

Diminishing toxicity of P25 TiO₂ NPs during continuous exposure to freshwater algae *Chlorella*

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Abstract

Nano-titanium dioxide (Nano-TiO₂) has been widely used in industrial manufacture and life science field due to its excellent physical and chemical properties in the past few decades, which makes it inevitably released into the aquatic environment. In freshwater ecosystem, *Chlorella* is the most commonly used species to study the effect brought by metal oxide nanoparticles. In this work, both the short term and long term effect of P25 TiO₂ on *Chlorella* were investigated. Here, we found that Nano-TiO₂ would cause serious damage to *Chlorella* in a short period of time, as confirmed by oxidative stress and algal cell morphology. However, different from the short-term condition, the damaging effect was gradually weakened along with the prolongation of exposure time of *Chlorella* to Nano-TiO₂ and the final observed inhibition rate of biomass was nearly zero. In addition, the other important finding of this work is that extracellular polymeric substance (EPS) has remarkably protective effect on algae cells. Algae cells with EPS removed were more vulnerable to nano titanium dioxide and exhibited more difficulties in returning back to normal growth compared with normal algae cells, which might be related to the attachment of nanoparticles to the cell surface.

1. Introduction

With the high-speed development of nanotechnology, nanomaterial is playing an increasingly important role in our daily life and has commonly been applied into the process of industrial manufacturing. Engineered nanoparticles (NPs) like Ag NPs, ZnO NPs, carbon nanotube and TiO₂ NPs are widely used (Jahan et al. 2017). Compared with other nanoparticles, large amount of TiO₂ NPs has been utilized in paints, plastics, sunscreens, solar cells, and food additives due to its unique characteristics such as shape, morphology and different crystal structure. (Bachler et al. 2015; Liang et al. 2000; Macwan et al. 2011; Nam et al. 2014). According to the report of Piccinno et al., TiO₂ (Piccinno et al. 2012) NPs, the highest production of engineered nanoparticles, exceeded 10⁴ tons in 2012. Most of the nanoparticles enter into the environment through wastewater discharge and waste disposal, which inevitably causes the concern about their release into the aquatic environment (Fekete-Kertész et al. 2016; Kiser M 2009). In this case, the enrichment behavior of NPs poses serious threat to aquatic biota (Thiagarajan et al. 2019).

Microalgae is a crucial part of the whole food chain and aquatic ecosystem since it is one kind of major primary producer (Becker 1995; Ramaraj et al. 2014). They are featured by simple structure, wide distribution, low cost, rapid propagation and stronger adaptability. Hence, the microalgae have been used as bio-indicator to evaluate the risk of toxicity of various pollutants (Rashid N 2017; Rodriguez-Morales et al. 2017; Xiong et al. 2017). Microalgae was used as a rational model system to study the ecotoxicological effects of nanomaterials because of its extensiveness (Yan et al. 2019). In recent years, many studies have evaluated the toxicity of TiO₂ NPs to microalgae individuals. Roy et al. (Roy et al. 2016) reported the various effects of P25 TiO₂ nanoparticles on two freshwater microalgae, *Chlorella* and *Scenedesmus*. Xia et al. (Xia et al. 2018) revealed that ocean acidification would enhance the toxicity effect of TiO₂ NPs to marine microalgae *Chlorella vulgaris*. Wu et al. (Wu et al. 2019) emphasized that

high concentrations of TiO₂ NPs contributed to the increased concentration of microcystin in water environment, which may cause damage to aquatic ecosystems. Jia et al. (Jia et al. 2018) evaluated the influence of TiO₂ NPs and multi-walled carbon nanotubes on the growth, cell components and morphology of freshwater diatoms. NPs released into an aqueous environment typically form suspensions that aggregate to varying degrees. Therefore, the stability and ecological toxicology behavior of NPs will exert a unique toxicological effect on the aquatic food web (Christian et al. 2008; Domingos et al. 2009).

In freshwater ecosystem, *Chlorella* is one kind of the most common algae species. Due to the primary producer status of microalgae in the aquatic environment, any changes at “g” level of the food web will affect organisms with a higher level of nutrition. Therefore, *Chlorella vulgaris* was selected to evaluate its toxicological feedback towards the exposure to P25 TiO₂ NPs. Cardinale et al. (Cardinale et al. 2012) reported that P25 TiO₂ NPs could inhibit the growth of *Chlorella* through affecting the metabolic process of *Chlorella*. Xia et al. (Xia et al. 2018) used marine microalgae *Chlorella* as the research object, which proved that ocean acidification would enhance the toxic effect of TiO₂ NPs on *Chlorella*. Roy et al. (Roy et al. 2016) emphasized that there is a significant interspecies difference in the toxicity of TiO₂ NPs to *Chlorella* cells under visible light compared with other algae, which may be associated with the bioavailability of TiO₂ NPs. Although many relative research on the toxicity of *Chlorella* exposure to TiO₂ NPs have been reported, almost no study focused on the effects of long-term growth of *Chlorella*.

Continuous interactions between nanoparticles and algae might exist in actual aqueous environment, and almost no relative study has been reported on this so far. No studies to date have been conducted to systematically investigated the interaction between P25 TiO₂ NPs and *Chlorella vulgaris* under continuous exposure, which would better reflect natural conditions. Hence, evaluating the toxicity of P25 TiO₂ NPs under a long-term toxicity examination is required to better understand the effects of nanoparticles in aqueous environmental matrix.

Accumulation of the NPs in the aqueous environment is a serious menace for the producers as well as the organisms at the higher levels of the food chain. An assessment of these aqueous pollutants in producing toxicity is crucial in keeping dynamic balance of water ecosystem. In this study, the behavior of TiO₂ NPs under the culture medium water was analyzed, meanwhile their complex effects on freshwater microalgae *Chlorella vulgaris* was also studied. This work will be the first to report the influence of EPS on the toxicity of nanomaterials with long-term exposure to *Chlorella vulgaris*. A preliminary characterization of TiO₂ NP was performed. The toxicity of TiO₂ NPs with and without EPS was evaluated by measuring chlorophyll and cell counting techniques. Oxidative stress determination through superoxide dismutase (SOD) and lipid peroxidation (LPO) tests further convinced the toxic aspects of NPs. In addition, electron microscopy analysis was performed to analyze the surface changes of algae after exposure to toxicants.

2. Materials And Methods

2.1. Materials

Nano titanium dioxide (TiO₂ NPs) Aeroxide® P25 was purchased from Sigma-Aldrich, Missouri, USA. According to the manufacturer, the average particle size of TiO₂ NPs is 21 nm with over 99.5% purity. The morphology and size of TiO₂ NPs were confirmed by transmission electronic microscopy (TEM; JEM-2100, JEOL, Japan). Initially, TiO₂ NPs dispersed in ultra-pure water were imaged by transmission electron microscopy (TEM) through depositing a 10 µL drop on formvar/carbon-coated copper grids and drying overnight, and micrographs were obtained at 200 keV. BG11 were procured from Haibo (Qingdao, China). All other chemicals used in the experiment were of analytical reagent grade and without further purification.

2.2. Stock suspensions of TiO₂ nanoparticles

200 mg of the nanoparticle powder were added to 100 mL of ultrapure water and sonicated for 30 min. The stock solution was stored at 4 °C and sonicated for 30 min before use.

2.3. Algal cultures

The algae, *Chlorella vulgaris* (FACHB-1068), were purchased from Freshwater Algae Culture Collection at the Institute of Hydrobiology (Wuhan, China). Algae in rapid growth phase were transferred into a conical flask containing BG-11 medium (Xiao et al. 2016). The cultures were maintained under 25 ± 0.5 °C (a photoperiod of 12:12 h light/dark cycle with a white fluorescent lighting). The flasks were shaken three times every day, which will help to keep the samples from precipitating and rearranged randomly in the whole system. The initial cell density was approximately 10⁶ cells/mL (Wu et al. 2019).

2.4. Aggregation and stability analysis of TiO₂ NPs in freshwater matrix

The varied TiO₂ hydrodynamic diameter (D_h) over time (t) was measured by dynamic light scattering (DLS) (Zetasizer Nano-S90, Malvern Instruments Ltd., UK) performed by a He-Ne laser at the wavelength of 633 nm. The sonicated TiO₂ NPs stock solution was added to the medium, which had been sterilized. The working concentration of TiO₂ NPs was 50 mg/L. For the experiment, 1.2 mL of the pre-prepared mixture suspension was introduced into a cuvette (Titan, Shanghai). The concentration of TiO₂ NPs in the DLS vial was maintained at 50 mg/L. The cuvette was vortexed for 1 s immediately prior to measurement. The D_h measurement was monitored over a time period from 1 to 180 min. During the DLS measurements, the scattered light intensity was detected by a photodetector at a scattering angle of 173°, with each autocorrelation function being accumulated over a period of 10 s. All cuvettes were used only once and all DLS measurements were conducted at 25 °C (Lin et al. 2016).

The sedimentation of TiO₂ nanoparticles in freshwater matrix was measured by UV-vis spectroscopy (752N, Xin Mao, Shanghai). TiO₂ NPs stock solution was sonicated for 30 min and then added in the freshwater matrix to obtain mixtures containing 50 mg/L TiO₂. The suspensions were measured at a

wavelength of 378 nm and the absorbance were recorded for 12 h at different time intervals. The sedimentation of TiO₂ was expressed by A/A_0 . Where A_0 is the initial absorbance and A denotes the absorbance recorded under different intervals during the 12 h experimental period.

2.5. Cytotoxicity assessment

2.5.1. Experimental set up

The growth inhibition examination was performed according to the OECD Guideline 201(No, 2011). In order to conduct continuous exposure to freshwater algae *Chlorella*, *Chlorella* culture was harvested at the stable stage of their growth (1.5×10^7 cells/mL). An appropriate amount of this algae was removed to inoculate in a new medium. The medium was mixed with an equal volume of culture medium containing an appropriate amount of TiO₂ nanoparticles. Simultaneously, *Chlorella* was harvested at the stable stage of their growth (1.5×10^7 cells/mL) and centrifuged at 6000 rpm for 20 min to remove soluble EPS. The pellet was obtained to inoculate in two mediums separately, one of which was a simple culture solution, another was culture solution mixed with TiO₂ NPs. All the experiments we built that obtain cultures at an initial cell density of 1×10^6 cells/mL and TiO₂ NPs concentrations at 50 mg/L. And a control group was set up in the absence of nanoparticles and no EPS removed. All samples were incubated for 30 days at 25 ± 0.5 ° C with a photoperiod of 12:12 h light/dark cycle under a white fluorescent light and a pH of 7.1 ± 0.5 . During the examination, 2 mL of algae suspension was taken for further cytotoxicity experiments from each sample at 1, 2, 3, 5, 7, 10, 15, 20, 25 and 30 days.

2.5.2. Algal growth assay

Aliquots of the algal suspension after interaction with/without TiO₂ NPs (50 mg/L) visible light and dark conditions were counted at least three times using a hemocytometer under a metallographic microscope (zeiss supra-55, Germany), and the density in each sample was calculated. The initial algal density was 1.6×10^6 cells/mL. The normal growth rate(R) of control algae was calculated as follows: $R(\%) = (C_0 - C_t) / C_0 \times 100\%$, where C_0 is the initial density and C_t denotes the density at the time t. The algae of test groups had a growth inhibition rate(IR), which was calculated as follows: $IR(\%) = (C - C_t) / C \times 100\%$, where C is the density of the control algal at time t and C_t represents the density of the test groups at the time t.

2.5.3. Evaluation parameter

Total protein, superoxide dismutase (SOD) and malondialdehyde (MDA) assays were performed according to previously reported methods(Xiao et al. 2016). The appropriate commercial kits were used to determine the total protein, SOD and MDA of algae, which were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The total protein was determined from the Bradford method (Bradford, 1976). The extent of lipid peroxidation can generally be determined by detecting the amount of MDA, through monitoring the color reaction of thiobarbituric acid (TBA method)(Janero 1990; Navarro et al. 2008). The ability of an organism to scavenge oxygen free radicals itself could be indirectly indicated

by the activity of SOD(Wang et al. 2010). A photochemical reduction method of nitroblue tetrazole was applied to determine the activity of SOD (NBT method)(Beyer and Fridovich 1987).

Morphological change on algae due to the exposure to toxicants were observed using SEM. Control samples with only algae, and algae treated with TiO₂ NPs (50 mM) with and without EPS were analyzed under scanning electron microscope (Zeiss supra55). After 72 h interaction, aliquots of algal suspension were placed on a piece of glass, air dried, and sputtered with gold before SEM analysis(Thiagarajan et al. 2019).

3. Result And Discussion

3.1. Characterization of TiO₂ NPs

SEM image Fig. 1 (a) showed that the TiO₂ nanoparticles are nearly spherical without obvious clumps. TEM image Fig. 1 (b) indicated the average size is near 20 nm, which is in accordance with the size of 21 nm provided by the manufacturer. The hydrodynamic particle size was found to be 233.6 ± 6.5 nm by DLS, and negative (-3.38 ± 0.11 mV) charge on TiO₂ surface in neutral aqueous solution. The larger hydrodynamic particle size of TiO₂ than the counterpart powder size may attributed to the formation of TiO₂ aggregate.

3.2 Aggregation and stability analysis of nanoparticles in freshwater matrix

In order to investigate the short-term toxicity effects of nano TiO₂ on algae cells, the short-term aggregation and sedimentation of P25 TiO₂ NPs were examined. The aggregation of TiO₂ NPs (50 mg/L) in the algal cell culture was studied in 3 hours. In the experiment, the algal culture medium with high salt content and abundant monovalent and divalent counter ions (Na⁺, Ca²⁺, Mg²⁺) can partly neutralize the NPs surface charge, the zeta potential was negative (TiO₂ NPs isoelectric point $\sim 6.2-6.4$) at which the present value of pH (i.e. 7.2). Therefore, NPs dispersed in media colliding had a high attachment efficiency with easily aggregation due to thermal movement. This phenomenon was certainly confirmed by DLS analysis (Fig. 2). The hydrodynamic diameter shows an increasing trend from 484.8 ± 50.8 μm to 2288.7 ± 231.3 μm within two hours. This study demonstrated that nano TiO₂ dispersion was extremely easy to form aggregates that reached micrometric size in the present media within 30 min. It was confirmed from the sedimentation experiment that the sedimentation rate of NPs was up to 80% within 6 hours, as shown in Fig. 3. In these cases, nano TiO₂ can easily forms large clusters and then gradually precipitates in the test vessel under the role of gravity, thus the amount of TiO₂ NPs in the suspension of the sample was reduced. Therefore, the way of agitation was selected to simulate the dynamic process, which helps to enhance the contact of algae cells with particles for a short-term toxicity test under the function of nano TiO₂.

3.3 Morphological variation of Chlorella cell

The effect of nano TiO₂ on the morphology upon the short time exposure of algal cells can be observed in SEM images. The morphological aberrations of vulgaris exposed to TiO₂ NPs were compared between control and treated cells were depicted in Fig. 4. It can be observed that the aggregated TiO₂ NPs adhere to the algal cell surface as the exposure time increased. Compared with the control group, the amount of chlorella attached to the surface of TiO₂ after removing EPS was relatively higher. The adhesion of TiO₂ NPs on the cell surface is one of the significant reasons leading to cell growth inhibition and death in a short period of time.

3.4 Effect of P25 TiO₂ nanoparticles on Chlorella algae growth

Fig.5 demonstrated that the growth inhibition effect was observed in the experimental group under suitable growth conditions for algae. The inhibition rates of the algae cells exposed to nano TiO₂ were detected to be 0.18, 0.36, 0.11 in the first three days. However, the rate was found to be decreased from 0.38 to -0.02 when the time was more than ten days. The observed growth inhibition of algae cells could remove algae-soluble EPS, and we found that algae cells without EPS were more susceptible to nano-TiO₂. In the absence of nano-TiO₂, the growth inhibition rate of the algae-removing algae was 0.36, 0.45, and 0.55 in the first three days, then a decreased trend was observed in the range of 0.55 to 0.04. A high inhibition rate within 1 to 10 days was maintained with EPS-removed algal cells exposed to nano TiO₂. Regularly after the tenth day, the inhibition rate also showed a decreasing trend in the range of 0.52 - 0.23. In the terms of biovolume, nano-titanium dioxide exhibits a great influence on algal cell growth, especially in the early stage. However, as the growth of algae cells matures, the toxicity of nanoparticles to algal cells is gradually reduced, which proved that the final growth inhibition rate was greatly reduced. Given the negligible dissolution of TiO₂ NPs in water (Dasari et al. 2013), the growth inhibition was mainly due to the attachment of nano TiO₂ to algae cells. It can be concluded that EPS has a protective effect on algae cells simultaneously. Under the same exposure conditions, algae cells without EPS were subjected to higher growth inhibition, which may be relate to the absence of EPS protection, and nanoparticles were more likely to adhere to the surface of algae cells.

3.4 Antioxidation and lipid peroxidation

Fig.6 demonstrated the variation of total protein content of chlorella exposed to nano TiO₂. Algae cells exposed to nano TiO₂ exhibited a higher total protein content than that without nano TiO₂. As we expected, the total protein content of the EPS-removed algae was lower than normal condition. Under 1 to 15 days of exposure to nano-titanium dioxide, the total protein content of algae cells was lower than that without exposure to nano TiO₂, whereas the total protein content of algae cells without nanoparticles were exceeded at 15 to 30 days. The photocatalytic action of nano TiO₂ can lead to cell membrane damage of the organism and eventually cell death by producing oxidative stress against the organism.

The algae secreted a variety of compounds with antioxidant function that help to resist the survival of toxic stress. This is why the algae cells exposed to TiO₂ NPs have a higher total protein content. The algae cells with EPS removed possibly damaged by TiO₂ NPs, which reduced biomass and resulted in less total protein compared with the algal cells without TiO₂ NPs.

The superoxide dismutase is the first and primary antioxidant enzyme against the oxidative threats. Figure 7 showed the varied production of SOD during the growth of chlorella as the function of TiO₂ NPs. We clearly understand that the production of chlorella SOD was reduced under the effect of TiO₂ NPs, thereby lowering the oxidative stress response of organism, which result in reduced biomass. At the same time, algae cells with EPS removed were more easily affected by TiO₂ NPs, so algae cells with EPS removed had minimal biomass when exposed to TiO₂ NPs. However, this effect was gradually weakened. As the organism continues to grow, the SOD secreted by the algae cells exposed to the TiO₂ NPs eventually reach a normal level.

Malondialdehyde (MDA) was a kind of peroxidation product of lipids and the accumulation of oxygen free radical possibly causes the peroxidation of lipid in algal cells. The change of MDA levels indicates the extent of oxidative damage in cells. As shown in Fig. 8, the level of MDA was significantly higher than the control group. This indicated that nano-titanium dioxide exhibits an oxidative damage to cells. In addition, it can be concluded that nano-titanium dioxide had a sustained damage effect to algae cells which was performed by comparison of MDA levels during 30 days.

4. Conclusion

In conclusion, TiO₂ NPs was highly ecotoxicity mainly due to the attachment of nanoparticles on the surface of algae cells. Meanwhile, a protective role of EPS on the growth of algae was observed. The algae cells with EPS removed were more easily damaged by nanoparticles. Furthermore, the damage of TiO₂ NPs to chlorella was reduced from the perspective of the entire growth cycle. After 25 days, the biomass and SOD activity of chlorella tend to be normal, indicating the negligible effect of nanoparticles on algae cells. This research will be helpful to further understand the biological toxicity of nanoparticles and the response mechanism of organisms. However, the natural water environment conditions are more complicated and more involved factors. It is necessary to further investigate the interaction mechanism of organisms with nanoparticles.

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Declarations

Declaration of Interest Statement

We declared that we have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Figures

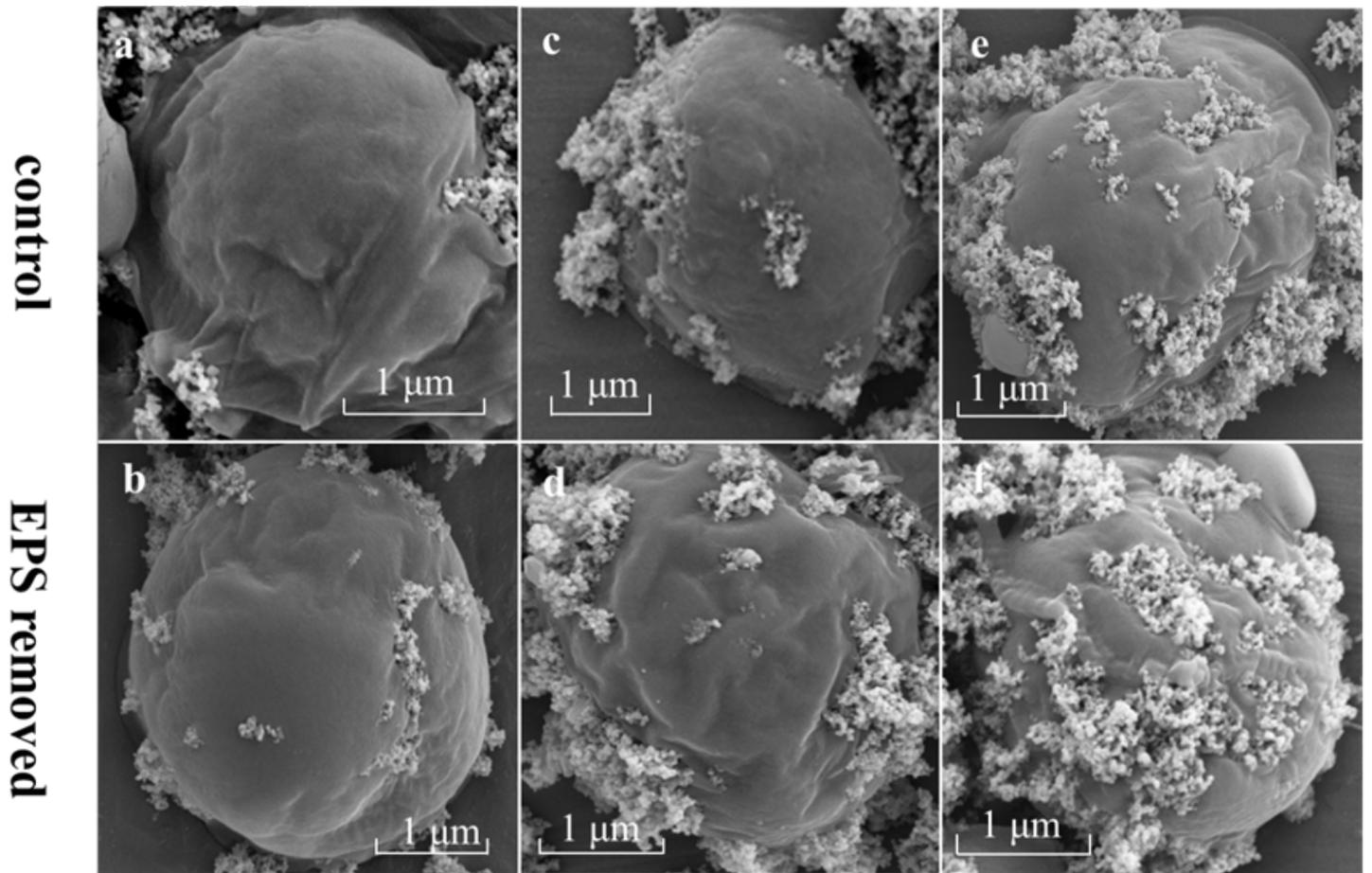


Figure 1

SEM images illustration of the ultrastructural changes in the Chlorella algae owing to the exposure of TiO₂ NPs. a and b are exposed for 24h, c and d are exposed for 48h, and e and f are exposed for 72h.

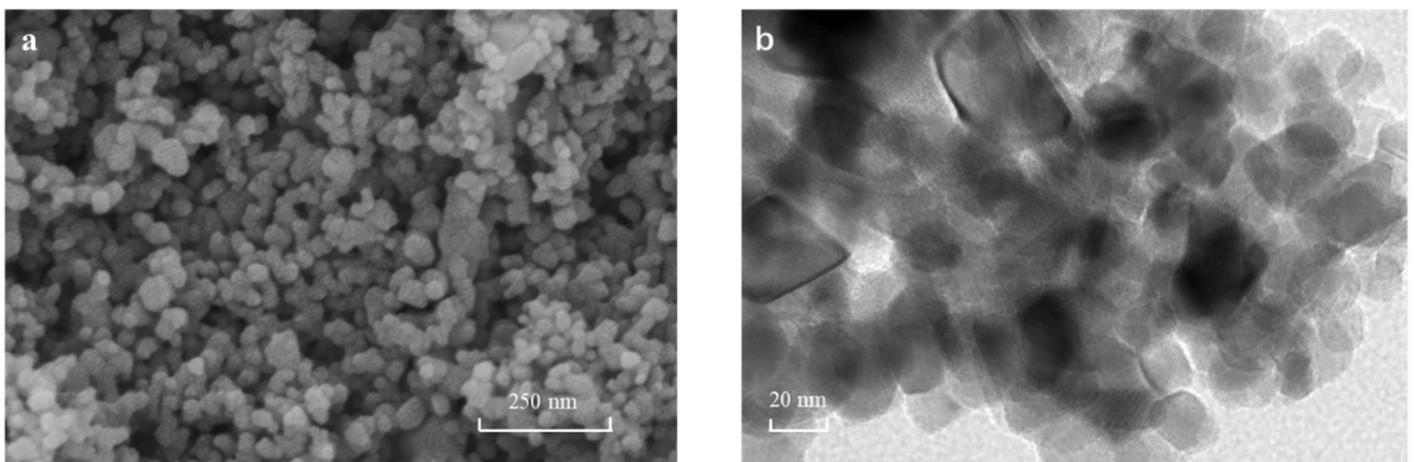


Figure 2

SEM (a) and TEM (b) images of TiO₂ NPs.

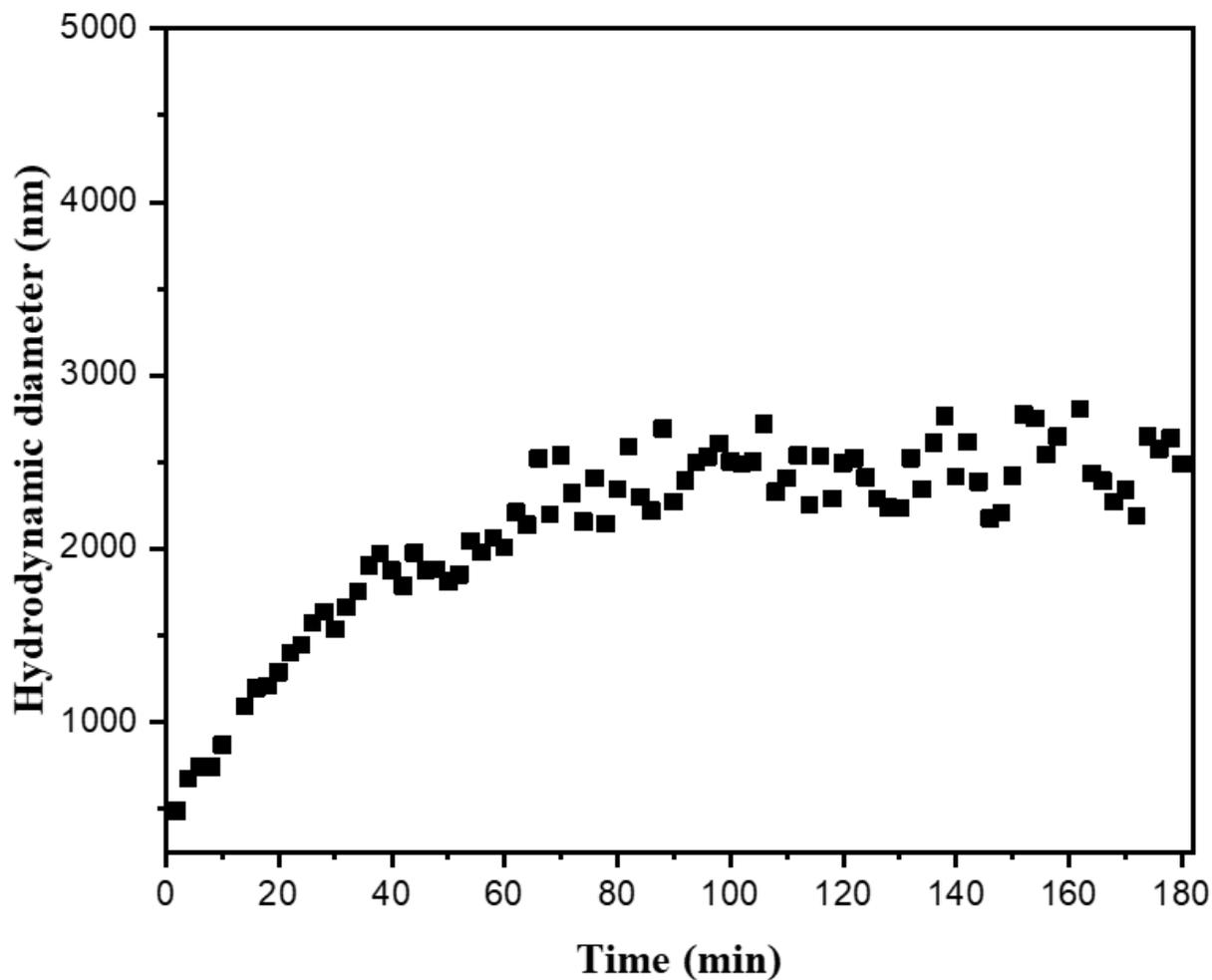


Figure 3

Aggregation of TiO₂ NPs (50 mg L⁻¹) monitored for 3 h by DLS.

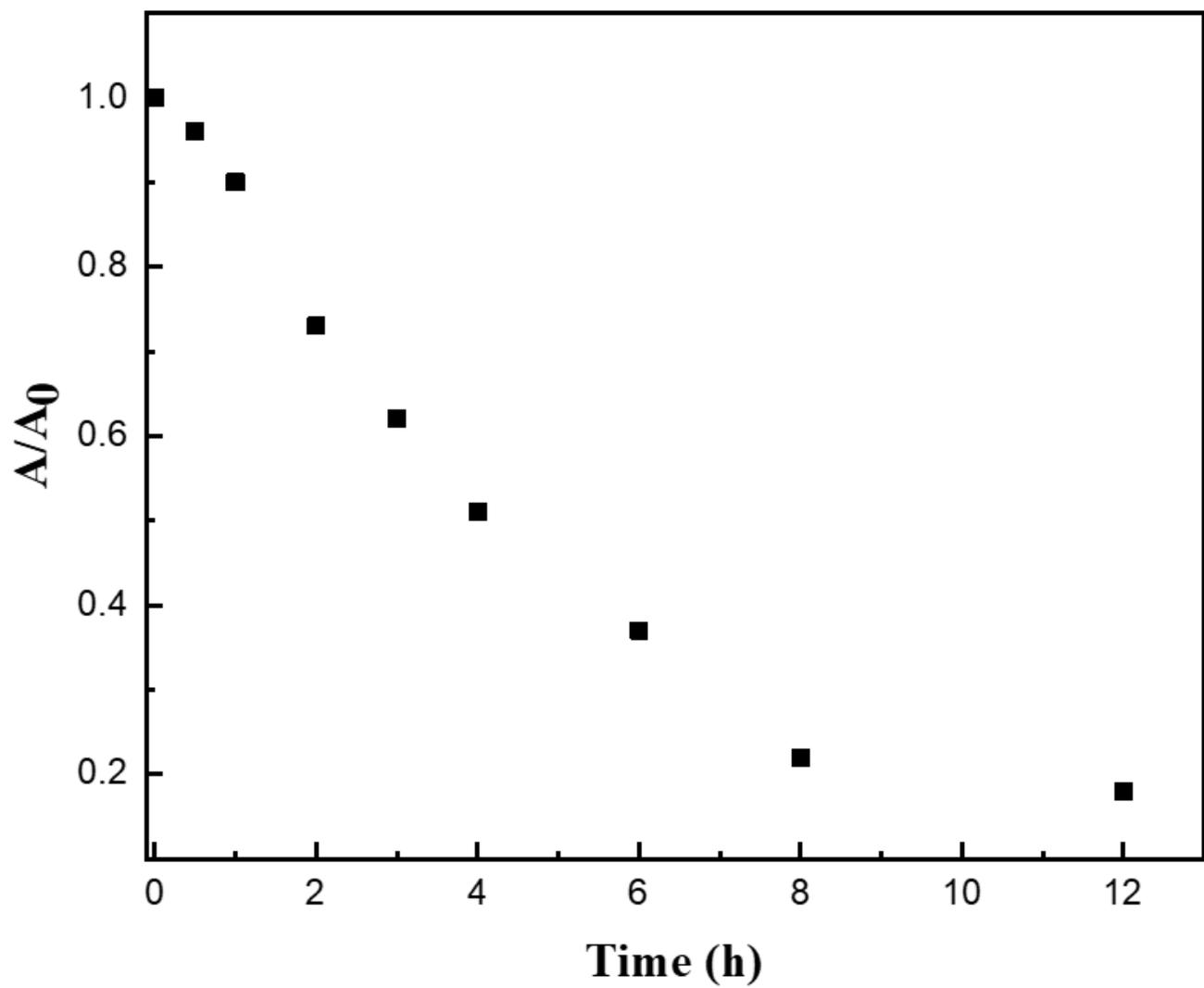


Figure 4

Sedimentation profile of 50 mg L⁻¹ TiO₂ NPs.

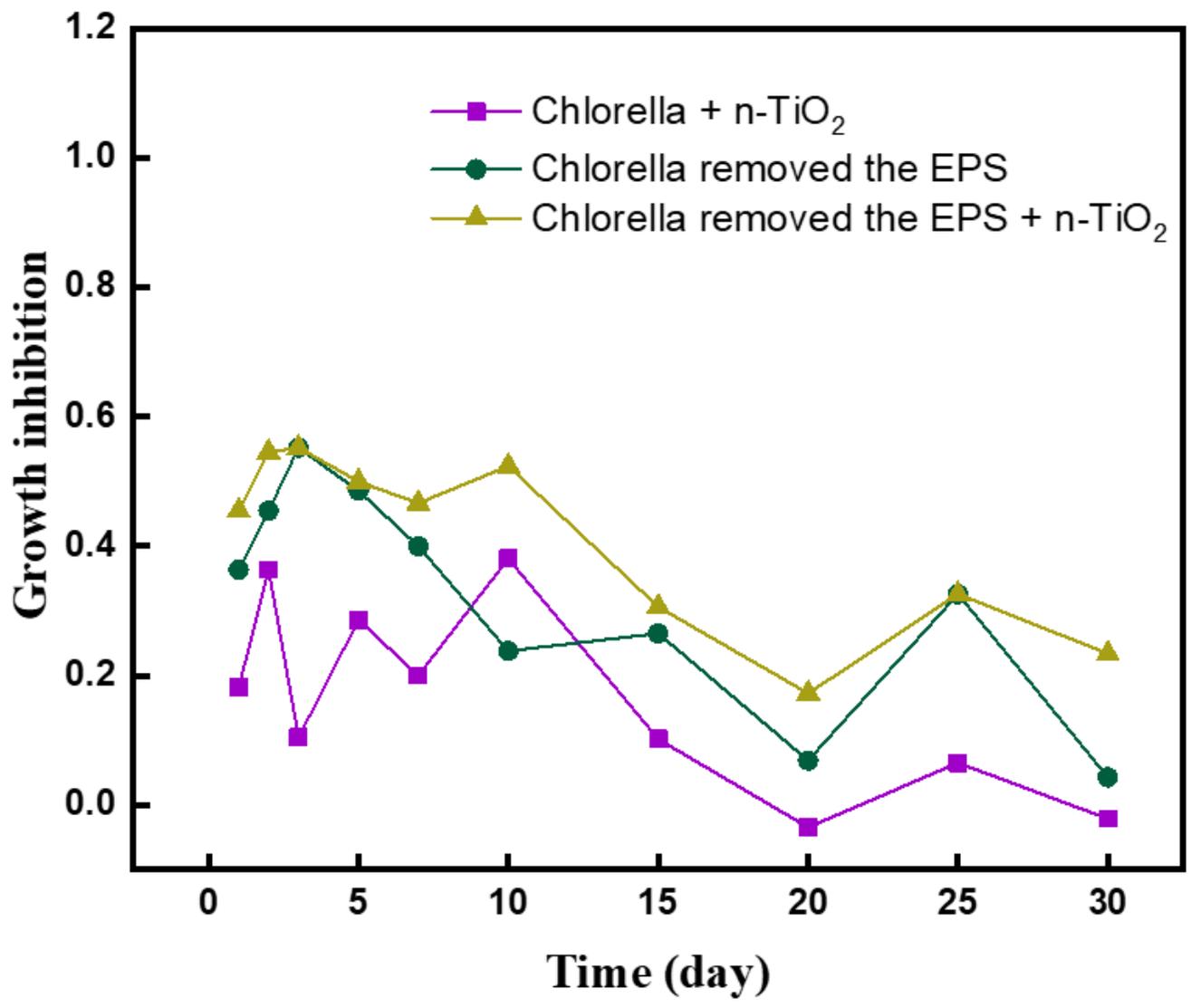


Figure 5

The growth inhibition of algae with/without nano TiO₂

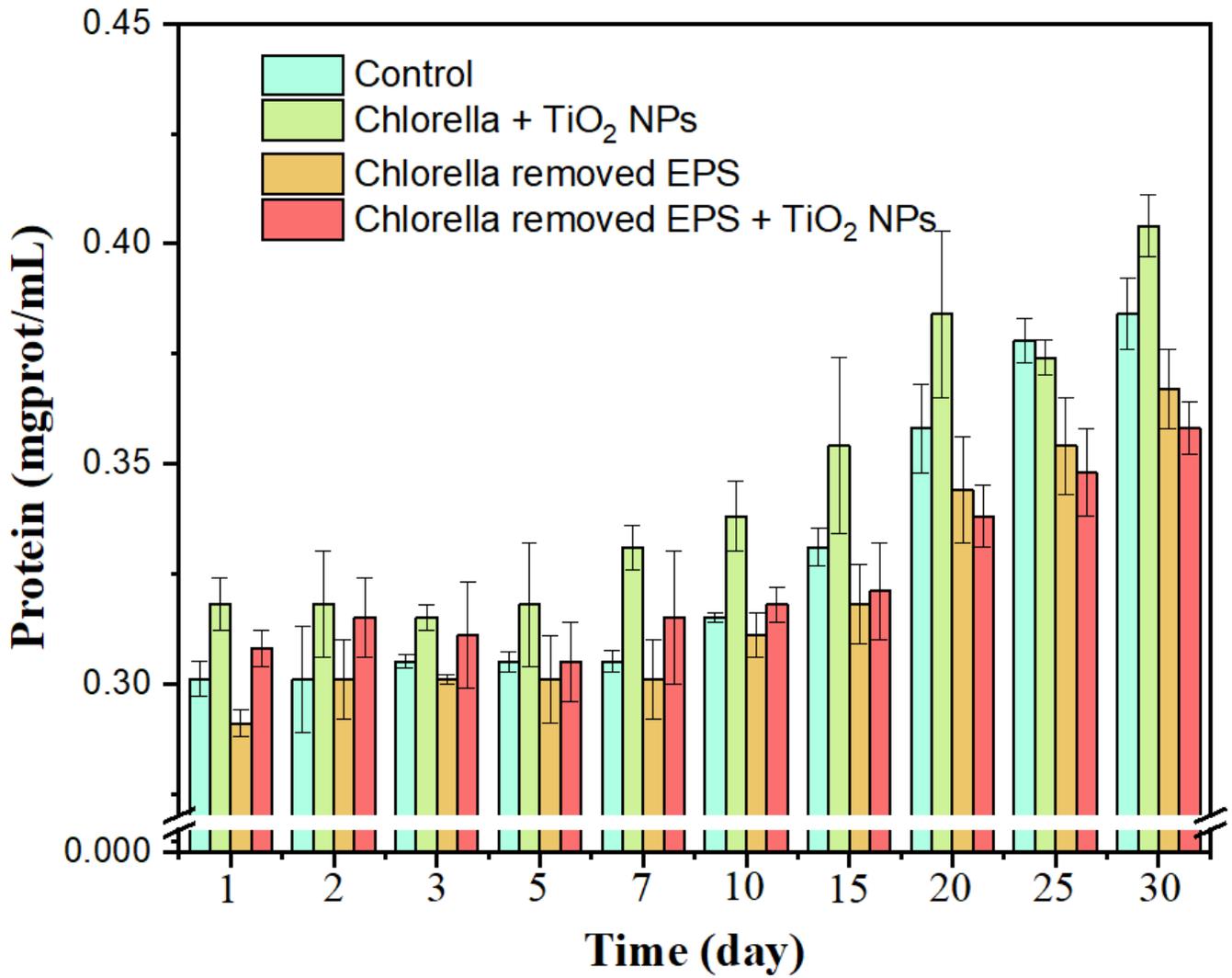


Figure 6

Total protein content of Chlorella grown for 30 days with/without TiO₂ NPs.

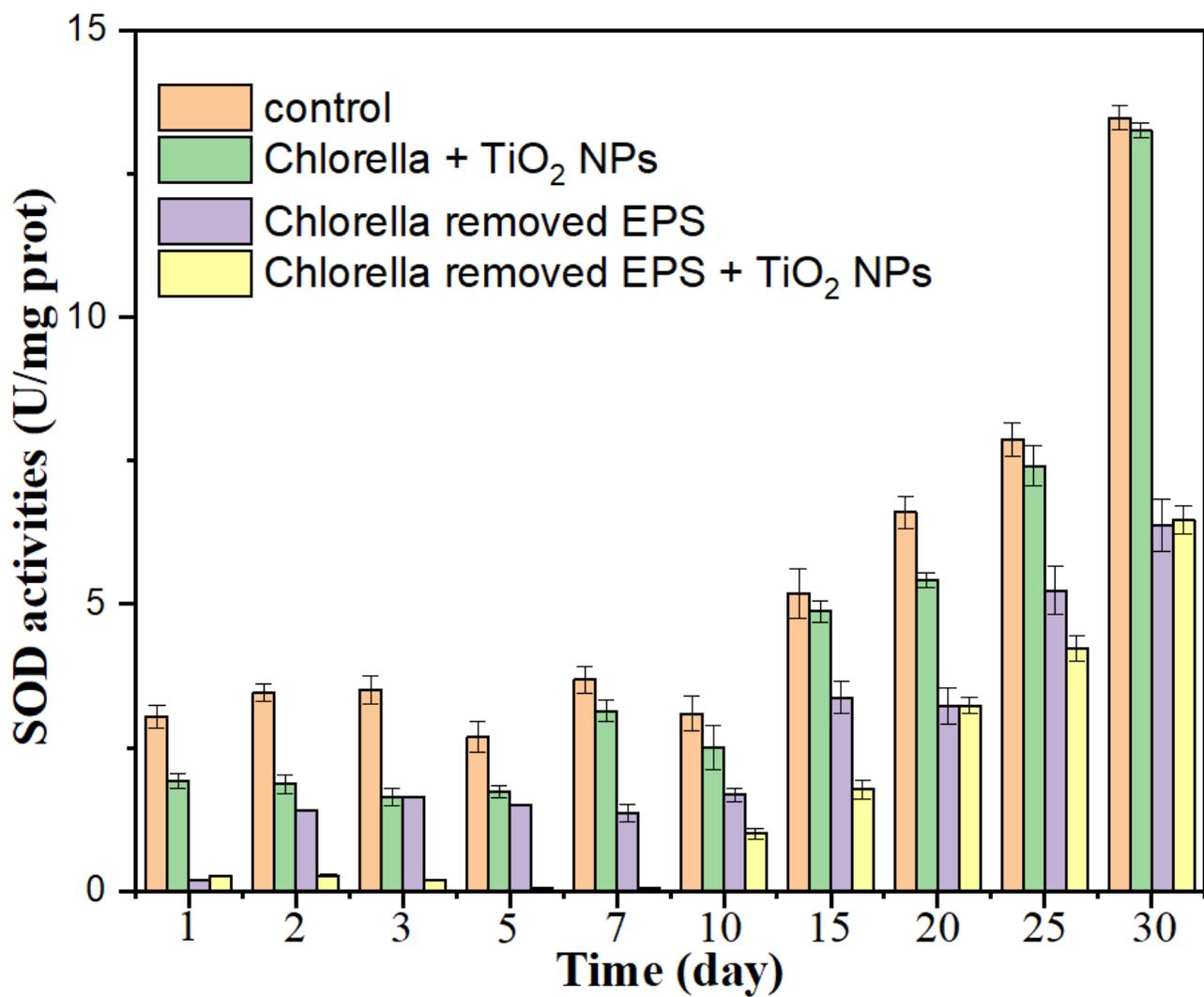


Figure 7

Relative levels of superoxide dismutase (SOD) detection exposed to 50mg/L TiO₂ NPs for 30 days.

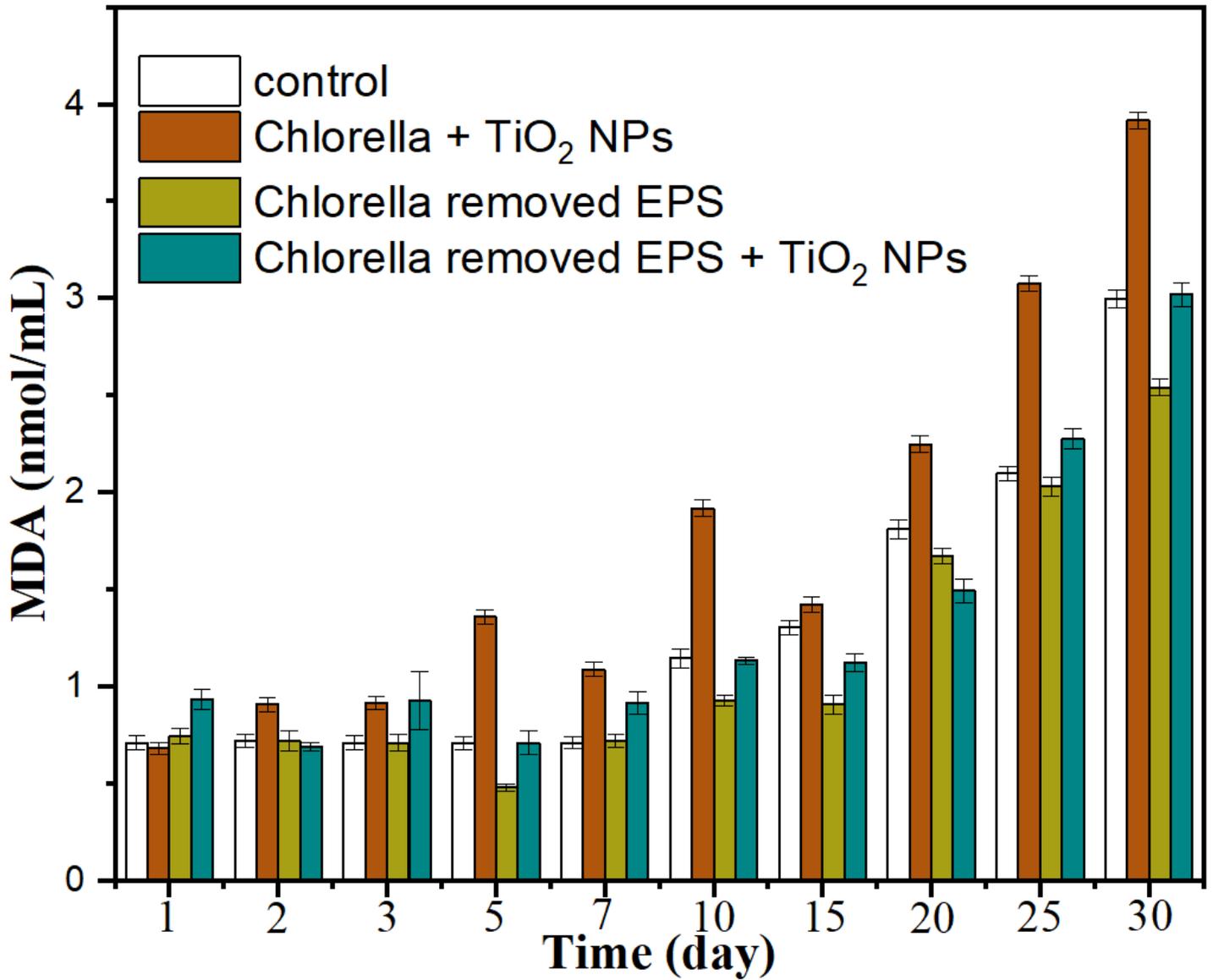


Figure 8

Malondialdehyde (MDA) of Chlorella grown for 30 days with/without TiO₂ NPs.