



Mechanism of nanotoxicity in *Chlorella vulgaris* exposed to zinc and iron oxide

Pallavi Saxena^a, Vinod Saharan^b, Prabhat Kumar Baroliya^c, Vinod Singh Gour^d,
Manoj Kumar Rai^e, Harish^{a,*}

^a Plant Biotechnology Laboratory, Department of Botany, Mohanlal Sukhadia University, Udaipur, 313 001, Rajasthan, India

^b Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, 313 001, Rajasthan, India

^c Department of Chemistry, Mohanlal Sukhadia University, Udaipur, 313 001, Rajasthan, India

^d Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur, India

^e Department of Environmental Science, Indira Gandhi National Tribal University, Amarkantak, Madhya Pradesh, 484887, India

ARTICLE INFO

Edited by Dr. A.M Tsatsaka

Keywords:

Algae
Aquatic-ecosystem
Antioxidant
Nanoparticles
Stress

ABSTRACT

Usage of nanoparticle in various products has increased tremendously in the recent past. Toxicity of these nanoparticles can have a huge impact on aquatic ecosystem. Algae are the ideal organism of the aquatic ecosystem to understand the toxicity impact of nanoparticles. The present study focuses on the toxicity evaluation of zinc oxide (ZnO) and iron oxide (Fe₂O₃) nanoparticles towards freshwater microalgae, *Chlorella vulgaris*. The dose dependent growth retardation in *Chlorella vulgaris* is observed under ZnO and Fe₂O₃ nanoparticles and nanoform attributed more toxicity than their bulk counterparts. The IC₅₀ values of ZnO and Fe₂O₃ nanoparticles was reported at 0.258 mg L⁻¹ and 12.99 mg L⁻¹ whereas, for the bulk-form, it was 1.255 mg L⁻¹ and 17.88 mg L⁻¹, respectively. The significant decline in chlorophyll content and increase in proline content, activity of superoxide dismutase and catalase, indicated the stressful physiological state of microalgae. An increased lactate dehydrogenase level in treated samples suggested membrane disintegration by ZnO and Fe₂O₃ nanoparticles. Compound microscopy, scanning electron microscopy and transmission electron microscopy confirm cell entrapment, deposition of nanoparticles on the cell surface and disintegration of algal cell wall. Higher toxicity of nanoform in comparison to bulk chemistry is a point of concern.

1. Introduction

Nanotechnology based-products inventory is growing rapidly. Current trend of using nano particles (NPs) has increased in almost every utility sector of mankind use [1]. Among the new promising metallic oxide NPs, zinc oxide (ZnO) has marked its place due to its amazing piezoelectric and pyroelectric characteristic properties [2]. Moreover, it possesses great excitation binding energy and a large band gap, wurtzite structure without center of symmetry which attributes for unique physiochemical properties [3]. Adding on greater surface per volume in

its nano form also enhances its exotic properties and makes it viable and resourceful for multiple usage at larger scale. Presently, ZnO NPs are in uses as UV-filters [4], antimicrobial agent [5], bio-remediating agent [6], nano-fertilizers component [7], food-packaging purposes [8], in electronics, in textiles [9], for biomedical purposes [10] and so on. Earlier studies revealed that the ZnO NPs are kinetically active and undergoes different transformations which in returns causes toxicological issues. Dissolution, aggregation, agglomeration, adsorptions are the prime factors associated with the ZnO NPs toxicity. Still a clear-cut relationship about the mechanism of toxicity has not been established

Abbreviations: ANOVA, analysis of variance; BG-11, blue green-11; BSA, bovine serum albumin; CAT, catalase; CDH, central drug house; DDW, double distilled water; IC₅₀, half maximal inhibitory concentration; Fe₂O₃, ferric oxide; FTIR, fourier-transform infrared spectroscopy; JCPDS, Joint Committee on Powder Diffraction Standards; LDH, lactate dehydrogenase; MDA, malondialdehyde assay; NADH, nicotinamide adenine dinucleotide (reduced form); NCBI, national center for biotechnology information; OD, optical density; NPs, nanoparticles; PBS, phosphate-buffered saline; PDI, polydispersity index; ROS, reactive oxygen species; SD, standard deviation; SEM, scanning electron microscopy; SOD, superoxide dismutase; TEM, transmission electron microscopy; UV, ultra violet; XRD, X-ray diffraction; ZnO, zinc oxide.

* Corresponding author.

E-mail address: harish.botany1979@gmail.com (Harish).

<https://doi.org/10.1016/j.toxrep.2021.03.023>

Received 13 November 2020; Received in revised form 23 March 2021; Accepted 26 March 2021

Available online 1 April 2021

2214-7500/© 2021 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

with respect to algae [11]. The testified toxicity is changed with respect to the species [12,13], particle nature [13] and test methods [14]. Albeit toxicological studies regarding the effects of ZnO NPs had been performed on different algae earlier but not in term of growth kinetics of algae [15–20]. Applications of iron oxide (Fe_2O_3) NPs are also increasing due to its use for different purposes like in agriculture as fertilizers [21], for waste-water management [22,23] for number of biomedical applications [24,25]. Large amount of usage inevitably enhances the release of higher quantity of iron-based NPs into the environment [26]. The ample amount of usage of these NPs will ultimately sink into the aquatic environment. They are entering into the aquatic ecosystem and induces toxicity to the flora and fauna [27]. Overall potential impact of these NPs release into the aquatic environment is still unknown which escalates the concern to evaluate the possible risks regarding the aquatic entities [28]. As algae constitute the key source of biomass production which supports all higher trophic levels of the aquatic body, therefore, they are an ideal model organism to assess the impact of these metal oxide NPs [12,13]. In addition to that, algae have low nutritional requirements and easy in handling. It also allows whole life-cycle assessment within short period of time. For that reason, to assess the impact of NPs, algae seem to be an epitomic monitoring tool [29,30]. The aim of this study is to do comparative analysis of toxicity of NPs and bulk form exposure on algal growth kinetics. In order to investigate the unresolved mechanism of toxicity of NPs, impact in terms of morphology, physiology and biochemistry has been evaluated after treating the algal cultures with different concentrations of respective NPs and their bulk counterpart.

2. Materials and methods

2.1. Algal culture establishment

Algal sample was collected from the Fatehsagar Lake, Udaipur, Rajasthan, India. Further, *Chlorella vulgaris* alga is isolated, purified and axenic culture is established through serial dilution and plating in BG-11 medium with 7.4 pH [31]. Algal cultures were maintained in 250 mL Erlenmeyer flasks at $25 \pm 1^\circ\text{C}$ under 14.5 Wm^{-2} light intensity. Algal cultures were sustained through frequent sub-culturing in fresh media after harvesting of samples during exponential phase of previous cultures after measuring the protein value. All the experiments were performed under the same culture conditions in triplicates.

2.2. Nanoparticle characterization and dispersion

ZnO and Fe_2O_3 NPs were purchased from Sigma-Aldrich (CAS No. 1314-13-2–030-013–007; CAS No. 1317-61-9) and their bulk counterparts from Central Drug House Private Limited (CAS: 1314-13-2; CAS No. 1309-37-1). Stock solutions for both nano and bulk form of the metal oxides (10 mg each) were prepared in 100 mL deionized water. Stock suspension of ZnO nanoparticles was sonicated for 30 min at 40 Hz, and for Fe_2O_3 NPs, sonication was performed for 45 min at 60 Hz by using Probe sonicator (Q-500, Qsonica, USA). From these stock solutions, working solutions were made in BG-11 medium for toxicological assessment as per the tolerance range of the algae. NPs within the BG-11 media were also characterized using dynamic light scattering (DLS) to estimate the particle size distribution and zeta potential (ZS90, Malvern, Instruments, UK).

2.3. Algal growth kinetics

Different concentrations of NPs and their bulk counterpart dispersed within the BG-11 medium were inoculated with algal inoculum ($100 \mu\text{g ml}^{-1}$ of protein value). Algal growth was evaluated on the basis of protein quantification [32,33] using bovine serum albumin as standard and absorbance of the samples were taken at 650 nm using UV–vis Spectrophotometer (Hitachi U2900). Protein content of treated cultures

was measured at intermittent interval starting from 5th day of inoculation, and after every fifth day.

2.4. Proline content

Algal suspensions were harvested after 25th days of treatment and suspended into 10 mL sulpho-salicylic acid [34]. Samples were centrifuged and 2 mL ninhydrin and 2 mL glacial acetic acid were added in 4 mL supernatant and placed on water bath for half an hour at 100°C . Lastly, 4 mL of toluene solution were added in each sample and shaken well. Red chromophore layer of toluene was formed at upper surface which was used to take OD at 520 nm using UV–vis Spectrophotometer maintaining toluene as control.

2.5. Chlorophyll and carotenoids content

Total chlorophyll content was measured according to Porra et al. [35] and carotenoids content were estimated through Lichtenthaler and Wellburn [36]. In brief, algal cells (10 mL) were harvested after 25th days of treatment from cultures after centrifugation at 7000 rpm for 10 min. Then supernatant was discarded and pellets were washed with DDW. After washing, 10 mL methanol were added in and shaken thoroughly. Then, samples were placed on water bath at 60°C for 15 min. Again, samples were centrifuged (4000 rpm for 5 min) and supernatant were collected to take OD at 663 nm and 645 nm. Remaining pellets were mixed in 10 mL DDW and placed at 4°C for 1 h and centrifuged (4000 rpm for 5 min). Supernatant was collected and OD is taken at 470 nm. Methanol was considered as blank during all above estimation. Further pigment content is calculated using following equations: Total chlorophyll = $17.76 (A_{646.6}) + 7.34 (A_{663.6})$, Carotenoids = $(1000 \times O.D_{470} - 2.86 \times \text{Chl}_a - 129.2 \text{Chl}_b / 245) \mu\text{g/mg}$.

2.6. Lipid peroxidation (Malondialdehyde Assay)

Lipid peroxidation was estimated by spectrophotometer method as described earlier [37]. In brief, 1.0 mL of algal cell suspension (after 25th days of treatment) was added in to 2 mL of trichloroacetic acid (20 %) and centrifuged for 45 min at 7000 rpm. Supernatant was collected and further 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 using UV–vis spectrophotometer.

2.7. Superoxide dismutase (SOD) enzyme activity

SOD activity was measured by adding 2 mL PBS buffer solution (0.5 M; pH 7.5) in 50 mg biomass of algal samples collected after 25th days of treatment. Samples were centrifuged for 10 min at 4°C at 13000 rpm and 100 μL supernatant was mixed with reaction mixture. Further, samples were incubated for 10 min at 37°C and absorbance were recorded at 560 nm [38].

2.8. Catalase (CAT) enzyme activity

To measure CAT activity after 25th day of treatment, 50 mg interacted algal biomass was mix in 2 mL PBS buffer (0.5 M; 7.5 pH). Samples were centrifuged at 12000 rpm at 4°C for 30 min and 100 μL supernatant was mixed in reaction mixture [39], containing 1.6 mL PBS (0.1 M, 7.8 pH), 100 μL EDTA (3 mM), 200 μL H_2O_2 (0.3 %). Absorbance was taken at 240 nm using UV–vis spectrophotometer and the activity was represented in terms of % decrease with respect to the control.

2.9. Lactate dehydrogenase (LDH) enzyme activity

Algal suspension after 25th day of treatment was centrifuged at 7000 rpm for 10 min and 100 μL supernatant was suspended in 100 μL of sodium pyruvate (30 mM) followed by addition of 2.8 mL of Tris–HCl

(0.2 M). Just before measuring the decrease in absorbance, about 100 μL of NADH (6.6 mM) was added and ten readings were measured at 340 nm using UV–vis spectrophotometer [40].

2.10. Microscopic analysis

To confirm the cytological damages, TEM (Tecnai, G-20 (FEI), USA) and SEM (Zeiss, Germany) analysis was performed. Algal cells treated with 5 mgL^{-1} of ZnO and 25 mgL^{-1} Fe_2O_3 NPs, bulk forms and control were harvested after 25 days, and used for TEM and SEM analyses. Ultrathin sections of the samples were obtained from ultramicrotome and shifted to copper grid for TEM analysis. For SEM study, air dried algal samples were subjected to gold sputter coating and analyzed.

2.11. Statistical analysis

All the experiments were conducted in triplicates. Mean, standard deviations, correlation and regression parameters were calculated using MS-Excel (office version 10.0). Statistically significant difference between control and treatment were analyzed using one-way ANOVA with the help of SPSS software version 17, with probability of error (P values) is taken as less than 0.5, for determining the significant differences.

3. Results and discussion

3.1. Nanoparticle characterization

Transmission electron microscopy (TEM) investigation uncovered

polymorphic shaped ≤ 100 nm sized ZnO NPs, while, Fe_2O_3 NPs exhibited 50 nm size with nearly spherical shape (Fig. 1). XRD peaks of the NPs and bulk counterpart were observed in accordance with the standard graphs database of Joint Committee on Powder Diffraction Standards (JCPDS) (Fig. 1). In DLS study, hydrodynamic size of ZnO and Fe_2O_3 NPs were 249.1 nm and 900 nm, respectively, whereas, zeta potential was -25.5 mV and -19.9 mV for ZnO and Fe_2O_3 NPs in the working media. Results indicated that NPs possess enough charge on them to keep themselves suspended with in the media and avoid agglomeration.

3.2. Algal growth kinetics

Growth kinetics of *Chlorella vulgaris* were evaluated under ZnO and Fe_2O_3 treatments (both NPs and bulk) by monitoring the algal growth up to 35 days on the basis of protein value. Analysis revealed that the nano form of ZnO and Fe_2O_3 particles were more toxic than their bulk counterparts as observed in change of growth kinetics (Fig. 2). Moreover, perniciousness was found directly proportional to the concentration under bulk as well as nano treatments in both ZnO and Fe_2O_3 [41, 42]. In case of ZnO, at the highest concentration i.e., 5 mgL^{-1} , 42 % reduction in protein-based growth-rate was observed under NPs exposure, while nearly 35 % reduction is observed under bulk chemical, in comparison to control on the 25th day. At lower dose, i.e., at 2.5 mgL^{-1} concentration, reduction in growth were almost same in both (nano and bulk form) which was about 21 % and 20 %, respectively. Whereas, at the lowest concentration of treatment, reduction was not found significant under nano as well as bulk. IC_{50} in case of nanoform was found to

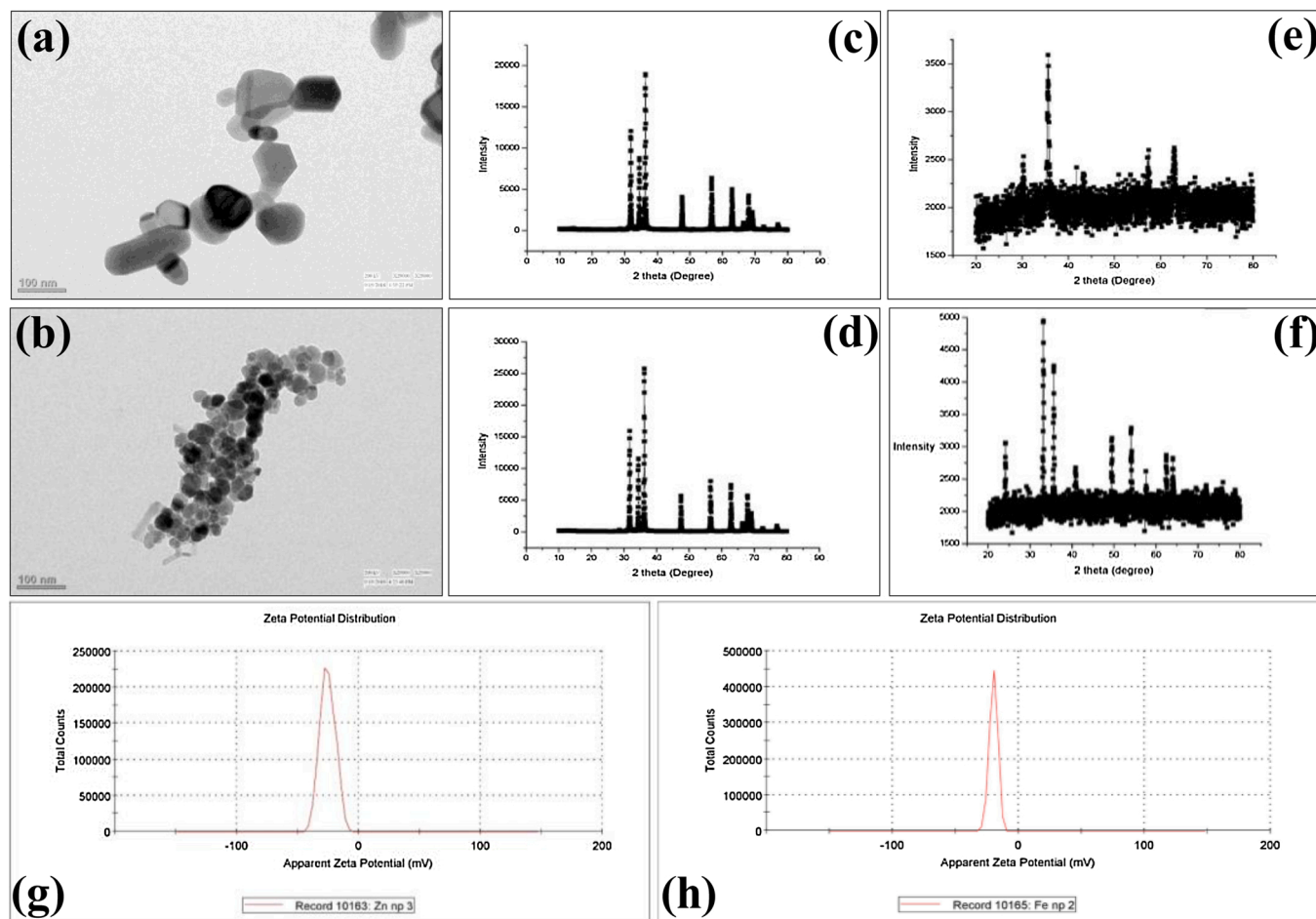


Fig. 1. Characterization of NPs. Transmission electron micrographs of ZnO (a) and Fe_2O_3 (b) NPs and XRD graphs of their nano and bulk forms respectively; ZnO (c and d) and Fe_2O_3 (e and f). Zeta Potential of ZnO (g) and Fe_2O_3 (h) NPs within the medium.

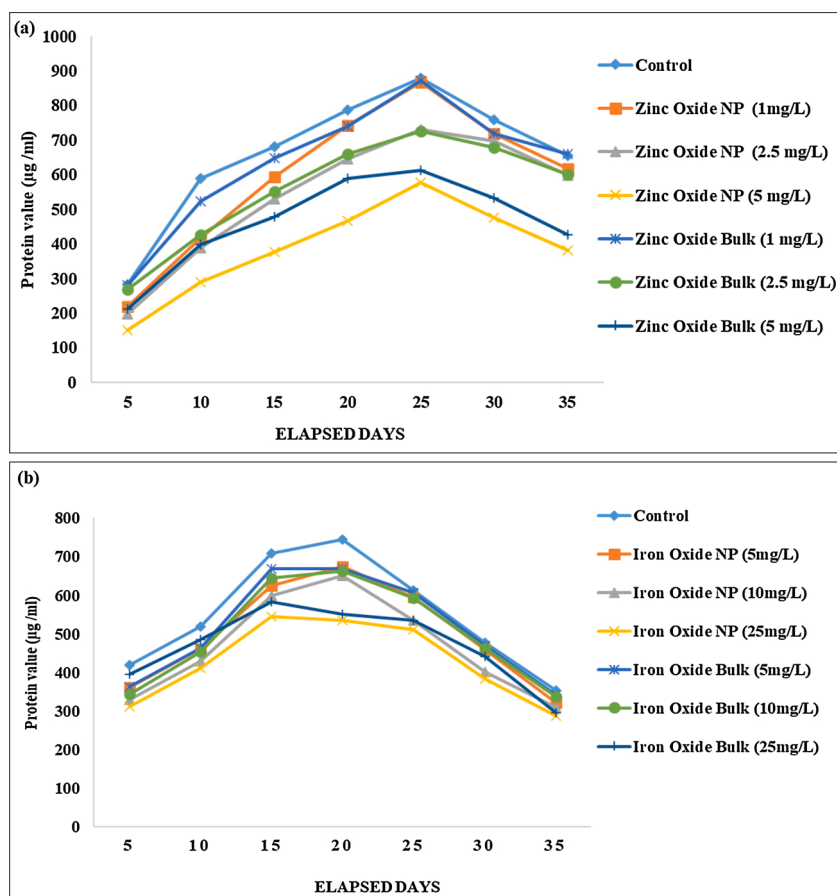


Fig. 2. Effect of ZnO (a) and Fe₂O₃ (b) NPs and their bulk counterparts on *Chlorella vulgaris* growth kinetics estimated via protein content.

be 25th day = 0.258 mg L⁻¹ and in case of bulk, IC₅₀ values was 1.255 mg L⁻¹. It is also found from the data that the inhibitory concentration was lowered down, with the respect to increase in the time of exposure, indicating that the time also plays a crucial role in attributing toxicity. Under Fe₂O₃ treatment, it is observed that 25 mg L⁻¹ concentration as sub-lethal for *Chlorella vulgaris*, as 26 % of the algal growth was found suppressed in nano-form, whereas, 25.44 % reduction is observed under bulk treatment in comparison to the control. Even at the lowest dosage of concentration i.e., 5 mg L⁻¹, 11 % and 9.4 % reduction in algal growth rates were examined. Likewise, at 10 mg L⁻¹, 12.5 % reduction under nano and 11 % less growth under bulk form were found. IC₅₀ values was observed at 12.99 mg L⁻¹ in case of nano and 17.88 mg L⁻¹ in case of bulk form of Fe₂O₃ treatment.

Under both metal oxide treatments, NPs exerted more toxicity and that was due to more interaction of NPs to algal surfaces as NPs having more surface area than its bulk forms [43]. Moreover, interactions were found dosage dependent as higher concentration increases the probability of more interactions of NPs towards algal cell surfaces [44,45]. In the case of ZnO, particles tend to lose the repulsive force with time, hence, tend to aggregate. Similar conceptions were also reported by few other studies performed under ZnO NPs [46–48]. Moreover, delayed in the onset of the exponential phase can also be accredited to the dissolution of ZnO NPs and its bulk form with in the algal culture media along with the aggregation under both of the cases. As the initial hours after the exposure, dissolution of zinc ions contributed toxicity within algal cells [49,50]. The dissolution of ZnO NPs is previously reported to be dependent on the initial exposure concentration [51]. Similarly, under Fe₂O₃ NPs treatment, NPs adhered to the algal cell surface and established the primary contact which might have hindered the ion exchange of the cell membrane. Due to this interference, it might have affected the ion transport and damaged the membrane. Due to the presence of

particles over the algal cells, it obstructed the nutrient exchange from the medium, which ultimately suppresses the metabolic activities and attributed to the toxicity [52,53]. Moreover, these interactions could play a vital role in developing oxidative stress (discussed below). Aggregation and adherence of NPs developed stronger interactions between the algal cell surface and particles due to which oxidative stress was observed within the treated algal cells [54]. The oxidative stress was mediated by the production and accumulation of ROS and later on confirmed through the various biochemical analysis and algal growth kinetics [55].

3.3. Proline content

Proline is known to play a role of antioxidant by mitigating ROS [56]. As ZnO NPs were reported to possess strong protein adsorption potential [57] which could lead to the elevated intracellular concentration of ZnO NPs within the algal cells and subsequently triggered the ROS production [58]. Increased level of proline content marked their role as ROS scavenger. Moreover, proline content increased in dose-dependent manner under both form (Table 1). Likewise, under the Fe₂O₃ NP treatments, much higher levels of proline content were examined in comparison to bulk counterpart (Table 2). Results clearly revealed that the size of the particle plays a vital role in attributing toxicity as far as proline parameter is concerned [59]. Increased proline abates the oxidative stress generated by ROS within the treated algal cells, which was clearly distinguished from untreated algal cells in both forms.

3.4. Chlorophyll and carotenoid content

Chlorophyll content was found significantly reduced under both bulk

Table 1Biochemical parameters analysis of *Chlorella vulgaris* under the treatment of ZnO NPs and its bulk counterpart.

ZnO treatment	Proline (µg/mL)	Total chlorophyll (µg mL ⁻¹)	Carotenoids (µg mL ⁻¹)	MDA (µmol/g)	LDH (nmol/min _{ml})	SOD (µ mg ⁻¹ prot)	CAT (µ mg ⁻¹ prot)
Control	0.04 ± 0.1	13.2674 ± 0.06	12.9200 ± 0.05	0.1005 ± 0.3	0.005 ± 0.2	2.55 ± 0.3	0.05 ± 0.1
ZnO NP (1 mg/L)	2.66 ± 0.1	9.1940 ± 0.5	15.7533 ± 0.08	5.3218 ± 0.3	10.26 ± 0.2	18.88 ± 0.3	6.26 ± 0.3
ZnO NP (2.5 mg/L)	5.60 ± 0.2	6.8437 ± 0.2	18.2866 ± 0.06	9.4080 ± 0.4	12.22 ± 0.3	27.44 ± 0.2	8.36 ± 0.2
ZnO NP (5 mg/L)	6.27 ± 0.1	3.6802 ± 0.3	22.7266 ± 0.05	13.5917 ± 0.3	19.69 ± 0.2	34.62 ± 0.3	11.79 ± 0.3
P value summary	P < 0.0001***	P < 0.0001**	P < 0.0001***	P < 0.001**	P < 0.0001***	P < 0.0001***	P < 0.0001***
Control	0.04 ± 0.1	13.2674 ± 0.06	12.9200 ± 0.05	0.1005 ± 0.3	0.005 ± 0.2	2.55 ± 0.3	0.03 ± 0.1
ZnO Bulk (1 mg/L)	1.75 ± 0.2	11.9075 ± 0.08	10.7266 ± 0.02	3.2280 ± 0.4	6.27 ± 0.3	10.86 ± 0.2	4.77 ± 0.2
ZnO Bulk (2.5 mg/L)	3.86 ± 0.1	9.9621 ± 0.01	14.3266 ± 0.03	7.3968 ± 0.2	9.44 ± 0.2	19.62 ± 0.2	5.87 ± 0.1
ZnO Bulk (5 mg/L)	4.22 ± 0.1	4.8790 ± 0.01	18.6533 ± 0.04	11.4880 ± 0.5	15.67 ± 0.2	29.54 ± 0.2	9.32 ± 0.1
P value summary	P < 0.0001***	P < 0.0001***	P < 0.0001***	P < 0.001***	P < 0.0001***	P < 0.0001***	P < 0.0001***

** Statistically highly significant difference.

*** Highly highly significant difference after analysis of variance.

Table 2Biochemical parameters analysis of *Chlorella vulgaris* under the treatment of Fe₂O₃ NPs and its bulk counterpart.

Fe ₂ O ₃ Treatment	Proline (µg/mL)	Total chlorophyll (µg mL ⁻¹)	Carotenoids (µg mL ⁻¹)	MDA (µmol/g)	LDH (nmol/min _{ml})	SOD (µ mg ⁻¹ prot)	CAT (µ mg ⁻¹ prot)
Control	0.108 ± 0.3	12.2674 ± 0.06	2.9200 ± 0.05	0.05 ± 0.1	0.006 ± 0.2	1.95 ± 0.3	2.55 ± 0.3
Fe ₂ O ₃ NP (5 mg/L)	8.325 ± 0.3	8.1940 ± 0.5	15.753 ± 0.08	3.26 ± 0.3	8.26 ± 0.2	6.88 ± 0.3	14.88 ± 0.3
Fe ₂ O ₃ NP (10 mg/L)	17.448 ± 0.4	7.8437 ± 0.2	12.286 ± 0.06	7.36 ± 0.2	10.22 ± 0.3	9.44 ± 0.2	17.84 ± 0.2
Fe ₂ O ₃ NP (25 mg/L)	25.581 ± 0.3	3.6802 ± 0.3	17.726 ± 0.05	9.79 ± 0.3	12.69 ± 0.2	14.62 ± 0.3	24.62 ± 0.3
P valueP value summary	P < 0.001**	P < 0.0001**	P < 0.0001***	P < 0.0001***	P < 0.0001***	P < 0.0001***	P < 0.0001***
Control	0.108 ± 0.3	12.2674 ± 0.06	2.9200 ± 0.05	0.05 ± 0.1	0.006 ± 0.2	1.95 ± 0.3	2.55 ± 0.3
Fe ₂ O ₃ Bulk (5 mg/L)	6.222 ± 0.4	10.9075 ± 0.08	9.7266 ± 0.02	2.77 ± 0.2	5.27 ± 0.3	3.86 ± 0.2	9.86 ± 0.2
Fe ₂ O ₃ Bulk (10 mg/L)	13.365 ± 0.2	9.9621 ± 0.01	11.3266 ± 0.03	6.87 ± 0.1	7.44 ± 0.2	5.62 ± 0.2	12.62 ± 0.2
Fe ₂ O ₃ Bulk (25 mg/L)	21.489 ± 0.5	5.8790 ± 0.01	15.6533 ± 0.04	8.32 ± 0.1	11.67 ± 0.2	9.54 ± 0.2	19.64 ± 0.2
P valueP value summary	P < 0.001***	P < 0.0001***	P < 0.0001***	P < 0.0001***	P < 0.0001***	P < 0.0001***	P < 0.0001***

** Statistically highly significant difference.

*** Highly highly significant difference after analysis of variance.

and NPs exposure as shown in Tables 1 and 2 [60,61]. Under NPs exposure even at the lowest concentration (1 mgL⁻¹) significant reduction in chlorophyll pigment was observed. Concentration dependent decrease was observed, in both forms, however, in case of NPs more decline of chlorophyll pigment was apparent than the bulk [62]. Similarly, under Fe₂O₃, reduction in chlorophyll content was observed depicting the impairment in photosynthetic machinery in the treated algal cells. As the dose for exposure was increased, decrease in the chlorophyll content was recorded for both forms [63,64]. Contrary, carotenoids content was increased after the exposure under both of the treatments. Carotenoids are known as efficient quenchers of triplet state photosensitizers, singlet oxygen and radicals [65] which strengthen our view that this may be the defense mechanism of algae cells when exposed to metal. Similar observations reported earlier during heavy metal stress in algae [66,17,18].

3.5. Lipid peroxidation (Malondialdehyde Assay)

Peroxidation of lipid molecules is an indication of oxidative stress. In the present investigation, increase in peroxidation with the increase in concentrations under both bulk and nano form of ZnO is reported (Table 1). Highest value of peroxidation was at 5 mgL⁻¹ under nano and bulk particles of ZnO. Dose-dependent nature of peroxidation was also observed in the previous studies [17,67,18]. Increased in both proline and lipid peroxidation with the increase in concentration pinpointing a correlation between ROS generation and their scavenging by proline. Moreover, lipid peroxidation has been reported to inactivate several essential enzymes and destabilizing the cell membrane which further diminishing the metabolic activity of the cells, therefore, became an elusive reason of growth retardation of algae under treatments [68]. In

Fe₂O₃ NPs, more peroxidation was found compared to bulk at the same concentration (Table 2). As explained earlier, higher surface area enables NPs to attach more to surface of algae and endowed membrane destabilization through lipid peroxidation via ROS production [17,69,70,18].

3.6. Lactate dehydrogenase (LDH) activity

Higher level of LDH enzyme activity is cue of cellular toxicity and under NPs treatments, higher LDH activity was observed, revealing that the NPs were more toxic than their bulk counterparts [71]. Membrane damage signifies the toxic effects generated after the exposure of ZnO/Fe₂O₃ NPs and its bulk counterpart [72]. Under NPs exposure, more membrane damage was apparent than the bulk. At similar concentration, NP treated cells was found to have higher LDH activity, in comparison to the both metal oxide bulk [51].

3.7. Superoxide dismutase (SOD) activity

In present investigation, dose dependent SOD activity was observed in the experiments. Even at the lowest concentration of NPs, enzymatic activity was found significantly higher than the bulk (Tables 1 and 2). Increase in SOD activity is directly related with the decrease in ROS content [73,74]. Higher activity of SOD converts O₂⁻¹ (superoxide) in to H₂O₂ (hydrogen peroxide). However, the amount of activity required to scavenge the ROS was not enough in the treated algae cells [38].

3.8. Catalase (CAT) activity

CAT enzyme activity was up-regulated during treatment with NPs

and its bulk counterpart in dose dependent manner as compared to untreated cells (Tables 1 and 2) [75]. Highest activity of CAT was observed at 5 and 25 mgL⁻¹ of ZnO and Fe₂O₃ NPs. Although H₂O₂ is less toxic compared to O₂¹ and based on the growth retardation of algae, we foresee that higher accumulation of H₂O₂ may have spurred toxic effect to growing algae cells [76,77]. Thus, higher activity of CAT which we measured in the treated cells not adequate to reduce the oxidative stress of the algae cells in NPs treatment.

3.9. Microscopic analysis

Compound microscopic images revealed change in shape and colour of treated algal cells in comparison to the untreated and ZnO treatments (Fig. 3). Moreover, aggregation of ZnO NPs as well as bulk particles around the algal cells were also observed. Similarly, under Fe₂O₃ treatment, aggregation and agglomeration were found in nano and bulk particles (Fig. 3). This phenomenon of shading effect was found to play a prominent role in case of Fe₂O₃ exposure in attributing toxicity [78,79]. Scanning electron microscopic (SEM) analysis revealed the detrimental effects after the exposure under both nano as well as bulk ZnO particles. Cell lysis and cell membrane damage is observed from the micrographs (Fig. 3). Earlier studies revealed that, with respect to the time, aggregation tendency of ZnO NPs increases as the repulsive force in between the particles tend to decrease [80]. Further, interrupted growth is also due to the reduced availability of nutrients. As the surface area of algal cell is occupied by ZnO particles, interface area for nutrient exchange from the medium is reduced [81,82]. Likewise, under Fe₂O₃ NPs, cell lysis and cell injury, is observed from micrographs taken under

25 mgL⁻¹ treatment of both nano and bulk particles. Transmission electron micrographs (TEM) showed significant changes in cell morphology (Fig. 3). Cell organelles were found collapsed, cell shapes were deformed and plasmolysis is observed and these changes are more pronounced in nano form of treatment. Similar, cellular damages where cell integrity may be persistent but disruption within the cells were prominent have been reported by Xia et al. [83]. Cells were collapsed as the internal machinery was completely found destructed under the exposure due to stress.

3.10. Conclusion

The present investigation uncovered that ZnO and Fe₂O₃ NPs induces the antioxidant defense system of the cell. However, biochemical defense machinery is not sufficient enough to cope up with the stress at higher doses of treatment. The sub-cellular organelle damage, membrane disintegration, and oxidative stress are the main reasons for toxicity of the NPs to the algal cell at tolerance threshold level. This investigation uncovered that nano form, in general, is more toxic to the *C. vulgaris* as far as ZnO and Fe₂O₃ compounds are concerned and it has interceded fundamentally through oxidative stress. The higher interaction of NPs due to more surface area in comparison to the bulk form, led to diminishing in the nutrient exchange interface of the algal cells. This led to a reduction in the growth rate of the algae. The shading effect also contributed to the reduced growth rate of algae. The membrane damage as observed by lipid peroxidation, LDH activity and loss in the chlorophyll content further deteriorated the growth of the algae. Moreover, exposure to these chemicals led to the generation of ROS and oxidative

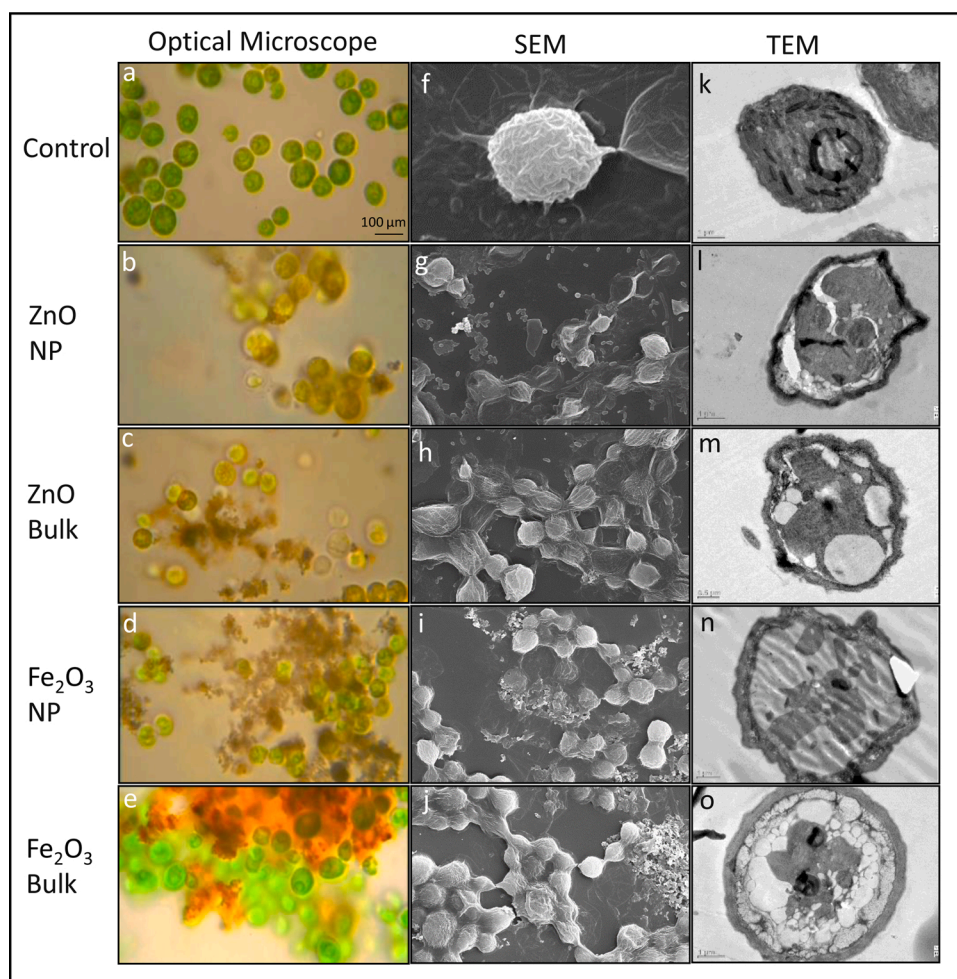


Fig. 3. Microscopic images of *Chlorella vulgaris* under control and different treatment of nano/bulk particles after 600 h. Optical microscopic images: (a) Control (b) ZnO NP treated cells (c) ZnO bulk treated cells (d) Fe₂O₃ NP treated cells (e) Fe₂O₃ bulk treated cells. Scanning electron microscopic images: (f) Control (g) ZnO NP treated cells (h) ZnO bulk treated cells (i) Fe₂O₃ NP treated cells (j) Fe₂O₃ bulk treated cells. Transmission electron microscopic images: (k) control (l) ZnO NP treated cells (m) ZnO bulk treated cells (n) Fe₂O₃ NP treated cells (o) Fe₂O₃ bulk treated cells. Treatment level for ZnO was 5 mgL⁻¹ concentrations (both NP and bulk) and for Fe₂O₃ was 25 mgL⁻¹ concentrations (both NP and bulk).

stress. Accumulation of proline, increase in activity of SOD and CAT indicated that the extent to which these are needed to cope with oxidative stress are not sufficient in the algal cells treated with NPs. These parameters ascertained the algal cells treated with NPs remained under higher oxidative stress in contrast to bulk treatment. Microscopic images disclosed the structural damages to the subcellular organelle of the algae due to treatment of ZnO and Fe₂O₃, which were more distinct in the nano form than bulk.

Conflict of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Pallavi Saxena: Investigation, Methodology, Writing - original draft. **Vinod Saharan:** Resources, Visualization, Data curation. **Prabhat Kumar Baroliya:** Resources, Validation. **Vinod Singh Gour:** Writing - review & editing. **Manoj Kumar Rai:** Formal analysis, Validation. **Harish:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgment

Contribution of Pallavi Saxena to this study was financially supported by University Grants Commission (UGC), New Delhi, India in the form of BSR meritorious fellowship [F.25-a/2013-14(BSR)/7-125/2007 (BSR)]. We wish to acknowledge SAIF, New Delhi for extending electron microscopy facilities. Financial support from Department of Science and Technology, New Delhi for laboratory infrastructure is also acknowledged (SERB File Number: EEQ/2020/000011). We are also thankful to Prof. N. Laxmi, Department of Physics, MLSU, Udaipur for providing us XRD facilities.

References

- [1] B. Bhushan, Springer Handbook of Nanotechnology, Springer, 2017.
- [2] Z.L. Wang, Zinc oxide nanostructures: growth, properties and applications, J. Phys. Condens. Matter 16 (2004) R829.
- [3] F. Piccinno, F. Gottschalk, S. Seeger, B. Nowack, Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world, J. Nanopart. Res. 14 (2012) 1109.
- [4] C.B. Ong, L.Y. Ng, A.W. Mohammad, A review of ZnO nanoparticles as solar photocatalysts: synthesis, mechanisms and applications, Renew. Sust. Energ. Rev. 81 (2018) 536–551.
- [5] A. Singh, N.B. Singh, S. Afzal, T. Singh, I. Hussain, Zinc oxide nanoparticles: a review of their biological synthesis, antimicrobial activity, uptake, translocation and biotransformation in plants, J. Mater. Sci. 53 (2018) 185–201.
- [6] E.A.S. Dimapilis, C.S. Hsu, R.M.O. Mendoza, M.C. Lu, Zinc oxide nanoparticles for water disinfection, Sustain. Environ. Res. 28 (2018) 47–56.
- [7] R. Raliya, V. Saharan, C. Dimkpa, P. Biswas, Nanofertilizer for precision and sustainable agriculture: current state and future perspectives, Agric. Food Chem. 66 (2017) 6487–6503.
- [8] M. Ejaz, Y.A. Arfat, M. Mulla, J. Ahmed, Zinc oxide nanorods/clove essential oil incorporated Type B gelatin composite films and its applicability for shrimp packaging, Food Packag. Shelf Life 15 (2018) 113–121.
- [9] N. Vigneshwaran, V. Prasad, A. Arputharaj, A.K. Bharimalla, P.G. Patil, Nano-zinc oxide: prospects in the textile industry, in: S. Ul-Islam, B.S. Butola (Eds.), Nanomaterials in the Wet Processing of Textiles, Volume 1, John Wiley & Sons, Hoboken, NJ, USA, 2018, pp. 113–134.
- [10] M. Khatami, R.S. Varma, N. Zafarnia, H. Yaghoobi, M. Sarani, V.G. Kumar, Applications of green synthesized Ag, ZnO and Ag/ZnO nanoparticles for making clinical antimicrobial wound-healing bandages, Sustain. Chem. Pharm. 10 (2018) 9–15.
- [11] N. Ye, Z. Wang, S. Wang, W.J. Peijnenburg, Toxicity of mixtures of zinc oxide and graphene oxide nanoparticles to aquatic organisms of different trophic level: particles outperform dissolved ions, Nanotoxicology (2018) 1–16.
- [12] X. Chen, C. Zhang, L. Tan, J. Wang, Toxicity of Co nanoparticles on three species of marine microalgae, Environ. Pollut. 236 (2018) 454–461.
- [13] K. Pikula, N. Mintcheva, S.A. Kulinich, A. Zakharenko, Z. Markina, V. Chaika, et al., Aquatic toxicity and mode of action of CdS and ZnS nanoparticles in four microalgae species, Environ. Res. 186 (2020), 109513.
- [14] V. Merdzan, R.F. Domingos, C.E. Monteiro, M. Hadioui, K.J. Wilkinson, 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 (2014) 316–324.
- [15] N.M. Franklin, N.J. Rogers, S.C. Apte, G.E. Batley, G.E. Gadd, P.S. Casey, Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl₂ to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility, Environ. Sci. Technol. 41 (2007) 8484–8490.
- [16] X. Peng, S. Palma, N.S. Fisher, S.S. Wong, Effect of morphology of ZnO nanostructures on their toxicity to marine algae, Aquat. Toxicol. 102 (2011) 186–196.
- [17] P. Chen, B.A. Powell, M. Mortimer, P.C. Ke, Adaptive interactions between zinc oxide nanoparticles and *Chlorella* sp, Environ. Sci. Technol. 46 (2012) 12178–12185.
- [18] P.J. Chen, S.W. Tan, W.L. Wu, Stabilization or oxidation of nanoscale zerovalent iron at environmentally relevant exposure changes bioavailability and toxicity in medaka fish, Environ. Sci. (Ruse) 46 (2012) 8431–8439.
- [19] J. Li, S. Schiavo, G. Rametta, M.L. Miglietta, V. La Ferrara, C. Wu, S. Manzo, Comparative toxicity of nano ZnO and bulk ZnO towards marine algae *Tetraselmis suecica* and *Phaeodactylum tricornutum*, Environ. Sci. Pollut. Res. 24 (2017) 6543–6553.
- [20] M. Bhuvaneshwari, V. Iswarya, S. Vishnu, N. Chandrasekaran, A. Mukherjee, Dietary transfer of zinc oxide particles from algae (*Scenedesmus obliquus*) to daphnia (*Ceriodaphnia dubia*), Environ. Res. 164 (2018) 395–404.
- [21] M. Rizwan, S. Ali, B. Ali, M. Adrees, M. Arshad, A. Hussain, A.A. Waris, Zinc and iron oxide nanoparticles improved the plant growth and reduced the oxidative stress and cadmium concentration in wheat, Chemosphere 214 (2019) 269–277.
- [22] A. Bedi, B.R. Singh, S.K. Deshmukh, A. Adholeya, C.J. Barrow, An *Aspergillus aculeatus* strain was capable of producing agriculturally useful nanoparticles via bioremediation of iron ore tailings, J. Environ. Manage. 215 (2018) 100–107.
- [23] O.M. Darwesh, I.A. Matter, M.F. Eida, Development of peroxidase enzyme immobilized magnetic nanoparticles for bioremediation of textile wastewater dye, J. Environ. Chem. Eng. 7 (2019), 102805.
- [24] L.S. Arias, J.P. Pessan, A.P.M. Vieira, T.M.T.D. Lima, A.C.B. Delbem, D. R. Monteiro, Iron oxide nanoparticles for biomedical applications: a perspective on synthesis, drugs, antimicrobial activity, and toxicity, Antibiotics 7 (2018) 46.
- [25] T. Vangijzegem, D. Stanicki, S. Laurent, Magnetic iron oxide nanoparticles for drug delivery: applications and characteristics, Expert Opin. Drug Deliv. 16 (2019) 69–78.
- [26] M. Mahmoudi, H. Hofmann, B. Rothen-Rutishauser, A. Petri-Fink, Assessing the *in vitro* and *in vivo* toxicity of superparamagnetic iron oxide nanoparticles, Chem. Rev. 112 (2011) 2323–2338.
- [27] M. Stefaniuk, P. Oleszczuk, Y.S. Ok, Review on nano zerovalent iron (nZVI): from synthesis to environmental applications, Chem. Eng. 287 (2016) 618–632.
- [28] A. Banerjee, A. Roychoudhury, Nanoparticle-induced ecotoxicological risks in aquatic environments: concepts and controversies, in: D.K. Tripathi, P. Ahmad, S. Sharma, D.K. Chauhan, N.K. Dubey (Eds.), Nanomaterials in Plants, Algae and Microorganisms, Academic Press, 2019, pp. 129–141.
- [29] J. Hou, Y. Wu, X. Li, B. Wei, S. Li, X. Wang, Toxic effects of different types of zinc oxide nanoparticles on algae, plants, invertebrates, vertebrates and microorganisms, Chemosphere 193 (2018) 852–860.
- [30] M. Dénél, N. Errien, P. Daniel, A. Caruso, F. Lagarde, Current methods to monitor microalgae-nanoparticle interaction and associated effects, Aquat. Toxicol. 217 (2019), 105311.
- [31] R. Rippka, J. Derelies, J.B. Waterbury, M. Herdman, R.V. Stainer, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, J. Gen. Microbiol. 111 (1979) 1–61.
- [32] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, Protein measurement with folin reagent, J. Biol. Chem. 193 (1951) 265–275.
- [33] D. Herbert, P.J. Phipps, R.E. Strange, The chemical analysis of microbial cell, in: J. R. Norris, D.W. Ribbons (Eds.), Methods in Microbiology, London Academic Press, London, 1971, pp. 209–344. Volume VB.
- [34] L.S. Bates, R.P. Waldren, I.D. Teare, Rapid determination of free proline for water-stress studies, Plant Soil 39 (1973) 205–207.
- [35] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, Biochim. Biophys. Acta Biomembr. 975 (3) (1989) 384–394.
- [36] H.K. Lichtenthaler, A.R. Wellburn, Determinations of Total Carotenoids and Chlorophylls a and B of Leaf Extracts in Different Solvents, 1983, pp. 591–592.
- [37] D.M. Metzler, M. Li, A. Erdem, C.P. Huang, Responses of algae to photocatalytic nano-TiO₂ particles with an emphasis on the effect of particle size, Chem. Eng. J. 170 (2011) 538–546.
- [38] K. Yilancioglu, M. Cokol, I. Pastirmaci, B. Erman, S. Cetiner, Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain, PLoS One 9 (2014), e91957.
- [39] R. Roy, A. Parashar, M. Bhuvaneshwari, N. Chandrasekaran, A. Mukherjee, Differential effects of P25 TiO₂ nanoparticles on freshwater green microalgae: *Chlorella* and *Scenedesmus* species, Aquat. Toxicol. 176 (2016) 161–171.
- [40] S. Pakrashi, S. Dalai, T.C. Prathna, S. Trivedi, R. Myneni, A.M. Raichur, N. Chandrasekaran, A. Mukherjee, Cytotoxicity of aluminium oxide nanoparticles

- towards fresh water algal isolate at low exposure concentrations, *Aquat. Toxicol.* 132 (2013) 34–45.
- [41] N.B. Hartmann, F. Von der Kammer, T. Hofmann, M. Baalousha, S. Ottofuelling, A. Baun, Algal testing of titanium dioxide nanoparticles-testing considerations, inhibitory effects and modification of cadmium bioavailability, *Toxicology* 269 (2010) 190–197.
 - [42] M. Samei, M.H. Sarrafzadeh, M.A. Faramarzi, The impact of morphology and size of zinc oxide nanoparticles on its toxicity to the freshwater microalga, *Raphidocelis subcapitata*, *Environ. Sci. Pollut. Res.* 26 (2019) 2409–2420.
 - [43] F. Chen, Z. Xiao, L. Yue, J. Wang, Y. Feng, X. Zhu, Z. Wang, B. Xing, Algae response to engineered nanoparticles: current understanding, mechanisms and implications, *Environ. Sci. Nano* 6 (2019) 1026–1042.
 - [44] S. Manzo, M.L. Miglietta, G. Rametta, S. Buono, G. Di Francia, Toxic effects of ZnO nanoparticles towards marine algae *Dunaliella tertiolecta*, *Sci. Total Environ.* 445 (2013) 371–376.
 - [45] F. Abdolapur Monikh, D. Arenas-Lago, P. Porcal, R. Grillo, P. Zhang, Z. Guo, M. G. Vijver, J.G.M. Peijnenburg W, Do the joint effects of size, shape and ecocorona influence the attachment and physical eco (cyto) toxicity of nanoparticles to algae? *Nanotoxicology* 14 (3) (2020) 310–325.
 - [46] L.J. Hazeem, M. Bououdina, S. Rashdan, L. Brunet, C. Slomianny, R. Boukherroub, Cumulative effect of zinc oxide and titanium oxide nanoparticles on growth and chlorophyll a content of *Picochlorum* sp., *Environ. Sci. Pollut. Res.* 23 (2016) 2821–2830.
 - [47] A. Oukarroum, I. Halimi, M. Sijaj, Cellular responses of *Chlorococcum* sp. Algae exposed to Zinc oxide nanoparticles by using flow cytometry, *Water Air Soil Pollut.* 230 (2019) 1.
 - [48] P. Saxena, Harish, Toxicity assessment of ZnO nanoparticles to freshwater microalgae *Coelastrum terrestre*, *Environ. Sci. Pollut. Res.* 26 (2019) 26991–27001.
 - [49] M. Li, Z. Luo, Y. Yan, Z. Wang, Q. Chi, C. Yan, B. Xing, Arsenate accumulation, distribution, and toxicity associated with titanium dioxide nanoparticles in *Daphnia magna*, *Environ. Sci. (Ruse)* 50 (2016) 9636–9643.
 - [50] F. Wu, B.J. Harper, S.L. Harper, Comparative dissolution, uptake, and toxicity of zinc oxide particles in individual aquatic species and mixed populations, *Environ. Toxicol. Chem.* 38 (2019) 591–602.
 - [51] M. Bhuvaneshwari, V. Iswarya, S. Archana, G.M. Madhu, G.S. Kumar, R. Nagarajan, N. Chandrasekaran, A. Mukherjee, Cytotoxicity of ZnO NPs towards fresh water algae *Scenedesmus obliquus* at low exposure concentrations in UV-C, visible and dark conditions, *Aquat. Toxicol.* 162 (2015) 29–38.
 - [52] Y. Wang, X. Zhu, Y. Lao, X. Lv, Y. Tao, B. Huang, Z. Cai, TiO₂ nanoparticles in the marine environment: physical effects responsible for the toxicity on algae *Phaeodactylum tricornutum*, *Sci. Total Environ.* 565 (2016) 818–826.
 - [53] Y. Tang, H. Xin, S. Yang, M. Guo, T. Malkoske, D. Yin, S. Xia, Environmental risks of ZnO nanoparticle exposure on *Microcystis aeruginosa*: toxic effects and environmental feedback, *Aquat. Toxicol.* 204 (2018) 19–26.
 - [54] S. Schiavo, M. Oliviero, J. Li, S. Manzo, Testing ZnO nanoparticle ecotoxicity: linking time variable exposure to effects on different marine model organisms, *Environ. Sci. Pollut. Res.* 25 (2018) 4871–4880.
 - [55] N. Fazelian, M. Yousefzadi, A. Movafeghi, Algal response to metal oxide nanoparticles: analysis of growth, protein content, and fatty acid composition, *Bioenergy Res.* (2020) 1–11.
 - [56] M. Choudhary, U.K. Jetley, M.A. Khan, S. Zutshi, T. Fatma, Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5, *Ecotoxicol. Environ. Saf.* 66 (2007) 204–209.
 - [57] M. Horie, K. Nishio, K. Fujita, S. Endoh, A. Miyauchi, Y. Saito, N. Nanashima, Protein adsorption of ultrafine metal oxide and its influence on cytotoxicity toward cultured cells, *Chem. Res. Toxicol.* 22 (2009) 543–553.
 - [58] T. Xia, M. Kovochich, M. Liong, L. Madler, B. Gilbert, H. Shi, A.E. Nel, Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties, *ACS Nano* 2 (2008) 2121–2134.
 - [59] S. Lopes, F. Ribeiro, J. Wojnarowicz, W. Łojkowski, K. Jurkschat, A. Crossley, S. Loureiro, Zinc oxide nanoparticles toxicity to *Daphnia magna*: size-dependent effects and dissolution, *Environ. Toxicol. Chem.* 33 (2014) 190–198.
 - [60] S.I.M. Houimli, M. Denden, B.M. Mouhades, Effects of 24-epibrassinolide on growth, chlorophyll, electrolyte leakage and proline by pepper plants under NaCl stress, *Eur. Asia J. Bio. Sci.* 4 (2010) 96–104.
 - [61] M. Shariati, A. Mallakin, F. Malekmohammady, F. Khosravi-Nejad, Inhibitory effects of functionalized indium doped ZnO nanoparticles on algal growth for preservation of adobe mud and earthen-made artworks under humid conditions, *Int. Biodeterior. Biodegr.* 127 (2018) 209–216.
 - [62] A. Sharan, S. Nara, Phytotoxic properties of zinc and cobalt oxide nanoparticles in algae, in: D.K. Tripathi, P. Ahmad, S. Sharma, D.K. Chauhan, N.K. Dubey (Eds.), *Nanomaterials in Plants, Algae and Microorganisms*, Academic Press, 2019, pp. 1–22.
 - [63] C. Saison, F. Perreault, J.C. Daigle, C. Fortin, J. Claverie, M. Morin, R. Popovic, Effect of core-shell copper oxide nanoparticles on cell culture morphology and photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas reinhardtii*, *Aquat. Toxicol.* 96 (2010) 109–114.
 - [64] F. Perreault, A. Oukarroum, L. Pirastru, L. Sirois, W. Gerson Matias, R. Popovic, Evaluation of copper oxide nanoparticles toxicity using chlorophyll fluorescence imaging in *Lemma gibba*, *J. Bot.* (2010).
 - [65] F. Wang, D. Banerjee, Y. Liu, X. Chen, X. Liu, Upconversion nanoparticles in biological labeling, imaging, and therapy, *Analyst* 135 (2010) 1839–1854.
 - [66] B.T. Bossuyt, C.R. Janssen, Long-term acclimation of *Pseudokirchneriella subcapitata* (Korshikov) Hindak to different copper concentrations: changes in tolerance and physiology, *Aquat. Toxicol.* 68 (2004) 61–74.
 - [67] Z.Y. Wang, J. Li, J. Zhao, B.S. Xing, Toxicity and internalization of CuO nanoparticles to prokaryotic alga *Microcystis aeruginosa* as affected by dissolved organic matter, *Environ. Sci. Technol.* 45 (2011) 6032–6040.
 - [68] E. Pinto, T.C. Sigaud-kutner, M.A. Leitao, O.K. Okamoto, D. Morse, P. Colepicolo, Heavy metal-induced oxidative stress in algae, *J. Phycol.* 39 (2003) 1008–1018.
 - [69] M. Auffan, W. Achouak, J. Rose, M.A. Roncato, C. Chanéac, D.T. Waite, J. Y. Bottero, Relation between the redox state of iron-based nanoparticles and their cytotoxicity toward *Escherichia coli*, *Environ. Sci. Technol.* 42 (2008) 6730–6735.
 - [70] T. Phenrat, T.C. Long, G.V. Lowry, B. Veronesi, Partial oxidation (“aging”) and surface modification decrease the toxicity of nanosized zerovalent iron, *Environ. Sci. Technol.* 43 (2008) 195–200.
 - [71] H. Zhang, Z. Ji, T. Xia, H. Meng, C. Low-Kam, R. Liu, S. Pokhrel, S. Lin, X. Wang, Y. P. Liao, M. Wang, Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation, *ACS Nano* 6 (2012) 4349–4368.
 - [72] H. Yang, C. Liu, D. Yang, H. Zhang, Z. Xi, 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 (2009) 69–78.
 - [73] A.M. Bhaduri, M.H. Fulekar, Antioxidant enzyme responses of plants to heavy metal stress, *Rev. Environ. Sci. Biotechnol.* 11 (2012) 55–69.
 - [74] A. Luis, L.M. Sandalio, F.J. Corpas, J.M. Palma, J.B. Barroso, Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling, *Plant Physiol.* 141 (2006) 330–335.
 - [75] A. Ševců, Y.S. El-Temsah, E.J. Joner, M. Černík, Oxidative stress induced in microorganisms by zero-valent iron nanoparticles, *Microbes Environ.* (2009), 1107220320–1107220320.
 - [76] S.P. Melegari, F. Perreault, R.H.R. Costa, R. Popovic, W.G. Matias, Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*, *Aquat. Toxicol.* 142 (2013) 431–440.
 - [77] T.Y. Suman, S.R. Rajasree, R. Kirubakaran, Evaluation of zinc oxide nanoparticles toxicity on marine algae *Chlorella vulgaris* through flow cytometric, cytotoxicity and oxidative stress analysis, *Ecotoxicol. Environ. Saf.* 113 (2015) 23–30.
 - [78] Z. Long, J. Ji, K. Yang, D. Lin, F. Wu, Systematic and quantitative investigation of the mechanism of carbon nanotubes’ toxicity toward algae, *Environ. Sci. Technol.* 46 (2012) 8458–8466.
 - [79] P.Y. Toh, B.W. Ng, C.H. Chong, A.L. Ahmad, J.W. Yang, C.J.C. Derek, J. Lim, Magnetophoretic separation of microalgae: the role of nanoparticles and polymer binder in harvesting biofuel, *RSC Adv.* 4 (2014) 4114–4121.
 - [80] M. Oliviero, S. Schiavo, G. Rametta, M.L. Miglietta, S. Manzo, Different sizes of ZnO diversely affected the cytogenesis of the sea urchin *Paracentrotus lividus*, *Sci. Total Environ.* 607 (2017) 176–183.
 - [81] V. Aruoja, H.C. Dubourguier, K. Kasemets, A. Kahru, Toxicity of nanoparticles of CuO, ZnO and TiO₂ to microalgae *Pseudokirchneriella subcapitata*, *Sci. Total Environ.* 407 (2009) 1461–1468.
 - [82] N. Gong, K.S. Shao, W. Feng, Z.Z. Lin, C.H. Liang, Y.Q. Sun, Biototoxicity of nickel oxide nanoparticles and bio-remediation by microalgae *Chlorella vulgaris*, *Chemosphere* 83 (2011) 510–516.
 - [83] B. Xia, B. Chen, X. Sun, K. Qu, F. Ma, M. Du, Interaction of TiO₂ nanoparticles with the marine microalga *Nitzschia closterium*: growth inhibition, oxidative stress and internalization, *Sci. Total Environ.* 508 (2015) 525–533.