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# Embryotoxicity and spermiotoxicity of nanosized ZnO for Mediterranean sea urchin *Paracentrotus lividus*

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## HIGHLIGHTS

- Sea urchins revealed very sensitive organisms to nZnO.
- nZnO toxicity for P.lividus could not be ascribable only to the ionic Zn.
- Sperm fertilization capability is not affected from tested Zn compounds.
- nZnO dispersed in ASW aggregate in μm size as function of particle concentration.
- Sea urchin developmental anomalies mainly evidenced nZnO toxicity.

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## ABSTRACT

The effect of nano ZnO (nZnO) upon the fertilization and early development of embryos of the Mediterranean sea urchin *Paracentrotus lividus* is reported herein for the first time. Zn ion ($ZnCl_2$) and bulk ZnO (bZnO) toxicity were assessed for comparison. The embryotoxicity tests showed a 100% effect already at 1 μM of nZnO (expressed as [Zn]) while bZnO and $ZnCl_2$ showed EC50s of 0.98 [0.88–1.19] μM [Zn] and 2.02 [1.97–2.09] μM [Zn], respectively. Noteworthy, the frequency of developmental defects for the three compounds was dissimilar and a specific trend for larval skeletal abnormality produced by nZnO was observed.

The sperm fertilization capability was only slightly affected by the tested chemicals while the effects were dramatic on the offspring quality of sperms exposed to ZnO compounds resulting in an early block of the regular larval development.

ZnO toxicity seems related not only to Zinc ions but also to some surface interactions of particle/aggregates with target organisms and/or with the seawater.



## 1. Introduction

Nanoparticles (NPs) of ZnO, having absorbing properties in the UV wavelength range, are commonly added to sunscreens and cosmetics [1,2]. During the life cycle of these commercial products, NPs may be released into the environment to become a novel possible contaminant to ecosystems. Currently no data regarding nanomaterial pollution in the environment can be found but the predicted environmental concentrations (PECs) are at present estimated to be in the region of ten nanograms per litre in surface waters [3]. Therefore, higher concentrations in the coastal marine environment could be present, especially during the summer season when a rapid release of sunscreen in the seawater can be expected, at least locally. In addition, industrial and urban effluents normally run out into estuarine or marine environments, thus coastal systems are likely to be the ultimate sink for any nanomaterial accidentally or purposely discharged into the environment [4]. It is well-known that the physicochemical characteristics of nanosized materials can be substantially different from those of traditional bulk materials of the same composition. This raises questions whether their reduced size might increase the bioavailability and/or the overall toxicity of these materials with respect to bulk counterparts [5]. The complex seawater environment could further complicate the problem, greatly affecting the aggregation and dissolution processes which produce both the potential toxic species: metal ions and nanoparticle aggregates.

Sea urchins are among the main marine organisms expected to be exposed to these new contaminants. Their gametes and embryos are often utilized to assess the toxicity of chemical compounds [6–8] due to their sensitivity and availability. Spermiotoxicity and

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embryotoxicity tests offer the possibility of comparing the effects of the same substance upon two different biological systems: sperms and embryos. Sperms consist of highly specialized single cell, which, when exposed to toxicants, can result in immediate damage, therefore reducing or suppressing their fertilization capacity and/or in a transmissible damage to offspring. Embryos represent a highly duplicative cell system which, when exposed to toxicants, become subject to adverse action on the delicate embryo developmental process.

Although nZnO revealed toxic in the aquatic environment for several organisms [9] little is known about its toxicity to marine biota, and to the best of our knowledge, studies about its effect upon the Mediterranean sea urchin's early development are not yet available. Recently, one study on the American sea urchin species *Lytechinus pictus* was published reporting a high toxicity effect of nZnO on the exposed embryos [10].

nZnO toxicity is generally ascribed to two general mechanisms: the generation of reactive oxygen species (ROS) [11], which can damage organisms through a variety of interrelated effects including lipid peroxidation and DNA damage [5,12] and the release of metal ions [13–15]. This last mechanism is often considered the main cause of nZnO toxicity in the aquatic environment but significant differences in toxicity of nanosized and bZnO have not been reported yet [16,17]. However, some studies show that, in complex matrices such as marine waters and soils, the toxic mechanisms cannot be restricted to the action of released metal ions but the presence of solid particles should also be considered for a full comprehension of the toxic impacts [18–20]. The aim of this research was to investigate the effects of nZnO upon early development, fertilization and offspring quality of the Mediterranean sea urchin *Paracentrotus lividus*. The findings were also compared to the toxic effects of Zn ions ($ZnCl_2$) to investigate how the $Zn^{2+}$ contributes to nZnO toxicity, and with bZnO to evaluate the toxicity linked to the nanosize.

## 2. Experimental

### 2.1. Chemicals

Zinc oxide (particle size <100 nm, surface area 15–25 $m^2$/g, purity >99%) and $ZnCl_2$ (purity >99.995%) were purchased from Sigma–Aldrich S.r.l. ZnO bulk powder for pharmaceutical formulation (particle size >200 nm, surface area 4–7 $m^2$/g, purity >99.9%) was purchased from Galeno S.r.l., Italy.

### 2.2. Test solution preparation

Stock solutions of nZnO, bZnO and $ZnCl_2$ with solid loading of 100 mg/L were made by dispersing each chemical in 100 mL of artificial seawater (ASW) [21] (pH 8.0, 0.22 μm filtered). The ZnO suspensions were prepared by bath-sonication for 30 minutes at 50 W. The stock solutions so obtained, were diluted with ASW to carry out the toxicological assays in concentrations ranging between 1 and 50 μM of Zn. The dilutions were briefly vortexed before the addition of the gametes or zygotes.

### 2.3. Particles and test solution characterization

As received materials were observed by Scanning Electron Microscopy on a LEO 1530. The specific surfaces area (SSA) of the ZnO dry powders were analyzed using the Brunauer, Emmett and Teller (BET) method on a Quantachrome, Autosorb-1instrument, recording $N_2$ adsorption/desorption isotherms at 77 K. Hydrodynamic diameters of nZnO and bZnO dispersions in seawater (at 10 and 100 mg/L) were characterized by Dynamic Light Scattering technique using the Zetasizer Nano ZS Malvern Instruments. This instrument employs a 4 mW He–Ne laser, operating at wavelength 632.8 nm with the measurement angle set at 173° using a Non-Invasive Back Scatter technology (NIBS). Samples were measured at a temperature of 25 °C.

The sedimentation process was measured recording the optical absorbance at 468 nm of nZnO and bZnO dispersions in ASW at 10 and 100 mg/L for 270 minutes For this measurement a home-made optical setup was used, built up by a photodiode light source peaked at 468 nm, coupled with an optical fibre and a monochromator coupled to a photodetector.

### 2.4. Test organisms

Adult *P. lividus* (Lamark) individuals were collected from the Tyrrhenian Sea (Bay of Naples 17 °C, Salinity 37.5‰, pH 7.9) by the staff of the Zoological station of Naples The sea urchins were then posed in an aquarium for 24 h at 18 °C (Salinity 38 ‰, pH 8.0). This rest time was necessary because the utilization of the organisms, right after their collection, produces a decrease of normal plutei in the control, probably due to the stress induced by the collection activity itself. Moreover, a sudden increase in temperature or salinity might not only induce spawning, but seriously harm the gametes [22].

### 2.5. Toxicity test

Gametes were harvested and embryos reared as described by Pagano et al. [8]. Spawning was induced in the sea urchins by injection of 1 mL of 0.5 M KCl through the perioral membrane. Eggs were collected by separately placing each spawning female in a different 250 mL beaker with ASW while "dry" sperm from each male was collected by an automatic pipette and stored in a sterile tube placed on ice. For each experiment, six individual females were selected for the egg quality (no immature forms, no debris, no fertilized eggs) and quantity. Males were selected for sperm motility (checked under the microscope) and quantity. Then, the best three male and three female gametes were pooled and filtered through nylon cheesecloth (∅ = 200 μm for eggs and 50 μm for sperm). The egg suspension (stock solution) was diluted in order to obtain the final concentration of 250–300 eggs/mL.

#### 2.5.1. Embryotoxicity test

Fertilization was carried out by adding 1 mL of pooled-sperm (1:1000 in ASW), to the egg suspension and by incubating it at 18 °C for 20 min The presence of the fertilization membrane was then verified in a random sample of 100 eggs. The excess sperm was removed by decanting zygotes and suspending them in ASW. A volume of the egg suspension corresponding to 250–300 fertilized eggs was treated with 10 mL of test solution. Three replicates for each treatment were prepared. The eggs were then incubated at 18 °C, for 48–50 h. After this period, 100 μL of 40% buffered formalin was added in each vessel and developmental abnormalities were determined in each replicate by direct observation of 100 individuals, randomly chosen. For each treatment schedule, 100 plutei were scored for the frequencies of: (1) normal larvae, according to their symmetry, shape, and size (N); (2) retarded larvae (R) having normal shape and symmetry but with reduced size (<1/2 N); (3) malformed larvae (P1), affected in skeletal and/or gut differentiation and/or pigmentation; (4) pre-larval arrest (P2), embryos unable to go to larval differentiation, as abnormal blastula or gastrulae [8,23]. Each test was performed at least three times.

#### 2.5.2. Spermiotoxicity test

Aliquots (10 μL) of concentrated sperm from the pool of three males were added to 10 ml of test solutions. After the exposure