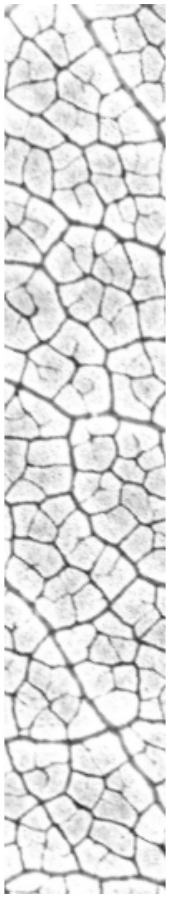

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Expression of copper toxicity in the rice-field cyanobacterium *Anabaena oryzae* Ind4

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Abstract
Effect of copper on the rice-field cyanobacterial isolate *Anabaena oryzae* Ind4 was analysed after a five-day exposure period. Copper affected vital parameters of the organism in a dose-dependent manner. The organism was to an extent tolerant to copper at 5 mg L^{-1} concentration; at this concentration cells showed comparable biochemical activities to that of the controlled cultures. However, beyond this level any increase in copper concentration led to significant adverse effects on all parameters of the organism studied. High copper levels led to severe morphological and ultra-structural changes in the cells distinctly visible under the scanning and transmission electron microscope.

Keywords: *Anabaena oryzae* Ind4, copper toxicity, carbon fixation, nitrogen metabolism, scanning and transmission electron microscope

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INTRODUCTION

Entry of wastewater into agricultural lands by way of seepage from industrial sites poses considerable threat of environmental and health hazards. Although wastewater is utilized for irrigation in several places because such a process helps in recycling of useful nutrients through the food chain, toxic elements (e.g. metals) present in the water severely compromise crop yield as well as endanger human and animal health due to bioaccumulation (Shakir et al. 2017). Many metals like copper, iron, manganese, nickel, zinc, etc. are essential as micronutrients for metabolic processes in living organisms, however several others like arsenic, cadmium, chromium, mercury and lead have no known physiological activity and are toxic even at trace levels of exposure (Qaiser et al. 2007). Besides, those metals essential to organisms have the capacity to become harmful at higher concentrations (Marschner 1986). Many toxic metals are major components of wastewater. Exposure to such contaminants for a prolonged period of time can cause significant damage to any exposed organism. Microorganisms that constitute the major biota of agricultural soil are the first set of organisms that gets exposed to metal contaminants when fields are irrigated with wastewater. Many constituents of the soil microflora are crucial in maintaining soil composition, health and in nutrient recycling (Komárek et al. 2008, Liu et al. 2013). However, damage done to this group of microbes goes unnoticed and remains to be assessed as the primary focus in agriculture based system is the principal crop. Disruption in the fine balance brought about in the overall crop land ecosystem by way of destroying beneficial microorganisms in the long run reduce soil quality, fertility and eventually affects crop yield (Liu et al. 2006, Mishra and Dhar 2004).

Cyanobacteria are one such group of microbes that contribute significantly to enhance soil quality and fertility as they are both photosynthetic and nitrogen fixing in nature. Their presence considerably enhances the carbon and nitrogen content of the soil which in turn aids in crop productivity (Aziz and Hashem 2003, Prasanna et al. 2014, Whitton 2000). It is a well-known fact that availability of fixed nitrogen is a limiting factor, especially in rice cultivation (Vaishampayan et al. 2001, Whitton 2000). Two third of the soil nitrogen worldwide (~ 180 million tonnes) is attributed to microbial activities mainly to cyanobacterial nitrogen-fixation (Kaushik 2014). Thus, a healthy cyanobacterial population is essential for better crop response especially in rice cultivation.

The state of Meghalaya has large reserves of coal which is regularly mined for commercial use. However, the process of mining is highly unplanned and unscientific, done by an obsolete technique known as *'rat-hole'* mining. Most of the mine tailings find their way to adjoining water bodies and agricultural fields. In addition, hilly terrains and heavy rainfall in the region compound the problem by accelerating the flow of these

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wastewaters. Rice, maize and potato are principal crops cultivated in Meghalaya (Directorate of Economics & Statistics, Government of Meghalaya, Shillong, India). Rice cultivation requires water-logged conditions where the uppermost part of the soil remains submerged under water. Therefore, water quality is a major determinant of the paddy field ecosystem. In mining areas paddy fields and low-lying water bodies serve as a sink to the above mentioned wastewaters in which deposition of several toxic metals at very high concentrations have been previously reported (Ahad et al. 2017, Diengdog et al. 2017). Toxic metals in these wastewaters alter the existing ecosystem by causing significant damage to crop plants and soil microflora. Many reports are available that have examined the adverse effects of metal polluted wastewaters used for irrigation on the quality and productivity of the principal crop (Balkhair and Ashraf 2016). However, their effect on the microflora that constitutes the crop field ecosystem is not documented. Metals are known to cause damaging effects on microbes including cyanobacteria on prolonged exposure as well as at higher concentrations (Ahad and Syiem 2018b). In this study we have looked into the effect of copper (one of the common mine tailing pollutants and commonly used pesticide) on *Anabaena oryzae* Ind4, a ubiquitously found rice-field cyanobacterium in the state. The copper uptake and distribution within the cyanobacterium was studied using atomic absorption spectroscopy. Copper mediated effects on morphology and ultra-structure were analysed using electron microscopes. The changes on carbon, nitrogen and energy metabolism on copper exposure were also evaluated. The present study is a comprehensive report on the antagonistic effects of an otherwise essential metal ion (i.e. Cu) on the cyanobacterium when present at higher concentration as well as when the exposure is chronic.

MATERIALS AND METHODS

Isolation, Maintenance and Molecular Identification of the Cyanobacterium

The cyanobacterium was isolated from a rice field near Mawkynrewin (25.43 °N, 91.99 °E), East-Khasi Hills district of Meghalaya, India and allowed to grow in freshly prepared BG-11_0 medium (pH 7.5) (Rippka et al. 1979). The culture was purified by repeated rounds of plating on 1.2% agar supplemented medium (Packer and Glazer, 1988). Single uni-algal colonies were transferred to 250 mL erlenmeyer flasks containing the same medium and allowed to grow under culture room conditions of temperature 25±2 °C with continuous light at a photon fluence rate of 50 µmol m^{-2} s^{-1}. Identity of the culture was established using PCR-based molecular technique. Genomic DNA was isolated from the cyanobacterium using MiniPrep Bacterial Genomic DNA method (Ausubel et al. 1999) and its quality was tested on a 1.2% agarose gel. Fragment of 16S rRNA gene from the isolated DNA was amplified by PCR and the purified amplicon was used for sequencing. Forward and reverse DNA sequencing reaction of the PCR amplicon was carried out in Xceleris Labs, Ahmedabad with CY106F(5´-CGG ACG GGT GAG TAA CGC GTG A-3´) and CY781R{equimolar mixture of CY781R(A) 5´-GAC TAC TGG GGT ATCTAA TCC CAT T-3´ and CY781R(B) 5´-GAC TAC AGG GGT ATCTAA TCC CTT T-3´} primers using BDT v3.1 Cycle sequencing kit on 3730xl Genetic Analyzer (Applied Biosystems, USA) (Nubel et al. 1997). Related sequences were retrieved from NCBI GenBank database using BLAST and based on maximum identity score first 10 sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 6 (Tamura et al. 2011).

Metal treatment

$CuSO_4 \cdot 5H_2O$ was used as the source of Cu in all experiments and working solutions of 5, 10, 15 and 20 mg L^{-1} in BG-11_0 medium were made using the same. Ten day old mid log phase cyanobacterial cells were used for all experiments and the culture concentration throughout the study was kept constant at 3 µg mL^{-1} of chlorophyll *a*. All experimental setups were kept in the culture room under fluorescent light at a photon fluence rate of 50 µmol m^{-2} s^{-1} with continuous illumination. Cells were harvested on day five by centrifuging at 2500 rpm for 5 min for conducting experiments.

Estimation of Photosynthetic Pigments

Chlorophyll *a* concentration was measured according to the method described by Mackinney (1941). Three mL cyanobacterial culture was centrifuged at 2500 rpm for 3 min. The supernatant was discarded and 3 mL methanol was added to the pellet. The solution was then vortexed and kept overnight at 4ºC to extract the total chlorophyll *a*. Following extraction, the mixture was centrifuged again at 2500 rpm for 3 min and absorbance of the resulting supernatant was read at 663 nm using a UV-visible spectrophotometer (SmartSpec Plus, BioRad, USA).

Determination of Phycobiliproteins

Three phycobiliproteins *viz*- **phycocyanin (PC)**, **allophycocyanin (APC)** and **phycoerythrin (PE)** were estimated according to Bennett and Bogorad (1973). Five mL culture was centrifuged at 2500 rpm for 3 min and the pellet washed twice in phosphate buffer saline, PBS (pH 7.5). After washing, the pellet was resuspended in 5 mL of the same buffer and solution was sonicated using Sonics Vibra cell sonicator (USA) fitted with a microprobe to disrupt the cyanobacterial cells. The supernatant collected after centrifugation at 45000 rpm for 45 min was used for phycobiliproteins estimation. Optical density of the supernatant was

measured at 615, 562 and 652 nm each using PBS as the blank.

Carotenoids

Carotenoids concentration was measured according to the method described by Morgan (1982). 3 mL cyanobacterial culture was centrifuged at 3000 rpm for 5 min. Three mL of 95% N, N - dimethylformamide was added to the pellet, mixed well and the tubes were incubated in dark for 5 min. Following incubation, the mixture was centrifuged again at 3000 rpm for 5 min and absorbance of the resulting supernatant was measured each at 461 and 663 nm using a UV-visible spectrophotometer.

Energy Metabolism (Photosynthetic Oxygen Evolving Complex Activity)

Light induced net oxygen evolution during photosynthesis and oxygen consumption during respiration is the measures of energy metabolism in living organisms. Thus, photosynthetic and respiratory rates were measured using a Clark-type oxygen electrode installed in a Plexi glass container with magnetic stirring (Rank Brothers, England) in order to assess energy status of the cyanobacterium under study. Cyanobacterial culture was added to sample chamber of the non-polarized electrode and each sample was equilibrated for 5 min with continuous stirring. The electrode was polarized and a linear rate of oxygen evolution was recorded under light supplied by a 100 W tungsten filament bulb, which was shielded from the sample by water bath acting as heat filter. Oxygen consumption was measured in dark with the chamber wrapped in aluminium foil. The rates of photosynthesis and respiration were expressed as nmol O_2 evolved or consumed $min^{-1}\mu g^{-1}$ chlorophyll *a*[26].

Nitrogen Metabolism

Estimation of total protein content

Protein was estimated according to Lowry et al. (1951). Three mL cyanobacterial culture was centrifuged at 2500 rpm for 3 min and the pellet was resuspended in 3 mL distilled water. Cells were disrupted by ultra-sonication using a Sonics Vibra cell sonicator (USA) fitted with a microprobe. The supernatant collected after centrifugation at 2500 rpm for 3 min was used for protein estimation. To 0.5 mL of the supernatant, 0.5 mL of distilled water and 5 mL of alkaline mixture were added and then vortexed. To this mixture, 0.5 mL of 1 mol L^{-1} Folin–Ciocalteu's phenol reagent was added and mixed well. The tubes were then incubated for 20 min at room temperature. The absorbance of the resulting solution was read at 750 nm against a blank and the protein concentration of the sample was estimated using a standard curve.

Nitrogenase activity

Nitrogenase activity was measured by acetylene reduction assay method as per Stewart et al. (1967). Acetylene gas at a final concentration of 10% (v/v) of air phase was injected into 15 mL tubes containing 5 mL cyanobacterial culture and incubated for an hour at 25 ºC under light with continuous shaking. A gas chromatograph (Varian 3900, The Netherlands) fitted with a Porapak T column (stainless steel column 6' × 1/8'', packed with a Porapak T of mesh size 80/100) and a flame ionization detector was used to estimate the amount of acetylene consumed by the cyanobacterial culture. Nitrogenase activity was expressed as nmol C_2H_2 consumed μg^{-1} chlorophyll *a* h^{-1}.

Glutamine synthetase (GS) activity

To determine the assimilation of nitrogen in cyanobacterial cells, GS activity was determined by assaying γ-glutamyl hydroxamate produced as per the method described by Sampaio et al. (1979). Cyanobacterial culture (2 mL) was centrifuged and the pellet was washed twice with 50 mmol/L Tris–HCL buffer (pH 7.5) and resuspended in 2 mL of the same buffer. The cells were broken by ultra-sonication to release the enzyme into the buffer. The assay mixture containing 40 mmol/L Tris–HCL buffer (pH 7.5), 3 µmol of$MnCl_2$, 20 µmol of potassium arsenate, 0.4 µmol of ADP (sodium salt), 60 µmol of hydroxylamine, and 30 µmol of glutamine was prepared. A 0.5 mL volume of the assay mixture was added to 0.5 mL of enzyme extract. After incubation in the dark for 10 min at 30 °C, the reaction was terminated by adding 2 mL of stop mixture (containing 10% FeCl3, 24% TCA, 6 mol/L HCl in distilled water). The final mixture was then centrifuged at 2500 rpm for 5 min and absorbance of the supernatant was read at 540 nm. The concentration of γ-glutamyl hydroxamate formed was estimated from a standard curve of the same. GS activity was expressed as nanomoles of γ-glutamyl hydroxamate formed per microgram of protein per minute.

Carbon Metabolism

Biomass

Biomass of the cells was expressed in terms of gram dry weight per mL of culture.

Carbohydrate estimation

Five mL culture was centrifuged at 2500 rpm for 3 min and the pellet resuspended in 5 mL distilled water. The solution was sonicated to disrupt the cyanobacterial cells. The supernatant collected after centrifugation at 2500 rpm for 3 min was used for carbohydrate estimation. To 1 mL of the supernatant, 4 mL of anthrone reagent (0.2% anthrone in concentrated H_2SO_4) was added and mixed well. The tubes were incubated in boiling water bath for 10 min. After cooling, the solutions were centrifuged at 2500 rpm for 3 min and absorbance of the resulting supernatant was read at 630 nm. A calibration curve was prepared using glucose solution (concentration range of 10-100 µg mL^{-1}) as the standard for determining carbohydrate content of the cyanobacterial cells (Roe 1955).

Morphological and ultra-structural analyses

Morphological and ultra-structural alterations in the cyanobacterial cells upon Cu exposure were studied using scanning and transmission electron microscopy, respectively. Electron microscopic studies were conducted as per standard protocols for biological samples at the Sophisticated Analytical Instrumentation Facility (SAIF), NEHU, Shillong. Samples for scanning electron microscopy (SEM) were subjected to primary fixation in 4% glutaraldehyde for 4 h and then washed in 0.1 M sodium cacodylate buffer with three changes of 15 min each at 4 °C. Sample dehydration was carried out using increasing amount of acetone at 4°C. The dried samples were then mounted on brass stubs and gold-coated (Fine coat ion sputter JFC 1100). Finally the samples were viewed under the scanning electron microscope (JEOL-JSM- 6360, Tokyo, Japan).

The initial steps up to dehydration was similar for transmission electron microscopy (TEM). Before dehydration, an additional step of secondary fixation of the samples was carried out for 1 h using 2% osmium tetroxide (OsO_4) in 0.2 M sodium cacodylate buffer. Dehydration was followed by clearing of the sample which was carried out using propylene oxide in two changes after every 30 min at room temperature. Infiltration was carried out in four steps:

Propylene oxide: Embedding medium (3:1)
overnight
Propylene oxide: Embedding medium (1:1)
1 h
Propylene oxide: Embedding medium (1:3)
1 h in vacuum
Pure Embedding medium
1 h in vacuum

Following infiltration, the samples were transferred into embedding molds and oriented. Pure embedding medium was poured into the molds and the molds were kept in an embedding oven at 50 °C for 24 h. For polymerization of the embedded molds, temperature of the oven was further raised to 60°C and maintained for 48 h. Each sample was cut into 60–90 nm sections using ultra-microtome MTX (Boeckeler Instruments, Tucson, AZ, USA) and the sections were stained using uranyl acetate and lead citrate and then viewed under the transmission electron microscope (JEOL- JEM-2100, 120 kV; Tokyo, Japan).

Cu removal and its distribution in the cell

Removal of Cu and its distribution in the cells were analyzed using GF-AAS (Analytik Jene AG Vario 6) (Nongrum and Syiem 2012). Cultures was treated with 5 mg L^{-1} for 24 h and centrifuged at 2500 rpm for 3 min and the amount of Cu^{2+} in the supernatant was analyzed. Percent Cu removal was calculated using the equation.

$$\% \, Cu^{2+} removal \; = \frac{(C_I - C_F)}{C_I} \times 100$$

where, C_I is the initial Cu supplied in the medium; C_F is the Cu concentration remained in the supernatant.

After the percent removal study was done, the pellet obtained to which 20 mL BG-11_0 medium was added, vortexed and centrifuged at 2500 rpm for 3 min. The supernatant was analyzed for precipitation of Cu^{2+}on the cell surface. Afterward, 0.1 Nof20 mL HCl solution was added to the pellet and left for 5 min after vortexing to desorb surface bound metal ions into the supernatant. The desorbed Cu^{2+}ions were determined in the supernatant collected after centrifugation. After this step, the residual pellet was again resuspended in 20 mL medium, ruptured by ultra-sonication (Sonics Vibra cell sonicator, VC-505, USA fitted with a microprobe) for 3 min and the resulting solution was analyzed using GF-AAS for the determination of Cu^{2+}internalized within 24 h in the cells.

RESULTS

Identification of the Cyanobacterium

The isolated cyanobacterium was identified as *Anabaena* sp. under light microscope (Olympus BX-53, Tokyo, Japan) (Desikachary 1959). The phylogenetic analyses of the organism was carried out by comparing amplified partial sequences of 16S rRNA gene with similar sequences deposited in GenBank (NCBI) and was named as *Anabaena oryzae* Ind4 (GenBank Accession No. KM596853).

Effect on Photosynthetic Pigments

A five day exposure to Cu modulated levels of various photosynthetic pigments at all concentrations tested. Although no adverse effect was noticed at lower concentrations of Cu tested, the increase in the pigment contents upon exposure to low Cu concentrations was less compared to that in the untreated cells indicating that the natural rate of pigment production in the treated cells were affected even at lower concentrations. At higher concentrations, Cu caused major reduction in the level of all pigments.

On day fifth of treatment, chlorophyll *a* concentration increased by ~71% in control and 62% in 5 mg L^{-1} Cu treated cells in comparison to initial Chlorophyll *a* concentration of day one. However, chlorophyll *a* concentration in 10, 15 and 20 mg L^{-1} Cu treated cells registered a reduction by ~ 16, 42 and 84%, respectively from the initial concentration. This indicated that not only the production of chlorophyll *a* was inhibited, Cu also initiated breakdown of existing chlorophyll *a* molecules at higher concentrations.

Carotenoids concentration was increased by ~ 140 and 116% in control and 5 mg L^{-1} Cu-treated cells. 10 mg L^{-1} Cu did not cause any significant change in the carotenoids levels on day 5 compared to the control whereas 15 and 20 mg L^{-1} Cu reduced the amount of carotenoids by ~ 53 and 86 %, respectively.

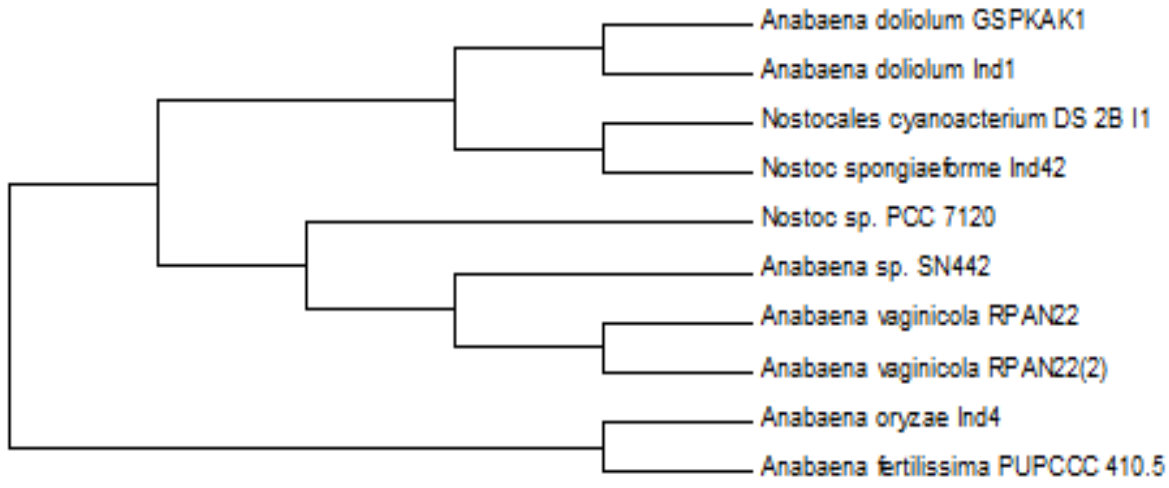

Fig. 1. Construction and analysis of phylogenetic tree for *Anabaena oryzae* Ind4

Table 1. Pigment concentration in Cu treated *Anabaena oryzae* Ind 4 cells within five days period of exposure. Cu treatment: 5, 10, 15 and 20 mg L^{-1}. All the values are expressed in mean ± SD; n = 3

		Chlorophyll *a* (µg mL^{-1})	Carotenoids (µg mL^{-1})	PC (µg mL^{-1})	APC (µg mL^{-1})	PE (µg mL^{-1})
Day 0	Initial	3.00 ± 0.052	0.77 ± 0.014	15.26 ± 0.24	11.35 ± 0.16	5.33 ± 0.038
Day 5	Control	5.13 ± 0.098	1.84 ± 0.022	34.79± 0.46	21.31 ± 0.30	9.26 ± 0.16
	Cu (5mg L^{-1})	4.87 ± 0.076	1.63 ± 0.022	28.32 ± 0.38	17.69 ± 0.22	9.14 ± 0.10
	Cu (10mg L^{-1})	2.53 ± 0.036	0.79 ± 0.016	21.56 ± 0.36	9.46 ± 0.20	4.83 ± 0.48
	Cu (15mg L^{-1})	1.74 ± 0.030	0.36 ± 0.010	18.11 ± 0.22	5.71 ± 0.074	3.38 ± 0.36
	Cu (20mg L^{-1})	0.48 ± 0.008	0.11 ± 0.004	8.54 ± 0.18	3.23 ± 0.052	1.24 0.026

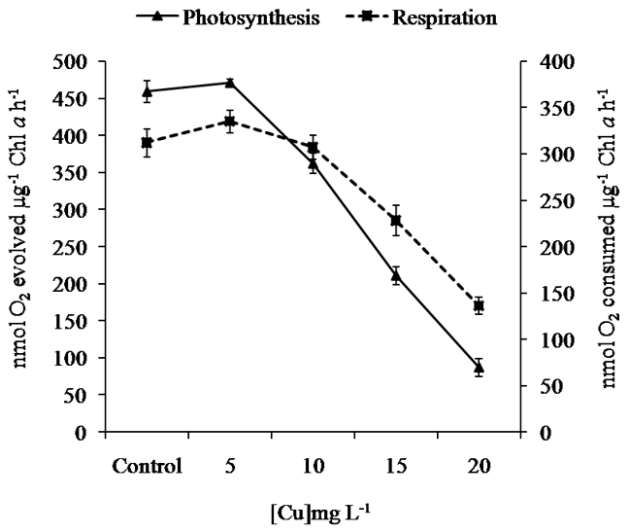

Fig. 2. Effect of different Cu concentrations (5, 10, 15 and 20 mg L^{-1}) on photosynthetic and respiratory electron transport activities of *Anabaena oryzae* Ind4. Control value on initial day was 326 (nmol O_2 evolved μg^{-1} chl *a* h^{-1}) for photosynthesis and 221 nmol O_2 consumed μg^{-1} chl *a* h^{-1} for respiration. Duration of exposure: 5 days. All the values are expressed in mean ± SD; n = 3

Least affected pigments were the accessory light harvesting complexes such as PC. PC concentrations increased by ~ 128, 86, 41 and 19% in the control and 5, 10 and 15 mg L^{-1} Cu treated cells respectively and declined by ~ 44% upon treatment in 20 mg L^{-1} Cu supplemented medium. APC levels increased by ~ 88 and 56% in control and 5 mg L^{-1} Cu-treated cells whereas their concentrations declined by ~ 17, 50 and 72% in 10, 15 and 20 mg L^{-1} Cu treated cells, respectively. PE levels increased almost by the same extent in control and 5 mg L^{-1} Cu treated cells (~ 73%). However, values drastically reduced by ~ 10, 36 and 77% upon exposure to 10, 15 and 20 mg L^{-1} Cu on day 5.

Energy Metabolism

There was an upsurge in both photosynthetic and respiratory electron transport chain activities at lower Cu exposure. Photosynthetic O_2 evolution was increased by ~ 44 and 11% on day 5 in 5 and 10 mg L^{-1} Cu treated cells as against ~ 41% increase in control cells. However, O_2 evolution was decreased by ~ 35 and 73% as Cu concentration in medium was from 15 and 20 mg L^{-1}, respectively. As was the case with photosynthetic electron transport chain activity, respiratory rates increased by ~ 12 and 30% and decreased by ~ 24 and 54% in cells treated with 5, 10, 15 and 20 mg L^{-1} Cu as against ~ 47% increase in the control cultures (**Fig. 2**).

Nitrogen Metabolism

The nitrogenase activity of the organism remain unchanged in cultures treated with 5 mg L^{-1} Cu for five days; however, nitrogenise activity progressively declined (~ 21 to 64%) as Cu concentration in the medium was increased from 10 to 20 mg L^{-1}.

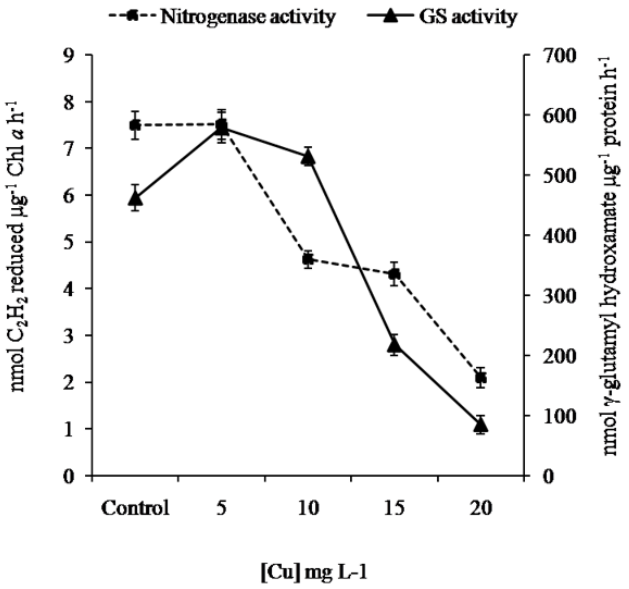

Fig. 3. Effects of Cu concentrations on nitrogenase and GS activity of *Anabaena oryzae* Ind4 at the end of five days. Control value on initial day was 6.2 (nmol of C_2H_2 reduced/µg of chl *a*/h) for nitrogenase activity and 257 nmol γ-glutamyl hydoxamate/µg protein/min for GS activity. All the values are expressed in mean ± SD; n = 3

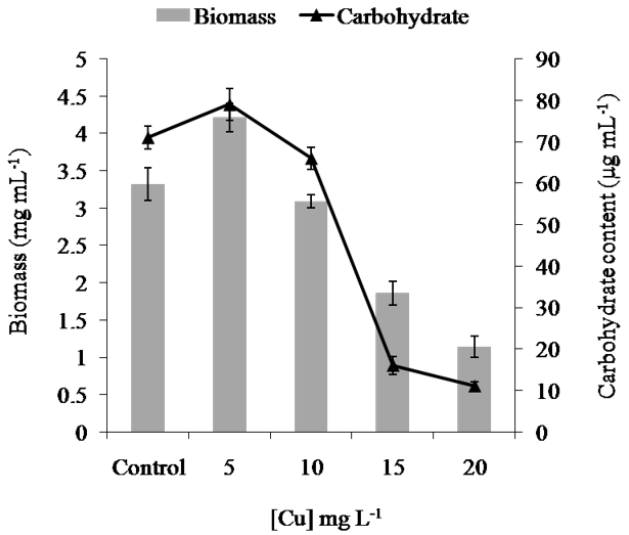

Fig. 4. Effect of Cu on biomass and carbohydrate content in the cyanobacterium *Anabaena oryzae* Ind4 within five days of exposure. Cu treatment: 5, 10, 15 and 20 mg L^{-1}. Control value on initial day was 2.1 mg mL^{-1} for biomass and 32.9 µg mL^{-1} for carbohydrate. All the values are expressed in mean ± SD; n = 3

GS activity recorded an increased by ~ 80 and 64% in 5 and 10 mg L^{-1} Cu treated cells on day 5 as against ~ 50% increased in the control. Any further increase in Cu concentration caused reduction in the GS activity (~ 33 and 74% in 15 and 20 mg L^{-1} Cu treated cells). Soluble protein content was enhanced by ~ 145 and 110% in cells exposed to 5 and 10 mg L^{-1} Cu as compared to 115% increased in the control cells. Presence of 15 and 20 mg L^{-1} Cu in the surrounding medium for 5 days resulted in ~ 15 and 55% drop in cell's soluble protein concentration (**Fig. 3**).

Carbon Metabolism

There was increase in biomass and carbohydrate at lower range of Cu treatment. Biomass increased by ~ 57 and 15% in 5 and 10 mg L^{-1} Cu treated cells as against an increase of ~ 24% in the control. However, biomass concentration was reduced by ~ 31 and 57% from the control value on day fifth in cells exposed to 15 and 20 mg L^{-1} Cu after 5 days.

Carbohydrate content increased by ~216 and 124 % in cells treated with 5 and 10 mg L^{-1} Cu respectively as against a control value of ~184%, whereas a decrease by ~36 and 56% from the control value of fifth day was observed upon 15 and 20 mg L^{-1} Cu exposure.

Morphological and Ultra-structural Changes

Scanning electron micrographs of untreated cells showed healthy cyanobacterial filaments that consisted of a large number of vegetative cells interspersed with heterocysts. Vegetative cells were smaller in size compared to heterocysts and all cells appeared turgid (**Fig. 5a**). 5 mg L^{-1} Cu concentration did not produce visible changes in the morphology after an incubation period of 5 days. Degree of stress (manifested as distortion in cell shape and size) increased with the increasing metal concentrations in the medium. Upon treatment with 15-20 mg L^{-1} Cu for 5 days, definite breaks in the cyanobacterial filaments at several places were observed in addition to the considerable elongation and shrivelling of individual cells (**Fig. 5c**; **d**).

Cross sections of untreated cyanobacterial cells under the transmission electron microscope showed healthy cells with intact thylakoid membranes and even cellular content (**Fig. 5e**). Cu at 5 mg L^{-1} did not cause produce noticeable changes in internal organization of the cells after a 5 day incubation period. Adverse effects became apparent at Cu concentrations above 5 mg L^{-1}. Gradual increase in degree of disintegration of the thylakoid membranes and appearance of intra-thylakoid spaces along with widening of the inter-membrane space were noticed in treatments starting from 10 mg L^{-1} Cu. Most prominent change upon Cu treatment was the appearance of a significant number of polyphosphate bodies within the cells. The size and number of polyphosphate bodies increased with the increase in Cu stress (**Fig. 5g**). Severe damage to all cellular components and complete disintegration of the inner membrane were observed in the cells treated with 20 mg L^{-1} Cu on day 5 (**Fig. 5h**).

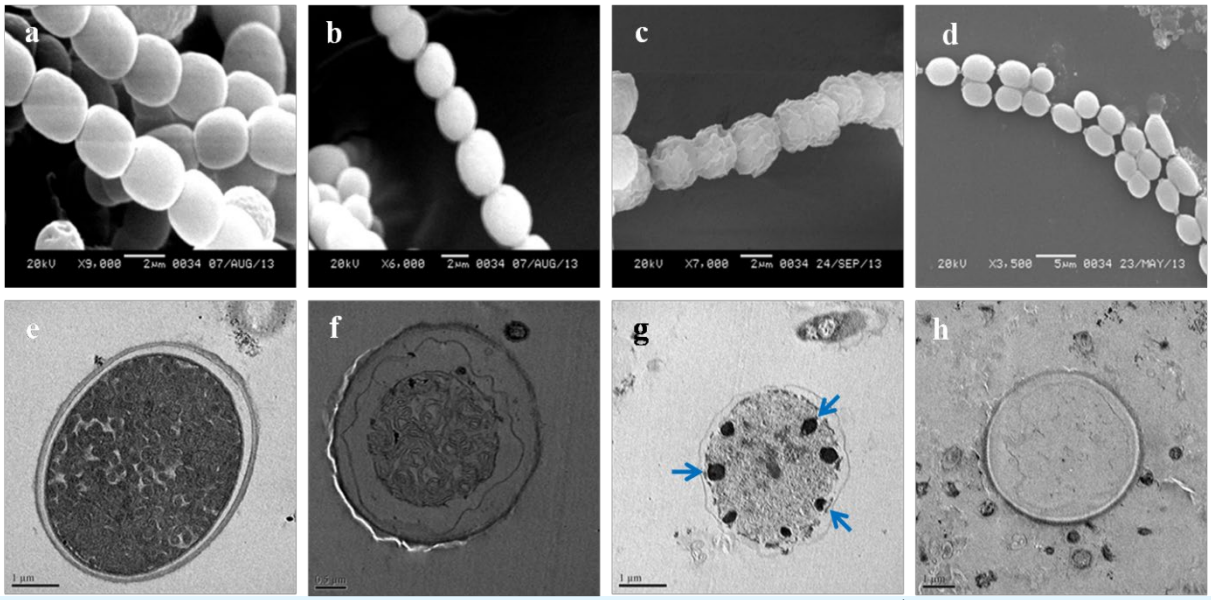

Fig. 5. Scanning electron micrographs (a) untreated cells, (b) cells treated with 10 mg L^{-1} Cu, (c) cells treated with 15 mg L^{-1} Cu (d) cells treated with 20 mg L^{-1} Cu. Transmission electron micrographs of (e) control cells, (f) cells treated with 10 mg L^{-1} Cu, (g) cells treated with 15 mg L^{-1} Cu (h) cells treated with 20 mg L^{-1} Cu. Arrows indicate polyphosphate bodies

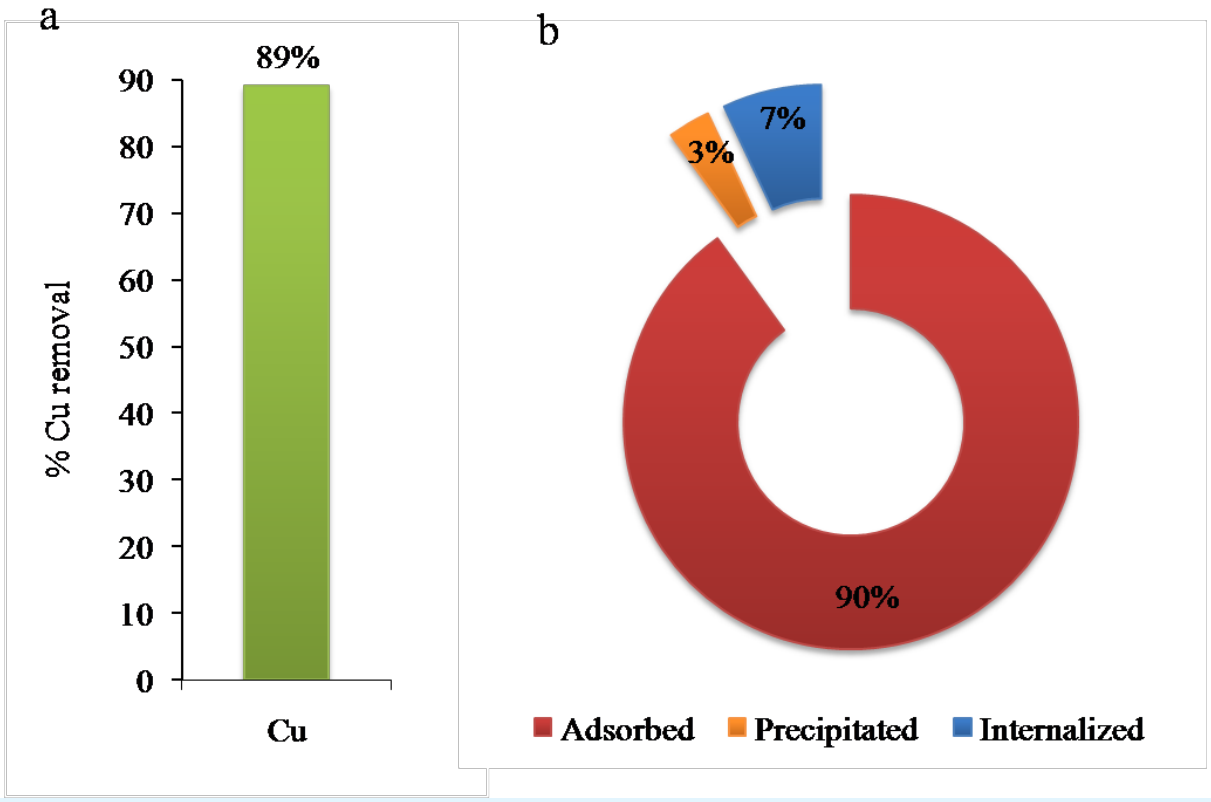

Fig. 6. Cu removal and its distribution on the cyanobacterial cell within 24 h are presented in the above figure. **a:** % Cu removal and **b**: cellular distribution of Cu

Cu Removal and Cellular Distribution

The organism was able to remove 89% (4.45 mg L^{-1}) of Cu from the medium supplemented with 5 mg L^{-1} Cu within 24 h (**Fig. 6a**). Of the total removed Cu, 90% of it was adsorbed on the cell surface, 3% was precipitated on cell surface and 7% was internalized.

DISCUSSION

Several references affirming the effect of metals on structure and physiology of cyanobacteria are available (Ahad et al. 2018, Dixit and Singh 2014, Micheletti et al. 2008, Prasad and Zeeshan 2005, Surosz and Palinska 2000, 2004, Singh et al. 2013). All these reports suggest that different cyanobacteria show different sensitivities to the same metal and on the other hand toxicity of different metals to the same cyanobacterial species may also vary (Fathi 2002). The present work attempted to study in detail the Cu induced changes in morphology, physiology and biochemistry of a cyanobacterial isolate from rice field environment where Cu in the form of $CuSO_4$ is added as general pesticide. Copper affected structure and physiology of the organism in a dose dependent manner which is in agreement with the findings of several other researchers (Ahad and Syiem 2018a, Arunakumara and Xuecheng 2009, Nongrum and Syiem 2012, Prasad and Zeeshan 2005, Surosz and Palinska 2000, 2004). Lower doses of Cu were stimulatory in most of the parameters studied. However increase in metal concentration in medium led to reduction in all vital activities of the organism. It has been reported that the response of an organism towards presence of metal ions in its surrounding involves a multi-faceted approach rather than a uni-directional one since cellular responses are a complex interplay of several inter-linked pathways crucial for the organism's survival (Fathi 2002, Gadd 2010). The effects seen on the physiological and biochemical parameters of the cyanobacterium *Anabaena oryzae* Ind4 under Cu stress were the net results of all factors working towards combating metal toxicity.

At 5 mg L^{-1} concentration, Cu induced most of the parameters studies. However, adverse effects of chronic exposure to Cu were visible beyond this concentration. Transmission electron micrographs showed noticeable changes in the internal organisation of cells exposed to 10 and 20 mg L^{-1} Cu. Both cytoplasmic and thylakoid membranes were adversely affected. There was disintegration of thylakoid membranes and appearance of intra-thylakoid spaces. These changes in cyanobacteria in response to metal toxicity have also been earlier reported by other researchers (Rangsayatorn et al. 2002, Surosz and Palinska 2000, 2004). Damage to thylakoid membranes may be the reason for loss in photosynthetic activity (**Fig. 2**) and reduction in pigment concentration (**Table 1**) since these membranes house all major components of the photosynthetic machinery. Disrupted electron transport chain may have aided in reduced photosynthetic oxygen evolution rates as observed during the study. This agrees with the findings of (De Vos et al. 1991, Surosz and Palinska 2004). It is a well established fact that plastoquinone, cytochrome b_6f complex and cytochrome c_6 in cyanobacteria are the three electron transport components commonly shared by both respiratory as well as photosynthetic electron transport chains. In addition, cellular respiration is known to occur both in the thylakoid as well as cytoplasmic membranes (Vermaas 2010); hence degradation of these membrane systems may have contributed towards reduction in the respiratory rates seen in the present study (**Fig. 2**).

Sinha et al. (2006) reported that energy status of the cells is severely compromised when photosynthetic and respiratory activities are affected as ATP and NADPH production are reduced. Our study also showed such negative effects upon Cu exposure on both photosynthetic and respiratory electron transport activities implying reduced energy status under Cu stress. This in turn is reflected in overall lowered metabolic functions such as reduction in nitrogenase and GS activities, in production of photosynthetic pigments, biosynthesis of carbohydrates and proteins and in total biomass production (**Fig. 2**; **3**; **4**). Biosorption and cellular distribution study (**Fig. 6**) showed that although Cu removal was mostly a surface phenomenon, 7% of the Cu bound on the cyanobacterial cells was internalized. Cu being a +2 metal ion (Cu^{2+}) thus can react with various biomolecules within the cells disrupting many biochemical functions and thereby bringing about imbalance in the cellular physiology that was reflected as decrease in production of various biomolecules and in reduced metabolic functions. Cells responded towards increase in the intercellular Cu concentration by increasing number of polyphosphate bodies (**Fig. 5g**). An observation earlier also recorded by Nagata et al. 2006. This was in order to sequester the excess Cu as their presence in high concentration in the cytoplasm of the organism leads to altered ionic balance in cells (Jensen et al. 1982, Keasling 1997, Surosz and Palinska 2004). Although under normal conditions, polyphosphate bodies serve as reserves for phosphate, increase in size and number of polyphosphate bodies are believed to be a common response by cyanobacteria to metal stress wherein the organism uses the excess phosphate to bind the positively charged metal ions and render them ineffective. The induction in most of the physiological parameters studied under the influence of lower dose of Cu (5 mg L^{-1}) could be due to a known phenomenon called hormetic effect where organisms respond to presence of lower doses of any harmful compounds/ threat by increasing their metabolic activities geared towards neutralizing the hazard.

CONCLUSION

The ability of cyanobacteria to fix atmospheric nitrogen and release nitrogenous compounds into the soil on their turn over makes them potent biofertilizer as this enhances soil fertility. In India, China, Vietnam, Thailand and Philippines, cyanobacteria are especially cultured and inoculated in paddy fields to increase crop

yield. Use of wastewater in irrigation of rice fields adjacent to industries is becoming a threat of bioaccumulation of toxic compounds in the rice grains as well as harming useful microbes in the fields. Copper sulphate is widely used as pesticide in crop cultivation and this study provided evidence that Cu is detrimental to beneficial microbes like cyanobacteria. Constant use of such chemicals over a period of time will destroy beneficial microbial population in agricultural lands compromising soil fertility. Thus, caution needs to be exercised while using chemical fertilizers/ pesticides in crop cultivation.

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