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The Effect of Titanium Dioxide Nanoparticles on *Haematococcus pluvialis* Biomass Concentration

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KEYWORDS

Algal biomass

Biomass concentration

Growth pattern

H. pluvialis

Titanium dioxide nanoparticles

ABSTRACT

The increased release of Titanium dioxide nanoparticles (TiO_2 NPs) into the aquatic ecosystem is caused by the augmented utilization of nanoparticles in personal care and household products. This has resulted in the contamination of marine, aquatic, and ground water resources, causing adverse impacts on the biota and flora, both in vivo and in vitro. The main purpose of this research was to examine the negative impacts of TiO_2 NPs on the bioaccumulation of *Haematococcus pluvialis*. The interaction and buildup of TiO_2 NPs on *H. pluvialis* were studied using scanning electron microscopy (SEM). The exposure of *H. pluvialis* to TiO_2 NPs with increasing concentrations (5–100 µg/mL) and time intervals (24 h to 96 h) impacted the biomass concentration of the microalgae. The SEM images provided evidence of changes in characteristics and impairment of the exterior of exposed cells. The findings revealed that the exposure of *H. pluvialis* to TiO_2 NPs resulted in a decline in biomass, which was dependent on the concentration and duration of exposure. The most severe adverse effects were observed after 96 hours of exposure, with a reduction of 43.29 ± 2.02% of biomass concentration. This study has demonstrated that TiO_2 NPs harm *H. pluvialis*, as evidenced by the negative impact on algal biomass resulting from the binding and buildup of these particles on microalga *H. pluvialis*. To sum up, the decline in algal growth is caused by the accumulation and interaction of TiO_2 NPs on microalgae scoring the adverse effects on the growth of *H. pluvialis* by TiO_2 NPs. The findings of this study call for novel screening methods to detect and eliminate TiO_2 NPs contamination in aquatic sources used for the cultivation of microalgae which may otherwise pose delirious effects to the consumers.

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1 Introduction

Nanotechnology focuses on the understanding and application of atoms and molecules smaller than 100 nanometres (nm). The nanoparticles' wide applications and unique features clearly explain the huge market potential for nanoparticles (NPs) (Banerjee and Roychoudhury 2019). The nanoparticles have been used in the pharmaceutical industry, water treatment, cosmetics, engineering, as a colourant in white plastics, pigments for food, and many other consumers and industrial products (Iswarya et al. 2015; Ziental et al. 2020).

Zinc oxide nanoparticles (ZnO NPs) and titanium dioxide nanoparticles (TiO_2 NPs) are the most commonly manufactured nanomaterials, with an estimated global output ranging from 10,000 to 88,000 tons per year. TiO_2 NPs are used in numerous fields with annual production exceeding four million tons annually. The production of TiO_2 NPs is still an increasing trend because of their vast range of uses, so the nanoparticle's release into the aquatic environment is inevitable (Bameri et al. 2022). Therefore, studying how TiO_2 NPs affect the environment, particularly aquatic creatures, is crucial. Microalgae have previously been employed as exemplary organisms for investigating the harmfulness of metallic oxide nanoparticles in the atmosphere (Djearamane et al. 2019). Several parameters can be used to indicate the presence of metallic oxide nanoparticles, including biomass and photosynthetic activities.

Haematococcus pluvialis is an important microalga with high nutritional importance. This freshwater microalga contain carbohydrates (30–40%), proteins (20–30%), fatty acids (7–25%), astaxanthin (>1.5%), carotenoids (>1.75%), and minerals. It is also one of the lushest natural sources of astaxanthin- a natural substance with potent antioxidant, anticancer, and anti-inflammatory properties (Harker et al. 1996; Dong et al. 2014; Hong et al. 2016; Matos et al. 2017). Furthermore, *H. pluvialis* holds high agricultural value as this microalga give colour to trout, farmed salmon, ornamental fish, prawns, and sea bream (Dore and Cysewski 2003). Therefore, they have been fed to the farmed aquaculture feeds. Environmental stress makes *H. pluvialis* cells highly sensitive and changes their morphology in response to environmental factors (Djearamane et al. 2019). The investigation was carried out to explore the influence of TiO_2 NPs on the biomass concentration of *H. pluvialis*. Consumption of microalgae nutritional supplements contaminated with TiO_2 NPs could potentially compromise the nutritional value and pose adverse health effects to consumers. Therefore, understanding the consequence of TiO_2 NPs on *H. pluvialis* would be crucial in assessing the ecological implications of TiO_2 NPs in waterways. These findings will also aid in the development of screening methods for TiO_2 NPs contamination in microalgae, which may alternatively cause negative health impacts to the consumers.

2 Materials and Procedures

2.1 Microalgae Cultivation

The stock culture was provided by UTEX1926 (University of Texas Culture Collection, Austin, TX, USA). *H. pluvialis* stock culture of 4mL was added to 200 mL of Bold's basal medium and was kept at room temperature (21 to 23°C) with a cool white fluorescent bulb providing 1200 lux illumination for 16 hours of daylight and 8 hours of darkness (Djearamane et al. 2018)

2.2 Microalgae's Exposure to TiO_2 NPs

TiO_2 NPs with particle size (23-35nm) were procured from Chemical Solutions, Malaysia, and used in this experiment. TiO_2 NPs stock solution of 200 µg/mL concentration was prepared in the appropriate culture medium to produce a homogeneous NPs suspension. TiO_2 NPs stock solutions were diluted with the BBM to prepare the working concentrations. Then, *H. pluvialis* cells from the eighth day of growth (exponential phase) with the initial optical density (OD) of 0.4 were exposed to NP concentrations over 96 hours in a 250 mL Erlenmeyer flask alongside NP-free controls. The influence of NPs (5, 10, 25, 50, 100 µg/mL) on the nutritional values of *H. pluvialis* was examined by comparing the NP exposed algal cells with the control cells at 24, 48, 72, and 96 hours. This investigation allowed us to assess the influence of NPs on the algal cells and how their nutritional values varied with NPs' exposure time (Djearamane et al. 2018).

2.3 Cellular Interaction of TiO_2 NPs on Algal Cells via SEM-EDX Analysis

The adsorption and accumulation of TiO_2 NPs in *H. pluvialis* biomass were analyzed using SEM-EDX to understand how TiO_2 NPs affected cell morphology. The algal cells were spun upon exposure to TiO_2 NPs, and the pellet was obtained. It was then washed twice with distilled water and 0.1X PBS and freeze-dried for further analysis. SEM-EDX was performed for the freeze-dried algal cells (JSM-6701F, Joel, Japan).

2.4 Effects of TiO_2 NPs on Algal Biomass Concentration

The algal biomass concentration of the test and the control were assessed using a spectrophotometer at an optical density of 680 nm (Gynesys 10S UV-Vis, Thermo Scientific, United States of America). An additional control with just the TiO_2 NPs for each concentration was also recorded and adjusted from the test reading to exclude the interference from TiO_2 NPs. BBM served as the blank for both the test and the controls. The trial and control results were expressed as a percentage of change in biomass and were used to calculate the trend of algal growth following the treatment with various concentrations of TiO_2 NPs at different time intervals.

$$\text{OD of the culture at 680nm} = OD_1 - OD_0 \quad \text{(eq. 1)}$$

OD_0 = OD of the medium with TiO_2 NPs only

OD_1 = OD of cell culture with TiO_2 NPs only

$$\% \text{ change in biomass} = \frac{(\text{OD 680 of negative control} - \text{OD 680 of test cell culture} \times 100)}{(\text{OD 680 of negative control})} \quad \text{(eq. 2)}$$

2.5 Statistical Analysis

All challenge tests were performed in triplicates (n = 3), and the data were conferred as mean ± standard error. The experimental results were tested for normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used for all analyses with significant values set at $p<0.05$. Tukey's post hoc test was used for multiple comparisons.

3 Results

3.1 SEM-EDX Analysis of Algal Cell Interactions with TiO_2 Nanoparticles

SEM image of *H. pluvialis* cells untreated and treated with TiO_2 NPs at 100 mg/mL is shown in Figure 1. The images demonstrate that cells in Figure 1A appeared as smooth cylindrical structures with undamaged cell membranes when they were not treated with TiO_2 NPs. The cells treated with TiO_2 NPs in Figure 1B show cell entrapment with NP clusters, aggregation of algal cells, and rupture of the cell membrane, which led to cell rupture and wrinkled cells of *H. pluvialis*. We have specifically reported the differences between the control and exposure at a concentration of 100 µg/mL TiO_2 NPs due to the significant changes observed in this particular concentration.

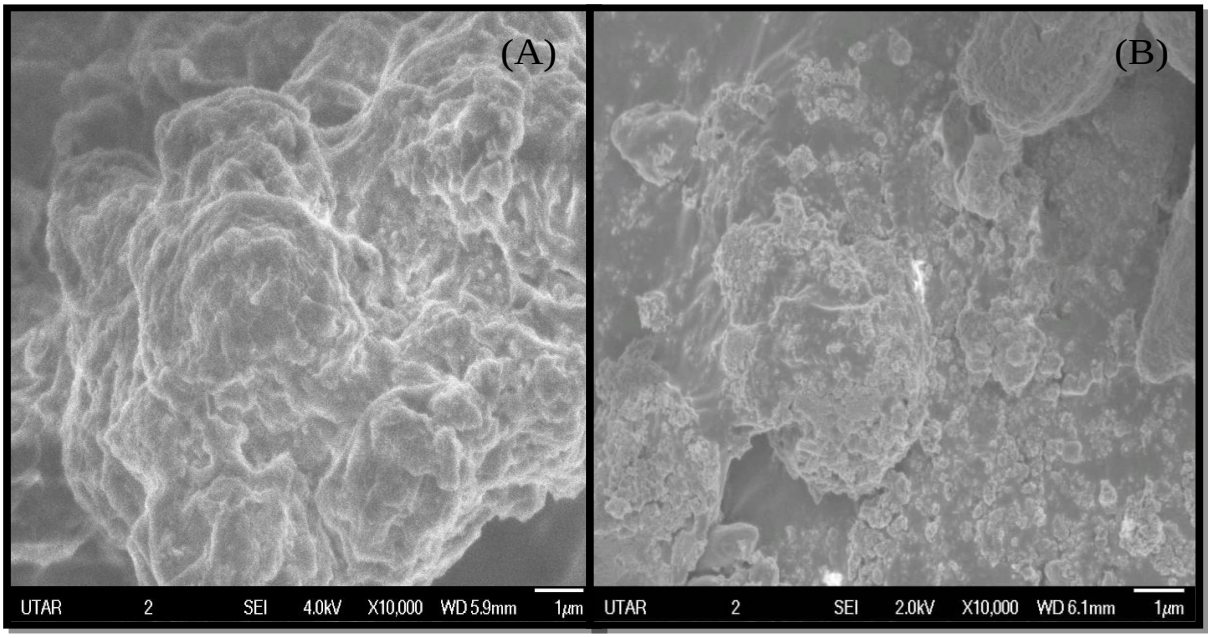

Figure 1 Scanning Electron Microscopy: (A) *H. pluvialis* cells without treating TiO_2 NPs at 96h with 10000X magnification; (B) *H. pluvialis* cells treated with 100µg/mL TiO_2 NPs at 96h with 10000X magnification.

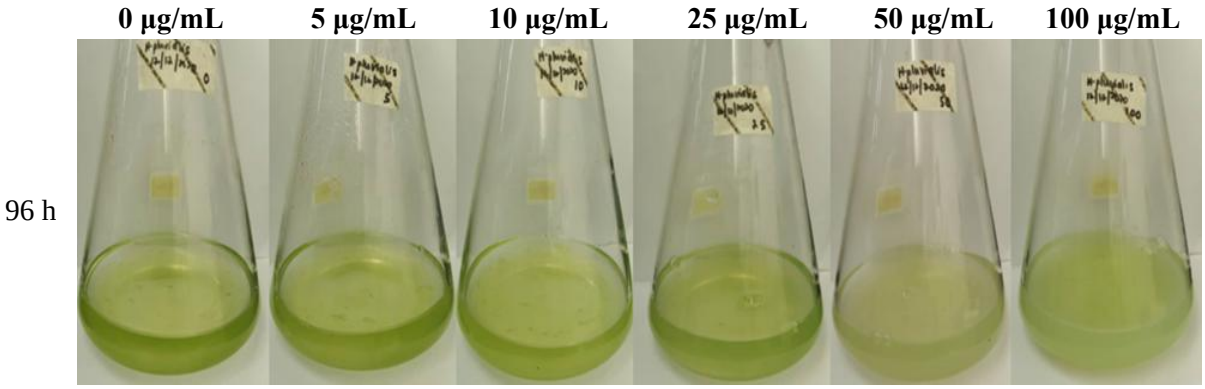

Figure 2 *H. pluvialis* cultures treated with different concentrations of TiO_2 NPs at 96 hrs

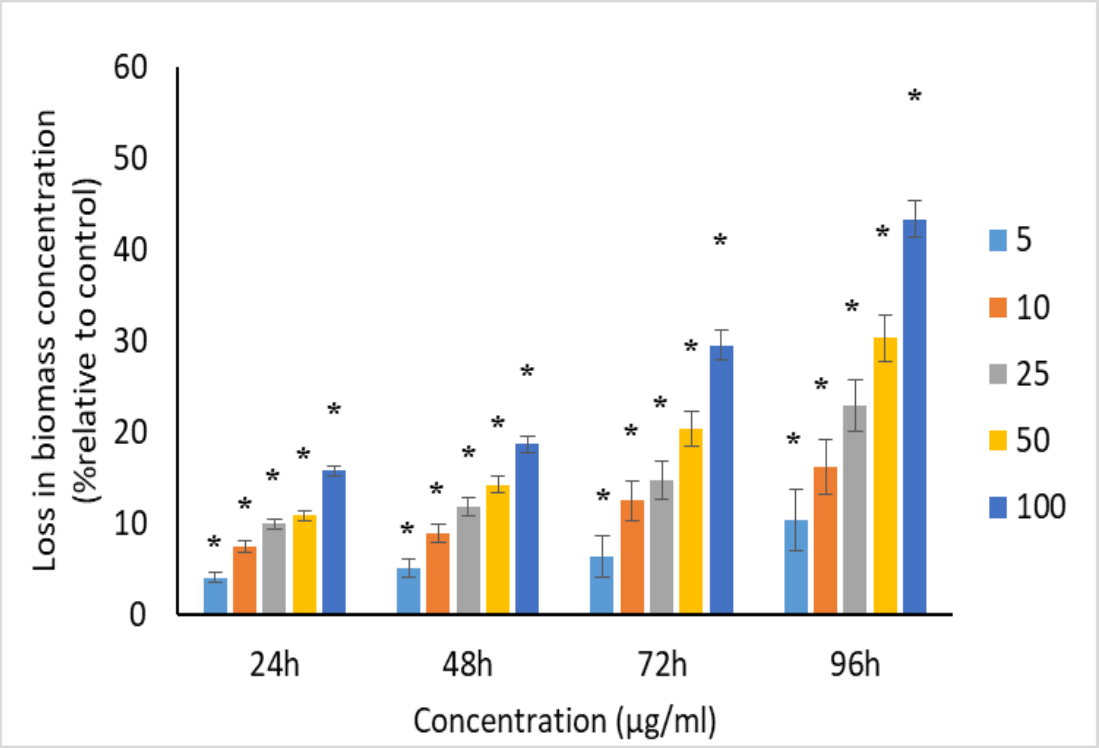

Figure 3 Plotted the mean values and standard deviation of the percentage loss in biomass concentration of *H. pluvialis* relative to the control after treatment with TiO_2 NPs from 24 to 96 h. The asterisk (*) indicates a significant difference at $p < 0.05$ between the control and TiO_2 NPs treated algal suspension for all the time intervals

3.2 Effects of TiO_2 NPs on Algal Biomass Concentration

When *H. pluvialis* culture was exposed to several TiO_2 NPs concentrations, as presented in Figure 2, a slight difference in the culture's colour was observed. As the concentration and exposure time increased, the intensity of the green colour decreased. This qualitative data indicates that the biomass concentration of *H. pluvialis* decreased with increased concentration and exposure time. Figure 3 depicts the percentage reduction of *H. pluvialis* biomass concentration relative to control when treated with different TiO_2 NPs concentrations ranging from 5 to 100 µg/mL for 24 to 96 hrs. A significant ($p < 0.05$) loss in biomass was detected for the tested concentrations of TiO_2 NPs between 24 and 96 hours. The highest loss in biomass occurred at 96 hours, with values reported as 10.42%, 16.15%, 22.87%, 30.29%, and 43.29% for 5, 10, 25, 50, and 100 µg/mL TiO_2 NPs respectively.

4 Discussion

SEM-EDX analysis was conducted on *H. pluvialis* cells exposed to TiO_2 NPs to determine the accumulation of the particles in the biomass and any resulting changes in cell morphology. Two pathways for metal ion uptake into algal cells were reported (Gupta et al. 2014). The initial step involved the adherence of metal ions onto the surface of the algae, after which the ions were taken up by cytoplasmic organelles and transported through the cell membranes. The current investigation used SEM-EDX to determine TiO_2 NPs' accumulation in the algal biomass, according to the mechanisms outlined by Gupta et al. (2014). Many researchers validated similar results (Dmytryk et al. 2014; Djearamane et al. 2018), where EDX analysis was carried out to show the accumulation of ZnO NPs, zinc, and Se within cells of *Spirulina platensis'* algal biomass.

Multiple layers of NPs adhering to and building up on the cell surface might interfere with food uptake and put photosynthetic bacteria under physical stress (Metzler et al. 2011). The study revealed that the size of TiO_2 NPs used in this research was much smaller than the pore diameter (ranging from 5 to 20 nm) in the cell walls of the microalgae (Navarro et al. 2008). Furthermore, it is suggested that most TiO_2 NPs adhered to the surface of *H. pluvialis*. It was noted that the physical barrier created by the NPs' surface attachment to the cells prevented the development of microorganisms that use sunlight for photosynthesis. Hazeem et al.(2016) suggested that when NPs adhere to the surface of cells and cluster together, it could cause physical harm and lead to changes in the cellular metabolic processes. These changes may restrict growth, lower biomass concentration, and affect photosynthetic compound production (Metzler et al. 2011).

To explore TiO_2 NPs' influence on the biomass of *H. pluvialis*, the changes in the algal biomass of *H. pluvialis* before and after exposure to TiO_2 NPs over 96 hours were evaluated using spectrophotometric techniques. Contradictory to our results, a previous study reported lesser impact that *H. pluvialis* biomass decreased by 18.1% on day 9 after being exposed to 100 µg/mL TiO_2 NPs. This could be due to the small size of the nanoparticles used in our research (Comotto et al. 2014). The toxicity of nanoparticles increases as their size decreases. According to a study by Xia et al. (2015), both bulk and nano titanium particles limited the growth of *Nitzschia closterium*. The calculated EC_{50} values were 88.78 ± 6.43 and 118.80 ± 12.78 mg/L for 21 and 60 nm TiO_2 NPs, respectively. That study also revealed that after exposing the cells for 96 hours, the 21nm TiO_2 NPs induced more toxicity than 60nm TiO_2 NPs and bulk particles of titanium. The cytotoxicity of TiO_2 NPs may also be influenced by their size, which is a factor in the study. The research conducted by Sendra et al. (2017) demonstrated that smaller Ag NPs had a greater inhibitory effect on the growth of *Chlamydomonas reinhardtii*. *C. reinhardtii* demonstrated over 50% growth suppression after 72 hours when subjected to 10 g/L of 4.5 and 16.7 nm Ag NPs, and the EC_{50} for Ag NPs was determined to be 10 g/L. Conversely, Ag NPs sized at 46.7 nm had an EC_{50} value of > 300 g/L. The growth of *H. pluvialis* is impeded by ZnO NPs, leading to declining algal biomass and cell viability. When *H. pluvialis* was exposed to different concentrations of ZnO NPs from 10 to 200 µg/mL for 72 hours, the microalgae's biomass concentration and cell viability reduced significantly. The extreme growth inhibition was seen at 96 hrs when 200 µg/mL of ZnO NPs resulted in a 49% drop in algal biomass and a 52% drop in cell viability compared to the control. Additionally, after 15 days of exposure, 100 µg/mL of 14 nm TiO_2 NPs reduced the biomass content of *S. platensis* by 74% (Comotto et al. 2014).

The form, shape, concentration, surface charge, and surface properties of nanoparticles exposed on microalgae may impact how quickly they grow (Cepoi et al. 2020). Many studies hypothesized that the formation of reactive oxygen species (ROS)(Djearamane et al. 2020; Suman et al. 2015; Xia et al. 2015) or mechanical damage to the cells produced by NPs was the cause of the reduction of cell development (Castro-Bugallo et al. 2014). Some factors cause the reduction in cell growth, for example, metal ion release (Lee and An 2013; Aravantinou et al. 2015; Suman et al. 2015), the effect of light shading (Sadiq et al. 2011), synergistic effects (Manzo et al. 2013) and also contact with the culture medium (Manier et al. 2013). One of the causes of growth inhibition is the degradation of cell membranes caused by exposure to NPs, which results in the unregulated release and absorption of electrolytes and consequently affect the photosynthetic system and the production of macronutrients (Anusha et al. 2017). The growth rate of microalgae *H. pluvialis* was affected when the metal ions interacted with the functional group of the microalgae surface (Balaji et al. 2014).

4.1 Limitations and moving forward

The current study did not report on the influence of TiO_2 NPs on the nutritional properties of *H. pluvialis*, including macromolecules and pigments. In the future, research can be performed to ascertain the impact of TiO_2 NPs on the nutritional properties of *H. pluvialis*. Further in-depth analysis is deemed essential to enhance the knowledge about the adverse effects of MNPs in aquatic environments that are used for commercial growth and cultivation of microalgae. It is also necessary to further create public awareness of using nanotechnologies to regulate commercial products that include nanomaterials in their production, as they may impede the ecological imbalance, directly and indirectly deteriorating human wellness. Furthermore, genomic studies can be carried out to analyze the impact of NPs on the synthesis of macromolecules and pigments.

Conclusion

To conclude, exposure of *H. pluvialis* to TiO_2 NPs resulted in a concentration and time-dependent buildup of NPs in the microalgae cells, leading to detrimental effects on the algal cells and biomass and severely damaging the cell surfaces and interiors. The findings of this research can assist in developing methods to detect TiO_2 NP contamination in *H. pluvialis*, enabling the production of high-quality nutritional supplements with uncompromised nutritional properties. Failure to do so may result in the consumption of TiO_2 NP-contaminated *H. pluvialis* supplements, which may not provide the intended nutritional benefits and may even pose health risks.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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