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Algal Response to Metal Oxide Nanoparticles: Analysis of Growth, Protein Content, and Fatty Acid Composition

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Abstract

This study was conducted to determine the impacts of ZnO-, CuO-, and Fe_2O_3-nanoparticles (NPs) on the growth, protein content, and fatty acid profile of the green microalga *Nannochloropsis oculata*. The growth of alga was inhibited by the increasing concentration of NPs in the order of CuO-NPs > ZnO-NPs > Fe_2O_3-NPs. Protein content went up in response to ZnO- and Fe_2O_3-NPs and decreased after exposure to CuO-NPs. The analysis of fatty acid profile revealed the negative effect of CuO- and Fe_2O_3-NPs on the content of saturated fatty acids (SFAs). By contrast, the content of SFAs was enhanced in the algal cells treated with ZnO-NPs. The level of unsaturated fatty acids (USFAs) was increased upon reflection to CuO-NPs, but it was reduced after treatment with ZnO- and Fe_2O_3-NPs. The analysis of biodiesel indicators showed that the cloud point (CP) can be increased considerably in the algal cells exposed to ZnO- and CuO-NPs. Intriguingly, the cold filter plugging point (CFPP) of biodiesel was remarkably elevated in the treated cells with Fe_2O_3-NPs. Taken all together, despite the toxicity caused by high concentrations of metal oxide NPs, NPs could raise the amount of CP and CFPP and improve the oxidative stability in *N. oculata* biodiesel.

Keywords *Nannochloropsis* · Metal oxide nanoparticles · Protein · Biodiesel · GC

Introduction

Nanomaterials have gained increasing attention because of their unique properties, including a large specific surface area and high reaction activity [1]. Correspondingly, NPs possess modified physicochemical properties compared to the bulk material [1, 2]. Metal oxide NPs belong to a family of nanomaterials that are frequently used in emerging technologies and have a variety of applications particularly in the manufacture of commercial products [3]. For example, ZnO-NPs have been applied in the cosmetic and sunscreen industry on account of their capacity to absorb, reflect, and scatter UV radiation [4]. Nanoscale ZnO materials are also potent antimicrobial agents when combined with materials such as surface coatings, paints, textiles, and plastics [5, 6]. CuO-NPs have potential to replace noble metal catalysts for carbon monoxide oxidation [7] and have been applied to sensing materials, glass, ceramics, and antimicrobials [8]. Fe_2O_3-NPs have wide applications as an essential component in different products like coatings, plastics, rubber, silicone, wear-resistant tools, and sealing materials [9, 10].

The low contents of metals are essential for the cellular functions of microalgae [11]. Zinc (Zn), copper (Cu), and iron (Fe) play important roles in photosynthesis and respiration in the structure of electron transport chain proteins [11–13]. However, high concentrations of Zn, Cu, and Fe are toxic, because their catalytic functions via the Fenton reaction may lead to the generation of hydroxyl radicals, which damage DNA, proteins, lipids, and other organic constituents of living cells [13, 14]. The release of metal oxide NPs in the environment makes them a threatening agent to living organisms in a way of toxicity [3]. The solubility of NPs is crucial for their different effects on ecosystems including toxicity. It has been proposed that their high stability may cause NPs to penetrate into cells and accumulate within organisms [15, 16].

The aquatic environment is the final sink of all pollutants, and there are numerous reports on the toxicity of NPs to aquatic organisms [17–19]. To study the effects of exposure to different concentrations of three types of metal oxide NPs (ZnO, CuO, and Fe_2O_3) and their potential impacts on aquatic systems upon environmental pollution, we selected the green

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microalga *N. oculata* as a model for research on aquatic microorganisms. Microalgae are sunlight-driven cell factories that contain natural products with high commercial importance like lipids, pigments, carbohydrates, vitamins, and proteins [20]. Some genera of microalgae such as *Nannochloropsis*, *Chlorella*, *Chlamydomonas*, *Schizochytrium*, and *Nitzschia* have received a great deal of interest owing to their capability for lipid accumulation and biodiesel production [21]. The average lipid content of algal cells varies between 1 and 70% of dry weight, but it can reach even higher amounts under certain circumstances. The potential oil yields for many microalgal species significantly exceed the oil production capacity of the largest oilseed crops such as soybean [22]. Lipids mainly consist of glycerol, carbohydrates, and saturated or unsaturated fatty acids (12 to 22 carbon atoms). The total content and the relative ratio of fatty acids in algae can be changed by the nutritional and environmental factors [23]. Fatty acids are important cellular components functioning as energy sources and structural components for membrane biosynthesis [24]. Unsaturated fatty acids (USFAs) determine the physical properties of the cell membrane [24]. It is well-known that higher plants and animals do not possess the enzymes for production of polyunsaturated fatty acids (PUFAs) with more than 18 carbons, and thus they have to get the acquired these PUFAs from food sources [25, 26]. Actually, fish oil is the common source of PUFAs, which are originated from microalgae in oceanic environments [25, 26].

Nowadays, extensive studies are focused on improving the synthesis and maximizing the production of valuable compounds using microalgae cultures. Microalgae are able to synthesize high contents of natural compounds especially under stress conditions. However, stress conditions can also have negative impacts on the microalgae growth [11, 21]. Although human activities and development of industry led to the release of metal oxide nanoparticles in aquatic ecosystems, the mechanisms of their impacts on living organisms remain to be elucidated. The marine microalga *N. oculata* has been reported to be a promising system for biofuel production due to its high lipids content and great growth rate. Thus, this algal species seemed to be a good model for research on the metabolism of fatty acids. Accordingly, the aim of the current study was to characterize the changes in growth, protein content, and fatty acid composition in *N. oculata* after exposure to different concentrations of ZnO-, CuO-, and Fe_2O_3-NPs.

Methods and Materials

Algal Culture

The cells of *N. oculata* was obtained from the Persian Gulf Institute of Ecological Research and cultivated in f/2 medium. To prepare the culture media, seawater was first filtered and then autoclaved for 20 min at 121 °C. Subsequently, macronutrients ($NaNO_3$ and $NaH_2PO_4.2H_2O$), trace metals (Na_2EDTA, $FeCl_3.6H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, and $Na_2MoO_4.2H_2O$), and vitamins (cyanocobalamin, thiamine-HCl, and biotin) were added to the sterilized seawater. The cells of *N. oculata* were inoculated using 50 mL of mother culture into 350 mL of liquid f/2 medium. The cultures were then uniformly mixed in 500 ml Erlenmeyer flasks, and an initial count of cell number in solution was performed using a Neubauer counting cell. The cultures were exposed to 26–28 °C and a 12:12 light-dark cycle of 5000 lx illumination [17].

Nanoparticles Dispersion and Characterization

ZnO-, CuO-, and Fe_2O_3-NPs were purchased from the nanomaterials pioneers company (Houston, TX, USA). A stock solution of the NPs (10 g/L) was provided and dispersed using sonication in an ultrasonic shaker, applying a constant time of 30 min. Different concentrations of NPs (5–200 mg/L) with three replicates were prepared and were utilized immediately after preparation (Fazelian et al. 2019). The size and shape of NPs as were determined by transmission electron microscopy (TEM) (Philips-em208) and X-ray powder diffraction (XRD) analysis (EQUINOX 3000, INEL, France). Hydrodynamic diameters of dispersed NPs in f2 medium at the concentration of 200 mg/L were characterized by dynamic light scattering (DLS) technique using the SZ-100 nanoparticle analyzer-Horiba.

Microscopic Analysis of *N. oculata* Exposed to NPs

Field emission scanning electron microscopy (FESEM) was used to examine the surface morphology of algae. After freeze-drying, the samples were covered with a thin layer of gold. The cells were observed by a Zeiss-Sigma VP-500, scanning FESEM [27].

Nanoparticle Exposure and Algal Growth Studies

The effects of ZnO-, CuO-, and Fe_2O_3-NPs on the growth rate of *N. oculata* were investigated in f/2 medium. For treatments, 4×10^4 cells mL^{-1} of *N. oculata* cultures (grown at 26–28 °C for 4 days) were exposed to different concentrations of NPs (5, 10, 50, 100, and 200 mg/L) for 72 h. The morphology of treated algal cells was studied using scanning electron microscopy (Zeiss-Sigma VP-500, Germany). The toxicity tests for NPs were carried out according to the modified guidelines of Organization for Economic Cooperation and Development as previously reported. The effect of different concentrations of NPs on the growth of *N. oculata* was recorded using cell

counts after 72 h, and the content of EC_{50} and EC_{90} for NPs was measured by Excel and R software [17].

MTT Test

The reduction of tetrazolium salts is actually a trustworthy method to examine cell proliferation. The yellow tetrazolium MTT is reduced by the action of dehydrogenase enzymes in viable cells to a blue-colored product [28]. In this assay, 500 μl of algal suspension with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg MTT in 1 ml salt phosphate buffer) was incubated for 4 h in darkness. The samples were centrifuged at 6000 rpm for 15 min, and the supernatant was discarded. The pellet was washed with 500 μl sea water. At the end of the experiment, 200 μl of dimethyl sulfoxide (DMSO) was added to the algal sample and stored for 2 h in darkness. After centrifugation, the supernatant was poured into microplate wells, and the absorbance of the samples was read at 570 nm by a microplate reader. Live control was considered as 100%, and the following calculations were used [17].

$$\%\text{Cytotoxicity} = 1 - \left(\frac{\text{mean absorbance of toxicant}}{\text{mean absorbance of control}}\right) \times 100$$

$$\%\text{Viability} = 100 - \%\text{Cytotoxicity}$$

Determination of Chlorophyll a

To determine the chlorophyll a concentration, the algae sample was obtained with centrifuge and immersed in acetone (85% V/V) for 24 h at 4 °C in darkness. The absorbance of the solvent was measured at 664 nm, 647 nm, and 470 nm with a spectrophotometer [29].

Determination of Protein Concentration

Protein estimation was carried out by the method of Bradford (1976) using bovine serum albumin as the standard reference [30]. For protein extraction, algal sample was homogenized in 0.05 M tris-sucrose buffer (pH = 7.5). The samples were centrifuged for 25 min in 10,000 g at 4 °C. The obtained extract was used for the measurement of protein concentration. Their absorption was read by a spectrophotometer at 595 nm.

Determination of Fatty Acid Profile by GC-MS

The detection of fatty acid methyl esters (FAMEs) was done using a gas chromatography-flame ionization detector (GC-FID) according to the method reported by Miller and Berger (1985) [31]. Initially, the solution containing the sample and NaOH (1.2 mol/L) in 50% methanol was boiled for 30 min. Then, the sample was acidified with HCl (10 mol/L), and methanolic BCl_3 (12%) was added and heated for 10 min. For the extraction of FAMEs, hexane/diethyl ether (1:1) was added. Then, NaOH (0.3 mol/L) was added to the FAMEs (organic extract). The FAME phase was evaporated completely using nitrogen flushing. FAME samples were investigated by a gas chromatograph (Varian, 3800) equipped with a fused silica capillary column BPX 70 (25 m × 0.32 mm, film thickness 0.25 μm) and a flame ionization detector. The run was carried out using a temperature gradient of 160–230 °C, with an increased rate of 1.5 °C min^{-1}. The identification of fatty acid (FA) was performed using external standards (SUPELCO F.A.M.E. Mix C4-C24).

Biodiesel Properties of FAME Profile

The carbon chain sizes and the number of double bonds are factors that influence some features of biodiesel such as saponification value (SV), iodine value (IV), cetane number (CN), cold filter plugging point (CFPP), and the oxidation stability. The amount of SV, IV, and CN of *N. oculata* was obtained by the following Eqs. (1–3) [32]:

$$\text{SV} = \sum(560 \times \text{N})/\text{M} \quad (1)$$

$$\text{IV} = \sum(254 \times \text{DN})/\text{M} \quad (2)$$

$$\text{CN} = 46.3 + (5.458/\text{SV}) - (0.225 \times \text{IV}) \quad (3)$$

where D is the number of double bonds, M is the fatty acid molecular mass, and N is the percentage of each fatty acid component of *N. oculata* oil.

The long-chain saturated factor (LCSF) was estimated by Eq. (4). This factor was directly used to calculate CFPP in Eq. (5) [32]:

$$\text{LCSF} = (0.1 \times C_{16}) + (0.5 \times C_{18}) + (1 \times C_{20}) + (1.5 \times C_{22}) + (2 \times C_{24}) \quad (4)$$

$$\text{CFPP} = (3.1417 \times \text{LCSF}) \quad (5)$$

The degree of unsaturation (DU) was assessed using the Eq. (6), as the level of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in the microalgae oil [33].

$$\text{DU} = \text{MUFA} + (2 \times \text{PUFA}) \quad (6)$$

Equation (7) was used to estimate the CP value based on the content of C16:0 in fatty acid profiles [33].

$$CP = (.526 \times C16) - 4.992 \quad (7)$$

Statistical Analysis

The experiments were performed in three replicates for all treatments. The results were examined using one-way analysis of variance (ANOVAs) followed by Duncan's multiple comparison tests. All statistical analyses were carried out using SPSS statistics 18, and $p < 0.05$ was considered to be significant.

Results

Characterization of NPs

The size and shape of ZnO-, CuO-, and Fe_2O_3-NPs were estimated using TEM and XRD analysis (Table 1). The studied metal oxide nanoparticles were spherical in shape. The particle size of ZnO-NPs was estimated to be 10–30 nm (99%), and the specific surface area (SSA) of NPs was 20–60 m^2/g. The particle size of CuO-NPs was 10–40 nm (99/9%) with SSA of 20 m^2/g. Fe_2O_3-NPs had an average particle size of 20–40 nm (98%) and SSA of 40–60 m^2/g, as reported by the manufacturer (Table 1).

The results of hydrodynamic diameter analysis revealed that ZnO-, CuO-, and Fe_2O_3-NPs had mean particle size of 745.9 ± 49.4, 493.0 ± 43.6, and 225.3 ± 11.7 nm, respectively (Table 1). These higher particle sizes of NPs in f2 medium designate the aggregation of NPs at the used concentrations in the culture medium.

Microscopic Analysis of *N. oculata* Cells

The probable presence and the form of chemical attachment of ZnO-, CuO-, and Fe_2O_3-NPs on the cell surface of algae were determined with SEM observations. In addition, the aggregation of NPs on the algae cell surface was evident in the SEM images (Fig. 1a–c).

The Growth and Chlorophyll a Content

Microalgal response to ZnO-, CuO-, and Fe_2O_3-NPs was assessed primarily based on the changes in growth and chlorophyll a content. The obtained results revealed that the applied NPs were toxic to *N. oculata* with EC_{50} of 153.72 mg/L, 116.981 mg/L, and 202.92 mg/L for ZnO-, CuO-, and Fe_2O_3-NPs, respectively (Table 2). Also, the EC_{90} values of ZnO-, CuO-, and Fe_2O_3-NPs were determined to be 325.69, 261.22, and 398.23 mg/L, respectively (Table 2). The results of MTT test showed that the cell viability was significantly decreased in the treated *N. oculata* cells with ZnO-NPs (10–200 mg/L), CuO-NPs (5–200 mg/L), and Fe_2O_3-NPs (10–200 mg/L) compared to the control samples (Fig. 2). A cell viability by 39.18%, 30.27%, and 54.42% (in comparison to the control medium) was observed after exposure to 200 mg/L ZnO-, CuO-, and Fe_2O_3-NPs, respectively.

The concentration of chlorophyll a was decreased significantly with the increasing concentration of NPs in the cultured cells (Fig. 3). The highest effect on the reduction of chlorophyll a content was observed after treatment with 200 mg/L of the used NPs ($p < 0.05$).

Protein Content

The content of protein showed a significant decrease in *N. oculata* cells after treatment with CuO-NPs, while ZnO- and Fe_2O_3-NPs significantly increased the protein content compared to the control sample (Fig. 4). The maximum effect on the enhancement of protein concentration was achieved by 100 and 200 mg/L of Fe_2O_3-NPs compared to the control sample ($p < 0.05$).

Analysis of Fatty Acid Profile

Our study showed that 100 mg/L of ZnO- and CuO-NPs and 200 mg/L of Fe_2O_3-NPs have significant influences on the fatty acids composition (Tables 3, 4). The effect of each nanoparticle on the content of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) was different (Table 4). The identified SFAs included C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, and C20:0 fatty acids, among which C16:0 (palmitic acid) and C18:0 (stearic acid) showed the uppermost quantities. MUFAs consisted of C14:1, C16:1, C17:1, C18:1, C20:1, C22:1, and C24:1 acids with C16:1 (palmitoleic acid) and

Table 1 Characteristics of the applied nanoparticles

Nanoparticles	Characteristics						
	Shape	Purity	True density (g/cm^3)	Specific surface area (m^2/g)	Color	Diameter (nm)	Hydrodynamic diameter (nm)
ZnO	Nearly spherical	99%	5.6	20–60	Milky white	10–30	745.9 ± 49.4
CuO	Nearly spherical	99%	6.4	20	Black	10–40	493.0 ± 43.6
Fe_2O_3	Spherical	98%	4.8–5.1	40–60	Black	20–40	225.3 ± 11.7

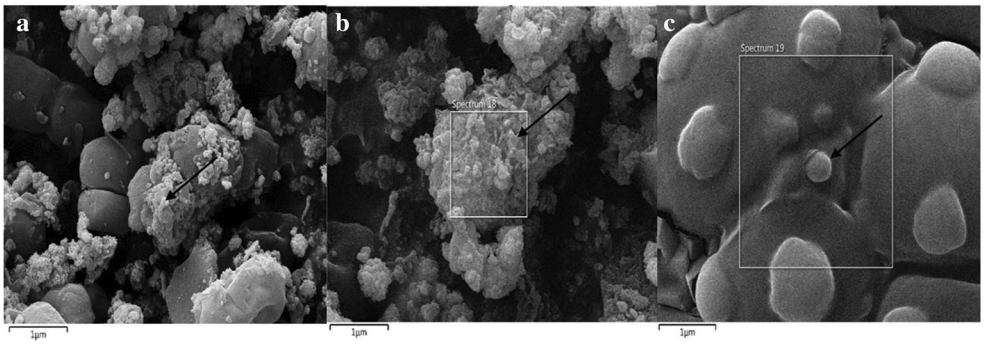

Fig. 1 SEM images of *N. oculata* exposed to ZnO- (**a**), CuO- (**b**), and Fe_2O_3-NPs (**c**). The aggregation of NPs has been shown in the SEM images by black arrows (**a**–**c**). Scale bar = 1 μ

C18:1 (oleic acid) as the two most abundant MUFAs. PUFAs were represented by C18:2 (linoleic acid) and C18:3 (linolenic acid) fatty acids. The content of linoleic acid was more than that of linolenic acid.

The level of SFAs was decreased in algal cells after exposure to CuO- and Fe_2O_3-NPs, while ZnO-NPs increased the content of SFAs compared to the control sample. The contents of individual SFAs, MUFAs, and PUFAs were variously altered in response to the treatment with different NPs (Table 3).

The effect of metal oxide NPs on the levels of C16 and C18 fatty acids has been presented in Table 3. These fatty acids have an important role in the quality of biodiesel. Regarding the obtained data, the percentage of 18:1, 18:2, and 18:3 fatty acids went up in the cells of *N. oculata* after treatment with Fe_2O_3-NPs, while CuO-NPs increased only the level of C18:1 and C18:3 (Table 3). In the treated algal cells with ZnO-NPs, the level of C18:1 fatty acid was declined, and the concentration of C18:2 and C18:3 acids were concurrently raised.

Intriguingly, ZnO- and Fe_2O_3-NPs decreased the percentage of MUFAs, while CuO-NPs increased the content of these fatty acids in *N. oculata* compared to the control condition (Table 4). On the other hand, opposite outcomes were obtained for PUFAs. Accordingly, the level of PUFAs was reduced by CuO-NPs and was enhanced by ZnO- and Fe_2O_3-NPs. Furthermore, the content of USFAs was increased in samples treated with CuO- and Fe_2O_3-NPs, while ZnO-NPs decreased the amount of USFA in comparison to the control (Table 4).

Table 2 EC_{50} and EC_{90}values of nanoparticles for *N. oculata*

Nanoparticles (mg/L)	EC	
	EC_{50}	EC_{90}
ZnO	153.723	325.692
CuO	116.981	261.229
Fe_2O_3	202.926	398.239

N. oculata was grown under different concentrations of ZnO-, CuO-, and Fe_2O_3-NPs. The microalgae were harvested after 72 h. The data represent means of three replicates and different letters indicate significantly different values ($p < 0.05$) between samples calculated by ANOVA

Estimation of Biodiesel Properties

The changes in biodiesel indices after exposure to applied NPs have been displayed in Table 5. The effect of ZnO-NPs on saponification value (SV) was positive, but CuO- and Fe_2O_3-NPs had a negative influence on the SV level. The iodine value (IV) was enhanced in response to ZnO- and Fe_2O_3-NPs treatments and was decreased by CuO-NPs. In a reverse direction, ZnO- and Fe_2O_3-NPs decreased cetane number (CN) of *N. oculata*, while CuO-NPs increased its value. Three metal oxide NPs increased the degree of unsaturation (DU) in *N. oculata*, and the highest quantity of DU (+ 17.5%) was achieved by using Fe_2O_3-NPs. The content of LCSF was decreased in the treated cells with ZnO- and CuO-NPs, while its level was considerably increased by exposure to Fe_2O_3-NPs. This parameter is related to CFPP and plays an important role in determining the cold response of produced biodiesel. The highest level of CFPP was observed in response to Fe_2O_3 (+ 123.34%). ZnO- and CuO-NPs decreased the level of CFPP compared to the control. CP level is another factor in the investigation of cold response. ZnO- and CuO-NPs increased the amount of CP by 31.76% and 14.28%, respectively, while Fe_2O_3-NPs decreased its level by − 64.19%. ZnO-NPs were the only NP that increased SFAs level of *N. oculata*. The changes in SFAs in response to ZnO-, CuO-, and Fe_2O_3-NPs were estimated to be by + 4.00%, − 2.59% and − 8.69%, respectively.

Discussion

The results of SEM analysis confirmed the presence and aggregation of ZnO-, CuO-, and Fe_2O_3-NPs on the cell surface of *N. oculata*. The algae cell wall usually contains macromolecules such as cellulose, matrix polysaccharides, and glycoproteins, which have NP-binding capacity [34]. After exposure to NPs, these macromolecules of cell wall have the possibility to interact with NPs [27]. NPs can pass through the cell membrane pores and enter the algal cell due to their small size and specific surface area [17, 34]. Also, large-sized NPs can

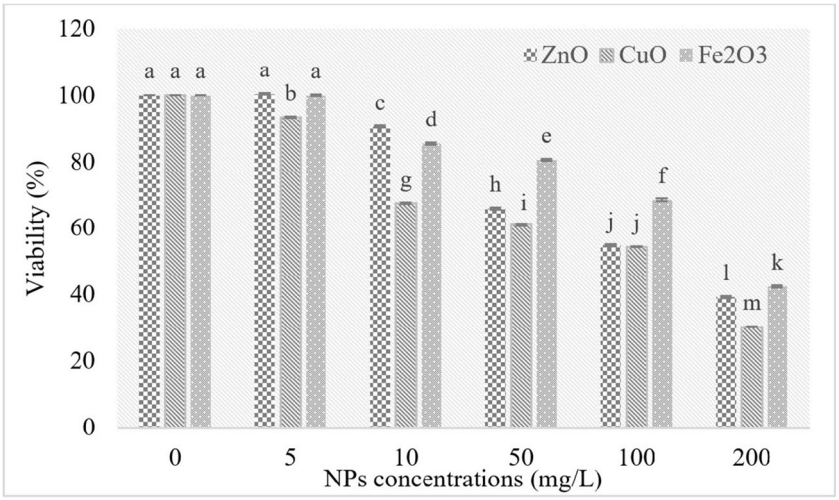

Fig 2 The calculated viability of *N. oculata* by MTT test in the control and treated *N. oculata* cells with ZnO-, CuO-, and Fe_2O_3-NPs ($p < 0.05$)

pass the plasma membrane through the protein channels or carriers and by endocytosis [2, 17]. The arrived NPs may affect the algae growth by interaction with macromolecules and organelles in the cells [17].

The bioavailability of NPs in the marine ecosystems depends on different parameters specially the aggregation rate and size of particles [35, 36]. Previous studies often reported that metal oxide NPs, particularly ZnO-NPs, are insoluble in pure water, while the solubility of these particles in a high ionic strength and high pH medium such as f/2 medium is very high [28, 37–39]. In the present study, the aggregation of the applied NPs was found by the DLS analysis. Similarly, the aggregation of ZnO-NPs in seawater at the concentration of 10 mg/L and their toxicity on the marine alga *Dunaliella tertiolecta* has been reported [37]. It was also shown that ZnO-NPs were able to form large aggregates in seawater (2.3 ± 1.6 μm) with toxic influences on marine organisms [38]. Moreover, ZnO-NPs were more toxic for the marine diatoms *Skeletonema costatum* and *Thalassiosira pseudonana* in comparison to bulk ZnO [38].

The tiny size of NPs is also an important factor in their bioavailability and toxicity. For example, 25-nm-sized TiO_2-NPs were more toxic than 100 nm TiO_2-NPs to *Desmodesmus subspicatus* [39]. Additionally, the toxicity of iron NPs to *Chlorella pyrenoidosa* has decreased by the increasing size of the NPs [9]. Based on our findings, the average size of the applied NPs appeared to be in a suitable range for possible uptake by cells of *N. oculata*. In such a way, the treatment of *N. oculata* cells with ZnO-, CuO-, and Fe_2O_3-NPs have been influenced the algal growth and quantity of vital compounds such as chlorophylls, proteins, and fatty acids. In the current study, the applied metal oxide NPs reduced the growth of *N. oculata*. The inhibition of algal growth was raised significantly with the increasing concentration of NPs. As a result, ZnO-, CuO-, and Fe_2O_3-NPs were toxic to *N. oculata* with relatively different EC_{50} and EC_{90} values for each NP. Likewise, EC_{50} values of CuO-NPs and Fe_2O_3-NPs were reported to be 150.45 mg/L and 214.44 mg/L for *Chlamydomonas reinhardtii* and *Scenedesmus obliquus*, respectively [10, 41]. These data are quantitatively in

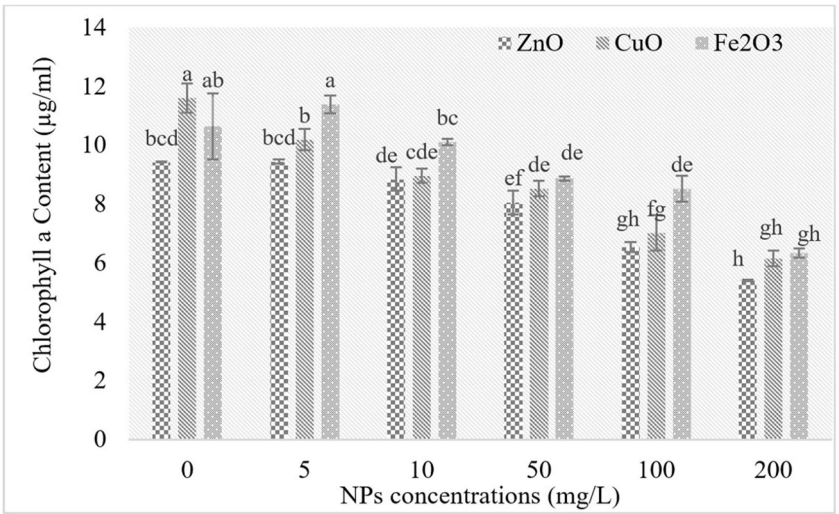

Fig. 3 Effect of ZnO-, CuO-, and Fe_2O_3-NPs on chlorophyll a content of *N. oculata* ($p < 0.05$)

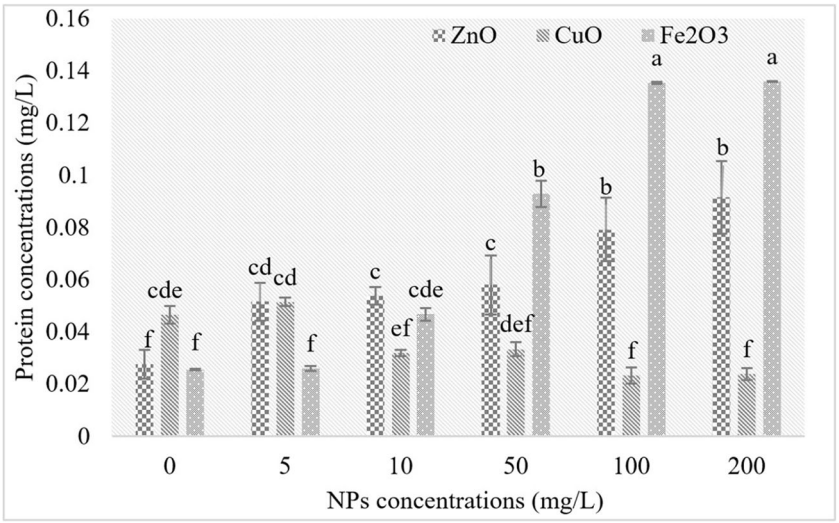

Fig. 4 Effect of ZnO-, CuO-, and Fe_2O_3-NPs on the protein content of *N. oculata* ($p < 0.05$)

comparable ranges with our findings on *N. oculata*. On the other hand, EC_{50} and EC_{90} values of CuO-NPs were expressed as about 17.37 mg/L and 38.9 mg/L for *Anabaena* sp. [42]. Other researches showed that the values of EC_{50} and EC_{90} vary greately between algal species depending on the structure and high surface to mass ratio as well as the nature of constitutive elements of NPs [40, 42].

According to the outcomes of MTT test, the order of toxicity of the used NPs in *N. oculata* was estimated as CuO > ZnO > Fe_2O_3. This finding was consistent with the results of cell counting using Neubauer chamber (EC_{50}), although the MTT test showed less toxicity of the NPs in *N. oculata*. Similar to our results, the cell viability of the green microalga *Chlorella vulgaris* was significantly reduced by 50–300 mg/L of ZnO-NPs [28].

The study of the toxicity of metal oxide NPs to the various organisms indicated that the toxicity of ZnO-NPs is higher than CuO-NPs. For instance, the highest toxicity among metal oxide NPs to aquatic ecosystems was revealed for ZnO-NPs [37]. In addition, the toxicity order of NPs for *Chlorella vulgaris* was reported as ZnO > Al_2O_3 > TiO_2 > CeO_2 and ZnO > NiO > CuO > TiO_2 > Fe_2O_3 [40–43]. However, in the present research, the toxicity of CuO-NPs to *N. oculata* was proved to be more than two other NPs. Likewise, Baek and An (2011) reported that CuO-NPs has the highest toxicity compared to ZnO-, NiO-, and Sb_2O_3-NPs in microorganisms [6].

Chlorophyll is one of the important photosynthetic pigments that can affect the growth of algae. The growth status of algal cells can be estimated by monitoring the intracellular contents of chlorophyll [17, 18]. A number of previous studies have demonstrated that application of CuO-NPs and TiO_2-NPs could decrease the chlorophyll content and growth rate of *N. oculata* and *Chlamydomonas reinhardtii* [17, 34]. The toxicity of NPs was mainly due to their oxidative effects on intracellular compartments such as the chloroplasts. Since chloroplasts are responsible for photosynthesis, a decrease in their function may result in a low growth rate [44]. It was described that excess iron amounts can accumulate high levels of ROS, which might have eventually oxidized the

Table 3 Fatty acid composition of *N. oculata* in control and treated cells with ZnO-, CuO-, and Fe_2O_3-NPs

	Fatty acids (mg/g.FAME)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
$Control_1$	22.84	17.22	8.18	28.22	9.08	4.21
ZnO-NPs	27.09	16.81	6.39	25.22	10.21	5.91
$Control_2$	13.89	20.12	2.17	7.42	5.66	1.05
CuO-NPs	14.52	15.75	1.81	10.65	3.75	1.99
$Control_3$	21.29	23.9	7.12	0.87	2.43	1.08
Fe_2O_3-NPs	13.73	17.59	12.38	6.5	4.87	2.75

Table 4 Composition of fatty acids of *N. oculata* obtained by gas chromatography

	Fatty acids (mg/g.FAME)				
	SFAs	MUFAs	PUFAs	USFAs	SFAs/USFAs
Control 1	39.71	45.44	13.29	58.73	0.67
ZnO-NPs	41.3	41.93	16.12	58.05	0.7
% Changes	+ 4	− 7.72	+ 21.29	− 1.15	+ 4.73
Control 2	22	36.14	6.71	42.85	0.51
CuO-NPs	21.43	39.33	5.74	45.07	0.47
% Changes	− 2.59	+ 8.82	− 14.45	+ 5.18	− 7.4
Control 3	36.57	36.61	3.51	40.12	0.91
Fe_2O_3-NPs	33.39	36	7.62	43.62	0.76
% Changes	− 8.69	− 1.66	+ 117.09	+ 8.72	− 16.13

SFAs saturated fatty acids, *USFAs* unsaturated fatty acids, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids

Table 5 Estimated biodiesel properties of the treated cells of *N. oculata* with NPs

	Biodiesel properties						
	SV	IV	CN	DU	LCSF	CFPP	CP
Control 1	209.22	71.75	30.18	72.02	7.04	5.65	7.02
ZnO-NPs	209.47	74.66	29.52	74.27	6.52	4.01	9.25
% Changes	+ 0.11	+ 4.05	− 2.18	+ 3.12	− 7.38	− 29.02	+ 31.76
Control 2	210.09	73.65	29.75	49.56	4.78	− 1.44	2.31
CuO-NPs	206.2	73.00	29.9	50.81	4.33	− 2.85	2.64
% Changes	− 1.85	− 0.88	+ 0.5	+ 2.52	− 9.41	− 97.91	+ 14.28
Control 3	210.57	55.35	33.87	43.63	7.84	8.18	6.2
Fe_2O_3-NPs	207.45	65.69	31.54	51.27	11.06	18.27	2.22
% Changes	− 1.48	+ 18.68	− 6.87	+ 17.51	+ 41.07	+ 123.34	− 64.19

chlorophyll and subsequently led to decreased chlorophyll content [17]. Therefore, lessening the content of chlorophyll might be one of the responsible factors for the reduced growth of *N. oculata*.

The protein content in *N. oculata* was increased after exposure to ZnO- and Fe_2O_3-NPs. An increase of protein content has also been reported for the treated *Scenedesmus obliquus* cells with Fe_2O_3-NPs [10]. Increase of protein quantity may act as a defense mechanism against abiotic stresses to prevent damage to algae cells [10]. Actually, antioxidant enzymes and molecular chaperons are important proteins, which play an essential role in protective mechanisms [10]. Moreover, the synthesis of metalloproteins and phytochelatins may confer an improved protection against toxicity of NPs [45]. Unlike ZnO- and Fe_2O_3-NPs, CuO-NPs reduced the protein amount of *N. oculata*. Similar results were reported in *Phaeodactylum tricornutum* (a marine diatom), in which TiO_2-NPs and CeO_2-NPs led to a decrease in the rate of protein synthesis [46]. Decrease of protein content is a common phenomenon in strict environmental stresses and may be due to the reduced protein biosynthesis or accelerated catalytic processes. The hydrolysis of proteins into amino acids probably is a cell adaptation to carbohydrate deficiency [47]. The decrease in the protein content in response to CuO-NPs may be due to the degradation of chlorophyll biosynthesis enzymes and the damage to photosynthetic protein structures in response to ROS production and oxidative stress caused by these NPs. The depletion of chlorophyll and protein was probably a responsible reason for the growth reduction and then the higher toxicity of CuO-NPs compared to the other NPs.

Microalgae produce a mixture of SFAs and USFAs with 12 to 22 carbons, some of which are from the ω3 and ω6 families [48]. High capacity for lipid production in algae has led to their use in biodiesel industry [32]. Microalgal oil can easily be transformed into biodiesel during the transesterification process. In the marine ecosystem, *N. oculata* has been cited as one of the best sources of oil to produce biodiesel owing to high biomass content and lipid accumulation [49]. It is well-known that the composition of algal fatty acids has a major impact on the quality of the biofuels, and SFAs/USFAs ratio determines the final quality of biodiesel [48]. Actually, the content of SFAs could increase oxidative stability of biodiesel in hot conditions [32]. In this study, CuO- and Fe_2O_3-NPs decreased the level of SFAs, while ZnO-NPs increased the amounts of SFAs. Also, the level of USFAs (MUFAs + PUFAs) was increased in samples exposed to CuO- and Fe_2O_3-NPs, but ZnO-NPs did not make any difference in the content of these fatty acids. Increase of PUFAs content in response to NPs has been observed in a number of previous studies. For example, TiO_2-NPs increased the amount of PUFAs in *Chlorella vulgaris* by the induction of oxidative stress [50]. In the current study, increased content of PUFAs was observed in *N. oculata* after exposure to ZnO- and Fe_2O_3-NPs. The increased concentrations of PUFAs play an important role against oxidative stress, because PUFAs may act as a scavenger of free radicals [50, 51]. In contrast, the content of PUFAs content was decreased in *Scenedesmus obliquus* in response to Fe_2O_3-NPs treatment [10]. This finding was similar to our results on the effect of CuO-NPs on PUFAs of *N. oculata*. Presumably, the reduction of PUFAs can also be an important factor controlling the oxidation of biodiesel compounds, and high level of PUFAs may decrease the final stability of biodiesel [10, 32].

C18 fatty acids are the most important fatty acids among USFAs (MUFAs and PUFAs). The influence of ZnO-, CuO-, and Fe_2O_3-NPs on C18 fatty acids was inconstant. In accordance with our finding using Fe_2O_3-NPs, a significant increase of oleic acid (18:1) in primary leaves of tomato in response to metal contamination was observed [52]. Similarly, an increased content of linoleic acid (18:2) in plants with Cd contamination was described [53]. It was also reported that linolenic acid (C18:3) is a stress signal and acts as a precursor to phyto-oxylypin biosynthesis [54]. Thus, the increase of C18:3 in the treated *N. oculata* with ZnO-, CuO-, and Fe_2O_3-NPs was probably a defensive response against the induced oxidative stress.

The parameters of biodiesel quality (i.e., SV, IV, CN, DU, LCSF, CFPP, and CP) could give a good insight into the quality of produced biodiesel from *N. oculata*. Previous studies have shown that there is a clear relation between LCSF and SV and CN values, and the higher LCSF may cause an increase in the values of SV and CN. On the other hand, IV and DU are indices of unsaturation and the oxidative stability of biodiesel decreases with their upsurge [32]. ZnO- and Fe_2O_3-NPs increased the IV and DU amounts of *N. oculata* biodiesel, while CuO-NP only increased the amount of DU. In this study, the content of USFA was increased in response to CuO- and Fe_2O_3-NPs. The USFA level is directly related to the amount of IV and DU, but in determination of the value of DU the role of PUFAs is higher than MUFAs. Thus, an increase of DU in response to ZnO-NPs can be due to an increase of PUFAs content in response to these NPs.

There is also a direct relation between the SFAs value and LCSF and CFPP. The ratio of SFAs/USFAs determined the biodiesel stability against oxidation. ZnO-NPs were the only NPs that increased the proportion of SFAs and SFAs/USFAs, while CuO- and Fe_2O_3-NPs decreased their quantity. Previous studies have shown that the increase of SFAs by the elevating amount of CFPP and CP improves biodiesel stability in tropical environments [32]. In the present investigation, ZnO- and CuO-NPs increased the CP level but decreased the levels of LCSF and CFPP. The increase of CP amount was probably due to the increase of C16:0, while the reduction of C18:0 and C20:0 fatty acids decreased CFPP amount after exposure to ZnO and CuO-NPs. An increase in the LCSF and CFPP proportion of biodiesel produced from *N. oculata* cells was observed in response to Fe_2O_3-NPs due to the increase in C18:0 and C20:0 fatty acids, while the levels of C16:0 and CP decreased in response to this NP. Accordingly, Fe_2O_3-NPs improved the quality of biodiesel produced from *N. oculata*, because it considerably enhanced the level of LCSF and CFPP. Taken together, the treatment of CuO- and Fe_2O_3-NPs increased the level of USFAs, which may be related to the increase of MUFAs and PUFAs content, respectively. Also, reduction of USFAs in response to the ZnO-NPs was due to the decrease of MUFAs. Eventually, increase of PUFAs can be improved by the oxidative stress caused by NPs, because these fatty acids have a ROS-removing function in *N. oculata*.

Conclusion

As a general conclusion, CuO-NPs showed high toxicity to algal cells resulting in decreased content of chlorophylls, proteins and PUFAs in *N. oculata*. By contrast, ZnO- and Fe_2O_3-NPs increased the amount of PUFAs and proteins. Also, CuO- and Fe_2O_3-NPs had a similar effect on the content of SFAs, while ZnO-NPs were the only NPs that increased the proportion of these fatty acids and consequently the ratio of SFAs/USFAs. In addition, upsurge of USFAs in response to CuO- and Fe_2O_3-NPs may be related to the increase of MUFAs and PUFAs contents, respectively. In comparison, ZnO-NPs were able to decline the quantity of these fatty acids due to the decrease of MUFAs. The results of biodiesel indicators showed that the applied metal oxide NPs improved the degree of unsaturation in *N. oculata*. Furthermore, ZnO- and CuO-NPs could increase the CP level, while Fe_2O_3-NPs caused a reverse response. Consequently, Fe_2O_3-NPs had a higher effect on the oxidative stability of biodiesel owning to the remarkable increase of CFPP.

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Compliance with Ethical Standards

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

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