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# COMPARATIVE STUDY OF COPPER NANOPARTICLES TOXICITY ON TWO FRESHWATER MICROALGAE SPECIES

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## Abstract

The objective of this study was to compare the toxicity of copper nanoparticles (CuNPs) on two freshwater microalgae species, namely the unicellular green algae *Chlorella vulgaris* and the cyanobacteria *Microcystis aeruginosa* KG. The experiments were performed using 0, 0.01, 0.05, 0.1, 1, and 5 ppm concentrations of CuNPs, which were added into a flask containing 145 mL culture medium for algae growth (CB) and 5 mL biomass of each algae. The capacity to generate biomass and change the cell structure of the two strains was measured in days: D0, D2, D6, and D10. CuNPs were synthesized via chemical reduction. The nanomaterial was characterized to have a spherical shape with an average size of around 30-40 nm and was well distributed without aggregating in solution. The results demonstrated that CuNPs have different effects on the growth of the two species of algae. CuNPs aggregated together to change the *C. vulgaris* cells, thereby increasing their biomass. On the contrary, at concentrations 1 and 5 ppm, *M. aeruginosa* KG strain was inhibited after 6 and 10 days. The techniques used to characterize material structure of CuNPs, namely SEM, TEM, EDX, and XRD, demonstrated that CuNPs were detected via the appearance of numerous small black dots on the CuNPs-treated cell with 10.36% and 0% Cu content for *M. aeruginosa* KG and *C. vulgaris*, respectively.

*Keywords*: *Chlorella vulgaris*, copper nanoparticles, effect, inhibition, *Microcystis aeruginosa* KG,

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## 1. Introduction

For many years, nanotechnology has been considered as a crucial technique that involves the synthesis and application of materials at nanometer dimensions (nm) (Swain et al., 2014). Nanomaterials possess some advanced characteristics in comparison to traditional materials because of their small size (>100 nm), which leads to a larger surface area over volume, and a crystalline structure that is highly reactive, creating a Plasmon surface resonance effect (Jayatissa et al., 2006; Kerli et al., 2021; Park et al.,

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2006). Thus, nanotechnology is applied in many domains, such as biotechnology, biomedical, pharmaceutical, and especially environmental pollution treatment, because of several advanced features in terms of functionality and size equivalent to biomolecules (Kerli et al., 2021; Shahzadi et al., 2021). However, the metal nanoparticles synthesized from these fields have been rapidly developed, causing increased risk to the human and ecological systems due to exposure (Khan et al., 2020; Kerli et al., 2021).

Nanomaterials can enter environmental compartments either intentionally or unintentionally, so it is necessary to pay attention to their potential effects on the environment. Some nanomaterials have been documented to be toxic for humans and various organisms not only due to direct contact but also due to prolonged exposure to the contaminated environment (Heinlaan et al., 2008; Khan et al., 2020; Wang et al., 2011; Zhang et al., 2009). Consequences include alterations in the community composition (Das et al., 2012), ecosystem metabolism (Colman et al., 2014), and nutritional cycle (Choi and Hu, 2009). The toxicity degree of nanoparticles might vary, depending on numerous factors, including particle type and size, organism species, synthesis process, and experimental method (Heinlaan et al., 2008; Zhang et al., 2009). Previous studies have revealed that the negative impact of nanomaterials on the environmental quality, aquatic animals, and aquatic vegetation vary greatly. For example, $TiO_2$ nanomaterial (20 g/L) impeded the development of *Vibrio fischeri*, *Daphnia magna* crustaceans, and *Thamnocephalus platyurus* (Heinlaan et al., 2008). According to Zhu et al. (2006), an increase in the concentration of $TiO_2$ increased the mortality of *Daphnia magna* upon exposure (Zhu et al., 2006). Similarly, the results from Hund-Rinkle et al. (2006) demonstrated that the nature of the nanoparticles determined the ecotoxicological effects on both algae and zooplankton.

Algae, as the primary producer, plays a significant role in the aquatic ecosystem by not only creating food chain and biomass in the water environment but also by contributing to the self-purification of polluted water (Chorus, 2010; Codd, 1994; Sankar et al., 2014). *Microcystis* is a genus of cyanobacteria found in brackish/saltwater and nutrient-rich water sources. Some of the strong, potent toxins produced by the *Microcystis* genus is found in drinking and bathing water sources, posing a major threat to human health as well as a severe threat to water resources worldwide, known as "water blooming" (Chorus, 2010; Codd, 1994). To ameliorate the increasing proclivity of "water blooming" in receiving waters, the prevention and control of cyanobacteria growth in aquatic environments are indispensable concerns that must be addressed. *C. vulgaris* are gram-positive, unicellular eukaryotes with cell walls comprising primarily cellulose (Rodea-Palomares et al., 2012). Due to the structure of these cells with a sporopollenin membrane and a high chlorophyll content, *C. vulgaris* green algae may absorb heavy metals, pesticides, insecticides, and other plant protection chemicals (Marambio-Jones and Hoek, 2010; Qian et al., 2016). Therefore, *C. vulgaris* is one of the model organisms generally used for examining the toxicity of nanomaterial (Oukarroum et al., 2012; Shirai et al., 1989; Zhang et al., 2009).

Copper (Cu) nanomaterial has been synthesized and utilized in various applications, such as semiconductor, catalysts, photovoltaic cells (Z. Wang et al., 2011), wood preservation, and antimicrobial textiles (Gabbay et al., 2006). It can be used to replace noble metal catalysts for carbon monoxide oxidation (Zhou et al., 2006), ecological monitoring, and wastewater treatment (Ingle et al., 2014; Rispoli et al., 2010). Due to its excellent thermal conductivity properties, the CuO nanofluid was used as a heat transfer material in mechanical equipments (Chang et al., 2005; Zhou et al., 2006). Cu is required by almost all living forms as an essential trace element; however, it becomes hazardous in unrestrained amounts. Cu toxicity has been extensively investigated for both aquatic and terrestrial organisms. Studies have suggested that CuO nanoparticles inhibit the growth of bacteria, protozoa, crustaceans, and algae (Bui et al., 2016; Fujiwara et al., 2006). Furthermore, Cu nanoparticles (CuNPs) have more special properties than their formulations (CuO and Cu) and are unstable in solution and air (Wang et al., 2011). Numerous recent studies have indicated that CuNPs can be toxic to unicellular algae growth by inhibiting or blocking the photosynthetic II electron transport processes, resulting in mitochondrial damage and a loss of membrane integrity with reactive oxygen species (ROS) formation, as well as a drop in the growth and pigment levels of cyanobacteria (Saison et al., 2010; Sankar et al., 2014). CuNPs restricted the growth of *M. aeruginosa* more strongly than other freshwater phytoplankton species, depending on the concentration of Cu and dissolved Cu in *M. aeruginosa* cells (Zeng et al., 2010). The downregulation of lipid peroxidation and the photosynthesis process by metal nanoparticles inhibits the *M. aeruginosa* algae cell growth (Aruoja et al., 2009). In addition, the size and forms of nanoparticles were also detected as determining factors influencing the growth of algae.

A number of concerns regarding the impact mechanism of nanoparticles in the environment and aquatic ecosystems need to be addressed (Römer et al., 2011). While numerous studies have been conducted, this concern is still inadequately explored. Hence, this research was conducted with an objective of assessing the toxicity of CuNPs on the growth and development of the green algae *C. vulgaris* and the cyanobacteria *M. aeruginosa* KG. The results from this study may facilitate a better understanding of the CuNPs' potential risks in aquatic environments and the mechanism of selective inhibition of Cu nanomaterial to control the "water bloom" of cyanobacteria *M. aeruginosa* KG.

## 2. Materials and methods

### 2.1. Algal cultures

The specimens of *C. vulgaris* and *M. aeruginosa* KG were retrieved from the Department of Environmental Hydrobiology, Institute of Environmental Technology, Vietnam. They were cultured in CB medium (culture medium for algae growth) at 25°C ± 2°C under fluorescent light (1000 lux, 14h light/8h dark). The CB medium was composed (mg L−1) of ingredients: 1-disodium glycerophosphate = 50; $KNO_3$ = 100; bicine = 500; $MgSO_4.7H_2O$ = 40; biotin = 0.0001; $Ca(NO_3)2.4H_2O$ = 150; thiamine hydrochloride = 0.01; vitamin B12 = 0.0001 with 3 mL of PIV micronutrients. The PIV medium contained $CoCl2.6H_2O$ = 0.4; $ZnSO_4.7H_2O$ = 2.2; $EDTA.2H_2O$ = 100; $Na_2MoO_4.2H_2O$ = 0.25; $MnCl_2.4H_2$ = 3.6 and $FeCl_3.6H_2O$ = 19.6. This medium was adjusted to pH 9 before autoclaving by adding NaOH 0.1M or HCl 0.1 M solutions. The initial algal cell number was $10^5$ $mL^{-1}$. All experiments were conducted using this medium.

### 2.2. Chemical synthesis and characterization of CuNPs

The CuNPs were synthesized using the chemical reduction method of $CuSO_4$ in an aqueous media with NaBH4 described by Marambio-Jones and Hoek, 2010. The morphology and size of the nanoparticles were determined by using a scanning electron microscope (SEM) (FESEM, S4800-Hitachi, Japan), transmission electron microscope (TEM) (JEOL-JEM1010, Japan), and X-ray diffraction (XRD) (BRUCKER D8-Advance 5005 diffractometer with Cu Kα radiation).

### 2.3. Experimental methods

#### 2.3.1. Experimental setup

Briefly, CuNPs were added into each flask containing 145 mL CB medium and 5 mL biomass *C. vulgaris* algae in order to the CuNPs concentration in every three flasks were 0 (control samples) and 0.01, 0.05, 0.1, 1, and 5 ppm (experimental samples). A similar procedure was performed with the cyanobacteria strain *M. aeruginosa* KG. Overall, there were 36 flasks (18 flasks for unicellular green algae *C. vulgaris* and 18 for the cyanobacteria *M. aeruginosa* KG) and each concentration was repeated thrice. The algal growth assays were performed under equivalent temperature as well as photoperiod, and was monitored for 10 days.

#### 2.3.2. Observation of the biomass-generating capacity

The biomass-generating capacity assays were performed as follow: the algal growth was assessed by measuring optical density of the culture suspension and analyzing Chlorophyll-a content (Lorenzen, 1967) in days D0, D2, D6, and D10. The algal cell density was counted using a Sedgewick–Rafter chamber under light microscope (Olympus BX51) (Burns et al., 2000). The inhibitory effectiveness (%) of algal growth was calculated using the following formula (Park et al., 2006; Usman et al., 2013) (Eq. 1):

$$Inhibition\ \ efficiency\ \ (\%) = \\ = [(control - treatment) / control)] \times 100 \quad (1)$$

#### 2.3.3. Observation of algae cells

The ultrastructure of the two algae cells and the elemental composition of the cell surface following CuNP exposure were analyzed by energy-dispersive X-ray spectroscopy (EDX) combined with SEM and TEM (JEM 1010, Japanese JEOL Company) (Duong et al., 2016). Briefly, the algae cells were concentrated and then embedded into agar. After fixation with 2.5% glutaraldehyde, the samples were exposed to osmium tetroxide for 2h. The samples were then treated with 1.0 percent $OsO_4$ for 1.5 hours before being dehydrated thrice in acetone. The specimen sections were stained with uranyl acetate and alkaline lead citrate before being examined using TEM and SEM–EDX.

### 2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. All the data of the three replicates in this study were averaged and the means ± SD (standard deviation) at the 5% probability level were calculated for analysis.

## 3. Results and discussion

### 3.1. Characteristics of CuNPs

Figures 1 and 2 depict the characteristics of CuNPs. The SEM image of the material CuNPs in Fig.1a indicates that Cu nanoparticles have a relatively homogeneous surface. Fig. 1b demonstrates that the TEM image of Cu nanoparticles have a relatively uniform size, estimated to be at 30–40 nm.

The XRD pattern (Fig. 2) shows that the Cu nanoparticles had face-centered cubic structures (FCC) with three diffraction peaks at (111), (200), and (220), corresponding to the angle 2θ = 43.3; 50.4 and 74.00 with high intensity (Marambio-Jones and Hoek, 2010; Soomro et al., 2014). There were no other diffraction peaks in the range 2θ from 0 to 800. These results indicate that the fabrication material are in crystalline form and pure.

### 3.2. The toxicity of the CuNPs

#### 3.2.1. Comparison of the biomass-generating capacity

##### 3.2.1.1. Chlorophyll-a content

The responses of different algae species to various toxic substances vary greatly (Boyle, 1984). *M. aeruginosa* was found to be more sensitive to nanomaterials than green algae (Heinlaan et al., 2008).

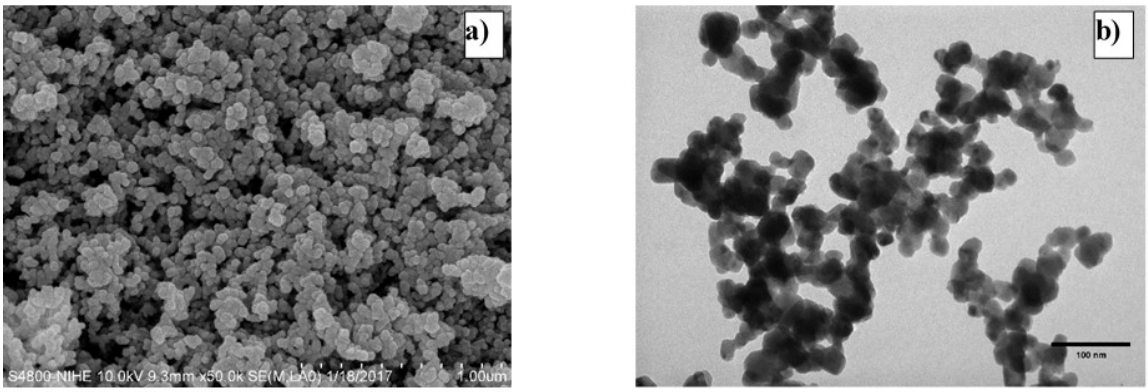



**Fig. 1.** SEM (a) and TEM (b) images of CuNPs crystalline prepared by the chemical reduction method

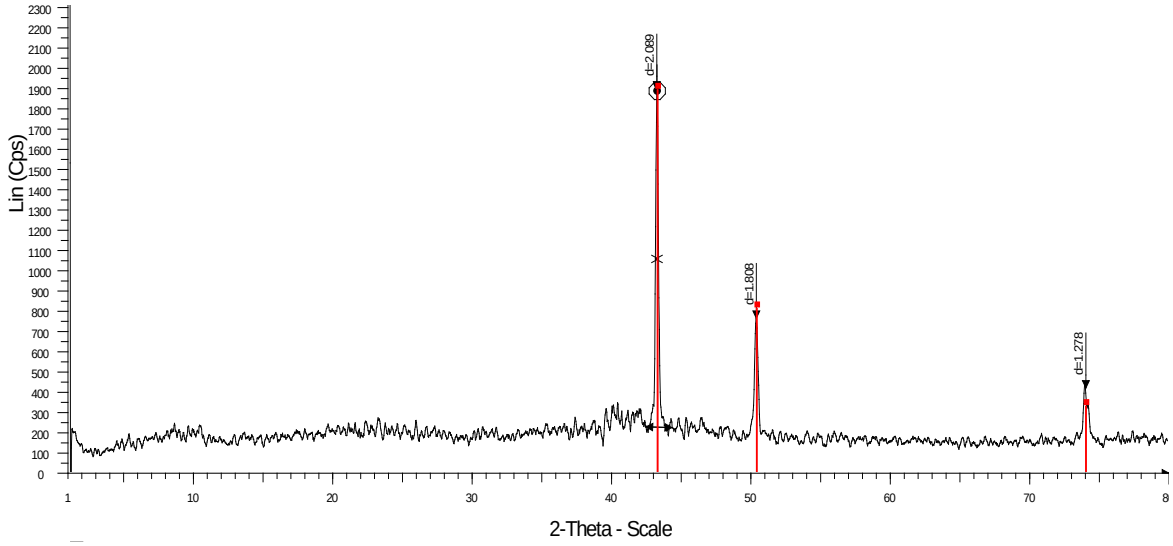



**Fig. 2.** XRD patterns of CuNPs crystalline prepared by the chemical reduction method

In the current study, CuNPs selectively affected algal growth rates. The photosynthetic activity of *M. aeruginosa* KG and *C. vulgaris* following the addition of Cu nanomaterial was measured using the UV-VIS (Ultraviolet-Visible) instrument.

The chlorophyll-a concentration of the two species was significantly different ($p < 0.05$). The concentration of chlorophyll-a of *C. vulgaris* under Cu nanomaterial treatments is shown in Fig. 3b. After 2 days of exposure, the concentration of chlorophyll-a at the different nanomaterial concentrations did not significantly change, but there was noteworthy accumulation after 6-10 days. The photosynthetic activity proliferated in the cells exposed to all the experimental concentrations (0.01, 0.05, 0.1, 1, and 5 ppm). The chlorophyll-a content at the starting day (D0) was 3.42 ± 0.81 µg/L, increasing to 69.8 ± 15.9 µg/L after 10 days (D10). After 2, 6, and 10 days of exposure, the photosynthetic activity of *M. aeruginosa* KG was not affected by Cu nanomaterial at 0.01, 0.05, and 0.1 ppm, as shown in Fig. 3a. When cells were exposed to higher concentrations of CuNPs, the chlorophyll-a content decreased from 2.24 ± 0.11 µg/L on day 0 (D0) to 1.73 µg/L and 0.43 µg/L after 2 days of exposure to the concentrations of 1 and 5 ppm, respectively.

*3.2.1.2. Cell count*

The inhibitory effect of Cu nanomaterial on the growth of two freshwater algae species was analyzed by direct cell counting (Fig. 4). The cell density of *M. aeruginosa* KG dramatically reduced after 2, 6, and 10 experimental days when the concentration of CuNPs was increased. For the concentrations 0.01, 0.05, and 0.1 ppm, CuNPs did not affect the growth of *M. aeruginosa* KG and the cell density increased linearly with the control experiment. However, the cell counts of *M. aeruginosa* KG significantly diminished after exposure to higher concentrations (Fig. 4a). By comparing the results between the control sample and each CuNPs treatment (the concentrations of 1 and 5 ppm), the current study demonstrated that the cell counts of *M. aeruginosa* KG from 132.8 ± 26.8 cell/mL (D0) decreased to 12.8 ± 3.35 cell/mL (D10). In contrast, the cell aggregation observed in the experimental testing samples of *C. vulgaris* spiked with CuNP exposure (Fig. 4b). The cell density in all treatments reached the control sample after the experimental duration; the observed values were 10.1 ± 2.5 cell/mL (D0) and 126.5 ± 14.9 cell/mL (D10). This finding indicated that CuNPs did not affect the

cell proliferation of the *C. vulgaris* strain.

*3.2.1.3. Efficiency of growth inhibition*

In the current study, CuNPs affected only the surface of *C. vulgaris* cells, slightly reducing its growth without causing cell death. Therefore, no significant toxicity was observed on *C. vulgaris* at all the concentrations of added CuNPs during the 10 experimental days. Both the cell counts and the concentration of chlorophyll-a increased linearly with the control sample and the percentage of growth inhibition reached to a maximum value of 43.4% with a concentration of 5 ppm of CuNP at the end of the experimental period (Fig.5b). On the contrary, the growth inhibition efficiency of CuNP on *M. aeruginosa* KG at concentrations 1.0 and 5 ppm were 90.1% and 93.7%, respectively (Fig.5a). The EC50 value calculated was 0.7159 mg/L, which is lower than the values found by authors Sankar et al. (2014); Zeng et al. (2010).

According to Sankar et al. (2014), the growth inhibition effectiveness of CuO nanomaterials on *M. aeruginosa* at concentrations 12.5 and 50 mg/L were 31% and 89.7%, respectively. *M. aeruginosa* algae were more adversely affected by CuNPs than other freshwater algae species due to its extreme sensitivity to Cu metal (Zeng et al., 2010). Moreover, the growth inhibition of CuNPs is dependent on the intracellular Cu concentrations and biotic agents. Metal toxicity is also related to its affinity with sulfur. Cu has a much higher affinity toward thiol compounds than other metals (Miao and Wang, 2007; Nies, 2003). The high sensitivity of *M. aeruginosa* to CuNPs toxicity can be explained via two primary mechanisms. The first mechanism involves the relatively low requirement for the micronutrient Cu of the cyanobacteria ancestors. The second mechanism involves the detoxification ability of bacteria by self-absorbing Cu metal in intracellular compartments, increasing the intracellular Cu concentration. These mechanisms could be relevant in explaining the findings of the current study. The current study found that increasing the concentration of CuNPs from 0.01 ppm to 5 ppm over a 10-day period had a significant effect on the development of the cyanobacterium *M. aeruginosa* KG. In fact, the sample containing CuNPs is higher in the cell leading to lower levels of detoxification of surplus internal Cu, and if the concentration of Cu is in excess, it may induce significant toxic effect by altering the photosynthetic pigment contents (Zhang et al., 2009). After exposure to Cu, the DNA fibrils could aggregate or cluster, leading to a reduction in the electron density of the nucleoplasm in *M. aeruginosa* cells (Verhoeven, 1979).

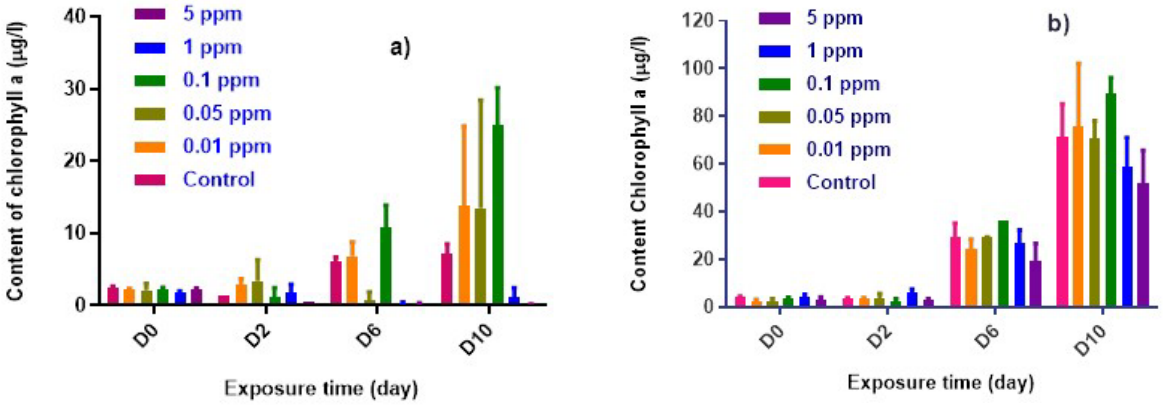



**Fig. 3.** The content of chlorophyll-a of *Microcystis aeruginosa* KG (a) and *Chlorella vulgaris* (b) after exposure to Cu nanomaterial at different concentrations (ppm); values are reported as the mean of three replicates ± SD (n = 3).

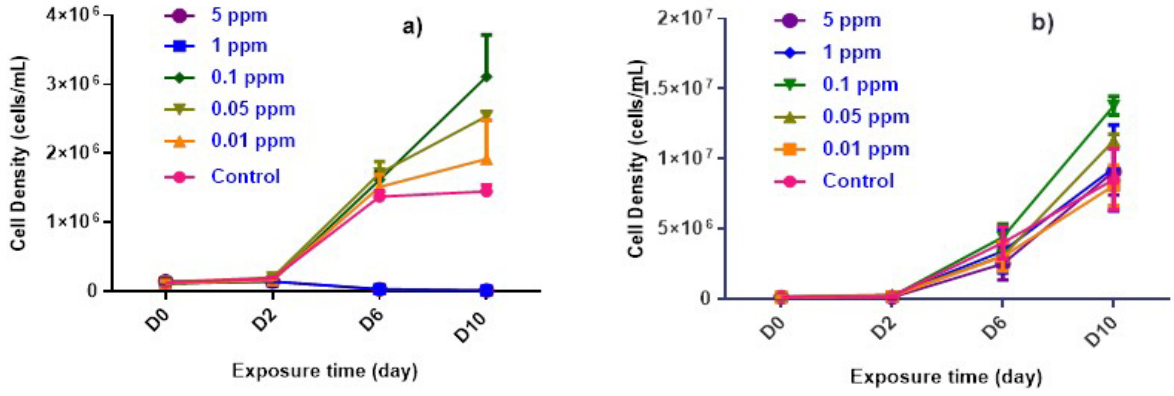



**Fig. 4.** The cell counting of *Microcystis aeruginosa* KG (a) and *Chlorella vulgaris* (b) versus time at different concentrations of CuNPs (ppm); values are reported as the mean of three replicates ± SD (n = 3)

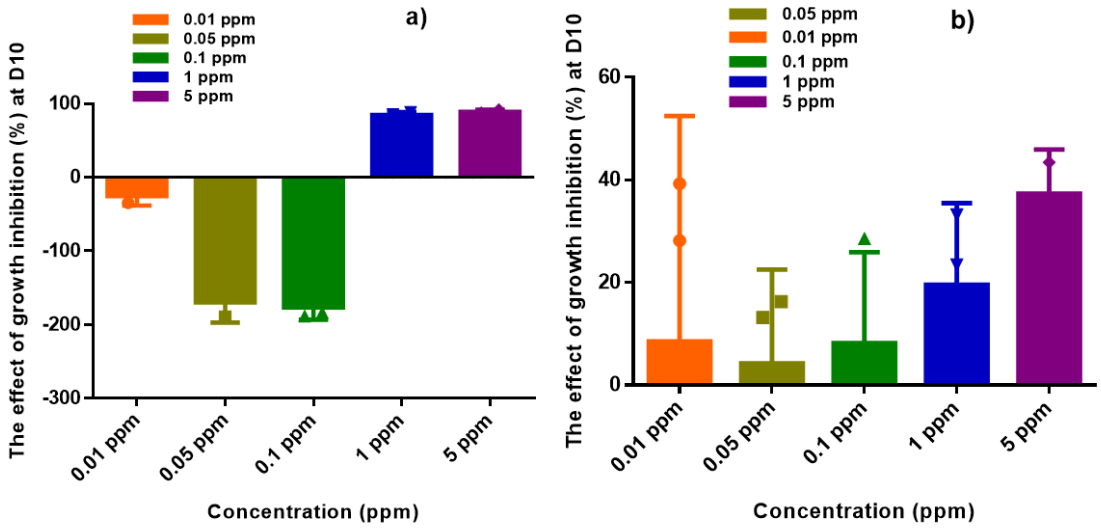



**Fig. 5.** The growth inhibition efficiency (%) of CuNPs on *Microcystis aeruginosa* KG (a) and *Chlorella vulgaris* (b) at D10; values are reported as the mean of three replicates ± SD (n = 3)

The finding from the current study regarding the toxicity of CuNPs toward *C. vulgaris* algae is in line with previous studies (Tubbing et al., 1994; Wang et al., 2008; Zhang et al., 2009; Fathi et al., 2020; Hashmi et al., 2021). Tubbing et al. (1994) have indicated that free Cu in the water was quickly complexed to carbonates and hydroxides, reducing its effect on the marine algae species. Zhang et al. (2009) and Hashmi et al. (2021) showed that the size of the material also had considerable impact on the growth inhibition. To illustrate, Cu ions and CuO bulk particles may have lower toxicity than CuNPs (Hashmi et al., 2021; Zhang et al., 2009). The recent finding demonstrated that the aggregation of Cu nanomaterial might reduce surface activity sites, thereby depleting its toxicity to C. vulgaris strain (Wang et al., 2008).

*3.2.2. Comparison of the toxicity of CuNPs by changes in the morphology and structure of the two algae cells*

*3.2.2.1. Changes in the morphology of the two algae cells*

The morphology of the two algae cells was analyzed to evaluate the toxic impact of CuNPs. Fig. 6 reveals that *M. aeruginosa* KG cells under the influence of CuNPs changed their shape and color (Fig. 6b), compared with the control sample (Fig. 6a). Meanwhile, the shape of *C. vulgaris* algae cells (Fig. 6d) was relatively similar to that of the control sample (Fig. 6c). The finding is consistent with the previous studies. *M. aeruginosa* KG is gram-negative, and its cell wall is made of peptidoglycan and lipopolysaccharide layers as an alternative to cellulose in green algae *C. vulgaris* (Park et al., 2010; Taylor et al., 2016). Therefore, the cell wall of *M. aeruginosa* KG strain is thinner, and the nanomaterials could permeate the cell faster and more easily than C. vulgaris green algae cells (Oukarroum et al., 2012). Moreover, the cell density of *C. vulgaris* during the CuNPs treatments (Fig. 6d) was reduced significantly compared with the control sample (Fig. 6c).

Our findings revealed that the photosynthetic activity of algae was significantly affected by CuNPs in the culture medium. This reduced photosynthetic activity could play a significant role in limiting algal growth and cell density. *C. vulgaris* has some effective mechanisms for minimizing the toxic effects of CuNPs that can explain similar cell density of this strain during the treatments and control. The algae cells of this strain would immediately start detoxifying through the induction enzyme system after adsorption of CuNPs (Bui et al., 2016; Qian et al., 2016). Moreover, superoxide dismutase (SOD) is one of the most important antioxidative enzyme that is capable of catalyzing the superoxide dismutation ($O_2^-$) into oxygen and hydrogen peroxide (Pereira et al., 2014; Yin et al., 2012; Fathi et al., 2020). The SOD enzyme activity and ROS production could play a vital role in ensuring the survival of *C. vulgaris* algae cells after exposure to CuNPs. Biologically relevant ROS includes superoxide anion radicals, hydroxyl radicals, singlet oxygen, and hydrogen peroxide ($H_2O_2$). SOD enzymes may protect C. vulgaris cells against ROS by lowering the steady state of superoxide anions (Fathi et al., 2020).

In addition, with a nano-scale size, nanoparticles tend to clump together and increase the ratio between volume and surface area, preventing $Cu_2^+$ ions from releasing, and limiting the toxicity of the material. Moreover, the coatings on the surface of the material (such as chitosan, PVP) may also be responsible for hindering the release of $Cu_2^+$ ions, which in turn results in less capping the CuNPs and reduced impact on the growth of *C. vulgaris* (Chao et al., 2021;Usman et al., 2013).

*Change in the structure of two algae cells*

Further tests performed using TEM and SEM–EDX methods concurred with our initial findings. Both *M. aeruginosa* KG and *C. vulgaris* cells were treated with CuNPs (1ppm) for 24 h.

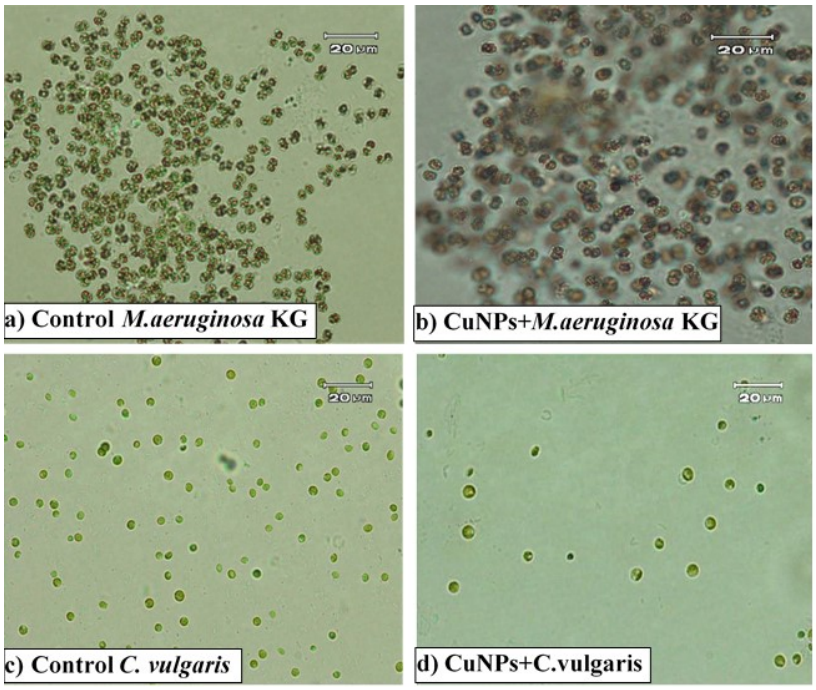



**Fig. 6.** Optical micrographs of *Microcystis aeruginosa* KG and *Chlorella vulgaris* cells in the control (a, c) and two algae cells after 24 hours of exposure to Cu nanomaterial 1 ppm (b, d). Bars, 20µm. Magnification 40×

Unlike the CuNP-treated samples, the TEM and SEM images of the control sample cells remained intact, and the form of two algal cells seemed to be spherical and smooth on the outside (Fig. 7a, 7c). However, the shape-changing of the two cells upon exposure to CuNPs could be visually noted. SEM (Fig. 8 a-b) and TEM (Fig. 7d) images of CuNPs-treated samples revealed that the cell wall was blurred, the membrane was thinner, and many small black dots were distributed in *M. aeruginosa* KG cells, whereas the green algae C. vulgaris sample did not show these features except for a marginally changed shape. *M. aeruginosa* KG cells contained around 10.36% of Cu (Fig. 9a), while *C. vulgaris* cells had almost 0% Cu (Fig. 9b). This finding may suggest that the black dots appearing in the *M. aeruginosa* KG cells could be because of CuNPs.

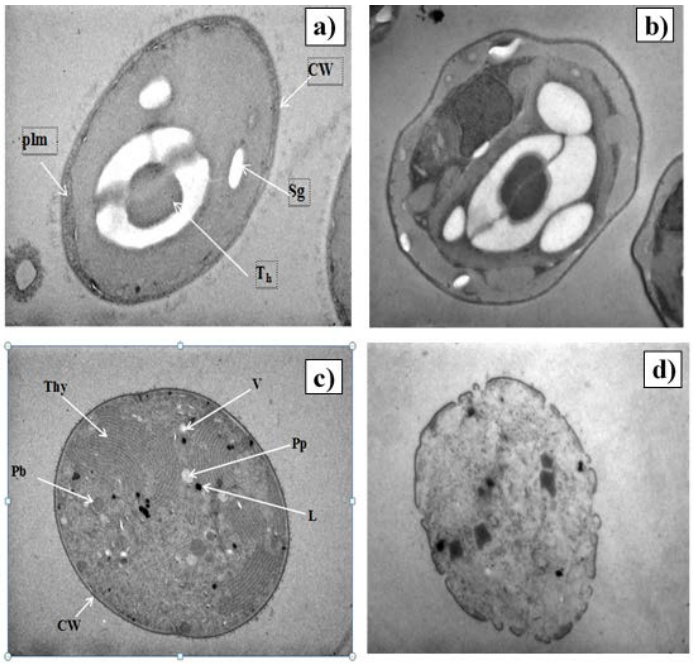



**Fig. 7.** TEM images illustrating the ultrastructural changes caused by the exposure of *Microcystis aeruginosa* KG and *Chlorella vulgaris* with the Cu nanomaterial at 1 ppm after 24 hours. *Chlorella vulgaris* and *Microcystis aeruginosa* KG cells in the control (a, c) and two algae cells after 24 hours of exposure with CuNPs (b, d). Many small black dots were distributing in *Microcystis aeruginosa* KG cells treated with Cu nanomaterial (CW - cell wall; Thy – Thylakoids; L - Lipid droplets; Pb - Polyhedral bodies; V – vacuoles; Pp - Polyphosphate bodies)

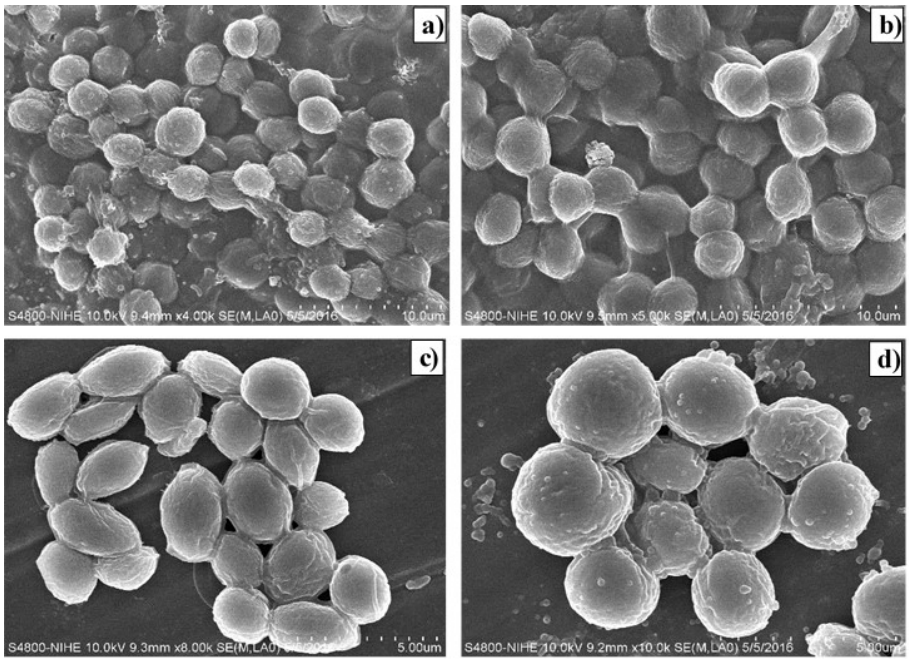
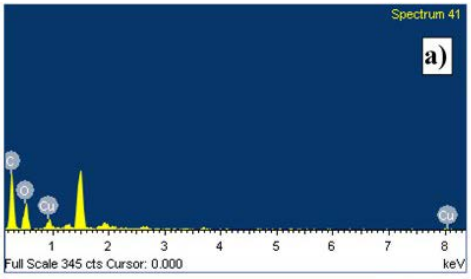



**Fig. 8.** SEM micrograph of *Microcystis aeruginosa* KG and *Chlorella vulgaris* before and after exposure to copper nanoparticles at 1 ppm for 24 hours. *Microcystis aeruginosa* KG (a, b) and *Chlorella vulgaris* (c, d)

| Element | Weight% | Atomic% |
|---|---|---|
| C K | 52.75 | 64.02 |
| O K | 36.89 | 33.60 |
| Cu L | 10.36 | 2.38 |
| Totals | 100.00 | |




| Element | Weight % | Atomic % |
|---|---|---|
| C K | 51.48 | 58.56 |
| O K | 48.52 | 41.44 |
| Cu L | 0.00 | 0.00 |
| Total | 100.00 | |

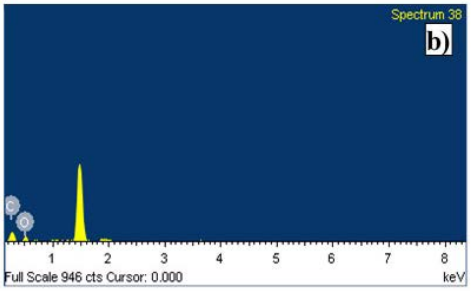



**Fig. 9.** The cell counting of *Microcystis aeruginosa* KG (a) and *Chlorella vulgaris* (b) versus time at different concentrations of CuNPs (ppm); values are reported as the mean of three replicates ± SD (n = 3)

Although our results differ from some published studies (Das et al., 2012; Hund-Rinke and Simon, 2006; Zhang et al., 2009), they are consistent with those of Chen et al. (2015) who demonstrated that the TEM image of the cell ultrastructure of *M. aeruginosa* treated by 0.06 and 0.07 ppm Cu ion was significantly damaged and the color of cultivated medium changed from green to yellow after 72 h. The size of nanomaterials is an important factor affecting the growth of algae. Some previous studies have indicated that only CuO nanoparticles with a size of less than 5 nm could penetrate through algal cells as the pore sizes of *M. aeruginosa* KG algal cell walls ranged from 5 to 20 nm (Das et al., 2012; Hund-Rinke and Simon, 2006; Zhang et al., 2009). In the current study, the effects of CuNPs on the two algae strains are different and the size of CuNPs was about 30–40 nm. However, TEM images still showed the black-dot region (Fig. 7d) and SEM–EDX results recorded the presence of CuNPs inside the *M. aeruginosa* KG cells (Fig. 9a). This demonstrates that Cu nanomaterials can penetrate inside the cell even though its material size is larger than that of algae cells. This can be due to the fact that the Cu nanomaterials in the current study were synthesized by the direct reduction method. After dissolving into the algae culture medium, CuNPs would rapidly release $Cu^{2+}$ ions that easily penetrated the inside of the cell and inhibited the

growth of *M. aeruginosa* KG algae. The CuO nanoparticle or dissolved Cu ion could easily accumulate in the inner algae cells, although the mechanism of this has not been clearly reported (Chao et al, 2021; Ivask et al., 2014; Zhang et al., 2009). The slow release of $Cu^{2+}$ due to the large size of nanoparticles, may possibly lead to the delayed toxic effects of the nanomaterials. At the concentrations of 0.01 to 0.1 ppm, the CuNPs did not affect *M. aeruginosa* KG growth. Nevertheless, at higher concentrations (1 and 5 ppm) and longer experimental time (6 and 10 days), CuNPs inhibited the growth of *M. aeruginosa* KG. Furthermore, it was observed that the excessive level of newly formed ROS could be considered as a primary cause of photosynthetic inhibition.

## 4. Conclusions

This study provided further evidence that CuNPs selectively inhibited the growth of two kinds of algae. Cu nanomaterials were a potential risk for *Microcystis aeruginosa* KG after 6 and 10 experimental days at two concentrations (1 and 5 ppm) and did not inhibit the growth of the *Chlorella vulgaris* strain, and the biomass of this strain at all the CuNPs concentrations (0.01, 0.05, 0.1, 1, and 5 ppm) increased linearly with the control sample. These findings contribute to a growing body of literature regarding the toxicity of nanomaterials usage in aquatic ecology system.

Additional experiments are required to increase our understanding of the toxicity of CuNPs in aquatic ecosystems. Experiments were performed on several other algae species to confirm whether the differential effects of nanomaterials depends on the cell structure of the two algae (cyanobacteria and unicellular algae) or the size of the nanomaterial or other environmental factors.

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