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Impacts of Intracellular Mercury Bioaccumulation on Biochemical Composition of *Anabaena Variabilis*

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Abstract: ***The uptake and accumulation of toxic heavy metal mercury by a fresh water planktonic cyanobacterium, Anabaena variabilis was checked out under laboratory conditions. The aim of this work was to investigate whether this fresh water planktonic alga could serve as an early warning system (or bioindicator) of increased input of Hg in the food chain. Further, the effects of this bioaccumulation was observed on different physiological parameters like growth measurements, chlorophyll, carotenoid, phycocyanin, carbohydrate, protein content and nitrate reductase activity of A. variabilis. The importance of NR activity as biological indicator (since A. variabilis is very sensitive even to the low concentrations of Hg^{2+}) for monitoring the effects of heavy metals on the cyanobacteria is to be perceived. The study also explores the possibility of using A. variabilis for bioremediation of Hg^{2+}.***

Keywords: Mercury uptake, bioaccumulation, bioindicator, pigments, nitrate reductase, physiology.

1. Introduction

Mercury contamination in water and soil has become a great environmental health issue due to its natural release and anthropogenic activities (Lindberg *et al.*, 2007). Hg containing compounds such as fertilizers, pesticides, lime, manure and soil amendments contribute a great deal to Hg contamination in aquatic as well as terrestrial environments (Han *et al.*, 2002). In aquatic environments, inorganic Hg, primarily in the water-column and at the water-sediment interface, is methylated by microorganisms, and the resulting methyl-mercury (MeHg) biomagnifies through the food chain (Stein *et al.*, 1996; Scheuhammer and Graham, 1999; Chen and Yang, 2012). Bioaccumulation of MeHg through the aquatic food chain can result in a risk of increased Hg exposure and toxicity to piscivorous wildlife and to humans who consume a fish based diet. Hg^{2+} is the predominant and bioavailable form of mercury (Heaton *et al.*, 2005; Han *et al.*, 2006). It occupies the prime position in the list of toxic heavy metals (Rai *et al.*, 1981). Its effects have been extensively studied by many workers (Stratton *et al.*, 1979; Barr 1986; Singh, 1987; Scheuhammer and Blancher, 1994; Cho and Park, 2000; Cargnelutti *et al.*, 2006; Zhou *et al.*, 2007; Ge *et al.*, 2009; Chen and Yang, 2012; Singh and Biban, 2013) in many plants.

The biomarker approach has been used extensively by ecotoxicologists for detecting exposure to and effects of environmental contamination. Cyanobacteria are the first photosynthetic oxygen-evolving gram negative prokaryotes which appeared during Precambrian era. They are characterized by high tolerance and can exist in various extreme conditions. Simultaneously, they are very sensitive and quickly respond to the pollution by heavy metals and other stress conditions (Murugesan and Ruby, 2005; Biban and Singh, 2012). They have developed natural methods and resistance towards heavy metals and their utilization for treatment of industrial effluents and metal recovery has been proved successful (Kiran and Kaushik, 2006; Wehrleim and Wettern, 1994). They are the most important biomass producers and play globally significant role in biogeochemical cycles of nitrogen, carbon and oxygen. These prokaryotes are excellent source of a wide range of biologically active compounds of significant values that has fascinated researches for their pharmaceutical, biotechnological and biomedical exploitation (Rastogi and Sinha, 2009). e.g. polyunsaturated fatty acids (PUFAs), β-carotene and other photopigments (carotenoids, chlorophyll and phycocyanin) that function as antioxidants, anti-inflammatory, neuro and hepatoprotective (Romay *et al.*, 2003) and antitumor compounds (Liu *et al.*, 2000; Pardhasaradhi *et al.*, 2003), polysulfated polysaccharides as antivirals (Ghosh *et al.*, 2004), sterols as antimicrobials and mycosporine-like amino acids (MAAs) and scytonemin as photoprotectants (Rastogi and Sinha, 2009). Not only this, they are also used as ingredients of food, cosmetics, natural dyes and fluorescent markers (Glazer *et al.*, 1976).

Nitrate reductase (NR) is the enzyme that is being used to help clean up the environment, and has great potential to be part of the solution to the global problem of excess nitrate and related nitrogen-nutrients in water sources (Campbell and Campbell, 1998). NR activity is the limiting factor when considering the growth and protein production of algae (Lau *et al.*, 1998). Each organism has different potential to accumulate NO_3^- from the environment due to variation in its NR characteristics (Berges, 1997). Since heavy metals and nitrate, both are harmful pollutants and often occur together, it is necessary to know the effects of heavy metals on the enzyme activity more so that the NR in algae can be used to treat the nutrient pollution. Nitrate reduction processes are perhaps most significant in maintaining the water quality by alteration of nitrate to nitrite (Awasthi and Rai, 2005).

So the present investigation was made to study the mercury uptake potential of *Anabaena variabilis*, and its effects on the physiological and biochemical composition along with its NR activity.

2. Materials and Methods

2.1 Experimental Organism

In this investigation, *Anabaena variabilis* was isolated from a fresh water religious pond, Brahma Sarowar of Kurukshetra (India), a place renowned for the great battle of Mahabharata as per Hindu mythology (Biban and Singh, 2012).

2.2 Culture Conditions

Cultivation of the planktonic blue green algae was done in 250 mL Erlenmeyer flasks containing 100 mL of Allen and Arnon's culture medium (Allen and Arnon, 1955). The laboratory conditions were maintained at a temperature of 25 ± 1^0C with the light intensity of 14.4 Wm^{-2} (18/6 h light/dark cycle).

2.3 Experimental Design

An aliquot of 10 mL of exponentially growing cyanobacterial cells was centrifuged and transferred to sterilized Erlenmeyer flasks supplemented with 100 mL of nutrient medium containing separately 0.0, 0.05, 0.1, 0.2, 0.5 and 1.0 µM of $HgCl_2$. The treated filaments were observed for short-term duration from the initial day of inoculation and then continued for the period of 14 days (at 0, 2, 4, 6, 8, 10, 12 and 14 days) to screen the effect of intracellular bioaccumulation of mercury on various physiological and biochemical growth parameters.

2.4 Parameters studied:

Hg^{2+} U*ptake:* Hg^{2+} uptake by *A. variabilis* was determined in a cold vapour atomic absorption spectrophotometer, Mercury Analyzer (Model, MA 5840, Electronics Corporation of India Limited, India; detection limit, 0.1 µg Hg^{2+}/L), in terms of quantifying the total intracellular built-up of Hg^{2+}. The 5.0 mL aliquots from these experimental suspensions withdrawn at desired intervals of 10 min. The cyanobacterial samples (pellets) were first digested in HNO_3 as per the method described in Standard methods for Examination of Water and Waste Water by APHA (19^{th} Edition, 1995).

The Hg^{2+} content of the acid digested samples was estimated as per the methodology described in the manual of Mercury Analyzer. All the control knobs present in the instrument were adjusted. The stopper was removed. A suitable aliquot of the blank, standard or sample solution were taken in the reaction vessel. Ten percent of the nitric acid was added to maintain a volume of 10 mL. Two mL of stannous chloride was also added and the stopper was replaced immediately. Magnetic stirrer was switched on and stirred vigorously for about 5 min and then adjusted to '0' and 100% Transmittance. The filter rod was left in the position. 'HOLD' mode of operation was switched on. The pump was started and air was allowed to purge through the reaction vessel. The absorbance was noted at 253.7 nm as early as possible within a min and switched back to 'NORMAL MODE'. The meter indication should be back to 100% Transmittance. The pump and the magnetic stirrer were switched off. Before each measurement it was adjusted with 0% and 100%. Measurements were repeated for standard also. The amount of mercury content was later on calibrated through the standard graph as obtained with $HgCl_2$. The Hg^{2+} content was quantified as µmol Hg^{2+} /mL culture.

1. **Growth Measurement:** Since cyanobacteria form uniform algal suspension, direct optical density measurements at 560 nm was a reliable growth parameter.
2. **Protein Content:** The protein content of the algae was measured by the method of Lowry *et al.*, 1951, modified by Herbert *et al.*, 1971.
3. **Carbohydrate Content:** This was done by following the method of Dubois *et al.*, 1956.
4. **Chlorophyll and Carotenoid Content:** The photosynthetic pigments were quantified using acetone by the method of Jenson, 1978.
5. **Phycocyanin Content:** The main accessory water soluble pigment, phycocyanin was measured following the method of Bennet and Bogorad, 1971.
6. **Nitrogenase Reductase Activity:** The NR activity of the cyanobacterium was observed by the method of Camm and Stein, 1974.

2.5 Statistical Treatments

The values presented are the means of three independent experiments with the bars showing standard errors in each case. The statistical analysis of the data presented was done as per the methodology dealt in detail (Singh and Singh, 1990; 1992a; 1992b). The data were verified for significance at a particular probability level, and the variance ratio (F) was calculated as F= Treatment mean square/Residual mean square. The correlation coefficient (r) was also calculated.

3. Results

The intracellular bioaccumulation of toxic heavy metal mercury (Hg^{2+}) and its impact on some physiological parameters of a cyanobacterium *Anabaena variabilis* was carried out during a 0-14 days exposure to 0.05-1.0 µM $HgCl_2$ under laboratory conditions. Fig.1 shows that Hg^{2+} uptake was time dependent ($F_{Days\ 8,\ 40} = 7.38$, $p< 0.05$) as well as concentration dependent ($F_{Hg^{2+}\ 5,\ 35} = 7.607$, $p< 0.05$). The correlation was also found significant between exogenous concentration and intracellular build-up of Hg^{2+} ($r= 0.91$, $p< 0.025$). The intracellular concentration of Hg^{2+} was found maximum in case of 1.0 µM Hg^{2+} treated cells throughout the experiment. As a result, on the 14^{th} day of experiment, the intracellular concentrations recorded were 0.045, 0.096, 0.184, 0.435 and 0.531 µmol Hg^{2+}/mL culture in case of 0.05, 0.1, 0.2, 0.5and 1.0 µM Hg^{2+} treated cultures showing 91.1 %, 96.2%, 92.2%, 87.1% and 53.3% efficiency for extracting Hg^{2+} from the medium. The impact of this bioaccumulated Hg^{2+} on different physiological experiments has been further explained in subsequent figures.

As we precede for the growth measurements of the organism, we have found that the maximum optical density (Fig. 2) was recorded as 0.91±0.03 in case of Hg^{2+}-less control cultures, which also exhibited maximum protein

content (Fig. 3) as 78.9±2.4 µg protein/mL culture on 14th day of observation. Both the parameters showed some tolerance to intracellularly accumulated mercury, but with increasing levels of Hg^{2+}, there was decrease in absorbance as well as protein content of the algae. While analyzing statistically, decrease in OD values (Fig.2: $F_{Hg^{2+}\ 6,\ 48} = 2.12$, $p<0.01$; $F_{Days\ 8,\ 48} = 3.8$, $p< 0.01$) was more significant than decrease in protein content (Fig. 3: $F_{Hg^{2+}\ 6,\ 48} = 1.05$, $F_{Days\ 8,\ 48} = 12.50$, $p< 0.05$; $r= 0.094$, $p< 0.01$).

The variation in photosynthetic pigment contents in shown in figures 4.1, 4.2 and 4.3. As per our observations, the effect of Hg^{2+} was more significant in case of phycocyanin content (Fig.4.3: $F_{Hg^{2+}\ 6,\ 48} = 89.58$, $p<0.01$, $F_{Days\ 8,\ 48} = 0.475$, $p<0.025$, $r= 0.61$, $p< 0.025$) than that in chlorophyll *a* (Fig.4.1: $F_{Hg^{2+}\ 6,\ 48} = 9.12$, $F_{Days\ 8,\ 48} = 7.63$, $p<0.05$, $r= 0.78$, $p< 0.025$) and carotenoid (Fig.4.2: $F_{Hg^{2+}\ 6,\ 48} = 15.47$, $F_{Days\ 8,\ 48} = 12.87$, $r= 0.681$, $p< 0.025$) contents respectively. The two factor ANOVA of the data showed that the effect of Hg^{2+} concentration was more prominent than that of the exposure time duration.

Figure 5 shows the impact of Hg^{2+} ions on the carbohydrate content of *A. variabilis*. The algae showed some increase in its carbohydrate content in case of 0.045 µmol Hg^{2+}/mL culture intracellular accumulation. However, further bioaccumulation showed severe reduction in its carbohydrate content.

The NR activity of the blue-green algae is represented in Fig. 6.1 and 6.2. The two-way ANOVA of the data shows that NR activity is a reliable bioindicator of Hg^{2+} pollution in the environment (Fig.6.1: $F_{Hg^{2+}\ 6,\ 48} = 45.48$, $p<0.01$, $F_{Days\ 8,\ 48} = 25.07$, $p<0.01$, $r= 0.96$, $p< 0.05$). The algae showed maximum NR activity with tolerance up to 0.184 µ mol Hg^{2+}/mL culture bioaccumulation. But, further increase in Hg^{2+} ions resulted in decrease in the NR activity. The presence of other heavy metals like Zn^{2+}, Cu^{2+} and Cd^{2+} (in equimolar combination with Hg^{2+}) also reduced the NR activity of the phytoplankton as shown in Fig. 6.2.

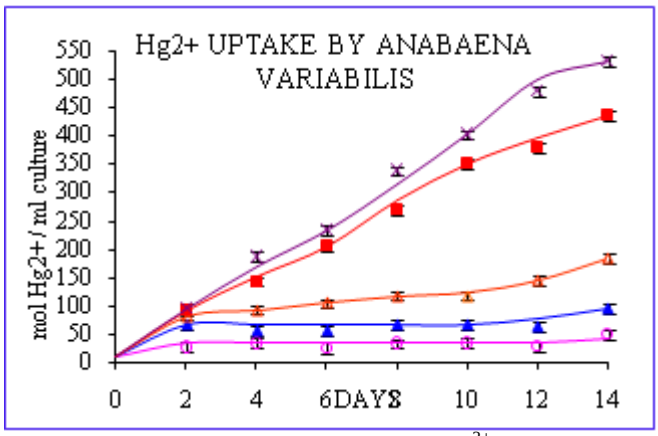

Figure 1: Intracellular accumulation of Hg^{2+} by *Anabaena variabilis* cells in 0.05 µM (o), 0.1 µM (▲), 0.2 µM (Δ), 0.5 µM (■) and 1.0 µM (x) of $HgCl_2$.Values are mean ±SE. $F_{Hg^{2+}\ 5,\ 40} = 7.607$, $F_{Days\ 8,\ 40} = 7.38$, $p< 0.05$; $r= 0.91$, $p< 0.025$.

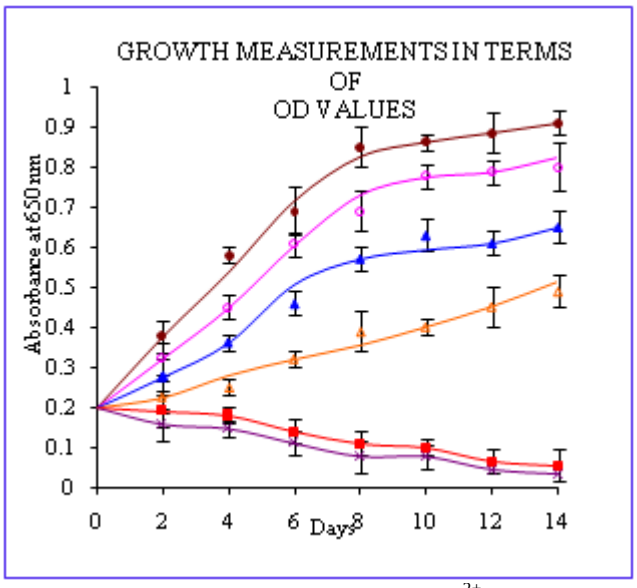

Figure 2: Impact of bioaccumulated Hg^{2+} on Optical Density measurements of *Anabaena variabilis* cultures: Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. $F_{Hg^{2+}\ 6,48}$= 2.12, $p<0.01$; $F_{days8,48}$= 3.8, $p<0.01$; $r= 0.92$, $p< 0.05$.

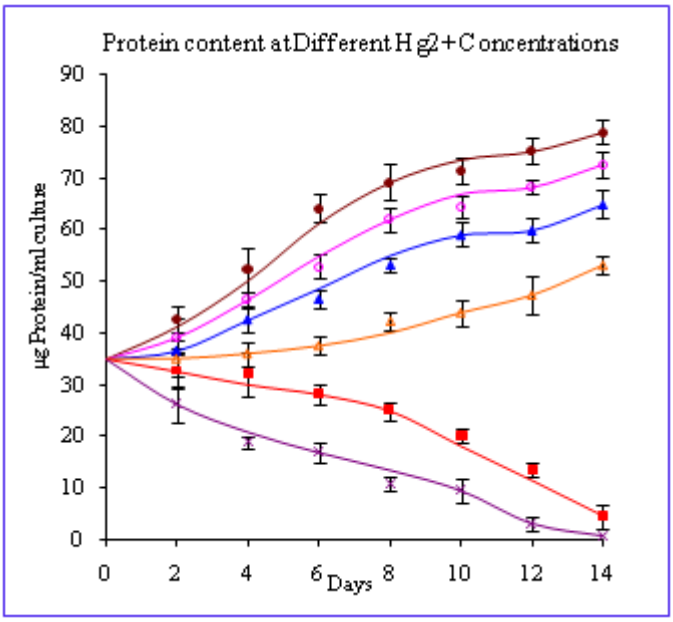

Figure 3: Impact of bioaccumulated Hg^{2+} on Protein content of *Anabaena variabilis* cultures: Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. ($F_{Hg^{2+}\ 6,48}$= 1.05, $p<0.05$; $F_{days8,48}$= 12.50, $p<0.05$; $r= 0.094$, $p< 0.01$).

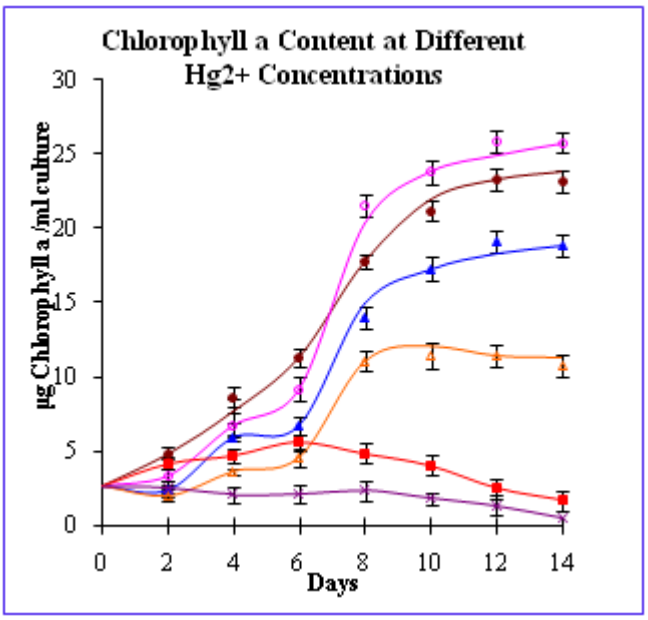

Figure 4.1: Variations in pigment Chlorophyll *a* content of *Anabaena variabilis* under the influence of Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. ($F_{Hg^{2+}6,48}$= 9.12, p<0.01; $F_{days8,48}$= 7.63, p<0.05; r= 0.78, p< 0.025).

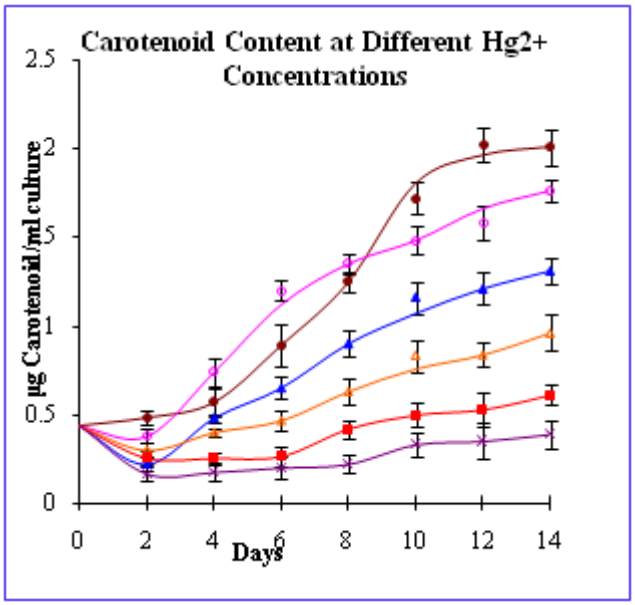

Figure 4.2: Variations in pigment Carotenoid content of *Anabaena variabilis* under the influence of Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. $F_{Hg^{2+}6,48}$= 15.33, p<0.025; $F_{days8,48}$= 12.5, p<0.025; r= 0.82, p< 0.025.

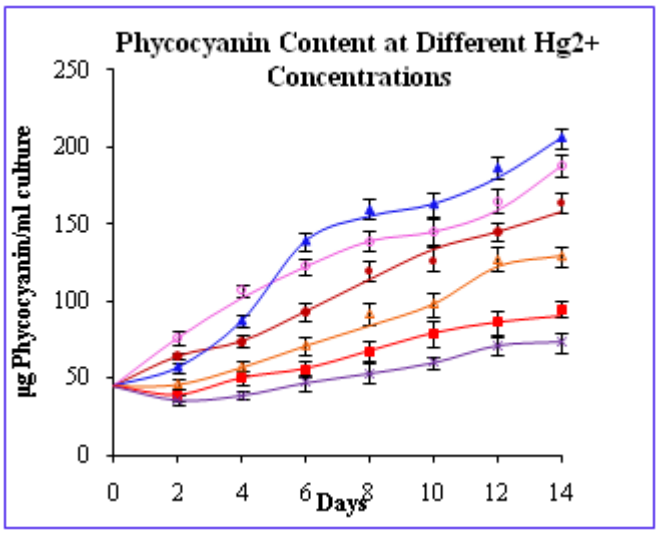

Figure 4.3: Variations in pigment Phycocyanin content of *Anabaena variabilis* under the influence of Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. $F_{Hg^{2+}6,48}$= 89.58, p<0.01; $F_{days8,48}$= 0.475, p<0.025; r= 0.61, p< 0.025.

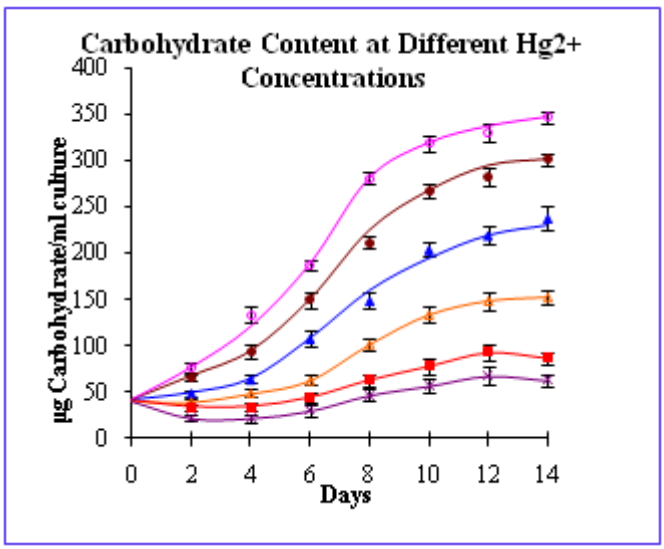

Figure 5: Impact of intracellular Hg^{2+} on Carbohydrate content of *Anabaena variabilis* cells during 0-14 days exposure to: Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. $F_{Hg^{2+}6,48}$= 23.35, p<0.01; $F_{days8,48}$= 17.01, p<0.05; r= 0.84, p< 0.05.

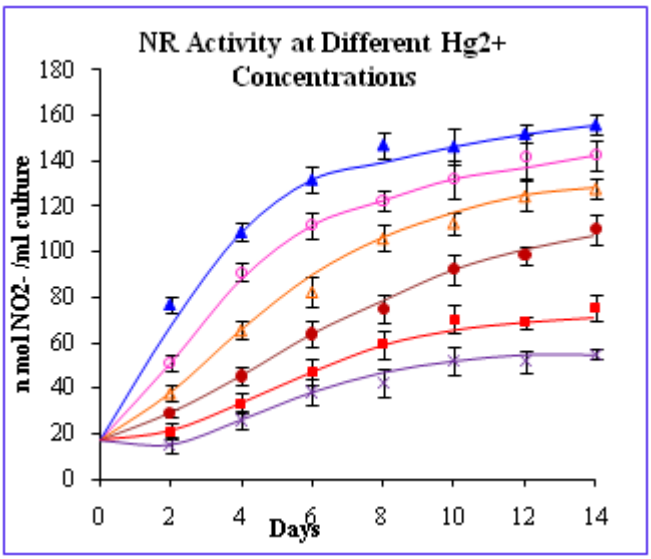

Figure 6.1: Impact of intracellular Hg^{2+} on Nitrogenase Reductase Activity of *Anabaena variabilis* cells during 0-14 days exposure to: Hg^{2+}-less control cultures (●), 0.045 µ mol Hg^{2+}/ml culture (o), 0.096 µ mol Hg^{2+}/ml culture (▲), 0.184 µ mol Hg^{2+}/ml culture (Δ), 0.435 µ mol Hg^{2+}/ml culture (■) and 0.531 µ mol Hg^{2+}/ml culture (x). Values are mean ±SE. $F_{Hg^{2+}6,48}$= 45.48, p<0.01; $F_{days8,48}$= 25.07, p<0.01; r= 0.96, p< 0.05.

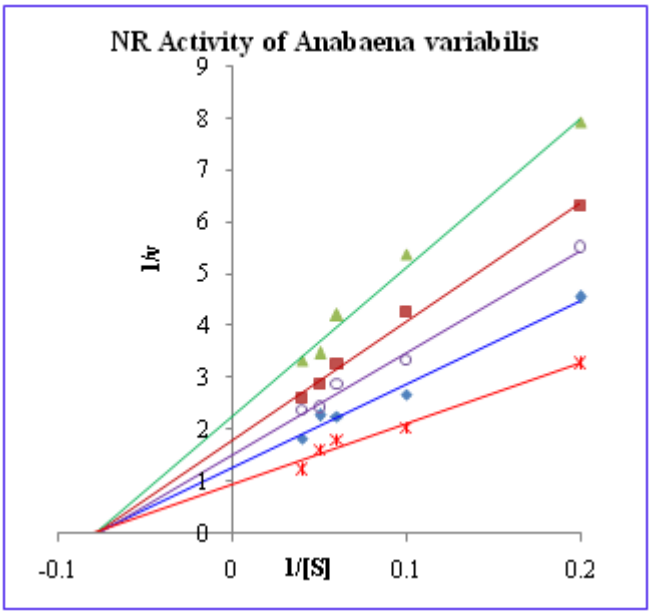

Figure 6.2: Double reciprocal plot for Nitrate Reductase Activity by *Anabaena variabilis* cells, interacting with some other heavy metals (0.5 µM, Extracellular, each) : Hg^{2+}-less control (●), Hg^{2+} -alone (■), Hg^{2+}+ Cu^{2+} (▲), Hg^{2+}+ Cd^{2+} (o), Hg^{2+}+ Zn^{2+} (x).

4. Discussion

Uptake of metals and metalloids can be harmLess (i.e. no effects), toxic or even beneficial (Awasthi, 2005). The bioassay studies on the cyanobacterium, *Anabaena variabilis* has clearly indicated that the organism to be very sensitive to very low concentrations of mercury (Murugesan and Ruby, 2005). The growth inhibitory effects of Hg^{2+} on plants have been directly linked to ultrastructural damage.

The effect of the metal ion is dependent on the varying degree of metal concentration in the cell and also the amount of ion that can transfer the cell membrane (Sinkiss, 1979). Biosorption studies on the Hg^{2+} uptake on a short term exposure showed its dependence on the metal concentration in the ambient medium. Hg^{2+} treatment reduced the amount of chlorophyll and resulted in breakdown of thylakoids. Furthermore, Hg^{2+}-stress inhibited the activity of NADPH:prochlorophyllide oxidoreductase (POR), which is responsible for the biosynthesis of chlorophyll (Lenti *et al.*, 2002). The metals were found to cause disruption of the thylakoid membranes in *Anabaena flos-aquae*, resulting in the degradation of the light harvesting pigment and thus decreasing their contents of the cells (Rai and Dubey, 1989).

The metal ions ability to enter a chemical reaction as a positively charged ion and their capacity to bind to the enzyme prosthetic group is an important reason behind the mechanism of enzyme inhibition. Metals like Zn^{2+}, Ni^{2+} and Cd^{2+} had interacted negatively with the NR activity of blue green algae, *Anacystis nidulans* and a green algae, *Chlorella vulgaris* in both free and immobilized states (Awasthi, 2005). The displacement of an essential metal ion forming the central and functional part of the enzyme protein may be one of the reasons for inhibition of NR by heavy metals, and secondly, interference with sulphydryl (-SH) group which often determine the secondary and tertiary structures of proteins. Besides, a reduced energy supply due to inhibition of photosynthetic electron transport and an indirect inhibition of uptake of substrate (NO_3^-) of energy may be other important reason. The sensitive histochemical based detection of Hg-triggered accumulation of ROS and oxidative injury can be used as biomarkers to indicate the toxicity in plants (Chen and Yang, 2012).

In plants, nitrate reductase (NR) and nitric oxide synthase (NOS) have been proposed as two enzymatic systems responsible for NO production. NO is involved in alleviation of heavy metals induced toxicity by directly regulating accumulation and translocation of heavy metals in plants. NO have been demonstrated to involve the regulation of Hg-induced oxidative stress and plant tolerance to algae. NO is shown to depress the generation of H2O2 and alleviate phytotoxicity by enhancing antioxidative capability (Wang and Yang, 2005). Heavy metals can regulate the generation of endogenous NO which is closely associated with the intrinsically physiological processes in plants.

Any stimulation in the ATP pool and availability of NADPH may stimulate ATP dependent processes like NR activity. Thus, enhanced photosynthetic activity indicates towards increase of the enzymatic activities.

5. Conclusion

Mercury inhibited synthesis of photosynthetic pigments and macromolecules. The blue green alga, *Anabaena variabilis* may be used as a bioindicator of Hg^{2+}-pollution in aquatic ecosystems, exploring its various physiological parameters (particularly the phycocyanin pigment content and nitrate Reductase activity). Being a tolerant species, *A. variabilis* can also be explored as a tool for bioremediation of mercury.

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