Effects of Copper Ion (Cu$^{2+}$) on the Physiological and Biochemical Activities of the Cyanobacterium Nostoc ANTH

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Abstract
The effects of copper sulphate on the cyanobacterium Nostoc ANTH were studied. The copper concentrations chosen for the study were 0.5, 1.0, and 5.0 mg/L. From the results, it was observed that removal of Cu$^{2+}$ ions by the organism was dependent on the external concentrations of Cu$^{2+}$ ions. Exposure to high concentrations of copper led to severe morphological changes in the cells, which were visible under transmission electron microscopy. Yet, complete elimination of copper from the growth medium led to poor growth, and ultimately the death, of the Nostoc ANTH cells. Cells exposed to low copper concentration (0.5 mg/L) in the medium showed comparable physiological and biochemical activities (such as growth, heterocyst frequency, nitrogenase activity, photosynthesis, and respiration) to that of the controlled cultures. However, these features were compromised as the copper concentration increased in the surrounding.

Keywords: Atomic absorption spectroscopy, Cu$^{2+}$ ions, Nostoc ANTH, Transmission electron microscopy

1. Introduction
In recent times, increasing amounts of various metals ions in the environment have shown undesirable effects on living organisms, including microbes that are absolutely necessary for processes such as geochemical recycling [1]. Metals are toxic, as they can interact with crucial functional groups that modify various cellular macromolecules, and disrupt membrane integrity, thus leading to reduction in cellular capacity in maintaining electrochemical gradients in the cells [2-5]. Concerns over environmental degradation in recent years have shifted considerable research efforts worldwide towards bioremediation and restoration of the environment using biological materials, especially microbes such as cyanobacteria in place of physical and chemical processes that are costly in the long run [6, 7]. In addition, using photosynthetic microbes is advantageous, as their energy requirements are directly met by sunlight. Besides, their presence in the environment causes no ecological imbalance.

In all living organisms copper is needed as micronutrient, since it plays an important role of essential cofactor for the functioning of various enzymes, such as cytochrome c oxidase, and superoxide dismutase. Copper is also required in electron transport activities [1, 8]. In cyanobacteria, copper is a component of plastocyanin. However, at higher concentrations (3.0 mg/L), copper is toxic to microorganisms [7, 9] and many copper-containing compounds, e.g., copper sulphate is commonly applied as a pesticide and algicide. It is often used to eliminate cyanobacterial growth in drains and water reservoirs. By virtue of being a redox metal, copper at high concentration can lead to increased production of intracellular reactive oxygen species (ROS), which becomes poisonous to the cell when their concentration goes beyond the cell’s capacity to scavenge these radicals [10, 11].

In the present work, the effect of divalent copper ion (Cu$^{2+}$) exposure was studied on the cyanobacterium Nostoc ANTH (isolated from Anthoceros punctatus). The chemical substance utilized to generate Cu$^{2+}$ in the experiments was CuSO$_4$. An atomic absorption spectroscopy (AAS) study was used to explore how much the organism accumulated Cu$^{2+}$ within it, and how the accumulated Cu$^{2+}$ affected the organism. Morphological changes were recorded using the transmission electron microscopy (TEM) study. Growth kinetics, heterocyst frequency, nitrogenase activity, photosynthesis, and respiration of Nostoc ANTH were examined under copper exposure in relation to controlled cultures, as measures of its physiological activities.

2. Materials and Methods
2.1. Isolation and Purification of Nostoc ANTH
Anthoceros punctatus gametophytic thalli were collected dur-
ing the month of September after the rainy season, from North-Eastern Hill University Campus (Shillong, Meghalaya, India). The thalli were washed, and later sterilized in 0.5% sodium hypochlorite solution. The microscopically visible cyanobacterial colonies were cut and plated on BG-11 \(_{m}\) medium with 1% agar. Plates were incubated in the culture room under fluorescent light (photon fluence rate of 50 \(\mu\)mol/m\(^2\)/sec) at 25°C. After three weeks of incubation, individual cyanobacterial colonies were picked up and transferred to a liquid medium. Plating and selecting processes were repeated, until unicellular suspensions were achieved. The identity of the cyanobacterial suspension was confirmed under light by microscope BX53 (Olympus, Tokyo, Japan) [12]. Free-living cultures were maintained for many generations in BG-11, medium [13]. All experiments were done on the sixth day. The BG-11, medium was prepared using macronutrients (K\(_2\)HPO\(_4\).3H\(_2\)O, MgSO\(_4\).7H\(_2\)O, CaCl\(_2\).2H\(_2\)O, citric acid, Ferric ammonium citrate, Na\(_2\)CO\(_3\), EDTA [disodium salt]) and micronutrients (H\(_3\)BO\(_3\), MnCl\(_2\).4H\(_2\)O, ZnSO\(_4\).7H\(_2\)O, Na\(_2\)MoO\(_4\).2H\(_2\)O, CuSO\(_4\).5H\(_2\)O, Co(NO\(_3\))\(_2\).6H\(_2\)O), and the pH was adjusted to 7.6.

2.2. Preparation of Stock Solution of CuSO\(_4\)_

A single metal aqueous stock solution of CuSO\(_4\) was prepared (1,000 mg/L), by dissolving the desired amount of CuSO\(_4\) (analytical reagent [AR] grade) in 1 L of BG-11, media. The solution was then filtered using a membrane filter.

2.3. Measurement of Growth

The media for maintaining \textit{Nostoc ANTH} contained CuSO\(_4\) at a concentration of 0.32 \(\mu\)M. Elimination of CuSO\(_4\) from the growth media led to poor growth and ultimate death of the \textit{Nostoc ANTH} cultures. However, for the present study this CuSO\(_4\) concentration was increased to 0.5, 1, and 5 mg/L, in order to estimate the effects of Cu\(^{2+}\) ions. The media for the control cultures continued to have CuSO\(_4\) at a concentration of 0.32 \(\mu\)M. A six day old culture of \textit{Nostoc ANTH} growing in the log phase was inoculated in the media, containing different concentrations of CuSO\(_4\) (0.5, 1, and 5 mg/L). Increases in chlorophyll-a concentrations [14], protein [15] and carbohydrate contents [16] were used as the measures of growth of the organism. Three milliliter cultures were taken for each of the measurements. Chlorophyll-a content was measured, after extracting the pigments in methanol, and reading the absorbance at 663 nm, as described by Mackinney [14] in 1941. Three milliliter of cultures was sonicated, and the total protein was estimated using Lowry’s spectrophotometric protein estimation method in the sample. Increase in carbohydrate content in the experimental cultures was measured using anthrone reagent against a standard curve.

2.4. Heterocyst Frequency and Nitrogenase Assay

Heterocyst frequency was calculated using an Olympus BX53 light microscope [17]. Nitrogenase enzyme activity was estimated as its ability to reduce acetylene in vivo by gas chromatography [18], using a gas chromatograph (Tracer 540; Teometrics, Austin, TX, USA) fitted with a Porapak ‘T’ column (Chromatography Research Supplies, Louisville, KY, USA) and a flame ionization detector.

2.5. Assay of Photosynthesis and Respiration

Oxygen evolution and consumption was measured by using a Clark-type oxygen electrode installed in a 3 mL plexiglass container with magnetic stirrer (Rank Brothers, Cambridge, England). Measurements involved adding 3 mL cyanobacterial culture to the sample chamber of the non-polarized electrode, and allowing each sample to equilibrate for 2 min with stirring. The electrode was then polarized, and a linear rate of oxygen evolution was obtained in light, with a photon fluence rate of 50 \(\mu\)mol/ m\(^2\)/sec [19]. Oxygen consumption was measured in the dark, with the chamber wrapped in aluminum foil.

2.6. Ultrastructural Studies Using Transmission Electron Microscopy

Four percentage glutaraldehyde was used to fix \textit{Nostoc ANTH} cells for TEM. Washing was done with 0.1 M sodium cacodylate buffer in three changes of 15 min each at 4°C. Post-fixation was carried out with 2% OsO\(_4\) in 0.2 M sodium cacodylate buffer, for 1 hr at 4°C. The specimen was then rinsed with 0.1 M buffer, in three changes of 15 min each at 4°C. Samples were dehydrated at 4°C in increasing percentages of acetone. Clearing was carried out using propylene oxide in two changes after every thirty min, each at room temperature. Infiltration was carried out in four steps using propylene oxide and embedding medium. Infiltrated samples were transferred into BEEM (BEEM Inc., West Chester, PA, USA) capsules or embedding molds, and oriented. Pure embedding medium was poured into the capsule or mold, and transferred into an embedding oven at 50°C; the samples were kept overnight for 24 hr. For polymerization of the embedded capsules, the temperature of the oven was further raised to 60°C, and maintained for 48 hr. Each sample was cut into 60–90 nm sections using ultramicrotome MTX (Boeckeler Instruments, Tucson, AZ, USA), and the sections were double stained using uranyl acetate and lead citrate. The sections were finally viewed in a JEM-2100, 120 kV TEM (JEOL, Tokyo, Japan).

2.7. Estimation of Cu\(^{2+}\) Ions Using Atomic Absorption Spectrophotometry

To separate cyanobacterial cells from copper supplemented media, the test solutions were centrifuged at 5,000 rpm. The supernatant was analyzed, using a PerkinElmer 3110 atomic absorption spectrophotometer (PerkinElmer, Waltham, MA, USA), for the amount of Cu\(^{2+}\) ions present. The amount recorded was subtracted from the total amount originally added, to arrive at the amount of Cu\(^{2+}\) ions removed by the cells. Desorption of Cu\(^{2+}\) ions adsorbed on the cell surface were achieved with 0.1 M EDTA, and the amounts desorbed were estimated using AAS. For determination of intracellular accumulation of Cu\(^{2+}\), the cells were sonicated using a Vibra-Cell ultrasonicator (Sonic & Materials Inc., Newtown, CT, USA) in 3 mL medium [6], and the ions released in the medium were calculated by AAS. Finally, cells were dried in a hot air oven at 40°C until constant weight was recorded, to determine the dry weight of the test organism under treatment.

3. Results and Discussion

3.1. Influence of Cu\(^{2+}\) Ions on Growth of Cyanobacterial Cells

The growth of \textit{Nostoc ANTH} cultures was measured as chang-
es in concentrations of chlorophyll-$\alpha$, protein and carbohydrate in the presence of Cu$^{2+}$ ions in the medium. The least affected component at the highest concentration used (5.0 mg/L CuSO$_4$) was the total protein content (reduced from a value of 38 µg/mL in the control cells to 25 µg/mL in the organism). The reduction in chlorophyll-$\alpha$ and carbohydrate content at 5.0 mg/L CuSO$_4$ was from 3.5 µg/mL in the control cells to 1.5 µg/mL, and from 99 µg/mL in the control cells to 43 µg/mL, respectively. The standard medium used for growing Nostoc ANTH (BG-110) contains CuSO$_4$ in the concentration of 0.32 µM. The lowest concentration used in this study was 0.5 mg/L CuSO$_4$, which corresponds to 2.0 µM. At this concentration, no noticeable change was observed in these parameters. The values were comparable to the control culture, in media supplemented with 0.5 mg/L. However, further increase in Cu$^{2+}$ ion concentration led to significant loss of growth in terms of all parameters studied, as seen from Fig. 1. Five milligram per liter copper concentration was highly detrimental to the growth of the organism.

### 3.2. Effect on Heterocyst Frequency, Nitrogenase Activity, Photosynthesis and Respiration

Both heterocyst frequency and nitrogenase activity were severely affected by copper treatment. At 1.0 mg/L CuSO$_4$ concentration, the heterocyst count decreased by 8%, which could not be counted at 5.0 mg/L CuSO$_4$ supplemented medium, as the filaments were completely fragmented, and heterocysts were seen disintegrated under the microscope. The effect was far more severe on nitrogenase activity. The presence of 1.0 mg/L CuSO$_4$ led to a reduction to 2.1 nmol C$_2$H$_2$ reduced/µg protein/hr, as against a control value of 3.09 nmol C$_2$H$_2$ reduced/µg protein/hr in this enzyme activity. No nitrogenase activity was recorded in medium supplemented with 5.0 mg/L CuSO$_4$ (Fig. 2). The effects on photosynthesis and respiration are presented in Fig. 3. The effect was more on respiration than on photosynthesis. Photosynthesis went down to ~105 nmol O$_2$ evolved/µg protein/hr in the presence of 5.0 mg/L CuSO$_4$, from ~138 nmol O$_2$ evolved/µg protein/hr recorded in the control cultures. However, in 5.0 mg/L CuSO$_4$, respiration dropped to ~101 nmol O$_2$ consumed/µg protein/hr in control cultures, to ~19 nmol O$_2$ consumed/µg protein/hr. This finding is similar to those from earlier reports [7, 20, 21]. Since heterocysts are sites of nitrogen fixation in the filamentous cyanobacteria where vegetative cells are responsible for providing carbohydrates to heterocysts for energy production, fragmentation of the filaments may have severely affected nitrogenase activity, as this had disrupted the transfer of the metabolites to the heterocysts. In addition, low respiratory and photosynthetic activities lead to a decrease in the concentration of ATP and NADPH$_2$ [22] that may have been responsible for reduced nitrogenase activity, as nitrogen fixation in cyanobacteria is an energy demanding process.

### 3.3. Ultrastructural Study by TEM

Control cells under TEM showed intact thylakoid membranes in Fig. 4(a). With CuSO$_4$ concentration at 0.5 mg/L in the medium, there were signs of shrinkage of the thylakoid membranes, and appearance of poly phosphate bodies as round, dark structures in Fig. 4(b), confirming their role in compartmentalizing metal ions within the cells, as reported by earlier researchers [7, 23]. The trapping of copper in these structures may be a defensive mechanism for detoxification of copper in this organism. By the sixth day of exposure to 1.0 mg/L CuSO$_4$, the thylakoid membranes shrunk further, leaving most parts of the cell volume empty in Fig. 4(c). The total cell content appeared in advanced stages of disintegration and breakdown, including the polyphosphate bodies, when the cells were treated with 5.0 mg/L CuSO$_4$ in Fig. 4(d). Thus, the drastic ultra structural changes seen in the

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**Fig. 1.** Concentrations of chlorophyll-$\alpha$, protein and carbohydrate in Nostoc ANTH cultures at different CuSO$_4$ concentrations in the growth media.

**Fig. 2.** Heterocyst frequency and Nitrogenase activity of Nostoc ANTH in different concentrations of CuSO$_4$.

**Fig. 3.** Effects of CuSO$_4$ concentration on respiration and photosynthesis in the Nostoc ANTH cells, C- control Nostoc ANTH cells.
treated cells, especially at 5.0 mg/L CuSO₄, clearly establish the toxicity of Cu²⁺ ions on the various cellular components of the Nostoc ANTH cells.

3.4. Cu⁺⁺ Sorption and Its Distribution in the Nostoc ANTH Cells

As shown in Fig. 5(a), concentrations of copper ions in the surrounding dictated its removal by Nostoc ANTH cells; a similar observation was also made by Mohamed [6]. With increasing metal concentrations, there was an increase in their removal from the medium. The removal percentage was always in the range of 50–60%, even though the concentration of copper ions in the surrounding media increased significantly from 0.5 to 5.0 mg/L, which is a ten-fold increase. This probably signifies the fact that there exists an equilibrium between the intracellular and extracellular Cu²⁺ ion concentrations. Further uptake of copper ions was initiated every time external concentrations of copper ions were increased, such that the removal percentage always was maintained between 50–60%. AAS studies revealed that ~58 mg of Cu²⁺ ions were removed per gram of Nostoc ANTH cells. Further analysis of the removed copper showed ~3.37 mg/g Cu²⁺ ions were precipitated on the cell surface which got dislodged when cells were vortexed in fresh media. A large amount (~39.3 mg/g) was adsorbed on the cell surface that needed desorption, using 0.1 M EDTA. It is already known that capsular polysaccharides of these microbes that are negatively charged are the main constituents that bind to the metal ions [6, 24-26].
To estimate the copper concentration that was accumulated intracellularly by the Nostoc ANTH cells, cells were sonicated in fresh media and Cu^{2+} ions were measured in the cell free supernatant, which yielded a value of ~13.3 mg/g in Fig. 5(b). From all these observations it can be inferred that the toxicity of increased copper ions may have been exerted at two levels. Firstly, binding of large amount of Cu^{2+} ions on the capsular polysaccharide may have hampered the uptake of the various nutrients necessary for cellular activities; and secondly, increase in the intracellular Cu^{2+} ions may have disrupted various enzymatic activities, and modified vital macromolecules crucial for living organisms.

3. Conclusions

Copper as micronutrient is required for proper functioning of the electron transport chain, which is essential for energy production in these organisms. Removal of copper by Nostoc ANTH from the medium was concentration dependent. 13.3 mg/g intracellular copper ions were enough to bring about breakdown in cellular components and metabolic processes, indicating this amount to be toxic to the organism. This may be due to the fact that copper, being a redox metal, may have increased production of intracellular reactive oxygen species (ROS) beyond the cell's capacity to scavenge these radicals, thereby negatively influencing deoxyribose nucleic acid, ribose nucleic acid, and proteins within the cells manifest by progressive destruction of cells and cellular functions.

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