Full Length Research Paper

Toxic effect of nano and bulk TiO₂ on growth, chlorophyll a content and oxidative stress of marine diatom *Chaetoceros muelleri*

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Received 10 February 2020; Accepted 17 March 2020

Abstract. Nanoparticles are widely used in many applications. The recent increase in nanomaterials usage has led to raising concerns about its health risks and environmental impact. In this study, we examined the toxicity effects of nanoparticles TiO₂ and bulk Ti on marine diatom *Chaetoceros muelleri*. We treated microalgae with equal amounts of nano and bulk TiO₂ in different concentrations include 0, 10, 50, 100, 200 and 300 ppm, then was investigated their growth in 96 h. Observations demonstrated growth inhibition in 50 mg/L from first exposure day for nano and bulk TiO₂. Furthermore, the chlorophyll a content strongly decreased in nano and bulk in TiO₂ in different days rather than control, respectively. As well as, results showed that, antioxidant values in nano-treated samples were higher than bulk in comparison control. In addition, we found that nano was more toxic than bulk. We aimed, indicating critical impacts of nTiO₂ on the ecosystem.

Keywords: nano TiO₂; bulk Ti; chlorophyll a content; growth inhibition; ROS production

1. INTRODUCTION

Nanomaterials are particles which their sizes are between 1 and 100 nanometers (Klaine et al., 2008). Nanomaterials are being increasingly used for commercial purposes such as fillers, catalysts, semiconductors, cosmetics, microelectronics, pharmaceuticals, and etc. (Farré et al., 2009). Nanomaterials properties are different compared with their parent compounds because of the atoms which are on the nanoparticles (NPs) surface, resulting in higher reactivity than bulk materials and provide areas which may bind and transport toxic chemical pollutants, as well as there is their being the toxic possibility by generating reactive radicals. Therefore, it is expected that NPs will have different biological effects than parent compounds (Oberdörster et al., 2005; Elder et al., 2007). Since oxide nanoparticles are among the most used nanomaterials due to their potential ecological effects, much attention was focused on the effects of nanomaterials on organisms in the aquatic system such as nano-TiO₂ (Klaine et al., 2008; Hewakuruppu et al., 2013). As the results showed the indirect effects of NPs, they are caused mainly by the physical restraints or the release of toxic ions (e.g., metal NPs) or ROS production, and the aggregation of NPs on the photosynthetic organism's surface may inhibit photosynthetic activity because of shading effects (i.e., reduced light availability) (Chen and Elimelech, 2007). Different studies also were illustrated Nano TiO₂ has an algal growth inhibition (Navarro et al., 2008a). As the algae are the primary producers in the aquatic environments and are widely spread in coastal ecosystems (Behrenfeld et al., 2006) also, their growth inhibition can be influenced by the effects of pollutants thus, in the aquatic risk assessment, algal growth inhibition assay is widely employed (Blinova, 2004; Navarro et al., 2008b). The algae play a considerable role in the aquatic ecosystems, not only producing biomass and organizing the basic nutrition for food chains but also contributing to the self-purification of polluted water. Therefore, the alga is one of the commonly used model organism for the toxicity test of toxins and nanoparticles as well (Ji et al., 2011).

2. MATERIALS AND METHODS

2.1. Materials preparation

Powdered TiO₂ nanoparticle (99.9% purity) with particle size 22 nm was purchased from Company pioneers of Iranian nanomaterials (nano-second). Bulk Ti was purchased from Razi Metallurgical Research Center. The preparing stock suspensions of nano and bulk TiO₂ were carried out by adding distilled water to nano and bulk TiO₂ powders and then, were dispersed by ultrasonicated for 30 mins then homogenized by vortex for 5 minutes. Finally, test suspensions were added to the f/2 medium (nano and bulk TiO₂: 0, 10, 50, 100, 200 and 300 mg/L).

2.2. Algae culture preparation

C. muelleri was purchased from the Persian Gulf and Oman Sea Ecology Research Institute, Iran. *C. muelleri* was cultured in f/2 medium in 500-mL Erlenmeyer flasks which all jars have been autoclaved before each examination. These flasks contained 100-mL algal stock. All of the flasks were placed on a transparent table that were shaken from below and constantly illuminated (12 h light:12 h dark) with cool white fluorescent light at 20-25 °C (Yap et al., 2004; López-Ruiz et al., 1995; Aruoja et al., 2009; Cardinale et al., 2012).

2.3. Toxicity tests

To determine the toxicity effects, the algae was 105 cells/ml with initial concentration, were treated with TiO₂ nano and bulk at selected concentrations (0, 10, 50, 100, 200 and 300 mg/L) in jars 250 ml which contain 100 ml algae. All of the culture mediums were placed on the same transparent table as mentioned above. Algal growth due to determining of IC₅₀ was measured daily to 96 h by hemocytometer under a microscope. All of the experiments were carried out in triplicate. In next phase (after determination of IC₅₀), again we exposed algae with suspensions in marked concentration (50 mg/L) for 15 days and monitored growth inhibition daily (Wang et al., 2016; Aruoja et al., 2009).

2.4. Chlorophyll a content measurement

Briefly, the algae were exposed to marked concentration of substances and their extractions which was added acetone 99% after centrifugation (15 minutes and 6000 rpm). Due to measuring chlorophylls content at third, sixth and ninth days, the absorption was measured at 630 and 664 nm using by UV-Vis spectrophotometer for chlorophyll a and c, respectively (as mentioned above). Then it was calculated according to the standard method and the equation was mentioned above (Wang et al., 2016; Aruoja et al., 2009; Cardinale et al., 2012).

2.5. Oxidative stress

The samples (treated and controls) prepared for antioxidant measurement after centrifugation (15 minutes and 6000 rpm) in 3rd, 6th and 9th day of exposure time. Then, added solutions to samples and incubated (20 min, 50 °C). Finally, after vortex for 2 minutes, the absorption samples measured by spectrophotometer in wavelength 700 nm (ascorbic acid was considered as standard) (Oyaizu, 1986; Saranya et al., 2014).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of the differences between values with 95% confidence intervals and the level of significance was accepted at $P \leq 0.05$. In the charts, values are shown as mean $\pm$ standard deviation of three independent experiments and in the table. To calculate standard deviations and draw tables and charts were used for Microsoft Excel 2016.

3. RESULTS AND DISCUSSION

3.1. Shading effect

Algal cell counts of control sample were calculated as presented in the Figs 1 and 2. The suspensions of nano and bulk TiO₂ may inhibit algal growth by avoiding of reaching light to cells for photosynthesis. As shown in Figs 3 and 4, cell counts descended in dose 50 ml/L in the first step and then, also decreased from fifth day in second step.

3.2. Effect of nano and bulk TiO₂ on algal growth

Growth parameters were negatively affected by concentrations (50 ppm) of nano TiO₂ and bulk Ti during 2 steps of experiments. At first step, the mortality rate increased to 50% for bulk Ti (Fig 6) and more for nTiO₂ (Fig 5) after 24 h of in microalgae in order to determine IC₅₀ during 96 h.

Figure 1. Imaging of cells of control samples of optical microscope (x10)

Figure 2. Imaging of cells of control samples of optical microscope (x250)

Figure 3. Cell counts of exposure of nano TiO_2 with control in 96 h in different concentrations

Figure 4. Cell counts of exposure of bulk Ti with control in 96 h in different concentrations

The growth inhibition rate was 0.42 and 0.85 in 50 mg/L for nano and bulk, respectively. While growth rate in control samples was 1.52 at the first day. However, reduction of growth was found negligible in concentrations of 10, 100 and 200 mg/L but in 300 ppm we observed an increasing trend during the 96 h that was out of our expectation whereas most of the previous researches demonstrated a relatively high level of toxicity with EC_{50} values about at high concentrations (Figs 5 and 6). This may be related to the formation of larger aggregates in the algal culture medium which may not penetrate to algal cells inside that resulted to create a lower opportunity for the collision between nano and bulk TiO_2 with algae cells. In addition to the above 4-day experiments, a 15-day-long observation term (second step) was also performed for 50 mg/L groups for nano and bulk TiO_2 in comparison with the control group. The growth inhibition rate of nano and bulk TiO_2 was measured each day, (as presented in Fig 7).

These findings were similar to other studies (Cardinale et al., 2012; Wang et al., 2016; Movafeghi et al., 2018; Manzo et al., 2015; Chen et al., 2015, 2012). Toxicity mechanism was performed by covered cell surfaces by the aggregates can induce a certain inhibition of the photosynthetic activity due to the reduction of the light availability (Navarro et al., 2008a). On the other hand, the alteration of the cell membrane due to the cell entrapment may affect cellular uptake of nutrients and energy transduction mechanisms (related to ATP synthesis) (Yeung et al., 2009; Hartmann et al., 2010).

Figure 5. Growth in first step of exposure to nTiO₂ with control in 96h in different concentrations

Figure 6. Growth in first step of exposure to bulk Ti with control in 96h in different concentrations

Figure 7. Growth in second step of exposure to nano and bulk TiO₂ with control in 15 days

3.3. Effects on chlorophyll a content

Actually, the content of photosynthetic pigments such as chlorophyll a is one of the most sensitive and efficient indicators of toxicity and grow status algae (Li et al., 2015). As shown in Fig. 8, chlorophyll a content. Chlorophyll a content, after 9 days treatment of nano and bulk TiO₂, at the concentration of 50 mg/L in comparison with control groups were measured. Measured chlorophyll a content from the 3rd day of exposure to 9th day in two materials had reduction trend. The reduction of chlorophyll a content was to 14%, 75% and 86% in third, sixth and ninth of exposure for nano TiO₂, respectively, as well as for bulk Ti, was observed no changing in chlorophyll a content after 3day of exposure but in 6 and 9 days reduced to 55% and 65% , respectively. The results determined, chlorophyll a content in samples exposed to nanoTiO₂ was significant statistically ($P < 0.05$) while, in the samples were exposed to bulk TiO₂ showed lower toxicity ($P < 0.05$).

Indirect physical effects, such as shading effects, were not investigated directly in this research. However, a reduction of chlorophyll a due to the presence of nano and bulk TiO₂ exposure was found, which might partly indicate the occurrence of shading effects (Wang et al., 2016; Aruoja et al., 2009). Many researchers believe that wrapped cells by NPs or the adsorption of NPs led to prevent of reaching to algal cells, consequently leading to growth inhibition (Aruoja et al., 2009; Chen et al., 2012; Xia et al., 2015).

Figure 8. Chlorophyll a content in 9 days of exposure of nano and bulk TiO₂ with control

3.4. ROS production and oxidative stress

As the results showed, nano and bulk TiO₂ aroused more active of the antioxidant defence mechanism and ultimately produce more antioxidants by cells to protect and defend cells and prevent oxidative stress by exposing algae cells to nano and bulk TiO₂ in different days and concentrations (Figures 9 to 11). Although, the amount of antioxidants production in the algae exposed to nano TiO₂ were more than bulk ($P < 0.05$). These results are also observed in other research which was conducted by Ghorbanpour et al. (2015). The close interaction between aggregates and cell membranes induced an oxidative stress as ROS production (Hartmann et al., 2010) that actually provoked damages of the algal cell exposed to 50 mg L⁻¹ of nano and bulk TiO₂. Furthermore, TiO₂ anatase reactivity resulting in an evident cytotoxicological effects (Hartmann et al., 2010; Manzo et al., 2015, 2013; Wang et al., 2016).

Figure 9. Amount of produced antioxidants of samples of samples in different concentrations in 3rd exposure time

Figure 10. Amount of produced antioxidants samples indifferent concentrations in 6th exposure time

The mechanism of TiO₂ toxicity seems mainly in association with ROS production, in response to the particles interaction with algal cells and in some cases to their internalization and aggregation in the chloroplast as well as due to the TiO₂ crystalline structure (Xia et al., 2015, Li et al., 2015; Iswarya et al., 2015).

Briefly, this is one of the first systematic studies on the effects of metal oxide nanoparticles and its bulk structure on algal growth in 2 steps and this is first study of nanotoxicology on *Chaetoceros* sp. As the food chain is an important way for NPs transmission to a high level of the food chain which organisms are absorbing and accumulating nanomaterials so we must be focused on NPS effects on the aquatic organisms.

Figure 11. Amount of produced antioxidants of samples in different concentrations in 9th exposure time

1. NMs= Nanomaterials, 2. NPs= Nanoparticles, 3. ROS= Reaction/Reactive Oxygen Species, 4. OD= Optical Density, 5. IC50= 50% Inhibition Concentration, 6. EC50= 50% Effect Concentration,

4. CONCLUSION

To our knowledge, this is one of the first systematic studies on effects of metal oxide nanoparticles and its bulk structure on algal growth in 2 steps and this is first study of nanotoxicology on *Chaetoceros* sp. In conclusion, released nTiO₂ into the marine environment will definitely accumulate and tend to settle to the bottom, but there is still a significant fraction of NPs which may stay in the water column and lead to risk to the health of the organisms living in the water column. In our investigation, the growth of the marine phytoplankton *C. muelleri* was determined to be inhibited growth and reduced chlorophyll a content by nano and bulk TiO₂. In addition to the nTiO₂ concentration, the exposure time maybe another prominent factor that impacts its effects. It was interesting to figure out that effects of nano and bulk TiO₂ were “high” on the first day in first step and 7th days in second step of exposure and then, with passing the time of exposure, it decreased gradually to a relatively “low” level, showing “high-low” two-step effects. The results suggested that although low toxicity in other concentrations was defined, the severe effects that occur on the first day of exposure should not be disregarded. Observed toxicity might be linked to the formation of large aggregates that trap algae cells and/or the adsorption of nTiO₂ on the surface of algae cells. Although direct physical effects such as cell wall damage was not studied directly, which was positively correlated to the growth inhibition resulted in algae entrapment. This conclusion did not exclude the actions of indirect physical effects such as shading effects. The other mechanisms such as the ratio of nTiO₂ to alga cells should be more attention.

In case of TiO₂, entrapped algal cells by aggregates of nano TiO₂ and bulk Ti may play a major role in toxicity. Lastly, other studies again demonstrate that solubility is a key factor in the toxicity of metal containing (nano) particles, at least for organisms. Thus, the solubility properties reported as N/A (not available or not applicable) in MSDS (Material Safety Data Sheet).

Acknowledgements: Authors are grateful to Persian Gulf and Oman Sea ecology Research Institute, Bandar-abbas, Iran for their help to prepare algal stocks and partly works.

Conflict of Interest: Authors declare that there is no conflict of interest.

REFERENCES

- Aruoja, V., Dubourguier, H.C., Kasemets, K., Kahru, A. (2009). Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*. Science of The Total Environment, 407(4), 1461-1468. <https://doi.org/10.1016/j.scitotenv.2008.10.053>
- Behrenfeld, M.J., O'Malley, R.T., Siegel, D.A., McClain, C.R., Sarmiento, J.L., Feldman, G.C., Boss, E.S. (2006). Climate-driven trends in contemporary ocean productivity. Nature, 444(7120), 752-755. <https://doi.org/10.1038/nature05317>
- Blinova, I. (2004). Use of freshwater algae and duckweeds for phytotoxicity testing. Environmental Toxicology, 19(4), 425-428. <https://doi.org/10.1002/tox.20042>
- Cardinale, B.J., Bier, R., Kwan, C. (2012). Effects of TiO₂ nanoparticles on the growth and metabolism of three species of freshwater algae. Journal of Nanoparticle Research, 14(8), 913. <https://doi.org/10.1007/s11051-012-0913-6>
- Chen, J., Li, H., Han, X., Wei, X. (2015). Transmission and Accumulation of Nano-TiO₂ in a 2-step food chain (*Scenedesmus obliquus* to *Daphnia magna*). Bulletin of Environmental Contamination and Toxicology, 95(2), 145-149. <https://doi.org/10.1007/s00128-015-1580-y>
- Chen, K.L., Elimelech, M. (2007). Influence of humic acid on the aggregation kinetics of fullerene (C60) nanoparticles in monovalent and divalent electrolyte solutions. Journal of Colloid and Interface Science, 309(1), 126-134. <https://doi.org/10.1016/j.jcis.2007.01.074>
- Chen, L., Zhou, L., Liu, Y., Deng, S., Wu, H., Wang, G. (2012). Toxicological effects of nanometer titanium dioxide (nano-TiO₂) on *Chlamydomonas reinhardtii*. Ecotoxicology and Environmental Safety, 84, 155-162. <https://doi.org/10.1016/j.ecoenv.2012.07.019>
- Elder, A., Yang, H., Gwiazda, R., Teng, X., Thurston, S., He, H., Oberdörster, G. (2007). Testing nanomaterials of unknown toxicity: an example based on platinum nanoparticles of different shapes. Advanced Materials, 19(20), 3124-3129. <https://doi.org/10.1002/adma.200701962>
- Farré, M., Gajda-Schrantz, K., Kantiani, L., Barceló, D. (2009). Ecotoxicity and analysis of nanomaterials in the aquatic environment. Analytical and Bioanalytical Chemistry, 393(1), 81-95. <https://doi.org/10.1007/s00216-008-2458-1>
- Ghorbanpour, M., Hatami, M., Hatami, M. (2015). Activating antioxidant enzymes, hyoscyamine and scopolamine biosynthesis of *Hyoscyamus niger* L. plants with nano-sized titanium dioxide and bulk application. Acta Agriculturae Slovenica., 105(1), 23-32. <http://dx.doi.org/10.14720/aas.2015.105.1.03>
- Hartmann, N.B., Von der Kammer, F., Hofmann, T., Baalousha, M., Ottofuelling, S., Baun, A. (2010). Algal testing of titanium dioxide nanoparticles—testing considerations, inhibitory effects and modification of cadmium bioavailability. Toxicology, 269(2), 190-197. <https://doi.org/10.1016/j.tox.2009.08.008>
- Hewakuruppu, Y.L., Dombrovsky, L.A., Chen, C., Timchenko, V., Jiang, X., Baek, S., Taylor, R.A. (2013). Plasmonic “pump–probe” method to study semi-transparent nanofluids. Applied Optics, 52(24), 6041-6050. <https://doi.org/10.1364/AO.52.006041>
- Iswarya, V., Bhuvaneshwari, M., Alex, S.A., Iyer, S., Chaudhuri, G., Chandrasekaran, P.T., Mukherjee, A. (2015). Combined toxicity of two crystalline phases (anatase and rutile) of Titania nanoparticles towards freshwater microalgae: *Chlorella* sp. Aquatic Toxicology, 161, 154-169. <https://doi.org/10.1016/j.aquatox.2015.02.006>
- Ji, J., Long, Z., Lin, D. (2011). Toxicity of oxide nanoparticles to the green algae *Chlorella* sp. Chemical Engineering Journal, 170(2), 525-530. <https://doi.org/10.1016/j.cej.2010.11.026>
- Klaine, S.J., Alvarez, P.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Lead, J.R. (2008). Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry, 27(9), 1825-1851. <https://doi.org/10.1897/08-090.1>
- Li, F., Liang, Z., Zheng, X., Zhao, W., Wu, M., Wang, Z. (2015). Toxicity of nano-TiO₂ on algae and the site of reactive oxygen species production. Aquatic Toxicology, 158, 1-13. <https://doi.org/10.1016/j.aquatox.2014.10.014>
- López-Ruiz, J., García, R.G., Soledad, M., Almeda, F. (1995). Marine microalgae culture: *Chaetoceros gracilis* with zeolitic product Zestec-56 and a commercial fertilizer as a nutrient. Aquacultural Engineering, 14(4), 367-372. [https://doi.org/10.1016/0144-8609\(94\)00011-Q](https://doi.org/10.1016/0144-8609(94)00011-Q)
- Manzo, S., Buono, S., Rametta, G., Miglietta, M., Schiavo, S., Di Francia, G. (2015). The diverse toxic effect of SiO₂ and TiO₂ nanoparticles toward the marine microalgae *Dunaliella tertiolecta*. Environmental Science and Pollution Research, 22(20), 15941-15951. <https://doi.org/10.1007/s11356-015-4790-2>
- Manzo, S., Miglietta, M.L., Rametta, G., Buono, S., Di Francia, G. (2013). Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*. Science of the Total Environment, 445, 371-376. <https://doi.org/10.1016/j.scitotenv.2012.12.051>

- Movafeghi, A., Khataee, A., Abedi, M., Tarrahi, R., Dadpour, M., Vafaei, F. (2018). Effects of TiO₂ nanoparticles on the aquatic plant *Spirodela polyrrhiza*: Evaluation of growth parameters, pigment contents and antioxidant enzyme activities. *Journal of Environmental Sciences.*, 64, 130-138. <https://doi.org/10.1016/j.jes.2016.12.020>
- Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Sigg, L. (2008a). Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology*, 17(5), 372-386. <https://doi.org/10.1007/s10646-008-0214-0>
- Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Behra, R. (2008b). Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. *Environmental Science and Technology*, 42(23), 8959-8964. <https://doi.org/10.1021/es801785m>
- Oberdörster, G., Oberdörster, E., Oberdörster, J. (2005). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives*, 113(7), 823-839. <https://doi.org/10.1289/ehp.7339>
- Oyaizu, M. (1986). Studies on products of browning reaction. *The Japanese Journal of Nutrition and Dietetics*, 44(6), 307-315. <https://doi.org/10.5264/eiyogakuzashi.44.307>
- Saranya, C., Hemalatha, A., Parthiban, C., Anantharaman, P. (2014). Evaluation of antioxidant properties, total phenolic and carotenoid content of *Chaetoceros calcitrans*, *Chlorella salina* and *Isochrysis galbana*. *Int. J. Curr. Microbiol. App. Sci.*, 3(8), 365-377. [10.1007/s10811](https://doi.org/10.1007/s10811)
- Wang, Y., Zhu, X., Lao, Y., Lv, X., Tao, Y., Huang, B., Cai, Z. (2016). TiO₂ nanoparticles in the marine environment: physical effects responsible for the toxicity on algae *Phaeodactylum tricornutum*. *Science of the Total Environment*, 565, 818-826. <https://doi.org/10.1016/j.scitotenv.2016.03.164>
- Xia, B., Chen, B., Sun, X., Qu, K., Ma, F., Du, M. (2015). Interaction of TiO₂ nanoparticles with the marine microalga *Nitzschia closterium*: Growth inhibition, oxidative stress and internalization. *Science of the Total Environment*, 508, 525-533. <https://doi.org/10.1016/j.scitotenv.2014.11.066>
- Yap, C.K., Ismail, A., Omar, H., Tan, S.G (2004). Toxicities and tolerances of Cd, Cu, Pb and Zn in a primary producer (*Isochrysis galbana*) and in a primary consumer (Pernaviridis). *Environment International*, 29(8), 1097-1104. [https://doi.org/10.1016/S0160-4120\(03\)00141-7](https://doi.org/10.1016/S0160-4120(03)00141-7)
- Yeung, K.L., Leung, W.K., Yao, N., Cao, S. (2009). Reactivity and antimicrobial properties of nanostructured titanium dioxide. *Catalysis Today*, 143(3), 218-224. <https://doi.org/10.1016/j.cattod.2008.09.036>