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Research Article

ZN TOLERANCE IN *ANABAENA VARIABILIS* MEGCH1: AN IN-DEPTH ANALYSIS OF CARBON AND NITROGEN ASSIMILATION; MORPHOLOGY AND ULTRASTRUCTURE

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ABSTRACT. The present work was conducted to check the effects of Zn exposure (10 - 100 µM) in the cyanobacterium *Anabaena variabilis* MEGCH1 over a period of seven days. The Zn concentration, even the lowest limit considered for the study, was substantially higher than that is generally found in coal mine contaminated wastewater. Most biochemical parameters in the organism showed marked tolerance towards Zn exposure up to a concentration of ~ 30 µM, although, in the presence of a higher Zn concentration, the organism showed substantial changes in its biomass, morphology, and ultrastructure, indicating the toxic nature of chronic Zn exposure. Similar toxicity was also evident in the entire C-fixation machinery, including the photosynthetic pigments, rate of photosynthetic and respiratory electron transport chain activities, and total carbohydrate content. There were negative impacts recorded on the heterocysts' frequency as well as on nitrogenase and glutamine synthetase enzyme activities that resulted in poor nitrogen fixation and assimilation. Consequently, the level of soluble protein content within the cells was also reduced. These adverse effects were reflected in an obvious decrease in total biomass production. The increase in the total proline content of the treated culture clearly indicated that the organism was under obvious stress under Zn exposure. The cyanobacterium's survival and performance, however, in the presence of significant Zn ions in its surroundings, indicated that the organism could be considered for bioremediation technologies.

Keywords: *Anabaena variabilis MEGCH1, zinc toxicity, carbon and nitrogen fixation and assimilation, SEM, TEM study.*

INTRODUCTION

One of the most significant issues in modern human society is environmental contamination [1]. The release of effluents from fast-growing industrial and mining activities, tanneries, dye and pigment factories, excess application of chemical fertilizers and pesticides, etc., is gradually polluting soil and water bodies with various contaminants [2]. In recent times, fast industrialization and urbanization have led to an increase in heavy metal contamination of the environment polluting the aquatic and terrestrial ecosystems [3]. Many heavy metals such as copper, nickel, and zinc are essential for life as they are involved in various metabolic processes in living cells. These are generally referred to as microelements or trace elements [4]. However, with an increase in their concentration in the environment, there is a consequent increase in the internalization of these metal ions

in the organisms such as microbes and plants that are directly in contact with the environment. Consequently, internalized metal ions undergo undesirable redox processes and/or bind incorrectly to the metal-binding sites of numerous enzymes, resulting in increasing ROS content and producing harmful effects. As a result, metal uptake in cells must be precisely balanced, and most organisms have developed strategies to respond to metal bioavailability by maintaining metal homeostasis [5]. Metal homeostasis is crucial in metal-polluted environments, protecting cells from metal overload, and maintaining vital functions including respiration, carbon metabolism, and photosynthesis [6]. The accumulation of heavy metals not only affects aquatic plants and aquatic animals but also has a significant impact on primary producers such as cyanobacteria. The compromised population number and health of such primary producers are reflected in the entire ecosystem *via* the food chain [7, 8].

Cyanobacteria are the oldest photosynthetic, gram-negative prokaryotes having the capacity to withstand diverse environmental conditions such as extreme heat, cold as well as heavy metal-contaminated surroundings [9]. Since they can endure high metal concentrations, they are suitable biological metal sorbents [10]. However, the effects of heavy metal exposure are not similar in all cyanobacteria, and different metal exposure results in a varied response in different cyanobacteria [5, 11] Studies on the effects of several heavy metals on various biological functions of different cyanobacteria have been conducted over the years in an attempt to generate data on the suitability of cyanobacterial strains for heavy metal bioremediation. Metals such as Fe, Zn, Cu, Mn, Mo, Ni, and Co play vital roles as cofactors to many enzymes and as structural components of proteins [12]. For instance, Fe, Cu, and Zn are present in superoxide dismutases, an enzyme that scavenges free radicals [13,14]. Zinc further aids various physiological functions in cyanobacteria where it helps to maintain protein structure and assists in CO_2 transfer and fixation in the enzyme carbonic anhydrase (CA). Additionally, zinc associates with the enzyme alkaline phosphatase (an enzyme that obtains phosphorus from organic phosphate esters) and is one of the components of zinc finger proteins needed for DNA transcription [15].

Although zinc is necessary for optimal growth, high zinc concentrations cause severe toxicity affecting the cyanobacteria's physiological and biochemical parameters such as photosynthesis and other essential cellular processes. Zn accumulation inside the cells not only affects the proteins, DNA, and lipids but also exhibits adverse effects on the cell number, ultrastructure, and morphology of the organism [16, 17]. The organism selected for the present study was isolated from a coal mining site where the effluent water contained a large number of metal ions in varying concentrations.

The physiological endurance of the organism to increased zinc concentration as well as changes in morphological, ultrastructural, physiological, and biochemical parameters were investigated to ascertain if this organism can be put forward as a zinc bioremediating agent in polluted environments. The organism's carbon and nitrogen fixation and assimilation and proline synthesis were also assessed to determine how zinc stress affects its growth.

MATERIALS AND METHODS

Culture condition of the organism

The cyanobacterium used in the present study was isolated from Chiehruphi, a coal mining site located in the East Jaintia Hills District of Meghalaya, India and identified as *Anabaena variabilis* MEGCH1 (GenBank accession No: **MF589225**). The isolate was grown in BG-11_0 media at pH 7.5, and maintained in the culture room under continuous light (photon fluence rate of 50 µmol m^{-2} s^{-1}), and at a temperature of 25±2 °C [18].

Zinc treatments

Zn^{2+} (1 mM) stock solution was prepared using zinc sulfate ($ZnSO_4.7H_2O$). A 10 - 100 µM concentration was made by diluting the stock solution appropriately.

Estimation of the photosynthetic pigments

Chlorophyll a concentration

The chlorophyll *a* content was measured according to Mackinney, [19]. Cell cultures were centrifuged at 2500 rpm for 3 min. 3 mL of methanol was added to the pellet and kept at 4°C overnight for chlorophyll *a* extraction. Next, it was again centrifuged at 2500 rpm for 3 min. Absorbance measurements were made at 663 nm spectrophotometrically against a methanol blank. Chlorophyll *a* concentration was calculated using the following formula and expressed as µg chlorophyll *a* μg^{-1} biomass.

$$\text{Chlorophyll } a = \text{OD}_{663} \times 12.63$$

Phycobiliproteins content

The contents of phycobiliproteins (phycocyanin [PC], allophycocyanin [APC], and phycoerythrin [PE]) were measured according to Bennett and Bogorad, [20]. After centrifugation of cell culture at 2500 rpm for 3 min, the pellet was washed twice with phosphate buffer saline and resuspended in the same buffer. The cells were then sonicated and centrifuged at 18000 g for 45 min. The supernatants were measured at optical densities 615, 562, and 652 nm using phosphate buffer saline as blank. PC, APC, and PE were calculated using the following formula and expressed as µg phycobiliproteins μg^{-1} biomass.

$$\text{PC} = \frac{\text{OD}_{615} \times (0.474 \times \text{OD}_{615})}{5.34}$$

$$\text{APC} = \frac{\text{OD}_{652} - (0.208 \times \text{OD}_{615})}{5.09}$$

$$\text{PE} = \frac{\text{OD}_{562} - 0.241[\text{PC}] - 0.849[\text{APC}]}{9.62}$$

Photosynthetic and respiratory electron transport chain (ETC) activities

Photosynthetic and respiratory ETC activity was assessed using a Clark-type oxygen electrode fitted in a 3 mL Plexiglass container with a magnetic stirrer (Rank Brothers, England) as described by Robinson et al. [21]. After the cell culture was added to the sample chamber of the non-polarized electrode, the sample was kept to equilibrate for 3 min with constant stirring. After that, the electrode was polarised. A linear rate of oxygen evolution was obtained under the illumination of a 100 W tungsten filament bulb, which was separated from the sample chamber by a water bath that functioned as a heat filter. The chamber was covered in aluminum foil as oxygen consumption was measured in the dark. The rates of oxygen evolution and consumption were calculated and expressed as nmol O_2 evolved or consumed per min μg^{-1} of biomass, respectively.

Estimation of carbohydrate content

The carbohydrate content was estimated according to the method described by Roe, [22] using glucose as a standard. After centrifugation of the cell culture at 3000 rpm for 3 min, the pellet was resuspended in distilled water. The mixture was then sonicated and centrifuged again at 3000 rpm for 5 min. The supernatant was used for carbohydrate estimation. Next, 4 mL of anthrone reagent (0.2 % in concentrated sulphuric acid) was added to 1 mL of the supernatant and mixed properly. After incubation in a boiling water bath for 10 min, the solution was centrifuged again at 3000 rpm for 5 min. The absorbance of the supernatant was read at 630 nm using a UV-Vis Spectrophotometer.

Heterocyst frequency and nitrogenase activity

Heterocyst frequency was calculated according to Wolk, [23] and expressed as a percentage of total cells. At least 500 cells were counted for each calculation.

The organism's nitrogenase activity was measured using the acetylene reduction assay method described by Stewart et al. [24]. Acetylene gas was introduced into 10 mL vials containing 5 mL of cyanobacterial culture at a final concentration of 10 % (v/v) air phase and incubated for one hour at 25±2 °C in light at a photon fluence rate of 50 μmol m^2 s^{-1} with constant shaking. The amount of acetylene reduction was estimated using a gas chromatograph (Varian 3900, The Netherlands) equipped with a Porapak T column (stainless steel column 6' x 1/8", packed with a Porapak T of mesh size 80/100) and a flame ionization detector. Nitrogenase activity was expressed as nmol C_2H_2 reduced μg^{-1} biomass h^{-1}.

Glutamine synthetase (transferase) activity

The glutamine synthetase (transferase) activity (GS activity) was evaluated by measuring the amount of γ-glutamyl hydroxamate generated, as described by Sampaio et al. [25]. Cell cultures were centrifuged at 3000 rpm, for 3 min. The pellet was washed in 50 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer. After sonication, 0.5 mL of an enzyme extract was added to an assay mixture (40 mM Tris-HCl buffer (pH 7.5), 3 μmol $MnCl_2$, 20 μmol potassium arsenate, 0.4 μmol ADP (sodium salt), 60 μmol hydroxylamine and 30 μmol glutamine) and incubated in the dark for 10 min at 30 °C. Next, the reaction was terminated by adding of 2 ml stop mixture (containing 10 % $FeCl_3$, 24 % TCA, and 6 N HCl in distilled water). The mixture was then centrifuged (2500 rpm, 5 min) and the absorbance of the supernatant was read at 540 nm. The amount of γ-

glutamyl hydroxamate produced was calculated using a standard curve. The activity of glutamine synthetase was expressed as nmol γ-glutamyl hydroxamate formed μg^{-1} protein min^{-1}.

Protein estimation

After centrifugation of the experimental culture at 3000 rpm for 3 min, the pellet was resuspended in distilled water and homogenized. The following reagents were used for protein estimation: (A) 2 % Na_2CO_3 in 0.1 N NaOH; (B) 1 % Sodium Potassium tartrate solution; (C) 0.5 % Cu_2SO_4 solution (D) Alkaline mixture – Solution A, B, and C in the ratio 98:1:1 (E) 1N Folin-Ciocalteu's phenol reagent. The homogenized culture was centrifuged at 2500 rpm for 3 min and 0.5 mL of the supernatant was mixed with 0.5 mL of distilled water, 5 mL of the alkaline mixture, and vortexed. Next, 0.5 mL of 1N Folin-phenol Ciocalteu's reagent was added to this mixture, thoroughly mixed, and incubated at room temperature for 20 min. Absorbance was read at 750 nm spectrophotometrically, and the protein concentration was calculated using a standard graph [26].

Proline estimation

The proline content was measured according to the method described by Bates et al. [27]. After centrifugation of the cell culture (3000 rpm, 5 min), the pellet was resuspended in 5 mL of 3 % sulphosalicylic acid and then sonicated. After the mixture was filtered through Whatman filter paper, 2 mL of acid ninhydrin reagent (90 mL glacial acetic acid + 24 mL orthophosphoric acid + 36 mL distilled water) and 2 mL of glacial acetic acid were added to the filtrate (2 mL) and incubated at 100°C for 1h. The reaction was terminated following the incubation period by placing the tubes in an ice bath. 4 mL of toluene was added and mixed vigorously to extract proline. Using a UV-visible spectrophotometer, the absorbance of the upper pink layer formed in each tube was measured at 520 nm. The concentration of proline was determined by calculating it against a standard curve made using a proline solution (25 - 250 M).

Scanning Electron Microscopy (SEM)

Control and Zn-treated cultures were prepared for SEM viewing by fixing the samples in 4 % glutaraldehyde for 4 h, followed by washing thrice in 0.1 M sodium cacodylate buffer for 15 min each at 4 °C. Dehydration of the samples was done with increasing concentrations of acetone (20 %, 40 %, 60 %, 80 %, 85 %, 90 %, 95 %, and 100 %) at 4 °C. Next, the samples were mounted on brass stubs and gold-coated, followed by viewing under a scanning electron microscope (JSM, 6300, JEOL, Tokyo, Japan).

Transmission Electron Microscopy (TEM)

Cyanobacterial cells were treated with 4 % glutaraldehyde at 4 °C for 24 h. The cells were then washed in sodium cacodylate (0.1 M) buffer three times each with 15 min intervals. Osmium tetraoxide (2 %) prepared in sodium cacodylate (0.2 M) buffer was added to the cells for post-fixation and further washed thrice in the same buffer for 15 min each. Next, the samples were dehydrated in acetone (10 - 90 %) at 4 °C, followed by clearing with propylene oxide for 30 min at room temperature, and then transferred to embedding molds. The embedding medium, Araldite CY212, was used (BEEM Inc., West Chester, PA, USA), and the molds were then incubated at 50 °C for 24 h, and the

temperature was further raised to 60 °C (48 h) for polymerization. Thin sections (60 - 90 nm) of the sample were cut using ultramicrotome MTX (Boeckeler Instruments, Tucson, AZ, USA). Following this, uranyl acetate and lead citrate were used to stain the samples before viewing them under a JEM-2100, 120 kV Transmission Electron Microscope (JEOL, Tokyo, Japan).

Statistical analyses

All the values were calculated in means ± standard deviation (SD). GraphPad Prism 5 software was used to perform the one-way analysis of variance (ANOVA) to check the significant difference between control and different treatments (Dunnett test).

RESULTS AND DISCUSSION

Effect of Zn treatment on CO_2 assimilation

Chlorophyll a and its accessory pigments

In the chronic presence of Zn (10-100 µM) in the growth medium for seven days, all concentrations studied exerted a negative impact on chlorophyll *a* production. The least reduction in the chlorophyll *a* content was 64 % at 10 µM, while a 100 µM exposure resulted in 93 % reduction (Fig. 1 a). A somewhat different result was obtained for the various phycobiliproteins (PC, PE, and APC) studied (Fig. 1 b). The PE content showed a significant increase of 148 % at 20 µM to 72 % at 60 µM Zn exposure from the control value, which dropped down drastically by 87.5 % when the cells were exposed to 100 µM Zn for seven days. Similarly, the PC content recorded an 11 % increase at 10 µM Zn exposure, but a subsequent increase in the concentration of Zn led to an increased reduction in PC content as the concentration of Zn was increased from 20 to 100 µM (9 %, 74.2 % and 95.8 % at 20, 60 and 100 µM). The most sensitive of all the accessory pigments was APC. It registered a decrease in its content in all the Zn concentrations studied (16.4 %, 81.8 % and 82.5 % at 20, 60 and 100 µM).

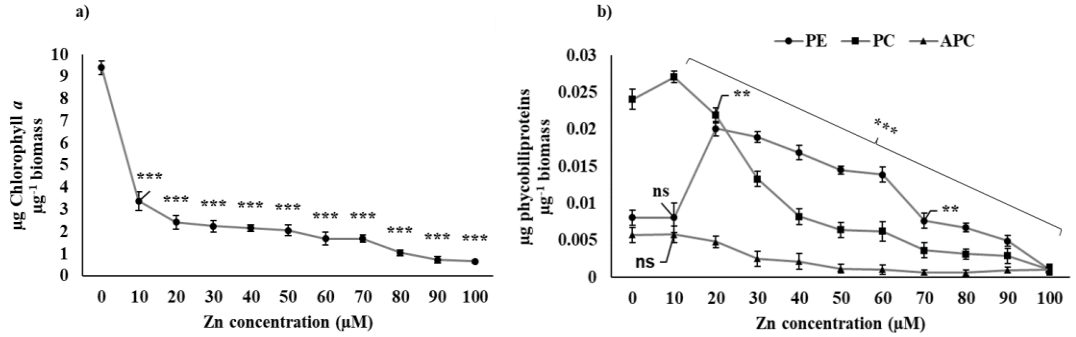


Fig. 1. *Effect of Zn treatment on (a) Chlorophyll a and (b) Phycobiliproteins (PC, APC & PE). All the values are in Mean±SD (N=3) and asterisks (*p<0.05, ** p<0.01 and *** p<0.001) above the histogram bars specify significance in differences between control and Zn^{2+} treated cells*

Assessment of photosynthetic and respiratory electron transport chain activities and estimation of carbohydrate content

The rate of photosynthetic electron transport chain activity measured in terms of O_2 evolution registered a 26 % increase in the presence of 10 µM Zn and declined from 30 µM exposure onwards, whereas compared to control, a reduction of 38 % at 60 µM and 68 % at 100 µM was observed (Fig. 2a). In contrast, the rate of catabolic activities of the culture represented by respiratory electron transport chain activity was evaluated in terms of O_2 consumption. This showed an increase up to 60 µM, with the highest increase of 45 % recorded at 40 µM. However, a reduction of 49 % from the control value at 100 µM exposure signified the toxic nature of Zn at high concentrations (Fig. 2 a). The estimation of cellular glucose concentration that is indicative of Calvin cycle activity indicated that Zn in all concentrations studied had a negative effect on this parameter (Fig. 2 b). There was already a considerable reduction of 23 % in carbohydrate content at 10 µM Zn exposure which became significant at 45 % in the presence of 60 µM and 53 % at 100 µM.

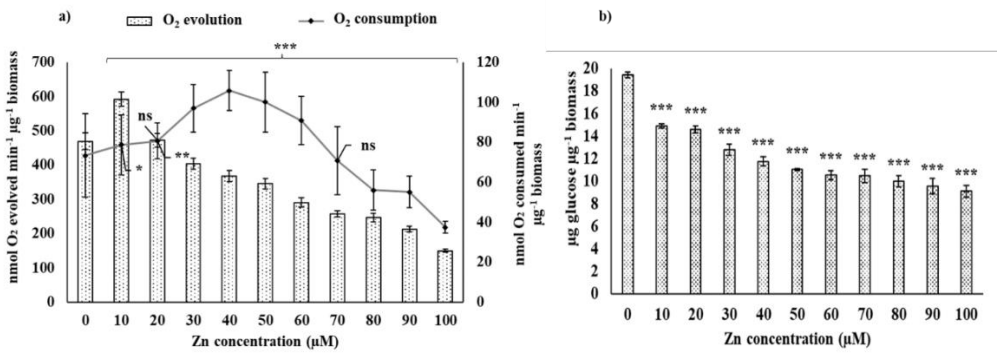


Fig. 2. *Effect of Zn treatment on (a) rates of photosynthetic and respiratory ETC activities as a function of O_2 evolution and consumption, respectively, and (b) carbohydrate content in Anabaena variabilis MEGCH1. All the values are in Mean±SD (N=3) and asterisks (*p<0.05, ** p<0.01 and *** p<0.001) above the histogram bars specify significance in differences between control and Zn^{2+} treated cells*

Effects of Zn treatment on N_2-fixation and assimilation

Heterocyst frequency, nitrogenase and glutamine synthetase (GS) activities, and total protein content

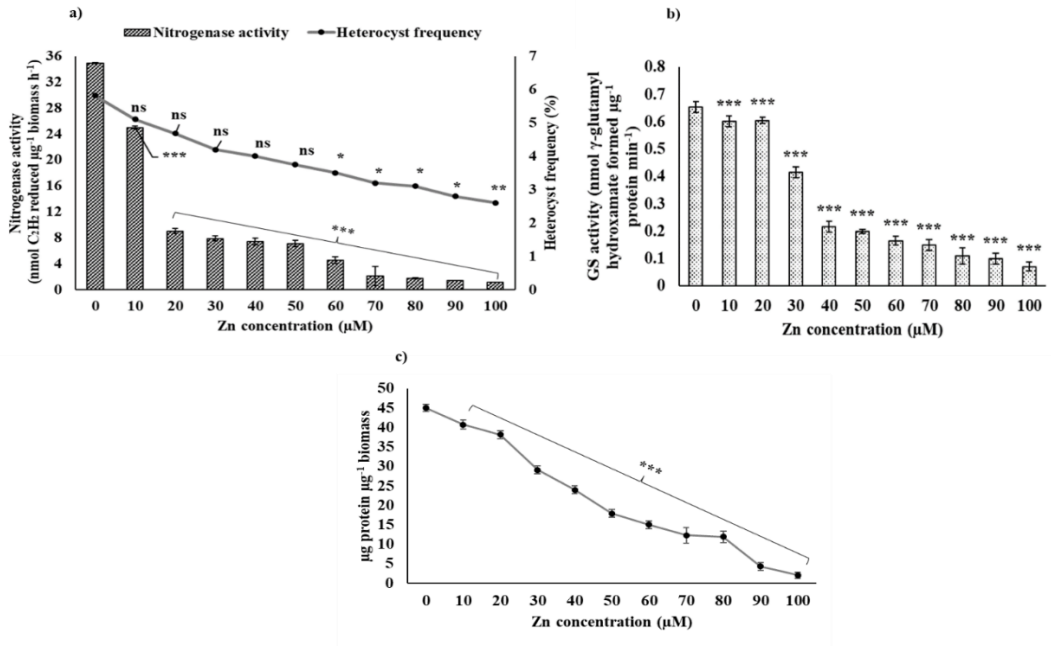


Fig. 3. *Effect of Zn treatment on (a) nitrogenase activity and heterocyst frequency (b) glutamine synthetase activity and (c) total protein content in Anabaena variabilis MEGCH1. All the values are in Mean±SD (N=3) and asterisks (*p<0.05, ** p<0.01 and *** p<0.001) above the histogram bars specify significance in differences between control and Zn^{2+} treated cells*

Exposure to variable doses of Zn showed a gradual decrease of 12 % at 10 µM to 55 % at 100 µM in the heterocyst frequency of the *Anabaena variabilis* MEGCH1 cells compared to control (Fig. 3 a). In contrast, the effect on the activity of the nitrogenase enzyme was more pronounced. A reduction of 28 %, 74 %, and 87 % were noted in the enzyme activity when the growth media were supplemented with 10 µM, 20 µM, and 60 µM Zn, respectively. Exposure to higher concentrations (70 - 100 µM) resulted in a severe drop in nitrogenase activity. The enzyme was almost shut down with a reduction of 94 % at 70 µM, 95 % at 80 µM, 96 % at 90 µM, and 97 % at 100 µM Zn exposure (Fig. 3 a). Fig. 3 b, shows the effect of Zn supplementation on the enzyme glutamine synthetase. Its activity decreased by 8 % at 10 µM and 75 % at 60 µM. However, an 11 % activity was still present in the presence of 100 µM Zn. A look at the total protein content showed a gradual decline of 9.5 % at 10 µM and 66.6 % at 60 µM. Exposure to 100 µM Zn resulted in an almost 95 % decrease in total protein content (Fig. 3 c).

Proline synthesis in response to Zn stress by Anabaena variabilis MEGCH1

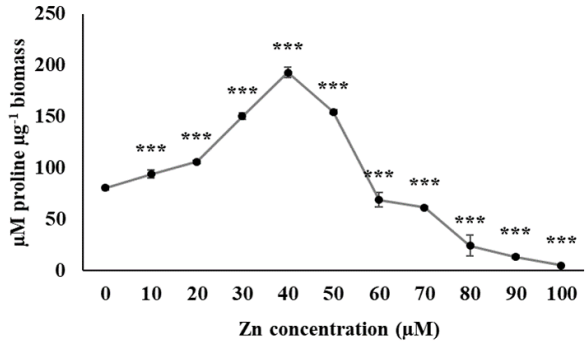


Fig. 4. *Effect of Zn stress on the proline content in Anabaena variabilis MEGCH1. All the values are in Mean±SD (N=3) and asterisks (*p<0.05, ** p<0.01 and *** p<0.001) above the histogram bars specify significance in differences between control and Zn^{2+} treated cells*

There was an increase in proline content with increasing Zn concentration in the growth medium up to 50 µM (25 % at 10 µM, 117 % at 20 µM, 85 % at 30 µM, 84 % at 40 µM, and 40 % at 50 µM). As concentration was increased beyond 50 µM, there was a decrease in total proline content from 30 % at 60 µM to 96 % at 100 µM (Fig. 4).

Zn induced morphological and ultrastructural changes in Anabaena variabilis MEGCH1

Analysis of morphological changes by Scanning Electron Microscopy

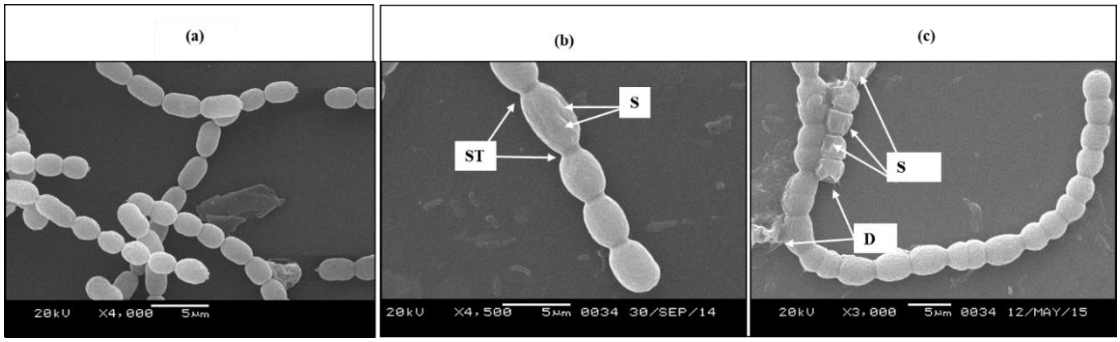


Fig. 5. *Scanning electron micrographs of (a) control Anabaena variabilis MEGCH1 cells, (b)10 µM Zn-treated Anabaena variabilis MEGCH1 cells, and (c) 100 µM Zn-treated Anabaena variabilis MEGCH1 cells (ST: Stretched, D: Disintegration, S: shriveling on the surface of the Zn treated Anabaena variabilis MEGCH1)*

Fig. 5a, shows healthy cyanobacterial cells under SEM. When cells were exposed to 10 µM Zn for seven days obvious stress was noticeable on the filament structure and on the individual cells. The connections between the cells were stretched, and shriveling of the individual cells due to osmotic stress was clearly visible (Fig. 5b). A 100 µM Zn

treatment led to prominent changes where many cells showed shriveling, and the smooth, rounded appearance of the cells within the filaments disappeared. The disintegration of some cells was also noticed (Fig. 5 c).

Ultrastructural studies of Anabaena variabilis MEGCH1 upon Zn treatment

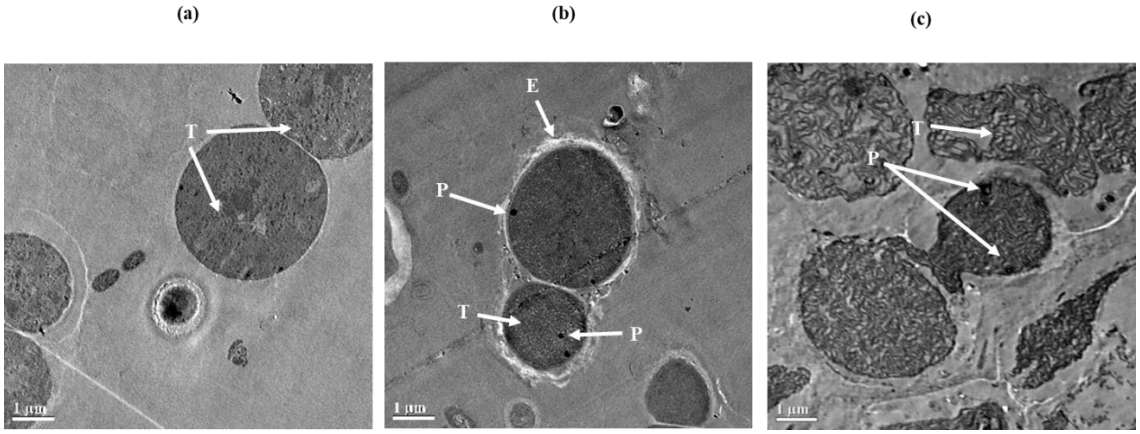


Fig. 6. *Transmission electron micrographs of (a) control Anabaena variabilis MEGCH1 cells, (b) 10 µM Zn treated Anabaena variabilis MEGCH1 cells, and (c) 100 µM Zn treated Anabaena variabilis MEGCH1 cells. (P: polyphosphate granules; T: thylakoids; E: exopolysaccharide layer)*

Changes in the ultrastructure of the organism were studied using TEM. Fig. 6a, shows the ultrastructure of healthy control cells. Under 10 µM Zn exposure, ultrastructural changes were negligible as the outer cell membrane and the thylakoids remained intact. However, there was a clear indication of exopolysaccharide cover around the cells. Polyphosphate granules also appeared in 10 µM Zn-treated cells (Fig. 6 b). Exposure to 100 µM Zn, the filament integrity was severely impacted, the cell membrane was disintegrated and thylakoid membrane distortion was highly visible (Fig. 6 c).

In cyanobacteria, many metals play essential roles in all stages of metabolism. In photosynthetic electron transport reactions taking place in the thylakoid membranes, various metal ions such as iron, copper, manganese, and magnesium act as cofactors for numerous proteins and pigment-protein complexes. Similarly, zinc plays a crucial role during photosynthesis, where it helps in CO_2 transfer and fixation in the enzyme carbonic anhydrase. The purpose of Zn^{2+} in the CA catalytic mechanism is to facilitate deprotonation of H_2O by generating the nucleophilic OH^-, which can then attack CO_2's carbonyl group to convert it to HCO_3 [28]. Although essential in various biochemical processes, metal ions can be potentially toxic in high amounts. Thus, the concentrations of various metal ions in living organisms are maintained within specific ranges by carefully adjusting the metal homeostasis [29, 30, 31].

In the present study, changes in the biochemical and physiological parameters in a cyanobacterium *Anabaena variabilis* MEGCH1 were analyzed under Zn exposure. As the organism was found thriving in a coal mining effluent water, it was of academic interest to study how Zn, which was one of the metal ions found in the effluent water (~13.9 µM), impacted the organism as well as to what extent the organism could tolerate

Zn in its surrounding. The WHO permissible limit for Zn in the surface water is 5 mg/L (which is equivalent to ~76.4 μM) [32, 33]. Therefore, for this study, the organism was exposed to much higher Zn concentrations (10 – 100 μM) in order to assess the organism's viability as well as changes brought about by such exposure with the idea that the organism might be a good candidate for bioremediation of heavy metal ions from wastewater. When the *Anabaena variabilis* MEGCH1 culture was treated with increasing zinc concentrations, the chlorophyll *a* and other photosynthetic light-harvesting pigments (PC, APC, PE) showed a substantial reduction in their content compared to control. Yet, even at 100 μM Zn exposure, the organism still retained all these pigments to a certain level (for e.g., 7 % of chlorophyll *a*, 12 % of PE, 4 % of PC, and 18 % of APC). A point to note is that such chronic exposure to high Zn concentrations had led not only to a reduction in the synthesis of the pigments, but it was also evident that Zn initiated the breakdown of existing pigment molecules as their amounts were lesser than what was estimated for the control cultures. Nevertheless, the presence of all pigments in the exposed cultures, even at 100 μM Zn, firmly established that the organism was highly resilient and was capable of bouncing back once such treatment was withdrawn. In line with the photosynthetic pigment status, 32 % of photosynthetic ETC activity and 51 % of respiratory ETC activity were still functional in the organism after seven days of 100 μM Zn exposure. Further, the organism could retain 47 % of the total carbohydrate content seen under the control condition. These observations signified that the organism was not fully compromised.

Among the various parameters of nitrogen fixation and assimilation studied, both nitrogenase and total protein content were severely affected. There was only ~ 5% nitrogenase activity left in the cultures exposed to 100 μM Zn for seven days. The protein content was also reduced by ~ 95 %. However, one must emphasize that in natural conditions, most soils contain different forms of fixed nitrogen that the organism can take up and use as sources for the synthesis of protein and other nitrogen-containing molecules. The nitrogen assimilating enzyme GS was still functional at ~ 11 % under 100 μM Zn treatment, and therefore, any fixed nitrogen taken up could be assimilated to different nitrogen-containing biomolecules in the organism. All such reductions seen in various parameters of carbon and nitrogen fixation could additionally be the result of an increase in intracellular Zn accumulation that hampered uptake of other metal ions and nutrients. As pointed out earlier, cyanobacteria require vital elements such as magnesium, potassium, calcium, phosphorus, iron, and copper for optimal functioning and metabolic activities. Thus, reduced uptake of these elements has a negative impact on cyanobacterial growth [34].

Despite the obvious adverse effects, the organism's remarkable tolerance to Zn exposure indicated that the organism by virtue of being present in mine effluent water, had developed strategies to endure high concentrations of metal ions such as Zn in its surrounding. These include secretion of an exopolysaccharide layer surrounding the cells that acted as a barrier to Zn ions and synthesis of polyphosphate bodies that are implicated in the internal sequestration of metal ions so that these are not available to disrupt various physiological and biochemical processes [35, 36]. Both the exopolysaccharide layer and the increased number of polyphosphate bodies were visible under the transmission electron microscopy study.

Although many parameters studied showed an immediate decrease upon Zn exposure, there was a noticeable increase of some parameters in the cultures exposed to lower concentrations of Zn. For example, PC was increased by 11 % at 10 μM Zn exposure.

The increase in PE was even more drastic. A 148 % increase in its content was recorded in the presence of 20 μM Zn from its value in the control culture. The upward trend remained up to an exposure to 60 μM Zn (72 % ↑). This phenomenon could be attributed to the already proven antioxidant capabilities of pigments such as phycobiliproteins [37] that were enhanced in response to increased oxidants produced during Zn exposure [38]. A similar upward surge was also noted in the activity of photosynthetic ETC, implying that a hormetic effect may have played a role where the organism responded to an incoming threat at lower Zn exposure by enhancing various biochemical activities [11, 17, 39]. The reduction in photosynthetic pigments seen under high Zn exposure could be due to the breakdown of already existing pigment molecules as well as due to low amino acid production as amino acids are the basic components of phycobiliproteins [40]. As seen in this study, the frequency of heterocysts (that host the nitrogenase enzyme), the nitrogenase and GS activities of the organism raised in a nitrogen-free medium ($BG11_0$) were significantly compromised (~ 55 %, ~ 95 % and ~ 89 % respectively) and therefore fixed nitrogen was highly restricted for any amino acid synthesis [16]. Again, any ammonia assimilation in the organism was further compromised as increased Zn translocation from the medium into the cells may have hampered uptake of other divalent ions such as Mg, Mn which are cofactors of GS, and surplus intracellular zinc may have overtaken the Mg or Mn ions (because of their low intracellular quantities) in binding to the thiol groups (S-H) of the enzyme, causing the electron transfer pathway to be disrupted and resulting in lower GS activity [16]. Consequently, this has led to highly reduced protein production (95 %↓) in the organism (Fig.3 c). The decrease in the photosynthetic pigments may have further added to reduced photon capture for photosynthesis, thus reducing the entire photosynthetic process that was reflected in lowered carbohydrate synthesis (Fig. 2 b) [41, 42]. The increase in the rate of respiration under treatment was observed up to 60 μM Zn exposure. This may be because the organism tried to raise the level of proton motive force (PMF) for enhanced ATP production needed to counter derogatory effects of Zn exposure by increasing electron flow from catabolic metabolism through the respiratory ETC. However, both photosynthetic and respiratory ETCs were compromised at higher Zn exposure. Reduction in the respiratory activity under high concentrations of Zn could further be due to the fact that Zn strongly inhibits complex I (NADH: ubiquinone oxidoreductase) by inhibiting either proton transfer to bound quinone or proton translocation [17, 43]. One of the stress-related changes in the metabolism is the synthesis and accumulation of the amino acid proline in plants and microbes [41, 44]. An increased proline content could be due to a boost in proline synthesis from glutamate, slowed down proline oxidation, and/or inability to incorporate proline into proteins [45]. Thus, an increase in proline content is indicative of stress experienced by an organism. A step-wise increase (25 % at 10 μM, 117 % at 20 μM, 85 % at 30 μM, 84 % at 40 μM, and 40 % at 50 μM of Zn treatment) indicated that the organism definitely experienced stress under chronic Zn exposure and synthesized proline in much higher concentration in response to the stress. A decrease in the proline content with increasing Zn concentration beyond 50 μM showed that the organism treated for 7 days in higher Zn concentrations was so compromised that it was unable to perform its repair activities (Fig. 4).

A look into the morphology through a scanning electron microscope exposed vital changes that occurred in the filament integration, structure as well as surface morphology upon Zn treatment. 10 μM Zn exposure led to minor changes in the organism in the form of cell elongation, stretching of the connection between two cells as well as shrinkage due

to osmotic stress throughout the entire cyanobacterial filaments (Fig. 5). Ultrastructural studies under TEM at the same concentration (10 µM) showed a significant presence of exopolysaccharide (EPS) layer surroundings the cells and an increased number of polyphosphate bodies that signified that the organism was experiencing the toxic presence of Zn in its vicinity and tried to protect itself by covering its individual cells with EPS and sequestering internalized Zn in the polyphosphate bodies (Fig. 6 b). 100 µM Zn exposure was highly toxic, and most cells lost their shape, size, and filament integrity. Ultra-structurally, at this exposure, most cells also lost their membrane integrity, and disintegration of the thylakoid membrane was highly visible. On the other hand, minor changes that were visible in the organism's morphology and ultrastructure at 10 µM exposure emphasized the fact that the organism is highly resilient to toxic levels of Zn in its surrounding that are normally found in wastewater and polluted soil.

The entire effect of Zn exposure on the cyanobacterium is depicted in Fig. 7.

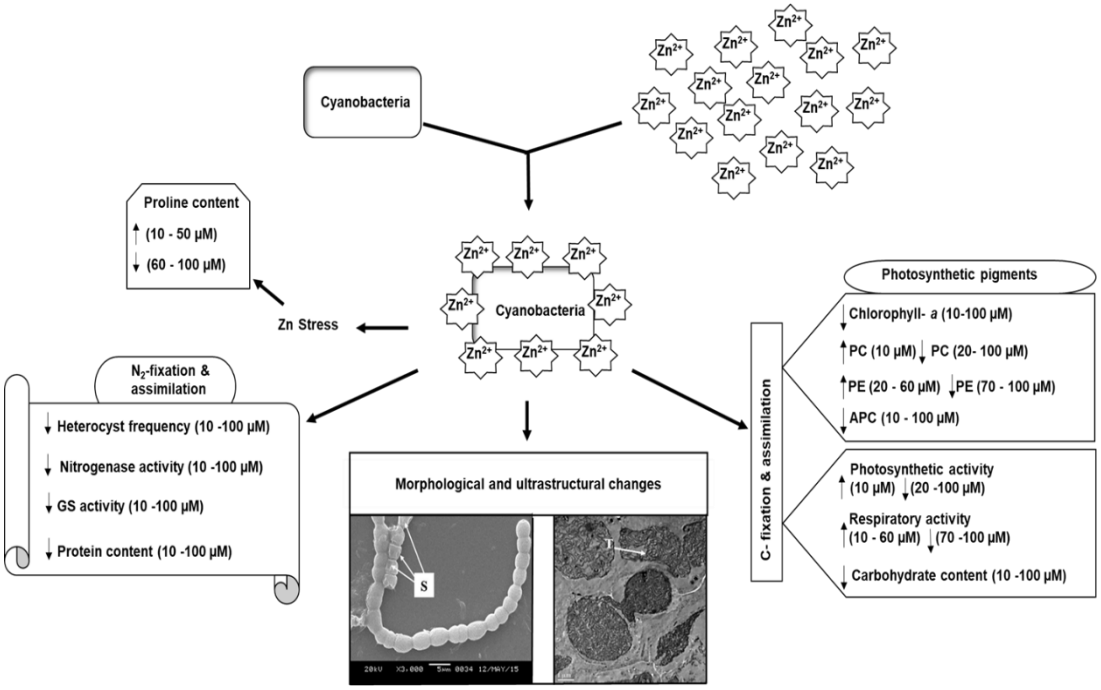


Fig. 7. *Schematic representation of chronic Zn exposure in the cyanobacterium Anabaena variabilis MEGCH1*

CONCLUSION

In conclusion, the tolerance and resilience shown by the cyanobacterium *Anabaena variabilis* MEGCH1 to chronic exposure to high Zn concentrations established the fact that this organism has realistic potential in Zn bioremediation from polluted soil and wastewater. A consortium made of such organisms with the potential to bioremediate explicit pollutants could therefore be very useful in the removal of specific contaminants from desired specimens. Thus, further research directed towards knowing the composition of pollutants of a particular wastewater system and designing a microbial consortium to remove each of these pollutants from that wastewater has the potential in reclaiming clean water bodies.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: M.B.S., Design: M.B.S., O.L.D., Data Collection or Processing: O.L.D., S.R., Analysis or Interpretation: M.B.S., O.L.D., S.R., Literature Search: O.L.D., S.R., Writing: M.B.S., S.R.

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