



Inhibition Effects of Cobalt Nano Particles Against Fresh Water Algal Blooms Caused by *Microcystis* and *Oscillatoria*

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Abstract: Cyanobacterial blooms deplete nutrients, reduce water clarity, exhaust carbon di oxide and produces secondary metabolites which negatively affect aquatic organisms and water quality. Control of algal blooms using metal nano particles is one effective method for the safety of water environment. Cobalt nano particles (CoNPs) were synthesized and tested against microalgae isolated from fresh water cyanobacterial blooms by assessing the effects on growth rate, biomass concentration, photosynthetic pigments concentration and antioxidant enzyme activity. *Microcystis* and *Oscillatoria* were identified as the predominant isolates from algal blooms and treated with varying concentrations (1, 2, 3, 4 and 5 mg·L⁻¹) of CoNPs. Steady decline in the growth rate of microalgae was observed at the end of 5 days indicating the toxicity of CoNPs on microalgal growth. At the end of cultivation period, 78% and 88% of reduction in biomass concentration of *Microcystis* and *Oscillatoria* were observed at 5 mg·L⁻¹ of CoNPs. The chlorophyll content was reduced from 1.53 to 0.24 mg·L⁻¹ in *Microcystis* and 1.63 to 0.29 mg·L⁻¹ in *Oscillatoria*. There was a 69.3% and 73.2% decrease in carotenoid content of *Microcystis* and *Oscillatoria* respectively. Both protein and carbohydrate contents of the microalgae were reduced with increasing concentration of nano particles. The decrease in Super oxide dismutase (SOD) activity with increased nanoparticle concentration reveals the formation of stress in the microalgae. The increasing GSH activity proved the effect of CoNPs on the activation of antioxidative enzymes to protect the cells. This study demonstrates the efficiency of cobalt nano particles (CoNPs) on inhibition of fresh water algal blooms thereby reducing the eutrophication problem.

Keywords: Microalgae, Nano Particles, Algal Bloom, *Microcystis*, *Oscillatoria*

1. Introduction

Microalgae utilize light, carbon di oxide and nutrients to produce biomass in water bodies which is consumed by planktonic herbivores. The rate of biomass production depends on irradiance and nutrient availability and if consumption does not keep pace with biomass productivity, excess growth occurs. Algal biomass accumulations lead to visible discoloration of the water and referred as blooms [1-3]. Blooms due to rapid growth, division rates, stable water column and higher nutrient upload leads to adverse tastes, odours and toxicity of affected water bodies [4]. Higher cell

densities of blooms rapidly deplete nutrients, increase turbidity, and exhaust CO₂ thus causing decline in biomass leading to decaying, odoriferous, unsightly scums. These scums contain variety of microorganisms which produce significant chemical and biological changes, release of toxic compounds, accelerated release of nutrients from sediments that aggravates eutrophication and blooms [5, 6].

Chlorophytes, dinoflagellates, diatoms and cyanobacteria are the major freshwater algal phyla that produce blooms [7, 8]. Among these, Cyanobacterial blooms may cause social,

economic and environmental problems [9-11]. Secondary metabolites produced by cyanobacteria are related to toxic effects on aquatic flora and fauna [12]. *Microcystis* and *Oscillatoria* are traditionally confined to nutrient enriched impoundments due to their unique adaptability to nutrient conditions. Bloom controlling strategies include mechanical, biological, chemical, genetic and environmental control. However, effective methods for preventing algal blooms are important for the safety of water environment.

Recently, metal nanoparticles are showing imperative role in the biological and environmental aspects [13]. Nanoparticles between 1 and 100 nm have been reported as toxic to algae [14-16]. Inhibitory effects of metal oxide nanoparticles against microalgae due to reactive oxygen species generation [17, 18], mechanical damage [19], light shading effect [20], interactions with nutrients [21] and other factors [22] were reported. Accordingly, the aim of this study was to determine the inhibitory effect of cobalt nano particles (CoNPs) on microalgae isolated from fresh water cyanobacterial blooms by assessing the effects on growth rate, biomass concentration, photosynthetic pigments concentration and antioxidant enzyme activity.

2. Materials and Methods

2.1. Collection and Identification of Microalgae

Algal blooms were collected from fresh water lakes and the samples were stored in a refrigerator until use and formalin (1/10) was used for preservation. 2-3 drops of algae rich water was transferred into the glass slide along with small bits of filaments, tufts and mats. The samples were teased as much as possible, covered with cover slip and observed under normal compound microscope. Identification of the algal samples following standard protocols described earlier [23, 24] revealed that *Microcystis* and *Oscillatoria* were the major isolates from the algal blooms.

2.2. Isolation and Sub Culturing

The serial transfer of single algal cultures into new medium was performed in a laminar air flow hood. An aliquot of the culture suspension was inoculated into fresh medium using a Pasteur pipette and 1 mL bulb. Cultures ($2-4 \times 10^4$ cells/ml) were maintained in a single room having approximately 500 square feet of floor space. The temperature of the room was kept at $20 \pm 1^\circ\text{C}$ using an air conditioner. The relative humidity was kept at $31 \pm 1\%$, the flasks were shaken by hand and randomly placed in a growth cabinet ($27 \pm 1^\circ\text{C}$, 12:12 h light/dark cycle, Philips TL 40W cool white fluorescent lighting, $140 \mu\text{mol photons/m}^2/\text{s}$) for 15-20 days. Aeration was done for 8 hours daily using aquarium motor. Cultivation was done simultaneously in open systems by exposing the culture bottles to natural sunlight with aeration.

2.3. Synthesis of Cobalt Nano Particles (CoNPs)

Cobalt nano particles were prepared using Cobalt

chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and hydrazine monohydrate as raw materials. A 20 ml solution of 10% $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ was added with 100 ml of sodium succinate (7.2 g) and heated at 70°C for 10 minutes. This was followed by addition of 10 ml of Polyvinyl alcohol (0.1 g) and drop wise addition of 30% hydrazine hydrate with constant stirring. The solution was filtered through Whatman filter paper, washed several times with deionized water and dried over night at 120°C temperature. The dried powder is analyzed by UV-Vis spectrophotometer.

2.4. Inhibition Assay

Cobalt nano particles (CoNPs) prepared as above was tested to determine the inhibitory effect on *Microcystis* and *Oscillatoria*. BG11 medium was used to cultivate the microalgae and batch cultures of isolated microalgae were grown in different concentrations ($1 \text{ mg} \cdot \text{L}^{-1}$, $2 \text{ mg} \cdot \text{L}^{-1}$, $3 \text{ mg} \cdot \text{L}^{-1}$, $4 \text{ mg} \cdot \text{L}^{-1}$ and $5 \text{ mg} \cdot \text{L}^{-1}$) of metal nano particles for a period of 5 days.

2.5. Growth Rate and Biomass Concentration

Specific growth rate (μ) of the microalgae was calculated according to the following formula.

$$\mu = \frac{\ln(N_t/N_0)}{T_t - T_0}$$

Where, N_t and N_0 are the dry cell weight concentration ($\text{g} \cdot \text{L}^{-1}$) at the end (T_t) and start (T_0) of log phase respectively.

Biomass ($\text{g} \cdot \text{L}^{-1}$) of microalgae grown in the presence of metal nanoparticles was determined by measuring the optical density of samples at 600 nm (OD_{600}) using UV-Vis spectrophotometer. Biomass concentration was then calculated by multiplying OD_{600} values with 0.6, a predetermined conversion factor obtained by plotting OD_{600} versus dry cell weight (DCW). DCW was determined gravimetrically by centrifuging the algal cells ($3,000 \times \text{g}$, 10 min) and drying.

$$\text{Biomass concentration} = \text{OD}_{600} \times 0.6 \quad (1)$$

2.6. Chlorophyll and Carotenoids Estimation

Chlorophyll contents of the microalga were estimated according to Becker [25] (1994). Algal cells were centrifuged and extracted with acetone overnight. The extract was centrifuged at $3000 \times \text{g}$ for 5 mins and the chlorophyll content in the supernatant were determined by measuring the optical densities at 645 and 663 nm in a spectrophotometer and then calculated using the following equation.

$$\text{Chl (mg/l)} = 8.02 \times \text{OD}_{663} + 20.21 \times \text{OD}_{645} \quad (2)$$

Carotenoids were determined by following the procedure of Whyte [26]. Algal cells were centrifuged and treated with centrifuging the algal cells and treated with KOH (60% w/w). The mixture was homogenized and warmed to 40°C for 40 mins and extracted using ethyl ether. The

solvent was evaporated followed by resuspending in acetone and the optical density was measured at 444 nm. Total carotenoids were calculated using the below equation.

$$Ct \text{ (mg/L)} = 4.32 \times OD_{444} - 0.0439 \quad (3)$$

2.7. Protein and Carbohydrate Assay

The extraction of proteins from microalgae was performed using alkali method. Aliquots of algal sample were centrifuged and 0.5 N NaOH was added to the pellet followed by extraction at 80°C for 10 mins. The mixture was centrifuged and protein content of the supernatant was estimated using Bovine Serum Albumin (BSA) as standard [27].

Cellular carbohydrates were estimated using the anthrone method described by Gerhardt et al., [28] after hot alkaline extraction [29]. Briefly, microalgal pellets were resuspended in distilled water and then heated in 40% (w/v) KOH at 90°C for 1 h. After cooling down, ice cold ethanol was added and stored at -20°C overnight followed by centrifugation. The pellet was resuspended in distilled water and then reacted with anthrone reagent. D-glucose was used as standard and the colour development was read at 578 nm in a spectrophotometer.

2.8. Superoxide Dismutase Assay

Superoxide dismutase (SOD) assay was carried out by subsequently pipetting the following solutions into the cuvette; 0.8 ml of Triethanolamine diethanolamine (100 mM), 40 µl NADPH (7.5 mM), 25 µl EDTA-MnCl₂ (100 mM/50 mM) and 0.1 ml of microalgal sample. The contents were mixed thoroughly and the absorbance was read at 340 nm over a 5 min period. Then added 0.1 ml of mercaptoethanol (10 mM) and the contents were mixed. The decrease in absorbance for about 20 min was measured to allow full expression of the chain leading to NADPH oxidation [30].

2.9. GSH Assay

The algal cells were pelleted by centrifuging at 8000 rpm for 10 mins and added with 2.5 ml of Na₂PO₄ (0.3M). A 0.25 ml of -5'-dithiobis [2-nitrobenzoic acid] (DTNB) reagent was added and the mixture was incubated at 37°C for 10 mins. After incubation, the absorbance was read at 412 nm in a UV-Vis spectrophotometer and the concentration of GSH was determined using the standard curve [31].

3. Results and Discussion

Microcystis and *Oscillatoria* were found as predominant microalgal species in this study (Figure-1a and 1b). The change in the colour of cobalt chloride solution indicated the synthesis of nano particles which were further characterized by UV-Vis spectroscopy. Nano sized cobalt exhibits a strong absorption due to collective oscillation of conduction of electrons under suitable radiation and this phenomenon is

known as Plasmon resonance. The Plasmon band of CoNPs was observed at 400-500 nm and the maximum absorption was found at 440 nm which confirmed the nano sized particles. The isolates were purified and cultivated in the presence of cobalt nano particles using BG 11 medium for a period of 5 days.

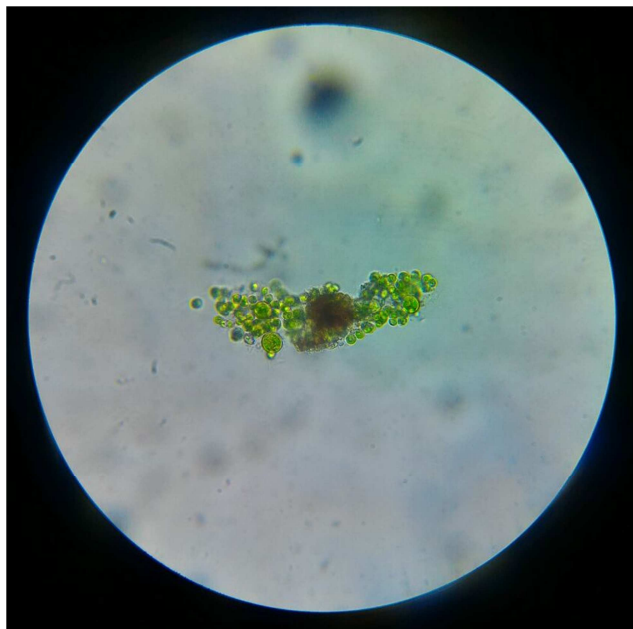


Figure 1a. *Microcystis* species.

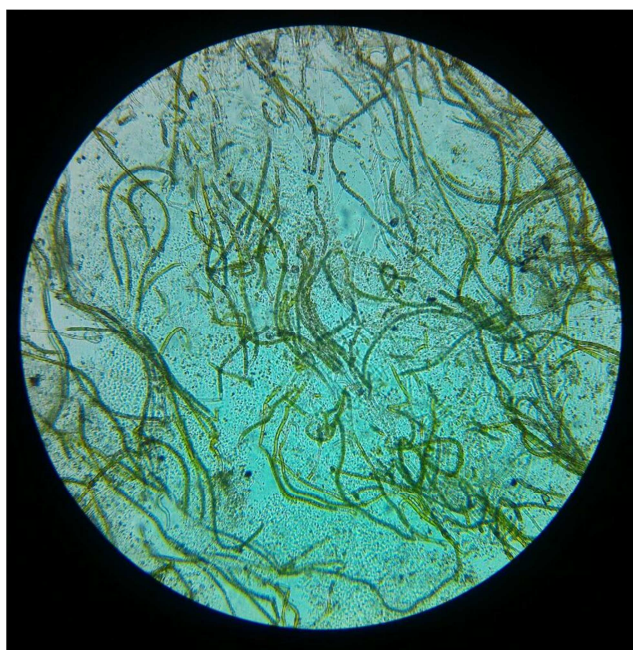


Figure 1b. *Oscillatoria* species.

In order to evaluate the effect of cobalt nanoparticle on growth of *Microcystis* and *Oscillatoria* strains, 3 ml of microalgal suspension with exponential growth phase was harvested from stock cultures and was added to 150 ml of BG11 medium. Microalgal cells were exposed to increasing nominal concentration of cobalt nano particles

including a control (1, 2, 3, 4 and 5 mg·L⁻¹). Growth medium without nano particles was served as control and the experiments were performed in triplicates. Specific growth rate is the measurement of microalgal growth and the presence of metal nanoparticle reduced the overall growth rate in both *Microcystis* and *Oscillatoria*. Steady decline in the growth rate of microalgae was observed at the end of 5 days (Figure-2 and 3) indicating the toxicity of CoNP on microalgal growth. In eutrophic water, *Microcystis* is reported as one of the most worldwide toxic bloom forming cyanobacteria [32]. Toxins produced by *Microcystis* affect both aquatic animals and humans [33]. Effect of metal nanoparticles on growth inhibition of *Microcystis* was reported earlier [34-36, 9]. Wang et al. [35] reported that 0.5 mg·L⁻¹ concentration of copper oxide nano particles produced toxicity on *Microcystis aeruginosa* where as Park et al. [34] described that 1 mg·L⁻¹ had significantly inhibited the algal growth. In this study CoNPs at a concentration of 1 mg·L⁻¹ was found inhibiting algal growth over a period of time.

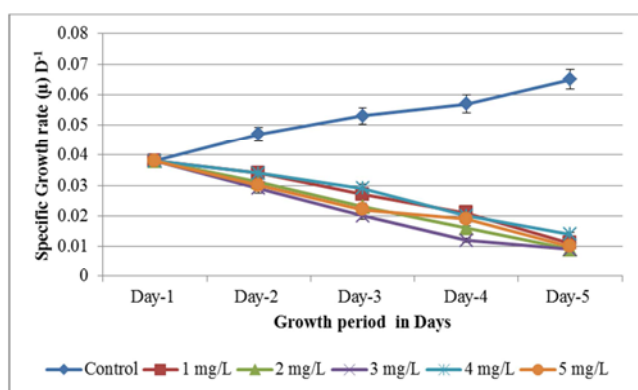


Figure 2. Specific growth rate of *Microcystis* at various concentrations of CoNPs.

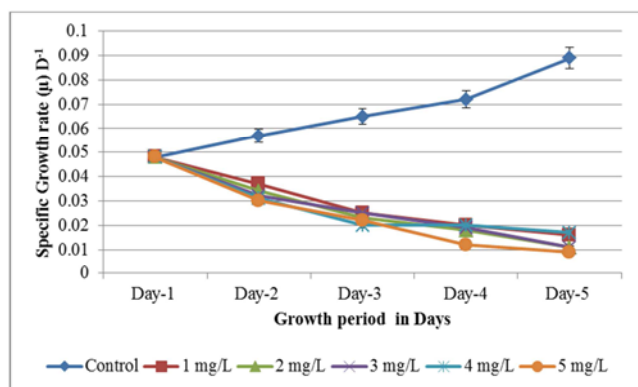


Figure 3. Specific growth rate of *Oscillatoria* at various concentrations of CoNPs.

The effect of CoNPs on biomass of *Microcystis* and *Oscillatoria* are represented in Figure-4 and 5. The experiments were performed for a period of 5 days with an initial biomass concentration of 0.065 mg·L⁻¹. CoNPs had significant effect on biomass content of the tested microalgae and at the end of cultivation period 0.014 mg·L⁻¹ was

observed in *Microcystis* which was 78% lesser than that of initial concentration. Similarly, increased CoNPs concentration reduced the biomass content of *Oscillatoria* which was 88% lower than initial concentration.

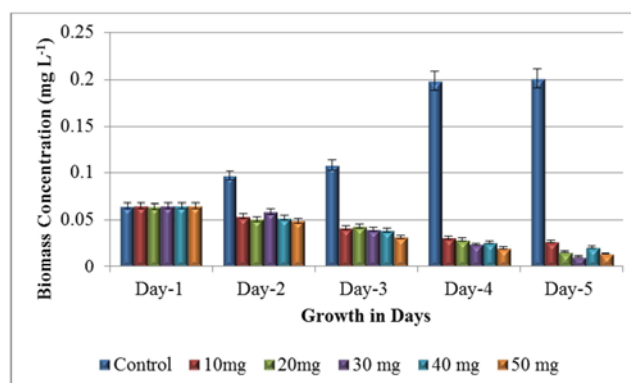


Figure 4. Biomass concentration of *Microcystis* at various concentrations of CoNPs.

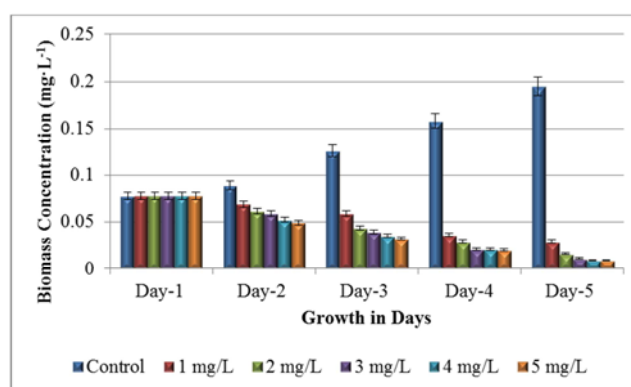


Figure 5. Biomass concentration of *Oscillatoria* at various concentrations of CoNPs.

Most reports demonstrated that increasing metal concentrations resulted in greater stress effect and reduction of algal growth rate was due to decrease in algal photosynthesis. This was supported in the present study where chlorophyll content was decreased with increasing nano particles concentration. The pigment concentration was reduced from 1.53 to 0.24 mg·L⁻¹ in *Microcystis* and 1.63 to 0.29 mg·L⁻¹ in *Oscillatoria* (Figure-6). The mechanism of toxicity of heavy metals to cyanophyta are not fully known but several heavy metals retard the flow of electrons in electron transfer reaction in mitochondria and chloroplast and thus can be expected to have a detrimental effect on respiration, photosynthesis and other processes related to it. The other mechanism proposed for the inhibition is the replacement of magnesium in the chlorophyll molecule, consequently cells accumulate protoporphyrin and synthesis of chlorophyll is blocked, this may be attributed to inhibition of reduction step in the biosynthetic pathways of this pigment. The results were similar for carotenoid contents where there was a 69.3% and 73.2% decrease in *Microcystis* and *Oscillatoria* respectively (Figure-7).

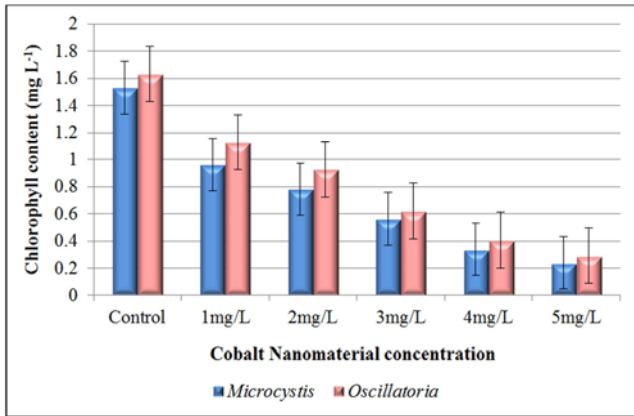


Figure 6. Chlorophyll content of algal bloom at various concentrations of CoNPs.

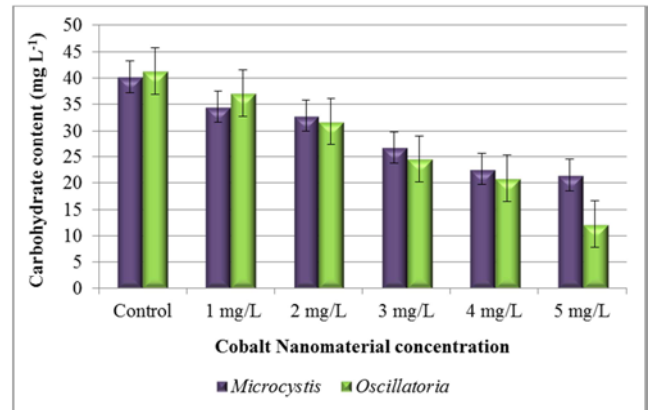


Figure 9. Carbohydrate content of algal bloom at various concentrations of CoNPs.

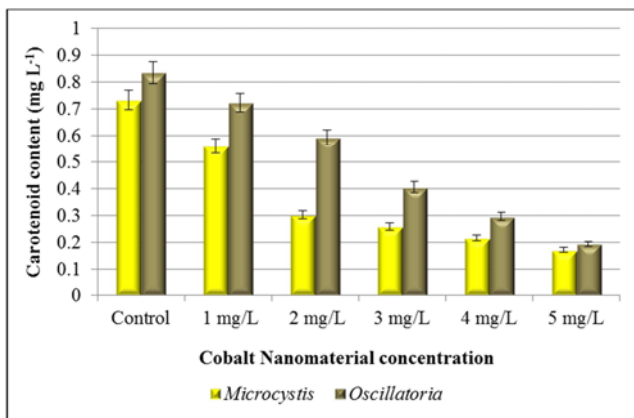


Figure 7. Carotenoid content of algal bloom at various concentrations of CoNPs.

Damage of cell membrane at high metal concentrations will lead to uncontrolled release/intake of electrolytes which may be responsible for the inhibition of growth. This might be linked with the synthesis of carbohydrates and proteins. This was confirmed by the data of proteins, where the trend in the accumulation of protein went parallel in most cases with the data of the photosynthetic pigments (Figure-8 and 9). This means that the efficiency of photosynthetic apparatus and the production of proteins and carbohydrates were closely associated and conclude that toxicity of metal nanoparticles disturbed both components.

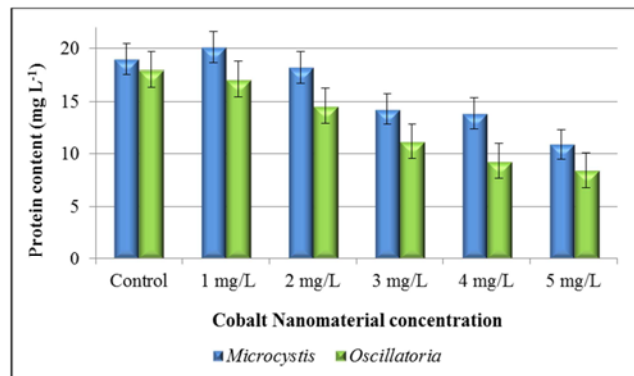


Figure 8. Protein content of algal bloom at various concentrations of CoNPs.

Metals also cause oxidative damage [37] and algal tolerance for metals partly depends on its defense responses to prevent oxidative damage [38]. Algae contain several enzymatic and nonenzymatic antioxidant defense systems to maintain the concentration of reactive oxygen species to protect cells from damage [39]. The primary scavenging enzymatic antioxidant defense system includes superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase [40]. In this study, the change in the antioxidant defense system was measured with SOD and GSH as biomarkers. SOD is an enzymatic antioxidant that can scavenge reactive oxygen species (ROS) generated by heavy metal stress. The change in SOD activity during the experiments is shown in Figure 10.

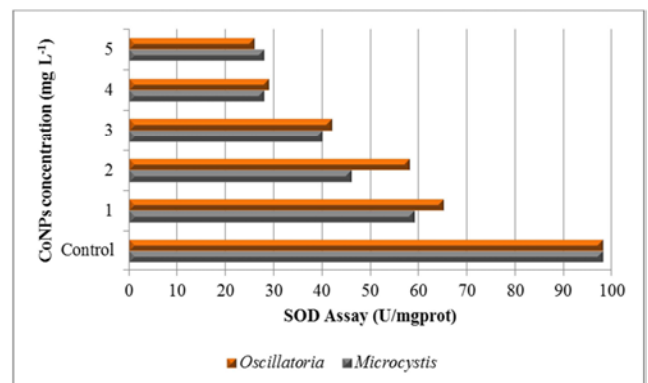


Figure 10. SOD Assay.

GSH is another major free radical remover and protect proteins from the oxidation of protein thiol groups [41]. Algae can respond to heavy metal stress by increasing the GSH concentration. The influence of GSH concentration by CoNPs is shown in Figure-11. As nanoparticles have a large surface area-to volume ratio, it is reported that NPs react strongly with cell compartments which increase free radical production and causing oxidative stress.

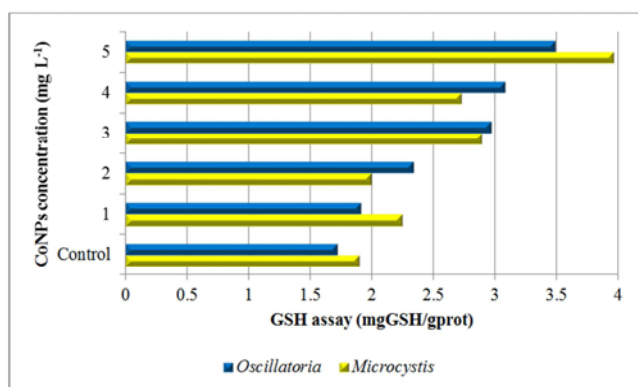


Figure 11. GSH Assay.

4. Conclusion

In conclusion, the inhibitory effect of CoNPs become greater with an increase in concentrations and suggested that the reduction in the growth rate of algae due to a decrease in algal photosynthesis caused by the inhibition of photosynthetic pigments and cellular components. The decrease in SOD activity with increased nanoparticle concentration reveals the formation of stress in the microalgae. The increasing GSH activity proved the effect of CoNPs on the activation of antioxidative enzymes to protect the cells. This study demonstrates the efficiency of cobalt nanoparticles (CoNPs) on inhibition of fresh water algal blooms thereby reducing the eutrophication problem.

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