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Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*

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HIGHLIGHTS

- nano ZnO is more toxic for the marine alga *D. tertiolecta* than its bulk counterpart.
- nano ZnO affects the algal growth rate starting from lower tested concentrations.
- Toxic effects of nano ZnO cannot be explained only by the free zinc ion action.
- The nano ZnO primary size affects the overall toxicity.

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ABSTRACT

Dose response curve and population growth rate alterations of marine Chlorophyte *Dunaliella tertiolecta* derived from the exposure to ZnO nanoparticles were evaluated. Bulk ZnO and ionic zinc were also investigated for comparison. At the same time, the aggregation state and particle size distribution were monitored. The evaluated 50% effect concentration (EC50 1.94 [0.78–2.31] mg Zn L^{-1}) indicates that nano ZnO is more toxic than its bulk counterpart (EC50 3.57 [2.77–4.80] mg Zn L^{-1}). Cross-referencing the toxicity parameters calculated for $ZnCl_2$ (EC50 0.65 [0.36–0.70] mg Zn L^{-1}) and the dissolution properties of the ZnO, it can be gathered that the higher toxicity of nano ZnO is most likely related to the peculiar physicochemical properties of the nanostate with respect to the bulk material. Furthermore growth rate of *D. tertiolecta* was significantly affected by nano ZnO exposure.
Our findings suggest that the primary particle size of the dispersed particles affect the overall toxicity.
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1. Introduction

Nanomaterials are an important emerging class of contaminants, with potential wide-ranging ecological impacts due to their small size and high reactivity. They often show different toxicity profiles compared with larger particles because of the peculiarities of the nanostate. In fact, the nanosize and huge surface area give nanoparticles (NPs) the potential to interact more efficiently with biological systems, producing toxicity (Manzo et al., 2011). Metal oxide NPs are commonly used for a range of applications and, in particular, ZnO nanoparticles (nano ZnO) are widely used in the production of pigments, semiconductors, sunscreens, and food additives. Nano ZnO has an inherently high risk of water contamination, and can reach high concentrations in surface waters posing a significant threat to aquatic ecosystems (Gottschalk et al., 2009).

Coastal systems are likely to be the ultimate sink for any nanomaterial and NPs, deliberately or purposely discharged into the environment (Klaine et al., 2008). Marine algae are highly diffused in coastal ecosystems (Behrenfeld et al., 2006) and so they are particularly susceptible to contaminants associated with anthropogenic pollution. The evaluation of NP effects upon marine phytoplankton is a necessary step to predict their potential impact on coastal marine food webs and on the whole ecosystems they support. How NPs move in seawater is still not well understood. It is well-known that NPs tend to aggregate in aquatic environments to form micrometer-sized particles and it is likely that this state of dispersion may reduce the influence of particle size, shape and surface properties on their ecotoxicity (Handy et al., 2008; Limbach et al., 2005).

As far as metal oxide NPs are concerned, the release of metal ions is one of the most investigated pathways. The dissolved Zn ions are considered as the most relevant toxic agents for nano ZnO in aqueous media (Aruoja et al., 2009; Franklin et al., 2007; Xia et al., 2008). Free Zn^{2+} appears toxic to many aquatic organisms (Eisler, 1993) including marine phytoplankton (Franklin et al., 2007; Miller et al., 2010). However, some studies reveal that the adverse impacts of nano ZnO could not be completely explained by their metal ion release (Maness et al., 1999) and that metal NPs may be more toxic than either their ionic forms or their parent compounds (Farré et al., 2009; Navarro et al., 2008). The point is that, at current knowledge, the observed ZnO toxicity cannot be attributed univocally to the release of zinc ions or to the nanosize as the peculiar surface interactions

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of these nanoparticles with target organisms and/or with the media (i.e. water, biological media, etc.) and the toxicity pathways are still far to be established (Miao et al., 2010; Miller et al., 2010).

In this study we focused on the ecotoxicological effect of nano ZnO towards the green alga *Dunaliella tertiolecta*, which is a marine unicellular chlorophyta commonly used in standard chronic algal toxicity testing, with the aim to establish the main toxicity parameters and to evaluate whether nano ZnO produced toxic effects which can be clearly ascribed to peculiarities of the nanostate. For each compound EC50, EC10, NOEC and lowest observed effect concentration (LOEC) were defined to provide biological criteria for the implementation of water quality standards to protect marine organisms. The EC10 value was considered in order to determine the concentration that induce the minimum effect (10%) statistically detectable and therefore to identify the more protective parameter for microalgae.

As far as the aggregation rate and size may be relevant for the effects of the dispersed particles in the marine ecosystem, the size distribution of ZnO particles was monitored at the same time of toxicity testing. Relying on *D. tertiolecta* we recorded dose response curves for exposure to nano ZnO and calculated for the first time its main toxicological parameters. Dose response curves were measured also for exposure to bulk ZnO in order to check any specificity in the nanosized material with respect to its bulk counterpart. The findings were also compared to the toxic effects of free Zn^{2+}, to specifically investigate the metal ion contributions to ZnO toxicity.

2. Materials & methods

2.1. Chemicals

Bare zinc oxide (ZnO cod. 544906, particle size 100 nm, surface area 15–25 m^2/g) and $ZnCl_2$ (cod. 429430, purity >99.995%) were purchased from Sigma-Aldrich. ZnO bulk powder for pharmaceutical formulation (particle size 200 nm, purity >99.9%) was purchased from Galeno S.r.l., Italy.

2.2. Organisms

D. tertiolecta (Chlorophyceae: Chlamydomonadales) (CriAcq Laboratory, Naples, Italy) algae, were maintained in sterilized standard medium (Guillard, 1975) made with artificial standard seawater (ASTM, 1998, pH 8.0, 0.22 μm filtered). To provide inoculant for experiments, microalgae were incubated under cool continuous white fluorescent lights (about 58 μmol photons m^{-2} s^{-1}) at 24 ± 1 °C with aeration for 5–7 days until log phase growth prevailed. Cell density was measured by hemacytometer.

2.3. Particle dispersions

Stock solutions of each testing material (nano ZnO, bulk ZnO and $ZnCl_2$) were prepared by dispersing 10 mg of the dry powders into 100 mL of Artificial SeaWater (ASW) to the final concentration of 100 mg L^{-1}. The zinc oxide suspensions were prepared by bath-sonication for 30 min at 50 W and aliquots of these were analyzed for particle size distribution. The stock solutions were properly diluted to carry out the toxicological assays in concentrations ranged between 0.01 and 10 mg Zn L^{-1}. The test suspensions were vortexed briefly before the addition of micronutrients and algae.

The solubility of nano and bulk ZnO in seawater was evaluated by analyzing the Zn^{2+} content of suspensions prepared with a strong excess of ZnO particles (that is with a solid loading much higher of any reported value of ZnO solubility in water or seawater). Nano and bulk ZnO (solid loading of 5 g L^{-1}) were mixed in standard seawater into glass bottles and placed onto a magnetic stirrer for around 70 h. These suspensions were then vacuum filtrated through 0.02 μm alumina membranes (Anodisc 47 mm, Whatman) for [Zn^{2+}] analysis.

2.4. Particle characterization

Nano and bulk ZnO as-received powders were observed by Scanning Electron Microscopy on a LEO 1530 (Fig. 1). The specific surface area (SSA) of both samples nano and bulk ZnO was analyzed using the Brunauer, Emmett and Teller (BET) method on a Quantachrome, Autosorb-1 instrument, recording N_2 adsorption/desorption isotherms at 77 K (Brunauer et al., 1938). The BET analysis was also used to confirm the primary particle size of the materials declared by the manufacturers. In fact, by the surface area BET is possible to calculate what is called the "BET radius" through the equation (Roelofs and Vogelsberger 2004):

$$r = 3/SSA_{BET} \quad \rho$$

where SSA_{BET} is the specific surface area of the particles as measured by the BET method and ρ is the particle density. This analysis showed that the average size of nano ZnO and bulk ZnO used was 100 nm and 200 nm respectively.

Hydrodynamic diameters of nano ZnO and bulk ZnO dispersions in seawater at 10 mg L^{-1} were characterized by DLS 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 size distribution of ZnO particles was monitored at the same time of toxicity testing.

The maximum amount of Zn ions that can be released was evaluated using the suspensions of nano and bulk ZnO in ASW. Dissolved Zn^{2+} was separated from ZnO particles by filtration of 20 mL of suspensions through a 0.020 μm membrane, acidified with HNO_3 (1%) and diluted 1:1000 with pure water for ICP-MS analysis. Measurements were performed on a spectrometer ICP-MS Elan 6000 (Perkin–Elmer).

2.5. Toxicity test

2.5.1. Algal growth inhibition test

Algal bioassays were performed according to IRSA-CNR (1978). All glassware was acid-washed, rinsed with purified water milliQ, and autoclaved before use. Algal cells (with a final density 10^3 cells mL^{-1}) were first filtered (0.22 μm) and rinsed three times with filtered autoclaved seawater. The algal cells were then added to each treatment and control (standard culture media, Guillard medium) together with nutrients deprived of metals, to avoid the addition of EDTA that would complex any free metal ions in the test solution. Test plates (10 mL) were kept in a growth chamber with continuous light (about μmol photons m^{-2} s^{-1}), at a temperature of 24 ± 1 °C for 4 days. The growth inhibition was expressed with respect to the control. The concentrations of the testing solutions were defined on the basis of a preliminary screening and were 2.28, 1.71, 1.14, 0.68, 0.23, 0.17, 0.11, 0.023 mg Zn L^{-1} for $ZnCl_2$, and 10, 7.5, 5, 3, 1, 0.75, 0.5, 0.1 mg Zn L^{-1} for ZnO (nanoparticles and bulk). Growth rate was calculated daily as: $\mu = (\ln N2 - \ln N1)/(t2 - t1)$ where N1 and N2 are the cell concentrations, at times t1 and t2, respectively.

2.5.2. Data analysis

Analysis of variance (ANOVA) was applied, using raw data, in order to test for significant differences in effects among treatments (significance level was always set at $p = 0.05$). When data did not meet the assumptions of normality and homoscedasticity, non parametric Kruskall–Wallis test was employed to compare individual treatments.

The EC50 was calculated using the Linear Interpolation Method (Inhibition Concentration procedure or ICp) (US EPA, 1993). The bootstrap method is used to obtain the 95% confidence interval, because standard statistical methods for confidence interval calculations are

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