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EFFECTS OF COPPER NANOPARTICLE EXPOSURE ON PHOTOSYNTHETIC PIGMENTS, ENZYME ACTIVITIES AND OXIDATIVE STRESS RESPONSE IN *SYNECHOCOCCUS ELONGATUS*

SRIVASTAVA R[1*], MISHRA RC[2] AND VERMA S[*]

1: Department of Botany, Mahakaushal University, Jabalpur, Madhya Pradesh, 482003

2: Department of Botany, Mahakaushal University, Jabalpur, Madhya Pradesh, 482003

[*]**Corresponding Author: Dr. Swati Verma; E Mail: r.srivastava2208@gmail.com**

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ABSTRACT

The effects of copper nanoparticles (Cu-Np) on the microalga *Synechococcus elongatus* BDU10144 were investigated. The results showed that Cu-Np treatment decreased the chlorophyll a content, phycobiliproteins, and total protein concentration in the microalga. In addition, Cu-Np treatment increased the lipid peroxidation and glutathione-S-transferase activity, and decreased the superoxide dismutase and catalase activity. These results suggest that Cu-Np treatment induced oxidative stress in the microalga, which could lead to cell damage and death.The findings of this study provide valuable insights into the potential toxicity of Cu-Np on microalgae and contribute to our understanding of the environmental implications of nanoparticle exposure.

Keywords: copper nanoparticles (Cu-Np), Synechococcus elongata, oxidative stress, chlorophyll a, phycobiliproteins, total protein, lipid peroxidation, glutathione-S-transferase, superoxide dismutase, catalase

INTRODUCTION

Aquatic habitats, such as lakes, rivers, and oceans, are highly susceptible to metal pollution due to the discharge of industrial waste, soil weathering, and urban mining into these water bodies, leading to consequential impacts on the aquatic biota **[1]**. Monitoring aquatic ecosystems is crucial as they provide essential support for

diverse organisms, including microorganisms, plants, insects, and fish, thus preserving a healthy biodiversity **[2]**. It should be noted that heavy metals, including copper, exhibit limited degradation and are instead assimilated or absorbed by water sediments and aquatic organisms, thereby contributing to metal pollution within their bodies **[3]**. Nanomaterials derived from abundant and affordable metals have gained significant attention due to their potential as alternatives to costly noble-metal catalysts employed in conventional chemical processes **[4]**. Nanoparticles (NPs) possess significant catalytic activity, making them valuable resources for chemical processes employed in industry and academia **[5]**. Recently, there has been a surge of research interest in copper nanoparticles (Cu-NPs), owing to their remarkable properties and extensive applications **[6]**. Copper is commonly present in substantial quantities in the vicinity of copper mines **[7] [8]**. Cu and Cu oxide NPs are currently manufactured at an annual rate of around 200 tons and find use in various fields such as antifouling paints, catalysis, batteries, electronics, polymers, and coatings **[9]**. These metal NPs often exhibit distinct activity compared to their bulk counterparts, primarily due to their unique quantum properties arising from different sizes and shapes **[10]**. However, their potential environmental impact and toxicity on organisms, particularly microalgae, remain a concern. Furthermore, apart from their industrial applications of NPs, the scientific community has shown a growing interest in the potential toxicological effects of nanoparticles (NPs) **[11]**. Recent studies have suggested that excessive exposure to copper, a commonly encountered metal, can trigger the overproduction of reactive oxygen species (ROS), leading to oxidative stress and damage to proteins, bio membranes, and nucleic acids **[12] [13]**. Therefore, in order to gain insights into the mechanism underlying copper toxicity in organisms, it is crucial to comprehend its predominance as a chemical and its behavior within the environment **[14] [15]**. Metals exhibit reactivity based on their solubility characteristics in an aqueous medium **[10]**. The release of free ions or complexes by metals can result in their absorption onto suspended particles present in the aquatic medium **[16]**. The behavior of metal constituents can vary within an aqueous system. In line with the previous statement, it is imperative to maintain copper concentrations in water bodies at low levels. Uncontaminated water typically exhibits copper concentrations as low as 0.5 to 1 µg/L **[17]**. One potential gap in the study is the lack of investigation into the specific mechanisms by which copper nanoparticles

(Cu Np) treatment affects *Synechococcus elongatus* BDU10144 **[9]**. While the study provides valuable insights into the negative impacts of Cu Np on photosynthetic pigments, oxidative stress markers, and antioxidant enzyme activity, it does not delve into the underlying molecular processes involved **[18]**. Understanding the molecular mechanisms of toxicity could provide a more comprehensive understanding of the effects of Cu Np on microalgae. Understanding the relationship between nanoparticles and microalgae is crucial for assessing the potential risks and implications for aquatic ecosystems **[19]**. In this study, the effects of Cu-Np treatment on *Synechococcus elongatus* BDU10144, a commonly studied microalgal strain. *Synechococcus elongatus* BDU10144 is a specific strain of the microalgal species *Synechococcus elongatus*. It is a cyanobacterium that belongs to the phylum Cyanobacteria **[20]**. This particular strain, BDU10144, has been used in studies to investigate the effects of various treatments and environmental factors on microalgae **[21]**. The study aimed to assess the impact of Cu-Np on various parameters, including photosynthetic pigments, antioxidant enzymes, and oxidative stress markers. The findings of this study provide valuable insights into the potential toxicity of Cu-Np on microalgae and contribute to our understanding of the environmental implications of nanoparticle exposure. Previous and current investigations have primarily aimed at understanding and characterizing specific metabolites, particularly phenolic compounds, and their functions during unfavorable circumstances. To further this understanding, *Synechococcus elongatus*, a unicellular freshwater green alga, was subjected to well-defined Cu nanoparticles (Cu-NPs). In addition to conducting fundamental toxicological assessments, such as evaluating growth, chlorophyll content, oxidation-reduction status, and copper accumulation in the algal biomass, the study sought to explore additional aspects related to the exposure of Cu-NPs.

Procurement of *Synechococcus elongatus* species

The vials containing *Synechococcus elongatus* BDU10144 cells were procured from the National facility for marine Cyanobacteria, Bharathidasan University, Palkalaiperur Tiruchiraoalli- 620024. This study was carried out according to the OECD guidelines and BBM medium (BOLD'S BASAL MEDIUM (MODIFIED) was prepared and used for development.

Estimation of Growth kinetics

Algal growth rate test was conducted in bath cultures in different flasks according to the standard procedure **[22]** using fresh

water *Synechococcus elongatus* spp. A cabinet with laminar air flow was prepared and was pre-sterilized with ultraviolet lamp for test performances. It should be noted that the required materials to prepare the culture medium were first sterilized under pressure of 0.1 MPa for 20 min and the control flasks were used as a comparative criterion. The tests were started in exponential growth phase and, the initial number of algal cells was about 1 × 105 cells/ml in each culture. The growth response of *Synechococcus elongatus* exposed to each of tested substances was determined by measurement of relative growth rate (after counting the cells) and reactive oxygen species (ROS). The cells were counted using a hemocytometer and the number of algae per ml was calculated according to the OECD guideline. Algae cells were counted at 24-h intervals and the relative growth rate (G) was calculated using the following equation.

$G=[NN0]*100G=[NN0]*100$

G = relative growth rate, N = Number of cells counted, N0 = Primary Cell Number.

Experimental K the growth constant coefficients (K) of algae for each group were calculated using the first order kinetic model equation in different time intervals and the mean of values of K are reported.

$K=Ln\ (N/N0)/t$

K = growth constant coefficient.

The values of K were calculated by plotting Ln (N/N0) against the time (plotting method), and the linearity of the plot was tested using the regression coefficient (R2).

Protein analysis

Protein analysis was conducted following the method of lowry **[23] [24]** by reacting 300 µL supernatant to 300 µL of Lowry D reagent then vortexed for 15 sec. Lowry D reagent was made in Institute laboratory (RANKEM). Sample of mixture was incubated for 10 min, reacted to 900 µL of Lowry E reagent then vortex for 30 sec. Then, the mixture was incubated for 45 min, and the absorbance was read using a spectrophotometer at λ 750 nm.

Superoxide dismutase analysis

Analysis of SOD activity was conducted following the method of **[24] [25] [26]**. This method reacted 1 mL of Tris-HCl buffer (pH 8.2) with 1 mL of aquabidest and 15 µL of algae extract supernatant and added with 10 µL of pyrogallol 2 mM. Absorbance measurements were performed with a spectrophotometer using a wavelength of 470 nm with time intervals of 3 min. Blank solutions were combination of pyrogallol and aquabidest (ratio 1: 1). The measured data were expressed as units per milligram of protein (1 unit was the amount of enzyme utilized to inhibit 50% of pyrogallol auto-oxidation per minute).

Catalase analysis

Catalase activity was measured according to modified Aebi and Lester (1984) method in Kasmiyati (2016) **[27]**. A total of 1.99 mL of phosphate buffer solution 50 mM pH 7.0 and 10 µL of supernatant sample was mixed. One milliliter of H2O2 solution (3% concentration) was added to mixture then incubated for a minute. Catalase enzyme activity was measured by a UV-VIS spectrophotometer at wavelength of 240 nm for 3 min (one-minute interval). Value of H2O2 extension coefficient is 40 mM cm-1. Enzyme activity is expressed in µmol H_2O_2 gram-1 fresh weight. Fresh weight of algae was determined by harvesting algae using centrifuge at 11000 g for 5 min (4°C) then they were weighed.

Ascorbate peroxidase analysis

APX activity was conducted following the method of **[28]** with modification. A mixture of 1.3 mL potassium phosphate buffer 0.05 mM pH 7.8 contained 0.1 mM EDTA was reacted with 10 µL supernatant, 800 µL 0.5 mM ascorbate and 800 µL H2O2 (3% concentration). Those were incubated for a minute then the decrease in absorbance was measured for 3 min (one-minute interval) at λ 290 nm using a spectrophotometer. The collected data were used to define the reaction rate for H_2O_2 independent of ascorbate oxidation.

Photochemical Quenching

Synechococcus elongatus microalgae were cultivated under sterile conditions using a liquid mineral medium in 1000-mL Erlenmeyer flasks. The flasks contained 400 mL of the medium, which excluded EDTA. The cultures were subjected to continuous illumination provided by warm white fluorescent lamps, with a photosynthetic photon flux density of 40-42 µmol m−2 s−1. Additionally, the cultures were stirred by shaking twice a day.

To initiate the experiment, the algal cultures were inoculated with Cu nanoparticles carboxylated with citric acid (nCu-Citr) or selenium nanoparticles carboxylated with citric acid (nSe-Citr), obtained from the Ukrainian State Scientific Research Institute 'Resource' (Kyiv, Ukraine). The nanoparticle concentrations ranged from 0.67 to 40 mg L−1 for nCu-Citr and from 0.07 to 4 mg L−1 for nSe-Citr. A control culture without nanoparticle addition was also maintained.

The determination of dry algal mass involved centrifugation (10 min at 1500×g) to concentrate the algae, followed by two washes with distilled water. The concentrated biomass was then dried at 105 °C until a constant weight was achieved.

The efficiency of photochemical reactions in photosystem II was assessed by measuring modulated chlorophyll a fluorescence at room temperature using the XE-PAM

fluorometer from Heinz Walz GmbH (Effeltrich, Germany). Before the measurements, all samples were dark-adapted for 10 minutes in the fluorometer cuvette, which was equipped with stirring capability. The measuring light flashes (2 Hz, 0.15 µmol m−2 s−1) were adjusted to ensure that photochemical charge separation in photosystem II reaction centers did not occur. The applied actinic light intensity was similar to that used during algae cultivation. Saturating pulses (5500 µmol m−2 s−1, 1 s) were employed to completely close all photosystem II reaction centers.

Various chlorophyll fluorescence parameters were calculated, including the maximal quantum yield of photosystem II photochemistry (Fv/Fm), the quantum yield of photosystem II photochemistry in the light-adapted state (Fv'/Fm'), photochemical quenching coefficients (qP and qL), non-photochemical quenching (NPQ), and the effective quantum yield of photosystem II electron transport (ΦPSII)

RESULT AND DISCUSSION

The undermentioned study gives insight to all the damages that can be experienced by micro algas of fresh water bodies and how much the contamination with nanoparticle effect aquatic food chain. The findings of this study suggest that Cu-Np treatment is hazardous to the microalgal strain *Synechococcus elongatus* BDU10144 and causes oxidative stress. The decrease in photosynthetic pigments and the increase in oxidative stress markers indicate that Cu-Np treatment interferes with the photosynthetic process in microalgae and damages the cells. The increase in SOD activity suggests that the microalgae are able to mount a defense against oxidative stress, but this defense may not be sufficient to prevent cell damage at higher Cu-Np concentrations.

1. **Chlorophyll a:**

A decrease in the Chlorophyll a content was observed with increase in the Cu Np in comparison to the control sample. This indicates that Cu Np is creating the toxic effect on the microalgal strain that is down regulating the production of Chlorophyll a. Similar results were obtained in a study by Silva *et al*., 2018 in which with increase in the Cu metal treatment decrease in the chlorophyll a content was observed. In another study Cu tolerance was assessed in *Odontella mobiliensis*, resulting in the reduction in chlorophyll concentration and growth rates within 72 h of treatment **(Figure 1) [8]**.

2. **Phycobiliproteins**

A significant decrease in the Phycoerythrin was obtained on increasing the Cu treatment. However, the PE concentration was low in all the treated samples in comparison to the control sample. Similar results have been obtained where the

phycoerythrin concentration decreased while increasing the Cu concentration from 50 to 100 µg/L in comparison to the control. The concentration of allophycocyanin increased considerably in the presence of Cu, this increase profile dependent on u concentration and exposure time. Supporting the present result, Yang *et al*., **[39]** reported that an increase in the metal treatment concentration and decrease in the APC was observed in *Spirulina platensis* **(Figure 2)**.

3. Total Protein

In the present study a slight decrease in the protein concentration was observed with increase in the Cu Np treatment. Similar results were seen where increase in the heavy metal treatment has resulted in the protein concentration **[38]**. In another study treatment of Cu(II) at a concentration ≥1.0 mg/L resulted in the decrease in the protein level **(Figure 3) [39]**.

4. Lipid Peroxidase and Glutathione-S-transferase Activity estimation

A significant increase in the Lipid peroxidase and Glutathione- S-transferase activity was observed with an increase in the Cu Np treatment. Previous studies reveal that lipid peroxidation is typically an indicator of (ROS) Reactive Oxygen Species caused by the cells under oxidative stress **[29]**. Morelli and Scarano, 2004 indicated that treatment of Copper in *Phaeodactylum tricornutum* resulted in an increase in the Lipid peroxidase Glutathione- S-transferase enzyme activity. In another study treatment with 3.0 µg mL^{-1} of Cu a significant increase in the enzyme activity was observed in *Chlorella vulgaris*. This resulted in the growth of this microalgae even at this high Cu concentration **(Figure 4)**.

5. Superoxide Dismutase and Catalase activity (Figure 5)

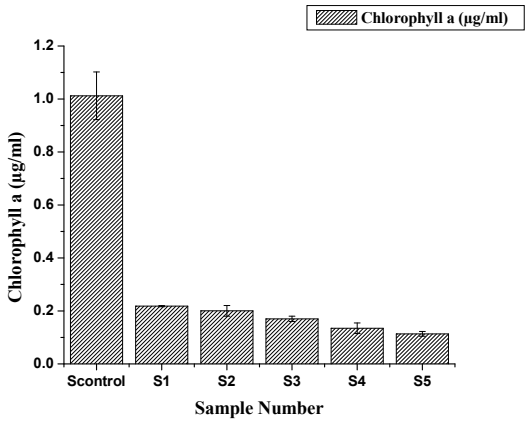

Figure 1: Effect of Cu Np on Chlorophyll a content of *Synechococcus elongatus* BDU10144 in $S_{control}$, S_1, S_2, S_3, S_4 and S_5 samples. Values are means ± SE of three replicates

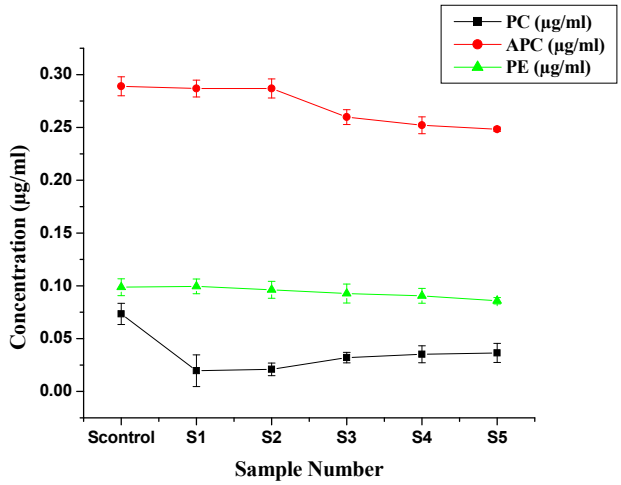

Figure 2: Effect of Cu Np on PC, APC and PE concentration of *Synechococcus elongatus* BDU10144 in $S_{control}$, S_1, S_2, S_3, S_4 and S_5 samples. Values are means ± SE of three replicates

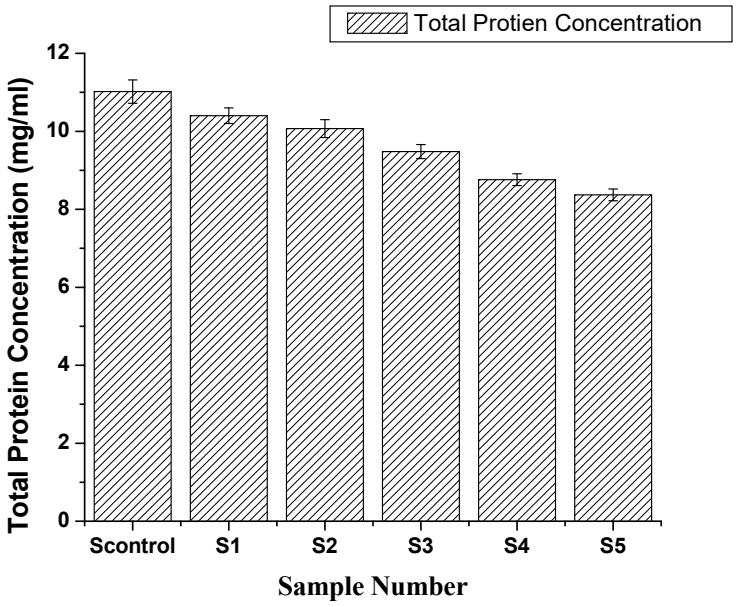

Figure 3: Effect of Cu Np treatment on total protein concentration of *Synechococcus elongatus* BDU10144 in $S_{control}$, S_1, S_2, S_3, S_4 and S_5 samples. Values are means ± SE of three replicates

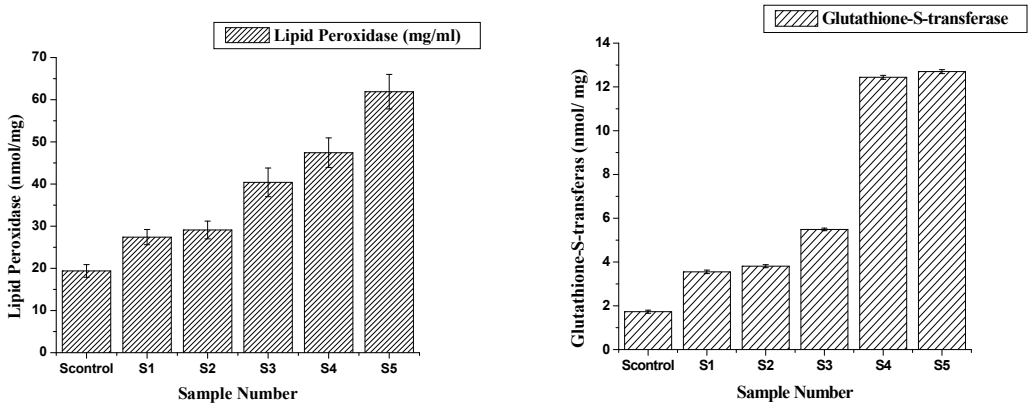

Figure 4: Effect of Cu Np treatment on Lipid peroxide and Glutathione- S-transferase activity of *Synechococcus elongatus* BDU10144 in $S_{control}$, S_1, S_2, S_3, S_4 and S_5 samples. Values are means ± SE of three replicates

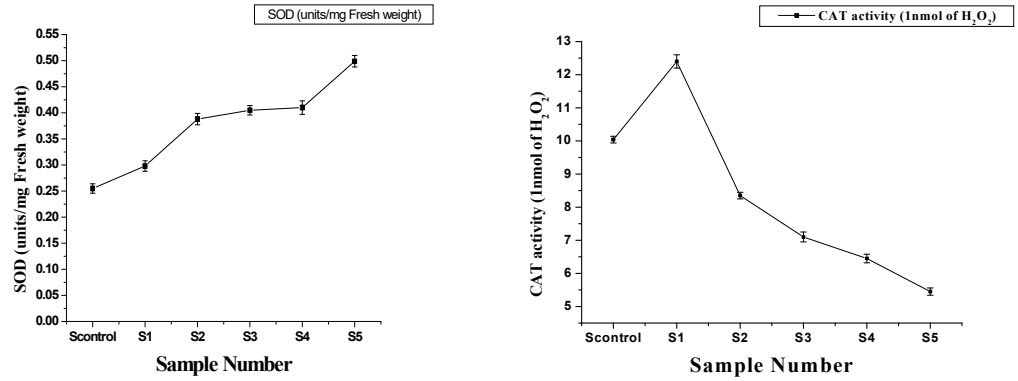

Figure 5: Effect of Cu Np treatment on Superoxide dismutase and Catalase activity of *Synechococcus elongatus* BDU10144 in $S_{control}$, S_1, S_2, S_3, S_4 and S_5 samples. Values are means ± SE of three replicates

Photochemical Quenching

The addition of copper nanoparticles to *Synechococcus elongatus* microalgae initially led to an increase in the Fv/Fm and Fv'/Fm' parameters, indicating the ability of both dark-adapted and light-adapted algal cells to convert light energy into chemical energy.

However, as the experiment progressed, the difference between the copper nanoparticle-treated samples and the control samples decreased. By the 24th day, only the samples treated with 4 mg L−1 copper nanoparticles exhibited higher Fv/Fm and Fv'/Fm' values compared to the control. In contrast, the addition of 2 mg L−1 copper nanoparticles did not result in significant differences in these parameters compared to the control.

The photochemical quenching coefficients, qP and qL, which represent the fraction of open photosystem II reaction centers and the proportion of light excitation energy used for electron transport, were not influenced by the concentration of copper nanoparticles. At the end of the experiment,

their values were reduced by 9-15% compared to the control.

These findings are consistent with those of **[30]**, who found that the addition of copper nanocarboxylates to Chlorella vulgaris microalgae initially led to an increase in the Fv/Fm and Fv'/Fm' parameters, but these effects were not sustained over time. The authors suggested that the initial increase in these parameters may be due to the activation of stress responses in the algal cells, which can temporarily improve photosynthetic efficiency. However, as the cells become more acclimated to the presence of copper nanoparticles, these stress responses are downregulated, and the photosynthetic efficiency returns to baseline levels.

The results of this study suggest that the addition of copper nanoparticles to *Synechococcus elongatus* microalgae can have a transient positive effect on photosynthetic efficiency. However, these effects are not sustained over time, and the long-term effects of copper nanoparticle exposure on algal photosynthetic processes are still unknown.

Further studies are needed to investigate the mechanisms by which copper nanoparticles affect photosynthetic efficiency in *Synechococcus elongatus* microalgae. These studies should also focus on the long-term effects of copper nanoparticle exposure on algal growth and development.

The treatment of Cu Np resulted in an increase in the SOD activity in the *Synechococcus elongatus.* In S5 with highest level of Cu Np treatment a highest SOD activity of 0.499 units/mg was obtained. Similar results were obtained when *Pavlova viridis* was treated with Cu and Zn was done. The authors suggested that the activation SOD antioxidant enzymes was enhanced to counteract the oxidative stress induced by the two metals (Li *et al*., 2006). Parallelly, a decrease in the CAT activity was also observed from 10.4 to 5.45 1nmol of H_2O_2. This significant reduction in CAT activity under Cu stress suggests that the test organism is unable to degrade the excess H_2O_2 radicals. These results indicate the oxidative damage caused by Cu-Np treatment in the *Synechococcus elongatus* cells. Similar results were seen in studies in which decrease in the CAT was observed on Cu treatment **[31, 32]**.

The effects of copper nanoparticles (Cu-Np) therapy on the microalgae *Synechococcus elongatus* BDU10144 were investigated in this study. The treatment caused several changes in a number of measures, which were observed. Firstly, the treatment resulted in a decrease in photosynthetic pigments, specifically a decrease in phycoerythrin concentration and

chlorophyll a content. This suggests that Cu-Np treatment interferes with the photosynthetic process in microalgae, which is crucial for their growth and survival. Secondly, the study observed an increase in lipid peroxidase and glutathione-S-transferase activity, indicating the generation of oxidative stress in response to Cu-Np treatment. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system. The increased activity of these enzymes suggests that Cu-Np treatment induces oxidative damage in the microalgal cells. Furthermore, the study found an increase in superoxide dismutase (SOD) activity, which is an enzyme involved in antioxidant defense. This increase in SOD activity can be seen as a protective response of the microalgae to counteract the oxidative stress caused by Cu-Np treatment **[33]**. There was also a negative impact on the photosynthetic pigments, as evidenced by the decrease in phycoerythrin concentration and chlorophyll content as also discussed by **[34]**. Lipid peroxidase and glutathione-S-transferase activity also increased, indicating the generation of oxidative stress. Superoxide dismutase, an enzyme involved in antioxidant defense, also saw an increase in activity also reported in study by Latifi **[33]**. The analysis of phycobiliproteins in the study showed a significant decrease in phycoerythrin (PE) concentration with increasing Cu Np treatment. The PE concentration was lower in all the treated samples compared to the control sample Cu-Np treatment is hazardous to the microalgal strain *Synechococcus elongatus* BDU10144 and causes oxidative stress **[34]**. Additionally, the concentration of allophycocyanin (APC) increased considerably in the presence of Cu, with the increase being dependent on the concentration and exposure time in *Spirulina platensis* **[35]**. These findings are consistent with previous studies that have reported the toxicity of copper nanoparticles on microalgae and cyanobacteria.

CONCLUSION

The study has shown that Cu-Np treatment can have a deleterious effect on the microalgae *Synechococcus elongatus* BDU10144. The treatment resulted in a decrease in photosynthetic pigments, specifically a decrease in phycoerythrin concentration and chlorophyll a content. This suggests that Cu-Np treatment interferes with the photosynthetic process in microalgae, which is crucial for their growth and survival. The study also observed an increase in lipid peroxidase and glutathione-S-transferase activity, indicating the generation of oxidative stress in response to Cu-Np treatment. Oxidative stress occurs

when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system. The increased activity of these enzymes suggests that Cu-Np treatment induces oxidative damage in the microalgal cells. Furthermore, the study found an increase in superoxide dismutase (SOD) activity, which is an enzyme involved in antioxidant defense. This increase in SOD activity can be seen as a protective response of the microalgae to counteract the oxidative stress caused by Cu-Np treatment.

The findings of this study suggest that Cu-Np contamination can have serious consequences for aquatic ecosystems. Microalgae are an important part of these ecosystems, and their decline could have a ripple effect on other organisms. Additionally, the oxidative stress induced by Cu-Np treatment could have negative health effects on humans and animals that consume contaminated water.

Further studies are needed to investigate the long-term effects of Cu-Np contamination on aquatic ecosystems and human health. However, the findings of this study suggest that Cu-Np contamination is a serious problem that needs to be addressed.

Microalgae are a vital part of aquatic ecosystems, and their decline could have a ripple effect on other organisms. For example, microalgae are the base of the food chain in many aquatic ecosystems, and their decline could lead to a decrease in the number of fish and other animals. The oxidative stress induced by nanoparticle contamination could have negative health effects on humans and animals that consume contaminated water. For example, oxidative stress has been linked to a number of health problems, including cancer, heart disease, and neurodegenerative diseases. Nanoparticles can persist in the environment for long periods of time, and they can be harmful to plants, animals, and humans. For example, nanoparticles have been found in drinking water, air, and soil.It is important to be aware of the potential consequences of nanoparticle contamination so that we can take steps to reduce exposure and protect our health and the environment

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