Poll Res. 38 (2) : 479-484 (2019)

ISSN 0257–8050

CYTOTOXIC EFFECTS OF ZINC OXIDE NANOPARTICLES ON *CHLORELLA VULGARIS*

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(Received 10 January, 2019; accepted 11 March, 2019)

ABSTRACT

Nanoparticles such as zinc oxide nanoparticles (ZnO NPs) have a wide range of applications in industrial and personal care products, but, at the same time, the presence of nanoparticles causes negative impact to aquatic ecosystem and the organisms within. In this study, the cytotoxic effect of ZnO NPs on fresh water microalga *Chlorella vulgaris* is reported. *C. vulgaris* cells were treated with 10, 50, 100, 150 and 200 mg/L of ZnO NPs for 72 h. Cytotoxicity effects of ZnO NPs were assessed by measuring the fluorescence emission of chlorophyll using fluorescent spectrophotometer, algal biomass by spectrophotometer and cell viability through counting viable cells at 24, 48 and 72 h. Exposure to ZnO NPs caused a decrease in chlorophyll content, cell viability and algal biomass. The cytotoxic effects increased as the concentration and exposure duration of ZnO NPs increased. Toxicity of ZnO NPs was indicated by reduction in the chlorophyll content, cell viability and biomass. This study showed the potential of *C. vulgaris* to be a bioindicator for ZnO NPs' toxicity in aquatic environment.

KEY WORDS: Zinc oxide nanoparticles, *Chlorella vulgaris*, Bioindicator, Aquatic environment

INTRODUCTION

Nanotechnology involves manufacturing of wide varieties of nanoparticles (NPs) with a broad range of industrial applications. Zinc oxide nanoparticles (ZnO NPs) are widely used nanoparticles among the rapidly expanding list of engineered nanoparticles in industrial and commercial products (Zhou *et al.*, 2014). ZnO NPs are the most commonly utilized nanoparticles in production of pigments, semiconductors, rubber, solar cells, chemical fibers, electronic devices, sunscreens and food additives (Dastjerdi and Montazer, 2010; Song *et al.*, 2010) because of their chemical stability, adsorption ability, UV absorption capacity and antimicrobial property (Osmond and Mccall, (2010). Extensive application of ZnO NPs in consumer products results in release of these particles into the aquatic environment through sewage of the industries and leading to the concerns of their potential toxicity to human and environmental health (Zhao and Castranova, 2011). Several studies have shown that ZnO NPs are toxic to algae (Xu *et al.*, 2013), bacteria (Li *et al.*, 2013), crustaceans (Blinova *et al.*, 2010) and fish (Zhu *et al.*, 2008). The large surface area of ZnO NPs gives an advantage of high electron density and high reactivity to interact with biomolecules which contributes to high bio-toxicity. In addition to physiochemical properties, ZnO NPs can also release free zinc ions which grounds for major toxic

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effects (Pisanic *et al*., 2009). The chemical reactions during the interaction of ZnO NPs with living cells cause oxidative stress and increase formation of reactive oxygen species (ROS) (De Berardis *et al*., 2010). Since microalgae are the base for aquatic food web and more sensitive to metallic contaminants than fish and invertebrates, they are the important organisms for monitoring water quality and aquatic toxicity (Franklin *et al*., 2007). Investigating the toxic effects of ZnO NPs on algae is of greater importance and can potentially lead to strategies to assess the potential adverse effects of engineered NPs in the environment (Maynard *et al*., 2006). Hence in this study, authors have used fresh water microalga *Chlorella vulgaris* as a model organism for evaluating the toxic effects of ZnO NPs from 24 h to 72 h by investigating the variations in cell viability, algal biomass, and chlorophyll content before and after treatment with ZnO NPs.

MATERIALS AND METHODS

Establishment of Algal Culture

Fresh water microalga *C. vulgaris* was obtained from Culture Collection of Algae and Protozoa (CCAP), United Kingdom. Algal cells were cultured in sterile Bold Basal Medium (BBM) at room temperature (23 ± 1° C) in an agitator with 120 rpm under T5 fluorescent light illumination with light intensity 2400 ± 100 lux under dark and light conditions maintained for 8 and 16 h respectively.

Preparation of Zinc Oxide Nanoparticles

Zinc oxide nanoparticles of 40 to 50 nm in diameter were purchased from Zhejiang Hongsheng Material Technology Co., China. The stock solution of ZnO NPs suspensions (500 mg/L) was prepared in algal culture medium BBM and sonicated for 30 minutes at 40 kHz to avoid aggregation of NPs in the solution (Zhou *et al.,* 2014).

Experimental Treatment of Algal Cells

For this experiment, *C. vulgaris* cells were taken from a 3–day–old culture with the aim of using the cells growing in an exponential growth phase (Petrescu *et al.,* 2013) with an initial cell density of 1 x 10^6 cells/mL (Asharani *et al*., 2008). A stock solution of ZnO NPs was diluted in 125 mL flasks to serial concentrations of 10 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L with 50 mL culture medium. The cells were cultured in 100 mL BBM in 250 mL Erlenmeyer flask with the presence of ZnO NPs. *C. vulgaris* cells were exposed for 72 h to the increasing concentrations of ZnO NPs from 10 - 200 mg/L. Algal cells without the exposure to nanoparticles were considered as the control. Experimental samples were kept with intermittent shaking at 8 h interval to prevent aggregation of cells. All treatments were analyzed for cytotoxicity at the interval of 24, 48 and 72 h in 3 replicates along with control cells.

Toxicity Assessment

Toxicity of ZnO NPs on *C. vulgaris* was assessed by analyzing the percentage of loss in cell viability, algal biomass and chlorophyll fluorescence emission at 24, 48 and 72 h.

Determination of Cell Viability and Growth Inhibition of Algal Cells

In order to investigate the cytotoxic effect of ZnO NPs on *C. vulgaris*, the growth inhibitory effect was studied using increasing concentrations of ZnO NPs (10-200 mg/L). At the end of each treatment time, aliquots of algal cell suspensions (ZnO NPs treated and untreated) were loaded into the cell count chamber (Neubauer, Marienfeld-Superior, Germany). Cells were counted in all 4 large corner squares under optical microscope (Nikon, Microphot-fxt, Japan) with high power lens (40 x). The number of intact cells without any distortion in shape and size was counted as viable cells (Iswarya *et al*., 2015). The percentage of growth inhibition in treatments was calculated in respect to control cells.

Algal Biomass Determination

The algal biomass was measured as an increase in absorbance at 685 nm in spectrophotometer (GeneQuant, GE, USA) using algal culture medium as blank. Experiments also included positive control (flask containing NPs in BBM medium with no algal cells) and negative control (flask containing algal cells in culture medium with no NPs). The negative control indicated algal growth in the absence of NPs. The absorbance value of positive control was subtracted from the experimental values (flasks containing algal cells and NPs in culture medium) to avoid the interference from the absorbance of ZnO NPs (Blair *et al.,* 2014).

Measurement of Chlorophyll Emission

Inhibition in the photosynthetic activity of algal cells

when treated with nanoparticles was estimated using fluorescence emission of chlorophyll as the biomarker for nanoparticles' toxicity on the photosynthetic system of algal cells (Barhoumi and Dewez, 2013. The chlorophyll content of algal cells was determined using spectrofluorometer (GloMax Multi Jr, Pormega Biosystems, USA). The intensity of the chlorophyll fluorescence emission was measured at an excitation wavelength 430 nm and an emission wavelength of 663 nm with the algal culture medium as blank.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance of the differences between toxic effects of ZnO NPs in different concentrations. The differences were considered significant when p <0.05.

RESULTS AND DISCUSSION

Algal Growth Inhibition

The cytotoxic effect of ZnO NPs was assessed through algal growth inhibition using viable cell counts. In the test cultures, with an increasing concentration of ZnO NPs, the cell viability decreased from 24 until 72 h with a gradual increase in the percentage of inhibition of viable cells. A statistically significant ($p < 0.05$) inhibition of algal cells was observed from 24 h for all tested concentrations of ZnO NPs with the highest growth inhibition reported at 72 h. The resultant values of growth inhibition at 72 h were 14.2%, 23.8%, 35.3%, 42.0% and 61.8% for 10, 50, 100, 150 and 200 mg/L of Zn ONPs. Further, results showed a typical concentration- and time-dependent growth inhibition of algal cells as the concentration of ZnO NPs and the duration of exposure was increased. For example, the progressive growth inhibition from 6.4 %, to 14.2 % was reported from 24 h to 72 h at 10 mg/L and the growth inhibition from 32.2 % to 61.8 % occurred at 200 mg/L from 24 h to 72 h respectively. The individual toxicity of increasing concentration of ZnO NPs (10 – 200 mg/L) for various treatments (24 h - 72 h) is presented in Fig. 1.

The reduction in actual number of viable cells for different treatments is presented in Fig. 2. From the results, it was evident that the number of algal cells died was dependent on the concentration and time of exposure to ZnO NPs. The highest reduction in cell viability from 316 x $10^4$to 120.8 x 10^4 cells / mL

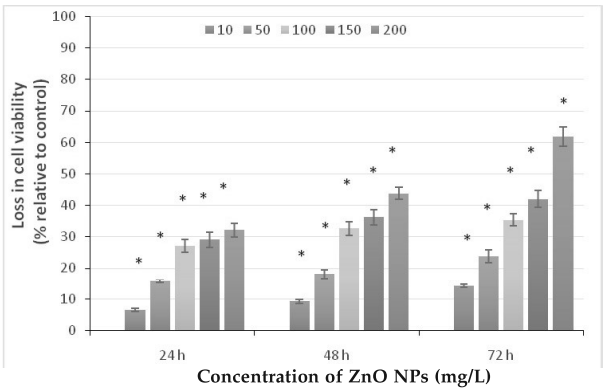

Fig. 1. Time and concentration dependent loss in cell viability of algal cells by the increasing concentrations of ZnO NPs from 10 to 200 mg/L for 24 h, 48 h and 72 h. * refers the significant difference at $p < 0.05$.

was found at 200 mg/L for 72 h. A similar phenomenon was reported by Tang *et al.*, (2013) and Suman *et al.*, (2015) that ZnO NPs exhibited concentration- and time-dependent cytotoxicity on cyanobacterium *Anabaena* sp. and *C. vulgaris* respectively. Lee and An, (2013) reported the growth inhibition of ZnO NPs on freshwater alga *Pseudokirchneriella subcapitata* and specified that the toxicity was solely due to release of Zn^+ from ZnO NPs. In addition, Manzo *et al.* (2013) revealed the growth inhibitory effect of ZnO NPs on marine alga *Dunaliella tertiolecta.*

Reduction in Algal Biomass

The significant reduction in algal biomass upon treatments with ZnO NPs was observed as shown in Fig. 3. A typical concentration- and time-dependent inhibitory effect of ZnO NPs on *C. vulgaris* was reported with a higher inhibitory value of 56.24 % at 200 mg/L for a prolonged exposure time of 72 h.

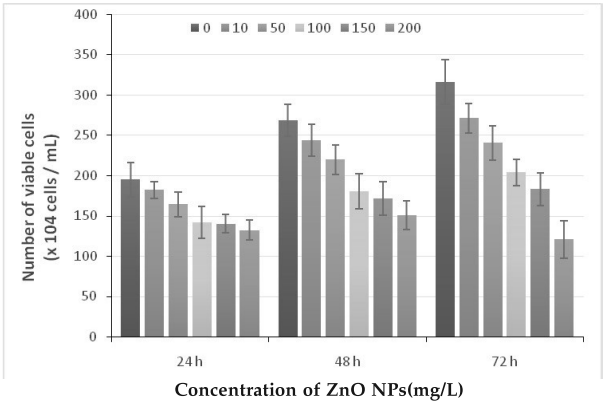

Fig. 2. Number of viable cells under various treatments with ZnO NPs for 24 h, 48 h and 72 h.

Previous studies have reported the dose- and time-dependent reduction in biomass of *C. vulgaris* using titanium dioxide NPs (Comotto *et al.,* 2014; Iswarya *et al*., 2015; Ji *et al*., 2011). Further, Sadiq *et al*., (2011)] demonstrated a significant reduction in biomass of *Chlorella sp*. by aluminum oxide NPs' toxicity.

Reduction in Chlorophyll Fluorescence Emission

The chlorophyll content of algal cells was assessed by the fluorescence emission of the chlorophyll. A

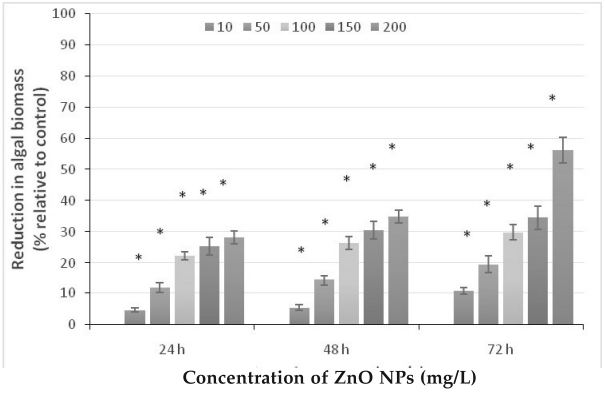

Fig. 3. Percentage of reduction in algal biomass with the increasing concentrations of ZnO NPs from 10–200 mg/L for 24 h, 48 h and 72 h. * refers the significant difference at $p < 0.05$.

concentration- and time-dependent decrease in the chlorophyll emission was noted, which confirmed the growth inhibitory effect of ZnO NPs with the increasing concentration and exposure duration of ZnO NPs as shown in Fig 4. The strongest effect on photosynthetic system was reported at 200 mg/L with the percentage of reduction in chlorophyll emission 39.0 %, 46.6 % and 68.2 % for 24, 48 and 72 h respectively. Similar findings were reported by Barhoumi and Dewez, (2013) and Iswarya *et al.* (2015) where the authors reported the strongest effect of iron oxide and titanium dioxide NPs respectively on photosynthetic electron transport chain of *C. vulgaris* with reduction in chlorophyll content of the algal cells. Furthermore, the authors have also recommended that the chlorophyll emission measurement can be used as biomarker for ecotoxicological assessment of NPs toxicity on *C. vulgaris* (Barhoumi and Dewez, 2013). Moreover, a study by Gong *et al.* (2011) reported a gradual decrease in chlorophyll content with increasing concentrations of nickel oxide NPs on *C. vulgaris*.

The suspension of nanoparticles can directly play a role in the growth inhibitory effect by occupying the surface of the algal cells and decreasing the

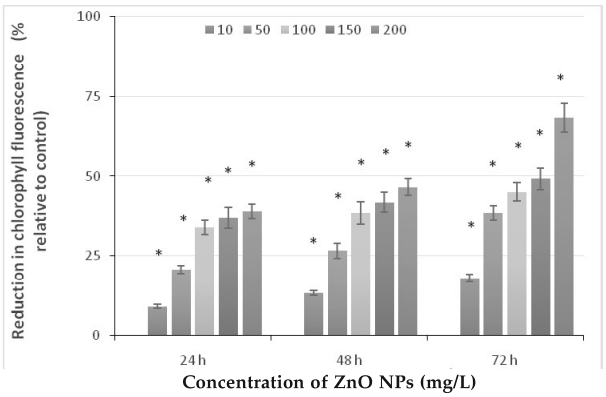

Fig. 4. Percentage of reduction in chlorophyll emission with the increasing concentrations of ZnO NPs from 10–200 mg/L for 24 h, 48 h and 72 h. * refers the significant difference at $p < 0.05$.

amount of light reaching the cells, and thus causing inhibition of photosynthetic activity (Navarro *et al.,* 2008) which results in growth inhibitory effect. Effective absorption of nanoparticles due to its large surface area can trigger greater growth inhibitory effect compared to micro-sized particles (Sadiq *et al*., 2011). Penetration of ZnO NPs into the cell envelope causes disruption of algal cell membrane which attributes to the cell growth inhibition and also the aggregation of ZnO NPs on algal cells could mechanically damage cell walls and membranes resulting in release of cellular contents into the extracellular space leading to cell death (Lin *et al*., 2009) which eventually result in decreased cell density and biomass. Algal cells develop physiological stress due to the toxicity of NPs and results in production of free radicals which in turn induce the formation of reactive oxygen species. The ROS could impair the photosynthetic system II activity leading to decrease in chlorophyll content or emission. The parameters based on fluorescence yield have been proposed to be a useful tool for the toxic evaluation of pollutants (Tang *et al.,* 2013).

CONCLUSION

In our study, we used cell viability and photosynthetic fluorescence as biomarkers to characterize the toxicity of ZnO NPs on *C. vulgaris* cells. Reduction in cell viability, biomass and the chlorophyll fluorescence related to photochemical reactions evidenced the potential source of cellular toxicity and proven to be sensitive biomarkers for ZnO NPs toxicity on *C. vulgaris*. This study showed the potential of *C. vulgaris* as the prospective

bioindicator for ZnO NPs toxicity with viable cell count, algal biomass and chlorophyll fluorescence emission as the biomarkers for toxicity testing.

ACKNOWLEDGEMENT

This research work was funded by Ministry of Education, Malaysia (Grant No. FRGS-1-2014-SG03-INTI-02-1) and Universiti Tunku Abdul Rahman Research Fund (UTARRF), (Grant No. IPSR/RMC/UTARRF/2016-C1/L3).

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