu SO_4*5H_2O (Merck Life Science) was prepared and then diluted to 50, 80, 100, 150, 200 µg/mL in double distilled water.

The W solution has been prepared according to the protocol reported in figure 7a, by adding the CNS powder to FNSW to obtain the final concentration of 1.25 g/L, which corresponds to the efficient concentration to remove heavy metals from contaminated seawaters (Fiorati et al., 2020). After 2 h of magnetic stirring at room temperature, the W solution was filtered through a 0.45 µm filter. The recovered filtered solution (wash 1 solution, W1 solution) was tested in the bioassays undiluted (dilution factor 1, DF 1) and diluted by applying the following dilution factors: 2, 5, 10, 20, 40, 80, 160 and 320 in *P. lividus*, and 2, 5, 10, 20, 40, 60, 80, 120 in *A. lixula.* Then, the recovered CNS powder was added to freshly FNSW, in the same volume of FNSW used to prepare the W1 solution, stirred for 2 h and filtered again through a 0.45 µm filter. The recovered filtered solution (wash 2 solution, W2 solution) was tested in the bioassays undiluted (DF 1) and diluted with the following dilution factors: 2, 5, 10, 20, 40, and 80 in *P. lividus;* and 2, 5, 10 and 20 in *A. lixula.* The procedure was repeated for the third time and the W3 solution was tested in the bioassays undiluted (DF 1) and diluted with the following dilution factors: 2, 5, 10, 20, 40, and 80 in *P. lividus*, and 2, 5, 10 and 20f in *A. lixula*.

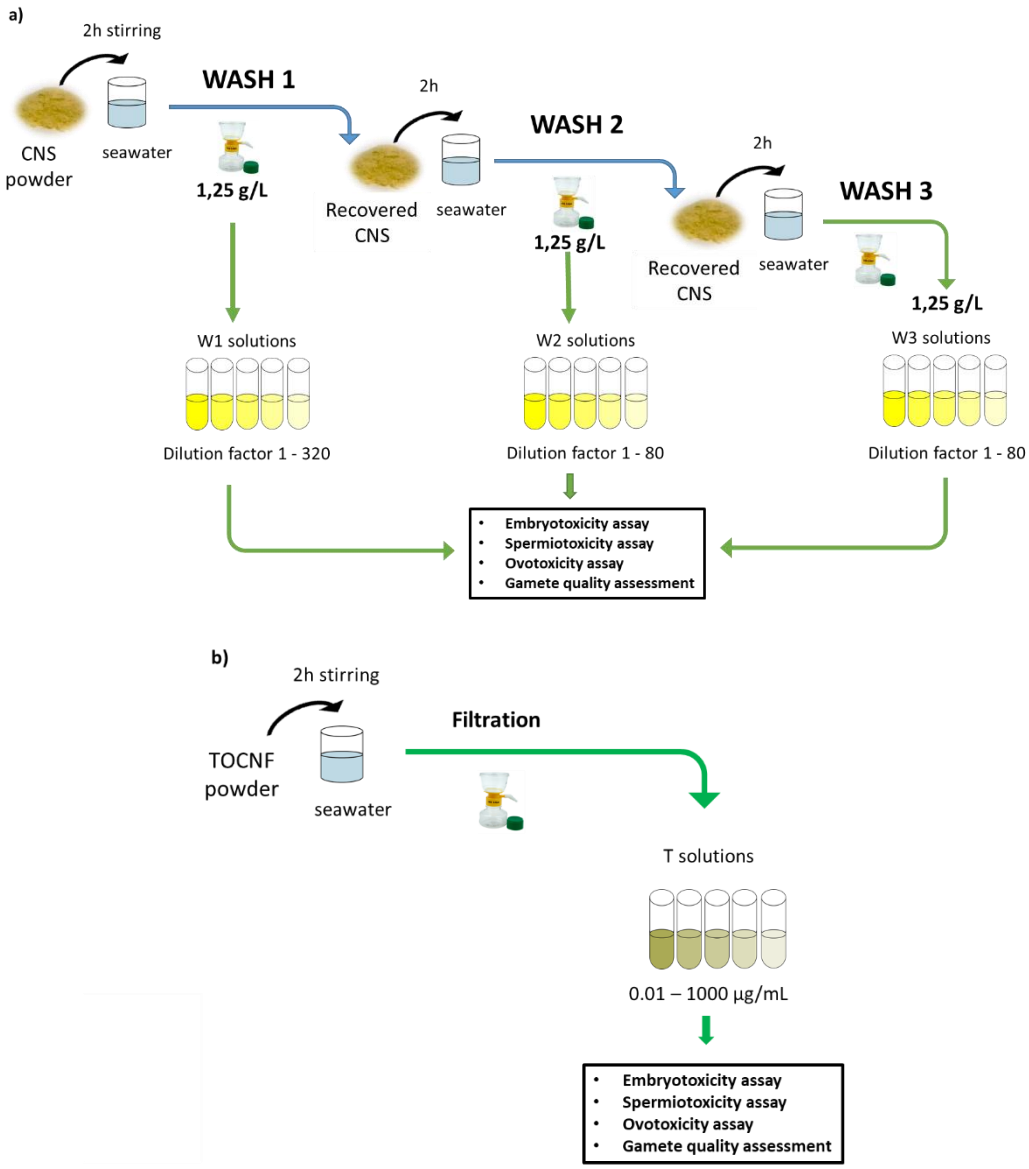


Figure 7. Schematic representation of the experimental setup adopted to prepare the "W solutions" (a) and "T solutions" (b). (a) The multi-washing protocol employed to prepare seawater exposed to Cellulose-based Nanosponges (CNS) "W solutions". The CNS powder was added to FNSW and stirred for 2 h at room temperature, filtered through a 0.45 µm filter and diluted in FNSW to obtain the W1 solutions. After this first wash, the CNS powder was recovered and added to FNSW to be washed a second time for additional two hours. Hence, the solution with the CNS powder was filtered through a 0.45 µm filter and diluted in FNSW to obtain the W2 solutions. To prepare the W3 solutions, the CNS recovered and washed twice were added to FNSW and stirred for 2 h at room temperature. Finally, the CNS powder washed three times was removed through a 0.45 µm filter and the obtained solution was diluted in FNSW obtaining the W3 solutions; (b) Protocol adopted to prepare the seawater exposed to TEMPO-oxidized cellulose nanofibers (TOCNF) "T solutions". The TOCNF powder was added to filtered seawater and stirred for 2 h at room temperature, then filtered through a 0.45 µm filter and diluted in seawater to obtain the T solutions. The solutions obtained applying these protocols were tested in the ecotoxicological bioassays reported in the black boxes, with *Paracentrotus lividus* and *Arbacia lixula*.

The TEMPO-oxidized cellulose nanofibers (TOCNF) were provided by the Politecnico of Milano and were produced by oxidizing the cotton fibres through the 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO)/NaClO/NaBr system (Pierre et al., 2017). The powder of TOCNF was added to FNSW to obtain a stock solution of 1000 μg/mL TOCNF, stirred for 2 h at room temperature, filtered through a 0.45 μm filter and diluted in FNSW to obtain the following TOCNF solutions (T solutions): 0.01, 0.1, 1, 10, 100, 1000 μg/mL (Figure 7b).
Citric acid solutions (CA solutions) were obtained after diluting a stock solution of 5000 μg/mL, prepared by dissolving the citric acid (Merck Life Science) in double distilled water, in FNSW to reach the following concentrations: 0.01, 0.1, 1, 10, 100, 1000 μg/mL.
To prepare the bPEI solutions, branched polyethyleneimine (25 kDa, Merck Life Science) was dissolved in double distilled water to obtain a stock solution of 5000 μg/mL that was magnetically stirred for 20 minutes at room temperature, to allow the solubilisation of bPEI fibres into water. The stock solution was used to prepare two unfiltered and filtered bPEI solutions. The unfiltered bPEI solutions were obtained by diluting the stock in FNSW at the final concentrations of 0.01, 0.1, 1, 10, 100, 1000 μg/mL. The filtered bPEI solutions were prepared by filtering the stock solution through a 0.45 μm filter and finally diluted in FNSW to obtain the same concentrations.

2.2.3. Gamete collection

According to the International standardised guidelines for conducting ecotoxicity tests with echinoids (ASTM, 2004), gamete spawning in sea urchins was induced by injecting 1 mL of 0.5 M KCl through the peristomal membrane, which results in an osmotic shock and, in turn, in gamete release. In females, eggs were collected in FNSW and preserved at 18±1°C until use. Egg quality was examined under a microscope (Zeiss Stemi 2000C) discarding the samples containing vacuolated, irregular, small, immature and fertilised eggs. As described in Sartori et al (Sartori et al., 2017), eggs were washed into a 1L beaker containing FNSW, in order to remove damaged eggs. In males, spermatozoa were collected dry directly from the gonopores with a pipette and stored in a 1.5 mL tube at 4°C. An aliquot of spermatozoa from each male was diluted in FNSW to preliminarily assess morphology and motility under a light microscope. Finally, a preliminary fertilisation test was carried out by adding a sub-sample of eggs to a small amount of sperm solution. Eggs not fertilised within 60 sec were discarded. Gametes of three animals that bypassed the initial check were pooled and counted as follows. An aliquot of dry sperm was diluted (DF 1000) in a solution of 50% distilled water and 50% FNSW, to reduce sperm flagellar movement. Subsequently, 5 μL of the

resulting suspension were loaded into the sperm counting chamber (Hawksley, UK) and observed under a microscope (ZEISS Axiophot, Germany) with a 40X objective.
Before performing egg count, eggs were washed at least three times in FNSW to remove the immature or damaged eggs present in the batch. Subsequently, 0.1 mL of the resulting suspension was used to count the eggs under a stereomicroscope (ZEISS Stemi 2000-C, Germany) with a 5X magnitude. The count was repeated at least five times to reach an accurate estimate of the egg concentration.

2.2.4. Embryotoxicity assay with *Paracentrotus lividus* and *Arbacia lixula*

In *P. lividus*, the embryotoxicity assay was performed according to the procedure described in Sartori et al., 2017. In particular, the selected eggs from three females were pooled and then fertilised with a sperm pool of three males (sperm/oocyte ratio of 50:1). After 20 minutes, the presence of the fertilisation envelope indicated that fertilisation occurred and that zygotes were formed. Then, 1 mL of fertilised egg suspension (1000 oocytes/mL) was transferred into a 6-well dish containing 9 ml of the test solution and incubated in a culture chamber at 18°C for 48h.
In *A. lixula*, different preliminary experiments were carried out to set up the embryotoxicity assay protocol (see supplementary materials, section S1, figure 1S). The spermatozoa of three males were mixed and used to fertilise a pool of eggs of three females with a sperm/egg ratio of 1000:1. After 20 min from fertilisation, zygotes were identified by the presence of the fertilisation envelope. Then, 1 mL of fertilised eggs (1000 embryos/mL) was transferred into 6-well plates containing 9 mL of the test solution and incubated in a culture chamber at 20°C for 48 h. In both species, at the end of the assay, embryos were fixed adding 4% glutaraldehyde in FNSW and 100 larvae were counted to discriminate between normal and abnormal plutei and calculate the percentage of normal plutei. Developmental abnormalities included: malformations in size, shape, symmetry, the integrity of the spicules, number or size of arms, and presence of the gut. Additionally, in *A. lixula* the presence of the skeletal "crown" at the base of the larva was also checked. The embryotoxicity test has been considered valid if it met the acceptability criteria, i.e. the percentage of normal plutei in the negative control was ≥ 80% (Sartori et al., 2017).

2.2.5. Data analysis and statistics

Each test was performed in triplicate and the assays were repeated three times. Data were checked for normal distribution by the Shapiro-Wilk test and variance homogeneity by Levene's test. After passing these tests, the one-way analysis of variance was performed and

followed by Fisher's least significant difference test for pair-wise comparison using the software Systat 11.0 (Systat Software Inc.). Percentage values were analysed after arcsine transformation to achieve normality. Differences were considered significant at a P value lower than 0.05 (*P<0.05) or 0.01 (**P<0.01). Data were expressed as mean ± standard error (SE).

2.3. Results

2.3.1. Cellulose-based Nanosponges-treated seawater effects on *Paracentrotus lividus* embryo development

The W1 solution negatively affected *P. lividus* embryo development. In particular, a total absence of normal embryos was observed in the W1 solution with a DF from 1 to 20. Compared to the control (90 ± 1.39%), the W1 solution with a DF of 40 and 80, significantly reduced the normal embryo percentages (81 ± 1.75% and 86 ± 0.67%, respectively; $P < 0.01$). On the contrary, the W1 solution with a DF of 160 and 320 did not significantly affect embryo development (89 ± 0.71% and 86 ± 0.71%, respectively) (Figure 8a). In the W2 solution diluted 1, 2 and 5 times no normal embryos at the pluteus stage were observed. Compared to the control (90 ± 1.29%), the W2 solution diluted 10 times significantly reduced the percentage of normal embryos up to 10 ± 1.38 % ($P < 0.01$). In the W2 solution diluted 20, 40 and 80 times, the normal embryo percentages did not significantly differ from the control (90 ± 1.37 %; 92 ± 1.36%; 93 ± 0.7 %; respectively) (Figure 8b). By exposing the zygotes to the solution W3, a significant impact on embryonal development was observed from the DF 1 to 5. In detail, after the exposure to the W3 solution diluted 1 and 2 times a total absence of normal embryos was observed. Compared to control (92 ± 0.46%), a significant reduction in normal embryos was also detected in the W3 solution diluted 5 times (83 ± 3.84%; $P < 0.01$). At DF >10, the embryotoxic effect was no longer observed (Figure 8c).

In the W solution, both the presence of abnormal pluteus larvae (Figure 9b) and a delay in embryo development were observed. In particular, in the W solutions with a DF 1 embryo development was blocked at the blastula stage (Figure 9e); whereas in the diluted W solution, embryo development was arrested at the early four-armed pluteus stage (Figure 9c) or gastrula stage (Figure 9d) depending on the DF applied.

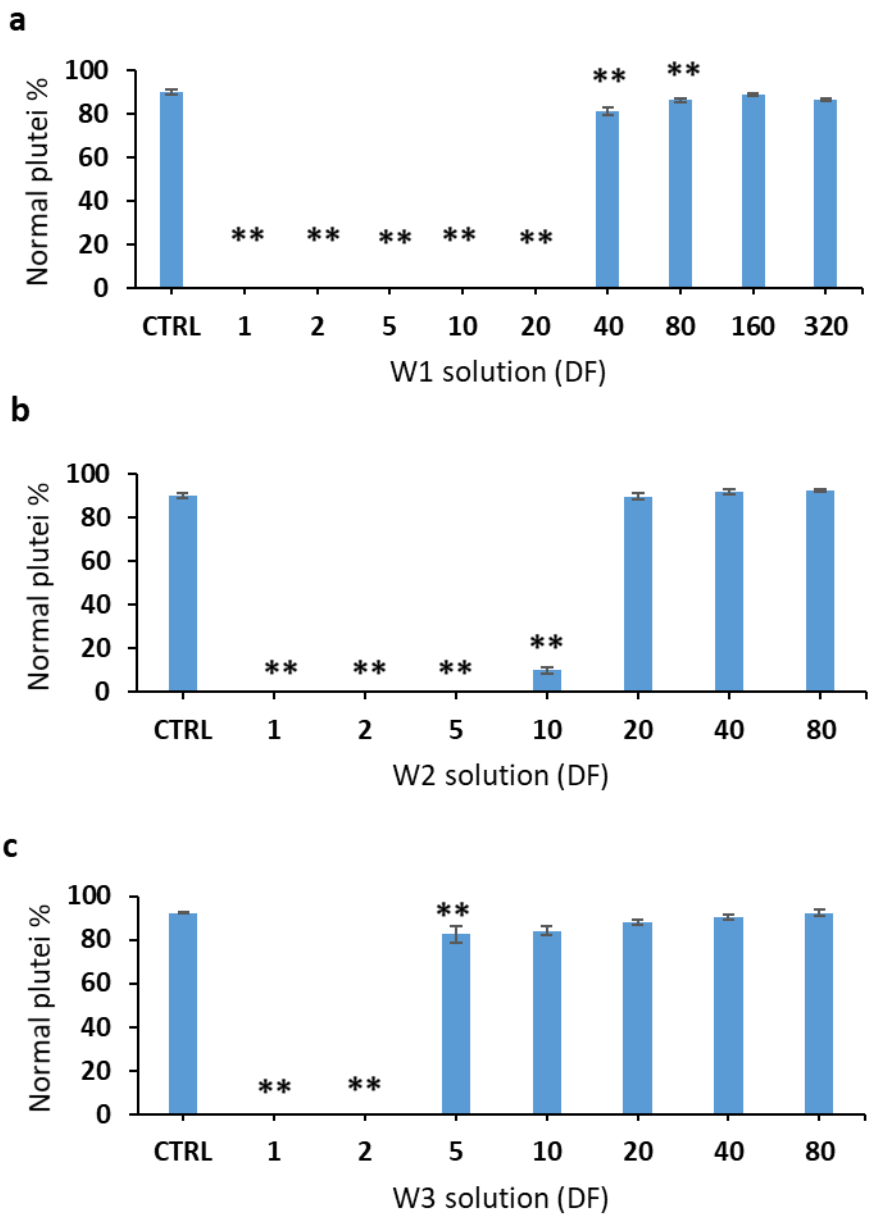


Figure 8. Embryotoxicity assay with *Paracentrotus lividus* for Cellulose-based Nanosponges treated seawater assessment. The graphs show the percentage of normal embryos at the pluteus stage developed into seawater conditioned with CNS powder (W solution). "W1 solution" (a) refers to seawater conditioned with CNS powder that was washed once. "W2 solution" (b) and "W3 solution" (c) respectively refer to seawater conditioned with CNS powder washed twice and three times. On the X-axis, the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). The significance level was set at $P< 0.05$*; $P< 0.01$** *vs* CTRL (control).

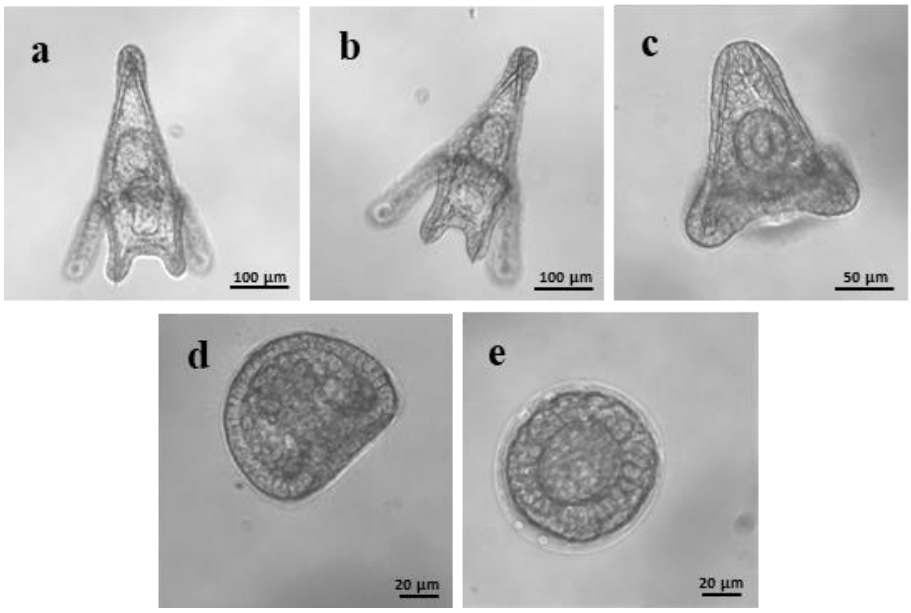


Figure 9. *Paracentrotus lividus* embryo development 48 hours post fertilisation in cellulose-based nanosponges solutions (W solutions). Morphological normal embryo at pluteus stage characterised by two pairs of arms called post-oral (the longest) and antero-lateral arms developed in filtered seawater (control) (a); abnormal pluteus larvae with crossed calcareous spicules at the apical end developed in W1 solution with DF higher than 40; in W2 solution with DF higher than 10 and W3 solutions with DF higher than 5 (b); delayed embryos blocked at: early four-armed pluteus larva stage developed in W1 solution with DF 40 and 80, W2 solution with DF 5, W3 solution with DF 2 (c); at gastrula stage developed in W1 solution with DF 20, W2 solution with DF 2, 5 and 10 (d); and blastula stage developed in W1 solution with DF of 1, 2, 5, 10, W2 and W3 solution with DF of 1 (e).

2.3.2. Cellulose-based Nanosponges-treated seawater effects on *Arbacia lixula* embryo development

The W solution negatively affected *A. lixula* embryo development. In particular, the absence of the normal embryos was observed in the W1 solution with a DF from 1 to 10. In the W1 solution diluted 20 and 40 times, a significant decrease of the normal embryo percentage was observed with respect to the control solution (control 93 ± 2.55 % *vs* 7 ± 2.55%, 58 ± 2.55%; $P < 0.01$). Increasing the DF of the W1 solution to 60, 80 and 120-fold, no significant differences were observed (85 ± 2.55%, 93 ± 2.55%, and 100 ± 4.12%, respectively) (Figure 10a).

In the W2 and W3 solutions with a DF of 1 and 2, no normal embryos at the pluteus stage were observed. The W2 and W3 solutions with a DF of 5 significantly reduced the percentage of normal embryos (71 ± 4.51% and 81 ± 4.03%; $P < 0.01$) with respect to the control (94 ± 4.03%). On the contrary, starting from a DF of 10, the W2 and W3 solutions

did not affect *A. lixula* embryo development (Figure 10b-c). As for *P. lividus*, also in *A. lixula*, the W solution affected the temporal progress of embryo-larval development (Figure 11).

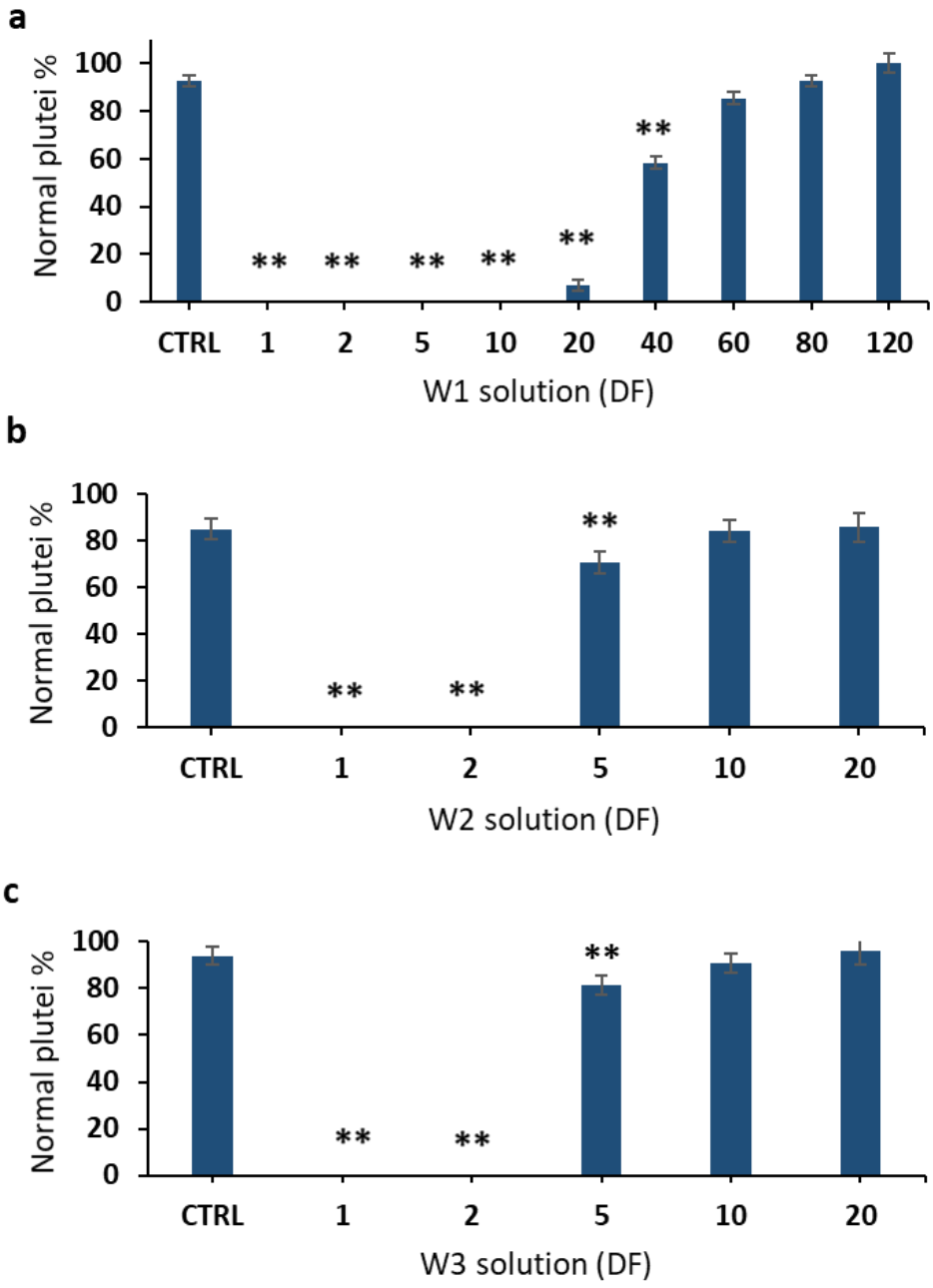


Figure 10. Embryotoxicity assay with *Arbacia lixula* for Cellulose-based Nanosponges treated seawater assessment. The graphs show the percentage of normal embryos at the pluteus stage developed into seawater conditioned with CNS powder (W solution). "W1 solution" (a) refers to seawater conditioned with CNS powder that was washed once. "W2 solution" (b) and "W3 solution" (c) respectively refer to seawater conditioned with CNS powder washed twice and three times. On the X-axis the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

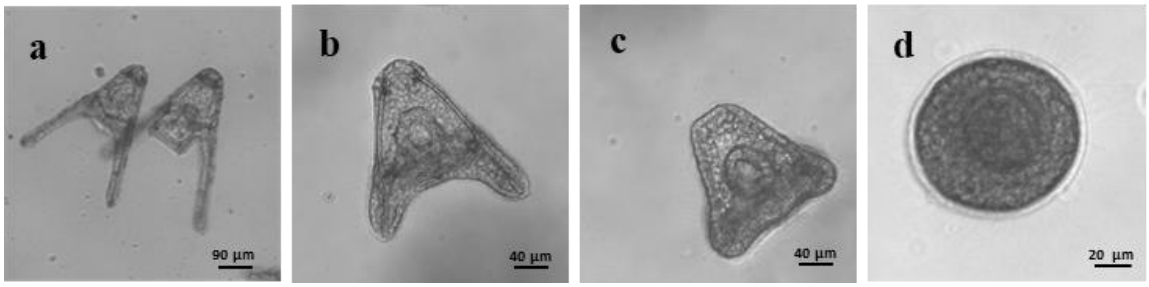


Figure 11. *Arbacia lixula* embryo development 48 hours post fertilisation in cellulose-based nanosponges solutions (W solutions). Morphological normal embryo at pluteus stage developed in filtered seawater (control) (a); early pluteus stage characterized by the absence of the antero-lateral arms developed in W1 solution with DF 20, 40 and 80, W2 solution with DF of 1, 2, 5, W3 solution with DF of 1, 2 (b); delayed embryos blocked at: early pluteus stage developed in W1 solution diluted 5 and 10 times (c); and blastula stage developed in W1 solution with DF of 1 and 2 (d).

2.3.3. Cellulose-based Nanosponges component exposure effects on *Paracentrotus lividus* embryo development

No significant effects on *P. lividus* embryo development were observed in T solution at concentrations from 0.01 to 100 µg/L. On the contrary, a significant decrease in the percentage of normal embryos was detected in 1000 µg/L T solution with respect to the control solution (control 89 ± 1.71% *vs* 77 ± 1.78%; $P < 0.01$) (Figure 12a).

In CA solution, the embryo development was not significantly affected at concentration up to 10 µg/mL. By increasing the citric acid concentration (100 and 1000 µg/mL), a significant decrease in the percentage of normal embryos was detected (58 ± 3.43% and 15 ± 3.43%, respectively; $P < 0.01$) compared to the control (98 ± 1.71%) (Figure 12b). The abnormal pluteus stage observed after exposure to T solution and CA solution resemble those reported in figure 9b.

For bPEI solution, two kinds were tested: unfiltered and filtered thought a 0.45 µm filter. In the latter case, the bPEI solution was filtered to simulate the protocol used for the preparation of the W solution. In the unfiltered and filtered 0.01 and 0.1 µg/mL bPEI solution, *P. lividus* embryo development was not significantly affected. On the contrary, at higher concentrations, the percentage of morphological normal embryos at pluteus stage significantly decreased (Figure 12c-d). In particular, at 0.5 µg/mL the presence of abnormal plutei resembled those reported in figure 9b was observed. By increasing the concentration of bPEI at 1 and 10 µg/mL, a delay of embryo development was observed, and the delayed embryos were similar to those showed in Figure 9c and 9d.

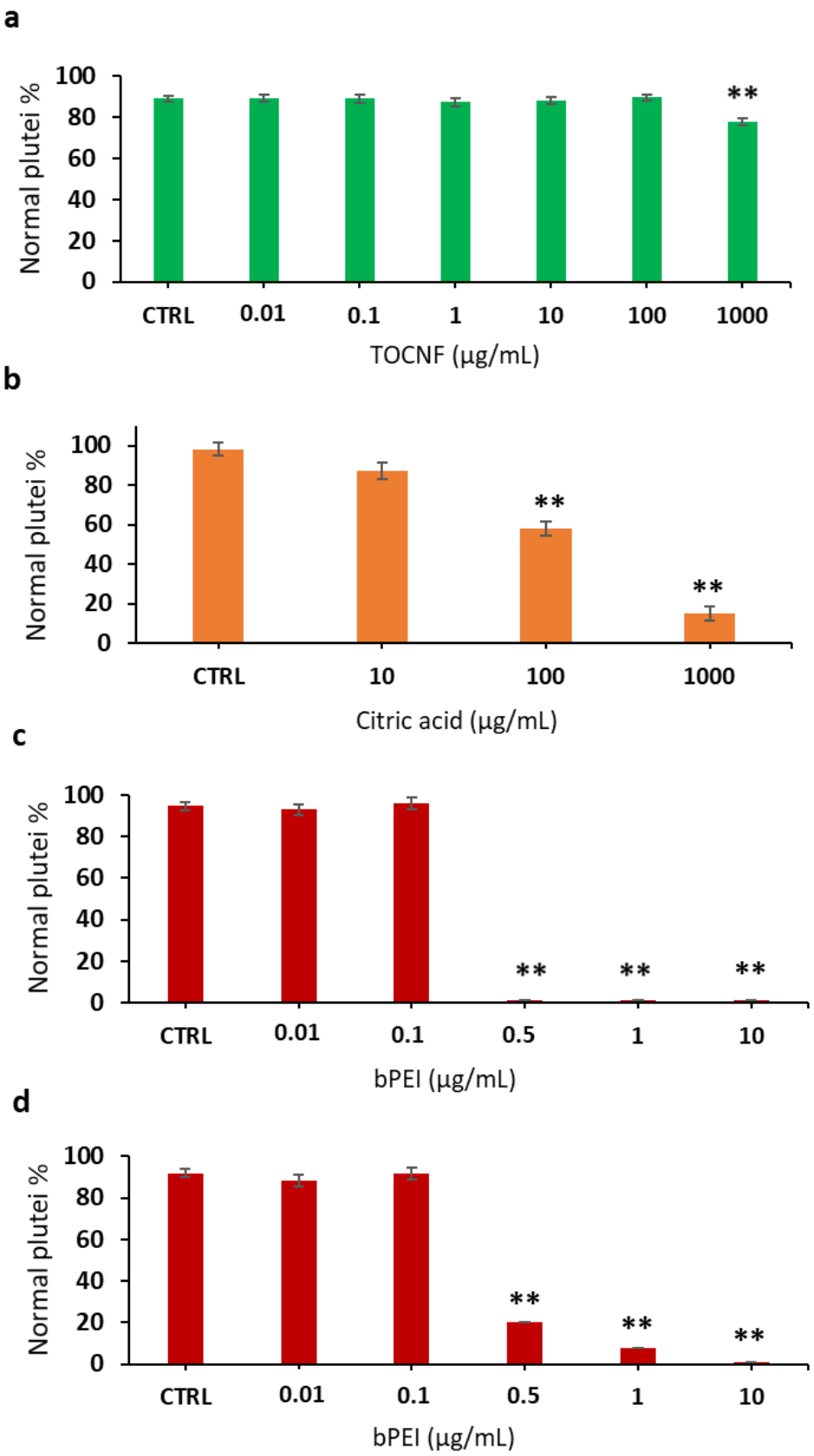


Figure 12. Embryotoxicity assay with *Paracentrotus lividus* for Cellulose-based Nanosponges component assessment. The graphs show the percentage of normal embryos at the pluteus stage developed into T solution (a), CA solution (b), bPEI solution (c), 0.45 µm filtered bPEI solution (d). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

2.3.4. Cellulose-based Nanosponges component exposure effects on *Arbacia lixula* embryo development

T solution in a range of 0.01-10 µg/mL did not affect embryo development of *A. lixula*. By increasing the TOCNF concentration, the normal embryo percentage significantly decreased at 100 µg/mL (81 ± 2.81%; $P < 0.01$) and collapsed at 1000 µg/mL (1 ± 2.81%; $P < 0.01$), compared to the control (92 ± 2.81%) (Figure 13a).

Similarly, CA solution affected *A. lixula* embryo development, compared to the control (95 ± 1.67%). In particular, at concentrations of 0.001 and 0.1 µg/mL embryo development was not significantly affected; whereas, increasing acid citric concentration, embryo development was significantly impaired at 10 and 100 µg/mL (32 ± 1.67% and 5 ± 1.67%, respectively; $P < 0.01$) until to observe the total absence of normal plutei at the highest tested concentration (1000 µg/mL) (Figure 13b).

The abnormal pluteus stage observed after exposure to CA solution resemble to those depicted in Figure 11b.

In the unfiltered bPEI solution, at the concentration of 0.01 µg/mL bPEI, the percentage of normal embryos did not differ from the control (control 95 ± 1.31% *vs* 96 ± 1.31%). However, 0.1 µg/mL bPEI significantly impaired embryo development (77 ± 1.31%; $P < 0.01$) that was totally inhibited at the highest tested concentrations (1-1000 µg/mL) (Figure 13c). Similarly, in the filtered bPEI solution, *A. lixula* embryo development was unaffected at the concentration of 0.01 and 0.1 µg/mL, but it was significantly affected at 0.5 µg/mL (82 ± 1.14%; $P < 0.01$) and totally inhibited at 1 and 10 µg/mL compared to the control (90±1.07%) (Figure 13d). At 0.5 and 1 µg/mL bPEI the embryo-larval development was arrested at early pluteus stage characterized by the absence of the antero-lateral arms, which looked alike those reported in Figure 11b. At 10 µg/mL the embryo development was arrested at early pluteus similar to those reported in figure 11c, characterized by the absence of the antero-lateral and of the post-oral arms. While starting from the 100 µg/mL the embryo development was arrested at blastula stage resembling those reported in figure11d.

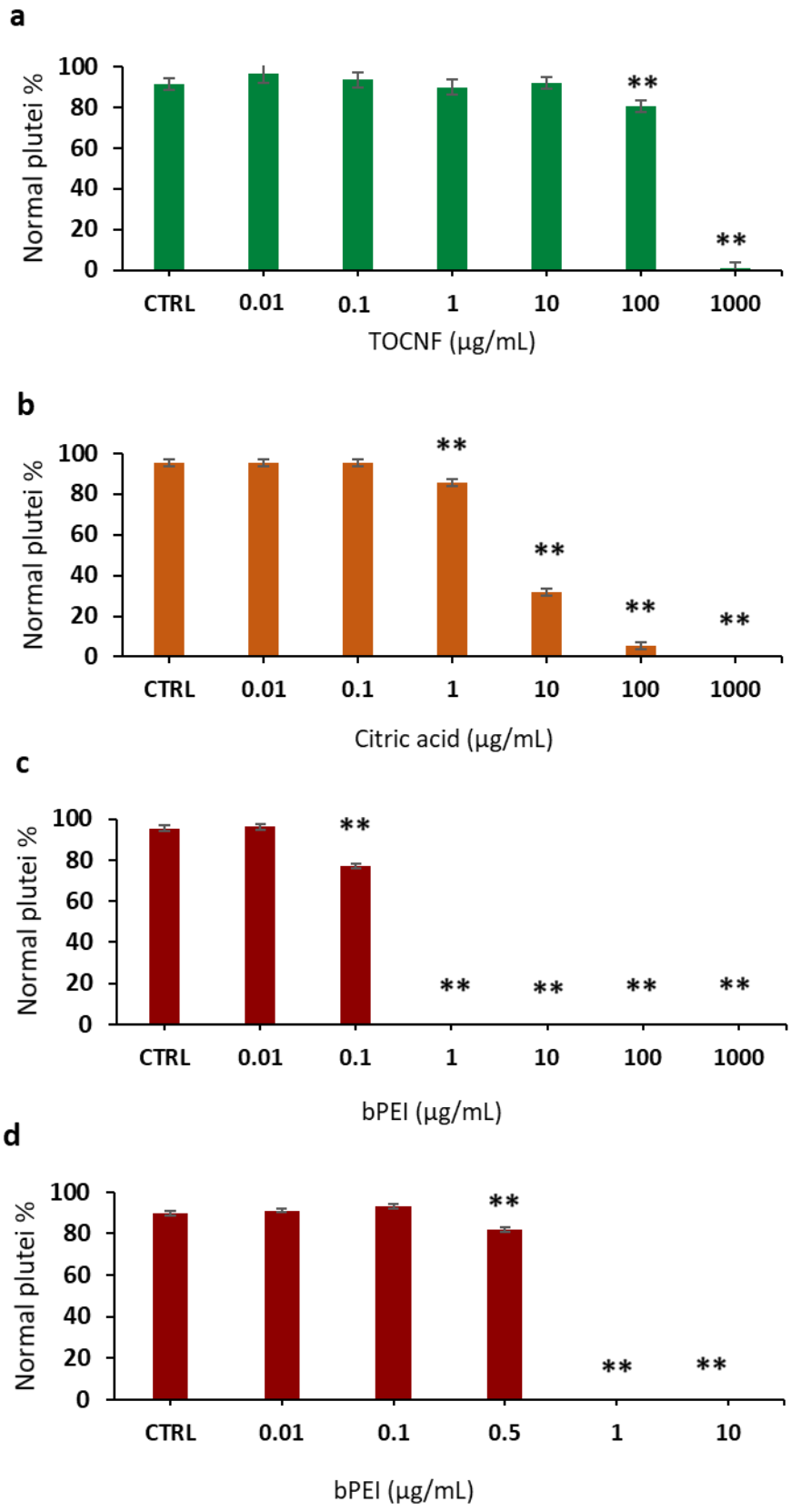


Figure 13. Embryotoxicity assay with *Arbacia lixula* for Cellulose-based Nanosponges component assessment. The graphs show the percentage of normal embryos at the pluteus stage developed into T solution (a), CA solution (b), bPEI solution (c), 0.45 µm filtered bPEI solution (d). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

2.4.Discussion

It has been widely demonstrated that marine organisms during the early life stages are more susceptible to pollution than adults. Therefore, focusing the ecotoxicological bioassays on embryos increases their performance by providing rapid, easy, cheap and sensitive ecotoxicity tests (Mohammed, 2013). Accordingly, the sea urchin embryotoxicity test presents these advantages and is proposed as a highly sensitive diagnostic tool. Nowadays, among the sea urchin Mediterranean species, *P. lividus* is the most consolidated biological model for monitoring marine environmental pollution and for assessing the effects of several contaminants. However, in recent years, overfishing due to the increased demand for sea urchin gonads for culinary purposes, the increasing marine pollution and climatic changes are affecting the *P. lividus* abundance in the Mediterranean Sea, including the Gulf of Naples. Thereby, the supply of adult *P. lividus* and, in particular, of mature *P. lividus* represented the major disadvantage in using this species as a test organism in the present project. Hence, *A. lixula* was selected as a possible suitable alternative for evaluating the ecosafety of CNS. Indeed, this species, together with the edible sea urchin *P. lividus*, is the most abundant echinoid in the Mediterranean Sea and is characterized by a comparable sensitivity to marine contaminants (Carballeira et al., 2012). Other advantages of using *A. lixula* are their better supply being less harvested for the fishery in the Mediterranean Sea since it is not an edible species and, from a biological point of view, its reproductive process is well described.

Although the embryotoxicity tests with *A. lixula* have been already performed by different research groups to evaluate the effects of different contaminants, a standardized procedure is not yet available since the experimental conditions significantly differ from each other (Arslan et al., 2007; Maisano et al., 2015; Bošnjak et al., 2011). Taking into account these previous studies, a set of preliminary tests has been carried out with *A. lixula* to define the suitable experimental conditions to establish a new embryotoxicity assay for ENMs. Particularly, to set the experimental conditions, changes in sperm:egg ratio, the number of embryos/mL, and the temperature of embryo culture have been tested using the reference toxicant to assess the biological quality of the assay. As result, the following experimental conditions allowed to obtain the highest percentage of normally developed plutei at 48 hpf (hours post fertilisation) in the control test: sperm:egg ratio of 1000:1; 100 embryos/mL in the test chamber; embryo development temperature of 20 °C (see supplementary materials, section S1, figure 1S). Moreover, the tests performed with the specific reference toxicants supported the sensitivity of the two sea urchin species employed. The calculated EC_{50} values, i.e. 67.69 μg/L in *P. lividus* and 92.17 μg/L in *A. lixula*, fell within the defined acceptability

ranges of 20-110 μg/L (see supplementary materials, section S3, figure 1S and 2S) (His et al., 1999; Fernández & Beiras, 2001; Novelli et al., 2002). In the present chapter, the embryotoxicity test with *P. lividus* and *A. lixula* has been performed to assess the impact of CNS and its components on embryo development, but also to verify the reliability of this bioassay in the assessment of ENM safety.

To date, the embryotoxicity test with *P. lividus* has been carried out to test the toxicity of different nanomaterials. In particular, this bioassay revealed that several NMs, such as AgNPs, carbon-based NPs, iron oxide, amine polystyrene NPs, Ni NPs and zinc oxide, were embryotoxic inducing embryo malformations and alteration of the normal progression of the development stages (Manno et al., 2013; Genevière et al., 2020; Manzo et al., 2013; Šiller et al., 2013). While, for *A. lixula,* embryotoxic effects have been reported only for two nanomaterials: CuO and Ag NPs (Giannetto et al., 2018; Burić et al., 2015).

In this project, the embryotoxicity test with *P. lividus* and *A. lixula* was performed to assess the potential toxicity of CNS leachate on embryo development. Indeed, the envisaged remediation process with CNS hypothesises CNS closed in bags that will act similarly to filters; therefore, CNS will not be directly released into seawater. The embryotoxicity tests revealed that the CNS leachate (W solution) impairs embryo development in both species, suggesting a potential release of chemicals into seawater from the nanostructured material tested. The observed alteration in the cleavage timing could be linked to aberrations in the mechanisms that regulate embryo development as the cell proliferation and differentiation. In the frame of the eco-design approach, which aims to develop an ecosafe ENM (Figure 1), a multi-washing protocol (W1, W2, W3) was adopted in order to reduce the embryotoxic effects. Data presented show that, in both *P. lividus* and *A. lixula,* the multi-washing of CNS followed by dilution increases the percentage of morphological normal embryo suggesting that this easy procedure can be introduced in the design of the CNS to obtain a safer ENM.

To verify if the multi-washing protocol alters the CNS remediation efficiency, the CNS absorption ability to Zn, with and without the multi-washing protocol has been tested. The analyses revealed that the CNS absorption ability did not change after the multi-washing protocol (data not shown).

The three constituents of CNS, i.e. TOCNF, citric acid and bPEI, have been tested one by one to verify which of them exerted the observed embryotoxic effects. In both sea urchin species, TOCNF resulted in a safe component showing an alteration of embryo development only at the highest tested concentrations, over 100 μg/mL in *A. lixula* and over 1000 μg/mL in *P. lividus*. CNF originates from cellulose, thereby they are natural, sustainable and biodegradable. Compared to non-nano cellulose, the nano-dimension of the CNF confers them new physicochemical characteristics, such as size, shape, surface area and charge,

which raises concerns about their ecotoxicity since these new properties may influence the modalities by which CNF interacts with a biological system. To date, the impact of CNF has been investigated in freshwater bacteria, algae, invertebrates and vertebrates, but not in marine organisms (Ogonowski et al., 2018; Pengiran et al., 2021; Harper et al., 2016; Ong et al., 2017). It has been demonstrated that CNF exposure (1 μg/mL) reduces the algal growth and cell viability as well as the intracellular ATP (Adenosine Triphosphate) levels and induces ROS generation in the freshwater green microalgae (Pereira et al., 2014). However, CNF did not affect vitality, morphology and swimming behaviour in fish and crustaceans (Pengiran et al., 2021; Ogonowski et al., 2018). The CNF can be extracted from the native celluloses by chemical and mechanical synthesis methods, which can influence their toxicity since each method introduces different physicochemical properties into the final cellulose material. A previous study evaluated the toxicity of CNF produced with different synthesis methods demonstrating that mechanically homogenised CNF resulted in higher ecotoxicity in zebrafish embryo development compared to fibres produced using the TEMPO process (Harper et al., 2016). In line with these previous results, the present thesis demonstrated that the impact of CNF prepared via TEMPO process on sea urchin embryo development is low, supporting the application of this synthesis method in CNF production to generate ENMs with a minimal hazard.

Citric acid is an intermediate of the Krebs cycle that serves as a substrate for biosynthetic processes in living organisms. Citrate and its esters are extensively applied as non-toxic plasticizers in various products from medicine to toys, food additives, and cosmetics (Bergfeld et al., 2011; Soccol et al., 2017). Recently, citric acid has also been employed as a crosslinking agent in the preparation of ENMs. Although the acute toxicity data available on citric acid are scarce, this reagent resulted relatively safe in the few marine species where it has been tested (Fiume et al., 2014). Differently, in the present study, the embryotoxicity test revealed that citric acid exposure affects sea urchin embryo development, from 100 μg/mL in *P. lividus* and 1 μg/mL in *A. lixula*. It is well known that citric acid forms stable chelate complexes with metal ions, such as calcium and magnesium. These ions play an essential role in the normal development of sea urchin embryos. Indeed, their deprivation has been proved to affect gastrulation, skeletogenesis and the development of the animal-vegetal axis (Martino et al., 2019). Thereby, the embryotoxic effects herein observed may be related to a possible reduction in the cell concentration of these ions due to their chelation by citric acid. Given the widespread application in several traditional industrial sectors as a cross-linker, the toxicity of citric acid toward other environmentally relevant species needs to be investigated.

The PEI (polyethyleneimine) is a synthetic and low cost polymer composed of repeating units of ethylene imine and amine groups with many biological and biomedical applications from drug delivery to water treatment. Based on different synthesised structures, it can be divided into linear and branched PEI, which differ in the amount and localization of amino groups. Indeed, the linear PEI is characterised by primary and secondary amino groups, whereas the bPEI contains also tertiary amino groups. Despite its increasing application, the safety of PEI is a concern since it has been associated with cytotoxicity, destabilisation of the plasma membrane and induction of apoptosis and necrotic cell death (Hunter & Moghimi, 2010). Furthermore, the PEI toxicity depends on the molecular branching and weight with the former being more toxic than the linear one (Almulathanon et al., 2018; Jones et al., 2013). Nevertheless, the toxic effects of PEI on aquatic organisms have been poorly investigated demonstrating that PEI coatings increased the toxicity of different NM (Schiavo et al., 2017). In the present thesis, the bPEI toxicity on marine invertebrate reproduction has been assessed for the first time demonstrating that it severely affects sea urchin embryo development. Indeed, the embryotoxicity tests revealed that bPEI caused abnormal embryo development at very low concentrations of 0.5 and 1 μg/mL, in *P. lividus* and *A. lixula* respectively. Moreover, the filtered bPEI solution exerted similar embryotoxic effects. Several studies indicated that PEI exhibited high cytotoxicity inducing necrotic cell death and apoptosis; nevertheless, the mechanism of PEI cell death induction is not yet elucidated. Apoptosis is a physiological process, which occurs during sea urchin embryo development playing a key role in shaping and sculpting the embryos and eliminating damaged or unnecessary cells. Changes in the level of apoptosis upon exposure to diverse contaminants have been reported in numerous invertebrates (Agnello & Roccheri, 2010). Thereby, it is possible to hypothesise that the embryotoxic effects of PEI herein observed may be due to an alteration of the sea urchin developmental program caused by PEI exposure that triggers the apoptotic process in normal cells.

The tests with the components of CNS, such as the bPEI, helped to elucidate the mechanism which drives the CNS toxicity in embryo development. Compared to the other components, bPEI seems to exert higher toxicity, by inducing an embryotoxic impact at lower concentrations than citric acid and TOCNF.

Therefore, the embryotoxicity induced by CNS could be mainly caused by the bPEI contained in the CNS leachate. It has been reported that PEI is able to induce membrane damage and activate the apoptotic program including the release of cytochrome c, the activation of caspase 3, and the alteration in mitochondrial membrane potential, in different human cell lines (Moghimi et al., 2005). Consequently, bPEI contained in the CNS could induce similar mechanisms in the embryonal cells by altering the apoptosis regulation and

driving the CNS toxicity in the aberrant embryos because apoptosis is involved in cell proliferation, differentiation, and morphogenesis (Agnello et al., 2015). Apart from these speculations, until today, the embryotoxicity here reported is the unique information about the CNS impact on sea urchin embryo development; and it represents a starting point for future studies to investigate the biochemical events that drive CNS toxicity.

Overall, the results presented in this chapter support the embryotoxicity test as a valuable and sensitive tool to assess the impact of CNS and indicate in *A. lixula* is a valid alternative species to *P. lividus* in assessing ENM toxicity.

CHAPTER 3

Ecosafety assessment of Cellulose-based Nanosponges and their components on fertilisation success

3.1. Introduction

The reproductive fitness of a species strictly depends on the ability of gametes to successfully meet, be activated and fuse with each other to start the fertilisation process. In broadcast spawners, as sea urchins, gametes are in direct contact with all organic and inorganic species dissolved in seawater, including contaminants that can impair their fertilisation competence. Sea urchin gametes are sensitive to several contaminants, consequently tests using fertilisation as an endpoint have been developed (Hudspith et al., 2017). Particularly, standard procedures are available for the sperm cell toxicity test, also known as the fertilisation test, a short-term assay used to assess the impact of stressors on sea urchin fertilisation success (Usepa, 2002). This test represents a relevant tool in ecotoxicological studies due to its sensitivity, reliability, low cost and rapidity of execution. In Europe, standard methods for fertilisation testing are available for the Mediterranean species *P. lividus* (Volpi Ghirardini & Arizzi Novelli, 2001; Sartori et al., 2017). On the contrary, the assay with *A. lixula* is not standardised and the literature does not report significant examples of ecotoxicological assessment with this species. Besides that, the efficiency of the test needs to be verified on ENMs, as well on ENMs for marine remediation purposes. Indeed, up to date, no information is available on its reliability in the assessment of ENMs exposure.

Starting from this rationale, in this chapter, the sperm cell toxicity test has been performed to assess the impact of the leachate of CNS on sperm fertilising ability in *P. lividus* and *A. lixula*.

Up-to-date, the assessment of the fertilisation competence of female gamete is not included in the standardised tests performed with sea urchins. Therefore, to establish also the risk of CNS leachate exposure in eggs, the ovotoxicity test has been developed both with P. *lividus* and *A. lixula*.

3.2. Materials and methods

3.2.1. Sperm cell toxicity test

Gametes of the two sea urchin species were collected as reported in the previous chapter. After collection, for both species, the spermatozoa of three males were mixed and then used to carry out the test. For *P. lividus*, the sperm cell toxicity test was performed according to the procedure described in (Sartori et al., 2017). After calculating the sperm concentration as described in the previous chapter, dry sperm was diluted in FNSW to obtain a sperm solution of $15x10^6$/mL. Then, aliquots of 0.1 mL of the sperm suspension were added to Petri dishes containing 9 mL of the test solutions (please see section 2.2.2) and incubated for 1 h at 18°C. To maintain the sperm/oocyte ratio at 15.000:1, 1 mL of oocytes (1.000 oocytes/mL) was added per each test chamber. After 20 min of incubation, the assay was stopped by adding a few drops of 4% glutaraldehyde in FNSW and at least 200 oocytes were counted under an inverted microscope (ZEISS Axiovert 100, Germany) with a 10X objective to discriminate between fertilised eggs, showing a completely raised fertilisation membrane, and unfertilised oocytes. Finally, the percentage of fertilised oocytes was calculated as the ratio between the number of fertilised oocytes and the total number of oocytes counted.
The spermiotoxicity test with *A. lixula* consisted of steps similar to those performed with *P. lividus*, but for this species, the sperm/oocyte ratio was fixed at 10.000:1. After several preliminary experiments (Supplementary materials, section S1, figure 2S), the fertilisation rate was determined at the two-cell stage due to the difficulty to observe the fertilisation envelope under a stereomicroscope. In both species, each test was performed in triplicate and repeated three times.

3.2.2. Ovotoxicity test

The procedure for the ovotoxicity test was set up after several preliminary experiments. After the collection of the eggs and their evaluation as described in the previous chapter, the eggs from three females were selected and mixed. Subsequently, 1 mL of this egg solution (1000 eggs/mL) were transferred to 6-well plates containing 9 mL of the test solutions and incubated in a culture chamber for 1 h at 18° in *P. lividus*, and 20°C in *A. lixula*. After incubation, 0.1 mL of sperm suspension were added to each well to achieve a sperm/oocyte ratio of 100:1 in *P. lividus*, and 1000:1 in *A. lixula.* After 20 and 90 min, respectively, a few drops of 4% glutaraldehyde in FNSW were used to stop the tests and count at least 200 eggs by distinguishing between fertilised and unfertilised eggs. Finally, the percentage of fertilised eggs was calculated as the ratio between fertilised oocytes and the total number of eggs counted. In both species, each test was performed in triplicate and repeated three times.

3.2.3. Experimental conditions and statistical analysis

Both the spermiotoxicity and ovotoxicity tests were performed with the experimental solutions described in section 2.2.2. Statistical analysis was carried out using the software Systat 11.0 (Systat Software Inc.) and performing a one-way variance analysis (ANOVA) followed by a parametric test. The mean comparison was realised through Least Significant Differences (LSD) and the minimum significance level was set at $P<0.05$. Data are presented as mean ± standard error (SE).

3.3 Results

3.3.1. Spermiotoxicity assay of Cellulose-based Nanosponges solution in sea urchins

The W solution weakly affected the sperm fertilising capability in the sea urchin *P. lividus*. Indeed, only in the W1 solution with a DF 1, the fertilisation rate significantly decreased compared to the control (control 94 ± 1.38% *vs* 37 ± 1.42%; $P < 0.01$); whereas, all the other dilutions of W solutions did not affect the sperm fertilising capability (Figure 14a). Similarly, the W2 solution with a DF 1 induced a significant decrease in the fertilisation rate (41 ± 2.45%; $P < 0.01$) compared to the control (90 ± 2.45%), while the other dilutions of W2 solution showed percentages of fertilised eggs similar to the control (Figure 14b).

For the W3 solution (Figure 14c), the same trend was observed. Compared to the control (90 ± 1.54%), a significant fertilisation rate reduction was observed after sperm exposure to W3 solution with a DF 1 (69 ± 1.54%; $P < 0.01$); whereas, in W3 solution diluted twice, five and ten times, the sperm fertilising capability was not significantly affected (88 ± 1.54 %, 86 ± 1.54%, and 91 ± 1.88%, respectively).

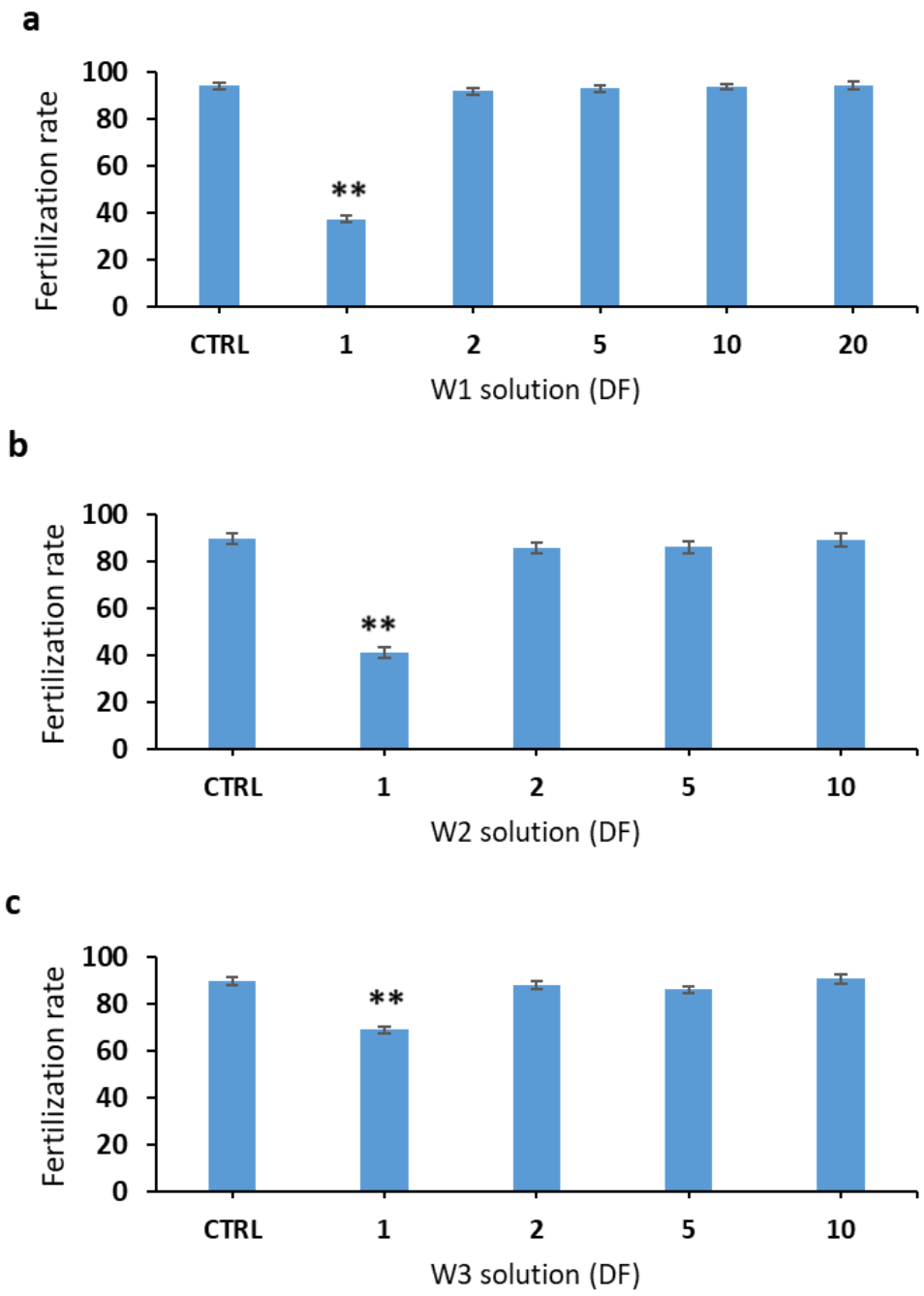


Figure 14. Spermiotoxicity assay with *Paracentrotus lividus* for Cellulose-based Nanosponges treated seawater assessment. The graphs show the percentage of fertilised eggs after sperm exposure to W1 solution (a), W2 solution (b), and W3 solution (c). "W1 solution" refers to seawater conditioned with CNS powder that was washed once. "W2 solution" and "W3 solution" respectively refer to seawater conditioned with CNS powder washed twice and three times. On the X-axis, the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). The significance level was set at $P< 0.05$* and $P< 0.01$** *vs* CTRL (control).

In *A. lixula*, the effect of sperm exposure to W solution showed a similar trend. In the W1 solution at the DF of 1, 2 and 5 a significant reduction in the percentage of fertilised eggs was observed (control 90 ± 1.57% *vs* 8 ± 1.57%; 12 ± 1.57%; 68 ± 1.57%, respectively; $P < 0.01$) (Figure 15a); whereas, the percentages of fertilised eggs in W1 solution at dilution higher than 10 times were comparable to those detected in control. After sperm exposure to W2 solution with a DF of 1 and 2, the percentage of fertilised eggs significantly decreased compared to the control (control 84 ± 3.4% *vs* 43 ± 3.4% and 72 ± 3.4%; $P < 0.01$) (Figure 15b). In the W2 solution with the DF higher than 5, the percentages of fertilised eggs were comparable to the control. A similar trend was observed for the W3 solution (Figure 15c).

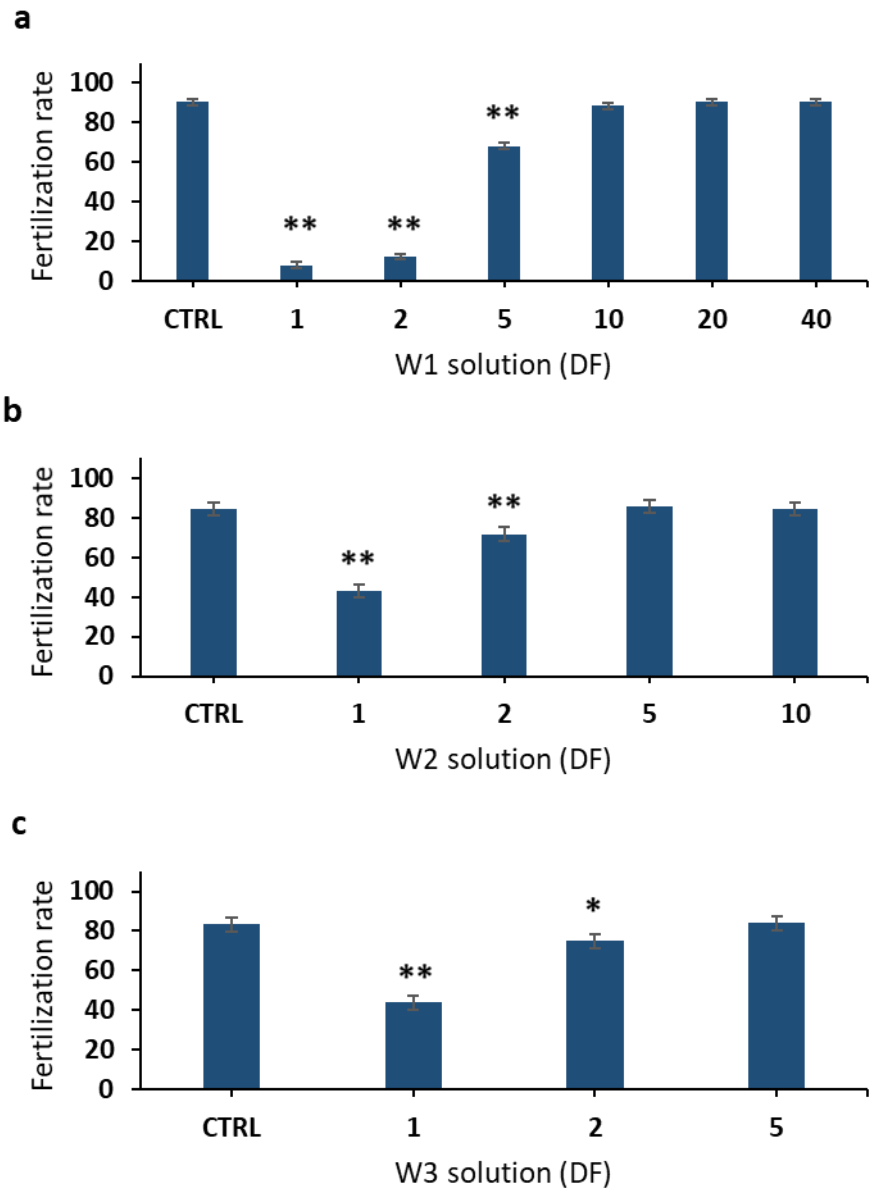


Figure 15. Spermiotoxicity assay with *Arbacia lixula* for Cellulose-based Nanosponges treated seawater assessment. The graphs show the percentage of fertilised eggs after sperm exposure to W1 solution (a), W2 solution (b), and W3 solution (c). On the X-axis, the dilution factors (DF) of the W solutions are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean

± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

3.3.2. Spermiotoxicity assay of Cellulose-based Nanosponges components in sea urchins

Regarding the components of CNS, T solution did not affect sperm fertilising capability in *P. lividus,* indeed the percentages of fertilised eggs were similar to the control (see supplementary materials, section S4, table 1S). Sperm exposure to 10 and 100 μg/mL CA did not significantly affect the fertilisation rate; whereas, 1000 μg/mL CA induced a significant decrease in the fertilisation rate (53 ± 16.27%; P < 0.01) compared to the control (81 ± 10.25%) (Figure 16a). For bPEI solution (Figure 16b), bPEI at the lowest tested concentration (0.01 μg/mL) did not affect the percentage of fertilisation. By increasing bPEI concentration, a significant reduction of the fertilization rate was observed at 0.1 μg/mL bPEI (control 89 ±1.8% *vs* 82 ±1.8%; P < 0.01) up to the total inhibition of fertilization in the highest tested concentrations. In spermatozoa exposed to filtered bPEI at the concentration of 0.01 μg/mL, the fertilisation rate was not affected (Figure 16c); whereas, the exposure to the highest tested concentrations (0.1- 10 μg/mL) caused a significant reduction of the sperm fertilising ability (91 ± 6.26%; 83 ± 6.26%; 39 ± 6.26%; 18 ± 6.26%; 0 ± 6.26%; P < 0.01) compared to the control (92 ± 6.26%).

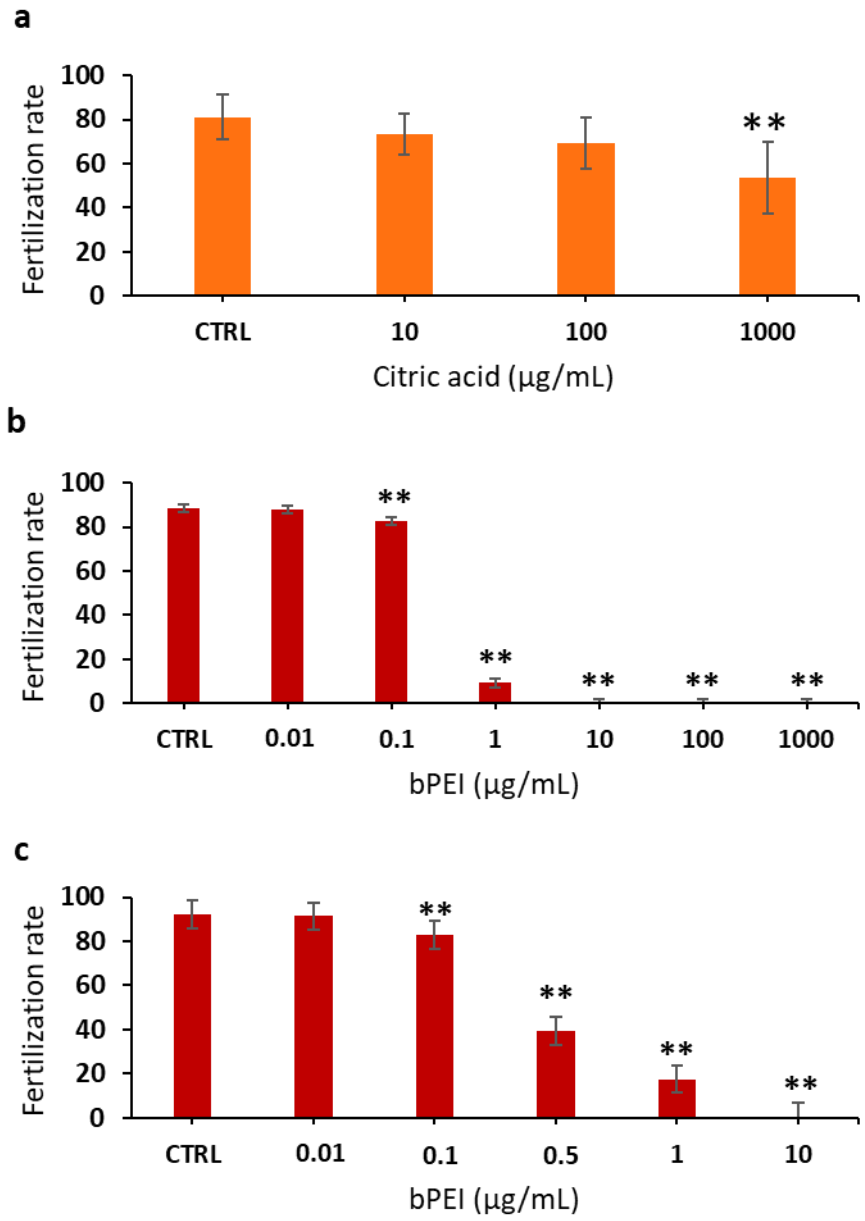


Figure 16. Spermiotoxicity assay with *Paracentrotus lividus* for Cellulose-based Nanosponges component assessment. The graphs show the percentage of fertilised eggs after sperm exposure to CA solution (a), bPEI solution (b), and 0.45 µm filtered bPEI solution (c). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

In *A. lixula*, the exposure of spermatozoa to T solution as well as CA solutions did not affect the fertilising capability of spermatozoa (see supplementary materials, section S4, table 2S and 4S).

The exposure of spermatozoa to 0.01 µg/mL of bPEI did not affect the sperm fertilizing capability; whereas, sperm exposure to 0.1 and 1 µg/mL bPEI concentrations significantly reduced the percentage of fertilised eggs to 78 ± 0.93% and 7 ± 0.93%, respectively (P <

0.01), in comparison to the control (84 ± 0.93%) (Figure 17a). The bPEI at a concentration of 10 µg/mL and higher caused the total absence of fertilised eggs.

Similarly, after sperm exposure to 0.01 µg/mL filtered bPEI, the percentage of fertilisation was comparable to the control; while, at the highest tested concentrations, i.e. 0.1-10 µg/mL filtered bPEI, a significant decrease in the fertilisation rate was observed (Figure 17b).

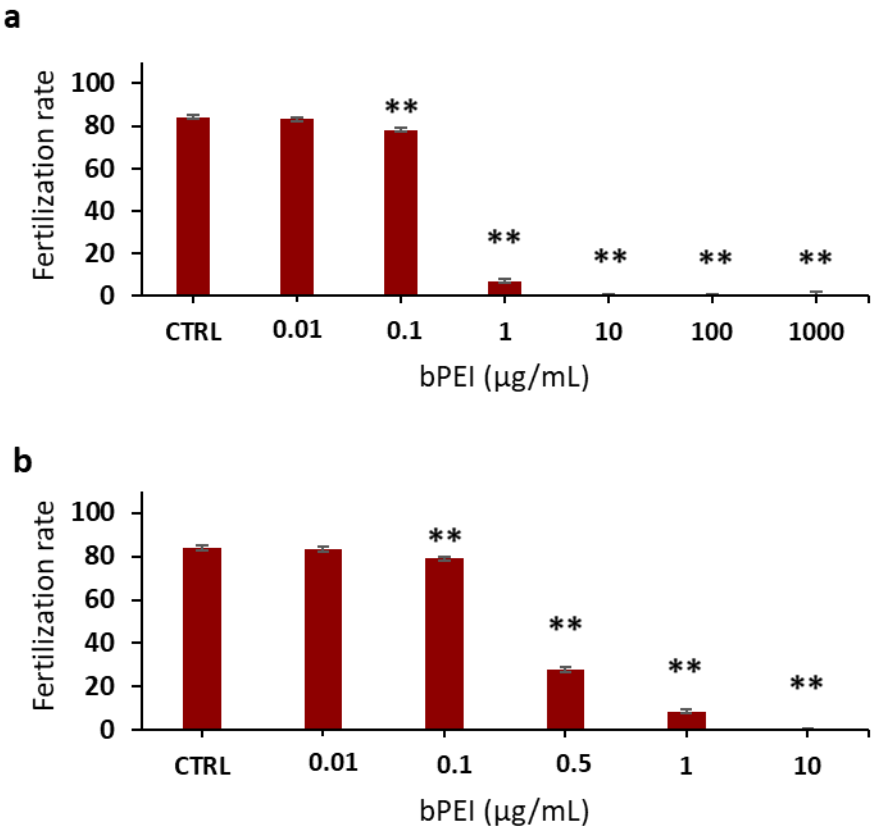


Figure 17. Spermiotoxicity assay with *Arbacia lixula* for Cellulose-based Nanosponges component assessment. The graphs show the percentage of fertilised eggs after sperm exposure to bPEI solution (a) and 0.45 µm filtered bPEI solution (b). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

3.3.3. Ovotoxicity assay of Cellulose-based Nanosponges solution in sea urchins

The W solution significantly affected the egg fertilisation competence in *P. lividus*. Indeed, the exposure of eggs to W1 solution with the DF of 1, 2 and 5 caused a significant reduction of the fertilised egg percentages (44 ± 3.97%, 52 ± 3.97%, 66 ± 3.97%, respectively; P < 0.01) compared to the control (80 ± 3.97%); whereas, by increasing the DF, the percentage of fertilised eggs was not affected (Figure 18a).

The egg exposure to W2 solution with a DF of 1 and 2 significantly decreased the percentage of fertilised eggs (47 ± 2.79%, 66 ± 2.79%, respectively; P < 0.01) compared to the control (80 ± 2.79%). By increasing the DF, no significant effects were detected (Figure 18b).

Similarly, the W3 solution at the DF of 1 and 2 decreased the percentage of fertilised eggs (52 ± 4.34%, 66 ± 3.34%, respectively; $P < 0.01$) compared to the control (80± 3.34%); whereas, after the exposure to W3 solution with the DF of 5 and 10, the oocytes' ability to be fertilised was no longer affected (Figure 18c).

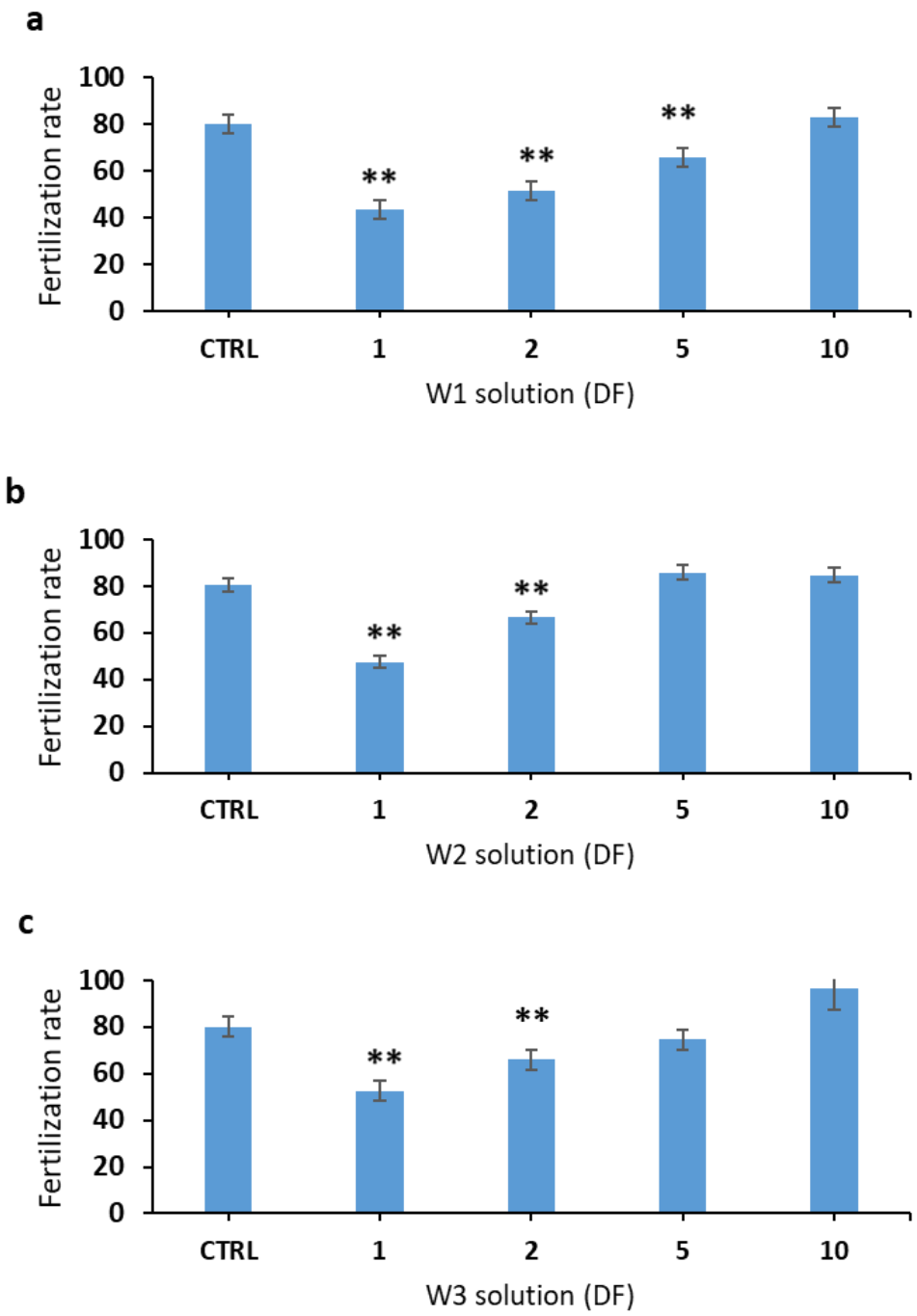


Figure 18. Ovotoxicity assay with *Paracentrotus lividus* for Cellulose-based Nanosponges treated seawater assessment. The graphs show the percentage of fertilised eggs after egg exposure to seawater conditioned with CNS powder (W solution). "W1 solution" (a) refers to seawater conditioned with CNS powder that was washed once. "W2 solution" (b) and "W3 solution" (c) respectively refer to seawater conditioned with CNS powder washed twice and three times. On the X-axis, the dilution factors (DF) of the W solutions are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

In *A. lixula*, W1 solution affected the oocyte fertilisation competence in a dose-dependent manner (14 ± 1.73% at DF 1; 38 ± 1.73% at DF 2; 67 ± 1.73% at DF 5; and 88 ± 1.73% at

DF 10 *vs* 96 ± 1.73%; P < 0.01). By increasing the DF at 20 and 40, this effect was no longer observed (Figure 19a). The W2 solution with a DF of 1 and 2 significantly affected the fertilization rate (control 97 ± 2.13% *vs* 29 ± 2.13%; 72 ± 2.13%; P < 0.01). Similarly, after the exposure to W3 solution at the DF of 1 and 2, the oocyte fertilisation competence was affected (control 96 ± 2.15% *vs* 30 ± 2.15%; 86 ± 2.15%; P < 0.01) (Figure 19b-c).

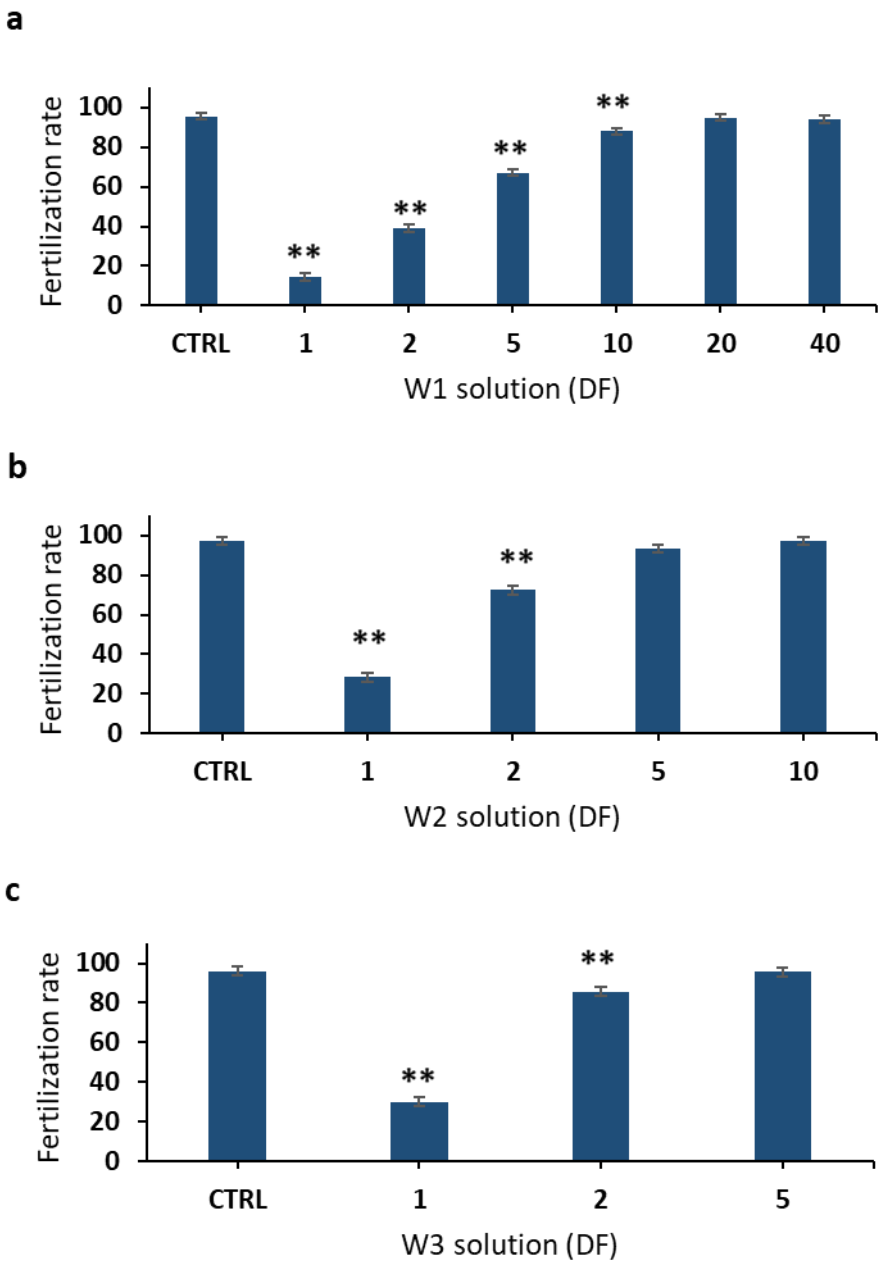


Figure 19. Ovotoxicity assay with *Arbacia lixula* for Cellulose-based Nanosponges treated seawater assessment. The graphs show the percentage of fertilised eggs after egg exposure to seawater conditioned with CNS powder (W solution). "W1 solution" (a) refers to seawater conditioned with CNS powder that was washed once. "W2 solution" (b) and "W3 solution" (c) respectively refer to seawater conditioned with CNS powder washed twice and three times. On the X-axis, the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). The significance level was set at P< 0.05* and P< 0.01** *vs* CTRL (control).

3.3.4. Ovotoxicity assay of Cellulose-based Nanosponges components in sea urchins

The exposure of *P. lividus* eggs to T solution (0.01–1000 µg/mL) as well as the CA solution did not significantly affect the fertilisation competence of eggs (supplementary materials, section S4, tables 1S and 3S).

The exposure of eggs to 0.01 and 0.1 µg/mL bPEI did not affect the percentage of fertilised eggs; otherwise, eggs exposure to 1 up to 100 µg/mL of bPEI induced a significant reduction of the fertilisation rate (control 97± 2.43% *vs* 26 ± 2.43%, 7 ± 2.43%, 8 ± 2.43%; $P < 0.01$) (Figure 20a). A similar trend was observed in bPEI filtered solution (Figure 20b). In particular, fertilisation rate was significantly decreased compared to the control (92 ± 3.21%), after exposure to 0.5, 1 and 10 µg/mL bPEI (66 ± 3.21%, 18 ± 3.21% and 1 ± 3.21%; $P < 0.01$).

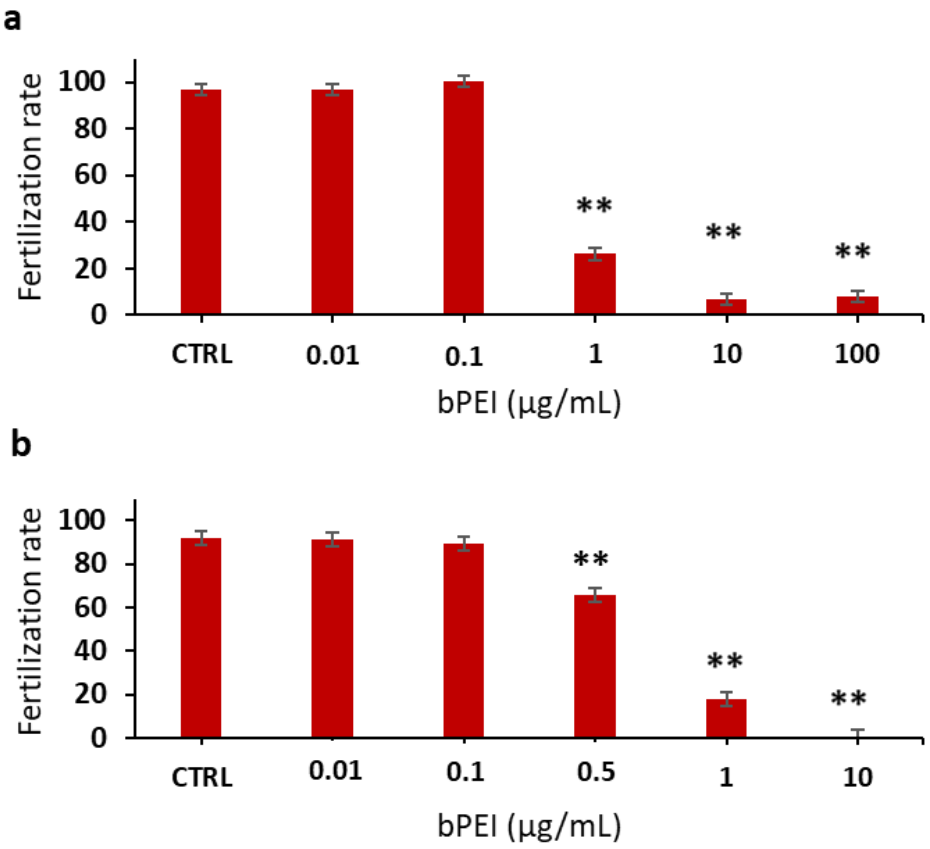


Figure 20. Ovotoxicity assay with *Paracentrotus lividus* for Cellulose-based Nanosponges component assessment. The graphs show the percentage of fertilised eggs after egg exposure to bPEI solutions (a) and 0.45 µm filtered bPEI solutions (b). On the X-axis, the solution concentrations are reported. Data are presented as mean ± standard error (SE). The significance level was set at $P< 0.05$ * and $P< 0.01$ ** *vs* CTRL (control).

In *A. lixula*, the fertilisation rate was not significantly affected by the egg exposure to T solution and CA solution (see supplementary materials, section S4, tables 2S and 4S).

Differently, the exposure of eggs to 1 µg/mL bPEI severely affected the fertilisation rate in *A. lixula* (control 84 ±1,48% *vs* 30 ±1.82%; $P < 0.01$) and a total absence of fertilised eggs was detected at the highest tested bPEI concentrations (Figure 21a).

Figure 21b shows the effects of egg exposure to the filtered bPEI solution. Also in this case, the fertilisation rate was not significantly affected at 0.1 µg/mL bPEI and significantly decreased after egg exposure to filtered bPEI concentrations greater than or equal to 0.5 µg/mL (45 ± 0.87% at 0.5 µg/mL; 43 ± 0.87% at 1 µg/mL and 0 ± 0.87% at 10 µg/mL; $P < 0.01$).

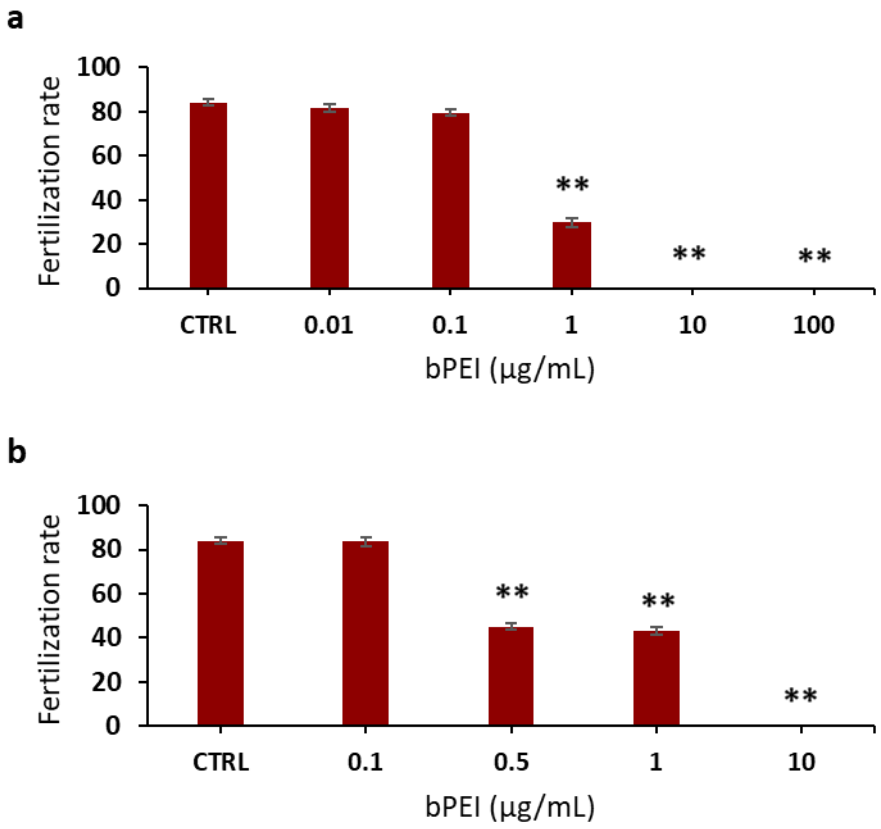


Figure 21. Ovotoxicity assay with *Arbacia lixula* for Cellulose-based Nanosponges component assessment. The graphs show the percentage of fertilised eggs after egg exposure to bPEI solution (a) and filtered bPEI solution (b). Data are presented as mean ± standard error (SE). The significance level was set at $P< 0.05$* and $P< 0.01$** *vs* CTRL (control).

3.4. Discussion

In the present chapter, the impact of CNS and its components on sperm and egg fertilisation competence in sea urchins have been investigated by means of spermiotoxicity and

ovotoxicity tests, respectively. Gamete fertilisation competence is the biological endpoint assessed in the spermiotoxicity and ovotoxicity tests. Indeed, the occurrence or not of fertilisation is a direct index of the impact of contaminants on the reproduction and survival of living organisms. The spermiotoxicity test has long been employed in *P. lividus* as a useful tool to assess the impact of different contaminants, including nanomaterials (Pagano et al., 2017). In particular, the spermiotoxicity test revealed that the sperm fertilising capability of the sea urchin *P. lividus* was not affected by the exposure to different NPs (SiO_2, Sn O_2, Ce O_2, Fe_3 O_4, Ag, Ti O_2, and Co NPs) as well as to nanosized ZnO NMs (Manzo et al., 2013). Similarly, the sperm fertilising capability of the sea urchin *Strongylocentrotus intermedius* was not impaired by carbon nanotubes, carbon nanofibers, silicon nanotubes, nanocrystals of cadmium and zinc sulphides, gold and titanium dioxide NPs (Pikula, et al., 2020b). On the contrary, the spermiotoxicity test has revealed that nZVI NPs impaired sperm fertilising ability in sea urchin *Psammechinus milliaris* (Kadar et al., 2013). To our knowledge, the spermiotoxicity test with the sea urchin has not been previously performed for testing the toxicity of seawater conditioned with nanostructured materials; therefore, it is not possible to compare the results herein reported with the toxicity of NPs reported in the literature.

Herein, the suitability of the spermiotoxicity test has been evaluated using the reference toxicants and, in both species, their EC_{50} fell within the defined acceptability ranges, as reported in figures 3S and 4S of the supplementary materials, section S3 (Nacci et al., 1986; Dinnel et al., 1987; Volpi Ghirardini & Arizzi Novelli, 2001; Lera & Pellegrini, 2006). Additionally, for *A. lixula*, some trials were previously performed to set the optimal experimental conditions for this species (see supplementary materials, section S1, figure 2S). Subsequently, the spermiotoxicity test has been applied to investigate the potential impact of CNS leachate and its components on the sperm fertilising ability in *P. lividus* and *A. lixula*. In both species, this test revealed that W1, W2 and W3 solutions at all the dilutions tested negatively affected the sperm fertilising ability, indeed fertilisation rate significantly decreased.

The reproductive success of living organisms relies on sperm but also on egg fertilisation competence. Therefore, the ovotoxicity test is aimed to assess the potential effects of contaminants on egg fertilisation competence and has been rarely applied to evaluate the effects of contaminants on marine organisms' reproduction. Up to date, this bioassay has been performed only with the sea urchin *S. intermedius* revealing a low inhibition of egg fertilisation after carbon and silicon nanotubes exposure (Pikula et al., 2020a). In the present thesis, the ovotoxicity test with *P. lividus* and *A. lixula* has been developed to assess ENM toxicity. Like the spermiotoxicity assay, the ovotoxicity assay is an acute test that measures the inhibition of fertilisation defined by the elevation of the fertilisation membrane around

the egg after exposure to ENM. In order to set up and validate a reliable protocol for the ovotoxicity test, some experimental trials have been performed to set the optimal conditions with *A. lixula* (see supplementary materials, section S1, figure 3S). Then, the acceptability of the ovotoxicity test has been assessed by using the specific reference toxicant in each species (see supplementary materials, section S3, figures 5S and 6S).

The ovotoxicity tests with the *P. lividus* and *A. lixula* revealed that the W1, W2 and W3 solutions, with low dilution factors, affected the egg fertilisation competence in both sea urchin species. Moreover, *A. lixula* showed high sensitivity to this assay since the W1 solution 10-fold diluted impaired the egg's capability to be fertilised.

The observed effects of CNS on fertilisation are clearly associated with chemicals that may leach into seawater being CNS removed by filtration. The CNS is composed of three components as TOCNF, acid citric and bPEI, which have been investigated one by one. TOCNF did not affect gamete fertilisation competence in both *P. lividus* and *A. lixula*, supporting the safety of cellulose nanofibers in sea urchins, as previously demonstrated in other aquatic species (Pengiran et al., 2021). Similarly, citric acid did not impair the ability of both gametes to fertilise or to be fertilised, except for the highest tested concentration (1000 μg/mL). In male gametes, this result was expected since citric acid is a fundamental component of the seminal plasma (Huang et al., 2013; Valdebenito et al., 2015). Among the components of CNS, the bPEI resulted in the most toxic compound. Each gram of CNS contains 440 mg of bPEI, which falls within the range of concentrations tested. Hence, in a hypothetical scenario, in which the total amount of bPEI content in a gram of sponge was released into seawater due to prolonged CNS use, an ecological risk for sea urchins may occur. Indeed, bPEI severely impairs the fertilisation competence of male and female gametes in both sea urchin species. Although the cytotoxicity of bPEI has been widely documented in animal cell lines including those of marine fish (Yoon et al., 2008), its toxicity on marine organisms has been scarcely investigated. It has been reported that bPEI exerted toxic effects in marine and freshwater algae, as well as in marine bacteria (Mikula et al., 2018; Fiorati et al., 2020; Mortimer et al., 2008). In the present project, the cytotoxicity of bPEI has been reported for the first time in sea urchin gametes and it can be *bona fide* responsible for the low fertilisation rate observed after CNS leachate pre-treatment of gametes in the spermiotoxicity and ovotoxicity tests. In conclusion, in the framework of the eco-design approach (Figure 1), data resulting from the bPEI ecotoxicology support the need to introduce modifications in the earlier phases of the CNS development to obtain a more eco-safe device for marine environmental remediation.

CHAPTER 4

Ecosafety assessment of Cellulose-based Nanosponges and their components on sea urchin gamete quality

4.1. Introduction

The reproductive success of the living organisms is strictly related to gamete quality defined as the ability of gametes to fertilise or to be fertilised and subsequently develop into a normal embryo (Bobe & Labbé, 2010). A good quality oocyte must be mature, presenting a species-specific size with a homogeneous cytoplasm without vacuoles or granules, and showing a good morphology and membrane integrity. Moreover, oocytes must contain optimal mitochondrial numbers and sufficient levels of ATP to produce good quality embryos after fertilisation (May-Panloup et al., 2007; Morici et al., 2007). Spermatozoa are highly specialised cells in their structures and functions possessing unique features such as the small size, the flagellum for movement and exclusive DNA and chromatin packaging. In marine species, the evaluation of oocyte quality is commonly based on morphological criteria, such as size, shape, transparency, chorion aspects, distribution and volume of lipid droplets, lipid content and floatability rate, as well as fertilisation and development success assessment (Bobe & Labbé, 2010; Valdebenito et al., 2015). Recently, different physiological markers, such as mitochondrial activity, the production of reactive oxygen species (ROS) and intracellular pH have been also reported as useful indicators of oocyte competence (Agnello et al., 2017; Torrezan-Nitao et al., 2018). Regarding sperm quality, it is commonly assessed by evaluating different parameters such as concentration, motility, morphology, vitality, mitochondrial activity, intracellular ROS levels, DNA damage as well as fertilising capability and offspring quality (Stampino et al., 2021; Gallo et al., 2021). To date, these quality parameters are also measured to investigate the exposure effects of different environmental stressors on gamete quality (Lettieri et al., 2021).

Gamete quality can be strongly influenced by environmental stressors. Particularly, in marine organisms with external fertilisation, such as sea urchins, gametes are directly exposed to environmental stressors, which can alter their quality and, in turn, fertilisation and embryo development success with severe consequences on species fitness and survival (Au et al., 2001; Lettieri et al., 2019). Alterations of gamete quality and, in turn, fertilisation competence and embryo survival, have been widely demonstrated after exposure to diverse physical and chemical contaminants (Gallo et al., 2020). Nonetheless, the literature lacks sufficient studies on the evaluation of gamete quality parameters after ENMs exposure.

In this chapter, different parameters of gamete quality that underlie the fertilisation and developmental competence, such as concentration, motility, morphology, mitochondrial activity, intracellular ROS levels, plasma membrane lipid peroxidation, have been evaluated for the first time to assess the potential impact of CNS leachate on gamete quality in *P. lividus* and *A. lixula*.

4.2. Materials and methods

4.2.1. Physiological assessment of gametes

Spermatozoa and eggs were exposed for 1 h at 18°C to the experimental solutions described in section 2.2.2. Then, diverse physiological parameters of gamete quality have been assessed by using fluorescent staining coupled with fluorescence spectroscopy. For each parameter, a specific fluorochrome has been employed and the specific fluorescence spectra were recorded at the spectrofluorometer (Shimadzu RF-5301, Tokyo, Japan) for spermatozoa and at the microplate reader (Tecan Infinite® m1000 pro) for the eggs.

The correct localization of each fluorochrome used to analyse the gamete quality parameters was assessed by confocal laser scanning microscopy. Briefly, sperm ($5x10^8$ spermatozoa/mL) and egg (500 egg/mL) aliquots were incubated with different fluorochromes and stained.

For spermatozoa, each aliquot was diluted 1:1 with a solution of glutaraldehyde (0.02 %) to block the sperm movement, mounted on a microscope slide and then observed under the confocal laser-scanning microscope (Zeiss LSM 510) with a 63X oil immersion objective.

For the eggs, preliminary experiments revealed that exposure to CNS and bPEI caused the egg adhesiveness to the bottom of the Petri dish, making it difficult to recover the eggs for the subsequent staining. Therefore, eggs were incubated in different supports: glass Petri dishes, plastic test tubes, and untreated multiwell plates, but the adhesiveness persisted. Finally, agarized multiwell plates were prepared to limit egg adhesion to the bottom of the 12-well plates.

4.2.1.1. Intracellular pH

The intracellular pH (pH_i) was evaluated by using the cell-permeant dye 2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Life Technologies, Milan, Italy), a membrane-permeant probe with a lateral group linked to fluorescein, which, inside the cell, is hydrolysed by cytosolic esterase into the intracellularly trapped indicator BCECF, whose fluorescence intensity is dependent upon the pH. For the sperm pH_i assessment, 200 µL aliquots of sperm suspensions ($1x10^6$ spermatozoa/mL) were

incubated in the presence of 5 µM BCECF-AM for 30 min in the dark at 18°C. Samples were centrifuged at 900x*g* at 4° C for 10 min, the pellet was resuspended in 200 µL FNSW and incubated for additional 30 min in the dark to allow the de-esterification of the fluorochrome. Finally, samples were centrifuged, the sperm pellet resuspended in 900 µL of FNSW and analysed to the spectrofluorometer in duplicate.

For the egg pH_i evaluation, the same procedure was performed except for the centrifugation. In the last step, egg samples were suspended in 600 µL FNSW, split into three wells of the 96-multiwell plates and finally analysed at the microplate reader.

BCECF-AM is a dual-excitation ratiometric pH indicator. Hence, for both gametes, the excitation wavelengths were set at 440 and 490 nm and the emission wavelength at 535 nm. The results were expressed, referring to the calibration curve, as a ratio between the emission intensity at 535 nm after excitation at 490 nm and the emission intensity at 535nm after excitation at 440 nm.

The stock calibration solution (290 mOsm) was prepared by dissolving KCl (135 mM) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (5mM) in double distilled water. Then, by using a bench pHmeter Mettler-Toledo™, the pH of the stock solution was adjusted using HCl or NaOH to obtain three solutions with the reference pH values of 6.5 - 7 - 7.5. An aliquot of the control sperm suspension was incubated in each of the three calibration solutions containing the ionophore nigericin (5 µM) (Merck Life Science).

The latter acts as K^+/H^+-antiporter suppressing the intra- and extra-cellular pH gradient in the presence of a depolarizing concentration of extracellular K^+. Hence, the fluorescent emissions expressed as ratiometric data were converted into pH_i values based on the linear regression analysis.

4.2.1.2. Mitochondrial activity

Mitochondrial activity was assessed through the analyses of the mitochondrial membrane potential (MMP) using the mitochondrial dye JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Life Technologies), a lipophilic dye able to permeate plasma membrane and accumulate as red fluorescent aggregates in mitochondria with high membrane potential, or, as a monomer, in mitochondria characterised by low membrane potential.

For the MMP evaluation in spermatozoa, 200 µL aliquots of sperm suspensions ($1x10^6$ spermatozoa/mL) were incubated with JC-1 (5 µM), in the dark, at 18°C for 30 min. Samples were centrifuged at 900x*g* at 4°C for 10 min, the pellet was resuspended in 200 µL of FNSW

and incubated for an additional 30 min under the same conditions. Finally, the pellets were resuspended in 900 µL of FNSW and analysed in duplicate at the spectrofluorometer.

For the MMP assessment in eggs, the same protocol was carried out except for the centrifugation. Egg pellets were suspended in 600 µL FNSW and split into three wells of the 96 multiwell plate in order to read them in triplicate using a microplate reader. As a positive control, a stained aliquot of the control sample was incubated in 5 µM CCCP (Carbonyl cyanide 3-chlorophenylhydrazone; Merck Life Science), a protonophore that inhibits the oxidative phosphorylation by increasing the proton permeability across the mitochondrial inner membrane and leading to the transmembrane potential dissipation. The resulting mitochondria membrane depolarization can be detected with the shift of JC-1 from aggregate to monomeric form.

The fluorescence emission spectra were recorded setting the excitation wavelength at 488 nm and the emission wavelength in the range of 500-620 nm. The MMP was calculated as a ratio of the fluorescence peak values at ~595 nm and ~525 nm.

4.2.1.3. Oxidative status

Intracellular reactive oxygen species (ROS)

To determine the intracellular ROS levels, two fluorochromes were used: the 2′,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Life Technologies,) and the dihydroethidium (DHE; Life Technologies) to specifically detect the hydrogen peroxide (H_2O_2) and the superoxide ion (O_2^-). The H_2DCF-DA is an apolar dye able to permeate cell membranes. Once in the cell, esterase enzymes remove the acetate groups producing the 2′,7-dichlorodihydrofluorescein (H_2DCF), which is polar and, therefore, unable to cross cell membranes. H_2DCF is oxidised mainly by H_2O_2 to the highly fluorescent 2′,7-dichlorofluorescein (DCF), whose fluorescence intensity is proportional to the intracellular ROS levels (Giorgio et al., 2007).

For H_2O_2 determination in spermatozoa, 200 µL aliquots of sperm suspensions ($5x10^6$ spermatozoa/mL) were incubated in H_2DCF-DA (10 µM), in the dark, at 18°C for 30 min. Samples were centrifuged at 900x*g* at 4° C for 10 min, the pellet was resuspended in 200 µL FNSW and incubated for additional 30 min in the same conditions. Subsequently, each sperm suspension was centrifuged, resuspended in 900 µL of FNSW and analysed in duplicate. Positive control was prepared by incubating an aliquot of the stained spermatozoa with hydrogen peroxide (25 µM) for 1 h. The emission spectra were recorded in a range of 500-560 nm, setting the excitation wavelength at 488 nm.

DHE (Life technologies) is a vital probe used for the detection of intracellular O_2^-. Inside the cells, DHE can be oxidised by O_2^- generating a specific product, the2-hydroxyethidium (2-OH-E^+) or by other ROS species forming nonspecific products as the ethidium (E^+) (Kalyanaraman et al., 2017). These two HE-derived products are fluorescent and their emission spectra overlap in the red range; however, it is possible to detect selectively the emission of 2OH-E by setting the excitation wavelength at 350 nm (Nazarewicz et al., 2013). For O_2^- assessment in spermatozoa, 200 µL aliquots of sperm suspensions ($60x10^6$ spermatozoa/mL) were incubated in 2 µM DHE, in the dark, at 18°C for 30 minutes and, then, resuspended in 900 µL of FNSW before the analysis in duplicate in the spectrofluorometer. The intracellular O_2^- level was specifically quantified by measuring the fluorescence intensity of 2OH-E setting the excitation and emission wavelengths at 350 nm and ~600 nm, respectively (Luchetti et al., 2009).

For ROS determination in eggs, the same staining and spectrofluorometric protocols used for the spermatozoa were carried out except for centrifugations. Egg pellets were suspended in 600 µL FNSW and split into three wells of the 96 multiwell plate in order to read them in triplicate using a microplate reader.

Lipid Peroxidation

The lipid peroxidation (LPO) of plasma membranes was detected by using the fluorescent dye C11-BODIPY$^{581/591}$, a fatty acid analogue that is easily incorporated in membranes, where it emits in red fluorescence (~595 nm) in absence of peroxidation or in green fluorescence (peak ~ 525nm) if peroxidation occurs.

For LPO assessment in spermatozoa, aliquots of 200 µL of sperm suspensions ($20x10^6$ spermatozoa/mL) were incubated in 5 µM C11-BODIPY$^{581/591}$ for 30 min in the dark at 18°C. Samples were centrifuged at 900x*g* at 4°C for 10 min, the pellet resuspended in 200 µL FNSW and incubated for additional 30 min. Sperm solutions were centrifuged, and the pellets were resuspended in 900 µL of FNSW and analysed in duplicate using a spectrofluorometer. The positive control consisted of an aliquot of the stained control sample exposed for 1 h in the dark, at 18°C, in a solution of ascorbic acid (750 µM) and ferrous sulphate ($FeSO_4$, 150 µM) that promotes peroxidation (Bansal & Bilaspuri, 2008).

The same staining protocol was carried on in the eggs, except for the centrifuge. In the last step, samples of eggs were suspended in 600 µL FNSW and split into three wells of the 96 multiwell plate in order to read them in triplicate using a microplate reader.

Fluorescence intensity was measured at an excitation wavelength of 488 nm and emission wavelengths of 500-620 nm. The peroxidation level was expressed by the ratio between the

green peak (~525 nm) and the sum of the two peaks detected in green (~525 nm) and red (~595 nm) wavelengths.

4.2.1.4. Sperm motility

To assess sperm motility, a small drop of sperm solution ($80x10^6$ spermatozoa/mL), previously exposed to the test solutions for 1 h at 18°C, was placed on a sperm counting chamber and a visual assessment was performed using a microscope with an objective 40X evaluating at least 5 visual fields. Sperm motility percentage was calculated as a ratio between the number of motile spermatozoa and the total sperm number.

4.2.2 Morphological assessment of gametes: Scanning electron microscopy (SEM)

Sperm and egg morphology was evaluated by using the scanning electron microscope (SEM). Following exposure to W solution (1.25 g/L), spermatozoa ($50x10^6$ spermatozoa/mL) and eggs (2000 eggs/mL) were fixed for 1 h at room temperature in 1% glutaraldehyde solution in 0.2M sodium cacodylate buffer (pH=7.2) and 20% FNSW. To preserve lipid membranes, specimens were washed once for 10 min in sodium cacodylate buffer, twice in distilled water and for 1 h at room temperature in 1% osmium tetroxide in distilled water. Dehydration was performed in ascending concentrations of ethanol (30, 50, 70, 90, and 100%). Finally, after being mounted on stubs, specimens were coated with palladium and examined under the scanning electron microscope (JEOL JSM 6700F microscope).

4.2.3. Statistical analysis

Statistical analysis of the gamete quality parameters was performed using Systat 11.0 (Systat Software Inc.).

The statistical evaluation of the treatments was obtained using parametric one-way analysis of variance followed by Fisher's least significant difference post hoc test performed to determine significant differences in treatment means. Differences were considered significant at a P value lower than 0.05 ($P \leq 0.05$) and 0.01 ($P \leq 0.01$). Data are reported as mean ± standard error (SE).

4.3. Results

4.3.1. Validation of fluorochrome localization in sea urchin gametes with the Confocal Laser Scanning Microscope (CLSM)

The specific localization of all the fluorochromes used for the gamete quality assessment has been verified with the Confocal Laser Scanning Microscope (CLSM). Figures 22 and 23 show the localization of fluorochromes in the spermatozoa and mature eggs, respectively.

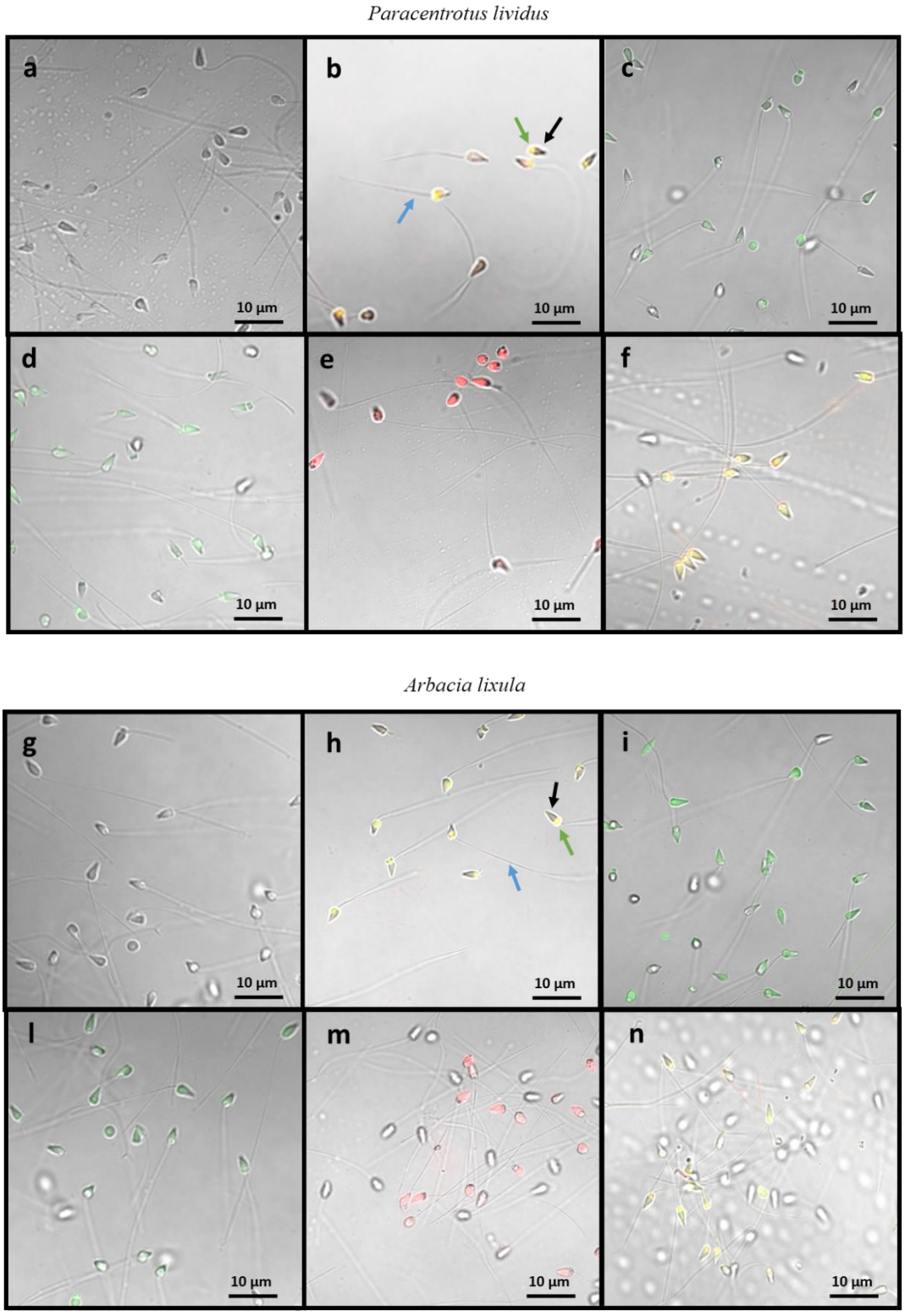


Figure 22. Confocal images of *Paracentrotus lividus* and *Arbacia lixula* spermatozoa unstained (a, g) and stained with the following fluorescent dyes: (b, h) JC-1, which is a MMP marker that localises in the mitochondria as red fluorescent aggregates (~595 nm) or green fluorescent monomers (~525 nm) depending on the high or low MMP, when excited at 488 nm; the co-localization of mitochondria characterised by high MMP and those with low MMP generates the orange fluorescence; (c, i) BCECF-AM is an intracellular pH marker that localises in the cytoplasm emitting in the green range once excited at 488 nm (d,l) H_2DCF-DA is a specific marker of hydrogen peroxide levels, it localises in the cytoplasm emitting in the green range (500- 560 nm) once oxidised by hydrogen peroxide and excited

at 488 nm; (e,m) DHE is a cytoplasmic marker of intracellular content of superoxide anions, it emits a red fluorescence (~600 nm) once oxidised by superoxide anions and excited at 488 nm; (f, n) C11-BODIPY$^{581/591}$ is a plasma membrane lipid peroxidation marker, therefore when it is excited at 488 nm it emits in red fluorescence (~595 nm) or in green fluorescence (~525 nm), respectively in absence and in presence of peroxidation. Arrows in the image indicate the anatomical structures of the spermatozoa: head (black arrow); mitochondria (green arrow); flagellum (blue arrow).

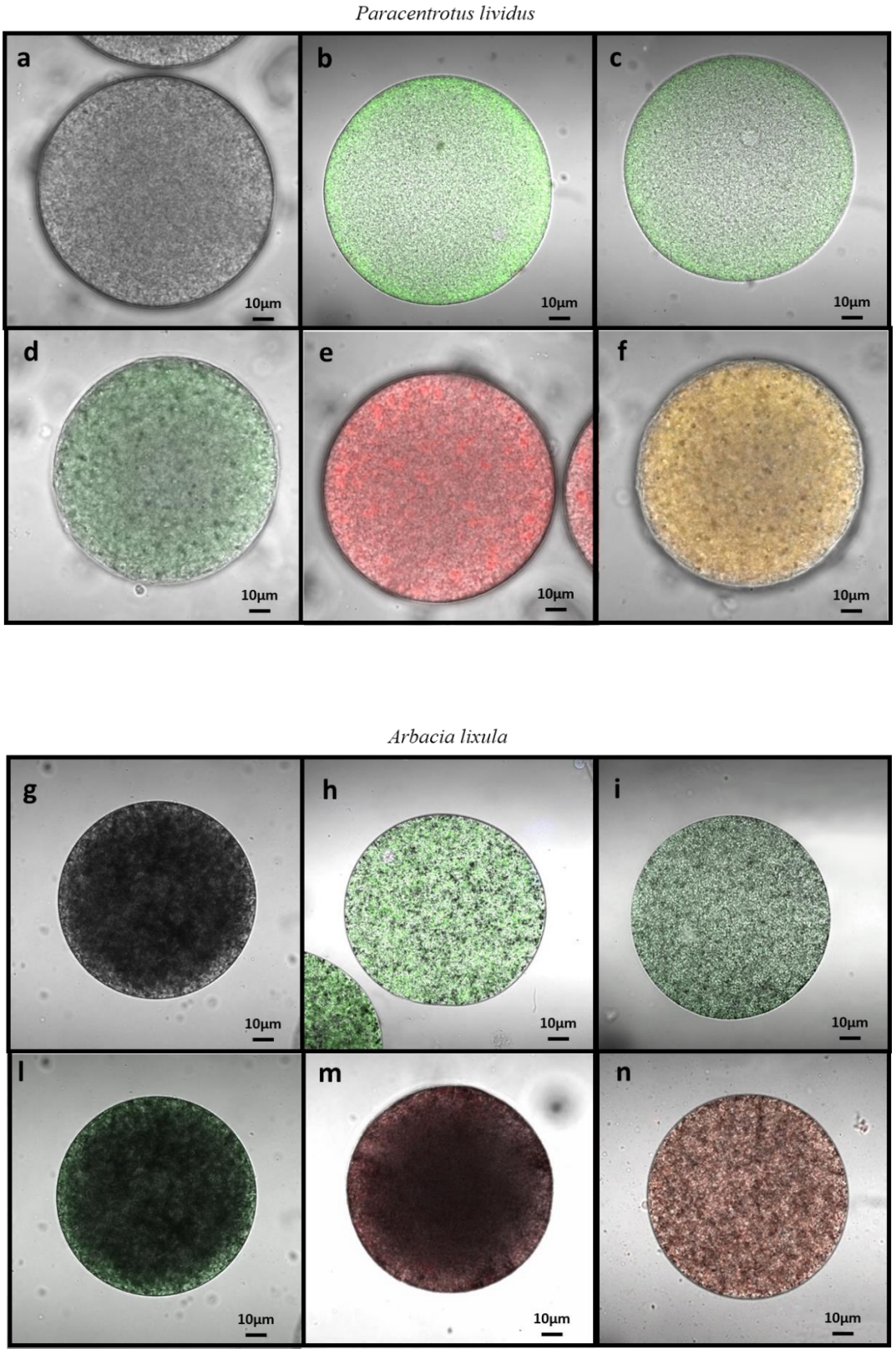


Figure 23. Confocal images of *Paracentrotus lividus* and *Arbacia lixula* eggs (a, g) unstained and stained with the following dyes: (b, h) JC-1 is a MMP marker that localises in the mitochondria as red fluorescent aggregates (~595 nm) or green fluorescent monomers (~525 nm) depending on the high or low MMP, when excited at 488 nm; mature eggs have a basal metabolism and thereby, a low mitochondrial activity, so only green fluorescence was observed; (c, i) BCECF-AM is an intracellular pH marker that localises in the cytoplasm emitting in the green range once excited at 488 nm (d, l) H_2DCF-DA is a specific marker of hydrogen peroxide levels, it localises in the cytoplasm emitting in the green range (500- 560

nm) once oxidised by hydrogen peroxide and excited at 488 nm; (e, m) DHE is a cytoplasmic marker of intracellular content of superoxide anions, it emits a red fluorescence (~600 nm) once oxidised by superoxide anions and excited at 488 nm; (f, n) C11-BODIPY$^{581/591}$ is a plasma membrane lipid peroxidation probe, after excitation at 488 nm it emits in red fluorescence (~595 nm) or in green fluorescence (~525nm), respectively in absence and in presence of peroxidation.

4.3.2. Sperm quality assessment after Cellulose-based Nanosponges exposure

4.3.2.1. Intracellular pH

In *P. lividus*, the intracellular pH of spermatozoa was not significantly affected by the exposure to CNS conditioned seawater, in all the tested solutions (see supplementary materials section S6, table 1S).

Similarly, in *A. lixula*, all tested solutions did not show any significant effect on the pH_i of spermatozoa (see supplementary materials section S6, table 2S).

4.3.2.2. Mitochondrial activity

In *P. lividus*, the exposure of spermatozoa to W solution affected the mitochondrial activity inducing a mitochondrial membrane hyperpolarization (Figure 24). The W1 solution with a DF 1 significantly increases the MMP of *P. lividus* spermatozoa (26.31 FI ± 1.67; $P < 0.05$), compared to the control (19.95 FI ± 2.14), while the W1 solution with the DF of 2, 5, 10, MMP values comparable to the control were recorded. Sperm exposure to W2 and W3 solutions did not significantly affect the MMP (supplementary materials section S6, table 1S).

As in *P. lividus*, the spermatozoa of *A. lixula* exposed to W1 solution showed an alteration of the MMP (Figure 24). In fact, the W1 solution with a DF 1 significantly increased the MMP, inducing a mitochondrial membrane hyperpolarization (22.92 FI ± 2.44; $P < 0.05$), compared to the control (15.08 FI ± 1.51); whereas the W solution at the DF of 2, 5, and 10, did not affect the MMP. Spermatozoa exposed to W2 solution with the DF of 1, 2, 5, and 10 showed MMP values similar to those recorded in the control. Finally, spermatozoa exposed to W3 solution, at all tested dilutions, presented MMP values comparable to the control (supplementary materials section S6, table 2S).

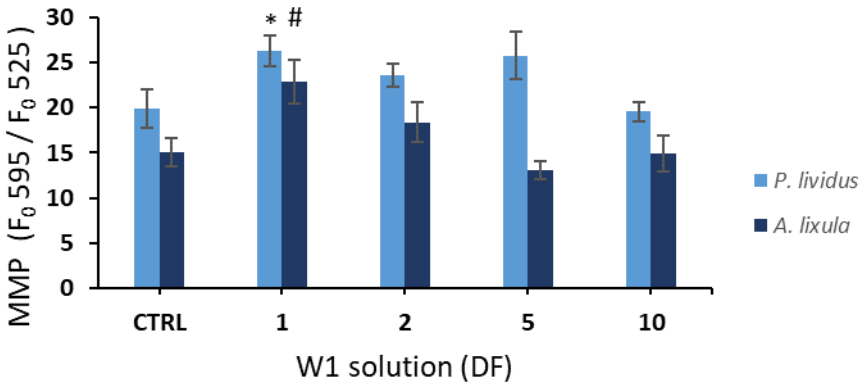


Figure 24. The graph shows the mitochondrial membrane potential (MMP) of sea urchin spermatozoa after exposure to W1 solution, i.e. seawater conditioned with CNS powder washed once. On the X-axis the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

4.3.2.3. Oxidative status

The oxidative status of spermatozoa was investigated by assessing the levels of two free radical species, i.e. the hydrogen peroxide and the superoxide anion, and the plasma membrane lipid peroxidation (LPO). As reported in Figure 25, in *P. lividus*, the levels of hydrogen peroxide significantly increased after exposure to W1 solution with DF of 1 compared to the control (control 334.51 FI ± 75.92 *vs* 593.84 FI ± 75.92; P < 0.05), while in the W1 solution with the DF of 2, 5 and 10 no significant differences were observed. Sperm exposure to W2 and W3 solution at all tested dilutions did not evidence any significant change in the levels of hydrogen peroxide compared to the control (supplementary materials section S6, table 1S).

In *A. lixula*, only spermatozoa exposed to W1 solution diluted once and twice showed a significant increase in hydrogen peroxide levels (367.80 FI ± 20.44 and 336.29 FI ± 20.90; P < 0.01 and P < 0.05) compared to the control (221.32 FI ± 21.84) but increasing the DF this effect was no longer observed (Figure 25). In sperm exposed to W2 and W3 solution, the levels of hydrogen peroxide were all comparable to the control (supplementary materials section S6, table 2S).

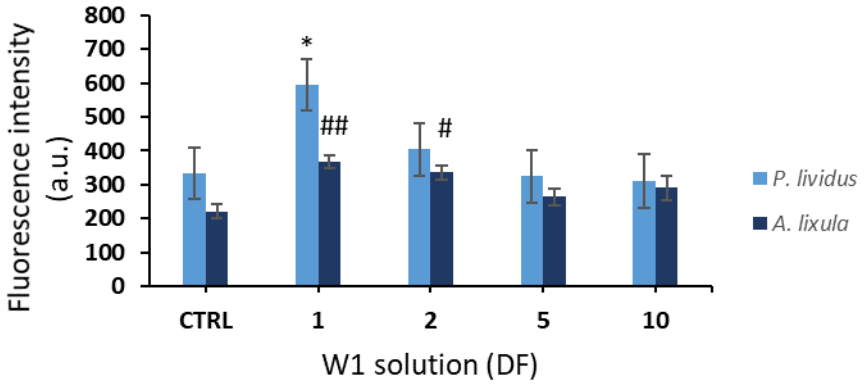


Figure 25. The graph shows the intracellular levels of hydrogen peroxide (H_2O_2) in sea urchin spermatozoa after the exposure to W1 solution, i.e. seawater conditioned with CNS powder washed once. On the X- axis the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

Regarding the levels of superoxide anion in *P. lividus* spermatozoa, they did not significantly differ from those recorded in the control (103.6 FI ± 9.87) after exposure to W1, W2 and W3 solution (supplementary materials section S6, table 1S).

Differently, in *A. lixula* spermatozoa exposed to the W1 solution with DF 1, the levels of superoxide anion (383.16 FI ± 26.50; P < 0.05) significantly increased with respect to the control (298.77 FI ± 17.25) (Figure 26), while for the diluted W1 solution and for all W2 and W3 solutions, no significant effect on superoxide anion levels were detected (see supplementary materials section S6, table 2S).

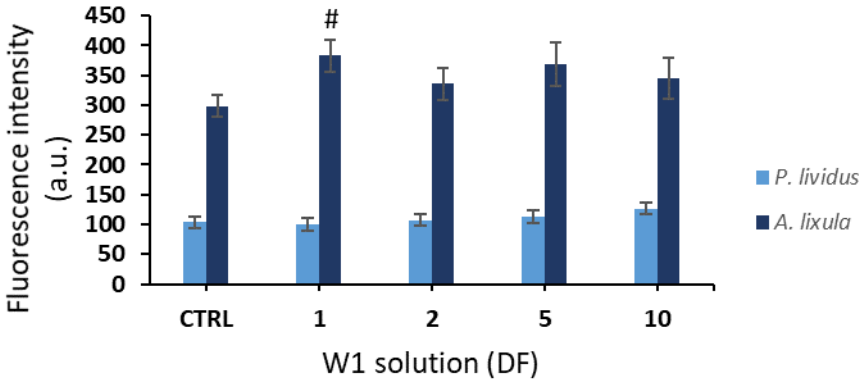


Figure 26. The graph shows the intracellular levels of superoxide anion (O_2^-) in sea urchin spermatozoa after the exposure to W1 solution, i.e. seawater conditioned with CNS powder washed once. On the X- axis the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant

differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

The exposure of *P. lividus* and *A. lixula* spermatozoa to W1, W2 and W3 solution, at all tested dilutions, did not significantly change the lipid peroxidation (LPO) levels compared to the unexposed (control) spermatozoa (see supplementary materials section S6, tables 1S and 2S).

4.3.2.4. Sperm motility

In *P. lividus*, the motility of spermatozoa was significantly reduced after exposure to W1 solution with the DF of 1, 2 and 5 (7 ± 6%; 18 ± 6%; 25 ± 6%; P < 0.01), compared to the control (69 ± 6%), while sperm exposure to W1 solution with the DF of 10 did not significantly affect sperm motility (54 ± 6%) (Figure 27). On the contrary, in W2 and W3 solutions, the sperm motility did not significantly differ from the controls for all tested DF (supplementary materials section S6, table 1S).

In *A. lixula*, the motility of spermatozoa was significantly reduced compared to the control (85 ± 4%) only after exposure to W1 solution with DF of 1 (53 ± 7%; P < 0.01) (Figure 27). The W2 and W3 solutions at all tested DF did not significantly affect the sperm motility (supplementary materials section S6, table 2S).

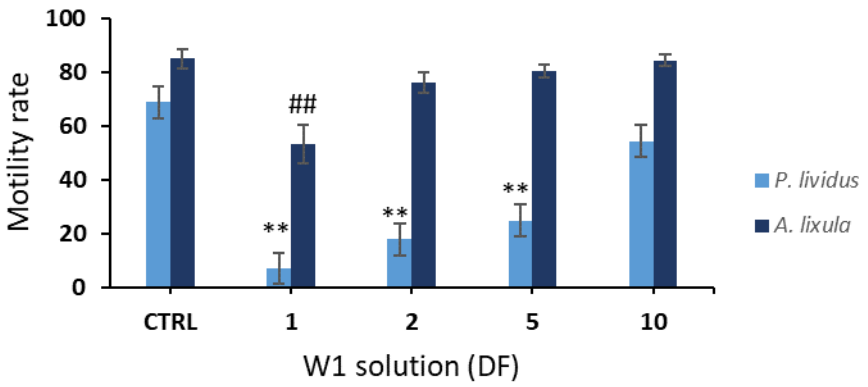


Figure 27. The graph shows the percentage of motile sea urchin spermatozoa after exposure to W1 solution, i.e. seawater conditioned with CNS powder washed once. On the X- axis the dilution factors (DF) of the W1 solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

4.3.3. Sperm quality assessment after exposure to Cellulose-based Nanosponges components

4.3.3.1. Intracellular pH

The CNS components tested one by one did not affect the pH_i in *P. lividus* and *A. lixula* spermatozoa (see supplementary materials section S6, table 3S and 4S).

4.3.3.2. Mitochondrial activity

In *P. lividus*, the exposure of spermatozoa to bPEI, CA and TOCNF did not affect the mitochondrial activity (supplementary materials section S6, table 3S). On the contrary, the spermatozoa of *A. lixula* exposed to bPEI solution showed an alteration of the MMP. In particular, 0.1, 1 and 10 µg/mL bPEI significantly increased the MMP (15.38 FI ± 0.14; 14.41 FI ± 0.80; 14.44 FI ± 0.70; respectively; $P < 0.01$ at 0.1 and 1 µg/mL; $P < 0.05$ at 10 µg/mL) compared to the control (10.20 FI ± 1.20) (Figure 28).
Meanwhile, in spermatozoa exposed to CA and T solutions, no significant differences in MMP values were observed compared to the controls (supplementary materials section S6, table 4S).

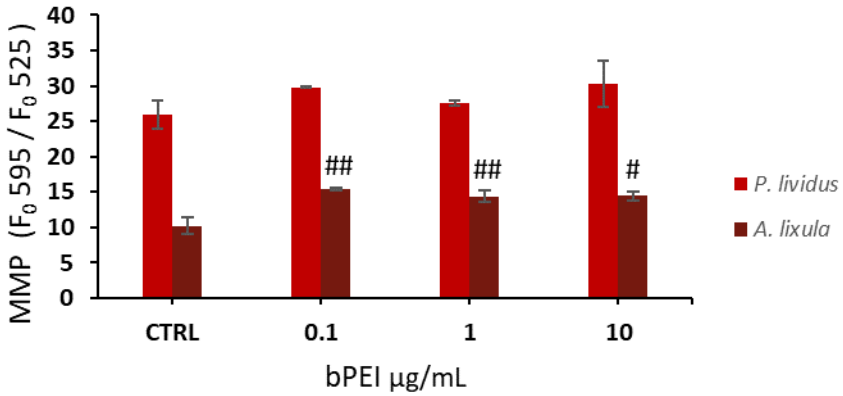


Figure 28. The graph shows the mitochondrial membrane potential (MMP) of sea urchin spermatozoa after exposure to bPEI. On the X- axis the concentrations of the bPEI solution are reported. Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 ($P< 0.05$); ** or ## indicate a significance level lower than 0.01 ($P< 0.01$).

4.3.3.3. Oxidative status

The hydrogen peroxide levels in *P. lividus* spermatozoa exposed to bPEI, CA and T solutions were not significantly affected at all tested concentrations (supplementary materials section S6, table 3S).

Differently, in *A. lixula*, sperm exposure to bPEI solution (0.1 to 10 µg/mL bPEI) significantly increased the levels of hydrogen peroxide (control 289.825 FI ± 19.3 *vs* 227.92 FI ± 31.47; 299.68 FI ± 29.67; 334.56 FI ± 24.66; $P < 0.05$ at 0.1 and 1 µg/mL; $P < 0.01$ at 10 µg/mL (Figure 29a). In the tested concentrations of CA solution, the spermatozoa evidenced a significant increase in hydrogen peroxide levels compared to the control spermatozoa (control 261.56 FI ± 8.79 *vs* 315.71 FI ±3.52; 343.43 FI ± 6.54; 322.54 FI ± 10.18; $P < 0.05$) (Figure 29b). On the other hand, the T solution did not affect hydrogen peroxide levels in spermatozoa (supplementary materials section S6, table 4S).

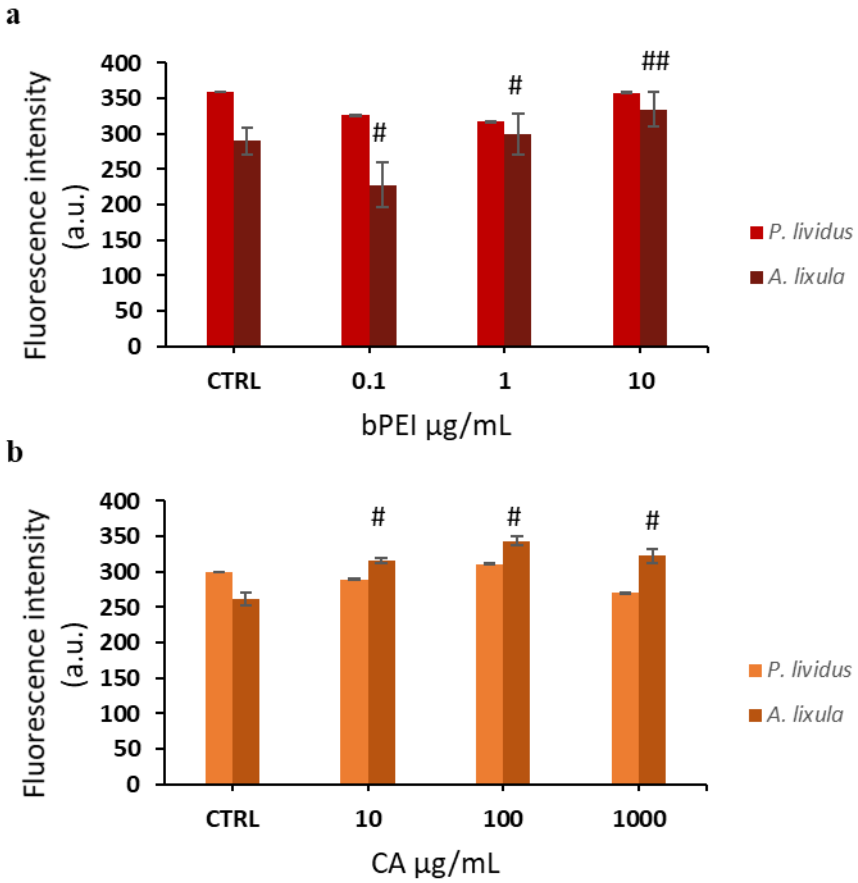


Figure 29. The graphs show the levels of hydrogen peroxide (H_2O_2) in sea urchin spermatozoa after exposure to bPEI (a) and CA (b). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 ($P< 0.05$); ** or ## indicate a significance level lower than 0.01 ($P< 0.01$).

The effects on superoxide anion levels after sperm exposure to bPEI, CA and T solutions in *P. lividus* are reported in the supplementary materials section S6, table 3S. In all solutions, at all tested concentrations, the levels of superoxide anion were similar to those recorded in control spermatozoa.

In *A. lixula*, no significant effects on superoxide anion levels after sperm exposure to CA and T solutions were observed at all tested concentrations (supplementary materials section S6, table 4S). Whereas, in bPEI solution, a significant increase in the levels of superoxide anion was observed after exposure to the concentration of 10 µg/mL bPEI (Figure 30).

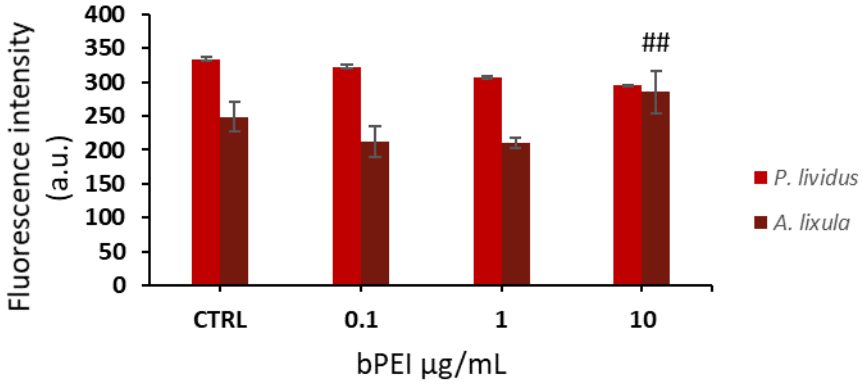


Figure 30. The graph shows the intracellular levels of superoxide anion (O_2^-) of sea urchin spermatozoa after exposure to bPEI. Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. ** or ## indicate a significance level lower than 0.01 (P< 0.01).

In both *P. lividus* and *A. lixula*, the sperm exposure to the bPEI, CA and T solutions did not significantly affect the LPO levels (supplementary materials section S6, table 3S and 4S).

4.3.3.4. Sperm motility

In *P. lividus*, the motility of spermatozoa was significantly affected by the bPEI solution (Figure 31). Sperm exposure to 1 and 10 µg/mL bPEI significantly reduced the sperm motility (45 ± 4.87% and 49 ± 0%, respectively; P < 0.01) compared to the control (64 ± 0.81%), while the exposure to CA and T solutions did not significantly affect sperm motility (supplementary materials section S6, table 3S).

Similarly, in *A. lixula*, the motility of spermatozoa was significantly affected only by bPEI solution (Figure 31) at the highest tested concentration, i.e. 10 µg/mL bPEI (29 ± 9.77%; P < 0.05) with respect to the control (82 ± 5.61%). While, the sperm motility was not affected by the exposure to CA and T solutions (supplementary materials section S6, table 4S).

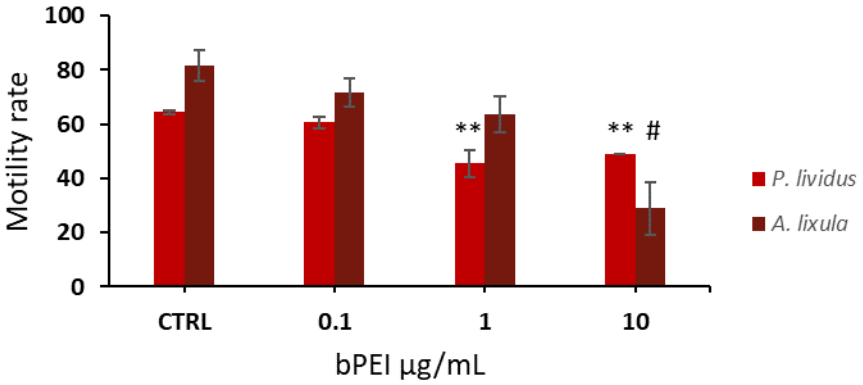


Figure 31. The graph shows the sperm motility of sea urchin after exposure to bPEI. Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

4.3.4. Egg quality assessment after Cellulose-based Nanosponges exposure

4.3.4.1. Intracellular pH

In both species, the pH_i of eggs was not significantly affected by the exposure to CNS conditioned seawater, in all the dilutions tested (see supplementary materials section S6, tables 5S and 6S).

4.3.4.2. Mitochondrial activity

In *P. lividus*, the exposure of eggs to W1 solution affected the mitochondrial activity inducing a mitochondrial membrane hyperpolarization (figure 32a). Particularly, the W1 solution with DF 1 significantly increased the MMP of *P. lividus* eggs (3.06 FI ± 0.30; P < 0.01), compared to the control (2.03 FI ± 0.30). In spermatozoa exposed to W1 solution with the DF of 2, 5, 10, the MMP values were similar to those recorded in control spermatozoa (1.67 FI ± 0.52; 2.88 FI ± 0.48; 2.11 FI ± 0.48).

Egg exposure to W2 solution induced a significant increase of the MMP, leading to the hyperpolarization of the mitochondrial membranes (figure 32b). Exactly, the MMP value in the control was 1.98 FI ± 0.23 while the egg exposed to W2 solution with the DF of 1 and 2 had MMP values of 3.33 FI ± 0.28 (P < 0.01) and 2.87 FI ± 0.19 (P < 0.05), respectively. Egg exposed to W2 solution with the DF of 5 and 10 had MMP levels comparable to the control. On the other hand, the MMP of eggs exposed to W3 solution was not significantly affected (supplementary materials section S6, table 5S). The eggs of *A. lixula* exposed to W

solutions did not show any significant alteration of the MMP (supplementary materials section S6, table 6S).

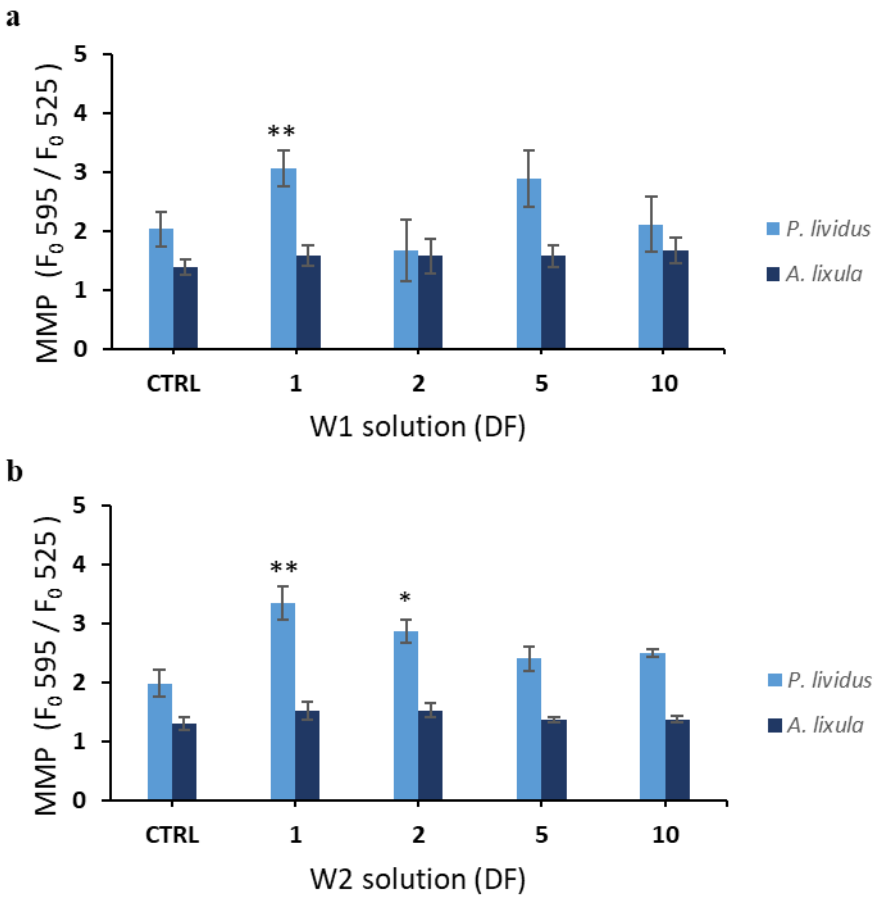


Figure 32. The graphs show the mitochondrial membrane potential (MMP) of sea urchin eggs after exposure to W solution. "W1 solution" (a) refers to seawater conditioned with CNS powder that was washed once. While, "W2 solution" (b) refers to seawater conditioned with CNS powder washed twice. On the X-axis the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

4.3.4.3. Oxidative status

As in the spermatozoa, the oxidative status of eggs was investigated by using three specific different dyes, to detect the levels of two free radical species, the hydrogen peroxide and the superoxide anion, and of the lipid peroxidation (LPO). As reported in figure 33, in *P. lividus*, the levels of hydrogen peroxide significantly increased in the W1 solution with DF 1 compared to the control (control 188.39 FI ± 13.78 *vs* 228.80 FI ± 13.78; P < 0.05). While the W1 solution with the DF of 2, 5 and 10 showed values comparable to the control (188.30 FI ± 20.25; 195.74 FI ± 20.25 and 202.13 FI ± 20.25). Egg exposure to W2 and W3 solution

did not affect the level of hydrogen peroxide compared to that of the control eggs (supplementary materials section S6, table 5S). In *A. lixula*, eggs exposed to W1, W2 and W3 solutions did not show any alteration of hydrogen peroxide levels compared to the control (supplementary materials section S6, table 6S).

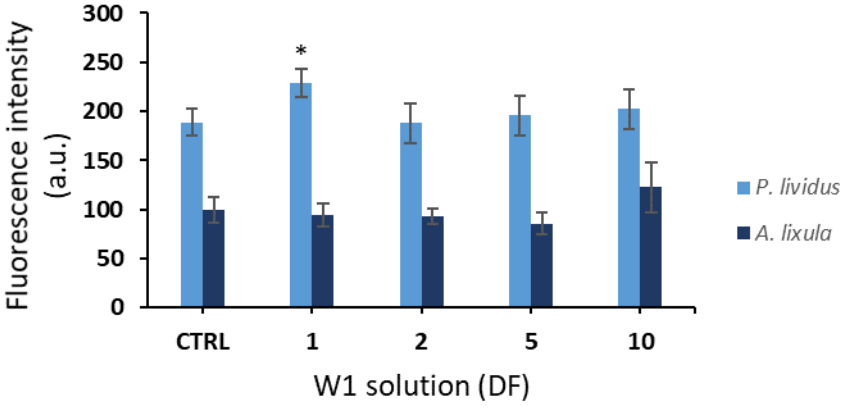


Figure 33. The graph shows the intracellular levels of hydrogen peroxide (H_2O_2) of sea urchin eggs after exposure to W1 solution, i.e. seawater conditioned with Cellulose-based Nanosponges powder washed once. On the X-axis the dilution factors (DF) of the W solution are reported. DF=1 corresponds to the highest concentration of CNS powder dissolved into FNSW (1.25 g/L). Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 (P< 0.05); ** or ## indicate a significance level lower than 0.01 (P< 0.01).

Regarding the levels of superoxide anion in *P. lividus* eggs, they did not significantly differ from the those recorded in the control (5.67 FI ± 0.63) after exposure to W1, W2 and W3 solutions (supplementary materials section S6, table 5S). Similarly, there was no alteration of superoxide anion levels compared to the control in *A. lixula* eggs exposed to the W1, W2 and W3 solutions (supplementary materials section S6, table 6S).
Additionally, the exposure of *P. lividus* and *A. lixula* eggs to W1, W2 and W3 solutions, at all tested dilutions did not significantly change the LPO levels compared to the unexposed (control) eggs (supplementary materials section S6, tables 5S and 6S).

4.3.5. Egg quality assessment after exposure to Cellulose-based Nanosponges components

4.3.5.1. Intracellular pH

Both in *P. lividus* and in *A. lixula,* egg exposure to the components of CNS (bPEI, CA and TOCNF solutions) did not affect the pH_i that was stable at all tested concentrations (supplementary materials section S6, tables 7S and 8S).

4.3.5.2. Mitochondrial activity

In *P. lividus*, the exposure of eggs to bPEI solution significantly affected the mitochondrial activity leading to the hyperpolarization of the mitochondrial membrane (Figure 34). Particularly, eggs exposed to 0.1, 1 and 10 µg/mL bPEI showed levels of MMP significantly higher than the control (control 2.37 FI ± 0.41 *vs* 2.61 FI ± 0.25; 3.03 FI ± 0.26; 3.19 FI ± 0.25; $P < 0.05$ at 0.1 and 1 µg/mL; $P < 0.01$ at 10 µg/mL). On the contrary, after the exposure to CA solution, at concentrations of 1, 10 and 100 µg/mL CA, the recorded MMP values in the exposed eggs were comparable to the control. Similarly, eggs exposed to T solution had MMP values similar to those recorded into the control (supplementary materials section S6, table 7S).

The eggs of *A. lixula* exposed bPEI solution showed an alteration of the MMP (Figure 34). In particular, in the eggs exposed to 0.1 and 1 µg/mL bPEI, MMP was not significantly affected (1.86 FI ±0.22; 1.56 FI ±0.11) compared to the control (1.77 FI ± 0.14). While, the MMP levels were significantly increased in the eggs exposed to 10 µg/mL bPEI (3.09 FI ± 0.49; $P < 0.05$). On the contrary, eggs exposed to the highest tested concentrations of CA and TOCNF (1000 µg/mL) had MMP values similar to the control (supplementary materials section S6, table 8S).

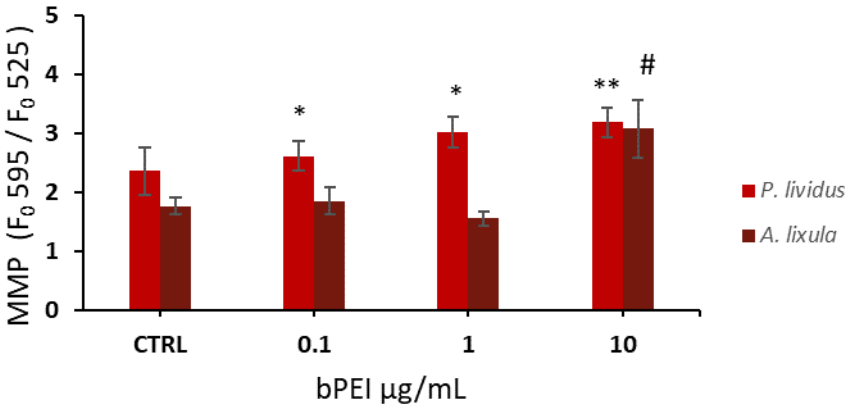


Figure 34. The graph shows the mitochondrial membrane potential (MMP) in sea urchin eggs after the exposure to bPEI. Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 ($P< 0.05$); ** or ## indicate a significance level lower than 0.01 ($P< 0.01$).

4.3.5.3. Oxidative status

The hydrogen peroxide levels in *P. lividus* and *A. lixula* eggs exposed to CNS components did not significantly change in comparison to the control (supplementary materials section

S6, tables 7S and 8S). Similarly, the egg exposure to CNS components did not affect the levels of superoxide anion, in both species (supplementary materials section S6, tables 7S and 8S). The effects on LPO levels after egg exposure to bPEI solution in *P. lividus* are depicted in Figure 35. LPO levels of the control eggs and of the eggs exposed to 0.1, and 1 µg/mL bPEI were similar. While, the exposure of eggs to 10 µg/mL bPEI induced a significant raise of LPO levels (38.43 FI ± 2.44; $P < 0.01$) compared to the control (26.53 FI ± 2.14).

On the contrary, the egg exposure to CA solution, at concentrations of 1, 10 and 100 µg/mL CA as well as to 1000 µg/mL TOCNF did not alter LPO levels compared to the control (supplementary materials section S6, table 7S).

In *A. lixula*, egg exposure to the highest tested concentration of bPEI solution significantly altered the LPO levels (Figure 35). Exactly, the LPO value in the control (50.34 FI ± 1.24) and in the eggs exposed to 0.1, and 1 µg/mL bPEI were similar, but the exposure of eggs to 10 µg/mL bPEI induced a significant raise of LPO levels up to 55.77 FI ± 1.53; $P < 0.05$. On the contrary, the eggs exposed to the CA and TOCNF at all tested concentrations showed similar levels of LPO than the control (supplementary materials section S6, table 8S).

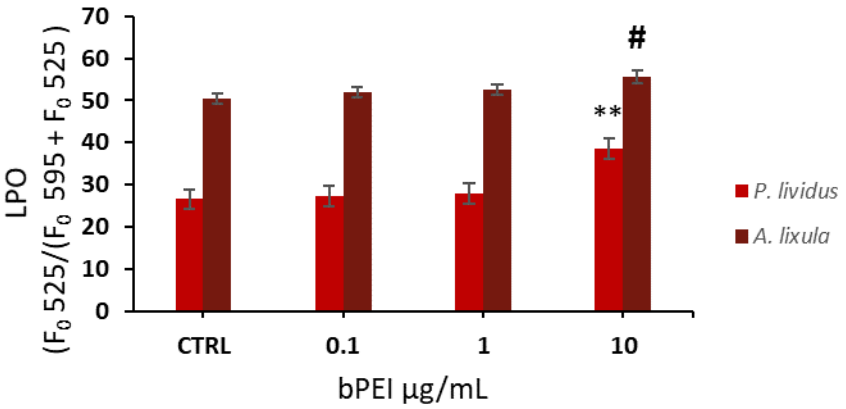


Figure 35. The graph shows the intracellular levels of lipid peroxidation (LPO) in sea urchin eggs after the exposure to bPEI. Data are presented as mean ± standard error (SE). Asterisks and hashes indicate significant differences compared to the control (CTRL), respectively in *Paracentrotus lividus* and *Arbacia lixula*. * or # indicate a significance level lower than 0.05 ($P < 0.05$); ** or ## indicate a significance level lower than 0.01 ($P < 0.01$).

4.3.6. Morphological assessment of gametes: Scanning electron microscopy (SEM)

The morphological analysis of gametes after exposure to CNS leachate was conducted by scanning electron microscopy (SEM). Spermatozoa of *P. lividus* and *A. lixula* showed the following peculiar structures: a conic head placed on the mitochondrial ring and a long flagellum (Figures 36 and 37). The exposure to W solutions (1.25 g/L) did not affect sperm

morphology but caused their entanglement. In the W2 and W3 solution, the number of sperm tails tangled was reduced and spermatozoa were homogeneously distributed on the stab surface, as in the control.

SEM analysis of *P. lividus* eggs exposed to W solutions (1.25 g/L) is reported in figure 38. The control eggs had a round shape, and a clean and smooth surface with the microvilli clearly visible. The exposure to the W1 solution altered the surface structure which shows some wrinkles. Microvilli were not visible and some solid material (CNS leachate) was attached to the egg surface. The presence of this material was observed also in the samples exposed to W2 solution, both on the egg surface and on the support. While in the eggs exposed to the W3 solution the amount of the CNS leachate was almost absent.

A similar effect was observed in *A. lixula* eggs (Figure 39), where the presence of CNS leachate attached to the egg surface gradually reduced from W1 to W3 solution. Also in this species, eggs preserved shape and size after exposure to W solutions; and the egg exposed to W3 solution showed a surface comparable to the control eggs.

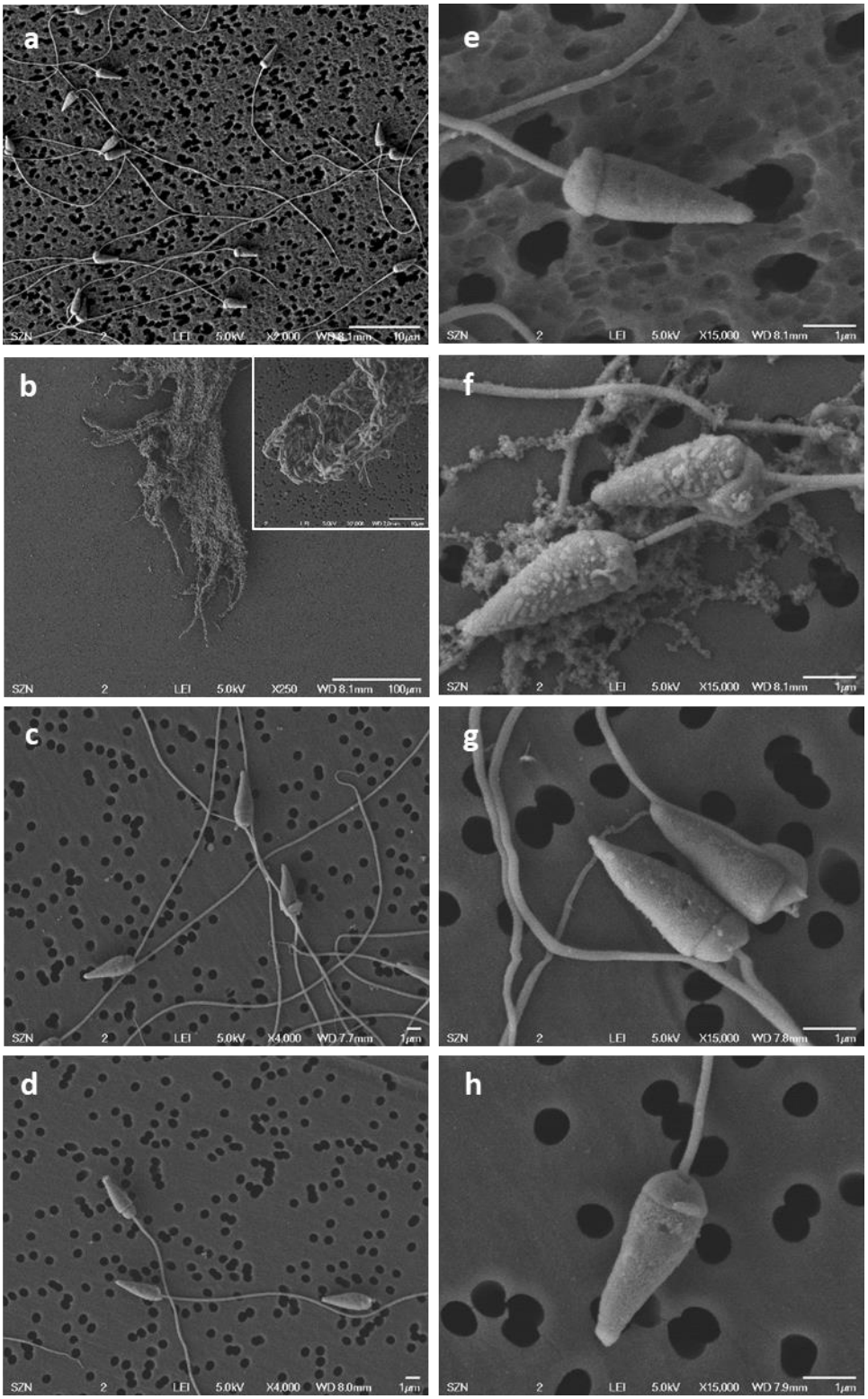


Figure 36. Scanning electron microscopy images of *Paracentrotus lividus* spermatozoa exposed for 1 hour to W solution (1.25 g/L). CTRL = FNSW (a, e), W1 solution (b, f), W2 solution (c, g), and W3 solution (d, h). The images e, f, g, h depict a higher magnification of the spermatozoa exposed to the same conditions.

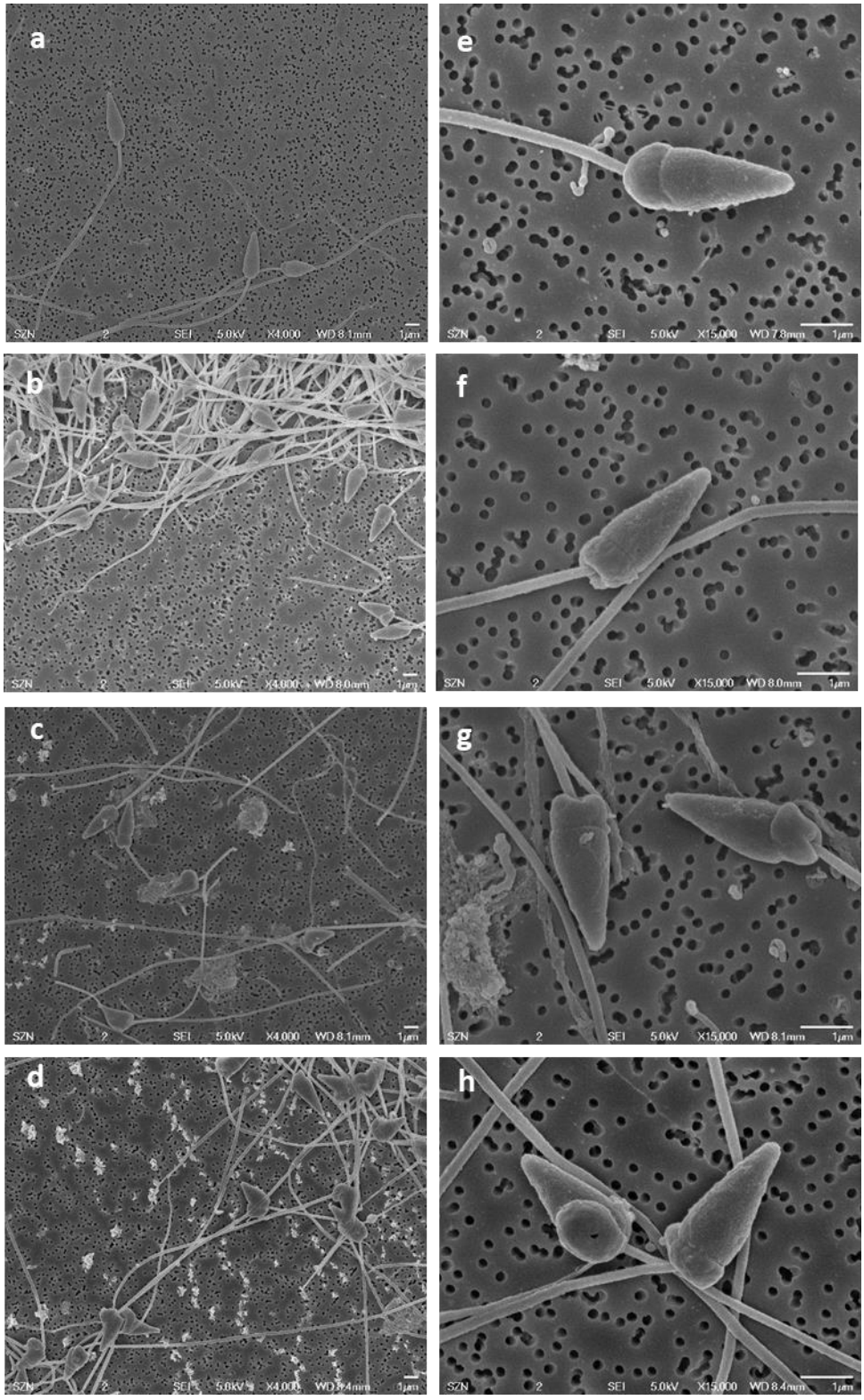


Figure 37. Scanning electron microscopy images of *Arbacia lixula* spermatozoa exposed for 1 hour to W solution (1.25 g/L). CTRL = FNSW (a, e), W1 solution (b, f), W2 solution (c, g), and W3 solution (d, h). The images e, f, g, h depict a higher magnification of the spermatozoa exposed to the same conditions.

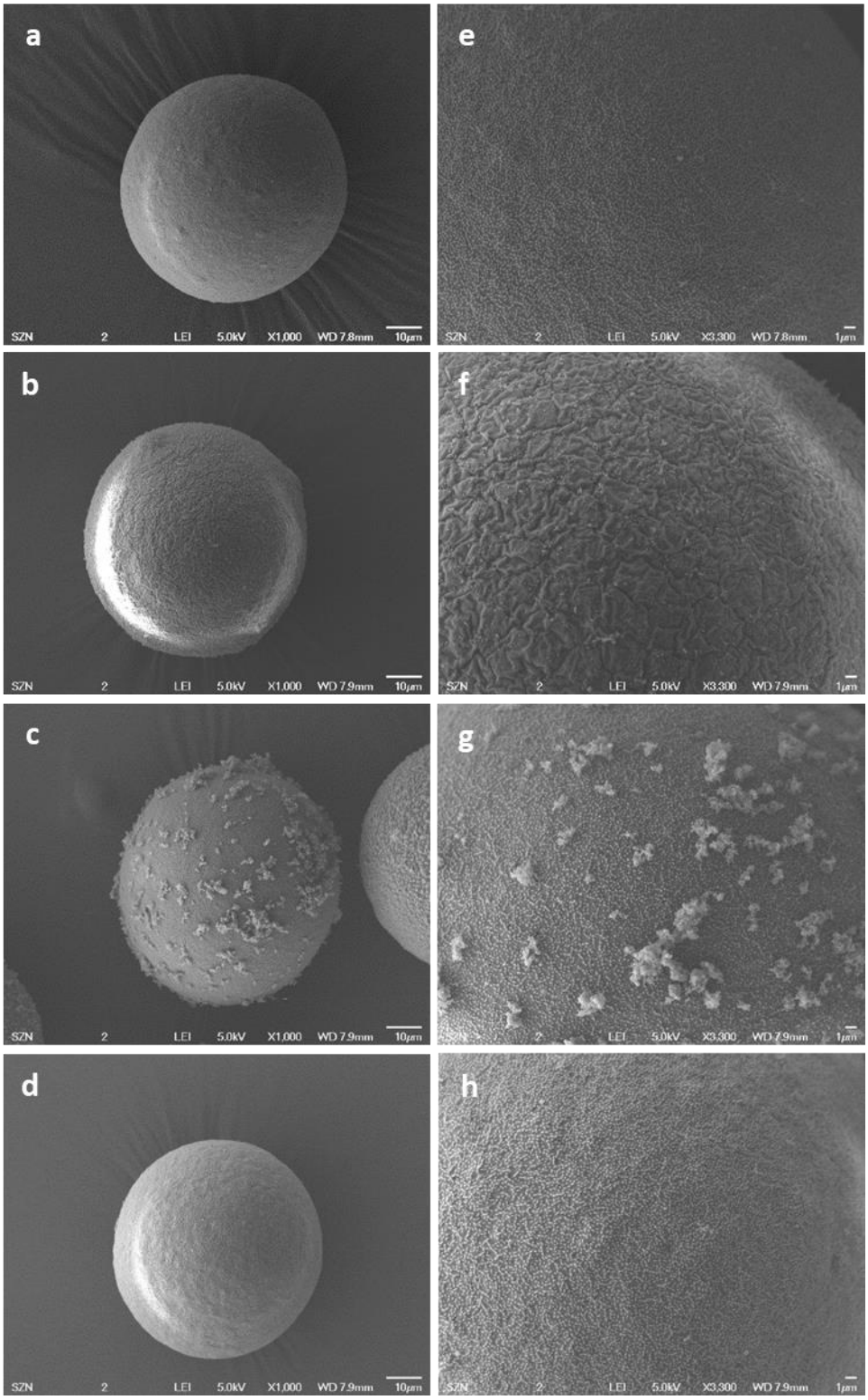


Figure 38. Scanning electron microscopy images of unfertilised *Paracentrotus lividus* eggs exposed for 1 hour to W solution (1.25 g/L). CTRL =FNSW (a, e), W1 solution (b, f), W2 solution (c, g), and W3 solution (d, h). The images e, f, g, h depict a higher magnification of the eggs exposed to the same conditions highlighting the microvilli of the plasmatic membrane.

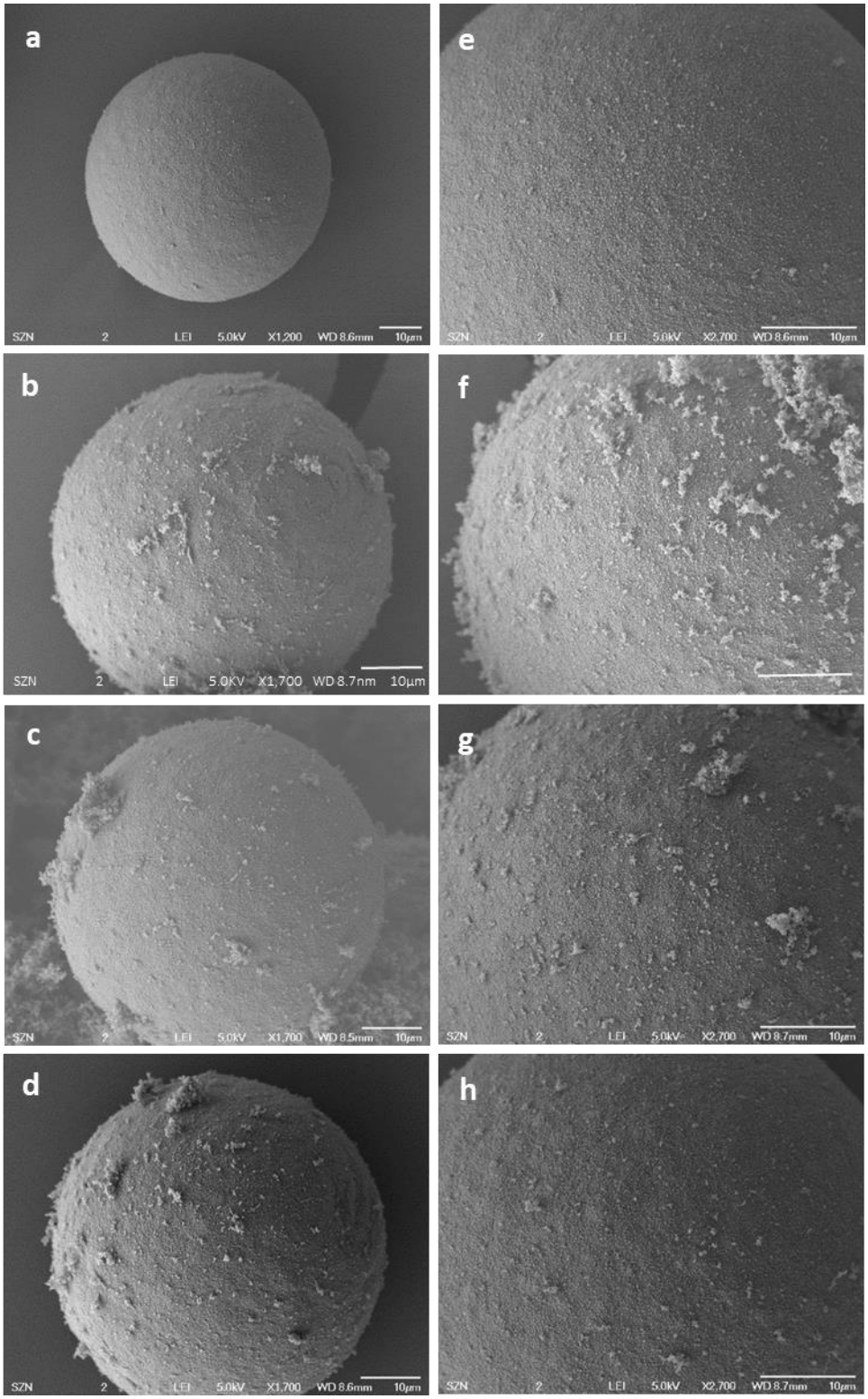


Figure 39. Scanning electron microscopy images of unfertilised *Arbacia lixula* eggs exposed for 1 hour to W solution (1.25 g/L). CTRL =FNSW (a, e), W1 solution (b, f), W2 solution (c, g), and W3 solution (d, h). The images e, f, g, h depict a higher magnification of the eggs exposed to the same conditions highlighting the microvilli of the plasmatic membrane.

4.4. Discussion

This chapter is focused on the assessment of the impact of CNS leachate and CNS components on the quality of sea urchin male and female gametes. The reproductive success of living organisms strictly depends on gamete quality, which can be estimated by assessing different morphological and physiological parameters, such as motility, mitochondrial activity, ROS level, intracellular pH, and DNA damage. Reproductive toxicity is the occurrence of adverse effects on the reproductive system, including gamete quality, resulting from exposure to contaminants (Usepa, 2002). Nevertheless, traditionally, reproductive toxicity tests estimate fertility and viable offspring as endpoints giving little attention to other endpoints and the mechanism of toxicity. Currently, the reproductive risk assessment process is beginning to expand this traditional approach with new tests and endpoints in order to define the mechanism of action.

In the present thesis, a new approach based on the evaluation of different gamete quality parameters as endpoints has been developed to screen the safety of ENMs. This approach has been previously employed to investigate the impact of different NPs on the sperm quality of *P. lividus* and of the ascidian *Ciona robusta* allowing the elucidation of the mechanism of their toxic action (Gallo et al., 2018; Gallo et al., 2019). Considering that the spermatozoa of these two species of sea urchins are similar in structure, shape and size, the protocols and the experimental conditions previously adopted with *P. lividus*; including temperature, the number of gametes and incubation time were used with *A. lixula* spermatozoa. Exceptionally, for motility, the sperm concentration was chosen to allow the correct count for the visual assessment. Indeed, a concentration of 80 * 10^6 spermatozoa allows having about 8 spermatozoa in each line of the counting chamber grid, reducing mistakes during the observation. Aiming to uniform the protocols; the temperature, the incubation time and the staining time were the same in both species and for both gametes. However, eggs are immotile cells and rapidly sink. Therefore, the 96-multiwell plate with a flat bottom was chosen as support for the fluorescence analyses, and some preliminary experiments were performed to check the best number of eggs to use (see supplementary materials section S5, figure 1S).

Here, this approach has been applied for the first time to investigate the potential risk of Cellulose-based Nanosponges' leachate on the quality of *P. lividus* and *A. lixula* gametes. Results herein reported demonstrated that CNS leachate affected some gamete quality parameters, such as the oxidative status and mitochondrial functionality, but not the pH_i. The pH_i is one of the well-studied parameters in the sea urchin spermatozoa. In the gonad, spermatozoa are characterised by a low pH (~ 7,3) that makes them quiescent and immotile

(Rothschild, 1948). Once released into seawater, through the spawning, the higher external pH increases the pH_i leading to the activation of the axonemal dynein, which uses the ATP produced in the mitochondria as substrate and consequently spermatozoa can swim (Rothschild, 1948; Shapiro et al., 1985; Lee et al., 1983; Cheung Lee et al., 1983). In sea urchin eggs, a pH_i value similar to the one measured in seawater has been reported (Holland & Cross, 1983; Payan et al., 1983). Differently, in male and female gametes exposed to seawater treated with CNS an increase in intracellular ROS levels has been detected. ROS production in male and female gametes is a physiological process. Indeed, at low and physiological controlled concentration, ROS play important roles in different processes (Du Plessis et al., 2015; O'Flaherty & Matsushita-Fournier, 2017) and are involved in the maintenance of gamete quality. Nevertheless, ROS overproduction has been linked to the induction of oxidative stress, which results in deteriorating the gamete quality (Frenzilli et al., 2001; Aitken et al., 2016). Thereby, the increase of intracellular ROS levels in the sea urchin spermatozoa and eggs can be related to a reduction in the fertilisation competence revealed by spermiotoxicity and ovotoxicity bioassays.

Mitochondrial functionality is another important trait of gamete quality related to cell viability and fertility (Qi et al., 2019). Indeed, mitochondria play a central role in cellular metabolism providing energy in the form of adenosine triphosphate (ATP) need for successful gamete maturation, meiosis completion, fertilisation and embryo development as well as for motility of spermatozoa. MMP is a key indicator of mitochondrial activity because it reveals the cell capability to generate ATP by oxidative phosphorylation. In this study, an increase in MMP has been detected in sea urchin gametes after CNS leachate exposure. Literature reports contrasting data on the correlation between MMP and ROS production (Ahamed, 2011; Ma et al., 2015; Ryu et al., 2014; Suski et al., 2012). The observed mitochondrial membrane hyperpolarization along with the intracellular ROS level increase are consistent with the accepted observation that ROS formation in mitochondria occurs at high membrane potentials (Suski et al., 2012). The high MMP and ROS overproduction may be due to the alteration of closure regulation of the mitochondrial permeability transition pore (MPTP) protein or inhibition of ATP synthase (Wojtczak et al., 1999). Indeed, the open-closure regulation of MPTP is essential for maintaining cell homeostasis, since it provides the release of ions or toxic compounds accumulated in the mitochondrial matrix, including ROS (Bernardi & Petronilli, 1996).

SEM analysis revealed that CNS leachate did not affect sperm morphology in both the tested species, but induced their entanglement, which was gradually reduced by applying the multi-washing protocol; therefore, it can be hypothesised that CNS leachate may have a sticky power that determines the tangle of tails, which probably prevents the interaction with the

eggs, and, in turns the fertilisation process as revealed by the spermiotoxicity test. The SEM analysis of the eggs of both species exposed to CNS leachate showed the presence on the egg surface of CNS components released into seawater and alteration of microvilli. Microvilli are a peculiar structure of eggs that increase the surface of the cell and play a main role in fertilisation. Indeed, in zone microvilli-free, spermatozoa are not able to attach and fertilise the eggs (Runge et al., 2007). Fertilisation is a result of a step-by-step process including binding and fusion of the gametes. Therefore, the presence of CNS leachate could cover the binding receptors (glycoproteins) of the egg membrane, precluding the binding between spermatozoa and eggs. This physical obstacle could impair the interaction between the gametes and as consequence, the entire process of fertilisation will be blocked.

Regarding the CNS constituents, the gamete quality assessment confirms the same scale of safety, TOCNF > CA > bPEI, observed for the ecotoxicological bioassays. Among the tested CNS components, the bPEI exerts similar adverse effects of CNS leachate on gamete quality confirming the previous hypothesis that the spermiotoxic and ovotoxic effects observed may be induced by bPEI, which is probably released into seawater by CNS.

Overall, in this chapter, it has been demonstrated that CNS affect sea urchin egg and sperm quality inducing an increase of the intracellular ROS level and mitochondrial activity as well as a modification of gamete surface, altering, in turn, gamete fertilisation competence.

CHAPTER 5

Conclusion

The present PhD project attempted to evaluate the suitability of sea urchin as a model species for assessing the potential environmental risks posed by CNS, i.e. nanostructured sponges developed for marine environmental remediation, in order to ensure their safe use. Sea urchins represent a suitable model for testing the toxicity of diverse contaminants. In particular, the sea urchin's early life stages have been widely demonstrated to be a highly sensitive and suitable marine *in vivo* model system for the ecotoxicological assessment of physical and chemical stressors. Currently, standard ecotoxicity tests are available and commonly used for conventional chemicals (Fernández, & Beiras, 2001; Novelli et al., 2002). Nevertheless, due to the unique properties of ENMs, the existing tests may not be appropriate to assess their toxicity. In the present study, the suitability of the standardised bioassays with the sea urchin *P. lividus*, such as the embryotoxicity and spermiotoxicity tests that, respectively, aimed to evaluate the potential effects of contaminants on sea urchin embryo development and sperm fertilising capability, in assessing the safety of CNS has been proved supporting their application for the ecosafety assessment of different ENMs. Moreover, in this study, a new bioassay, named ovotoxicity essay, has been developed. This assay aimed to assess also the potential effects of CNS on egg fertilisation competence since male and female gametes may not be equally sensitive as emerged for this study. In fact, unlike spermatozoa whose fertilizing capability was affected only by the CNS with a DF 1, the fertilization competence of the eggs was altered also by the CNS with higher DF suggesting that the sea urchin eggs are more sensible to CNS than spermatozoa and that these assays represent a fundamental tool to screen female gamete in the framework of a reproductive risk assessment of ENMs.

In the present study the sea urchin *A. lixula*, which cohabits with *P. lividus*, has been introduced as a possible suitable alternative biological model in the ecotoxicity testing of nanostructured materials because, in the last decades, the *P. lividus* population have been decreasing and, in turn, their availability in the Mediterranean Sea.

The experimental condition of embryotoxicity, spermiotoxicity and ovotoxicity tests such as sperm:egg ratio, temperature and duration, have been successfully set up since, up to date, the literature lacks this information or reports different conditions.

The ecotoxicity data of the three bioassays performed indicate that the sea urchin species *A. lixula* and *P. lividus* have a similar sensitivity to CNS supporting the use of *A. lixula* in the ecotoxicological toxicity tests. Additionally, standardised bioassays with *P. lividus* (embryotoxicity and spermiotoxicity tests), commonly employed to assess the impact of

traditional contaminants, are sensitive and valuable tools also for assessing the environmental impact of nanoengineered materials for marine environmental remediation.

Comparing the sensitivity of the different life stages tested in this study, the embryos showed a higher sensitivity to all compounds herein tested, than both gametes. Nevertheless, the toxicity tests with gametes are fundamental since they give complementary information on the reproductive toxicity of nanoengineered materials. The ovotoxicity test reveals that CNS leachate affects female gametes causing a reduction in their fertilisation competence. Furthermore, this test is more sensitive than the spermiotoxicity test. Hence, this bioassay can be recommended not only in the screening of nanoengineered material toxicity but also to assess the negative effects of other stressors.

The current approach to the risk assessment in reproductive toxicity relies on the results of acute and sub-chronic toxicity tests. However, one of the objectives of the reproductive risk assessment is to expand the tests, primarily focused on fertility endpoints, to the mechanisms of action. An emerging complement to the traditional approach may be the inclusion of new parameters to assess gamete quality, as reported in the present project.

Overall, the ecotoxicity data indicate that CNS developed for marine environmental remediation are able to affect sea urchin reproduction due to a release of chemical additives from the manufacturing process. Hence, in the framework of eco-design approach, these data suggest a re-design of CNS in order to obtain a safer device.

The research here reported contributes to improving the methodology used for the ecotoxicological assessment of ENMs. Indeed, the tests performed and developed herein follow the 3R's principle for animal research (replacement, reduction, refinement). The employment of *A. lixula* as a replacement for *P. lividus* supports the safeguard of *P. lividus* populations and helps to expand the scientific knowledge on other sea urchin species. Both the bioassays and the gamete quality assessment are performed by using the gametes, limiting the pain for the animal to the injection for the spawning. Furthermore, the gametes picked up from one animal are sufficient to perform the bioassays and the gamete quality multi-parametric approach by reducing the number of animals to be employed. Finally, for an efficient environmental risk assessment of ENMs for remediation, the ecotoxicological screening of ENMs and their components as part of their production process is encouraged to obtain ENMs safe for the marine environment.

Supplementary materials

Section S1. Preliminary experiments to set up biological assays with the sea urchin *Arbacia lixula*

In order to set up the experimental conditions for the embryotoxicity, spermiotoxicity and ovotoxicity assays with *A. lixula*, some preliminary tests were performed. Indeed, in literature few studies describing these assays with *A. lixula* are available (Visconti et al., 2017; Gianguzza et al., 2014; Giannetto et al., 2018; Carballeira et al., 2011; Maisano et al., 2015) reporting different experimental conditions (sperm:egg ratio, number of embryos/mL, temperature of culture) or, in some cases, were completely missing (Table 1S).

Table 1S The table shows the experimental conditions tested on *Arbacia lixula*. (hpf = hours post fertilisation; mpf = minutes post fertilisation).

Experimental conditions			**References**
	Embryotoxicity test		
Sperm: egg ratio	**Temperature**	**Block of the test**	
1:100	18 °C	72 hpf	Maisano et al., 2015
--	20, 24, 26 °C	48 hpf	Visconti et al., 2017
1:100	18°C	72 hpf	Giannetto et al. 2018
--	--	72 hpf	Carballeira et al., 2011
	Spermiotoxicity test		
--	20, 24, 26, 27 °C	4 hpf	Gianguzza et al., 2014
1: 20000	--	20 mpf	Carballeira et al., 2011
--	20, 24, 26 °C	4 hpf	Visconti et al., 2017

Based on the little information available in the literature, preliminary embryotoxicity, spermiotoxicity and ovotoxicity assays were carried out to set up the optimal temperature of embryo culture (18°C or 20°C) and the sperm: egg ratio (100:1, 500:1 or 1000:1), which allow obtaining a high percentage of morphological normal pluteus after 48 hours post fertilisation (hpf). Unlike spermiotoxicity and embryotoxicity assays with *P. lividus,* which were blocked 20 minutes after fertilisation when the fertilised eggs are surrounded by a well visible fertilisation envelope, with *A. lixula* these assays have been stopped 90 minutes post fertilisation at 2-cell embryo stage because the fertilization envelope was not clearly visible in this species making difficult the distinction between fertilised and unfertilised egg. The acceptability threshold for all the tests was ≥ 80% of normal plutei/fertilised eggs in the

negative control. All the preliminary tests were performed by using copper, which is recommended as a reference toxicant for these species. Particularly, embryos, spermatozoa and eggs were exposed to 50, 80, 100, 150 and 200 µg/L of copper sulphate ($CuSO_4 *5H_2O$) in *A. lixula*. Each assay was performed in triplicate and repeated three times

The embryotoxicity preliminary test revealed that the acceptability threshold of 80% of normal embryos in the control 48 hpf has been achieved using the sperm:egg ratio of 1000:1 and performing the embryo culture at 20°C (figure 1S).

The spermiotoxicity tests showed that the fertilisation rate met the acceptability threshold of 80% fertilised eggs in the control using the sperm:egg ratio of 10000:1 at 20°C (figure 2S).

For the ovotoxicity tests, the temperature was set at 20°C and three sperm:egg ratios were tested.

As shown in figure 3S, only the sperm: egg ratio of 1000:1 allowed to reach and overcome the acceptability threshold of 80% of fertilised eggs in the control. Therefore, it was chosen as the ratio to perform the ovotoxicity assay.

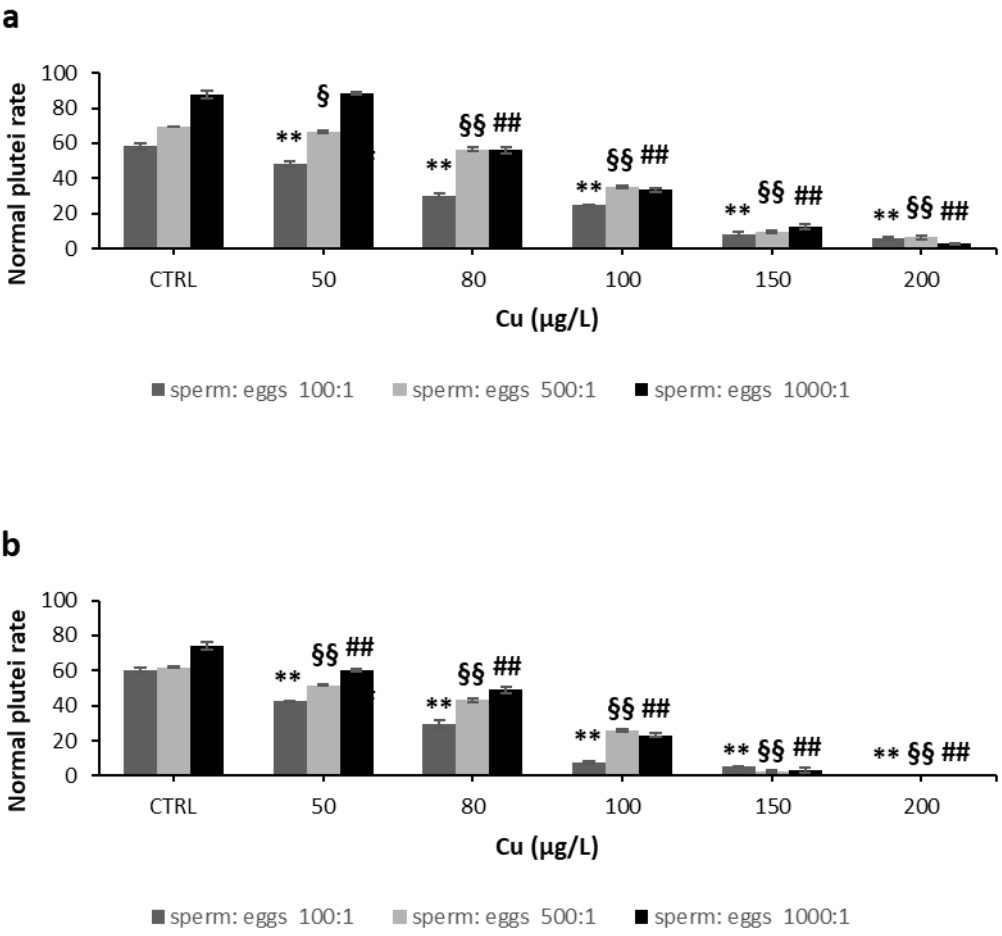


Figure 1S *Arbacia lixula* embryo development 48 hours post fertilization at different temperature and sperm: egg ratio. a) embryotoxicity test carried out using the sperm:egg ratio of 100:1, 500:1, and 1000:1 and incubating embryos at 20°C (a) and 18°C (b). The acceptance threshold of the embryotoxicity test was ≥ 80% of normal pluteus larvae. Data are presented as normal plutei rate (%) ± standard error (SE).
The symbols *, §, and # indicate significant differences compared to the control (CTRL), respectively in the sperm:egg ratio of 100:1, 500:1, and 1000:1. *, § or # indicate a significance level lower than 0.05 ($P < 0.05$); **, §§ or ## indicate a significance level lower than 0.01 ($P < 0.01$).

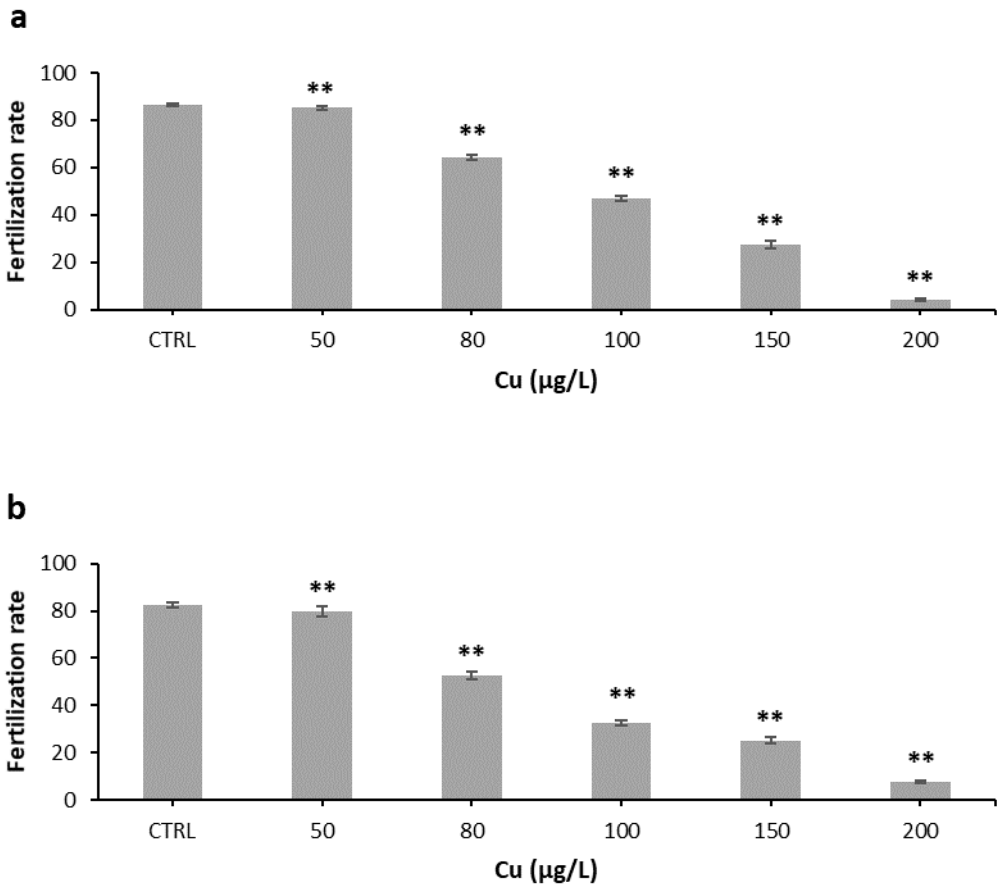


Figure 2S *Arbacia lixula* spermiotoxicity test at different temperatures. Fertilised eggs that reached the two-cell stage 90 minutes after fertilisation. During this time, that correspond to the duration of the spermiotoxicity test, the embryos were exposed at 20°C a); and at 18°C b). The acceptance threshold of the spermiotoxicity test was ≥ 80% of fertilised eggs in the control. Data are presented as fertilisation rate (%) ± standard error (SE). The significance level was set at P< 0.05*; P< 0.01** *vs* CTRL (control).

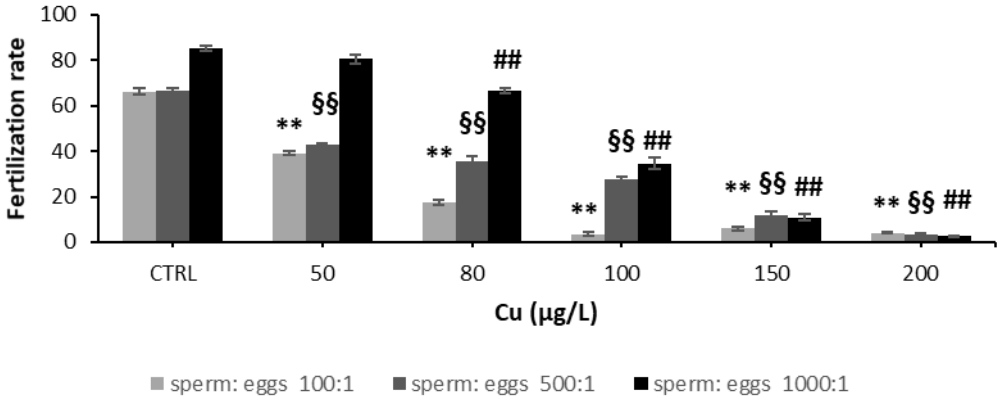


Figure 3S *Arbacia lixula* ovotoxicity test at different sperm: egg ratio. The ovotoxicity test was performed at 20°C and blocked 90 minutes after the fertilisation. The graph shows the percentage of fertilised eggs that reached the two-cell stage within this time. The acceptance threshold of the ovotoxicity test was ≥ 80% of fertilised eggs in the control. Data are presented as fertilisation rate (%) ± standard error (SE). The symbols *, §, and # indicate significant differences compared to the control (CTRL), respectively in the sperm:egg ratio of 100:1, 500:1, and 1000:1. *, § or # indicate a significance level lower than 0.05 ($P< 0.05$); **, §§ or ## indicate a significance level lower than 0.01 ($P< 0.01$).

Section S2. Preliminary assays to set up the experimental conditions of ovotoxicity test with the sea urchin *Paracentrotus lividus*

Up to date, the ovotoxicity test, which allows testing potential effects of nanomaterial on the ability of eggs to be fertilized, has never been performed. Therefore, preliminary tests were performed to set up the best experimental conditions for this assay. As for the spermiotoxicity test with *P. lividus*, the temperature and the duration of the test were set at 18°C and 20 minutes post fertilisation, respectively, whereas two different sperm: egg ratios were evaluated such as 50:1, usually employed in the embryotoxicity assay, and the sperm: eggs ratio of 100:1. The copper nitrate ($Cu(NO_3)2*3H_2O$) was used as reference toxicant at the following range of concentrations 20, 50, 70, 100 and 150 µg/L. Three biological replicates of the assay were performed with three analytical replicates. The acceptance threshold of the ovotoxicity test was ≥ 80% of fertilised eggs in the control. As reported in figure 1S, the preliminary tests revealed that the sperm: eggs ratio of 50:1 did not meet the acceptance threshold, therefore this ratio was excluded as a possible experimental condition. Differently, the sperm: eggs ratio of 100:1 was able to meet the acceptance threshold and was chosen as the best experimental condition for the ovotoxicity assay.

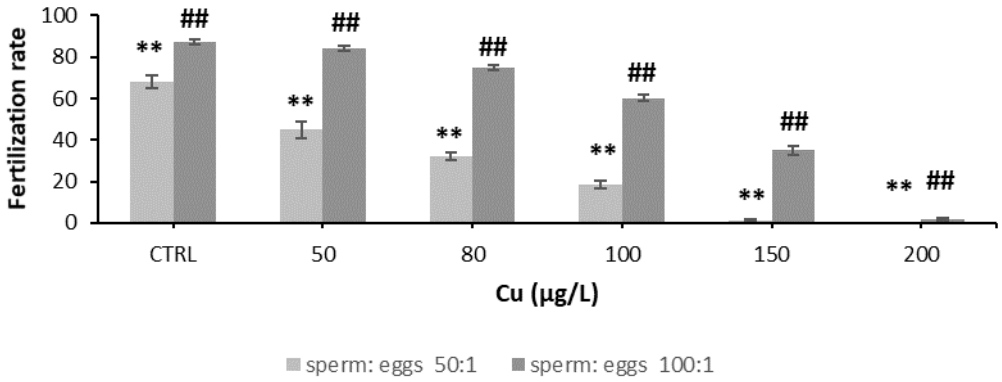


Figure 1S. *Paracentrotus lividus* ovotoxicity test at different sperm: egg ratio. The graph shows the percentage of fertilised eggs 20 minutes after fertilisation by using the sperm: egg ratio of 50:1 and 100:1. The acceptance threshold of the ovotoxicity test was ≥ 80% of fertilised eggs in the control. Data are presented as fertilisation rate (%) ± standard error (SE). The symbols * and # indicate significant differences compared to the control (CTRL), respectively in the sperm:egg ratio of 50:1 and 100:1. * or # indicate a significance level lower than 0.05 ($P< 0.05$); ** or ## indicate a significance level lower than 0.01 ($P< 0.01$).

Section S3. Estimation of sea urchin sensitivity to the reference toxicant

To estimate the sea urchin sensitivity, embryos, spermatozoa and eggs were exposed to a range of concentrations of copper, which is recommended as a reference toxicant for these species. The copper nitrate ($Cu(NO_3)_2*3H_2O$) concentrations tested with *P. lividus* were 20, 50, 70, 100 and 150 µg/L. While, for *A. lixula* the copper sulphate ($CuSO_4*5H_2O$) concentrations tested were 50, 80, 100, 150 and 200 µg/L. Tests were performed in three biological replicates, the statistical analysis was performed with GraphPad Prism software package, version 9 (San Diego, CA), which was also used to calculate the 50% effective concentration values (EC_{50}).

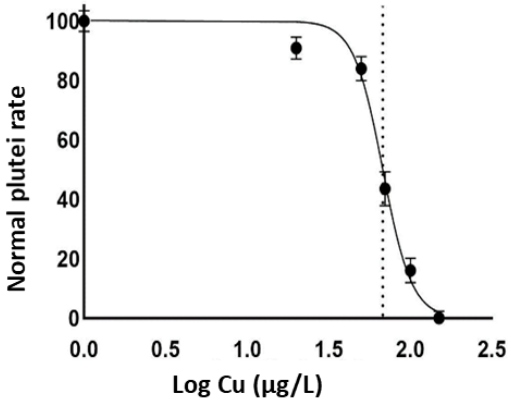


Figure 1S Effect of copper nitrate ($Cu(NO_3)_2*3H_2O$) on *Paracentrotus lividus* embryo-larval development. In the graph, the Log EC_{50} is depicted by the dashed line, corresponding to the EC_{50} of 67.69 µg/L.

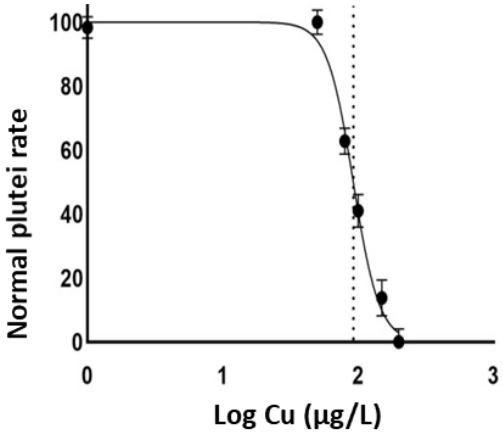


Figure 2S Effect of copper sulphate ($CuSO_4$ $*5H_2O$) EC_{50} on *Arbacia lixula* embryo development. In the graph, the Log EC_{50} is depicted by the dashed line, corresponding to the EC_{50} of 92.17 µg/L.

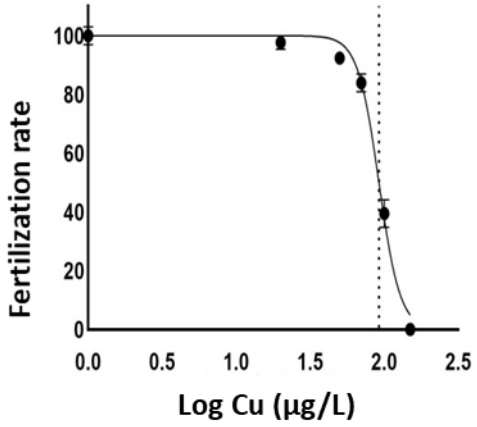


Figure 3S Effect of copper nitrate ($Cu(NO_3)_2*3H_2O$) on *Paracentrotus lividus* spermatozoa. In the graph, the Log EC_{50} is depicted by the dashed line, corresponding to the EC_{50} of 92.14 µg/L.

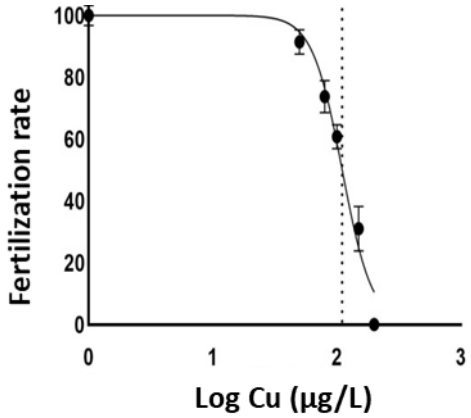


Figure 4S Effect of copper sulphate ($CuSO_4 *5H_2O$) EC_{50} on *Arbacia lixula* spermatozoa. In the graph, the Log EC_{50} is depicted by the dashed line, corresponding to the EC_{50} of 110.5 µg/L.

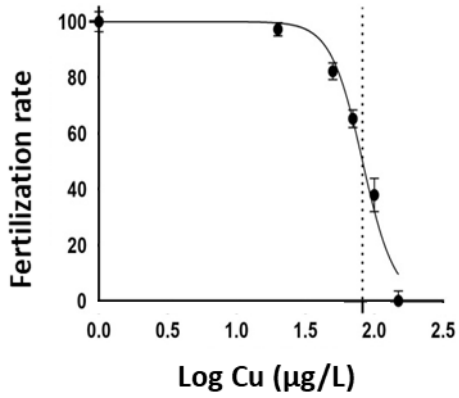


Figure 5S Effect of copper nitrate ($Cu(NO_3)_2*3H_2O$) on *Paracentrotus lividus* eggs. In the graph, the Log EC_{50} is depicted by the dashed line, corresponding to the EC_{50} of 82.23 µg/L.

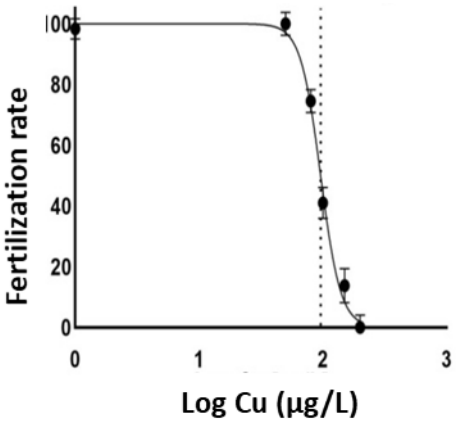


Figure 6S Effect of copper sulphate ($CuSO_4$ *$5H_2O$) on *Arbacia lixula* eggs. In the graph, the Log EC_{50} is depicted by the dashed line, corresponding to the EC_{50} of 95.92 µg/L.

Section S4. Ecosafety assessment of CNS and its components on gamete fertilisation competence

Spermatozoa and eggs of *P. lividus* and *A. lixula* were exposed to a range of concentrations of TOCNF and citric acid. Then, they were used to carry out the spermiotoxicity and ovotoxicity tests. Each test was performed in triplicate and the assays were repeated three times. Data are presented as fertilisation rate (%) ± standard error (SE). Since data are expressed as percentage values and do not show a continuous distribution ($\pm\infty$), they were converted into arcosen before performing the statistical analysis with parametric tests using the software Systat 11.0 (Systat Software Inc.). To compare all treatments to the control group, one-way analysis of variance (ANOVA plus Fisher LSD post-test) was performed. Differences were considered significant at a P value lower than 0.05 (*P<0.05) or 0.01 (**P<0.01).

Table 1S Effect of TOCNF on *Paracentrotus lividus* eggs and spermatozoa. Statistical analyses did not reveal any significant change compared to the control. FR (Fertilization rate).

TOCNF (µg/mL)	**Spermiotoxicity test FR (%)**	**Ovotoxicity test FR (%)**
CTRL	85.30 ± 1.57	78.87 ± 1.15
0.01	85.56 ± 1.92	78.21 ± 1.42
0.1	85.77 ± 1.92	82.46 ± 1.42
1	86.73 ± 1.92	80.52 ± 1.42
10	86.81 ± 1.92	81.06 ± 1.15
100	84.54 ± 1.57	78.77 ± 1.15
1000	86.48 ± 1.57	81.72 ± 1.15

Table 2S Effect of TOCNF on *Arbacia lixula* eggs and spermatozoa. Statistical analyses did not reveal any significant change compared to the control. FR (Fertilisation rate).

TOCNF (µg/mL)	**Spermiotoxicity test FR (%)**	**Ovotoxicity test FR (%)**
CTRL	82.85 ± 1.34	81.37 ± 0.95
0.01	82.81 ± 1.34	80.99 ± 1.70
0.1	81.09 ± 1.34	82.32 ± 1.70
1	82.54 ± 1.34	81.40 ± 1.70
10	82.71 ± 1.34	80.92 ± 0.95
100	82.19 ± 1.34	80.92 ± 0.95
1000	80.94 ± 1.34	80.00 ± 0.95

Table 3S Effect of citric acid on *Paracentrotus lividus* eggs. Statistical analyses did not reveal any significant change compared to the control. FR (Fertilisation rate).

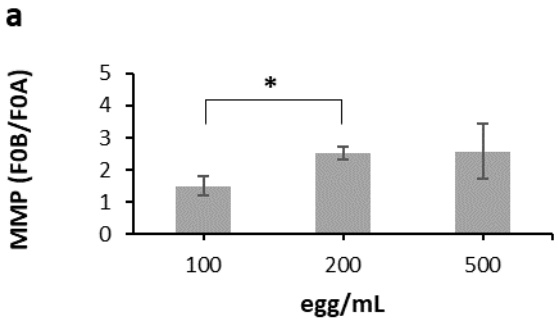
Citric acid (µg/mL)	**Ovotoxicity test FR (%)**
CTRL	71.59 ± 1.41
10	72.90 ± 3.16
100	70.95 ± 3.16
1000	65.40 ± 2.71

Table 4S Effect of citric acid on *Arbacia lixula* eggs and spermatozoa. Statistical analyses did not reveal any significant change compared to the control. FR (Fertilisation rate)

Citric acid (µg/mL)	**Spermiotoxicity test FR (%)**	**Ovotoxicity test FR (%)**
CTRL	100.00 ± 3.55	99.00 ± 3.66
10	97.60 ± 3.58	97.29 ± 3.70
100	98.51 ± 3.55	97.70 ± 3.63
1000	98.54 ± 3.52	97.89 ± 3.66

Section S5. Preliminary experiments to set up the experimental conditions of egg quality assessment

The egg quality assessment has been performed by employing fluorescence staining coupled with a fluorescence microplate screen assay. In particular, the microplate-based approach involved the distribution of the eggs at small volumes, 200 µl, in wells of a plate, which were subsequently analysed. Thereby, in order to set up the optimal number of eggs for well, after staining with JC-1 to evaluate the mitochondrial membrane potential (MMP), C11-BODIPY$^{581/591}$ for the lipid peroxidation assessment, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to estimate the hydrogen peroxide (H_2O_2) intracellular levels, the dihydroethidium (DHE) for the intracellular content of superoxide anions (O_2^-) evaluation, and the 2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-carboxyfluoresce in acetoxymethyl ester (BCECF-AM) to determinate the intracellular pH (pH_i), 100, 200, and 500 eggs/mL were transferred to a 96-well plate for spectrofluorometric analysis. Statistical analysis of the egg quality parameters was performed with Systat 11.0 (Systat Software Inc.) by using parametric one-way analysis of variance followed by Fisher's least significant difference post hoc test. Data are reported as mean values ± standard error (SE), and differences between groups were considered significant at a P value lower than 0.05 ($P<0.05$) and 0.01 ($P<0.01$). The obtained results revealed that 500 eggs/mL for each well was the optimum egg concentration to perform the spectrofluorometric analysis in *P. lividus.* Particularly, except for LPO and pH_i assessment in which the values did not change based on egg concentrations (Figure 7S b and e), the MMP and ROS values significantly increased by enhancing the egg concentration (figure 7S a, c, d) suggesting that 100 and 200 egg/mL were not sufficient to estimate the analysed quality parameters and may induce an underestimation of the egg quality. Additionally, since the *A. lixula* eggs have a similar shape, structure and size to those of *P. lividus*, this optimum egg concentration was also employed for the egg quality assessment in *A. lixula*.



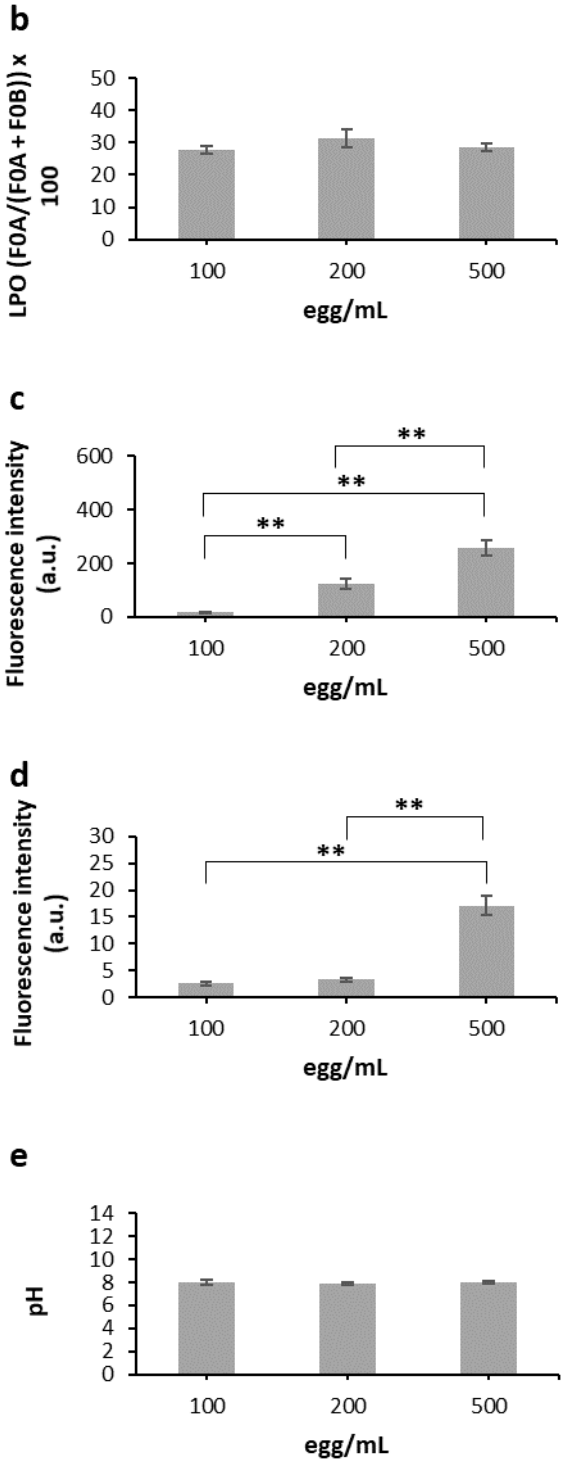


Figure 1S. Egg quality in *Paracentrotus lividus* using different egg concentration. After staining eggs at different concentrations (100, 200, and 500 egg/mL) were transferred to the wells of a 96-well plate and analysed at the spectrofluorimeter to assess a) the mitochondrial membrane potential (MMP) calculated as the ratio between the intensity fluorescence peaks at ~595 (F_0B) and ~525 (F_0A) nm; b) lipid peroxidation (LPO) calculated as the ratio between the intensity fluorescence peak at ~525 nm (F_0A) and the sum of both peaks (F_0A ~525 + F_0B ~595); c) intracellular levels of hydrogen peroxide (H_2O_2) expressed as arbitrary units (a.u.) at the fluorescence peak; d) intracellular content of superoxide anions (O_2^-) evaluated as arbitrary units (a.u.) at the fluorescence peak; e) intracellular pH (pH_i) resulting from the calibration curve as the ratio between the two fluorescence emission peaks recorded at 535 nm after excitation at 490 nm and 440 nm. Data are presented as mean values ±

standard error (SE). * and ** indicates a significance level lower than 0.05 (P< 0.05) and 0.01 (P< 0.01), respectively.

Section S6. Ecosafety assessment of CNS and its components on sea urchin gamete quality

In both species, spermatozoa and eggs were exposed to W solution, bPEI solution, CA solution, TOCNF solution. DF refers to the dilution factor of W1, W2 or W3 solutions. Statistical analysis was performed using Systat 11.0 (Systat Software Inc.). The statistical evaluation of the treatments was obtained using parametric one-way analysis of variance followed by Fisher's least significant difference post hoc test performed to determine significant differences in treatment means. Data are reported as mean values (± SE) of intracellular pH (pH_i) evaluated by using the cell-permeant dye 2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-carboxyfluoresce in acetoxymethyl ester (BCECF-AM), mitochondrial membrane potential (MMP) evaluated by JC-1, intracellular levels of hydrogen peroxide (H_2O_2) evaluated by 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), intracellular content of superoxide anions (O_2^-) evaluated by dihydroethidium (DHE), lipid peroxidation (LPO) evaluated by C11-BODIPY$^{581/591}$, and sperm motility (motility). The numbers highlighted in bold are significantly different compared to the control. Differences were considered significant at a P value lower than 0.05 ($P<0.05$) and 0.01 ($P<0.01$).

Table 1S Sperm quality assessment after W solution exposure in *Paracentrotus lividus.*

	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO ($F_0A/(F_0A + F_0B)) \times 100$	Motility (%)
W1 solution						
CTRL	10.42±0.02	19.95±2.14	334.5±75.92	103.6±9.87	62.64±1.00	69.00±5.89
DF 1	10.45±0.03	**26.31±1.67 P = 0.01**	**593.8±75.92 P = 0.018**	100.1±9.87	61.57±1.00	**7.05±5.89 P = 0.00**
DF 2	10.44±0.05	23.62±1.31	404.6±78.40	107.1±9.87	60.41±1.00	**17.94±5.89 P = 0.00**
DF 5	10.51±0.05	25.79±2.58	325.2±78.40	113.1±9.87	60.02±1.00	**24.86±5.89 P = 0.00**
DF 10	10.41±0.12	19.57±1.04	311.2±78.40	126.7±9.87	60.65±1.00	54.45±5.89
W2 solution						
CTRL	12.23±0.45	18.72±3.01	244.1±0.07	81.80±1.36	56.30±1.39	53.98±4.50
DF 1	12.22±0.41	22.76±2.60	264.0±0.11	80.39±1.30	61.22±2.25	53.17±5.15
DF 2	12.41±0.38	25.29±2.27	268.5±0.13	83.41±1.74	57.63±2.96	54.88±9.59
DF 5	12.12±0.29	22.97±2.84	278.6±0.16	78.57±1.55	55.25±1.33	48.51±5.47
DF 10	12.34±0.40	17.96±1.57	262.2±0.13	80.44±1.18	56.73±1.09	45.42±6.51
W3 solution						
CTRL	10.95±0.52	19.48±3.48	252.1±0.08	81.05±1.36	57.78±2.07	51.99±7.92
DF 1	12.00±0.59	19.26±2.33	276.9±0.09	78.87±1.70	55.37±1.01	47.18±1.00
DF 2	11.99±0.59	18.33±1.69	277.0±0.15	79.44±1.60	53.95±1.84	48.78±5.95
DF 5	12.12±0.47	15.56±1.98	270.1±0.08	83.93±1.90	54.53±2.35	48.58±3.54
DF 10	11.91±0.40	15.77±1.10	243.3±0.10	83.00±1.70	57.06±3.26	39.58±2.78

Table 2S Sperm quality assessment after W solution exposure in *Arbacia lixula.*

	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO ($(F_0A/(F_0A + F_0B)) \times 100$)	Motility (%)
W1 solution						
CTRL	9.33±0.08	15.08±1.51	221.3±21.84	298.8±17.25	52.27±2.25	85.19±3.61
DF 1	9.50±0.13	**22.92±2.44 P = 0.01**	**367.8±20.44 P = 0.002**	**383.2±26.5 P = 0.048**	50.51±2.46	**53.32±6.96 P = 0.000**
DF 2	9.12±0.07	18.35±2.21	**336.3±20.90 P = 0.011**	335.5±25.96	47.98±2.64	76.29±3.75
DF 5	9.13±0.12	13.10±1.04	263.8±24.06	368.1±36.47	52.21±3.04	80.46±2.39
DF 10	9.14±0.17	14.89±1.96	290.0±36.89	344.8±34.61	49.87±3.03	84.39±2.16
W2 solution						
CTRL	10.85±0.09	15.41±0.75	268.7±2.18	282.7±1.40	76.2±1.53	82.49±0.82
DF 1	11.13±0.04	19.10±1.28	290.2±0.73	357.3±2.38	60.82±1.08	80.09±1.92
DF 2	11.09±0.11	19.00±0.50	293.0±3.85	323.7±1.13	72.18±1.87	81.14±2.15
DF 5	10.83±0.05	18.48±1.35	276.3±1.72	337.7±0.66	76.07±1.84	83.33±0.00
DF 10	10.88±0.12	23.24±1.57	270.7±2.49	316.7±1.67	72.83±0.71	83.33±0.00
W3 solution						
CTRL	10.02±0.10	25.51±2.54	179.8±0.71	363.0±0.25	52.34±2.93	75.57±0.57
DF 1	10.14±0.12	26.30±3.00	200.3±0.81	404.5±1.11	50.39±3.01	74.93±1.75
DF 2	10.37±0.23	31.01±1.24	226.8±3.25	379.5±0.80	47.08±0.50	73.38±0.28
DF 5	10.69±0.04	25.80±1.58	185.7±1.45	395.0±1.06	46.41±0.83	77.56±2.23
DF 10	10.56±0.03	31.56±1.20	224.8±1.66	408.5±1.41	48.72±2.10	77.04±0.60

Table 3S Sperm quality assessment after exposure to CNS components in *Paracentrotus lividus*

(µg/mL)	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO ($F_0A/(F_0A + F_0B)) \times 100$	Motility (%)
bPEI						
CTRL	11.42±0.62	25.94±1.94	359.4±0.54	334.1±2.52	52.41±5.00	64.39±0.81
0.1	11.43±0.75	29.79±0.07	326.2±0.67	322.6±3.46	51.20±5.45	60.61±2.23
1	11.64±0.81	27.55±0.33	317.3±0.53	307.3±1.79	50.66±4.41	**45.47±4.87 P = 0.006**
10	11.16±0.62	30.25±3.27	358.4±0.24	294.9±0.55	49.57±5.53	**48.98±0.00 P = 0.000**
Citric acid						
CTRL	11.09±0.24	14.48±2.58	299.9±0.30	236.2±1.14	58.92±2.34	76.39±1.15
10	11.91±0.25	21.57±0.74	289.5±0.35	221.2±1.62	58.19±1.32	77.25±3.13
100	11.42±0.30	21.02±3.85	311.1±0.35	222.5±4.22	58.15±1.62	72.94±1.43
1000	11.78±0.23	24.82±2.96	269.9±0.37	215.8±1.35	59.03±3.04	68.64±1.70
TOCNF						
CTRL	10.73±0.62	16.47±0.40	285.1±54.53	246.9±27.40	58.9±1.25	65.94±6.12
1000	10.33±0.36	18.57±0.50	306.9±30.99	292.1±15.77	57.11±1.51	74.49±9.17

Table 4S Sperm quality assessment after exposure to CNS components in *Arbacia lixula.*

(µg/mL)	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO ($(F_0A/(F_0A + F_0B)) \times 100$)	Motility (%)
bPEI						
CTRL	9.33±0.05	10.20±1.20	289.8±19.3	249.0±21.23	56.97±0.59	81.72±5.61
0.1	9.21±0.07	**15.38±0.14 P = 0.004**	**227.9±31.47 P = 0.042**	211.5±22.76	60.61±1.67	71.65±5.11
1	9.28±0.10	**14.41±0.80 P = 0.001**	**299.7±29.67 P = 0.026**	210.4±8.099	56.53±1.07	63.66±6.81
10	9.28±0.05	**14.44±0.70 P = 0.028**	**334.6±24.66 P = 0.000**	**285.6±31.14 P = 0.001**	46.19±1.18	**28.94±9.77 P = 0.014**
Citric acid						
CTRL	9.33±0.05	16.98±2.02	261.6±8.79	257.4±33.36	56.88±0.82	81.72±5.61
10	9.19±0.06	17.05±0.21	**315.7±3.52 P = 0.014**	224.7±28.34	59.97±2.72	82.70±10.82
100		14.21±2.91	**343.4±6.54 P = 0.001**	249.4±31.37		61.38±2.34
1000		17.69±1.43	**322.5±10.18 P 0.007**	248.4±10.58		63.42±9.55
TOCNF						
CTRL	9.33±0.05	9.68±1.11	215.2±18.79	257.4±33.36	56.97±0.59	81.72±5.61
10	9.26±0.06	13.47±1.64	294.3±28.23	290.9±45.02	54.43±0.86	91.39±1.55
100		10.24±1.16	289.5±23.57	249.0±21.09		88.01±1.81
1000		12.97±1.54	292.5±29.16	306.6±14.06		74.72±6.02

Table 5S Egg quality assessment after W solution exposure in *Paracentrotus lividus.*

	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO ($(F_0A/(F_0A + F_0B)) \times 100$)
W1 solution					
CTRL	8.27±0.07	2.03±0.30	188.4±13.78	5.67±0.63	28.60±1.33
DF 1	8.22±0.07	**3.06±0.30 P = 0.013**	**228.8±13.78 P = 0.033**	5.67±0.63	27.98±1.33
DF 2	8.25±0.14	1.67±0.52	188.3±20.25	5.33±0.59	27.63±2.59
DF 5	8.19±0.14	2.88±0.48	195.7±20.25	6.00±0.67	32.68±2.59
DF 10	8.04±0.14	2.11±0.48	202.1±20.25	7.33±0.81	28.22±2.59
W2 solution					
CTRL	8.06±0.15	1.98±0.23	228.4±27.49	6.44±0.17	16.11±0.98
DF 1	8.09±0.09	**3.33±0.28 P = 0.000**	221.3±65.78	5.89±0.19	16.7±0.92
DF 2	8.30±0.11	**2.87±0.19 P = 0.019**	210.6±46.64	6.22±0.26	17.8±1.28
DF 5	8.00±0.04	2.40±0.20	212.2±58.63	6.11±0.19	16.15±0.58
DF 10	8.23±0.10	2.49±0.07	232.3±51.60	5.89±0.25	16.16±1.51
W3 solution					
CTRL	7.38±0.22	1.64±0.13	228.4±27.49	6.44±0.17	16.11±0.98
DF 1	7.65±0.30	2.38±0.28	224.6±15.33	6.56±0.32	16.67±0.92
DF 2	7.36±0.18	1.76±0.35	199.8±65.58	5.89±0.25	17.80±1.28
DF 5	7.31±0.19	2.19±0.15	251.0±14.35	6.78±0.21	16.15±0.58
DF 10	7.09±0.19	1.93±0.17	224.8±44.24	5.89±0.25	16.16±1.51

Table 6S Egg quality assessment after W solution exposure in *Arbacia lixula.*

	pH	**MMP (F_0B/F_0A)**	**H_2O_2 (A.U.)**	**O_2^- (A.U.)**	**LPO ($F_0A/(F_0A + F_0B)$) x 100**
W1 solution					
CTRL	7.88±0.07	1.38±0.14	99.6±12.75	7.60±0.30	99.58±2.11
DF 1	8.00±0.08	1.58±0.18	94.67±11.53	8.40±0.53	94.67±1.41
DF 2	8.04±0.11	1.57±0.30	93.08±8.34	8.07±0.44	93.08±1.39
DF 5	7.99±0.12	1.57±0.18	85.75±11.30	8.13±0.35	85.75±1.27
DF 10	8.02±0.15	1.66±0.21	122.6±25.76	7.73±0.24	122.6±1.30
W2 solution					
CTRL	7.83± 0.11	1.30± 0.11	114.5±69.36	4.33± 0.14	64.59± 2.09
DF 1	7.97±0.06	1.51±0.15	115.9±57.80	4.58±0.32	64.04±2.99
DF 2	7.91±0.06	1.52±0.12	87.90±35.41	4.42±0.28	64.01±2.30
DF 5	7.85±0.09	1.36±0.05	101.2±49.24	4.75±0.21	64.38±2.28
DF 10	7.75±0.11	1.37±0.06	90.67±23.07	5.00±0.29	63.33±2.42
W3 solution					
CTRL	7.83± 0.11	1.26± 0.13	114.5±69.36	4.33±0.14	64.59± 2.09
DF 1	7.56±0.23	1.60±0.07	92.1±33.95	4.58±0.30	64.29±2.32
DF 2	7.63±0.23	1.52±0.21	92.1±45.21	4.83±0.26	61.88±2.32
DF 5	7.93±0.22	1.19±0.03	95.5±43.04	4.75±0.21	61.56±1.56
DF 10	7.89±0.19	1.22±0.06	111.2±78.81	4.58±0.18	64.28±1.94

Table 7S Egg quality assessment after exposure to CNS components in *Paracentrotus lividus.*

(µg/mL)	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO (F0A/(F0A + F0B)) x 100
bPEI					
CTRL	8.05±0.17	2.37±0.41	290.1±25.98	1.57±0.48	26.53±2.14
0.1	7.92±0.20	**2.61±0.25 P = 0.052**	298.5±25.98	1.55±0.48	27.37±2.44
1	8.01±0.20	**3.03±0.26 P = 0.002**	301.8±27.76	2.38±0.48	28.04±2.44
10	8.45±0.21	**3.19±0.25 P = 0.000**	341.4±36.74	2.18±0.45	**38.43±2.44 P = 0.000**
Citric acid					
CTRL	8.09±0.10	2.37±0.41	290.1±25.98	2.78±0.58	23.31±1.52
10	7.91±0.10	2.05±0.28	310.2±29.96	2.60±0.46	25.34±1.52
100	7.80±0.12	1.25±0.28	260.5±29.96	2.80±0.53	21.63±1.52
1000	7.63±0.10	1.94±0.28	244.3±29.96	2.73±0.58	21.21±1.52
TOCNF					
CTRL	8.05±0.11	2.58±0.85	325.7±51.27	2.67±0.45	34.46±.89
1000	7.72±0.11	2.54±0.85	343.1±40.28	2.83±0.57	37.46±5.62

Table 8S Egg quality assessment after exposure to CNS components in A*rbacia. lixula*.

(µg/mL)	pH	MMP (F_0B/F_0A)	H_2O_2 (A.U.)	O_2^- (A.U.)	LPO ($(F_0A/(F_0A + F_0B)) \times 100$)
bPEI					
CTRL	8.41±0.05	1.77± 0.14	165.5±21.00	8.05±0.46	50.34±1.24
0.1	8.66±0.04	1.86± 0.22	137.8±17.82	7.61±0.48	52.05±1.28
1	8.46±0.05	1.56± 0.11	118.9±18.32	7.88±0.47	52.47±1.20
10	8.23±0.14	**3.09± 0.49 P = 0.008**	118.9±18.17	7.80±0.45	**55.77±1.53 P = 0.008**
Citric acid					
CTRL	8.41±0.05	1.77± 0.14	165.5±21.00	8.05±0.46	50.34±1.24
1000	8.55±0.07	1.62± 0.17	149.5±21.48	7.76±0.50	53.16±1.51
TOCNF					
CTRL	8.41±0.05	1.77± 0.14	165.5±21.00	8.05±0.46	50.34±1.24
1000	8.63±0.07	1.72± 0.19	128.5±23.52	7.55±0.46	53.02±1.51

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Abbreviations

2-OH-E^+ = 2-hydroxyethidium

ATP = Adenosine Triphosphate

BCECF = 2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-carboxyfluorescein

BCECF-AM = 2',7'-bis-(2-192 carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester

bPEI = Branched Polyethyleneimine

CA solution = Citric acid solution

CA = Citric Acid

CCCP = Carbonyl cyanide 3-chlorophenylhydrazone

CNF = Nanofibers of Cellulose

CNS = Cellulose-based Nanosponges

DCF = 2′,7-dichlorofluorescein

DF = Dilution Factor

DHE = Dihydroethidium

E^+ = Ethidium

EC50 = Effective Concentration to affect 50% of embryo

ENMs = Engineered Nanomaterials

$FeSO_4$ = Ferrous Sulphate

FNSW = Natural Seawater Filtered through a 0.22 μm nitrocellulose filter

GCs = Germinal Cells

GO = Graphene Oxide

GO-Fe_3O_4 = Graphene Oxide-Iron Oxide

GSR = Green and Sustainable Remediation

H_2DCF = 2′,7-Dichlorodihydrofluorescein

H_2DCF-DA = 2′,7-Dichlorodihydrofluorescein Diacetate

H_2O_2 = Hydrogen Peroxide

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPF = hours post fertilisation

JC-1 = 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide

LCA = Life Cycle Assessment

LPO = Lipid Peroxidation

MMP = Mitochondrial Membrane Potential

MPTP = Mitochondrial Permeability Transition Pore

NMs = Nanomaterials

NOM = Natural Organic Matter

NPs = Nanoparticles

$nTiO_2$ = Titanium Dioxide NPs

nZVI = Nanoscale Zero-Valent Iron

PAHs = Polycyclic Aromatic Hydrocarbons

PB = Prussian Blue

PEI = Polyethyleneimine

PGCs = Primordial germ Cells

pHi = Intracellular pH

ROS = Reactive Oxygen Species

SE = Standard Error

SEM = Scanning Electron Microscope

T solution = Seawater exposed to TOCNF

TEMPO = 2,2,6,6 Tetramethylpiperidinyloxyl

TOCNF = TEMPO-Oxidised Cellulose Nanofibers

TOUS-CNF = TEMPO-Oxidised And Ultra-Sonicated CNF

USEPA = U.S. Environmental Protection Agency

W solution = Seawater exposed to CNS

W1 solution = Wash 1 solution; seawater exposed to CNS washed once

W2 solution = Wash 2 solution; seawater exposed to CNS washed twice

W3 solution = Wash 3 solution; seawater exposed to CNS washed three times