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Toxicity and trophic transfer of P25 TiO_2 NPs from *Dunaliella salina* to *Artemia salina*: Effect of dietary and waterborne exposure

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ABSTRACT

The recent increase in nanoparticle (P25 TiO_2 NPs) usage has led to concerns regarding their potential implications on environment and human health. The food chain is the central pathway for nanoparticle transfer from lower to high trophic level organisms. The current study relies on the investigation of toxicity and trophic transfer potential of TiO_2 NPs from marine algae *Dunaliella salina* to marine crustacean *Artemia salina*. Toxicity was measured in two different modes of exposure such as waterborne (exposure of TiO_2 NPs to *Artemia*) and dietary exposure (NP-accumulated algal cells are used to feed the *Artemia*). The toxicity and accumulation of TiO_2 NPs in marine algae *D. salina* were also studied. *Artemia* was found to be more sensitive to TiO_2 NPs (48 h LC_{50} of 4.21 mg L^{-1}) as compared to marine algae, *D. salina* (48 h LC_{50} of 11.35 mg L^{-1}). The toxicity, uptake, and accumulation of TiO_2 NPs were observed to be more in waterborne exposure as compared to dietary exposure. Waterborne exposure seemed to cause higher ROS production and antioxidant enzyme (SOD and CAT) activity as compared to dietary exposure of TiO_2 NPs in *Artemia*. There were no observed biomagnification (BMF) and trophic transfer from algae to *Artemia* through dietary exposure. Histopathological studies confirmed the morphological and internal damages in *Artemia*. This study reiterates the possible effects of the different modes of exposure on trophic transfer potential of TiO_2 NPs and eventually the consequences on aquatic environment.

1. Introduction

P25 TiO_2 nanoparticles (TiO_2 NPs) are photocatalysts, which find applications in cosmetics, sunscreens, paints, water purifying system, and self-cleaning agents (Browning et al., 2014; Robertson et al., 2010). Occurrence of both anatase and rutile forms increases the photo catalytic activity of TiO_2 NPs (Hurum et al., 2003). Their release into the marine environment is inevitable and can occur due to urban activities, surface runoff, sewage and waste discharge, and coatings on marine structures (Zhu et al., 2016). Nanoparticles can significantly contribute to the marine ecotoxicity irrespective of their aggregation and agglomeration in seawater (Keller et al., 2010). The predicted levels of TiO_2 were about 1.6 μg L^{-1} in surface water, 1.2 mg L^{-1} in water treatment plant effluent, and 6 mg kg^{-1} in sediments (Gottschalk et al., 2013). The risk evaluations of nanomaterials suggested that organisms at both lower and higher trophic levels are greatly affected (Wang et al., 2017).

As model test species, marine algae *D. salina* and crustacean *Artemia* have been used in the current investigation to study the effect of TiO_2 NPs in artificial sea water medium (ASW). Marine algae, being highly susceptible to engineered nanomaterials, can be utilized as a pollution indicator in marine systems owing to their high bioaccumulation ability (Barhoumi and Dewez, 2013). Only a few prior studies have reported the toxicity of P25-type NPs towards marine algae. Miller et al. (2012) have analysed the growth inhibition in four algal species (*Thalassiosira pseudonana*, *Skeletonema costatum*, *Dunaliella tertiolecta*, and *Isochrysis galbana*) by TiO_2 NPs. Sendra et al. (2017b) reported the toxicity of sunscreens and TiO_2 NPs towards *Chaetoceros gracilis*, *Amphidinium carterae*, *Pleurochrysis roscoffensis*, and *Nannochloropsis gaditana*. Under direct sunlight, sunscreens without NPs were found to be less toxic compared to those with TiO_2 NPs. A comparative study of TiO_2 particles in both NP and bulk forms towards marine algae *Phaeodactylum tricornutum* and freshwater algae *Chlamydomonas reinhardtii* was carried out by Sendra et al. (2017a).

Utilizing *Artemia* as an experimental model in ecotoxicological testing is noteworthy, considering the advantages it possesses namely, their short life span, compliance to various salinity and temperature conditions, and as it is a non-selective filter feeder (Nunes et al., 2006).

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Owing to their filter feeding behaviour, ability to accumulate metals, and sensitivity to environmental pollutants, *Artemia* is often used as a bio-indicator (Viarengo et al., 2007). Previous studies about the effects of TiO_2 NPs on *Artemia* showed significant accumulation of NPs in both *Artemia* nauplii and adults. Higher concentrations (LC_{50} > 100 mg L^{-1}) and longer exposure duration caused mortality in both nauplii and adults (Ates et al., 2013). Clemente et al. (2014) reported 48 h EC_{50} of 285 mg L^{-1} for anatase and rutile mixture of $nTiO_2$ NPs in the presence of visible light. Under UV illumination, EC_{50} decreased to 4 mg L^{-1}, highlighting the significance of UV in exacerbating the toxic effects. Absence of light decreased the toxicity effects of $nTiO_2$ NPs on *Artemia franciscana* larvae, whereas in the case of starvation condition, toxicity increase for both $nTiO_2$ and $TiCl_4$ was reported (Minetto et al., 2017)

Toxicity of nanoparticles in the lower trophic level may greatly affect the organisms at higher levels via food web. Thus, it is important to study the transfer of nanomaterials across the trophic levels. Uptake–depuration studies of TiO_2 NPs in *Chlamys farreri* were performed for waterborne and dietary exposure. Waterborne exposure of the particles reportedly resulted in an increased content of NPs in the gills, digestive gland, and mantle of scallops compared to the dietary exposure (Wang et al., 2017). A study of trophic transfer of Ag NPs from *Artemia* nauplii to marine *Oryzias melastigma* showed a trophic transfer efficiency of less than 6% for a 28-day treatment period (Wang and Wang, 2014). The lack of information on the trophic transfer of TiO_2 NPs from algae to *Artemia* necessitated further investigation.

Herein, we report the toxicity of TiO_2 NPs on algae (*D. salina*) and subsequent trophic transfer to marine crustacean *Artemia*. The influence of two different modes of exposure such as waterborne (nanoparticles exposure to *Artemia* via aqueous media) and dietary exposure (NP-accumulated algal cells are used to feed the *Artemia*) on the mortality, uptake, and trophic transfer of TiO_2 NPs was evaluated.

2. Materials and methods

2.1. Materials

Titanium dioxide nanoparticles (Aeroxide P25, particle size: 21 nm (TEM), ≥ 99.5% trace metals basis) and 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich. Hydrogen peroxide solution 30% w/v (H_2O_2) and nitroblue tetrazolium chloride (NBT) were purchased from SDFCL (Mumbai, India). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and hydroxylamine hydrochloride were obtained from Hi-Media Pvt. Ltd (Mumbai, India).

2.2. Test organism

Marine algae *Dunaleilla salina* was procured from CMFRI, Mandapam, Tamil Nadu, India, and the culture was grown along with supplements (Supplemental Table 1) at 23 ± 2 °C (day/night rhythm of 16 h/8 h, under white fluorescent lighting with a photon concentration of 40.5 μmol m^{-2} s^{-1}). *Artemia* brine shrimp cysts were obtained from Ocean Star International Inc., USA, and stored at 4 °C. Prior to hatching, the brine shrimp cysts were hydrated in deionized water for 12 h at 4 °C. Sinking cysts were rinsed with distilled water. Approximately, 1 g of pre-cleared cysts was incubated in 2 L of sterilized natural seawater in a round-bottom glass tank at 30 ± 1 °C. Aeration and light illumination were provided using an aquarium air pump and a fluorescence lamp (10 W, 0.44 mW/cm^2), respectively. Hatching of *Artemia* brine shrimp cysts commenced within 24 h of incubation. The hatched nauplii were transferred into fresh seawater medium, and the 48-h-old nauplii were used for toxicity studies. Toxicity assessment of nanoparticles was performed according to OECD (2004) guidelines. All the experiments were carried out using ASW.

2.3. Stability and aggregation

The stability and aggregation of nanoparticles (0.1, 1, and 10 mg L^{-1}) after being sonicated (20 kHz, 750 W, 20 min) in artificial seawater were analysed using dynamic light scattering method (90 Plus Particle Size Analyzer, Brookhaven instruments Corporation, USA) at different time intervals of 0, 24, and 48 h.

2.4. Effect of TiO_2 NPs on algae

Exponential phase algal cultures were harvested and centrifuged at 7000 rpm for 10 min at 4 °C. The pellet obtained was washed with sterilized ASW. A series of concentrations of nanoparticles (0.1, 1, and 10 mg L^{-1}) was allowed to interact with the algal cells of 0.1 optical density at 610 nm (2.5 × 10^6 cells/mL) under visible light conditions. Interaction was carried out for 72 h according to OECD guidelines (OECD, 1984). MTT assay was performed to determine the cellular viability, wherein yellow colour MTT was reduced to purple colour formazan in the mitochondria of viable cells (Pakrashi et al., 2013). After an interaction period of 72 h, 500 μL of the sample was mixed with 20 μL of MTT solution (5 mg in 1 μL PBS) and incubated in dark for 4 h. After incubation, the samples were centrifuged at 8000 rpm for 8 min. The pellet obtained was washed with 500 μL of ASW, and 200 μL of dimethyl sulfoxide was added. The absorbance was measured at 570 nm using a microplate reader (ELISA plate reader, Biotek, Powerwave XS2).

2.5. Chlorophyll estimation

Chlorophyll, a photosynthetic pigment, is essential in determining the growth of algae and is an important parameter in determining the cellular viability after nanoparticle interaction (Fargasova, 2001). Ten mL of cell suspension was centrifuged at 7000 rpm for 10 min at 4 °C. The pellet was washed using ASW and suspended in 1 mL N, N-dimethyl formamide (DMF) and incubated in dark for 24 h. After the incubation time period, the samples were centrifuged again, and the supernatant containing extracted chlorophyll was measured at 475, 649, and 665 nm using an UV–Vis spectrophotometer (Hitachi, U-2910, Japan).

Chlorophyll content in algal cells was measured at two different stages of interaction: I) After 48 h exposure of algal cells to different concentrations of TiO_2 NPs (0.1, 1, and 10 mg L^{-1}), the chlorophyll a, b, and total content were measured, II) Chlorophyll content was measured during the dietary exposure study. Briefly, algal cells were initially exposed to different concentrations of TiO_2 NPs (0.1, 1, and 10 mg L^{-1}) for 48 h. Then, *Artemia salina* were fed with algal cells (*Dunaliella salina*) that were pre-treated with TiO_2 NPs. After the 48 h of feeding, the remaining algal cells in the medium were measured for chlorophyll content.

2.6. Toxicity, uptake, and depuration of TiO_2 NPs on Artemia: waterborne exposure

The 48-h-old hatched *Artemia* nauplii were interacted with nanoparticles of different concentrations (0.1, 1, and 10 mg L^{-1}) for 48 h. After the interaction period, the numbers of live nauplii were counted under an optical microscope (Zeiss Axiostar Optical Microscope, USA). No feed was provided to *Artemia* during the course of the experiment. For the nanoparticle uptake study, interacted *Artemia* nauplii were washed with Milli-Q water and transferred to pre-weighted tubes. The samples were dried at 70 °C in a hot-air oven. The uptake of TiO_2 NPs was determined by acid digestion (HNO_3) of dried nauplii and subsequent analysis for TiO_2 concentration using a graphite furnace method (Analyst 400, PerkinElmer). Threshold for the graphite furnace technique for Ti measurement is 10 μg/L. For accumulation study, the interacted nauplii were transferred to fresh ASW medium to evacuate