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TiO_2 nanoparticles in seawater: Aggregation and interactions with the green alga *Dunaliella tertiolecta*

Elisabetta Morelli[a,*], Edi Gabellieri[a], Alessandra Bonomini[a,b], Danika Tognotti[a], Giacomo Grassi[b], Ilaria Corsi[b]

[a] Institute of Biophysics, National Research Council, 56124 Pisa, Italy
[b] Department of Physical, Earth and Environmental Sciences, University of Siena, 53100 Siena, Italy

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) have been widely employed in industrial applications, thus rising concern about their impact in the aquatic environment. In this study we investigated the chemical behaviour of TiO_2 NPs in the culture medium and its effect on the green alga *Dunaliella tertiolecta*, in terms of growth inhibition, oxidative stress, ROS (Reactive Oxygen Species) accumulation and chlorophyll content. In addition, the influence of exopolymeric substances (EPS) excreted by the microalgae on the stability of NPs has been evaluated. The physicochemical characterization showed a high propensity of TiO_2 NPs to form micrometric-sized aggregates within 30 min, large enough to partially settle to the bottom of the test vessel. Indeed, an increasing amount of TiO_2 particles settled out with time, but the presence of EPS seemed to mitigate this behaviour in the first 6 h of exposure where the main effects in *D. tertiolecta* were observed. TiO_2 NPs did not inhibit the 72-h growth rate of *D. tertiolecta*, nor affected the cellular chlorophyll concentration in the range 0.01–10 mg L^{-1}. The time-course of ROS production showed an initial transient increase of ROS in TiO_2 NP-exposed algae compared to the control, concomitant with an enhancement of catalase activity. Interestingly, intracellular ROS was a small fraction of total ROS, the highest amount being extracellular. The occurrence of cell-mediated chemical transformations of TiO_2 NPs in the external medium, related to the presence of EPS, has been evaluated. Our results showed that carbohydrates were the major component of EPS, whereas proteins of medium molecular weight (20–80 kDa) were preferentially bound to TiO_2 NPs, likely influencing their biological fate.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) are extensively used in a wide range of industrial applications and consumer products such as water treatments, photocatalysis, solar cells, self-cleaning paints, cosmetics and sunscreens (Handy et al., 2008; Mu and Sprando, 2010; Botta et al., 2011; Piccinno et al., 2012). Many applications are based on the ability of these metal-oxide NPs to absorb UV-light and to exhibit photocatalytic properties (Wold, 1993; EPA, 2009). The wide spread use of TiO_2 NPs has raised concerns about their impact on the aquatic environment and human health (Klaine et al., 2008). Moreover, the use of these NPs in new marine nanotechnologies, such as pollution remediation systems, has increased the need to understand their fate and sustainability for the marine environment (Corsi et al., 2014). Actually, no data on the amount of TiO_2 NPs in marine waters are available, but the predicted environmental concentrations in surface waters are in the order of a few μg L^{-1} (Minetto et al., 2014). Despite the importance of the marine environment, most of papers concerning the effects of TiO_2 NPs on aquatic biota have been carried out on freshwater rather than on marine organisms (Minetto et al., 2014). Since coastal waters and sediments are the final sink for TiO_2 NPs, a great variety of marine organisms, like crustaceans, molluscs and algae, may be exposed to them (Matranga and Corsi, 2012). Among these, microalgae represent the major primary producers in marine ecosystems, and are of great importance for the maintenance of the aquatic ecosystem. In this perspective, marine microalgae, which are widespread in coastal waters and are particularly sensitive to pollutants, can be used as a model for the studies of aquatic risk assessment of nanomaterials (Amiard-Triquet et al., 2015). Several studies have shown that TiO_2 NPs can cause adverse effects to aquatic organisms, such as fishes (Paterson et al., 2011), invertebrates (Canesi and Corsi, 2016; Della Torre et al., 2015; Zhu et al., 2011; Galloway et al., 2010), and bacteria (Blaise et al., 2008; Cherchi and Gu, 2010). Only a few recent studies have addressed the interactions of these NPs with marine phytoplankton. A marked

* Correspondence to: Institute of Biophysics, Area della Ricerca del CNR, via Moruzzi, 1, 56124 Pisa, Italy.
E-mail address: elisabetta.morelli@pi.ibf.cnr.it (E. Morelli).

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variability of the effects is apparent from the literature, depending on the species studied and the experimental conditions, such as NPs size and type, ionic strength of the culture medium and UV irradiation (Castro-Bugallo et al., 2014; Xia et al., 2015; Sendra et al., 2017a). Indeed, Miller et al. (2012) reported that TiO_2 NPs inhibit the growth rates of *Thalassiosira pseudonana*, *Skeletonema marinoi*, *Dunaliella tertiolecta* and *Isochrysis galbana* only under UV irradiation.

Usually, the effect of TiO_2 NPs in algae is described by parameters reflecting the physiological state of the cells, such as growth rate, photosynthetic pigment content, antioxidant response, assimilation rate of NPs (Chen et al., 2012; Xia et al., 2015; Li et al., 2015; Manzo et al., 2015; Wang et al., 2016; Schiavo et al., 2016). Direct toxic effects, such as membrane and cellular damages, have been described in *Phaeodactylum tricornutum* (Wang et al., 2016), in *Nitzschia closterium* (Xia et al., 2015) and in the freshwater green alga *Chlamydomonas reinhardtii* (Chen et al., 2012) in response to high levels of TiO_2 NPs exposure. Indirect effects of NPs can be caused by the aggregation of NPs on cell surface which could reduce light availability for photosynthesis (shading effect), and/or the uptake of nutrients through the cell surface (Aruoja et al., 2009; Chen et al., 2012; Li et al., 2015). Another well described mechanism of TiO_2 NPs toxicity is the production of reactive oxygen species (ROS), because of their photocatalytic properties and interaction with organisms or biomolecules present in the environment (Von Moss and Slaveykova, 2014).

Studies aiming to address the toxicity of NPs cannot be separated from the knowledge of the chemical behaviour of these NPs in a complex medium, such as seawater (Handy et al., 2008). In the aquatic environment, NPs generally tend to aggregate as a function of size, surface charge, pH of the medium, as well as of the presence of dissolved organic matter (Klaine et al., 2008; Keller et al., 2010). In particular, in seawater, the high ionic strength can effectively shield the repulsive forces among charged NPs, thereby favouring the aggregation processes. In particular, TiO_2 NPs aggregate very quickly in seawater, within a few hours (Keller et al., 2010), with consequent fast sedimentation rates. The aggregation process, common to a large variety of NPs, reduces the exposure of organisms living in the water column and increases the risk for benthic and filter-feeding organisms (Klaine et al., 2008; Morelli et al., 2013; Baker et al., 2014; Zhou et al., 2016). Moreover, recently it has been shown that heteroaggregation of TiO_2 NPs with microalgae can play an important role in the toxicity mechanisms of these nanoparticles, especially at high ionic strength (Sendra et al., 2017b).

A few studies have shown that TiO_2 NPs toxicity is related not only to their physicochemical properties (i.e. surface charge, size, shape, photocatalytic activity), but also to the environmental conditions (i.e. pH, ionic strength, natural organic matter). One of the main components of natural organic matter is constituted EPS excreted by bacteria and phytoplankton. EPS are ubiquitous in the marine environment. They are mainly composed by polysaccharides and proteins, with a variable composition depending on species and environmental conditions (Verdugo et al., 2004). EPS are amphiphilic biopolymers which can assemble to form marine gels, but can also play a key role in the interaction between NPs and marine organisms. Recent papers have shown that EPS can interact with NPs, thus affecting NPs aggregation and /or degradation processes (Quigg et al., 2013). The interaction of EPS derived from phytoplankton with NPs in the marine environment has been described for quantum dots (Zhang et al., 2012), copper-based NPs (Adeleye et al., 2014) and silver engineered NPs (Miao et al., 2009) but, to our knowledge, no study has been performed with TiO_2 NPs. Moreover, no information is available about the chemical characterization of biomolecules, such as proteins, which could adsorb on NPs, and consequently affect their biological fate.

The aim of the present study was to investigate the effect of TiO_2 NPs on the green alga *Dunaliella tertiolecta*, in terms of growth rate, chlorophyll content, oxidative stress and ROS accumulation. We used Dynamic Light Scattering (DLS) to monitor the behaviour of TiO_2 NPs suspensions in the culture media both alone and conditioned with EPS, in order to assess their stability in seawater during the exposure experiment. In addition, we focused on the chemical characterization of EPS and their interaction with NPs, in order to improve our understanding on their role in TiO_2 NPs toxicity.

2. Materials and methods

2.1. Preparation and behavioural characterization of TiO_2 NPs

Nanosized titanium dioxide (TiO_2 NPs) Aeroxide® P25 was supplied by Evonik Degussa (Germany). According to the manufacturer, TiO_2 NPs have an average primary size of 25 nm and are composed of anatase: rutile 80%:20% crystalline phase (purity > 99.5%). Brunauer, Emmet and Teller (BET) specific surface area is $50 \pm 15\ m^2\ g^{-1}$. The pristine nanopowder was employed for dispersions as received, with no surface modifications. Stock dispersions were prepared suspending TiO_2 NPs powder in ultrapure Milli-Q water (Millipore, Bedford, MA, USA) up to a concentration of 5000 mg L^{-1} by tip sonication (45 min at 100 w, 50% cycle off) in an ice-cool bath. Initially, NPs dispersed in Milli-Q water were imaged through transmission electron microscopy (TEM) by deposition of a 10 µL drop on formvar/carbon-coated copper grids and overnight drying, and micrographs were acquired at 200 keV.

NPs stocks were stored in dark at room temperature up to 24 h and diluted in f/2 algal medium (salinity 38‰, pH 8), in order to achieve 10 mg L^{-1} working dispersions, which were bath-sonicated for 15 min prior to behavioural analysis. In order to assess the role of green algae exopolymeric substances (EPS) on TiO_2 NPs behaviour, such working dispersions were prepared in f/2 medium conditioned by culturing *D. tertiolecta* for 96 h (final cell density, $1{\cdot}10^6$ cells mL^{-1}), which was then removed by centrifugation and 1.2 µm filtration, prior to test TiO_2 NPs dispersion. The concentrations of carbohydrates and proteins in this algae-conditioned culture medium were 0.93 and 0.45 mg L^{-1}, respectively. The aggregation of TiO_2 NPs in standard f/2 and algae-conditioned f/2 medium was assessed via Dynamic Light Scattering (DLS) on a Zetasiser Nano ZS90 (Malvern Instruments Ltd., Malvern, UK), operating with 90° backscattering angle and a 639 nm wavelength laser. Hydrodynamic diameter (Z-average) growth and Polydispersity Index (PdI) were monitored every 10 min for 2 h. Each measurement consisted of three independent runs, all made of 11 sub-runs. The dispersion's electrostatic stability in both f/2 algal media was assessed measuring the NPs' surface charge (ζ-potential) and reported as an average of three measurements. In addition, the dynamic sedimentation process was followed spectrophotometrically over 72 h from dispersion, using a well-established procedure (Keller et al., 2010; Della Torre et al., 2015), on a Perkin-Elmer Lambda 650 UV–Vis spectrophotometer. First, a calibration curve was obtained by measuring absorbance at 269 nm of a range of TiO_2 dispersions of known concentration. The lowering of suspended TiO_2 concentration due to settling was estimated through absorbance measurements at 0, 1, 2, 4, 6, 24, 48 and 72 h after dispersion. For each time point, absorbance was recorded in triplicate, from volumes withdrawn in the upper layer of dispersions (< 1 cm from the surface), which were left stand unmoved in 50 mL Falcon tubes, at RT, for the whole experimental duration in order to avoid perturbation and resuspension. The sedimentation profile was then achieved plotting normalized concentration values (i.e. C/C_0, where C_0 is the initial concentration at 0 h and C refers to specific time points) vs. time, expressed as mean ± SD.

2.2. Exposure experiments

The unicellular green alga *Dunaliella tertiolecta* was obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, U.K.. Stock cultures were maintained in axenic conditions, in a growth chamber at 21 ± 1 °C and fluorescent daylight (100 µmol photons $m^{-2}\ s^{-1}$) in a 16:8 light-dark cycle photoperiod. Culture