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Effect of Copper on cyanobacterium "*Phormidium fragile*" in the walls of historical monuments

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Abstract: A biodegradation of stone monuments, statures and historic building remains a worldwide problem. Some species of Chlorophyta (green algae) and Cyanobacteria are considered the pioneering inhabitants in the colonization of stone. An attempt was made to study "Effect of heavy metal copper on the cyanobacterium "*Phormidium fragile*" was conducted in the laboratory to test the effect of growth and biochemical composition. Copper was applied to the medium in the form of copper sulphate ($CuSO_4.5H_2O$) in different concentrations (200, 400, 600, 800, 1000 ppm) in which the micoalgae can grow. The microalgae suspension was analyzed. Growth was measured in terms of chlorophyll 'a' content and it was decreased in all the concentrations (200, 400, 600, 800, 1000 ppm) of copper. The carbohydrate content was gradual decrease from lower to higher concentration. Protein content was decreased with increased concentration of copper 200 to 1000 ppm. The lipid content was also decreased. Our result suggests that copper have greater toxicity effect on microalgae growth and its usage limits biodeterioration.
KeywordS: Cynobacteria, copper, chlorophyll, protein, amino acid, lipid.

Introduction and Experimental.

The effect of biological organism in the deterioratoion of stones monuments, rocks and archeological remains is a worldwide problem (1). A wide variety of organisms have been identified on limestone and other types stone monuments in tropical environments ranging from fungi, algae to protozoa. The microbiota on building stones develops in various ways due to the environmental conditions (2). Cyanobacteria are photolithoautotrophic organisms, may grow on the surface of the stone (epilithicphototrophs) cause aesthetic damage to stone monuments by creating variously colored microbial films on their surfaces or may penetrate some millimeters into the rock (endolithic phototrophs) giving them an unattractive appearance (3). These organisms release some biomolecules, they may cause mechanical stresses to the mineral structure as a result of shrinking and swelling cycles of these colloidal biogenic slimes inside the pore system, leading to the alteration of pore size and distribution. Due to the continuous presence of moisture is responsible for the predominant growth of microalgae over other organisms and the subsequent formation of carbonate crusts and oxalates which can seriously damage the calcareous stone. Cyanobacteria may also play a direct role in supporting the growth of heterotrophic organisms such as fungi and other bacteria, which lead to acid decay of calcareous materials by producing acids (4).

In order to inhibit the microbial growth on stones, various methods can be employed such as physicals, mechanicals, biologicals, or biochemical's, even if those based on active principles in solution (chemicals) are the more frequently applied (5). Many inorganic and organic chemical compounds have been used, as biocide

agents, to eliminate the biodeteriogens from cultural objects. Biological growth can be controlled by using Pesticides. These chemicals have a biocide action with a specific toxicity for the microbes. These are classified in different ways depending on their chemical nature or their mode of action. In addition to the pesticide, other coformulants, such as carriers or additives to improve the efficiency of the product or to accelerate its application, are present in the formulation of chemicals. These coformulants could have negative effects on the objects to be treated. Disinfectants are chemicals that destroy vegetative forms but are not always effective against survival resistant or quiescent phase structures. A problem with the use of pesticides is the persistence of the product in the soil or water. This problem is especially prevalent with other biocide compound like herbicides that are applied or dispersed in external environments, with high risk for water and soil contamination (6).

Many authors have used several chemical products having biocide characteristics, in order to eliminate the biodeteriogens developing on stone monuments or rock sites. These products are commercially available both as active principle or formulates and cover a wide range of chemical classes, from very simple inorganic compounds such as Na and Ca hypochlorite to very complex organic ones such as the Quaternary Ammonium Compounds (Preventol R50, Neo-Desogen) (7). Furthermore, they can possess a strictly specific mode of action such as the urea derivatives (Diuron, Karmex) that block the photosynthetic process, or a broad toxic spectrum like the Organotin compounds (TBTO). The inhibition of fungal and bacterial growth was done by the treatment of external walls with an algicidal substance. Any biocide intended for use on historic monuments and rock sites must be not only effective against biological growths but at the same s time cause no damage to the stone material either by direct action or by leaving deposits on it which may result in successive damage (8). Moreover, copper is also one of the essential micronutrients for algal growth. It is involved in numerous physiological functions, mainly those which participate in electron flow, catalyze redox reactions in mitochondria and chloroplasts (9). However, in excessive quantities copper becomes toxic as it interferes with photosynthetic and respiratory processes, protein synthesis and development of cell organelles (10). Specifically excess copper can cause chlorosis, damage to plasma membrane permeability, leading to ion leakage (11). Hence efforts have been made to establish the toxic level of copper on algal growth in the present study.

Microalgal strain and culture

The samples for microalgae isolation were collected aseptically from different location of Brihadeeswarar temple compound walls at Thanjavur. Samples were transferred to a 100 mL conical flask containing 20 mL of sterilized BG-11 medium and then incubated on a rotary shaker at 27ºC and 120 rpm under illumination using white LED (20 W) at intensities of 10000 lux by maintaining a 12-12 light/dark cycle at 27 ºC for four weeks (12). Every two days, the flasks were examined for algal growth using an optical microscope, with serial dilutions being made in BG-11 medium from flask showing growth. Subcultures were made by streaking and restreaking on BG11 medium with 2 % agar. The plates were incubated at 27ºC under illumination by maintaining same condition for two weeks. The purity of the culture was confirmed by repeated plating and by regular observation under a microscope and the colonies were segregated based on their morphological features using light microscope. Single colony of the two different isolates was picked up with the help of micropipette, thoroughly washed with sterile BG-11 medium and allowed to grow in the BG-11agar medium (Y) and incubated under light intensity, by maintaining a 12-12 light/dark cycle at 27 ºC(13).

An aliquot of cultured cells (2 mL) was harvested in the mid of exponential phase by centrifugation (10,000g for 5 min) in a sterile microcentrifuge tube. The genomic DNA was extracted by using xanthogenate method.

PCR Amplification of 16s rRNA Gene

Quality and quantity of DNA preparations were checked by standard spectrophotometry and the DNA template were prepared in a concentration of 25 ng/µL and used for PCR reactions (14). In this study the 16S rRNA gene region of the isolates were used for the amplification of the primers. Lyophilized primers such as 1R (AGAGTTTGATCCTGGTCAG) and 740R (TCTACGCATTTCACCGCTAC) were prepared at 100 pmol concentration with deionized water. The amplifications system contained 200 µM of dNTP with 0.3 µM of primers, 1.25 U of Taq DNA polymerase template, 1X PCR buffer and 100 ng of template DNA. Polymeric Chain Reaction (PCR) reaction was performed in thermocycler using a PCR program involved 35 cycles of 5 min denaturation at 95ºC, 1 min primer annealing at 65ºC and 60 s at 72 ºC for extension with final extension at 72 ºC for 5 min (15). The PCR products were separated on agarose gels and stained with ethidium bromide and the gels were documented with a gel documentation system. The PCR was performed twice to check the reproducibility.

Effect of Copper Sulphate

To test the effect of Copper on growth, dominant algal species from the isolation of different micro algal species was treated with different concentrations of Copper, in the form of Copper Sulphate ($CuSO_4$).

Molecular weight of copper sulphate - 249.68
Atomic weight of copper - 63.54 (Or) 63.54 mg of copper is present in 249.68 mg of copper sulphate
Therefore,
100 mg of copper = **2**49.68×100/63.54
=392.95 mg of $CuSO_4$ = 392.95 mg/$CuSO_4H_2O$/1000 ml

Stock solution of Copper Sulphate was prepared in sterile media (BG-11 medium) from which different concentrations of Copper Sulphate were prepared viz., 200, 400, 600, 800, 1000 ppm and control respectively. Exponentially growing microalgal sp was inoculated into 100 ml of each test solution taken in 250 ml conical flasks. This experiment was conducted for 28 days in a culture room, illuminated with white fluorescent light (10,000 lux) by maintaining a 12-12 light/dark cycle at 27 ºC.

Growth measurement

After the inoculation, growth rate was measured in term of chlorophyll 'a' as biomass components at initial (0) day 4th, 8th, 12th, 16th, 20th, 24th and 28thdays. For measurements of chlorophyll 'a' , A known volume of culture was centrifuged at 5000 rpm for 5 min and the pellet was treated with known volume of methanol (80%) and kept in water bath for 1 h at 60ºC in dark with occasional shaking. The mixture was centrifuged, at 5000g for 10 min, and concentrations of chlorophyll-a (CCHL-a) in the supernatant were determined according to Mckinney's1941(16)

Chlorophyll 'a' = A_{663} X 12.63
Where A_{663}=Absorbance at 663 nm
12.63=Correction factor

All the growth analysis viz., carbohydrate, protein, and lipid content calculated at the end of 21 st day Estimation of carbohydrates followed by Dubois et al., 1956,(17). Estimation of protein followed by Lowery et al., 1951,(18) and Estimation of lipid followed by Sato and Murata (19).

Result and Discussions

The colonies of cyanobacteria collected from Brihadeeswarar temple compound walls Thanjavur, India initially cultured in BG-11 medium were brought to the laboratory and purified by serial dilution fallowed by plating. Two isolates of the cyanobacterial culture were selected based on their purity, growth rates and were segregated based on their morphological features. The two isolated cyanobacterial cultures of S1, S2 were identified as the genera *phormidium, Scytonema* respectively, by morphological examination under a microscope based on cell shapes, color, colony morphology. The colonies of S1 are filmentous, distinctly green, trichrome constricted at the cross walls, not granulate, end cell attenuated. The colonies of S2 are filamentous, olive green, barrel shaped, trichrome constricted at the cross walls, terminal parts of branches cylindrical, with rounded apical cell, middle parts of trichomes elongated (20).

In order to further confirm the identity of the isolates, DNA from *Phormidium sp, Scytonema* sp was extracted. The isolated DNA from microalgae was amplified by the primers. In each case, PCR products of the expected sizes appeared as intense bands on agarose gels. The sizes of the amplified PCR products were approximately 1200bp. According to the 16S rRNA sequences, we concluded that microalgal isolates S1,S2 were closely related to *Phormidium fragile, Scytonema* mille, based on sequence similarities, respectively (21).

The Effect of different concentrations of $CuSO_4$ on the growth and biochemical constituents such as carbohydrate, protein, and lipid of *Phormidium fragile* are shown in (Figure 1 to 4). The effect of different concentrations of $CuSO_4$ on the cynobacterium *Phormidium fragile* revealed that the effect of chlorophyll- a, carbohydrates, protein, and lipid decreased with increased concentrations of $CuSO_4$ from 200 ppm to 1000 ppm. Similar observations were reported by Khalil (1997) in *P. fragile* exposed to mercury (22). Heavy metals stress caused reduction in growth, photosynthetic pigments and biological compositions.

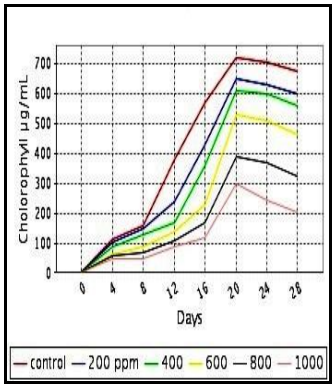

Figure – 1Effect of copper sulphate on Cholorophyll content

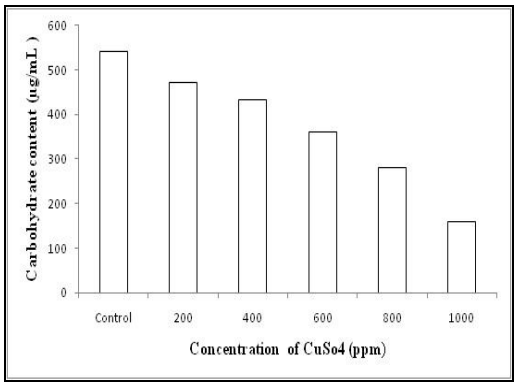

Figure – 2 Effect of copper sulphate on carbohydrate content

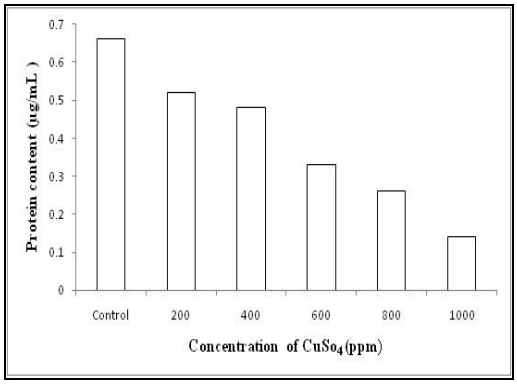

Figure – 3 Effect of copper sulphate on Protein content

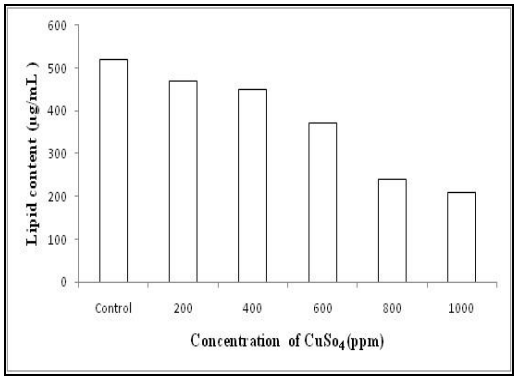

Figure – 4 Effect of copper sulphate on Lipid content

In the present study, the growth of *Phormidium fragile* was measured in terms of chlorophyll 'a' and found to be influenced by the $CuSO_4$ at the different concentration viz 200, 400, 600, 800, 1000 ppm. Different concentrations of $CuSO_4$ (200, 400, 600, 800, 1000) were exhibited similar responses but in control the growth

was increased up to 20th day and thereafter growth was decreased. Maximum growth was observed in control and decreased growth at 20th day and after they reached to decline phase.

Table 1. Effect of cupric sulphate-supplementation on chlorophyll, protein, lipid, carbohydrate content by P.fragile.

Concentration of $CuSo_4$ (ppm)	Cholorophyll µg/mL	Protein (µg/mL)	Lipid (µg/mL)	Biomass concentration (g %)
Control	675	0.66	520	0.60
200	597	0.52	470	0.55
400	560	0.48	450	0.46
600	462	0.33	370	0.32
800	321	0.26	240	0.12
1000	204	0.14	210	0.05

Composition (carbohydrate, protein, and lipid) of *Phormidium fragile* at the different concentration of $CuSO_4$ is shown in figure 2, 3, 4 and Table1. There was a gradual reduction in the level of carbohydrate content in control, 200, 400, 600, 800, 1000 ppm. Maximum carbohydrate content was observed in the control, 200, 400 ppm treated cultures (540,472,432 µg/ml) and minimum was observed in the 1000 ppm on 28th day (160 µg/ml) (Fig 2). Some researchers suggested that the metallic compound may cause disruption of thylakoid membrane resulting in the decrease of chlorophyll pigments(23). Higher concentrations of $CuSO_4$ showed maximum inhibition of the total carbohydrate content of test algae. Most studies indicated that acclimation or adaptation to higher metal concentrations is accompanied by the potential for increased tolerance by adjusting physiological or biochemical mechanisms(24). Earlier studies showed that metal efflux was an important mechanism to regulate the intracellular metal content in bacteria (25). Furthermore, the internal metals were found to be partitioned in different subcellular compartments, which may affect the tolerance capability (26).

There was a gradual reduction in the level of protein content in control, 200, 400, 600, 800, 1000 ppm on 28th day. Maximum protein contents (0.66 µg/ml) was observed in the control and the minimum was observed in 1000 ppm on 28th day (0.14 µg/ml) (Fig 3). The protein content was decreased with increased concentration of $CuSO_4$. Singh and Rai (1989) observed reduction in protein content of *Chlorella vulgaris* exposed to chromium. This reduction was suggested to be due to the blocking of sulfohydrexyl groups by metallic compound. There was a gradual decrease in the level of lipid content in control, 200, 400, 600, 800, 1000 ppm. Maximum lipids were observed in the control, 200, 400, 600, 800 ppm concentrations of $CuSO_4$ treated cultures, which were 520, 470, 450, 370, 240 µg/ml and minimum was observed in 1000 ppm (210 µg/ml) (Fig4).The lipid content was decreased at higher concentration.

From the present study, it is clear that the copper influence the blue green alga *Phormidium fragile* by reducing its growth and biochemical compositions. Further research on this type of organism with different compounds is needed to consider and use this compound as a biocide to control the microbial growth.

Conclusion

The presence of cyanobacterial and algal biofilms on stone surface can be considered biodeteriogenic. So In this study, it is clear that the $CuSO_4$ influence the blue green alga by reducing its growth and biochemical compositions. *Phormidium fragile* have an important role in the disfigurement of monuments and stone works of art. Moreover, there are several references in the literature that point to direct decay mechanisms caused by these photosynthetic microorganism.

References

1. Jimenez, C.S., Deposition of airborne organic pollutants on historic buildings, Atmosphere Environment, 1993, 27B, 77-85.
2. Lamenti, G., Tiano, P. and Tomaselli, L., Biodeterioration of ornamental marble statues in the Boboli Gardens (Florence, Italy), Journal of Applied Phycology, 2000,12, 427-433.

3. Danin, A. and Caneva, G. Deterioration of limestone walls in Jerusalem and marble monuments in Rome caused by cyanobacteria and cyanophilous lichens, International Journal of Biodeterioration and Biodegradation,1990, 26, 397-417.
4. Gaylarde, C. and Gaylarde, P.M., Biodeterioration of external painted walls and its control, 2000, Brazil, 4.
5. Richardson, B.A., Control of microbial growth on stone and concrete, Elsevier Applied Science, 1998, 12, 101-107.
6. Saiz-Jimenez, C., Deposition of airborne organic pollutants on historic buildings, Atmosphere Environment, 1993, 27B, 77-85.
7. Tiano, P., Accolla, P. and Tomaselli, L., Biocidal treatments on algal biocoenosis. Control of the lasting activity,Science and Technology for Cultural Heritage,1994, 3, 45-55.
8. Allsopp, C. and Allsopp, D., An updated survey of commercial products used to protect materials against biodeterioratio, Biodet. Bull, 1983,19, 99-146.
9. Hansch, R. and Mendel, R., Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe,Ni, Mo, B, Cl), Curr. Opin. Plant. Biol., 2009,12, 259-266.
10. Upadhyay, R.K. and Panda, S.K., Copper-induced growth inhibition, oxidative stress and ultrastructural alterations in freshly grown water lettuce (Pistia stratiotes L.), C. R. Biologies, 2009, 332, 623–632.
11. Bouazizi, H., Jouili, H., Geitmann, A. and Ferjani, E.EI., Copper toxicity in expanding leaves of Phaseolus vulgaris L.: antioxidant enzyme response and nutrient element uptake, Ecotox.Enviro.Safe,2010, 73, 1304–1308.
12. Kanz, T. and Bold, H.C., Physiological Studies, Morphological and Taxonomical Investigation of Nostoc and Anabaena in Culture, Austin (TX): University ofTexas; 1969. Publication No. 6924.
13. John, D.M., Whitton, B.A. and Brook, A.J., The freshwater algal flora of the British Isles an identification guide to freshwater and terrestrial algae, Cambridge: Cambridge University Press; 2003, 39- 43.
14. Ward, D.M., Weller, R. and Bateson, and M.M., 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community, Nature,1990, 345, 63-65.
15. Rasmussen, U. and Svennig, M.M., Fingerprinting of cyanobacteria based on PCR with primers derived from short and long tandemly repeated repetitive sequences, Applied Environmental Microbiology,1998, 64, 265-272.
16. McKinney, G., Absorption of light by chlorophyll solutions, J. Biol. Chem, 1941, 140, pp. 315-322.
17. Dubois, M., Gilles, R.A., Hamilton, F.K., Robers, P.A. and Smith, F., Calorimetric method for determination of sugar and related substances, Anal. Chem, 28, 1956, 350-366.
18. Lowery, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin-phenol reagent, J. Bio. Chem., 1957, 193, 269-275.
19. Sato, N. and Murata, M., Membrane Lipids.,In: Methods of Enzymology (eds.) Packer, L. and A.N. Glazer,1957, 167, 251-259.
20. Klein, S., Chapman, D.J. and Garber, E.D., Chemotaxonomy of the Oscillatoria– *Phormidium* complex, Biochemical Systematic and Ecology, 1973, 1, 173-177.
21. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y., Generic assignments, strain histories and properties of pure cultures of cyanobacteria, Journal of General Microbiology,1979,111, pp. 1-61.
22. Khalil, Z., Toxicological response of a cynobacterium, *Phormidium fragile*, to mercury, Water Air Soil Polut., 1977, 98(1-2),179-185.
23. Singh, C.B. and Rai, L.C.,Impact of chromium and cadmium on certain physiological variables of *Chlorella vulgaris* isolated from river Ganga, boil. Journal. I.,2000, 1(2), pp. 15-24.
24. Soldo, D. and Behra, R., Long-term effects of copper on the structure of freshwater periphyton communities and their tolerance to copper, zinc nickel and silver, Aquatic Toxicology., 2000, 47, 181-189.
25. Nies, D.H., Efflux-mediated heavy metal resistance in prokaryotes, FEMS Microbiology Reviews, 2003, 27(2-3), 313-339.
26. Wang, W.X. and Rainbow, P.S., Influence of metal exposure history on trace metal uptake and accumulation by marine invertebrates, Ecotoxicology and Environmental Safety, 2005, 61(2), 145-159.

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