Toxic effects of different particle size ZnO NPs on marine microalgae *Chlorella* sp.

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**Abstract:** Zinc oxide nanoparticles (ZnO NPs) are widely used and will inevitably be released into the marine environment, which will have a serious impact on the microalgae in the ocean, and the toxicity of the NPs is closely related to its size. In this study, it was found that small particles of ZnO are more toxic to photosynthesis. The content of SOD and MDA are significantly increased, indicating that oxidative stress has occurred and the cell membrane has been destroyed. The results show that the NPs with smaller size had the higher toxicity to microalgae.

1. Introduction

In recent years, with the rapid development of nano materials, it has been widely used in electronic equipment, energy, cosmetic and biomedicine[1-3]. ZnO NPs are the third highest annual production of nanoparticles (550 tons per year) after silica and titanium dioxide in worldwide[4]. Nanotechnology environmental health and safety (nano EHS) is an important consideration for the acceptance and promotion of these emerging technologies[5]. ZnO NPs will be released into the environment and will eventually enter our aquatic organisms from these nano-based products[6]. The ocean is the final confluence area for any substances released into the environment which ZnO NPs will inevitably be brought into it. In coastal ecosystems, microalgae can perform photosynthesis and as the foundation of the aquatic food web, any change in microalgae may affect higher nutrient levels[7, 8]. Particle size may be an important factor in determining the impact of NPs on biological systems[9]. Numerous studies have shown that the toxicity of NPs is closely related to particle size[10, 11]. Generally speaking, smaller nanoparticles have higher specific surface area and more toxicity[12]. If ZnO NPs are small enough, they can enter the nucleus through the nuclear hole or internalize through the cell wall hole during cell division[13]. However, some studies have reached the opposite conclusion. Sharma et al[14] reported that the DNA damage of 29 nm Ag NPs exposed to fish for 7 days was higher than that of 18 nm Ag NPs exposed to the same concentration. Little study is known about the size dependence of the toxicology of ZnO NPs in marine microalgae. As such, in our study we attempt to explore the toxicity of ZnO NPs with different particle sizes to the marine green alga *Chlorella* sp.

2. Materials and Methods

2.1 Algal strain and culture medium

Nanopowders of ZnO (CAS:1314-13-2, 99.8% metals basis, 30±10 nm,90±10 nm,200 nm) were purchased from Macklin Industrial Corporation, China. The concentration of this experiment is 10 mg/L. Marine algae *Chlorella* sp. was obtained from the OUC, all microalgae were cultivated in an f/2 medium.
Guillard and Ryther, 1962) made with sterile seawater (filtered by a 0.45 um membrane) from Qingdao, China. The microalgae were cultivated in a 3 L Erlenmeyer flask at 20±1℃ under cool, continuous white fluorescent lights (4,000 LUX) with a 12-h light 12-h dark cycle, and shaken twice per day to prevent the sedimentation of the algae.

2.2 Content of chlorophyll a
After 96 h, collecting the algae cells at 4000 r/min, remove the supernatant, add 4 mL of 80% acetone to the precipitated algae mud, ultrasonically break for 3 min, after breaking, centrifuge the cells at 5000 r/min, take the supernatant for 10min, take 80% acetone as a blank reference, Measure the absorbance at 663 nm and 646 nm.

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\text{Chlorophyll a Ca (mg/L) = 12.21A}_{663}-2.81A_{646}
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2.3 Enzymatic activity and lipid peroxidation
To evaluate the oxidative stress caused by the ZnO NPs, the intracellular antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT), in algal cells were measured after exposure to different size ZnO NPs after 96h. The algal cells were harvested by centrifugation 5000 r/min 10 min at 4 ℃ and suspended in 2 mL phosphate-buffered saline solution (PBS; pH 7.2) for subsequent ultrasonic disruption by sonication in an ice bath. After centrifugation 8000 r/10 min at 4 ℃, the supernatant was collected for measurements of enzyme activity, CAT level and lipid peroxidation. The MDA content, SOD and CAT activities were determined using commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s instructions.

2.4 Data analysis
The experimental results are expressed in the form of mean value ± standard deviation (mean ± SD). The statistical software SPSS 19 and one-way analysis of variance (one-way ANOVA) should be used to analyze the differences between the control group and the exposure experimental group. \( P < 0.05 \) indicates a significant difference between the control groups.

3. Result and discussion

3.1 Content of chlorophyll a
The content of photosynthetic pigments (including chlorophyll a) is an effective indicator of the growth status of algae \[15\]. In order to further understand the effect of ZnO NPs on the marine microalga Chlorella, the chlorophyll a content of NPs treatment was measured. The chlorophyll in the microalgae cells was significantly reduced after exposure to nano-ZnO compared with the control, while the microalgae exposed to zinc ions had the lowest chlorophyll content, indicating that nano-zinc oxide had a certain effect on the photosynthesis of microalgae. This may be due to the fact that ZnO NPs aggregated by adsorbing on the algae cells, and the aggregates adhere to and shield the algae cells, resulting in a reduction in the available light and reducing the utilization of light \[16\]. Chen \[17\] used SEM micrographs to confirm the adsorption of NP on the surface of algae (Chlorella sp.), which induced severe cell damage to algae cells. There is no significant difference in the effect of different particle size of ZnO NPs on the photosynthesis of microalgae, because the aggregate size of the different particle size of ZnO NPs after 96 h are similar, so the effect on the photosynthesis of algae cells is not obvious. However, the content of chlorophyll a in microalgae was significantly reduced under zinc ion exposure, indicating that zinc ions have a more significant impact on microalgae photosynthesis and produce stronger toxic effects. The toxic effects of ZnO NPs are not only caused by the released zinc ions.
Fig. 1 The content of chlorophyll-a in the microalgae cells after 96 h. The data are presented as mean ± SD, n = 3

3.2 Oxidative stress

Fig. 2 The SOD activity, CAT activity, MDA level under different sizes of ZnO NPs in microalgae at 96 h. The data are presented as mean ± SD, n = 3

In antioxidant enzymes, SOD neutralizes superoxide anions (O$_2^-$) because they are catalytically converted to hydrogen peroxide (H$_2$O$_2$), which can be reduced by CAT and GPx enzymes to convert H$_2$O$_2$ to H$_2$O [18]. The potential of ZnO NPs to induce oxidative stress was evaluated by measuring SOD in algae cells. The results are shown in Fig 2. that SOD is obviously induced at 30 nm, indicating that excessive ROS is produced, causing oxidative stress. Suman et al. [19] reported similar results. As the particle size increases, SOD gradually decreases, and the SOD produced by algae cells is not obvious when they are exposed to bulk ZnO. Indicating that the smaller particle size will result more oxidative damage to the microalgae, which is similar to the results of Saxena [20]. In this experiment, the change
trend of CAT activity of Chlorella vulgaris is similar to that of SOD. The increase of cat is caused by the increase of its reaction substrate (H$_2$O$_2$), that is, the accumulation of SOD products. ZnO NPs with smaller particle size produce more ROS for algae cells, so the content of SOD and cat is significantly higher. Therefore, in this experiment, it can be found that ZnO NPs with smaller particle size produce stronger oxidative stress on algae cells.

To further examine the lipid peroxidation of algal cell membranes exposed to ZnO NPs directly, the MDA level of the algae were measured as well. MDA usually indicates the degree of oxidation of the biofilm. Malondialdehyde is the main product of the lipid peroxidation process of the cell membrane, and its content directly reflects the degree of damage to the cell membrane. If the cell membrane is oxidized, its permeability and fluidity will change, which may increase the transmembrane capacity of Zn$^{2+}$[21]. In this study, the MDA level of the microalgae cell treated with ZnO NPs was significantly higher than that of the control, indicating that the ZnO NPs caused the lipid peroxidation of algal cells and destroyed the microalgae cell membranes, which is consistent with Tang’s results [8]. 30 nm ZnO NPs has the most significant impact on the microalgae, indicating that the smaller the particle size, the greater damage to microalgae cell membranes. When the size of ENP decreases, the ratio of surface area to mass or volume increases, which in turn affects other physical and chemical properties, such as the reactivity of surface atoms and electrical and optical properties [22]. If the ZnO NPs are small enough, they can enter the nucleus through the nuclear pore or be internalized through the cell wall pores during cell division [13, 23].

4. Conclusion
Our results show that the physical and chemical properties of the particles play an important role in the developmental stages of algae. This study clearly shows that the photosynthesis of algae under exposure to smaller nanoparticles is extremely inhibited and marine microalgae exposed to smaller NPs produced stronger oxidative stress. The result reveal that the toxic effect is partly caused by the release of zinc ions but not all of it depends on this factor. In addition, ZnO NPs in seawater are extremely unstable and will aggregate and release ions. The effect of such factors on the toxicity of nanoparticles should also be considered during research.

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