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Interaction of TiO_2 nanoparticles with the marine microalga *Nitzschia closterium*: Growth inhibition, oxidative stress and internalization

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HIGHLIGHTS

- Inhibition of marine microalgae by TiO_2 NPs and bulk particles was evaluated.
- Aggregation of TiO_2 NPs and bulk particles was observed in marine algal test medium.
- TiO_2 NPs induced damage to algal cell membranes as detected by flow cytometry.
- Increased TiO_2 nanotoxicity to algal cells was caused by internalization of NPs.

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ABSTRACT

The toxicity of TiO_2 engineered nanoparticles (NPs) to the marine microalga *Nitzschia closterium* was investigated by examining growth inhibition, oxidative stress and uptake. The results indicated that the toxicity of TiO_2 particles to algal cells significantly increased with decreasing nominal particle size, which was evidenced by the 96 EC_{50} values of 88.78, 118.80 and 179.05 mg/L for 21 nm, 60 nm and 400 nm TiO_2 particles, respectively. The growth rate was significantly inhibited when the alga was exposed to 5 mg/L TiO_2 NPs (21 nm). Measurements of antioxidant enzyme activities showed that superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities were first induced and subsequently inhibited following exposure to 5 mg/L TiO_2 NPs. The depletion of antioxidant enzymes with a concomitant increase in malondialdehyde (MDA) levels and reactive oxygen species (ROS) posed a hazard to membrane integrity. A combination of flow cytometry analysis, transmission electron microscopy and Ti content measurement indicated that TiO_2 NPs were internalized in *N. closterium* cells. The level of extracellular ROS, which was induced by TiO_2 NPs under visible light, was negligible when compared with the intracellular ROS level (accounting for less than 6.0% of the total ROS level). These findings suggest that elevated TiO_2 nanotoxicity in marine environments is related to increased ROS levels caused by internalization of TiO_2 NPs.

1. Introduction

The production of engineered nanomaterials has increased exponentially over the past several years. As a result of this increase, engineered nanoparticles (NPs) are inevitably released into aquatic systems from the common sources, including personal care products, urban and industrial sewage and anti-fouling components of paints—eventually reaching the ocean (Handy et al., 2008; Klaine et al., 2008; Matranga and Corsi, 2012). Consequently, coastal waters are expected to represent the ultimate sink for NPs (Canesi et al., 2012).

The stability of NPs is mainly related to their physicochemical properties (e.g., size, charge, and coating) and environmental factors (e.g., ionic strength and dissolved organic materials) (Liu et al., 2014; Zhao et al., 2014). For example, multi-walled carbon nanotubes can be stably suspended in water as a result of dissolved organic materials. Humic acids (HAs) greatly enhanced suspension of both P- and C-MWCNTs. The suspension enhancement was attributed to HA sorption, which increased electrostatic repulsion and steric hindrance between individual MWCNTs (Zhou et al., 2012). However, the high ionic strength can result in aggregation of TiO_2 NPs (Zhu et al., 2014). Thus, the dispersion of NPs in seawater is likely to be more difficult than that in freshwater. It is reasonable to assume that the mechanisms by which NPs exert toxic effects on marine organisms are more complex than the mechanisms by which NPs exert toxic effects in freshwater. Therefore, findings from NP toxicity investigations in fresh water (Li et al., 2014a, 2014b; Ma et al., 2014; Zhao et al., 2013) must be taken into account but cannot be extrapolated to marine biota that exist in seawater.

Among the various types of NPs, TiO_2 NPs are currently of great interest. TiO_2 NPs are widely used for many applications such as sunscreens, paints, coatings, solar cells, and photocatalytic water purification (Botta

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et al., 2011). For Europe and USA, the highest predicted environmental concentrations in surface water were projected for TiO_2 NPs compared with other NPs such as ZnO NPs, Ag NPs, carbon nanotubes (CNTs), and fullerenes (Gottschalk et al., 2009). The concentration of TiO_2 NPs (20 and 300 nm) released from painted facades was reported to be as high as 3.5×10^8 particles/L in runoff water (Kaegi et al., 2008). Much attention has been devoted to the risk of increased TiO_2 NP release on various ecosystems (Cañas-Carrell et al., 2011; Li et al., 2014a, 2014b; Wallis et al, 2014). Importantly, recent eco-toxicological data showed that TiO_2 NPs were toxic to marine organisms, such as invertebrates (Barmo et al., 2013; Canesi et al., 2010; Zhu et al., 2011a, 2011b), cyanobacteria (Cherchi and Gu, 2010), and polychaetes (Galloway et al., 2010). Miller et al. (2010) reported that TiO_2 NPs had no measurable effect on the population growth rates of marine phytoplankton (*Thalassiosira pseudonana*, *Skeletonema marinoi*, *Dunaliella tertiolecta* and *Isochrysis galbana*). On the contrary, the same group (Miller et al., 2012) reported that TiO_2 NPs can be phytotoxic to the same marine phytoplankton under natural levels of ultraviolet radiation. However, these authors did not characterize intracellular and extracellular reactive oxygen species (ROS) production. Furthermore, they did not examine the impact of TiO_2 NPs on membrane permeability. As such, the mechanism of TiO_2 NP toxicity on marine phytoplankton remains unclear. In this study, we focus on the cytotoxicity of TiO_2 NPs on marine phytoplankton, a key primary producer of the food web within the marine ecosystem. *Nitzschia closterium*, a marine eukaryotic unicellular diatom, was selected because it is widely present in the marine ecosystem and is a high-quality food for the culture of bivalves. The aim of this study was to investigate (1) the growth inhibition of marine microalgae caused by TiO_2 NPs compared with their bulk counterparts; (2) the mechanism by which TiO_2 NPs exert cytotoxic effects on algae cells, which was determined by examining antioxidant enzyme activities and ROS generation over time; and (3) the possibility that the NPs are internalized within cells. These results will enhance our understanding of the mechanism by which TiO_2 NPs exert toxic effects on marine organisms. In addition, this work will provide essential data for studying the downstream effects of NPs on the microalgae-bivalve food chain in future studies.

2. Materials and methods

2.1. Materials

TiO_2 particles of three different sizes were used in this study: 21 nm NPs were acquired from Sigma Aldrich Company Ltd., China, while 60 nm NPs and 400 nm particles were obtained from Aladdin Reagent Inc., China. All the experiments were carried out using a TiO_2 NP stock suspension (1000 mg/L) prepared in marine algal f/2 medium. A 30 min sonication (200 W; 4×10^4 Hz) was performed to homogenize the stock suspensions immediately before further use. The size distribution and zeta potential of TiO_2 NPs and bulk particles (BPs) in the algal test media were measured with a particle size analyzer (Nano ZS Malvern Instruments, USA). The crystalline structure of the powder was examined using X-ray diffraction (XRD; Bruker D8 Advance TXS, Germany). In addition, the morphology of all the particles was observed using a transmission electron microscope (TEM, JEM-2100, JEOL, Japan).

2.2. Algal growth assays

The unicellular marine diatom *N. closterium* was purchased from the Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, China. During the growth inhibition experiments, the algal cells were cultured in f/2 medium under 3500 lx light intensity with a 12:12 light–dark cycle (temperature: 20 ± 1 °C; salinity: 33.5 ± 0.5; pH: 7.8 ± 0.1). The light source in the tests was a cool fluorescent tube emitting light in the visible spectrum with a wavelength range of 400–700 nm. Stock solutions of TiO_2 NPs and BPs were added to the algal medium for treatment at different concentrations (5, 10, 20, 40, 80, and 100 mg/L for 21 nm TiO_2 NPs; 10, 20, 40, 80, 160, 240, and 360 mg/L for 60 nm TiO_2 NPs; and 20, 40, 80, 160, 320, and 500 mg/L for TiO_2 BPs). Algae (1×10^6 cells/mL) in the exponential phase of growth were exposed to TiO_2 particles of three different sizes, and the algal biomass measurements were performed from 0 to 96 h. All treatments were performed in triplicate. Cells were counted using a microscope (Olympus AX70, Japan), and in each sample, the algal cells were counted a minimum of three times. The concentration of TiO_2 suspended in the medium was measured using UV spectrophotometry and calculated according to the method described by Federici et al. (2007).

2.3. SEM and TEM imaging

A scanning electron microscope (SEM, HITACHI S-4800) was used to observe the morphology of algae after TiO_2 exposure. Transmission electron microscopy (TEM, JEM-2100, JEOL, Japan) was employed to characterize the size and morphology of TiO_2 NPs as well as the interaction between TiO_2 NPs and algal cells. After exposure to NPs for 24 h, all algal cells were fixed using 2.5% glutaraldehyde. Then, the samples were post-fixed in 1% osmic acid for 1 h and washed three times with phosphate-buffed saline (PBS, pH 7.2). The samples were dehydrated in increasing concentrations of acetone (30, 50, 70, 80, 90, and 100%; 20 min each time) at room temperature. EPON812 resin was used to permeate and impregnate the samples for 5 h. Ultrathin sections were made and placed on nickel grids for TEM observation and energy dispersive spectroscopy (EDS, INCA100, Oxfordshire, UK) analysis.

2.4. Enzymatic activity, lipid peroxidation and reactive oxygen species

Time-dependent determination of the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), lipid peroxidation (LPO) and ROS generation in algal cells was conducted after exposure to TiO_2 NPs (5 mg/L) for 2, 6, 12, 24, and 48 h. The algal cells were collected by centrifugation (5000 rpm) for 5 min at 4 °C, and the supernatant was removed for extracellular ROS determination. Meanwhile, the ROS levels of the algal culture medium in the absence or presence of 5 mg/L TiO_2 NPs were determined after 2 h of exposure. The harvested algae were suspended in 2 mL of buffer solution (PBS, pH 7.2) and immediately disrupted by sonication (Ningbo Haishu Kesheng Ultrasonic Equipments Co., Ltd, KS-500F, China) for 3 min with a 3 s pause after each 3 s pulse in an ice bath. The cell homogenate was centrifuged at 2500 rpm for 10 min at 4 °C, and the supernatant was used for the measurement of enzyme activities, malondialdehyde (MDA) level and intracellular ROS generation.

The activity of SOD was measured based on its ability to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide radicals generated with xanthine/xanthine oxidase. One unit of SOD activity (U) is defined as the amount of protein that inhibits the rate of NBT reduction by 50% in 1 mL of the reaction solution (Sun et al., 1988). CAT activity was determined according to Claiborne (1985) by measuring the initial rate of decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption over 1 min. POD activity was assayed using guaiacol as a hydrogen donor by measuring the change at 470 nm over 1 min as described by Chance and Maehly (1955). The enzyme activities were calculated per mg of protein. Protein concentrations in the cell extracts were determined at 595 nm using the method developed by Bradford (1976), with bovine serum albumin as the standard. Enzyme assays were conducted using a BIO-RAD iMark microplate reader (Bio-RAD, Hercules, CA, USA). The LPO level was determined in terms of MDA (a product of lipid peroxidation) content, which was measured using the thiobarbituric acid (TBA) reaction as described previously (Erdelmeier et al., 1998). ROS generation was measured according to the method described