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CAPABILITY OF Cu_2O NPs/DIATOMITE MATERIAL IN GROWTH INHIBITION AND REMOVAL OF *Microcystis aeruginosa* IN AQUACULTUTE CULTIVATION

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Abstract. The application of nano materials in disinfection has been considered as a promising technology in the world. However, high cost is one of the main disadvantages of this method. Therefore, the investigation of new nanoparticles with high efficiency and cheap is target of scientists. In this study, Cu_2O nanoparticles (Cu_2O NPs) with an average particle size of ~27.4 nm deposited on diatomite (Cu_2O NPs/diatomite), a new low-cost material was used for growth inhibition and bloom prevention of cyanobacteria in eutrophic waters. Diatomite collected from natural mines was used in the experiments. *Microcystis aeruginosa* strain originated from Bac Lieu shrimp pond was isolated and cultured in Z8 medium. Experiments were conducted by exposing *M. aeruginosa* to Cu_2O NPs/diatomite and diatomite at concentrations of 0.1, 0.2 and 0.5 g/L for 72 and 120 h, respectively. The investigation results showed that Cu_2O NPs/diatomite could remove *M. aeruginosa* cells much more efficiently than diatomite. The straight line equation obtained from linear relationship between Cu_2O NPs/diatomite concentration and the inhibition growth rate percentage (I_{GR}) showed that at a concentration of 0.115 g/L, this nano material could inhibit 100 % of *M. aeruginosa* in the culture medium after 72 h. Hence Cu_2O NPs/diatomite was recommended as a promising material for removing cyanobacterial bloom in aquaculture cultivation.

Keywords: Cu_2O, cyanobacteria bloom, diatomite, *Microcystis aeruginosa*, nanoparticles.

Classification numbers: 1.3.3, 2.4.2, 3.3.1.

1. INTRODUCTION

Global warming creates conditions for cyanobacteria to develop, causing eutrophication and imbalance of aquatic ecosystems, threatening water resources and human health [1]. Cyanobacteria appear more and more in many countries around the world and they are quickly predicted to be a global problem [2]. Cyanobacteria strains such as *Anabaena*, *Microcytis*, *Oscilatoria*, etc. are capable of producing toxins into the environment, of which the most common strain is *Microcystis aeruginosa* (*M. aeruginosa)* which produces toxin microcystin. Therefore, the control of cyanobacterial growth is essential for water quality protection and safety of aquatic ecosystems. There have been several reports on the presence and toxicity of cyanobacteria in the lakes of Vietnam. In the North, the occurrence of *Anabaena* sp. and *Microcystis* has been confirmed in Hoan Kiem Lake and Nui Coc reservoir which produced the toxins concentration as high as 0.91 μg/L and 46.0 μg/L, respectively [3]. In the South, the genus *Microcystis* was detected in Tri An reservoir with concentrations up to 0.64 mg/g dried weight of the scum samples [4].

A lot of technologies have been applied for controlling the development of cyanobacteria. Common approaches to the treatment of cyanobacteria are based on physical processes (ultraviolet irradiation, membrane, ultrasonic, etc.), chemical processes (coagulation, H_2O_2, metal salts, etc.) and their combined ones (UV/H_2O_2-Fe) [5 - 9]. However, besides the advantages, these well-known methods have shown considerable disadvantages, such as generation of secondary pollutants, high treatment costs, etc. [10 - 12]. Therefore, some biological candidates such as natural enemies and plant solvent extracts were also chosen to control cyanobacteria in the lakes. Specifically, Thuy and colleagues elucidated that the aqueous extracts from *Eupatorium fortune* Turcz plant could inhibit the growth of phytoplankton and *Microcystis* as 34.5 % of their community in the sample after 14 days of testing [12]. However, it is necessary to develop other applicable technologies to treat cyanobacteria not only at the laboratory scale but also in the lakes and reservoirs with high efficiency, low cost and safety for the environment.

In recent years, nano materials have been known as emerging reagents for application in environmental engineering because of their small dimension and quick reaction. They have been widely used in water disinfection and algae boom controlling in practice [10]. In particular, some known nanoparticles for inhibiting the growth, attacking and disrupting cell membranes of harmful bacteria such as *Escherichia coli*, *Staphylococus aureus*, *Vibrio parahaemolyticus*, etc. have been studied. Metal nanoparticles such as Cu_2O NPs [13], Fe_3O_4 NPs [14], TiO_2 NPs [15], ZnO NPs [16], Ag NPs [17], etc. have been widely used for controling and eliminating the toxin of *M. aeruginosa*. The use of Ag NPs (1 mg/L) could remove 98.7 % of *M. aeruginosa* in the test samples, with the EC_{50} determined to be 0.0075 mg/L [17]. It was reported that nano-TiO_2 performed well to remove *Selenastrum bibraianum* after 96 h [18]. The effectiveness of copper nanoparticles in inhibiting *M. aeruginosa* has been also confirmed [19]. Some clay minerals were also used as flocculants to remove cells and toxins of the strain *M. aeruginosa*, such as diatomite [20], montmorillonite [13] and bentonite [21]. Among the materials, diatomite was known as a geological sediment containing natural amorphous silica, possessing unique properties as high porosity (80 - 90 %), excellent adsorption capacity, low cost and environmental friendliness [22]. In this study, Cu_2O NPs/diatomite was synthesized and figured out to be a good material for the growth inhibition and removal of *M. aeruginosa.*

2. EXPERIMENTAL

2.1. Materials and chemicals

Diatomite (~66.3 % SiO_2) was supplied by a diatomite company in Phu Yen province. Cu_2O NPs/diatomite was synthesized by chemical reduction. Cu_2O NPs have an average particle size of ~27.4 nm and a characteristic peak λ_{max} at 430 nm. Cyanobacteria were obtained in a shrimp pond in Bac Lieu province. The chemicals used in the experiment including H_3BO_4, $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $Co(NO_3)_2.6H_2O$, $VOSO_4.6H_2O$, $AL_2(SO_4)_3K_2SO_4.2H_2O$, $NiSO_4(NH_4)_2SO_4.6H_2O$, $Cd(NO_3)_2.4H_2O$, $Cr(NO_3)_3.7H_2O$, $Na_2WO_4.2H_2O$, KBr, KI were supplied by Merck, Germany. Distilled water was used in all experiments.

2.2. Synthesis of Cu_2O NPs/diatomite

Before carrying out the experiments of cyanobacterial controlling, the synthesized Cu_2O NPs/diatomite was fabricated using hydrazine hydrate as the reduction agent. The fabrication was processed by the following steps (Fig. 1): (1) 10 g of pure diatomite was added to a Becher containing 30 mL of deionized water and stirred for 30 min for dispersion. After that, 10 mL of 2 % copper sulfate solution was added to the mixture. The obtain solution was continuously stirred for 30 min at room temperature for complete immobilization of Cu^{2+} ions in solution; (2) the pH of the obtained solution was adjusted to 6 with 5 % NH_4OH solution. Then, 5 mL of 8 % hydrazine solution was slowly added to the solution and stirred continuously for 1 - 2 h for the reduction of Cu^{2+} to Cu^{+} (Cu_2O nano), and the color of solution would change to yellowish-orange indicating the presence of Cu_2O NPs/diatomite; (3) the solid part was filtered and washed with deionized water until pH value reached neutral; (4) the product was vacuum-dried at 60 ^oC until the weight remained constant. The product was then ground to fine powders for further use.

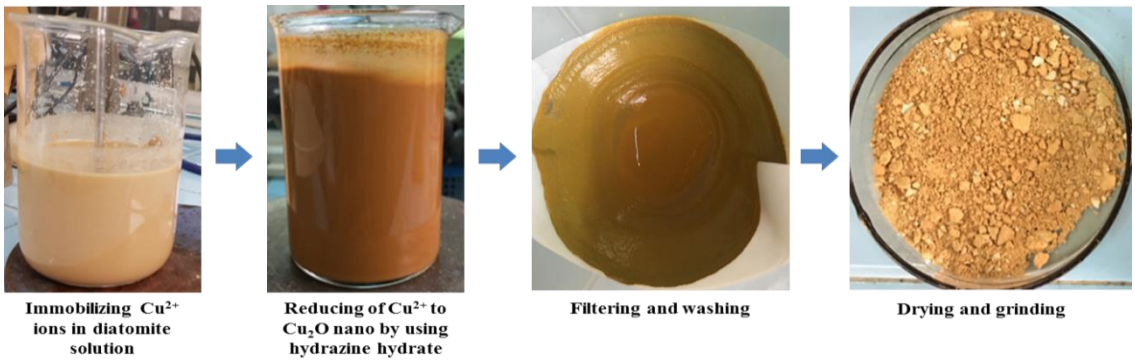

Figure 1. The diagram of Cu_2O NPs/diatomite synthesis.

2.3. Sampling, isolation, and biomass culture of cyanobacteria

Cyanobacteria samples were collected from a shrimp pond in Bac Lieu province using a 25 µm phytoplankton net. Fresh samples were brought to the culture room and isolated under Olympus BX51 optical microscope (Olympus, Tokyo, Japan) at magnifications ×100 - 400 by using a Pasteur pipette with the tip elongated under an alcohol lamp. Isolation was performed by observing the sample under the microscope, then the colony of *M. aeruginosa* (Fig. 2) was suctioned into the Pasteur pipette, rinsed several times with distilled water or with prepared and pre-sterilized medium, and then transferred into a glass tube containing Z8 medium [23]. The isolated algae samples were labelled and named. The selected samples were transfered to Erlenmeyer flasks with Z8 medium [24]. Micronutrients for cyanobacteria culture were as follows: H_3BO_4 (3.1 g), $MnSO_4.4H_2O$ (2.23 g), $ZnSO_4.7H_2O$ (0.22 g), $(NH_4)_6Mo_7O_{24}.4H_2O$

(0.088 g), $Co(NO_3)_2.6H_2O$ (0.146 g), $VOSO_4.6H_2O$ (0.054 g), $Al_2(SO_4)_3K_2SO_4.2H_2O$ (0.474 g), $NiSO_4(NH_4)_2SO_4.6H_2O$ (0.198 g), $Cd(NO_3)_2.4H_2O$ (0.154 g), $Cr(NO_3)_3.7H_2O$ (0.037 g), $Na_2WO_4.2H_2O$ (0.033 g), KBr (0.119 g), KI (0.083 g), distilled water (1 L). The medium was sterilized in an autoclave at 2.0 atm (120.23 ^oC) for 15 min. All cultures were grown on a 12 h - 12 h light - dark cycle at a temperature of 27 ± 1 °C under a light intensity of 3,000 lux.

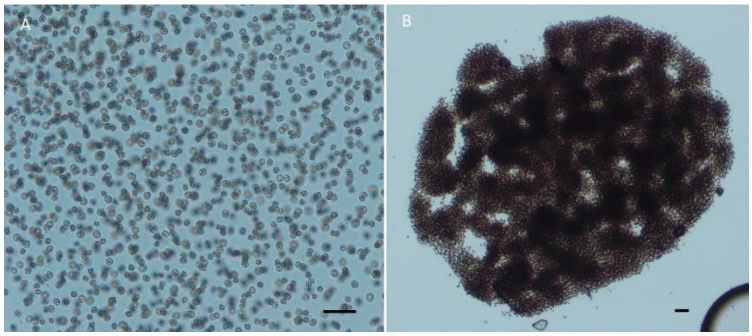

Figure 2. Photo of the *M. aeruginosa.* (A) cell shape, (B) photo of a *M. aeruginosa* colony. Scale bar: 20 µm.

2.4. Exposure of Cu_2O NPs/diatomite and diatomite to *M. aeruginosa* culture medium

Cu_2O NPs/diatomite and diatomite with the concentrations of 0.1, 0.2, and 0.5 g/L were spiked directly into the *M. aeruginosa* culture flasks containing 300 mL of Z8 medium each. Then the living culture stocks of *M. aeruginosa* were added to the solution to achieve an initial concentration of 5×10^4 cells/L. The culture flasks without Cu_2O NPs/diatomite and/or diatomite were used as control experiments. The investigation was carried out in the laboratory condition as mentioned above, and pH was recorded as in the range of 7.0 - 8.0 without adjustment. The cell density of *M. aeruginosa* was monitored at 72 h and 120 h intervals using a Neubauer Counting Chamber.

To investigate the effect of Cu_2O NPs/diatomite on inhibiting the growth of *M.aeruginosa*, the specific growth rate of the bacteria and the inhibition percentage were calculated as follows [25]:

$$\mu_{x-y} = \frac{\ln C_y - \ln C_x}{t_y - t_x} \quad (1)$$

$$I_{GR} = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad (2)$$

where: μ_{x-y} was the specific growth rate in the time from x to y; $t_y - t_x$ the exposure duration time; C_x and C_y the cell concentrations at time x and y, respectively. I_{GR} (%) the inhibition growth rate percentage of *M. aeruginosa* rate; μ_T and μ_C average specific growth rates of algae in the treatments and the controls, respectively.

3. RESULTS AND DISCUSSION

3.1. The sensory evaluation of *M. aeruginosa* treatment by Cu_2O NPs/diatomite

Figure 3 showes the growth observation of *M. aeruginosa* strain in the culture medium containing Cu_2O NPs/diatomite with the concentrations of 0.1, 0.2, and 0.5 g/L and control medium after 72 h and 120 h exposure.

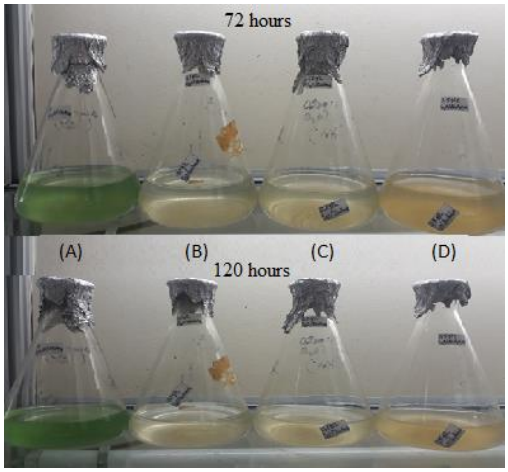

Figure 3. The *M. aeruginosa* exposure at different Cu_2O NPs/diatomite concentrations after 72 h and 120 h: (A): Control ; (B): 0.1 g/L ; (C): 0.2 g/L ; (D): 0.5 g/L.

In the Erlenmeyer flasks containing Cu_2O NPs/diatomite, the color of flasks was changed from blue to yellow-brown with increasing the content of this nano material (Figure 3D). In contrast, the control experiment showed that the color intensity of *M. aeruginosa* community was increased overtime. This result indicated an increase in the concentration of cyanobacteria in the solution (Figure 3A). From the sensory evaluation, it could be assumed that the presence of Cu_2O NPs/diatomite in the culture medium has caused the growth inhibition of cyanobacteria.

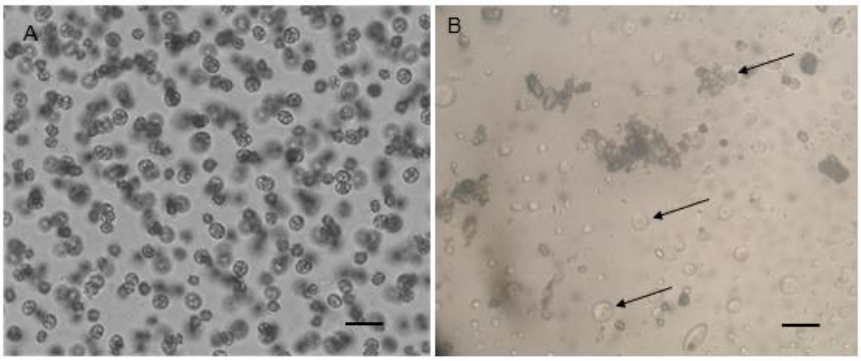

Figure 4. TEM images of *M. aeruginosa* strain. (A) Control; (B) Exposure to Cu_2O NPs/diatomite at a concentration of 0.1 g/L after 72 h. Arrows indicate the single cell of *M. aeruginosa* after exposure to Cu_2O NPs/diatomite. Scale bar: 10 µm.

For more qualitatively, the transmission electron microscopy (TEM) images of *M. aeruginosa* before and after exposure to Cu_2O NPs/diatomite at a concentration of 0.1 g/L after 72 h were taken (Fig. 4). It was clearly illustrated that after the exposure to Cu_2O NPs/diatomite, almost circle cells of *M. aeruginosa* were disappeared or the cell membrane was blurred and remained only some tiny residue in the drop of water. The TEM results demonstrated that

several individuals, discrete cells were degraded during the experiment. In addition, some *M. aeruginosa* cells tended to regroup, possibly related to the flocculation of the cells under the influence of Cu_2O NPs/diatomite. This proved that *M. aeruginosa* cells have lost their integrity and damaged, the cell membranes showed the signs of destruction [26, 27]. Thus, the ability to remove *M. aeruginosa* cells of Cu_2O NPs/diatomite after exposure was clearly revealed. Singh and colleagues indicated that the integration of Ag NPs and NaOCl could make a strong effect on the membrane integrity and then increased cell lysis rates of *M. aeruginosa* [28]. Other authors have also demonstrated that magnetic Fe_3O_4 NPs could cause cell integrity; decrease the protein, intracellular, polysaccharide concentration in cyanobacterial cells, resulting in the destroy of *M. aeruginosa* cells [29]. The inhibition of nano-TiO_2 on algal growth by the damaging of the lamellar organization of thylakoids and the photosynthetic process was observed in a previous study [18]. Therefore, it could be concluded that the nanoparticles attacked barrier cell protection, further affected organelles and compounds inside the cells, causing metabolic disturbances, and hence killed the microorganisms.

3.2. The effect of Cu_2O NPs/diatomite on the growth of *M. aeruginosa*

Figure 5 shows the concentration of *M. aeruginosa* in the culture medium during the retention time. It was illustrated that the presence of diatomite at the concentration of 0.1; 0.2 and 0.5 g/L in the medium could slightly hamper the growth of this algae, and the inhibition was not significantly different among the diatomite doses (Fig. 5A). The inhibition of *M. aeruginosa* by Cu_2O NPs/diatomite was clearly observed during the operation period (Fig. 5B).

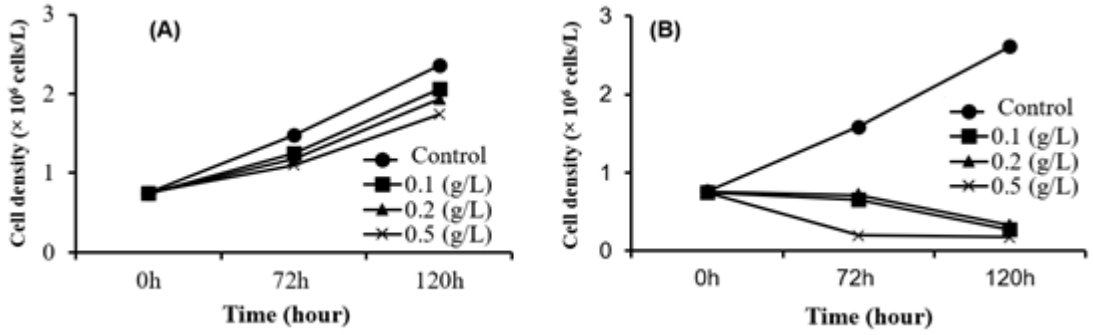

Figure 5. The growth of *M. aeruginosa* during time course: (A): Adding diatomite, (B): Adding Cu_2O NPS/diatomite.

The concentration of *M. aeruginosa* in the solution was not much changed after 72 h and decreased after that until the end of the investigated time (120 h) at the dose 0.1 and 0.2 g/L Cu_2O NPs/diatomite. Significantly, at a concentration of 0.5 g/L Cu_2O NPs/diatomite, the *M. aeruginosa* community was sharply destroyed from 0 to 72 h and stable after that until 120 h exposure. From 0 h to 72 h testing time, the cell density of *M. aeruginosa* in the medium of 0.5 g/L nano material dropped from 0.747×10^6 to 0.200×10^6 cell/L while algae community was developed from 0.760×10^6 to 2.615×10^6 cell/L in the control test. The obtained result indicated that Cu_2O NPs/diatomite caused strong effects on the growth of *M. aeruginosa* in the culture medium. So far, there has not been any report on the application of Cu_2O NPs for removing *Microcystis* genus of cyanobacteria in Viet Nam. The effect of some types of nanoparticles on controlling the growth of cyanobacteria was revealed in previous studies. Li and colleagues used the Cu NPs/biochar composite paralyzed at 550 ^oC and 750 ^oC to inhibit the growth of *M. aeruginosa* [19]. Another research found out that the half growth inhibition

concentration of the nano-TiO_2 to *M. aeruginosa* was reached at 9.1 mg/L [18]. Other authors demonstrated the potential of Ag NPs and NaOCl in removing cyanobacteria [28]. It was observed that cell growth of *M. aeruginosa* decreased when the concentration of Ag NPs increased. The maximum *M. aeruginosa* removal efficiency was achieved at a concentration of Ag NPs of 1 mg/L [17]. Therefore, it was reliable to confirm the effectiveness of nano materials in the treatment of algae. However, different materials applied to control the growth of microorganisms at variable experimental conditions could give different results.

3.3 The inhibition of Cu_2O NPs/diatomite on the growth of *M. aeruginosa*

Based on the observed data on cell numbers, the specific growth rate of *M. aeruginosa* during the experiment was calculated (Eq. 1) and then the inhibition rate (Eq. 2) could be determined. The plots of I_{GR} versus diatomite or Cu_2O NPs/diatomite concentration are shown in Figure 6. It was clear that those parameters had a rather good linear relationship with high correlation coefficient (R^2) values. In addition, these trendlines with positive value of slopes have indicated that the concentrations of diatomite or Cu_2O NPs/diatomite were directly proportional with I_{GR} values. Interestingly, the slope of trendline in case of using Cu_2O NPs/diatomite (453.480) was much bigger than that in the diatomite case (44.112). This result demonstrated that the Cu_2O NPs/diatomite has much more inhibited the growth of *M. aeruginosa*. From the line equation, it was predicted that the Cu_2O NPs/diatomite could inhibit 100 % of *M. aeruginosa* in the culture medium at a concentration of 0.115 g/L after 72 h of testing time. In a previous study, it was reported that the Ag NPs could cause 100 % inhibition to the growth of *Pseudokirchneriella subcapitata* at a concentration of 50 µg/L [25]. Thus, the result was expected to bring about the chances for application of much cheaper Cu_2O NPs/diatomite in the growth inhibition of microorganisms.

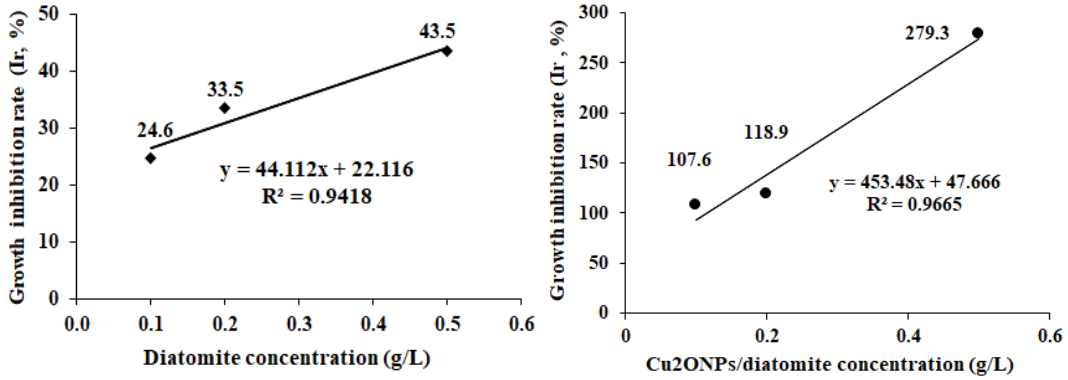

Figure 6. Growth inhibition rate of *M. aeruginosa* in experimental plots: (A): diatomite, (B): Cu_2O NPs/diatomite

4. CONCLUSION

The treatment of cyanobacteria (*M. aeruginosa* strain) by using Cu_2O NPs/diatomite material was investigated at the laboratory scale. The removal of *M. aeruginosa* was evaluated based on the sensory and quantitative analysis. The flask color and TEM images indicated that *M. aeruginosa* were destroyed after 72 h exposure to Cu_2O NPs/diatomite in the culture medium. The Cu_2O NPs/diatomite showed promising results of removing cyanobacteria at a concentration of 0.5 g/L compared with the diatomite-based and control experiment. The

inhibition growth rate percentage was calculated to be 100 % after 17 h of testing time by using 0.115 g/L Cu_2O NPs/diatomite. Thus, it was highly suggested to apply Cu_2O NPs/diatomite for removing *M. aeruginosa* and other microorganisms. The nano Cu can be considered as a low cost, high efficient, and safe material for the aquatic environment. It is necessary to process further trials for controlling eutrophication in lakes and reservoirs as well as in other aquaculture cultivation media.

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CRediT authorship contribution statement. Nga Thi Dinh: Methodology, Writing& Editing manuscript, Supervision. T. Thuy Nhung Dang, Thanh Luu Pham, T. Hoang Yen Tran: Data collection, data analysis, preparing – original draft; Dinh Tuan Phan: Funding acquisition; Visualization, Supervision; Quoc Hien Nguyen: Review & Editing.

Declaration of competing interest. The authors declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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