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RESEARCH ARTICLE

Toxicity assessment of ZnO nanoparticles to freshwater microalgae *Coelastrella terrestris*

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Abstract

Commercial usage of ZnO nanoparticles has increased recently due to its versatile applications, raising serious environmental concern because of its ultimate release of nanoparticles in aquatic ecosystem. Therefore, it is important to understand the impact of ZnO nanoparticle toxicity especially on algal flora, which is the primary producer in the aquatic food chain. In the current study, algal growth kinetics was assessed after the exposure of zinc oxide nanoparticles and its bulk counterpart to *Coelastrella terrestris* (Chlorophyceae). Zinc oxide nanoparticles were found to be more toxic ($y = 34.673x$, $R^2 = -0.101$, 1 mg L^{-1} nanoparticle (NP)) than bulk ($y = 50.635x$, $R^2 = 0.173$, 1 mg L^{-1} bulk) by entrapping the algal cell surface. Higher toxicity may be due to oxidative stress within the algal cell as confirmed through biochemical analysis. Biochemical parameters revealed stressful physiological condition in the alga under nanoparticle exposure, as lactate dehydrogenase release (18.89 ± 0.2 NP; 13.67 ± 0.2 bulk), lipid peroxidation (0.9147 ± 1.2 NP; 0.7480 ± 0.8 bulk), and catalase activity (4.77 ± 0.1 NP; 3.32 ± 0.1 bulk) were found higher at 1 mg L^{-1} in the case of nano-form. Surface adsorptions of nanoparticles were observed by SEM. Cell organelle damage, cell wall breakage, and cytoplasm shrinkage were found as responses under toxic condition through SEM and TEM. Toxicity was found to be influenced by dose concentration and exposure period. This study indicates that nano-form of ZnO is found to be more toxic than bulk form to freshwater alga.

Keywords Algae · Stress · Toxicological effects · TEM · Zinc oxide nanoparticles

Abbreviations

ANOVA	Analysis of variance
BG-11	Blue Green-11
BSA	Bovine serum albumin
CAT	Catalase
CDH	Central drug house
FTIR	Fourier-transform infrared spectroscopy
LDH	Lactate dehydrogenase
MDA	Malondialdehyde assay
NADH	Nicotinamide adenine dinucleotide hydrogen
NCBI	National Center for Biotechnology Information
NP	Nanoparticle
PBS	Phosphate-buffered saline
PDI	Polydispersity index
SD	Standard deviation
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
UV	Ultraviolet
ZnO	Zinc oxide

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Introduction

Globally, ZnO nanoparticle is the third highest annually produced nanoparticles (550 tons per year) after silica dioxide and titanium dioxide, respectively (Piccinno et al. 2012). ZnO nanoparticles possess exotic piezoelectric and pyroelectric properties, a wide bandgap, high exciton binding energy, and wurtzite structure lacking the center of symmetry; these properties make them unique and suitable for versatile usage (Wang 2004). A comprehensive range of ZnO nanoparticle

applications covers almost every mainstream sector of mankind needs like the following: as nano-fertilizers and as other nano-agrochemicals in agriculture (Raliya et al. 2017); as UV filter/absorber in textiles and cosmetics (Lu et al. 2015); as nano-photocatalyst for wastewater treatment and in electronics (Sharma et al. 2017); as nano-composite film in the food industry for packaging purposes (Ejaz et al. 2018); and as an antimicrobial agent (Alswat et al. 2016) and biosensors (Lupan et al. 2016) in pharmaceuticals for targeted drug delivery (Rasmussen et al. 2010; Ghaffari et al. 2017) and even for other biomedical purposes (Mishra et al. 2017). From these nano-based products, ZnO nanoparticles will eventually get released into the environment and ultimately sink into our aquatic bodies (Wang et al. 2018). Even models predicting the environmental fate of nanoparticles concluded the necessity to evaluate the toxicity of nanoparticles including the aquatic flora and fauna (Sun et al. 2016). For that reason, it is very imperative to assess the impact of ZnO nanoparticles on aquatic ecosystems. As alga is the primary source of food in aquatic ecosystem, any sort of damage to algal flora could lead to the disturbance in the complete food chain and ultimately to the aquatic ecosystem (Nowack and Bucheli 2007). Alga is the integral component of the aquatic ecosystem and being the primary producer, its impact would affect the whole food chain of the aquatic ecosystem; therefore, algae are the ideal model organisms to examine the effect of nanoparticles (Cattaneo 2018; Espinasse et al. 2018).

Earlier studies reported that a ZnO nanoparticle concentration between 0.06 and 100 mg L^{-1} imparts toxicity to most of the algae (Franklin et al. 2007; Miao et al. 2010; Chen et al. 2012; Li et al. 2017; Bhuvaneshwari et al. 2018). The reported toxicity is found different regarding the different species, particle nature, and test methods (Merdzan et al. 2014). ZnO nanoparticles are kinetically very active and undergo numerous transformations rapidly that prominently influence itsThe two prime factors imparting toxicity on the basis of earlier toxicological impact. The two prime factors imparting toxicity on the basis of earlier studies were dissolution and aggregation (Chen et al. 2012; Ma et al. 2013). Reactive oxygen generation production is also thought to be an important factor in mediating toxic responses (Klaine et al. 2008; Fu et al. 2014). Reduction in photosynthetic pigment (Hazeem et al. 2016), algal growth inhibition (Manzo et al. 2013; Li et al. 2017), and lipid peroxidation (Ji et al. 2011; Suman et al. 2015) as responses to ZnO nanoparticles have also been reported earlier. But the straightforward relationships between these factors, responses, and exposed organism have not yet been established. This avenue is further being explored to understand the overall impact of ZnO nanoparticles to the algal physiology. The purpose of the study is to monitor the ever-lasting impact of ZnO nanoparticles on *Coelastrella terrestris* which could help us predict the role of algae in aquatic nano-ecotoxicology. In the present study, we comparatively assessed the effect of ZnO nanoparticles and bulk form on *Coelastrella terrestris* on growth kinetics. We have monitored the dynamics of toxicity over a period of 25 days; therefore, this is relatively a long-term study exploring the impact of ZnO exposure on the life cycle of *Coelastrella terrestris.* We have tried to establish a straightforward relationship between the dose concentration and the number of exposure days by estimating IC_{50}. Overall changes bought by the inclusion of ZnO in nano- and bulk form during the growth of the algae are monitored and the metabolic, physiological, and morphological changes under the treatment during the growth phases are assessed.

Material and methods

Algal cultures

Axenic culture of *Coelastrella terrestris* (NCBI accession number: MK294227.1) has been established in lab-made BG-11 media (pH 7.4; Rippka et al. 1979) in 250-mL Erlenmeyer flasks and cultures were kept at 25 ± 1 °C temperature and 14.5 W m^{-2} light intensity from the samples collected from Fateh Sagar, a freshwater lake in Udaipur, Rajasthan. Cultures were maintained on a regular basis through frequent media change under the same conditions and were subjected to harvest during their exponential phase after measuring the protein value. All the experiments were carried out under the same experimental conditions in triplicates.

Chemicals, particle dispersions, and nanoparticle characterization

ZnO nanoparticles and the bulk counterpart were purchased from Sigma-Aldrich (CAS: 1314-13-2-030-013-007) and Central Drug House Private Limited (CAS: 1314-13-2), respectively. The size of the ZnO nanoparticles was confirmed further through TEM (Tecnai, G-20 (FEI), USA) and FTIR analysis (Bruker Alpha Model, laser class-1). Stock solutions for both ZnO nano- and bulk forms were prepared with 10 mg in 100 mL deionized water. In the case of ZnO nanoparticles, stock suspension was sonicated for 30 min at 40 Hz by using a Probe sonicator (Q-500, Qsonica, USA.). Further, dilutions were made in BG-11 medium for toxicological assessment between 0.1- and 1-mg L^{-1} concentrations. Test suspensions were vortexed mildly before use. One milligram per liter of ZnO nanoparticle concentration was used further to determine the zeta potential of suspended nanoparticles and hydrodynamic diameters through dynamic light scattering using Zetasizer Nano (ZS90, Malvern).

Algal growth kinetics

Protein values from homogenously growing axenic algal cultures in the abovementioned conditions were assessed using a protocol given by Lowry et al. (1951) as modified by Herbert et al. (1971) by taking absorbance at 650 nm using a UV-Vis spectrophotometer (Hitachi U2900). From OD, subsequent protein values were calculated using BSA as standard. To estimate the growth rate of algae, 1 mL of algal suspension with a protein value of 100 μg mL^{-1} was further harvested from established cultures and inoculated in 100 mL of freshly prepared BG-11 medium having different concentrations of ZnO nanoparticles and its bulk counterpart. From these newly established cultures, protein contents were assessed regularly starting from 96 h, after every fifth day. IC_{50} values were also calculated to analyze the growth rates under the exposure conditions.

Biochemical parameters

All the biochemical analyses were performed on the 25th day of the experiment. Day 25 was chosen because in the growth curve, the highest protein value was detected on that day and after that, a decline was observed. Therefore, to assess the impact of treatment on the physiological condition of the algae, the 25th day was chosen for further assessment.

Estimation of chlorophyll and carotenoids Extraction of pigment was done in methanol and their relative amounts were estimated using equations as per Mackinney (1941): 13.42 × A_{665} = μg chlorophyll mL^{-1} and 200× A_{420} = μg carotenoids mL^{-1}.

Lipid peroxidation (MDA) One milliliter of interacted cell suspensions was added to 2 mL of trichloroacetic acid (20%) and centrifuged for 45 min at 7000 rpm. The supernatant was added to 3 mL of 2-thiobarbituric acid (0.5%) and heated for 10 min in boiling water bath. After cooling, absorbance was measured at 532 nm as per Metzler et al. (2011).

Lactate dehydrogenase assay To quantify membrane damage, lactate dehydrogenase (LDH) assay was performed (Wacker et al. 1956; Pakrashi et al. 2013). One milliliter of each algal cell suspension under various treatments was centrifuged at 7000 rpm for 10 min. About 100 μL of supernatant was collected and added to 100 μL of 30 mM sodium pyruvate followed by 2.8 mL of 0.2 M Tris-HCl. And just before measuring the decrease in absorbance, about 100 μL of 6.6 mM NADH was added. Ten readings at 340 nm were measured using UV-Vis spectrophotometer.

Superoxide dismutase activity Algal cell suspensions were prepared by adding 2 mL (0.5 M) PBS buffer solution (pH 7.5) in 50 mg biomass harvested from treated algal cultures. Samples were centrifuged for 10 min at 4 °C at 13000 rpm. One hundred microliters of the supernatant was collected and mixed with reaction mixture prepared as per Yilancioglu et al. (2014). This was incubated for 10 min at 37 °C and absorbances were recorded at 560 nm.

Catalase activity Catalase (CAT) enzyme helps in decomposing hydrogen peroxide into water and oxygen. To quantify the activity, 50 mg of interacted algal biomass was harvested and suspended in 2 mL (0.5 M) PBS buffer (pH 7.5). Samples were centrifuged at 12000 rpm at 4 °C for 30 min. One hundred microliters of supernatants was collected and mixed with reaction mixture as per details given by Roy et al. (2016). CAT activity was represented in terms of percent decrease with respect to the control.

Microscopic analysis

Comparative microscopic analyses of algal cells under treatments were done repeatedly using a compound microscope (Olympus CH20i). Furthermore, to study the algal morphology and particle localization, untreated and treated (1-mg L^{-1} concentration of ZnO treatments of both bulk and nano-forms) algal cells were analyzed through TEM (Tecnai, G-20 (FEI), USA) and SEM (EVO 18, Zeiss, Germany). For TEM analysis, blocks were prepared, ultrathin sectioning were done, and sections were loaded on copper grids, whereas for SEM, algal cell drops of each sample were coated, air-dried via gold sputtering, and subjected to analyses.

Statistical analysis

All the experiments were done in triplicates. Mean and standard deviation were calculated using MS Excel (office version 10.0) and values are shown as mean ± SD in Table 1. Correlation and regression equation were estimated using MS Excel. Statistically significant differences between control and treatment were analyzed using a one-way ANOVA with the help of Prism Software (version 3.02), as P values less than 0.5 were assumed as the significant differences.

Results and discussion

Nanoparticle characterization

Transmission electron microscopy analysis revealed polymorphic shape and size (≤ 100 nm) of ZnO nanoparticles (Fig. 1a). The transmittance peaks at 518.48, 480.38, and 424 wavenumber cm^{-1} of ZnO nanoparticles were comparable with the standard graph of ZnO (Fig. 1b). Sonication was essential as particles have tendency to aggregate in aqueous form

Table 1 Biochemical parameter analysis of *Coelastrella terrestris* under different treatment levels

ZnO treatment (conc. in mg L^{-1})	MDA content	Total chlorophyll (μg mL^{-1})	Carotenoids (μg mL^{-1})	LDH (nmol/min_{mL})	SOD (unit mg^{-1}_{prot})	CAT (unit mg^{-1}_{prot})
Control	0.2008 ± 0.3	13.3484 ± 0.02	9.1066 ± 0.04	0.002 ± 0.2	3.65 ± 0.3	0.03 ± 0.1
0.1 NP	0.5428 ± 0.6	11.3264 ± 0.02	8.9066 ± 0.05	7.26 ± 0.3	16.89 ± 0.2	2.96 ± 0.1
0.5 NP	0.7180 ± 0.8	5.1130 ± 0.03	5.4866 ± 0.07	15.32 ± 0.2	24.54 ± 0.3	3.76 ± 0.2
1 NP	0.9147 ± 1.2	4.2228 ± 0.03	3.8933 ± 0.05	18.89 ± 0.2	31.62 ± 0.3	4.77 ± 0.1
F	1.001	72460	8301	316800	7367000	72040
P value	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
P value summary	**	***	***	***	***	***
Control	0.2008 ± 0.3	13.3484 ± 0.02	9.1066 ± 0.04	0.002 ± 0.2	3.65 ± 0.3	0.03 ± 0.1
0.1 bulk	0.4060 ± 0.4	12.3911 ± 0.04	8.7266 ± 0.06	1.27 ± 0.3	5.77 ± 0.2	0.77 ± 0.2
0.5 bulk	0.6368 ± 0.6	8.5082 ± 0.03	7.9200 ± 0.05	10.54 ± 0.2	17.67 ± 0.2	2.87 ± 0.1
1 bulk	0.7480 ± 0.8	7.5778 ± 0.03	6.3333 ± 0.03	13.67 ± 0.2	27.44 ± 0.3	3.32 ± 0.1
F	1.001	29480	2444	148800	555300	32490
P value	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
P value summary	***	***	***	***	***	***

(Angel et al. 2013). Zeta potential of suspended ZnO nanoparticles was observed to be − 24.6 mV in stock solution prepared within deionized water (Fig. 2b). The dynamic light scattering study showed the hydrodynamic average size of ZnO nanoparticles within the deionized water was 252 nm in diameter with PDI value of 0.164 (Fig. 2a) and showed a slow and clear tendency to aggregate further as the size is much greater than the actual size of ZnO nanoparticles analyzed through TEM. The reason behind the aggregation is the increase in ionic concentration which subsequently decreases the repulsive forces between the ZnO nanoparticles. Hence, in aqueous medium, hydrodynamic sizes usually greater than the actual particle sizes were found (Bian et al. 2011).

Algal growth kinetics

Growth of *Coelastrella terrestris* was found adversely affected under the ZnO nanoparticles and its bulk counterpart treatments. However, analyzing the growth pattern through protein content, it was observed that the nano-form of ZnO is more toxic to the *Coelastrella terrestris* than its bulk form. Toxicity in both cases, nano-form and bulk form, was found to be dose-dependent. It is clearly evident that the increase in dosage concentration leads to the increase in toxicity similar to the previously reported study (Schiavo et al. 2016). To assess the growth rate, estimation of protein is one of the important and useful parameters (Turhani et al. 2005). Protein as a parameter has also been found useful in assessing the toxicity induced by heavy metal and nanoparticles in few earlier published reports (Rai et al. 1992; Harish et al. 2008; Gong et al. 2011). Further, ZnO NPs were found to exhibit a strong adsorption capability for proteins; therefore, estimating the protein is critical to understand the toxicity level (Horie et al. 2009). Earlier studies also concluded the toxicological responses because of Zn^{+} ion dissolution which depends on the adsorption potential of proteins resultant into deprived growth rate in algae (Manzo et al. 2013; Suman et al. 2015). On the 25th day, when highest protein level was marked by control, a 27% reduction in growth rate under bulk ($y = 50.635x$, $R^2 = 0.173$) and a 54% reduction in growth rate under nano-form ($y = 34.673x$, $R^2 = -0.101$) at 1 mg L^{-1} of treatment level were observed in terms of protein level. In other treatment concentrations also, reduction in soluble protein under nano-form was higher compared with the bulk was observed (Fig. 3). Even at the lowest concentration (0.1 mg L^{-1}), a significant reduction (25%) in protein content was observed in the case of nano-form ($y = 26.302x + 166.58$; $R^2 = 0.1846$), whereas in the bulk, the reduction was almost negligible ($y = 47.095x + 149.63$; $R^2 = 0.3557$). A remarkable difference under the nano- and bulk treatments was also observed at 0.5 mg L^{-1} where a 35% reduction ($y = 17.764x + 144.3$, $R^2 = 0.1226$) in nano-form and a 27% reduction ($y = 36.385x + 130.43$, $R^2 = 0.262$) in bulk were detected. Reduced level of proteins clearly showed that the nano-form of particles is toxic in comparison with the bulk. Moreover, IC_{50} values were also calculated in both of the cases. IC_{50} calculated on the 25th day revealed that the nano-form is more toxic ($IC_{50} = 0.255$ mg L^{-1}) in comparison with the bulk form ($IC_{50} = 0.455$ mg L^{-1}). It means that lower concentration of nano-form is required to induce a 50% growth inhibition and higher dose of bulk is required to induce the same level of inhibition. Results clearly revealed that the nano-form of ZnO nanoparticles is more toxic than the bulk form. IC_{50} growth rate parameter is more appropriate scientifically and provides better interpretations between the comparative studies (Bergtold and Dohmen 2011).

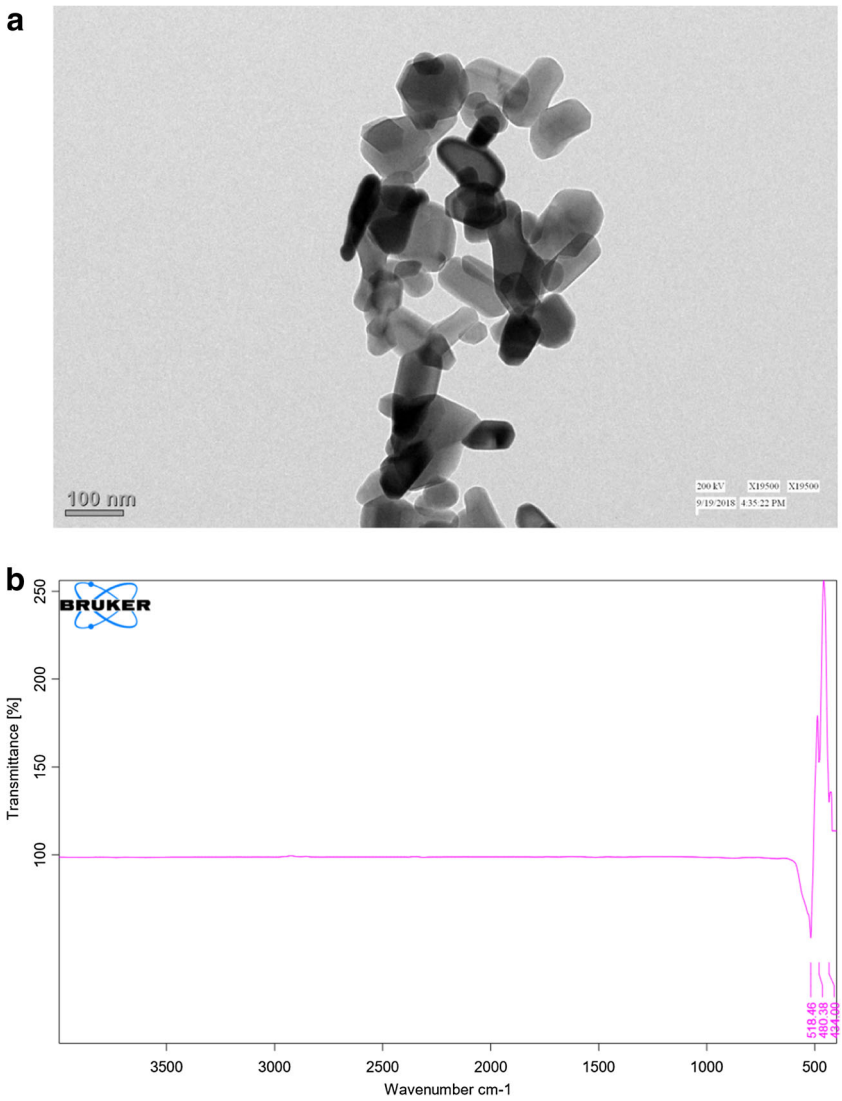

Fig. 1 Zinc oxide nanoparticle characterization: **a** TEM micrograph and **b** FTIR spectrum

As the nano-form of particles possesses greater surface area to volume ratio which might be one of the key factors in attributing toxicity to the algae, smaller size leads to the stronger interaction with the algal cell and alters the cell wall thickness (Kim et al. 2016). In fact, intrusion of nanoparticles is much easier in the case of newly formed algal cells in comparison with the mature ones (Dash et al. 2012) which might be the reason of retarded growth rate response in the case of nanoparticle treatments. Inherent pores of algal cell also suggested allowing nanoparticle internalization. It was reported that more pores must be induced after nanoparticle exposure. It produces oxidative stress and leads to disruption of the cell (Navarro et al. 2008; Xia et al. 2015; Taylor et al. 2016; Tripathi et al. 2017). The size of the particle plays a prominent role in toxicological pavements (Franklin et al. 2007). Earlier studies done on ZnO nanoparticles reported that different particle shapes and sizes of the same metal oxide result into a different toxicity level (Peng et al. 2011; Samei et al. 2019). Likewise, Manzo et al. (2013) revealed that the bulk ZnO particles were less toxic than its nanoparticles for *Dunaliella tertiolecta*. In the present study, the lowest concentration (0.1 mg L^{-1}) of bulk treatment was not observed to be harmful to the algae, whereas the same concentration in nano-form was found generating a negative impact on algal growth. However, at the 1-mg L^{-1} concentration, both were detected affecting the growth adversely but the toxicity was found more in the case of nanoparticles in comparison with the bulk. Similar results were observed in the case of two marine algae, *Tetraselmis suecica* and *Phaeodactylum tricornutum*, while assessing the ZnO nanoparticles and bulk form toxicity (Li et al. 2017). The surface of algal cells was observed to be occupied by ZnO nanoparticles (Fig. S1), which could have obstructed the nutrient exchange between the growth medium and algal cell (Bhattacharya et al. 2010). A similar phenomenon was

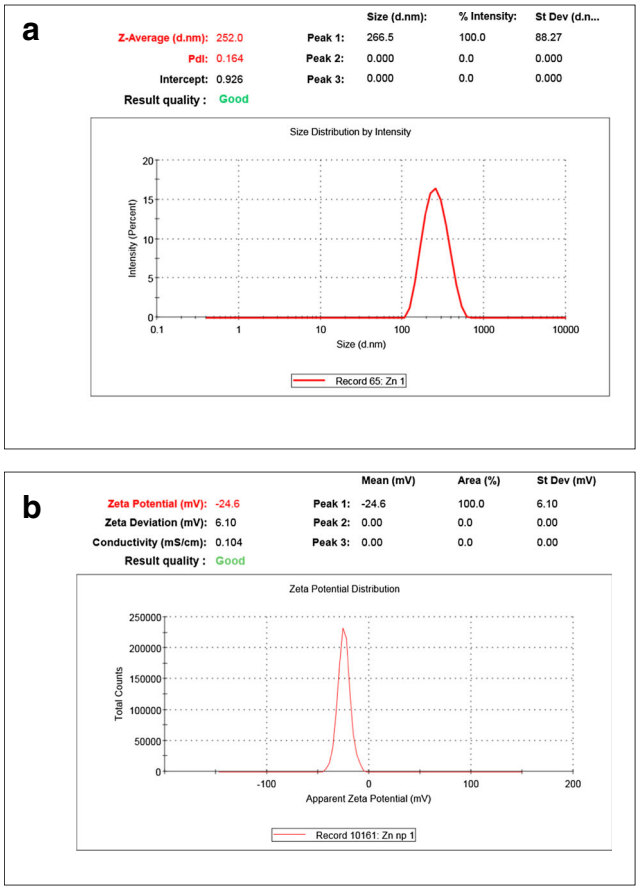

Fig. 2 **a** Hydrodynamic size nanoparticles measured via dynamic light scattering of zinc oxide nanoparticle stock solution in deionized water. **b** Zeta potential of suspended nanoparticles

observed by Metzler et al. (2011) in assessing the effect of titanium dioxide nanoparticle on algal growth. Moreover, aggregates entrapping algal cells must have reduced the availability of light which could have affected the cell growth activity (Fig. S1). Similarly, Huang et al. (2005) also concluded that the inhibition of algal cell growth is due to the adsorption of titanium dioxide nanoparticles on algal cell surface. Interestingly, with the increase in dosage concentration, we observed delays in the growth pattern at the onset of exponential phase in both bulk and nano-forms of ZnO. Merdzan et al. (2014) reported ZnO nanoparticle aggregation in correlation with the dissolution phenomenon and concluded that both the processes could simultaneously contribute to produce toxicological impact. Moreover, earlier dissolution was found to damage the cell wall and destructive cell organelles, which was also apparent in the present study after microscopic analysis, which will be discussed later. Moreover, the toxicological attributes were also because of the time of the exposure. As the duration of exposure was extended, aging of particles also occurred which also influences the toxicity in the case of nanoparticles (Schiavo et al. 2018). The overall impact of ZnO nano-form and bulk form is found negative on the protein content of algal cells which in terms of growth was suppressive.

Biochemical parameters

Toxicity assessment by evaluating biological endpoints like chlorophyll, enzyme activities, and peroxidation reveals the oxidative stress of the algae and helps in correlating the mechanism, imparting the toxicity. Therefore, it is important to determine the biochemical parameters (Chen et al. 2019).

Chlorophyll and carotenoid content Significant deduction in chlorophyll content was observed after the exposure of ZnO nano- and bulk counterpart at 0.5- and 1-mg L^{-1} concentrations (Table 1). At the lowest concentration of 0.1 mg L^{-1}, no significant changes in chlorophyll were detected in both nano- and bulk samples. Chlorophyll content was found more sensitive under treatments as it was earlier detected in stress conditions reported by Houimli et al. (2010) whereas contrastingly carotenoid content was not found much reduced. This supports the earlier studies reporting that metal stress in algae responded in carotenoid synthesis to quench oxidative stress (Nikookar et al. 2005; Wang et al. 2010).

Lipid peroxidation (MDA) Oxidative stress could be detected by the quantification of lipid peroxidation, as the membrane lipid damage indicates the stress conditions (Sayeed et al. 2003). A dose-dependent increase in the lipid peroxidation was observed in all concentrations exposed, except in bulk at the 0.1-mg L^{-1} concentration. A similar dose-dependent response was reported in the earlier study conducted by Wang et al. (2011). In nano-form, increase in lipid peroxidation was also reported by Chen et al. (2012). Peroxidation levels were found much higher in the case of nano-treatments in comparison with its bulk counterpart as a biomarker of oxidative stress indicating that nano-form is more toxic.

LDH release Lactate dehydrogenase leakage is one of the parameters to measure cytotoxicity induced by nanoparticles (Yang et al. 2009). LDH release was found to be enhanced with the dosage concentration under nanoparticle treatment, whereas, at lowest concentration (0.1 mg L^{-1}) of bulk, no such release was detected (Table 1). However, at the same concentration, ZnO nano-form was observed to be more cytotoxic to the algae than its bulk counterpart via LDH analysis. At 1 mg L^{-1} of nano-form concentration, substantial membrane damage was notified indicating the toxicity as reported earlier (Zhang et al. 2012).

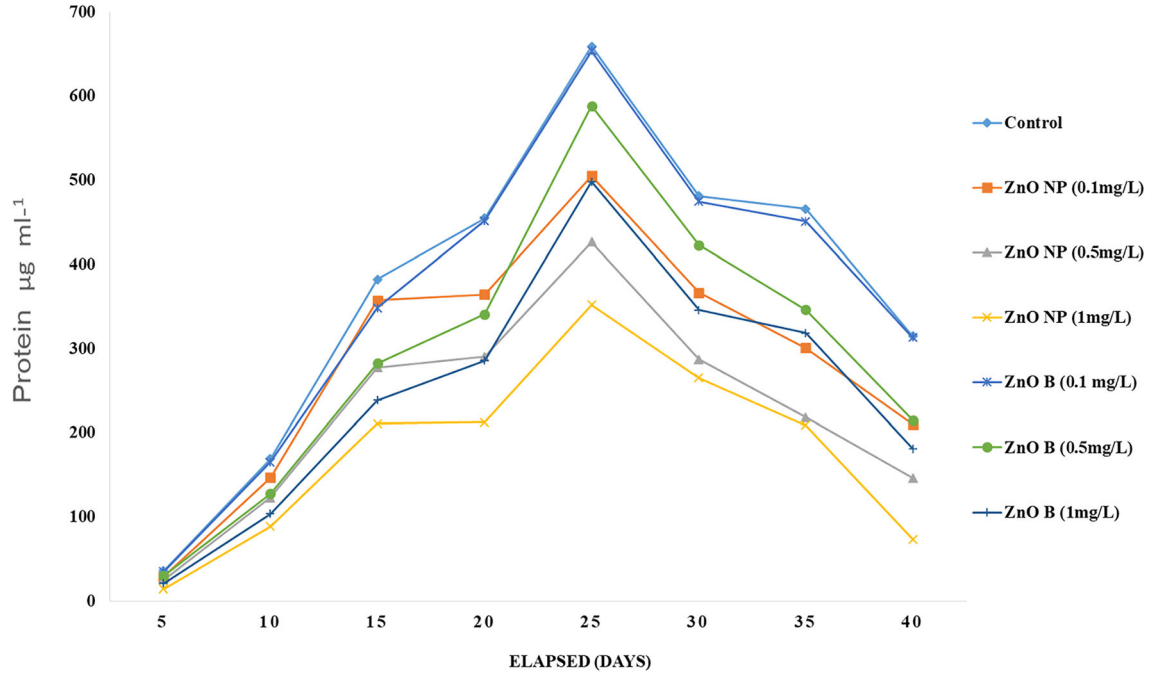

Fig. 3 Effect of zinc oxide nanoparticles and its bulk counterpart on *Coelastrella terrestris* growth kinetics measured via protein content

SOD activity Enzymatic activity was reflective even at lower concentrations of treatment. The maximum activity detected in nano- and bulk form was 31.62 ± 0.3 and 27.44 ± 0.3 unit mg^{-1}_{prot} at 1 mg L^{-1}, respectively. Enzymatic activity observed was directly proportional to dose concentration. Superoxide dismutase (SOD) activity clearly showed stressful physiological condition in algae after the exposure of ZnO in nano- and bulk forms as reported earlier (Suman et al. 2015).

CAT activity Catalytic activity was found increasing in linear manner with respect to the dosage concentrations. However, under nano-treatments, catalase activity was more profound than the bulk treatments. At the 1-mg L^{-1} ZnO nanoparticle exposure, the value 4.77 ± 0.1 unit mg^{-1}_{prot} was obtained which is the highest among all, whereas at the same concentration, 3.32 ± 0.1 1 unit mg^{-1}_{prot} was observed in bulk form. Only under the 0.1 mg L^{-1} bulk, no significant activity of catalase was noticed.

Microscopic analysis

Preliminary screening of algal cells done by compound microscope embarked a difference between the cultures grown under nano- and bulk treatments and provided us supportive evidences (Fig. 4). In comparison with the control, particles of ZnO along with media particles were found aggregated under both of the cases which must have hindered the nutrient availability required by the algal cells to grow. Peculiar morphological proofs and lack of motility of *Coelastrella terrestris* allowed an easier aggregation of ZnO around the cells. Another observation was that with the increase of dosage concentrations in both of the cases, aggregation was found to increase. This reduced the availability of light to the algal cells which have obstructed the growth (Aruoja et al. 2009; Gong et al. 2011).

Similar results were evident by SEM analysis (Fig. 5). One more trend was observed that in the case of nano-form, surface adsorptions of ZnO nanoparticles were observed (Fig. S1) as reported earlier (Li et al. 2017). It could be the reason why nano-form is more toxic than bulk as it covers larger surface area of algal cell via forming large aggregates and stops the exchange between the cell and the media and light. Moreover, aggregation increases the direct interaction of ZnO nano- and bulk forms with the algal cell surface which could be the reason of cytological damages manifested after the TEM analysis under both treatments (Xia et al. 2015). TEM results revealed that cell organelles after the exposure collapsed and fragmented (Fig. 5). Cell wall was found ruptured; cytoplasm was shrunken. Overall analysis showed that the long-term exposure of ZnO nanoparticles induces cytological abnormalities to which the growth of the algae was affected.

Conclusion

Our results revealed that physiochemical nature of the particles plays an important role with respect to the development phase of the algae. This study clearly revealed that the growth

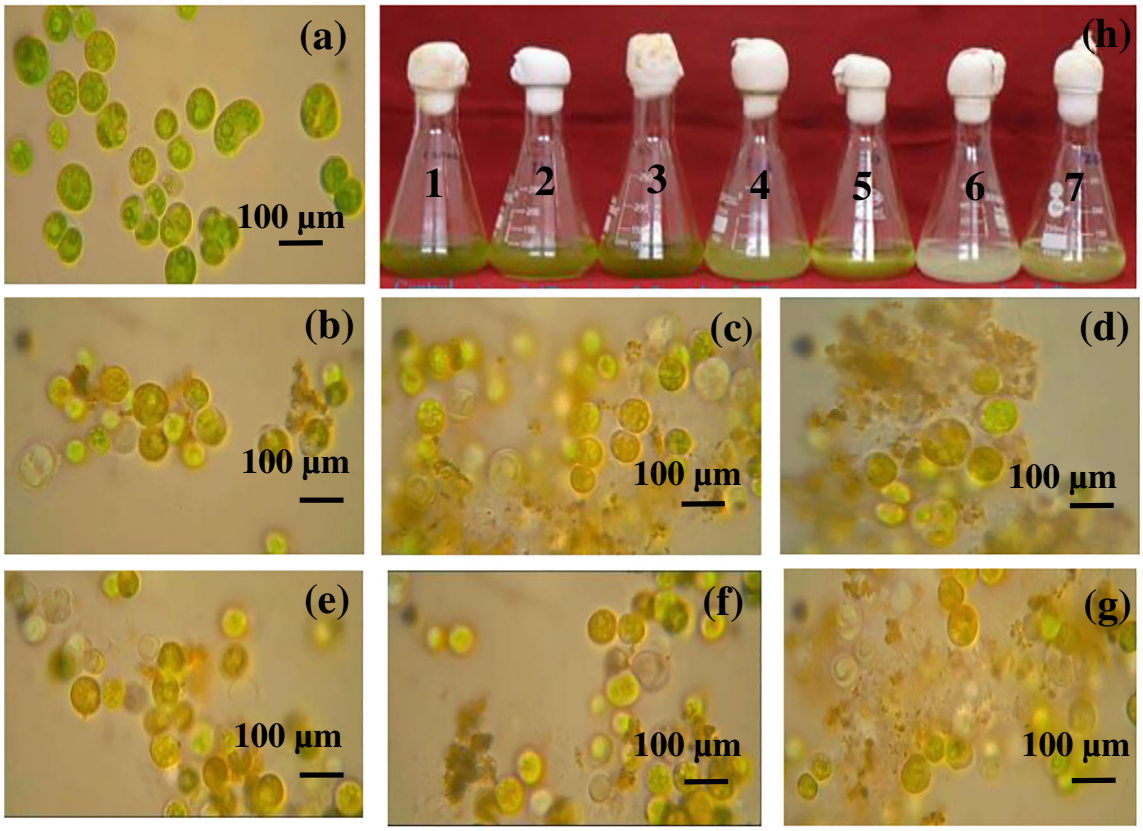

Fig. 4 Compound microscopy images of *Coelastrella terrestris* under control and under different treatment concentrations of zinc oxide nanoparticles and its respective bulk form after 600 h: **a** control or untreated algal cells; **b**–**d** zinc oxide nanoparticle–treated algal cells with 0.1-, 0.5-, and 1-mg L^{-1} concentrations, respectively; **e**–**g** zinc oxide bulk–treated algal cells with 0.1-, 0.5-, and 1-mg L^{-1} concentrations, respectively; **h** algal cultures under treatment along with control (1 control; 2, 3, and 4 represent 0.1-, 0.5-, and 1-mg L^{-1} zinc oxide nanoparticle treatments, respectively; 5, 6, and 7 represent 0.1-, 0.5-, and 1-mg L^{-1} zinc oxide bulk treatments, respectively)

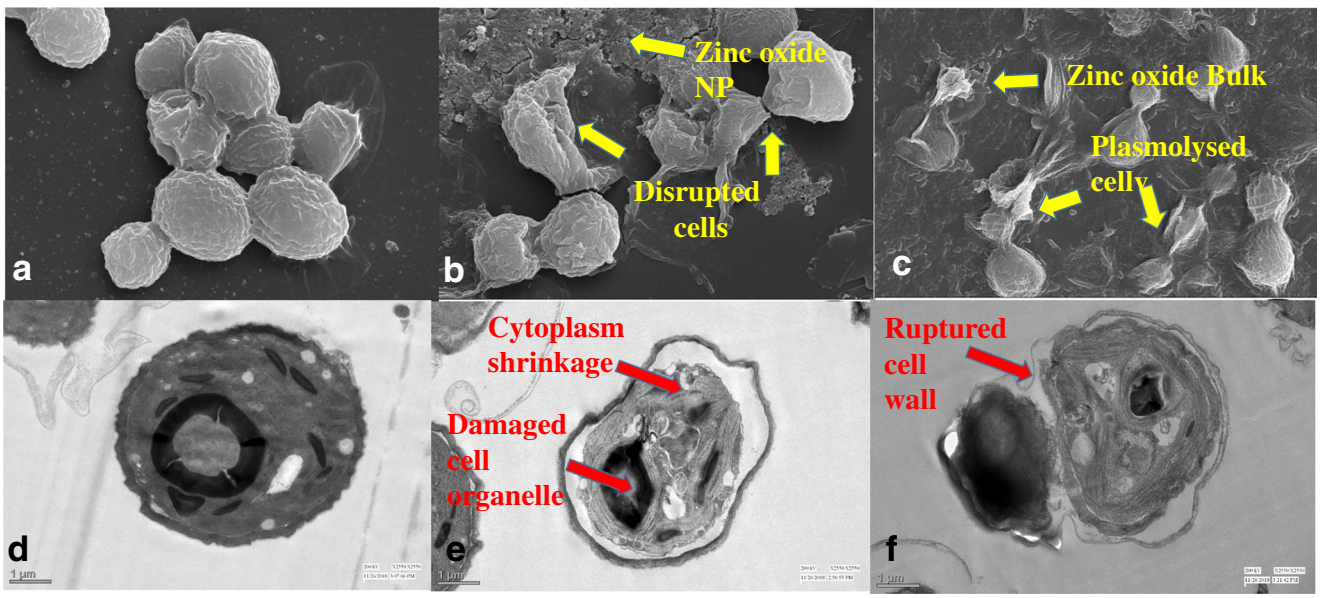

Fig. 5 Scanning electron microscopy: **a** *Coelastrella terrestris* control cells, **b** algal cells treated with zinc oxide nanoparticles, **c** algal cells treated with zinc oxide bulk counterpart (arrows representing detrimental effects of treatments). Transmission electron microscopy: **d** *Coelastrella terrestris* control cells, **e** algal cells treated with zinc oxide nanoparticles, **f** algal cells treated with zinc oxide bulk counterpart; both micrographs were taken after 25 days of exposure treated with 1 mg L^{-1}

rate of algae was suppressed. The nano-form of ZnO was found remarkably toxic in comparison with its bulk counterpart. Toxicity was found directly proportional to the dosage concentration under both nano- and bulk treatments, and aggregation was found to play a vital role in attributing toxicity. To develop a better understanding regarding the nature of aggregation with respect to the media and exposure time, more investigation needs to be done.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alswat AA, Ahmad MB, Saleh TA, Hussein MZB, Ibrahim NA (2016) Effect of zinc oxide amounts on the properties and antibacterial activities of zeolite/zinc oxide nanocomposite. Mater Sci Eng C 68:505–511

Angel BM, Batley GE, Jarolimek CV, Rogers NJ, (2013) The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. Chemosphere 93(2):359–365

Aruoja V, Dubourguier HC, Kasemets K, Kahru A (2009) Toxicity of nanoparticles of CuO, ZnO and TiO_2 to microalgae *Pseudokirchneriella subcapitata*. Sci Total Environ 407:1461–1468

Bergtold M, Dohmen GP (2011) Biomass or growth rate endpoint for algae and aquatic plants: relevance for the aquatic risk assessment of herbicides. Integr Environ Assess Manag 7:237–247

Bhattacharya P, Lin S, Turner JP, Ke PC (2010) Physical adsorption of charged plastic nanoparticles affects algal photosynthesis. J Phys Chem C 114:16556–16561

Bhuvaneshwari M, Iswarya V, Vishnu S, Chandrasekaran N, Mukherjee A (2018) Dietary transfer of zinc oxide particles from algae (*Scenedesmus obliquus*) to daphnia (*Ceriodaphnia dubia*). Environ Res 164:395–404

Bian SW, Mudunkotuwa IA, Rupasinghe T, Grassian VH (2011) Aggregation and dissolution of 4 nm ZnO nanoparticles in aqueous environments: influence of pH, ionic strength, size, and adsorption of humic acid. Langmuir 27:6059–6068

Cattaneo AG (2018) Nanotoxicological evaluation in marine water ecosystem: a detailed review. In: Kumar V, Dasgupta N, Ranjan S (eds) Environmental toxicity of nanomaterials, 1st edn. CRC Press, pp 61–90

Chen P, Powell BA, Mortimer M, Ke PC (2012) Adaptive interactions between zinc oxide nanoparticles and *Chlorella* sp. Environ Sci Technol 46:12178–12185

Chen F, Xiao Z, Yue L, Wang J, Feng Y, Zhu X, Wang Z, Xing B (2019) Algae response to engineered nanoparticles: current understanding, mechanisms and implications. Environ Sci Nano 6:1026–1042. https://doi.org/10.1039/C8EN01368C

Dash A, Singh AP, Chaudhary BR, Singh SK, Dash D (2012) Silver nanoparticles on growth of eukaryotic green algae. Nano Micro Lett 4:158–165

Ejaz M, Arfat YA, Mulla M, Ahmed J (2018) Zinc oxide nanorods/clove essential oil incorporated type B gelatin composite films and its applicability for shrimp packaging. Food Pack Shelf 15:113–121

Espinasse BP, Geitner NK, Schierz A, Therezien M, Richardson CJ, Lowry GV, Ferguson L, Wiesner MR (2018) Comparative persistence of engineered nanoparticles in a complex aquatic ecosystem. Environ Sci Technol 52:4072–4078

Franklin NM, Rogers NJ, Apte SC, Batley GE, Gadd GE, Casey PS (2007) Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility. Environ Sci Technol 41:8484–8490

Fu PP, Xia Q, Hwang HM, Ray PC, Yu H (2014) Mechanisms of nanotoxicity: generation of reactive oxygen species. J Food Drug Anal 22(1):64–75

Ghaffari SB, Sarrafzadeh MH, Fakhroueian Z, Shahriari S, Khorramizadeh MR (2017) Functionalization of ZnO nanoparticles by 3-mercaptopropionic acid for aqueous curcumin delivery: synthesis, characterization, and anticancer assessment. Mater Sci Eng C 79:465–472

Gong N, Shao KS, Feng W, Lin ZZ, Liang CH, Sun YQ (2011) Biotoxicity of nickel oxide nanoparticles and bio-remediation by microalgae *Chlorella vulgaris*. Chemosphere 83:510–516

Harish, Sundaramoorthy S, Kumar D, Vaijapurkar SG (2008) A new chlorophycean nickel hyperaccumulator. Bioresour Technol 99(9):3930–3934

Hazeem LJ, Bououdina M, Rashdan S, Brunet L, Slomianny C, Boukherroub R (2016) Cumulative effect of zinc oxide and titanium oxide nanoparticles on growth and chlorophyll a content of *Picochlorum* sp. Environ Sci Pollut Res 23:2821–2830

Herbert D, Phipps PJ, Strange RE (1971) The chemical analysis of microbial cell. In: Norris JR, Ribbons DW (eds) Methods in microbiology, volume VB. London Academic Press, London, pp 209–344

Horie M, Nishio K, Fujita K, Endoh S, Miyauchi A, Saito Y et al (2009) Protein adsorption of ultrafine metal oxide and its influence on cytotoxicity toward cultured cells. Chem Res Toxicol 22(3):543–553

Houimli SIM, Denden M, Mouhandes BM (2010) Effects of 24-epibrassinolide on growth, chlorophyll, electrolyte leakage and proline by pepper plants under NaCl-stress. Eur Asia J Biosci 4:96–104

Huang CP, Cha DK, Ismat SS (2005) Progress report: short-term chronic toxicity of photocatalytic nanoparticles to bacteria, algae, and zooplankton. EPA Grant number: R831721./http://cfpub.epa.gov/ncer_abstracts/indexcfm/fuseaction/display.abstractDetail/abstract/7384/report/0S

Ji J, Long Z, Lin D (2011) Toxicity of oxide nanoparticles to the green algae *Chlorella* sp. Chem Eng J 170:525–530

Kim DY, Vijayan D, Praveenkumar R, Han JI, Lee K, Park JY, Chang WS, Lee JS, Oh YK (2016) Cell-wall disruption and lipid/astaxanthin extraction from microalgae: *Chlorella* and *Haematococcus*. Bioresour Technol 199:300–310

Klaine SJ, Alvarez PJJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem 27:1825–1851

Li J, Schiavo S, Rametta G, Miglietta ML, La Ferrara V, Wu C, Manzo S (2017) Comparative toxicity of nano ZnO and bulk ZnO towards marine algae *Tetraselmis suecica* and *Phaeodactylum tricornutum*. Environ Sci Pollut Res 24:6543–6553

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin reagent. J Biol Chem 193:265–275

Lu PJ, Huang SC, Chen YP, Chiueh LC, Shih DYC (2015) Analysis of titanium dioxide and zinc oxide nanoparticles in cosmetics. Food Compos Anal 23:587–594

Lupan O, Cretu V, Postica V, Ahmadi M, Cuenya BR, Chow L, Adelung R (2016) Silver-doped zinc oxide single nanowire multifunctional nanosensor with a significant enhancement in response. Sensors Actuators B Chem 223:893–903

Ma H, Williams PL, Diamond SA (2013) Ecotoxicity of manufactured ZnO nanoparticles–a review. Environ Pollut 172:76–85

Mackinney G (1941) Absorption of light by chlorophyll solution. J Biol Chem 140:315–322

Manzo S, Miglietta ML, Rametta G, Buono S, Di Francia G (2013) Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*. Sci Total Environ 445:371–376

Merdzan V, Domingos RF, Monteiro CE, Hadioui M, Wilkinson KJ (2014) The effects of different coatings on zinc oxide nanoparticles and their influence on dissolution and bioaccumulation by the green alga, *C. reinhardtii*. Sci Total Environ 488:316–324

Metzler DM, Li M, Erdem A, Huang CP (2011) Responses of algae to photocatalytic nano-TiO2 particles with an emphasis on the effect of particle size. Chem Eng J 170:538–546

Miao AJ, Zhang XY, Luo Z, Chen CS, Chin WC, Santschi PH, Quigg A (2010) Zinc oxide–engineered nanoparticles: dissolution and toxicity to marine phytoplankton. Environ Toxicol Chem 29: 2814–2822

Mishra PK, Mishra H, Ekielski A, Talegaonkar S, Vaidya B (2017) Zinc oxide nanoparticles: a promising nanomaterial for biomedical applications. Drug Discov Today 22:1825–1834

Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao A-J, Quigg A, Santschi PH, Sigg L (2008) Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17:372–386

Nikookar K, Moradshahi A, Hosseini L (2005) Physiological responses of *Dunaliella salina* and *Dunaliella tertiolecta* to copper toxicity. Biomol Eng 22:141–146

Nowack B, Bucheli TD (2007) Occurrence, behavior and effects of nanoparticles in the environment. Environ Pollut 150:5–22

Pakrashi S, Dalai S, Prathna TC, Trivedi S, Myneni R, Raichur AM, Chandrasekaran N, Mukherjee A (2013) Cytotoxicity of aluminium oxide nanoparticles towards fresh water algal isolate at low exposure concentrations. Aquat Toxicol 132:34–45

Peng X, Palma S, Fisher NS, Wong SS (2011) Effect of morphology of ZnO nanostructures on their toxicity to marine algae. Aquat Toxicol 102:186–196

Piccinno F, Gottschalk F, Seeger S, Nowack B (2012) Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. J Nanopart Res 14:1109

Rai UN, Tripathi RD, Kumar N (1992) Bioaccumulation of chromium and toxicity on growth, photosynthetic pigments, photosynthesis, in vivo nitrate reductase activity and protein content in a chlorococcalean green alga *Glaucocystis nostochinearum Itzigsohn*. Chemosphere 25(11):1721–1732

Raliya R, Saharan V, Dimkpa C, Biswas P (2017) Nanofertilizer for precision and sustainable agriculture: current state and future perspectives. Agric Food Chem 66:6487–6503

Rasmussen JW, Martinez E, Louka P, Wingett DG (2010) Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. Expert Opin Drug Deliv 7(9):1063–1077

Rippka R, Derelies J, Waterbury JB, Herdman M, Stainer RV (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1–61

Roy R, Parashar A, Bhuvaneshwari M, Chandrasekaran N, Mukherjee A (2016) Differential effects of P25 TiO_2 nanoparticles on freshwater green microalgae: *Chlorella* and *Scenedesmus* species. Aquat Toxicol 176:161–171

Samei M, Sarrafzadeh MH, Faramarzi MA (2019) The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga, *Raphidocelis subcapitata*. Environ Sci Pollut Res 26:2409–2420

Sayeed I, Parvez S, Pandey S, Bin-Hafeez B, Haque R, Raisuddin S (2003) Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. Ecotoxicol Environ Saf 56:295–301

Schiavo S, Oliviero M, Miglietta M, Rametta G, Manzo S, (2016) Genotoxic and cytotoxic effects of ZnO nanoparticles for *Dunaliella tertiolecta* and comparison with SiO_2 and TiO_2 effects at population growth inhibition levels. Sci Total Environ 550:619–627

Schiavo S, Oliviero M, Li J, Manzo S (2018) Testing ZnO nanoparticle ecotoxicity: linking time variable exposure to effects on different marine model organisms. Environ Sci Pollut Res 25:4871–4880

Sharma N, Jha R, Baghel S, Sharma D (2017) Study on photocatalyst zinc oxide annealed at different temperatures for photodegradation of eosin Y dye. J Alloys Compd 695:270–279

Suman TY, Rajasree SR, Kirubagaran R (2015) Evaluation of zinc oxide nanoparticles toxicity on marine algae *Chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis. Ecotoxicol Environ Saf 113:23–30

Sun TY, Bornhöft NA, Hungerbühler K, Nowack B (2016) Dynamic probabilistic modeling of environmental emissions of engineered nanomaterials. Environ Sci Technol 50:4701–4711

Taylor NS, Merrifield R, Williams TD, Chipman JK, Lead JR, Viant MR (2016) Molecular toxicity of cerium oxide nanoparticles to the freshwater alga *Chlamydomonas reinhardtii* is associated with supra-environmental exposure concentrations. Nanotoxicology 10:32–41

Tripathi DK, Tripathi A, Shweta SS, Singh Y, Vishwakarma K, Yadav G, Sharma S, Singh VK, Mishra RK, Upadhyay RG, Dubey NK, Lee Y, Chauhan DK (2017) Uptake, accumulation and toxicity of silver nanoparticle in autotrophic plants, and heterotrophic microbes: a concentric review. Front Microbiol 8:7

Turhani D, Cvikl B, Watzinger E, Weißenböck M, Yerit K, Thurnher D, Lauer G, Ewers R (2005) In vitro growth and differentiation of osteoblast-like cells on hydroxyapatite ceramic granule calcified from red algae. J Oral Maxillofac Surg 63(6):793–799

Wacker WE, Ulmer DD, Vallee BL (1956) Metalloenzymes and myocardial infarction: malic and lactic dehydrogenase activities and zinc concentrations in serum. N Engl J Med 255:449–456

Wang ZL (2004) Zinc oxide nanostructures: growth, properties and applications. J Phys Condens Matter 16:R829–R858

Wang GH, Hao ZJ, Huang ZB, Chen LZ, Li XY, Hu CX, Liu YD (2010) Raman spectroscopic analysis of a desert cyanobacterium *Nostoc* sp. in response to UV-B radiation. Astrobiology 10:783–788

Wang ZY, Li J, Zhao J, Xing BS (2011) Toxicity and internalization of CuO nanoparticles to prokaryotic alga *Microcystis aeruginosa* as affected by dissolved organic matter. Environ Sci Technol 45: 6032–6040

Wang Z, Vijver MG, Peijnenburg WJ (2018) Multiscale coupling strategy for nano ecotoxicology prediction. Environ Sci Technol 52(14): 7598–7600

Xia B, Chen B, Sun X, Qu K, Ma F, Du M (2015) Interaction of TiO_2 nanoparticles with the marine microalga *Nitzschia closterium*:

growth inhibition, oxidative stress and internalization. Sci Total Environ 508:525–533

Yang H, Liu C, Yang D, Zhang H, Xi Z (2009) Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. J Appl Toxicol 29:69–78

Yilancioglu K, Cokol M, Pastirmaci I, Erman B, Cetiner S (2014) Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PLoS One 9:e91957

Zhang H, Ji Z, Xia T, Meng H, Low-Kam C, Liu R, Pokhrel S, Lin S, Wang X, Liao YP, Wang M (2012) Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. ACS Nano 6:4349–4368

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