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ORIGINAL ARTICLE

Effects of titanium nanoparticles on the photosynthesis, respiration, and physiological parameters in *Dunaliella salina* and *Dunaliella tertiolecta*

Fatemeh Ghazaei[1] · Mansour Shariati[1]

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Abstract

The development of nanotechnology and the upsurge of interest in titanium dioxide (TiO_2) nanoparticles, especially the anatase and rutile crystalline phases, in consumer products such as paint and sunscreen, has polluted the aquatic environment and had adverse effects on living organisms, especially algae. Microalgae help to preserve the aquatic ecosystem. Accordingly, the present study investigated the effects of anatase and rutile TiO_2 nanoparticles on the growth, photosynthetic pigment (chlorophyll), photosynthesis, and respiration rate of two algae species, *Dunaliella salina* (at NaCl concentrations of 1.5 and 0.5 M) and *Dunaliella tertiolecta* (at NaCl concentrations of 0.5 and 0.17 M). Treatment with 50, 100, 150, and 200 ppm of TiO_2 and nano-TiO_2 revealed that nano-TiO_2 inhibited the growth and decreased the specific growth rate, chlorophyll, and photosynthesis of both algal species. The rate of decrease was significantly lower at higher concentrations of NaCl in both species; however, the greatest significant difference was observed at lower concentrations of NaCl in the anatase phase. The respiration rate increased for 2 weeks but, especially at lower concentrations of NaCl, the rate of increase declined at higher concentrations after exposure to both substances, especially in the anatase phase. The findings reveal that nano-TiO_2 has a toxic effect on *Dunaliella* algae and its effect depends on the concentration of NaCl. The toxic effect was shown to decrease at higher concentrations of NaCl.

Keywords *Dunaliella salina* · *Dunaliella tertiolecta* · NPs · Photosynthesis · Respiration · Toxicity

Introduction

Metal oxide nanoparticles have been developed at the industrial level and used for medicine, engineering, science, biology, and water treatment (Nel et al. 2009; Patra et al. 2012; Shipway et al. 2000; Zhang 2003). Because the industrial applications of nanomaterials have increased, their release into the aquatic and terrestrial environments has increased. The direct release of TiO_2 NPs into the surface water in aged paint is about 16 μg/L (Kaegi et al. 2008). The environmental concentration of TiO_2 NPs in surface water has been modeled at less than 1 μg/mL (Gottschalk et al. 2009). Nanoparticles (NPs) are produced or manipulated particles and materials having at least one external nanometric dimension of 1 to 100 nm (ISO 2008). Engineered nanomaterials possess unique physical and chemical characteristics which can be attributed to their small size, complex chemical composition, surface structure, and shape (Nel et al. 2006). Titanium dioxide nanoparticles (TiO_2 NPs) are commonly produced nanomaterials (Piccinno et al. 2012; Furman et al. 2013). They are used extensively in products such as sun blocks, coloring, and surface coatings (Fisher and Egerton 2001; Kaida et al. 2004). They also are used to study air, soil, and water decontamination (Esterkin et al. 2005; Choi et al. 2006). The crystalline phases of TiO_2 are the anatase (tetragonal), rutile (tetragonal), and brookite (orthorhombic) (Cho et al. 2013). Among these, rutile is the most prevalent and natural form and constitutes a crucial percentage of heavy minerals. Because rutile shows the highest refractive indices, it is deployed in optical elements and is applied as a construct for refractory ceramics, pigments, etc. (Winkler 2003; Yu et al. 2013). The brookite phase is scarce in nature and does not have significant economic importance (Allen et al. 2009). However, anatase is commonly deployed in organic photovoltaics as an electron collection layer (Small et al. 2012). It is also used as

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✉ Mansour Shariati
mansour_shariati@yahoo.com; mansour@sci.ui.ac.ir

[1] Department of Biology, University of Isfahan, Hezar jarib st, Isfahan, Iran

catalytic support to produce nanotubes and nanoribbons (Gregory et al. 2008). Because of their high energy-absorption characteristic, both the rutile and anatase phases are used as sun blocks and in coloring, plastics, paper, food, and electronics (Ferguson et al. 2005; Mueller and Nowack 2008; Wang et al. 2006; Winkler 2003).

Rutile and anatase are allotropic forms of TiO_2 NPs with different surface properties and reactivity. The reactivity and the cytotoxicity potential of TiO_2 NPs are controlled by their stability in experimental systems. The stability of TiO_2 NP colloidal suspension in aquatic systems is affected by the particle dimensions (especially the surface/volume ratio), concentration, and crystalline structure. For example, rutile is lipophilic and can initiate apoptosis, while anatase is hydrophilic and induces cell necrosis and cell membrane damage. Studies have shown that anatase is more cytotoxic than rutile (Wang et al. 2008; Chen et al. 2012). It shows greater photocatalytic activity than rutile, which causes oxidation reactions on the surface of particles and allows formation of highly reactive hydroxyl and hydroperoxide radicals which cause membrane damage to algal cells (Dalai et al. 2013; Clement et al. 2013).

At high concentrations of TiO_2 NPs, their toxicity can produce different biological behaviors in organisms, such as decreased growth rate, inhibition of photosynthetic ability, and reduction of cell viability (Dalai et al. 2013; Navarro et al. 2008a, b; Li et al. 2015). Given that TiO_2 NPs are widely used, they find their way into the aquatic environment and affect aquatic life, especially algae, which are primary producers in the aquatic context (Kahru and Dubourguier 2010; Hall et al. 2009; Ji et al. 2011). Lubick (2008) and Navarro et al. (2008a, b) reported that, when NPs interact with algae, the aquatic toxicity of the nanomaterials is affected. This is caused mostly by the release of toxic ions, physical restraints, generation of reactive oxygen species (ROS), and cell membrane damage. The physical restraints include the shading effect from an increase in NPs (Navarro et al. 2008a, b). Nanoparticles may decrease the accessibility of light for photosynthesis to algal cells (Schwab et al. 2011; Comotto et al. 2014), hampering their growth, photosynthesis, and enzymatic activity (Aruoja et al. 2009; Chen et al. 2012; Ma et al. 2013; Melegari et al. 2013; Wang et al. 2008; Wong et al. 2010). It has been reported that, when the TiO_2 NPs are applied to algal cells, the aggregation of several layers of nano-TiO_2 on the surface of the cells may impede the nutrient transport and cause physical stress (Ji et al. 2011; Metzler et al. 2011). Cardinale et al. (2012) examined the toxicity of TiO_2 NPs on *Chlorella vulgaris*, *Scenedesmus quadricauda*, and *Chlamydomonas moewusii*. Their findings showed a decrease in the gross primary production of these algae; however, the rate of decrease differed according to the species.

Microalgae are necessary for maintenance of the aquatic ecosystem, are the first level of the food chain for generating oxygen and organics, and affect the nutrition of phytoplankton; thus, they are used as a model for estimating the aquatic risk caused by nanomaterials (Brunet et al. 2009). *Dunaliella* is a single-celled green marine alga belonging to the phylum Chlorophyta. *Dunaliella* species are able to survive at 0.17 M of salinity to NaCl-saturated medium (Borowitzka 1981) in ecological environments such as the sea, salt marshes, and saline soil (Wu et al. 2016). In food chains, *Dunaliella* is a primary food for fish and shrimp such as Artemia, which live in saltwater (Hosseini Tafreshi and Shariati 2009).

The TiO_2 nanoparticle is one of the most widely used particles in several industries. Most experiments on the effect of TiO_2 NPs on microalgae are on fresh water species. There are limited reports highlighting the effects of ecological toxicity of TiO_2 NPs on microalga from saline habitat. On the other hand, one barrier to the study of NPs on cells is the cell wall. Thus, it seems necessary to perform further research on the toxic effects of TiO_2 nanoparticles on microalgae from saline habitat in particular halotolerant *Dunaliella* species, a model organism without a cell wall, with nutritional and economic value and a cosmopolitan distribution in marine coastal waters, salt lakes, and salt marshes (Ben-Amotz and Avron 1983).

The present study is the first of its nature to evaluate the toxicity of anatase and rutile NPs, which may have inherent differences in the toxic effects, towards two different species of *D. salina* and *D. tertiolecta*, grown in their optimum salinity 1.5 M NaCl and 0.17 M NaCl, respectively. The cell morphology, specific growth rate, chlorophyll synthesis as well as photosynthetic and respiration activity were determined to evaluate the toxicity of TiO_2 NPs on algae. The results will be useful to realize the potential risks of TiO_2 NPs on salt aquatic ecosystem.

Investigating the effect of different concentration salinities on each species is not objective of our study, but it may be hypothesized that titania NPs (anatase and rutile), rather than affecting the metabolism of microalga, can interact with salt in the medium (French et al. 2009). The salinity of culture between two species of *Dunaliella* cells used in our experiment is too high (1.5 M compared to 0.17 M), which may affect the interpretation of the results. As *Dunaliella* cells are halotolerant and not halophyte (Borowitzka and Brown 1974), the tolerance range of cells can be trained to higher or lower salt concentrations by serial transfer to lower or higher salinities (Brown and Borowitzka 1979). To better understand the effect of interaction between salinity and TiO_2 NPs, rather than the direct effect of TiO_2 NPs, two species also cultured in same salinity (0.5 M NaCl), in addition, 0.17 M and 1.5 M NaCl for *D. tertiolecta* and *D. salina*, respectively. For obtaining the same salinity condition for both species, which had low difference in growth rate and chlorophyll content compared to optimum NaCl concentration of each species, *D. salina* and *D. tertiolecta* cells cultured and adapted to variety of salinity by serial transfer to low or high salinities.

Materials and method

Algal cultures and growth

The *D. salina* Teod. UTEX 200 and *D. tertiolecta* (UTEX LB999) were provided from the algae culture collection at the University of Texas at Austin. The cultures were grown in Johnson modified medium (Shariati and Lilley 1994) at 1.5 M (optimal) for *D. salina* and 0.17 M (optimal) for *D. tertiolecta* (Brown and Borowitzka 1979). The cultures were kept at temperature of 25 °C, 150 μmol photons m^{-2} s^{-1} of light under a 16 h/8 h light/dark photoperiod, and continuous shaking (100 rpm). In the exponential growth phase of the cultures, they were inoculated into the 250-mL Erlenmeyer flasks containing 100 mL of fresh medium and the treatments were applied.

To study the interaction between concentration of salt in the medium and TiO_2 NPs, both species cultured in the medium containing same NaCl concentration (0.5 M NaCl). The cells were cultured and trained in variety of salinities (0.25, 0.5, 0.75, 1.0, and 1.5 M and 0.17, 0.5, 0.75, and 1.0 M), for *D. salina* and *D. tertiolecta*, respectively, by serial transfer to lower or higher salinities and the best concentration was selected. In each transfer to new different salinity, the cells were kept for 2 weeks in mentioned condition. After reaching to the final desired salinity, the cells sub-cultured twice.

Chemicals

Bulk dry titanium (IV) dioxide (TiO_2), as the control and nanopowder (anatase: < 25 nm, > 99% trace metal basis and rutile: < 30 nm, 99.9% trace metal basis), was obtained from US Research Nanomaterials.

Characterization of nano-TiO_2

To determine the particle size, shape, and surface morphology of the TiO_2 NPs, transmission electron microscopy (TEM; Philips CM30) was used. The crystal structure of the nano-TiO_2 was analyzed by X-ray diffraction (XRD; D8-Advance Bruner). The average particle size of the nano-TiO_2 was calculated based on Scherrer's equation (Bo et al. 2010) as $L = k\lambda / \beta\cos\theta$, where L is the average size of the nanoparticles, k is equal to 0.89, λ is the X-ray wavelength (0.154 nm), β is the peak broadening, and θ is the angle of the peak maximum.

Stock preparation of TiO_2 nanoparticles

A stock solution of TiO_2 and NPs (anatase and rutile) at 2500 ppm was prepared in Milli-Q water. The TiO_2 and NP suspension was sonicated for about 30 min and shaken for 2 min. The stock was added to Erlenmeyer flasks to reach final concentrations of 50, 100, 150, and 200 ppm. A fresh stock solution of TiO_2 NPs was used for all experiments.

Confocal laser scanning microscopy

In order to determine the three-dimensional structure of the algae cells, confocal laser scanning microscopy (CLSM) was used. The control cells and cells treated with 150 ppm of TiO_2, anatase and rutile, were collected after 72 h of reaction. Next, 500 μL of the sample was stained with 500 μL of propidium iodide (PI) for 5 min at 25 °C in the dark and then centrifuged at 200 rpm for 2 min at 4 °C (Pakrashi et al. 2013). PI is a fluorescent compound that binds to nucleic acids and cannot cross the membrane of live cells (Ormerod et al. 1992). The stained cells were washed thrice with 2× saline-sodium citrate (SSC) buffer to remove the unbound dye and were observed by CLSM using a BP emission filter at 565–615 nm and a LP excitation filter at 543 nm (Leica TCS SP5, Germany). The setup CLSM was adjusted for deletion of chlorophyll fluorescence, which could have affected the results.

Cell number and pigment analysis

The cell number was used to assess the effect of TiO_2 NPs on the growth of algae after 3, 7, 10, 13, 17, and 21 days of treatment. A hemocytometer under light microscopy was used to determine the number of cells (Schoen 1988). To extract chlorophyll, 1 mL of alga cell suspension was centrifuged at 13,000*g* for 1 min. Next, 1 mL of 80% acetone (*v*/*v*) was added to the pellet, mixed and vortexed for 5 min under dark conditions, then the cell suspension was centrifuged at 13,000*g* for 5 min. The supernatant was removed and the chlorophyll concentration (μg/cell) was evaluated spectrophotometrically using the Arnon (1949) method.

The specific growth rate (μ) was calculated by enumerating the cells by the following formula (OECD 2011):

$$\mu = \mathrm{lnXn–lnX0}/(\mathrm{tn−t0})$$

where X0 is the average number of cells at t0, Xn is the average number of cells at tn, and μ the specific growth rate.

Inhibition percentage of chlorophyll synthesis for each treatment was calculated as (OECD 2011)

$$\%\mathrm{Ir} = (\mathrm{lc−lr})/\mathrm{lc} \times 100$$

where %Ir is the inhibition percentage, lc is the chlorophyll synthesis in untreated cells (control), and lr is the chlorophyll synthesis in treated cells.

Photosynthesis and respiration measurements

Harvesting the experimental cultures for photosynthesis

About 30 mL of algal culture was centrifuged at 500*g* (SANYO MSE MISTRAL 3000i) for 15 min at 25 °C. The supernatant was removed and the pellet was re-suspended in a phosphate buffer, containing 0.25 mM KH_2PO_4, 0.25 mM K_2HPO_4, 0.2 mM $MgCl_2$, 5 mM $NaHCO_3$, and NaCl based on the culture medium and the pH was adjusted to 7.5. The specified amount of TiO_2 and nano-TiO_2 was added to the re-suspended culture in phosphate buffer to obtain final concentrations of 50, 100, 150, and 200 ppm of TiO_2, anatase, and rutile. One milliliter of the algal suspension containing different concentrations of TiO_2 and nano-TiO_2 was transferred to the measuring vessel of the O_2 electrode, and the photosynthesis (oxygen evolution) and respiration (oxygen uptake) were evaluated on days 0 and 14 of the experiment.

Measurement of O_2 evolution and uptake

Photosynthesis and respiration were evaluated polarographically using a Clark-type O_2 electrode (Hansatech Ltd., UK) in water-jacketed reaction vessels (Delieu and Walker 1972) at 28 °C. The O_2 electrode was attached to a chart recorder with a speed of 5 mm/min. To provide illumination for photosynthesis, a projector (Hansatech Ltd., UK) with a 100 W bulb containing photosynthetically active radiation (PAR) was deployed and the light beam was projected through an infrared filter (to absorb the heat of the light) and a spherical focusing lens (a round-bottom flask filled with water) to obtain 500 μmol photon m^{-2} s^{-1}. For measuring the respiration, the reaction vessel was covered with a black box. Oxygen evolution of photosynthesis and oxygen uptake of respiration were measured at days 0 and 14 for 15 min each and were calculated as O_2 mg Chl^{-1} min^{-1}.

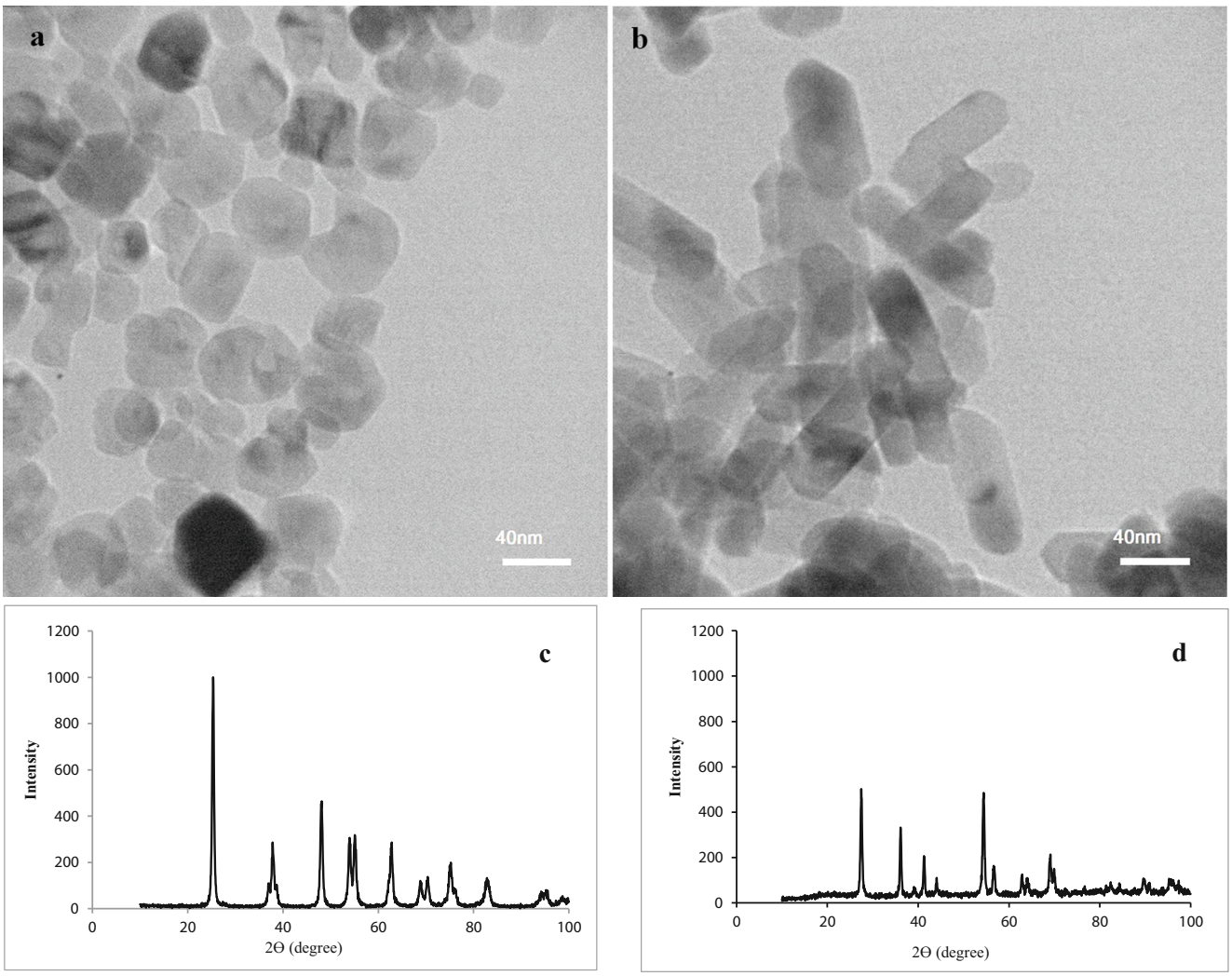

Fig. 1 Transmission electron microscopy images of **a** anatase and **b** rutile nanoparticles and show that the anatase NPs have the cubical and spherical shape and rutile NPs were rod-shaped. XRD spectra of samples of **c** anatase and **d** rutile nanoparticles

Statistical analysis

The experiments were done in three independent replicates for all treatments. Means and standard deviations (SD) were calculated for each treatment. Significant differences between the control, TiO_2, and NPs were calculated using ANOVA with Tukey test (p values < 0.05).

Results

Primary characterization of nano-TiO_2

The transmission electron microscopic of nano-TiO_2 was carried out to confirm the morphology and the primary size of nanoparticles. TEM image (Fig. 1) confirms that the primary size of NPs is under 50 nm and shows that the anatase NPs have the cubical and spherical shape (Fig. 1a). As shown in Fig. 1b, rutile NPs were rod-shaped. Figure 1c, d shows the XRD pattern of the anatase and rutile NPs. This spectrum shows several sharp and several small peaks, indicating the crystalline structure of nano-TiO_2. The average grain size, calculated based on Scherrer's equation, was about 25 nm for anatase and 30 nm for rutile, indicating the nanostructure of the two nanoparticles.

The TEM images of TiO_2 nanoparticles in the presence of NaCl (Fig. 2) show no morphological changes in primary characterization of nanoparticles but aggregate and aggregate sizes have changed. NaCl changes the formation of TiO_2 NPs from nanometer sized to larger-sized aggregates (micron sized), and in the presence of high concentrations of NaCl (1.5 M) (Fig. 2b), the size of NP aggregation is bigger than 0.5 M of NaCl (Fig. 2a).

Confocal laser scanning microscopy

The images of the confocal laser scanning microscopy (CLSM) show the toxic effect of TiO_2, anatase, and rutile on *D. salina* (Fig. 3) and *D. tertiolecta* (Fig. 4) cells. In both algae cells, the untreated cells (Figs. 3a, 4a) were not stained, which is due to the undamaged cell membrane. When the cells were treated with the TiO_2 and NP_S, stained cells were observed, demonstrated red fluorescence, showed scatter nucleus, and nuclear materials were diffused into the cytoplasm (Fig. 3b–d, 4b–d).

The effect of TiO_2 NPs on the specific growth rate and chlorophyll synthesis of *D. salina* and *D. tertiolecta*

To determine the similar concentration of NaCl in the medium for two species with high differences in salt concentration of their medium, when the cell adapted to a variety of salinities (0.25, 0.5, 0.75, 1.0, and 1.5 M and 0.17, 0.5, 0.75, and 1.0 M), for *D. salina* and *D. tertiolecta*, respectively (not shown), the results indicated that in *D. tertiolecta*, in 0.5 M NaCl, cell growth and chlorophyll content declined about 20–25% and in salinity higher than 0.75 M NaCl, the reduction in cell growth and chlorophyll content was high. In *D. salina* in 0.5 M NaCl, cell growth and chlorophyll content decreased about 15–20% and in salinity in 0.75 and 1 M NaCl was very close to 1.5 M NaCl. Therefore, 0.5 M NaCl was selected.

The effect of two types of TiO_2 NPs (anatase and rutile) on the specific growth rate of the two algae species of *D. salina* and *D. tertiolecta* at different salinities is shown in Fig. 5. According to the results, the TiO_2 and TiO_2 NPs showed concentration-dependent cytotoxicity toward the algal cells in the two algae species and at all salinity conditions; however, both of the NPs are more toxic than TiO_2, especially in concentrations more than 100 ppm. In *D. salina* (Fig. 5b) and *D. tertiolecta* (Fig. 5c) cultured at 0.5 and 0.17 NaCl, respectively, anatase was more effective than rutile. In all applied concentrations of TiO_2 and TiO_2 NPs, no significant differences were observed in *D. salina* (Fig. 5a) and *D. tertiolecta* (Fig. 5d) at 1.5 and 0.5 NaCl,

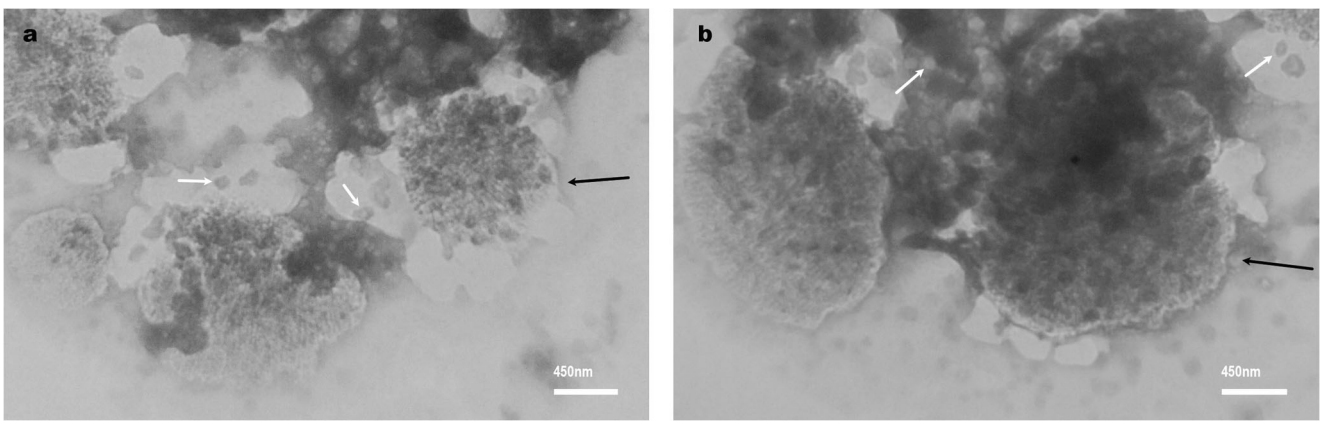

Fig. 2 Transmission electron microscopy images of TiO_2 nanoparticles in presence of **a** 0.5 M NaCl and **b** 1.5 M NaCl. White arrow: primary characterization of nanoparticles, black arrow: formation of TiO_2 NPs from nanometer-sized to micron-sized aggregates

respectively. When the TiO_2 and TiO_2 NPs were added to the culture of *D. salina* and *D. tertiolecta*, cell aggregation was observed (not shown) and it was higher in TiO_2 NPs than TiO_2.

The percentage of the inhibition of chlorophyll synthesis after its normalization with the control cells, after 2 weeks, was calculated (Fig. 6). Result reveals that TiO_2 and TiO_2 NPs showed concentration-dependent cytotoxicity on chlorophyll synthesis. Any increase in all applied concentration of treatments is concomitant with an increase in the inhibition of chlorophyll synthesis in all conditions (Fig. 6a–d). In *D. salina* at 1.5 M NaCl (Fig. 6a), there are no differences between different kinds of titanium. While in other conditions, there are significant differences between nano-TiO_2 and TiO_2, in which anatase has a more severe effect than rutile. When the optimum salinity of medium for *D. salina* decreased from 1.5 M NaCl to 0.5 M NaCl, the effect of nano-TiO_2 increased, whereas, increasing the salinity of medium from the optimum salinity of 0.17 M NaCl for *D. tertiolectra* to 0.5 M NaCl caused a decrease in the toxicity of nanoparticles.

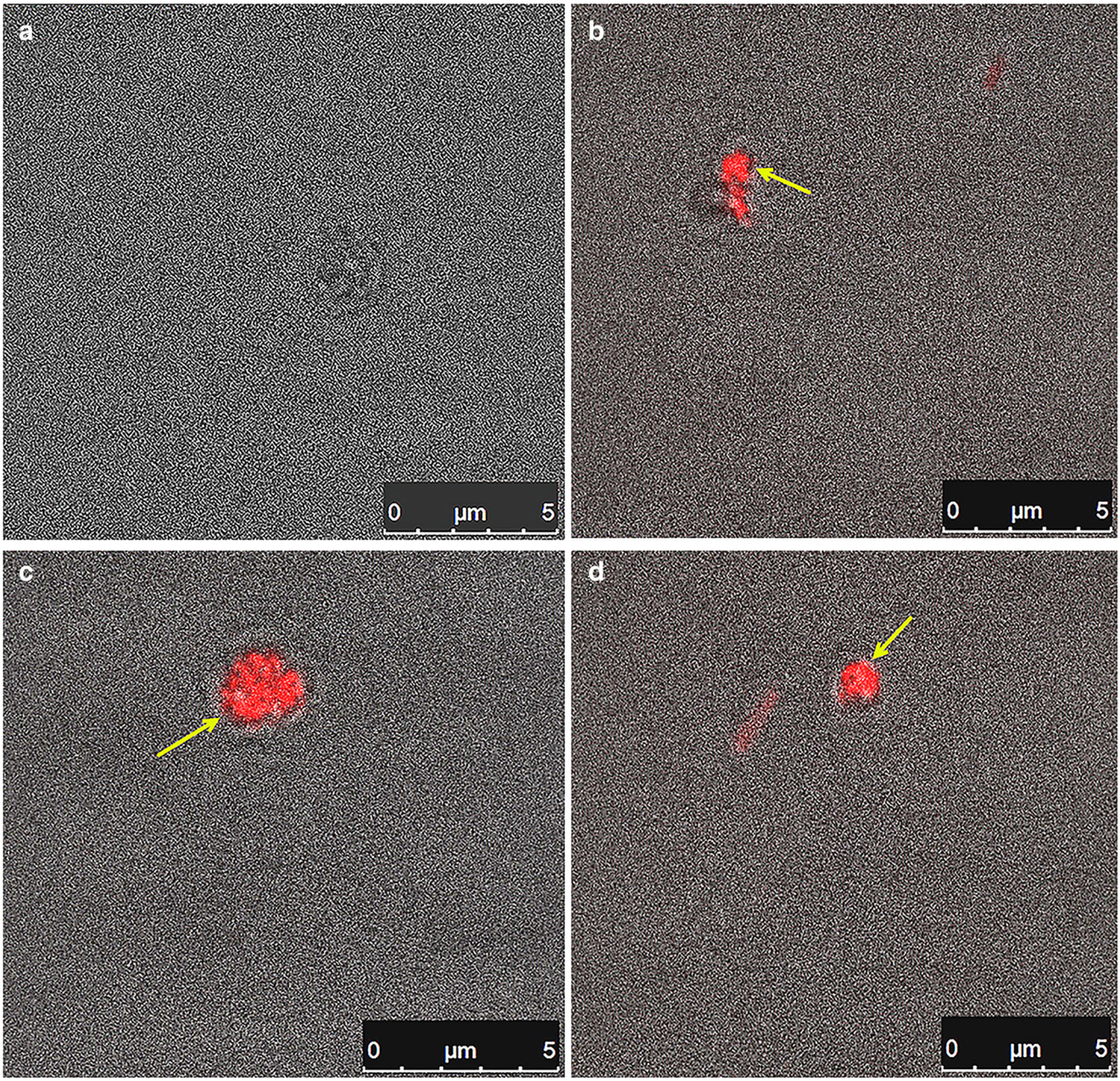

Fig. 3 Confocal laser scanning micrographs of untreated and treated *D. salina* cells. **a** Untreated cell; **b** TiO_2-treated cells; **c** anatase-treated cells; **d** rutile-treated cells. Arrows show nuclear damage and release of nuclear contents into the cytoplasm

The effect of TiO_2 NPs on the net photosynthesis and respiration in *D. salina* and *D. tertiolecta*

Net photosynthesis (oxygen evolution) and respiration (oxygen uptake) of the two algae were evaluated at the beginning and after 2weeks of the experiment under different concentration of TiO_2 and TiO_2 NPs. In all conditions, any increase in TiO_2 and nano-TiO_2 concentration led to a decrease in the photosynthesis after 14 days. The higher decrease was observed in the presence of the two nano-TiO_2 than TiO_2, in *D. salina* at 0.5 M NaCl (Fig. 7b) and *D. tertiolecta* at 0.17 M NaCl (Fig. 7c), and the most considerable decrease was especially caused by anatase. In *D. salina*, cells cultured at 1.5 M NaCl (Fig. 7a) and *D. tertiolecta* cells cultured at 0.5 M NaCl (Fig. 7d), nano-TiO_2 declined the net photosynthesis compared to the TiO_2, while no considerable difference was identified between anatase and rutile.

As shown in Fig. 8, after 14 days of applying different concentrations of TiO_2 and nano-TiO_2 on *Dunaliella* cells, an increase in the respiration was observed in all conditions. The intensity of increase declined in higher concentrations of TiO_2 and nano-TiO_2. In *D. salina* grown at 1.5 M NaCl (Fig.

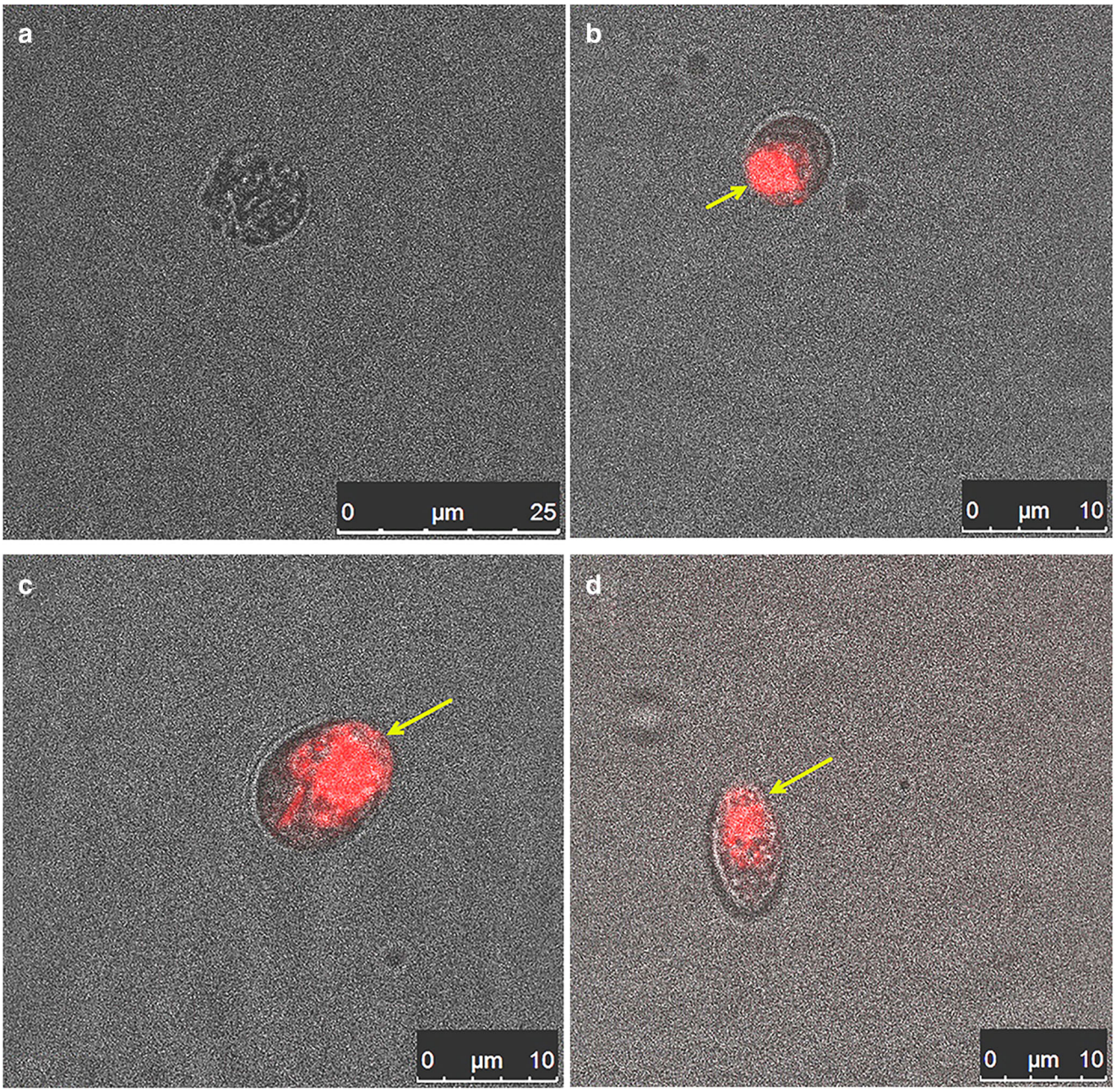

Fig. 4 Confocal laser scanning micrographs of untreated and treated *D. tertiolecta* cells. **a** Untreated cell; **b** TiO_2-treated cells; **c** anatase-treated cells; **d** rutile-treated cells. Arrows show scattered nucleuses and release of nuclear contents into the cytoplasm

8a) and *D.tertiolecta* at 0.5 M NaCl (Fig. 8d), there were no significant differences between nano-TiO_2 and TiO_2 and a low difference was observed between the applied concentrations of the two TiO_2 and nano-TiO_2. While in *D. salina* grown in 0.5 M NaCl (Fig. 8b) and *D. tertiolecta* in 0.17 M NaCl (Fig. 8c), the two nano-TiO_2 NPs, in particular anatase, were more effective compared to TiO_2.

Discussion

TiO_2 nanoparticles are common engineered nanomaterials that are used to produce different materials. Studies have shown that nano-TiO_2 are more toxic than TiO_2 because of their shape, surface area, size, and crystal structure (Dalai et al. 2013; Li et al. 2015). The TEM images confirmed that anatase and rutile are spherical and rod-shaped, respectively. This also has been reported by Iswarya et al. (2015). The results of the current study also confirm that anatase is smaller than rutile.

The unicellular green algae *Dunaliella* was used to investigate the effect of TiO_2 NPs. Brown and Borowitzka (1979) investigated the optimal salinity condition for *Dunaliella* growth and reported values of 1.5 M and 0.17 M NaCl for *D. salina* and *D. tertiolecta*, respectively. The results of the current study confirmed that when *D. salina* cells were transferred from 1.5 M (optimal) to 0.5 M NaCl (acclimated), and *D. tertiolecta* cells were transferred from 0.17 M (optimum condition) to 0.5 M NaCl (acclimated), the growth rate decreased.

CLSM images substantiate the toxic effect of TiO_2 micro- and nanoparticles on algae cells. The scatter shape of the nucleus in the treated cells as compared to the definite shape of nuclease in the control suggests a TiO_2 NPs genotoxicity potential and nucleus-specific (DNA) action on algal cells (Dalai et al. 2013).

Iswarya et al. (2015) reported that diffusion of the nucleus can be attributed to the interaction with nanoparticles in the nucleus of the cell and is indicative of their genotoxicity. It seems that both TiO_2 and NPs damage the cell membrane and

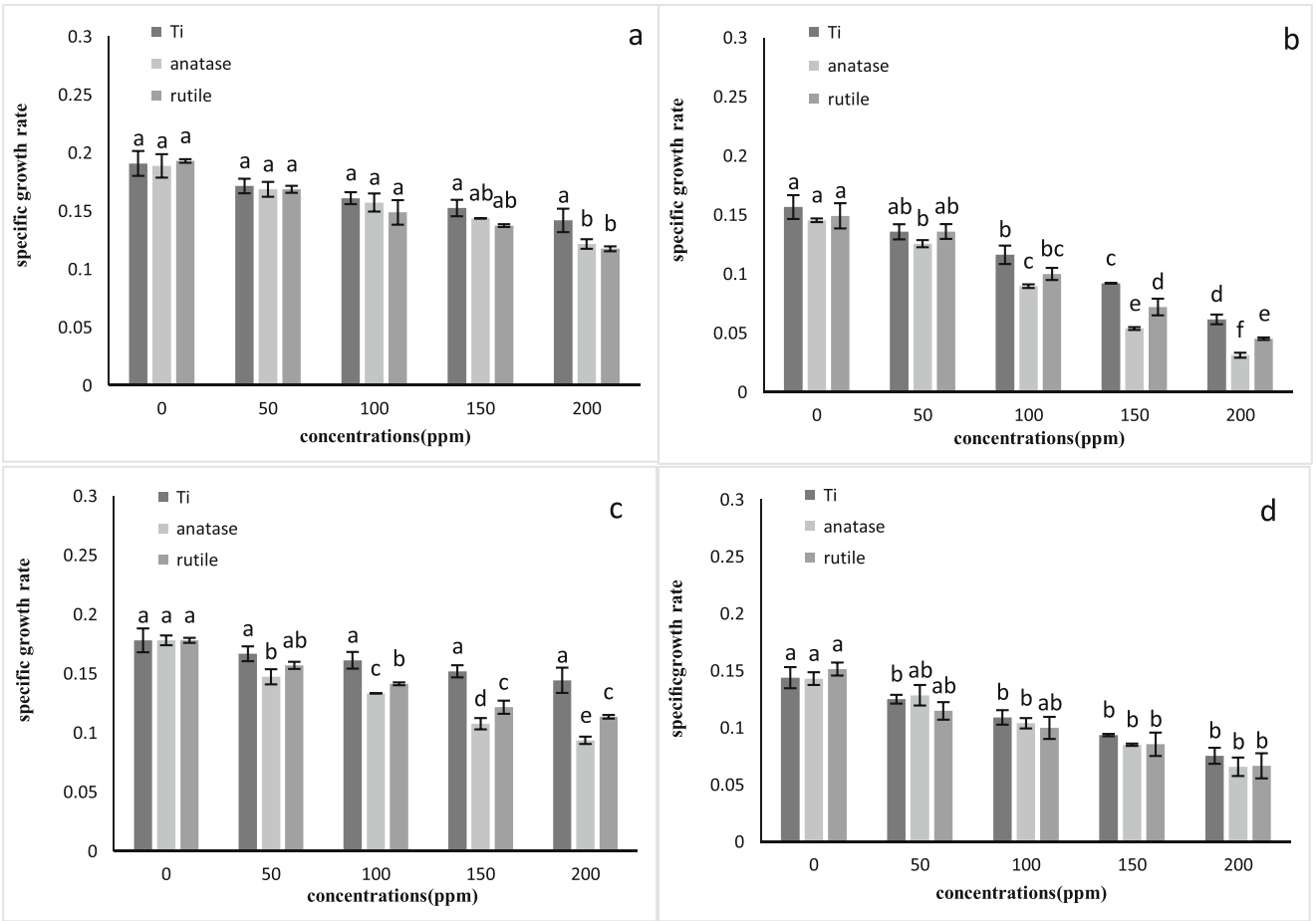

Fig. 5 Effects of different concentrations of TiO_2 nanoparticles (anatase and rutile) and TiO_2 (as control), on the specific growth rate of *D. salina* grown at **a** 1.5 M, **b** 0.5 M NaCl and *D. tertiolecta* grown at **c** 0.17 M, **d** 0.5 M NaCl. Each value represents the mean ± standard deviation of three replicates. Different small letters indicate significant differences between various treatment conditions at $p < 0.05$ (according to Tukey test)

enter the cell, causing damage to the nucleus in the *Dunaliella* species used. Concentration-dependent inhibition of algal growth was observed at all concentrations of TiO_2 and nano-TiO_2 in both algal species. The results corroborate the greater effect of nano-TiO_2 on both algal species than TiO_2. In general, NPs enhance physical, chemical, and electrical characteristics because of their size and large surface area per given mass (Karakoti et al. 2006; Chernyshev 2009). Although concentration-based inhibition of the algal specific growth rate, chlorophyll concentration and photosynthesis were found among both anatase and rutile NPs individually, the difference between them was not statistically significant for *D. salina* at 1.5 M and *D. tertiolecta* at 0.5 M NaCl. However, the greatest decrease was found for anatase NPs in *D. salina* at 0.5 M and *D. tertiolecta* at 0.17 M NaCl, which demonstrates the increased toxicity of anatase. It was demonstrated that the crystal structure of nano-TiO_2 led to cytotoxicity and toxic reactions, which indicates that anatase TiO_2 is more toxic than rutile TiO_2 (Sayes et al. 2006; Clement et al. 2013). Investigation of TiO_2 cytotoxicity towards algae (Ji et al. 2011; Dalai et al. 2013) indicates that ROS is responsible for this nanotoxicity by causing membrane damage to algal cells. The toxicity of oxide nanoparticles to *Chlorella* sp. has been reported by Ji et al. (2011) and suggests that aggregation of nanoparticles entrapped in the algal cell plays a role in nanotoxicity.

A decrease in chlorophyll synthesis was observed at all concentrations of TiO_2 and nano-TiO_2 in both algal species. Studies have indicated that the aggregation of nanoparticles on the surface of algae cells decreases accessibility to light and that shading disturbs energy transmission (Hartmann et al. 2010; Kulacki and Cardinale 2012). This decreases chlorophyll production, which in turn may affect photosynthesis and the growth rate.

Photosynthesis in *D. salina* and *D. tertiolecta*, like the growth rate and pigment content, was affected by nano-TiO_2 treatment. As expected, the presence of nano-TiO_2 decreased photosynthesis. Chlorophyll is a primary photosynthesis pigment essential for cellular algal performance; thus, one reason for the observed decrease in photosynthesis is the decrease in

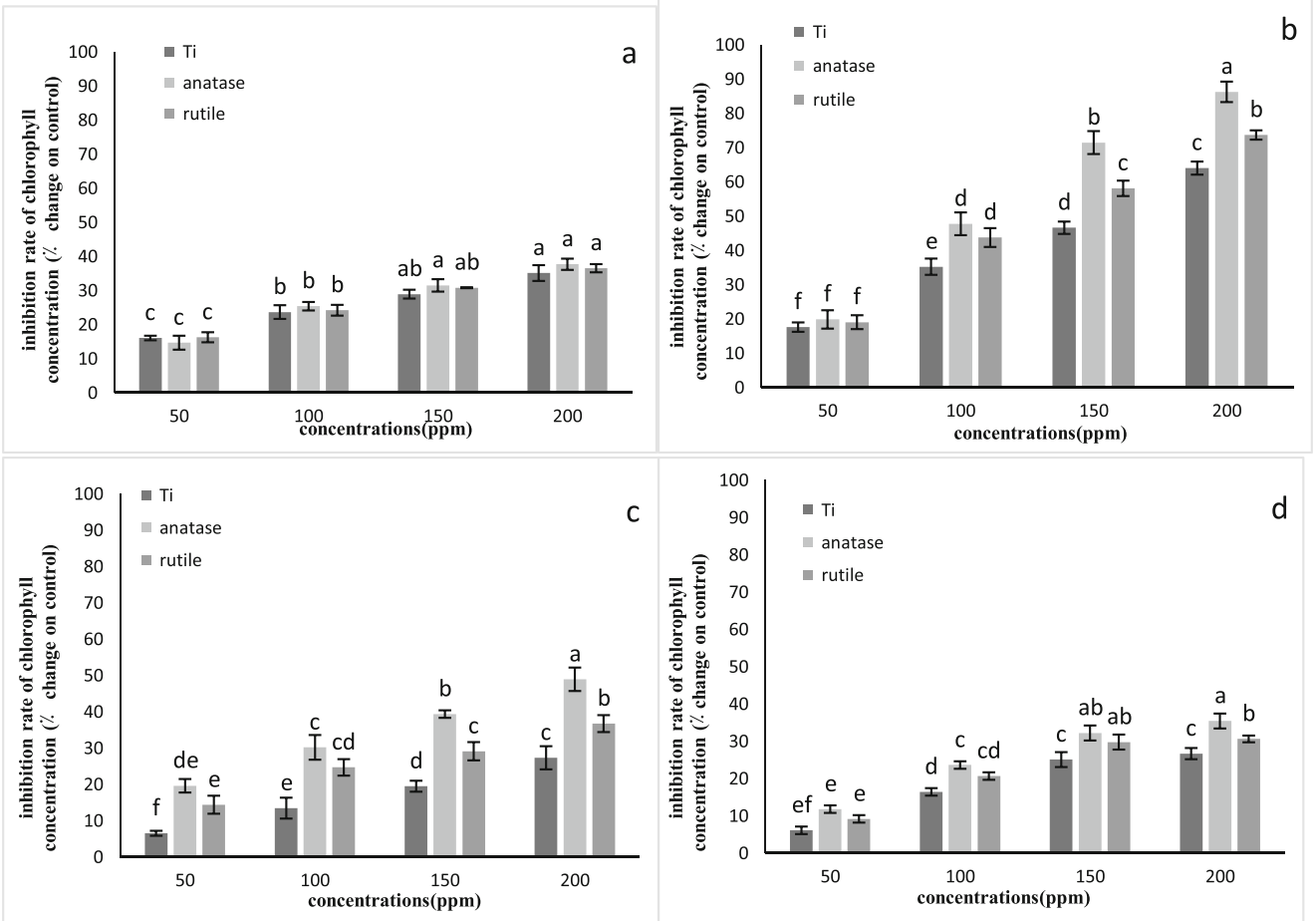

Fig. 6 The percentage of inhibition rate of chlorophyll concentration in control after 2 weeks of exposure to different concentrations of TiO_2 (as control), anatase and rutile in *D. salina* suspensions grown at **a** 1.5 M and **b** 0.5 M NaCl and in *D. tertiolecta* grown at **c** 0.17 M and **d** 0.5 M NaCl. Each point represents the mean ± standard deviation of three replicates. Different letters indicate significant differences between various treatment conditions at $p < 0.05$ (according to Tukey test)

chlorophyll concentration. It has been reported that in *C. vulgaris*, the presence of nano-TiO_2 significantly inhibited photosynthetic activity. This is related to damage to the reaction center of PS II, which inhibits photosynthesis and the electron transport chain (Middepogu et al. 2018). Decreased activity of ATPase, along with decreased generation of ATP and glucose, has been reported in *C. vulgaris* after the application of NPs-TiO_2 (Middepogu et al. 2018). It seems that, in *Dunaliella* cells, the reduction in photosynthesis caused by TiO_2 and nano-TiO_2 could be involved in decreases in material and energy metabolism that cause inhibition of algal growth. This was more pronounced in nano-TiO_2 because of its structure.

The current results showed that in the cells subjected to TiO_2 and nano-TiO_2, as photosynthesis decreased, respiration increased after 2 weeks. A reduction of the intensity of the increase in respiration was concomitant to an increase in the concentration of TiO_2 and nano-TiO_2 in the medium. It has been reported that nTiO_2 stimulated respiration in *C. vulgaris*, leading to reduced growth (Cardinale et al. 2012). In response to TiO_2 stress, some of the carbon fixation in photosynthesis, instead of being used for growth, was used to increase respiration in order to produce more energy to cope with stress. This could be another reason for the decreased growth rate in *Dunaliella* cells under treatment by NPs. Increased membrane permeability after treatment with TiO_2 NPs has been reported. Dalai et al. (2013) found that TiO_2 NP adsorption onto the cell surface facilitated the uptake of the NPs and injured the cell membrane, leading to NP internalization. They then interacted with the internal organelles, mainly chloroplasts and mitochondria, and distorted the internal organelles. It seems that, in the current study, in *Dunaliella* cells, as the concentration of TiO_2 and nano-TiO_2 in the medium increased, the chloroplast and mitochondria membrane were damaged and metabolic activities such as photosynthesis and respiration were inhibited.

The interaction of nano-TiO_2 and NaCl may affect their performance, irrespective of the effect of stress induced by the nanoparticles (French et al. 2009; Xiang et al. 2013). The TEM images of TiO_2 show that although high concentrations of NaCl do not change the primary characterization of nanoparticles, they change the formation of TiO_2 NPs from

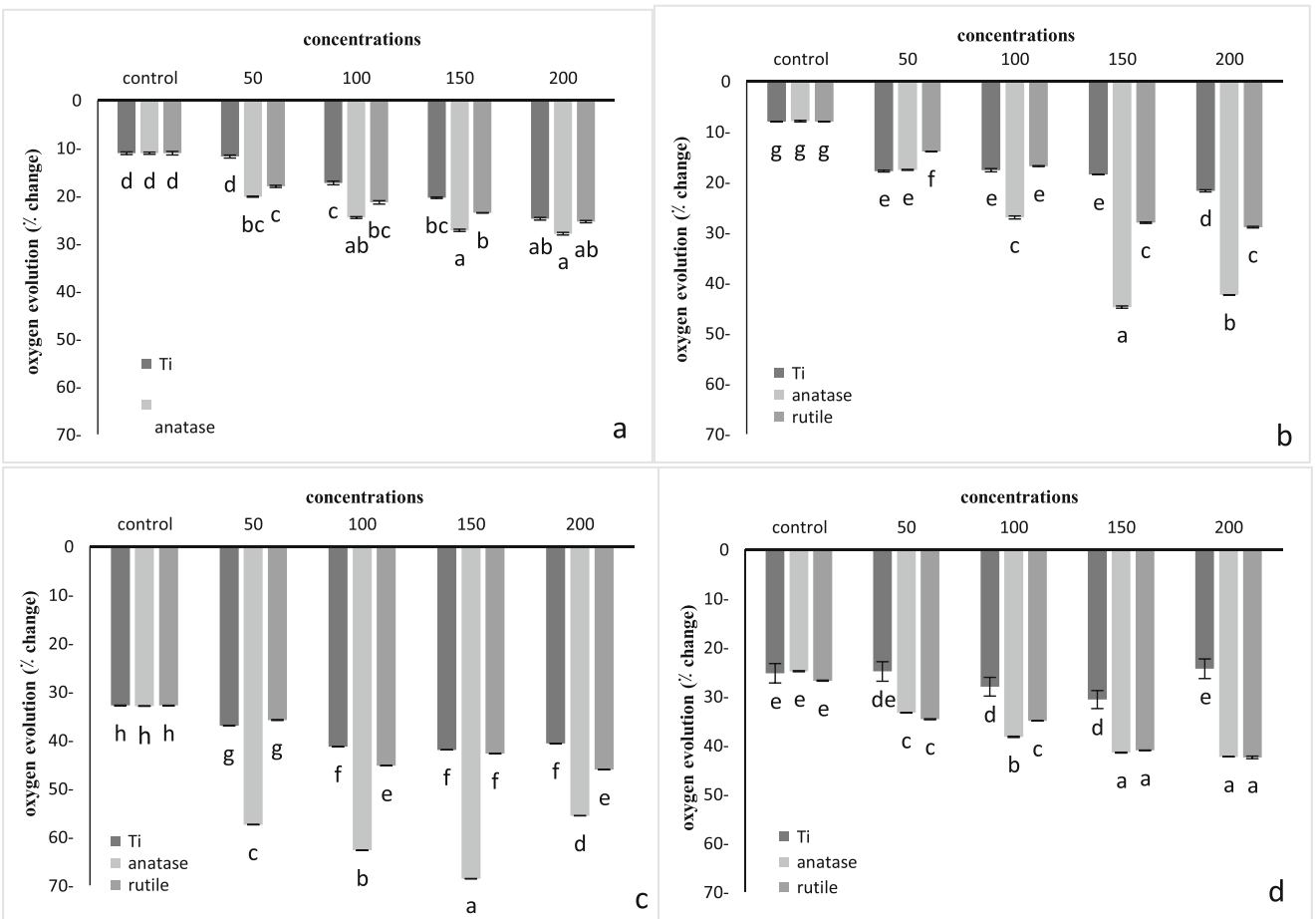

Fig. 7 Percent change of photosynthesis in day 14 and in the beginning day of experiment at different concentrations of TiO_2, anatase, and rutile in *D. salina* grown at **a** 1.5 M and **b** 0.5 M NaCl and in *D. tertiolecta* grown at **c** 0.17 M and **d** 0.5 M NaCl. Each point represents the mean ± standard deviation of three replicates. Different small letters indicate significant differences between various treatment conditions at $p < 0.05$ (according to Tukey test)

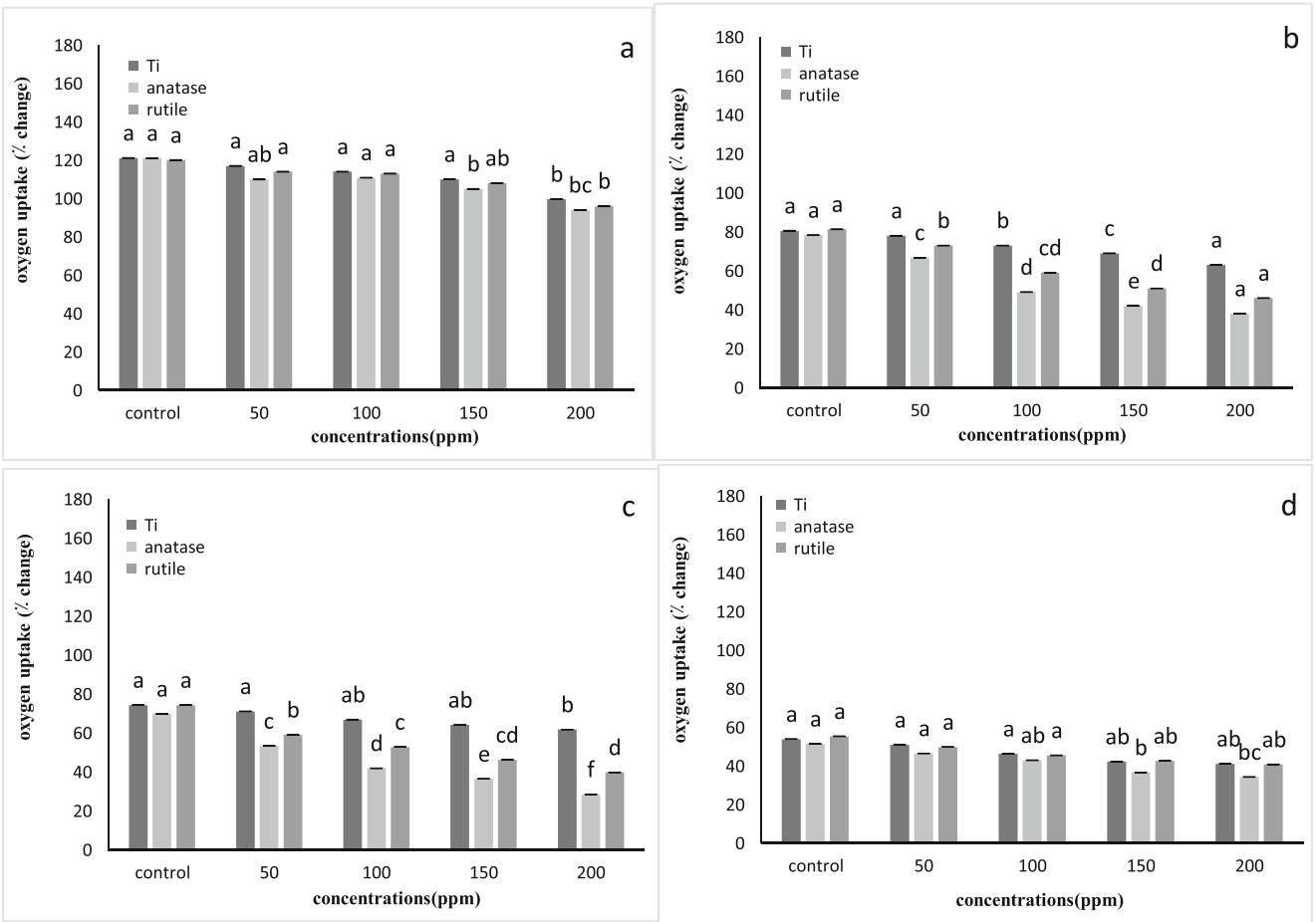

Fig. 8 Percentage of changes in respiration in day 14 and in the beginning day of experiment at different concentrations of TiO_2, anatase, and rutile in *D. salina* grown at **a** 1.5 M and **b** 0.5 M NaCl and in *D. tertiolecta* grown at **c** 0.17 M and **d** 0.5 M NaCl. Each point represents the mean ± standard deviation of three replicates. Different letters indicate significant differences between various treatment conditions in each strain at $p < 0.05$ (according to Tukey test)

stable aggregates (nanometer sized) to larger-sized aggregates (micron sized) which in turn reduced the effect of TiO_2 NPs. French et al. (2009) reported a similar agglomeration of NPs on the interaction with NaCl. Our results revealed that, under most conditions in both species, as the NaCl concentration decreased in the culture media, from 1.5 to 0.5 M NaCl and from 0.5 to 0.17 M NaCl in *D. salina* and *D. tertiolecta*, respectively, the nano-TiO_2 (particularly anatase) were more effective. These clearly show the interaction between salinity in the medium and TiO_2 NPs. Therefore, different responses to TiO_2 NPs between two species are not species dependent and probably are due to differences between different salt concentrations in their medium.

The metal-metal antagonist effect was found to decrease the adverse effects of metals such as Ni, Mo, Mn, and Cu, on the growth rate and photosynthetic activities of *Scenedesmud quadricauda* alga (Fargasova 2001). Sharma (2009) noted that the presence of cations in surface water and soil played an important role in the aggregation of nano-TiO_2. Toxicological studies have shown that the toxicity of nanoparticle determined by their size, aggregation, and agglomeration influenced the transport of nano-TiO_2 in the medium (Sager et al. 2007). Thus, the NaCl-nTiO_2 interaction may have influenced the bioavailability and efficiency of nano-TiO_2 on *Dunaliella* cells based on the NaCl concentration in the mixture, aside from NP stress and shading effects.

Conclusions

This study confirmed the ecotoxicological effect of anatase and rutile of TiO_2 NPs towards *D. salina* and *D. tertiolecta* green algae at two concentrations of NaCl. The growth rate, chlorophyll content, photosynthesis, and cell membrane integrity were strongly affected by the crystallinity of the nanoparticles. CLSM showed that the NP interaction with algal cells damaged the cell membranes. The size and structure of TiO_2 micro- and nanoparticles affected toxicity. In fact, compared with the micro-sized particles, the TiO_2 nanoparticles exhibited the highest toxicity and anatase particles showed

significantly more toxicity than rutile particles because of the allotropic form of anatase. The present research also provides evidence for the toxic effect of nano-TiO_2 on *Dunaliella* algae that depends on the concentration of NaCl in culture (aquatic environment). The toxic effect of NPs decreased at higher concentrations of NaCl. Given that TiO_2 NPs are widely used in a variety of consumer products, the findings of the present study should be considered when determining the potential effects of NPs on aquatic life. This work investigated the underlying mechanisms of the toxicity of nano-TiO_2 towards green marine alga and paved the way for in-depth studies in nanoecotoxicology to determine the effects of nanoparticles on microalgae and their risks to natural environments.

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