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Effects of copper oxide nanoparticles on the growth of *Chlorella vulgaris*

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ABSTRACT

The increase of copper oxide nanoparticle (CuO-NP) utilization in industry during recent years has resulted in their entry into aquatic ecosystems. In light of this fact, we have studied the toxicity of CuO-NPs at various concentrations on *Chlorella vulgaris* using an algal growth inhibition test (OECD201). *Chlorella vulgaris* was grown in positive Zander (Z-8 + N) media in a growth chamber. After reaching to the logarithmic growth phase, the algae were exposed to various concentrations of CuO-NPs at intervals of 24, 48 and 72 hours. Algal cell numbers were counted daily and the data were analyzed by Probit analysis. Some parameters such as: the effective concentration (EC_{10}, EC_{50}, EC_{90}), no observed effect concentration (NOEC), specific growth rate (μ), doubling time (G) and percentage growth inhibition (I %) were calculated. The values of EC_{10} = 12.588, EC_{50} = 43.699, EC_{90} = 152.019 and NOEC = 4.3699 mg/L were obtained after 72 hours. The results showed significant differences between control and treatments at specified intervals of cell density and percentage of growth inhibition ($P < 0.05$). In addition, chlorophyll and carotenoid content in treated cells were significantly lower compared to those in control samples; a decrease in the efficiency of photosynthesis is very probably a major mechanism in the reduced growth and viability of *C. vulgaris* exposed to CuO-NPs.

Keywords: Toxicity of nanoparticles; Percent inhibition of growth; Specific growth rate; Photosynthetic pigments

Introduction

Several industries such as mining, textile, electro−plating, and metallurgy release heavy metals into aquatic ecosystems. They are persistent environmental pollutants that cannot be degraded or destroyed (1). Recent developments in nano-technology may lead to release of new copper containing materials (species) such as CuO nanoparticles (CuO-NPs) into the environment and could potentially influence aquatic ecosystems. Because of the rapid development of nanotechnology, nanomaterials with various diameters and shapes have been manufactured and increasingly utilized in some industrial commodities and products (2). Algae, a group of organisms providing the basic nutrition for the food chain, are generally used as

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model organisms to investigate heavy metal toxicity (3). CuO-NPs are being used as biocide (4, 5). Also, core-shell CuO-NPs are being used as components of pigments, often as antifouling agents (6), protecting sea-going vessels from slime fouling, weeds and crustaceans.

The introduction of these nanoparticle (NP) pigments, however, comes with risks that have not yet been well evaluated. For example, the utilization of such pigments may cause strong deteriorative effects on different trophic levels of aquatic ecosystems, effects that are not the intended aims (7). Currently, there is limited information about the risk of exposure to NPs and, therefore, the risks may be underestimated, especially considering the probability that the levels of exposure to nanoparticles are high (2, 3). The possibility that aquatic ecosystems are adversely affected by nanoparticle contamination has motivated diverse research efforts aimed at assessing the bioaccumulation and toxic effects of NPs in microalgae (8). To ensure the safe application of CuO-NPs, a broad knowledge of the functions of CuO-NPs (and other NPs) in the environment is needed.

The toxicity of CuO-NPs has been studied extensively in a number of organisms (9). Results showing toxicity were explained partly by the release of Cu ions from CuO-NPs; and the power of NPs to attach to cells, and the uptake of NPs by organisms (4, 10, 11). The green algal species, *Chlorella vulgaris*, which is mainly dispersed in fresh waters, is a suitable species for biological toxicity tests (12, 13, 14). In toxicity examination of nanoparticles, C. vulgaris is one of the preferred model organisms (15). On the other hand, C. vulgaris is the dominant species of algae and the base of food chain in fresh water ecosystems (16) which are often exposed to contaminant toxicity.

In this study we aimed to evaluate the toxicity of CuO-Nps through assays of growth parameters and the measurement of CuO-NPs sequestration by the native C. vulgaris in Guilan (North Iran) wetlands.

Materials and Methods

Nanoparticles

CuO-NPs produced by US Research Nanomaterials Company were used. The size of CuO-NPs used was 40nm. A 2000 mg/L stock solution was used for preparing appropriate concentrations of the nanoparticles for the assays (Table 1 and Figure 1) **.**

Table 1. CuO nanoparticle characteristics

Feature	Amount, size or state
Purity	99%
Color	Black
Particle size	40 nm
Specific area	~20 m^2/g
Morphology	nearly spherical
Bulk Density	0.79 g/cm^3
True Density	6.4 g/m^3

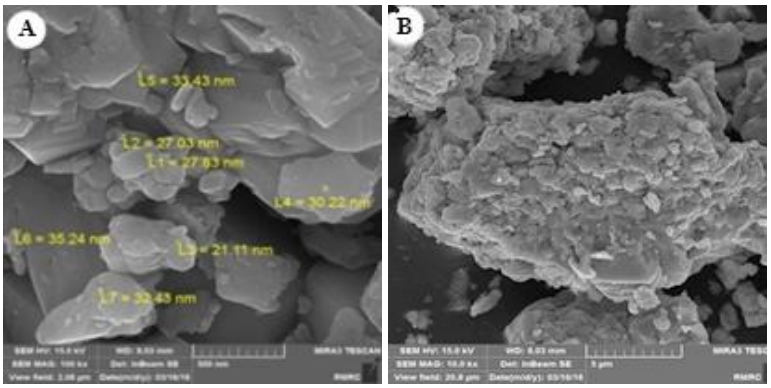


Figure 1. SEM of CuO-NPs. A. nm scale. B. µm scale

Preparation of culture medium and cultivation of algae

Chlorella vulgaris was collected from National Inland Water Aquaculture Institute (Bandar Anzali, Prov. Guilan, North Iran) and was plated on solid Zander culture medium (17, 18). Zander culture media were prepared using six main stock solutions including $NaNO_3$ (1 g/L); $FeCl_3$ (0.005 g/L); Na_2CO_3 (0.1 g/L); 2Ca (SO4). 5H2O (0.008 g/L); KH_2PO_4 (0.5 g/L); $MgSO_4$. $7H_2O$ (0.1 g/L); HCl Na_2EDTA (0.075 g/L); $MnCl_2$. $4H_2O$ (0.001 g/L); Na_2SiO_3. $9H_2O$ (0.1 g/L); H_3BO_3 (0.19 g/L); $Ca(NO_3)_2$. $6H_2O$ (0.1 g/L); MnCl2.4H2O (0.001 g/L); KBr (0.03 g/L); $Al_2(SO_4)_3$. $18H_2O$ (0.556 g/L); $ZnSO_4$. $7H_2O$ (0.023g/L); $CuSO_4$. $5H_2O$ (0.01g/L); $KI(NH_4)_6$ (0.03 g/L); and MO_7O_{27}. $4H_2O$ (0.008 g/L) (17, 18). The pH of the culture medium was adjusted to 6.8, temperature 24 ± 2°C, the photoperiod to 12 hours light. The cultures were exposed to illumination with intensity of ~60mol of photons m^{-2} s^{-1}. The culture medium was sterilized in an autoclave at 121°C for 15 min, and then the bottled media was refrigerated at 6°C.

Preparation of main treatments

Suspensions of 0, 10, 20, 40, 60, 80 and 160 mg/L nanoparticles in three replicates were prepared by ultrasonic mixing (using a Misonix Sonicator 3000, Iran) for 60 minutes. These suspensions released 0, 0.017, 0.116, 0.131, 0.140, 0.209 and 0.249 mg/L Cu^{2+} as assayed by atomic absorption flame spectrometry (Varian Spectra AA 220FS, Varian Spectra Co., Australia). To assay the effects of the nanoparticle preparations, 4×10^4 cells from the main stock of *Chlorella* were added to 40 mL of the treatment solutions. This number of cells was obtained by dilution of primary algal stock under completely sterile conditions. Then mixtures were then incubated at 24 ± 2 °C. The light program was 12 hours darkness and 12 hours light, controlled by a timer. These conditions were maintained constantly during the 72 hours of the experiment.

Determination of growth inhibition in Chlorella

Growth inhibitory effects were assayed according to the OECD201 method (19). At 24, 48 and 72 hours after the start of the experiment, a drop was removed from the cultures using Pasteur pipettes and the cells were counted on a Neubauer chamber under an optical microscope (20). After counting and recording the data, the average number of cells in top and bottom squares was calculated and then the number of cells was determined using following formula:

$$\text{Cell density per (mL)} = \text{average cells counted in the large square} \times 10^4 \times \text{dilution factor} \quad [1]$$

To determine the statistical significance of differences among the treatments at various concentrations of algal cells and control samples, one-way ANOVA was used. For identification of distinctions between each known level of treatment, the t-test was used.

$$NOEC \text{ (no observed effect concentration)} = EC_{50}/EC_{10} \quad [2]$$

EC: The effective concentration

Values of μ (growth rate per hour), G (doubling time per hour) and I (percent inhibition) were calculated by Fogg and Thake (17) equations (Equations 3, 4 and 5).

$$\mu = ln\, x_1 - ln x_0\, (t_1 - t_0)^{-1} \quad [3]$$

x_1 *is the biomass concentration at time* t_1

x_0 *is the biomass concentration at time* t_0

$$G = ln2\mu - 1 \quad [4]$$

$$I\% = (\mu c - \mu t)/\mu c \quad [5]$$

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SE-SEM, MIRA3 TESCAN) was used to investigate the effect of nanoparticles on the shape and size of cells in *C. vulgaris* and to image the surfaces. Imaging of the surfaces of control and NP-treated cells was performed on cells exposed to the 26.2 mg/L NP concentration (EC_{30} = 26.2 mg/L).

Determination of chlorophyll a contents

Algal cells were collected according to the method of Eijckelhoff and Dekker (21). Samples were extracted in 10 mL of 100% methanol in the dark at 4°C for 24 h. The extracts were then centrifuged at 3000 rpm for

10 min. Absorbance of the supernatant was measured at 647 and 664.5 nm against 100% methanol. The following formulae (21) were used for calculation of chlorophyll a (Chla) and beta-carotene (β Car) contents:

$$Chla = ((12.25 \times OD\ 663\ nm) - (2.79 \times OD\ 647\ nm)) \times Dilution\ factor \quad [6]$$

$$\beta\ Car = ((-0.43 \times od412 + 0.251 \times od431 - 4.376 \times od460 + 13.12 \times od480) \times 536/1000)) \times Dilution\ factor \quad [7]$$

Results

As shown in Figure 2A, the lag phase growth of *Chlorella* lasted 4 days before entering the logarithmic phase, which continued until the 16 day; the stationary phase finally started after 16 days. Nanoparticle stress was initiated by exposing the cells in the exponential growth phase (days 6 to 16) to the particles. This time frame was chosen to ensure that the cells were actively metabolizing (19). Algal growth under different concentrations of CuO-NPs showed that increasing concentrations of CuO-NPs reduced growth of *C. vulgaris* (Figure 2B). 24 hours after initiating the experiment, the number of cells in the cultures decreased with increasing concentrations of nanoparticles; the reduction in cell numbers was significant in cultures exposed to 160 mg/L of CuO. According to t-test analysis, the numbers of cells under CuO-NPs treatments were significantly different from control at each 24 hour time point assayed. In addition, specific growth rate was reduced (Figure 3A). This reduction is significant compared to the control at concentrations higher than 60 mg/L after 72 hours. In contrast, the growth rate continuously increased in control samples throughout the experiment.

Doubling time (*G*) is the time required for doubling the number of cells. This parameter decreased in control samples within 72 hours (indicating that cell growth was accelerating), but it significantly increased in NP-treated cells with increasing concentrations of nanoparticles (Figure 3B). Percentage of growth inhibition (*%I*) increased for all treatments with increasing concentration and time of treatment (Figure 3C) compared to the control. The effective concentration for 72h EC_{50} and 72h EC_{90}, were 43.699 and 152.0197 mg/L respectively (Table 2).

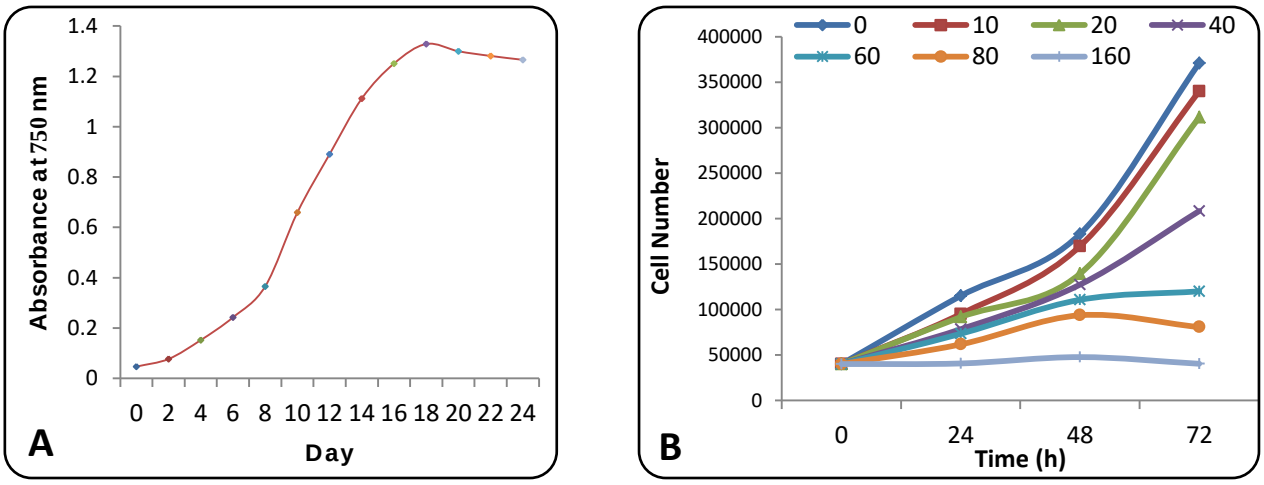
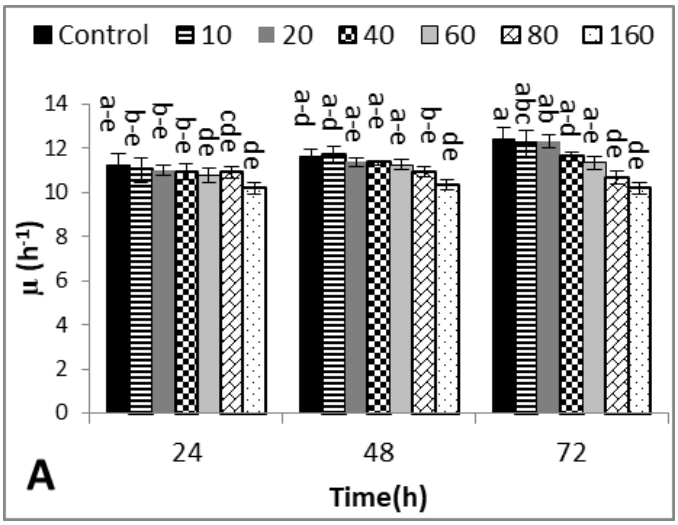
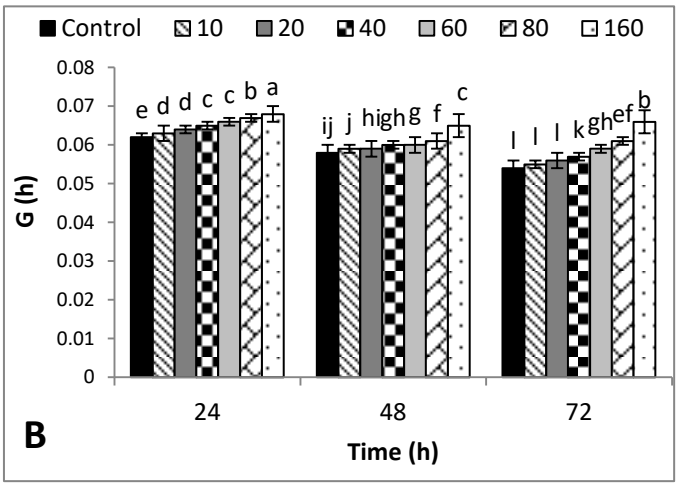


Figure 2. A. Growth curves of microalgae *Chlorella vulgaris* at 25±2°C under illumination with intensity of ~60 mol of photons m^{-2} s^{-1} with a photoperiod of 12 hours of light and 12 hours of darkness in the aerated growth chamber. B. Average of *Chlorella* cell number at different time points and different concentrations of CuO-NPs (mg/L). The data represent the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to t-test with $p<0.05$.





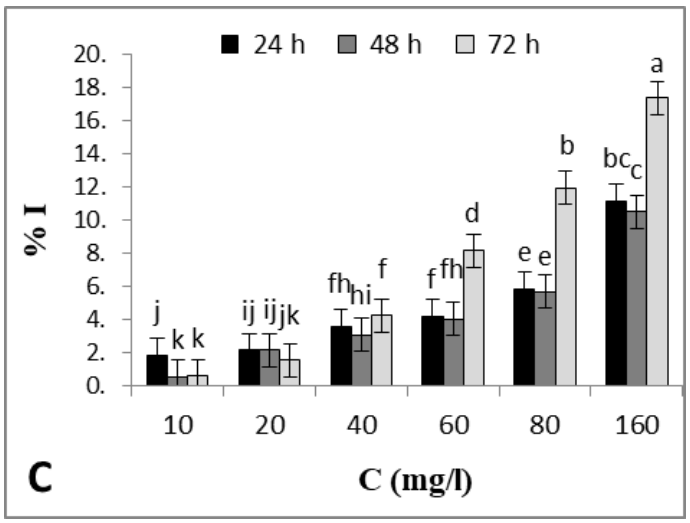


Figure 3. A. Specific growth rates in *Chlorella vulgaris* exposed to CuO-NPs (mg/L). B. Doubling time (G) during the experiment. C. Percentage of specific growth inhibition (%I). The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to t-test with $p<0.05$.

Table 2. The values of EC 10, EC50, EC 90 and NOEC

Time (h)	EC*$_{10}$ (mg/L)	EC$_{50}$	EC$_{90}$	NOEC**
24	5.78	137.02	3244	13.42
48	12.35	77.76	489.2	7.77
72	12.58	43.39	152.1	4.36

* EC: The effective concentration
** NOEC: No observed effect concentration

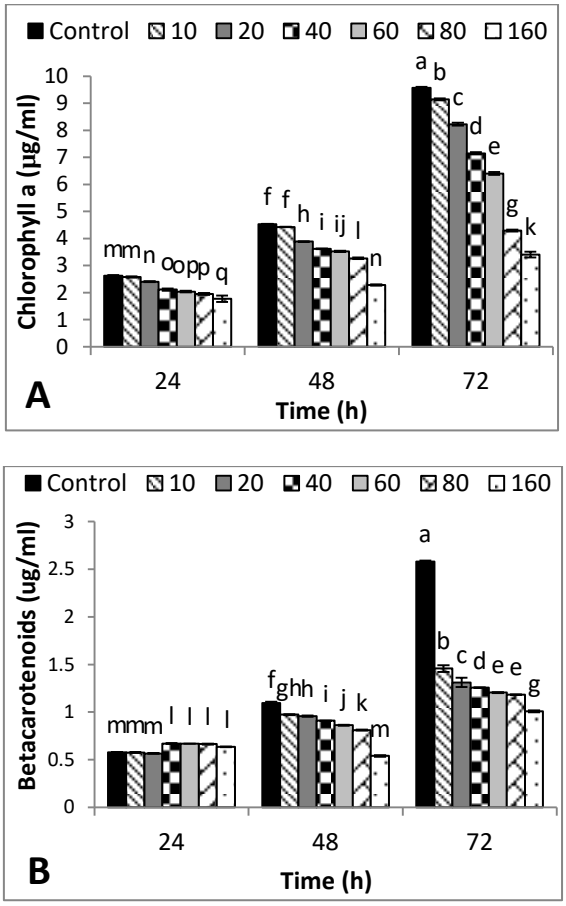


Figure 4. A. Changes of chlorophyll a. B. Changes of β-carotene in *Chlorella vulgaris* (µg/ml) in different concentrations of CuO-NPs (mg/L) within 72 hours. The data represents the average of three replicates ± standard error (SE), respectively. Different letters indicate significant differences among treatments according to t-test with p<0.05.

Statistical analysis showed, also, that treatment with CuO-NPs had a significant impact on chlorophyll content of the test samples and with increasing concentrations of nanoparticles, in each case decreased in treated cells compared to control (Figure 4A). This difference between all the treatments was particularly strong 72 hours after exposure to stress.

In addition, the amount of β-carotene significantly increased compared to control at NP concentration of 40 mg/L after 24 hours. However, after 48 and 72 hours exposure to stress, β-carotene content decreased relative to the control, and this difference was large after 72 hours (Figure 4B).

Electron microscopy was employed to gain some insight into the mechanism of growth inhibition in the NP-exposed algae. Generally, electron microscopic images showed that the nanoparticles bound to each other and, more relevantly, to the surface of the algal cells. It caused altering the appearance of cells through disruption of direct physical interactions between them and increasing the number of lysed cells (Figure 5A-E). In addition, CuO-NPs often formed bridges between the cells and increased cell aggregation in areas where the nanoparticles had accumulated.

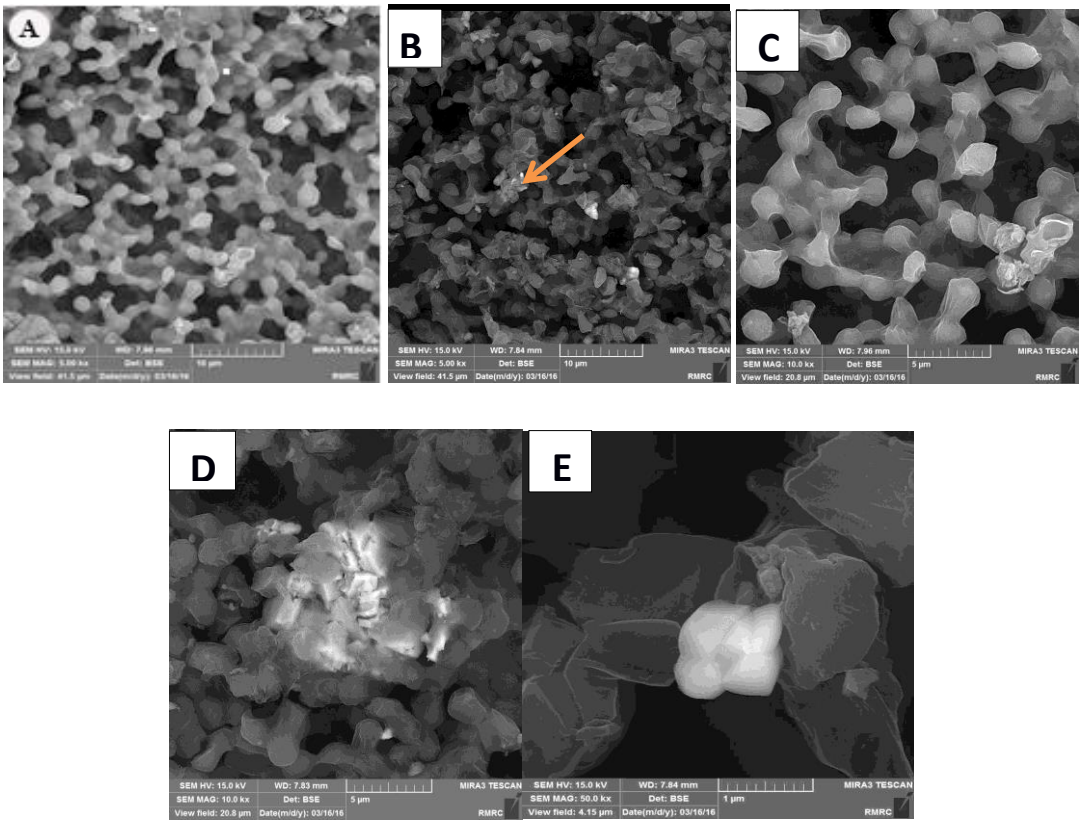


Figure 5. SEM images of *Chlorella vulgaris* after 72 hours exposure to CuO-NPs at concentration of 26.2 mg/l. A, C. Control samples. B, D, E. *Chlorella* cells treated with CuO-NPs. Arrow indicates CuO.

Discussion

Results of this research show that the "specific growth rate (μ)" decreased and "doubling time (G)" increased with increasing CuO-NPs concentrations, similar to toxicity of ZnO-NPs on *Chlorella vulgaris* and *Scenedesmus dimorphus* (16). As a result of toxic effects of Cuo-NPs, the lowest rate of growth compared with controls is observed after 72 hours at concentrations of 80 and 160 mg/L. Some researchers attribute the toxicity of NPs to the release of metal ions (22, 23). Aruoja et al. found that Cu^{2+} was the main cause of toxicity of CuO-NPs to *Pseudokirch-neriella subcapitata* (24). Although some studies indicate that CuO-NPs induce *Chlamydomonas reinhardtii* to yield oxygen radicals ROS and that the concentration of ions may well underlie the toxicity of CuO-NPs (25). Another research found that Cu^{2+} content in fronds of *Landoltia punctata* exposed to CuO-NPs was four times greater than in fronds exposed to an equivalent dose of dissolved copper (5). That CuO-NPs aggregated with *C. vulgaris* and precipitated, is clear in Fig. 5. Furthermore, exposure to CuO-NPs reduced the growth of *C. vulgaris*, perhaps as a result of shading. Similarly, studies revealed that Ag nanoparticles could directly affect the surface of *C. vulgaris* cells and form large agglomerations. While 50 ng of Ag is likely incapable of entering the cells, it can act as the bridge for cells to attach with each other and, thus, speed up cell aggregation (15). The toxicity of N-SiO_2 to *Scenedesmus* may be caused by the absorption of nanomaterials to the surface of algal cells (22). Binding of the particles to the surface of *Chlorella* sp. and *Scenedesmus* by N-Al_2O_3 nanoparticles has been shown to reduce the utilization rate of light, which may well be the cause for slower growth (26). Thus, the condensation effect of CuO-NPs on *C. vulgaris* may be one of the possible explanations for its toxicity. Other effects are likely operative as well. It was shown that the Ag^+ released by Ag nanoparticles led to the toxicity of *Thalassiosira*, while no toxicity was found after the free Ag^+ was infiltrated by membrane or complexed with mercaptan. Most Ag

nanoparticles, however, made nontoxic aggregates larger than 0.22 µm diam. in sea water (27), again suggesting that shading may be the major mechanism of growth inhibition by NPs. Photosynthesis is a significant influential factor in algal growth. Chlorophyll a (Chl a) is the central basis of photosynthesis, and the Chl a content of the cells is a strong indicator of the growing condition of algae. Fig. 7 shows how the concentrations of CuO-NPs affect the Chl a contents of the cells at 72 h.

The highest concentration of Chl a was found in *C. vulgaris* control group, but in the treated groups of this species, Chl a decreased by time expansion and increasing CuO-NPs concentrations (Fig. 4A). The amount of β-carotene decreased significantly after 48 hours in all concentrations compared to control; although after 24 h the amount was increased due to oxidative stress caused by CuO-NPs. Carotenoids have an important role in the non-enzymatic defense system of plants but it seems that they cannot be maintained for a long time as shown by their decrease after 48 h, due to damage to biosynthesis pathways. The decrease in Chl a and carotenoid content is a usual event in plants exposed to nanoparticles, as has been previously reported in algae (28). The degradation of pigments is usually the result of oxidation induced by contaminants. The values of Chl a were significantly variable when the concentrations of CuO-NPs reached 4.36 mg/L or above, showing toxicity. CuO-NPs proved to have a damaging effect on the pigment content of *C. vulgaris*. As was shown previously (29), increasing the concentration of nanoparticles was associated with reductions in chlorophyll content. This effect may be due to inhibition of chlorophyll synthesis (29). The impact of CuO-NPs on the toxicity and its following oxidative stress to green alga *Chlamydomonas reinhardtii* was investigated previously (8). It was observed that CuO-NPs caused growth inhibition and a significant reduction in carotenoid levels. Analysis of esterase activity indicated a reduction in cell metabolic activity as CuO-NP concentration increased. CuO-NPs induced a rise of reactive oxygen species, as well as lipid peroxidation of cellular membranes after 72 h of exposure, in comparison to control. Investigation of CuO-NPs uptake indicated that they are present in different sites of *C. reinhardtii* cells (3).

One study on the toxicity of aluminum nanoparticles in *Chlorella kesslari* reported that LC90 was 0.6, 8.2, and 7.4 mg/L for 5, 26, and 78 nm nanoparticles, respectively; with toxicity decreasing as nanoparticle size increased (30). An investigation on *Chlorella vulgaris* and *Dunaliella tertiolecta* concluded that the effect of 50 nm silver nanoparticles for 24 hours at 0-10 mg/L concentration resulted in extensive compression of the algal cells. In addition, algal chlorophyll was severely reduced. Silver nanoparticles had growth inhibitory effects in both algal species and dramatically decreased cell survival (15).

The scanning electron micrographs of *C. vulgaris* cells showed that they got denser after CuO-NPs absorption and their external surfaces started to fracture compared with the cell surfaces of cells lacking any contact with these particles. The composition of the cell wall was probably affected by the metal ions. Silver nanoparticles have been observed to cross plasma membrane into the cytosol, leading to the destruction of organelles such as the endoplasmic reticulum, and reducing the activity of photosystem II, leading ultimately to a decrease in Chl a, in photosynthesis and also dry weight. This leaded to reduced cell growth and survival, but not surprisingly (30). The toxicity of nanoparticles is inversely proportional to the size and form of Cu^{2+}> N-CuO> M-CuO, respectively, in *Chlorella* sp. (29). Reducing the particle size increased surface to volume ratio and thus increased attraction forces between particles, aiding the agglomeration of nanoparticles. In addition, it was shown that copper oxide nanoparticles attached to algal cells (29). An electron micrographic survey of *Chlorella* cells exposed to copper found that cell concentrations after copper adsorption were higher, and the cell surfaces had begun to fracture in comparison with the smooth surface of cells unexposed to the metal (32). It can reasonably be argued that these changes were caused by heavy metals attachment to functional groups such as amino, amide, carboxyl and hydroxyl, located on algal cell surfaces (33, 34). Furthermore, the shading effect of CuO-NPs on *C. vulgaris* affected photosynthesis and cell growth (15).

Conclusion

CuO-NPs have significant toxic and growth inhibition effects on *C. vulgaris*. Data analysis showed nanoparticles have wide effects on growth of *C. vulgaris* and there is a direct relationship between the concentration of nanoparticles and their toxicity on the algae. With increasing nanoparticle concentration, some key factors such as chlorophylls, carotenoids and some other growth factors of *C. vulgaris* decreased significantly.

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