A laboratory study on effects of some metals on biochemical parameters of freshwater Alga *Anabaena ambigua*

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Abstract : The disturbance of aquatic ecosystems due to metal pollution from various sources such as industrial and domestic, leads to loss of biodiversity as well as increases the bioaccumulation and magnification of toxicants in the food chain. The inhibitory and stimulatory effects of heavy metals depend on concentration. Different organisms, however, have different sensitivities to the same metal and the same organisms may be more or less damaged by different metals.

The present investigation was carried out to study biosorption potential and also observe the biochemical response of alga Anabaena ambigua to six selected metals. Microalgae Anabaena ambigua was obtained from National Chemical Laboratory (NCL), Pune. The selected microalgae was tested for Chromium (Cr), Copper (Cu), Iron (Fe), Manganese (Mn), Selenium (Se) and Zinc (Zn) removal from aqueous solution. For the assessment of biosorption of metals by alga Anabaena ambigua the concentration of all the six metal ion species in the absorption medium was varied between 5 and 25 mg/L. The residual concentration of metals from the absorption medium was determined by using UV spectrophotometer after seven days of incubation period. The laboratory experiments were conducted for the assessment of biochemical responses of metals in algal species at various concentrations (0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) for fifteen days of exposure. Stimulatory effects and inhibitory effects were studied by calculating 50% inhibitory concentration (IC50) value.

IndexTerms - Algal biomass, percent bioremoval, heavy metal ions, stimulatory effects, inhibitory effects, UV Spectrophotometer.

I. Introduction

Several living organisms including bacteria, fungi, algae etc. are used as biological indicators and also used to control the pollution. As far as aquatic environment is concerned algae are considered as a good bioindicators (Albergoni et al., 1980; Favero et al., 1996; Markert and Oehlmann, 1998). The wide distribution of algae makes them a good candidate in monitoring of pollution by using a simple models (Rachlin et al., 1984; Piccinni and Gutierrez, 1995).

Metals are considered as a major water pollutants and acts as more toxic due to their bioaccumulation behavior. Metals can accumulate in algae through two ways, firstly by physical – chemical way which is a fast process and secondly, by cellular uptake which is a slow process. During metal removal by any living organisms, metals either binds on their surface or they uptake the metals I inside their body. In this process microalga plays prime role (Fraile et al., 2005; Leborans and Novillo, 1996; Rollemberg et al., 1998; Solisio et al., 2008). As they have high absorption capacity and minimal cost, application of algal biomass for metal bioremediation, gaining more importance. In addition, the metals adsorbed onto the cell surface can be successfully recovered through desorption by using certain chemicals including EDTA, HCL etc. (Costa and Franca, 1998; Gupta and Rastogi, 2008b). But at the same time it is very important to study the effects of these metals on micro algae.

Algae are widely present in both the aquatic ecosystems i. e. freshwater as well as marine environments. Some algae are microscopic and are visible only with the aid of light microscope. Algae also form base of the food chain and hence very important in aquatic environment both in terms of fundamental ecology and as a natural resource for human. There are very rare places on the earth where algae cannot be found (Pelczar et al., 1986).

Need of study

Heavy metal pollution affects the presence of planktonic algae, when entered in an aquatic ecosystem through various sources like industrial effluent, agricultural runoff, sewage discharge, sediments etc. (Whitton, 1984; Seidl et al., 1998; Nayar et al., 2004). The lake diatoms from Italian lake (Lake d'orta) are affected due to metal pollution (Cattaneo et al., 1998). Some metals leads to cancer causing hereditary changes in the genome (Paul, 2009).

Herein we report the effects of Cr, Cu, Fe, Mn, Se and Zn on the photosynthetic parameters of fresh water algae *Anabaena ambigua*. The effect of different metal concentration was also studied.

II. RESEARCH METHODOLOGY

2.1 Anabaena ambigua cultivation

The starting culture was obtained from the National Chemical Laboratory (NCL), Pune. *Anabaena ambigua* in log phase used in the experiment was inoculated in the Fog's medium at p^H 7.5. The medium was sterilized by autoclaving at 121°C for 15 minutes. Medium was stored at 4°C until inoculation. Culture was grown in liquid media in 2 L glass Erlenmeyer flasks and incubated at 25°C in a growth chamber with a light and dark cycle of 8 hrs and 16 hrs at 3000 – 3500 lux, light intensity provided by cool white day light fluorescent tube lamps.

2.2 Experimental setup for toxicological study

A stock solution (1000 mg/L) of all the selected metals was prepared in double distilled water. Three different sets of flasks (250 ml) along with one control were prepared each containing 100 ml of nutrient medium and sterilized by autoclaving at 121^0C and 15 lb pressure for 15 minutes. Cooled at room temperature and metal solution was mixed aseptically in flasks for preparation of different concentrations. One ml of algae culture (one month old) was inoculated in each flask and incubated for 15 days under white fluorescent light of 2000 lux with 16/8 hours light and dark photoperiod at 28 ± 2^0C in temperature controlled culture chamber.

2.3 Estimation of chlorophyll

Chlorophyll content was estimated by Arnon's method. 10 ml of sample was taken and centrifuged at 6000 rpm for 15 minutes. Supernatants were discarded, while pellets extracted with 5 ml of 80% aqueous acetone for at least 6 hours at 4^0C temperature. The tubes were wraped with aluminum foil and kept in dark. The samples were centrifuged again and the supernatants were used for measuring the optical density at 663 nm and 645 nm against 80% acetone as a blank by spectrophotometer. Total chlorophyll was calculated for each sample using the Arnon's formula (Arnon, 1949).

2.4 Estimation of protein

Protein contents were estimated by the Lowry method using Bovine Serum Albumin (BSA) as standard. The pellets remaining after chlorophyll extractions were dissolved in 0.1 N NaOH, centrifuged at 6000 rpm for 15 minutes. 0.2 ml of supernatant was mixed with 2.1 ml of working solution – I (1% $CuSO_4$ + 1% Na-K-Tartrate + 2% Na_2CO_3 in 0.1 N (NaOH). After 10 minutes, 0.2 ml of 50% diluted Folin – Ciocalteu's reagent was added. Absorbance was recorded after 30 minutes at 750 nm by spectrophotometer against a blank having no protein (Lowry *et al.*, 1951).

2.5 Estimation of total carbohydrate

The total carbohydrate contents were estimated by the Anthrone method. 10 ml samples were centrifuged at 6000 rpm for 15 minutes. Pellets were separated and extracted with 80% ethanol. On further centrifugation, the supernatants were used for total carbohydrate estimation. 0.5 ml of supernatants was added to 2.5 ml anthrone reagent in ice bath. Then the tubes were boiled in water bath at 100^0C for 10 minutes. After cooling, the absorbance were recorded at 620 nm using spectrophotometer, against a blank having no carbohydrate (Dubois *et al.*, 1956).

2.6 Estimation of starch

The starch contents were estimated by the Anthrone reagent method. The pellets remained during total carbohydrate estimation, were used for starch estimation. The pellets were extracted with 52% perchloric acid for 30 minutes at 0^0C centrifuged and supernatants were diluted upto 15 times. 1 ml of diluted sample was mixed with 2 ml cold anthrone reagent in ice bath. Boiled for 10 minutes at 100^0C in water bath, cooled and recorded the absorbance at 630 nm by using spectrophotometer against a blank having no starch. Calculated the starch content by multiplying with 0.9 to the values obtained from standard curve (Dubois *et al.*, 1956).

2.7 Estimation of free amino acids

The total free amino acids were estimated by the Ninhydrin method (Moore and Stain, 1948). 10 ml of samples taken and centrifuged at 6000 rpm for 15 miutes. Supernatants were discarded while pellets extracted with 5 ml of 80% ethanol. 0.1 ml of extract was mixed with 1 ml of ninhydrin solution and 0.9 ml of distilled water. The tubes were boiled in water bath for 20 minutes. 5 ml of diluent was mixed and after 15 minutes recorded the absorbance at 570 nm by spectrophotometer against a blank by taking 0.1 ml of 80% ethanol instead of the extract.

III. RESULTS AND DISCUSSION

Micronutrients are essential for proper growth, development and metabolism in living organisms. Some metals also acts as a micronutrients like copper, manganese, iron, zinc, cobalt etc. (Beveridge and Doyle, 1989; Bruins *et al.*, 2000). But if present in higher amount, these metals have adverse effects on environments. In aquatic ecosystem metal ions are easily absorbed by fishes, algae, vegetables due to their solubility and hence accumulate in human body through food chain (Azimi *et al.*, 2017). Metal toxicity becomes serious issue as they show adverse effects like reproductive abnormalities, fetal death, neurological disorders (Huang *et al.*, 2018). Hence it becomes necessary to study the toxic effects of metals on living things. In the present work, toxicity study includes the changes in total chlorophyll, total protein, total carbohydrate, total starch and total free amino acids contents of *Anabaena ambigua*, in presence of individual different concentrations of chromium, copper, iron, manganese, selenium and zinc after fifteen days of incubation period was investigated.

Table. 1: Effects of metal concentrations on total chlorophyll content of *Anabaena ambigua*

Conc. (mg/L)	Chromium	Copper	Iron	Manganese	Selenium	Zinc
Control	0.0284± 0.00020	0.0285± 0.00012	0.0286± 0.00024	0.0286± 0.00024	0.0283± 0.00020	0.0284± 0.00024
0.1	0.0346± 0.00021	0.0297± 0.00017	0.0394± 0.00020	0.0383± 0.00021	0.0574± 0.00024	0.0593± 0.00017
0.5	0.0293± 0.00020	0.0264± 0.00017	0.0374± 0.00028	0.0315± 0.00034	0.0716± 0.00020	0.0384± 0.00033
1.0	0.0205± 0.00021	0.0196± 0.00018	0.0295± 0.00033	0.0266± 0.00021	0.0515± 0.00020	0.0275± 0.00034
2.0	0.0144± 0.00029	0.0146± 0.00024	0.0245± 0.00029	0.0215± 0.00016	0.0494± 0.00028	0.0175± 0.00024
3.0	0.0107± 0.00016	0.0093± 0.00017	0.0184± 0.00016	0.0145± 0.00024	0.0473± 0.00016	0.0084± 0.00028
4.0	0.0094± 0.00029	0.0075± 0.00029	0.0155± 0.00020	0.0044± 0.00024	0.0253± 0.00024	0.0044± 0.00030
5.0	0.0085± 0.00012	0.0053± 0.00020	0.0085± 0.00028	0.0014± 0.00033	0.0144± 0.00029	0.0027± 0.00016

Data are mean ± S.D. of three replicates per treatment.

Chlorophyll is an important pigment playing important role in the photosynthetic activity for primary productivity. Many studies have demonstrated the effects of metals on chlorophyll content by inhibiting the uptake and transportation of other important elements, which are important for synthesis of pigments. It is also well known that the algal cells exposed to heavy metals may lead several biochemical changes (Rocchetta *et al.*, 2006). The results of this investigation also exhibits that the inhibitory and stimulatory effects of metals depend on the concentrations.

The data given in table 1, exhibits the effects of different metal concentrations on total chlorophyll contents of algae. The data expresses that the total chlorophyll was inhibited 50% (IC_{50}) at 2.0 mg/L, 2.0 mg/L,4.0 mg/L, 3.0 mg/L, 5.0 mg/L and 2.0 mg/L concentrations of Cr, Cu, Fe, Mn, Se and Zn resp.

The decline in chlorophyll content might be caused due to reduction in the synthesis of chlorophyll possibly by increasing chlorophyllase activity. It happens due to the destruction in chloroplast membrane and deactivation of electron transport in photosystem I (Sen and Mondal, 1987). At higher concentration of heavy metals, chlorophyll synthesis will be inhibited because heavy metals inhibits the enzymes which are responsible for chlorophyll synthesis (Prasad and Prasad, 1987). In case of zinc, its toxicity results from the binding of zinc to SH groups and disruption of the enzyme structure (De-Filippis and Pallaghy, 1994). The present results demonstrated concentration dependent effects of metals on chlorophyll formation, being stimulated by lower concentration and inhibited by higher concentration (Jain *et al.*, 2007). The stimulation of chlorophyll synthesis is associated with the formation of polychelatins (PCs), which plays detoxification role (Prasad, 2004).

Table. 2: Effects of metal concentrations on total protein content of *Anabaena ambigua*

Conc. (mg/L)	Chromium	Copper	Iron	Manganese	Selenium	Zinc
Control	0.4125± 0.00020	0.4125± 0.00020	0.4126± 0.00024	0.4136± 0.00016	0.4126± 0.00024	0.4123± 0.00017
0.1	0.4836± 0.00024	0.4833± 0.00024	0.5195± 0.00029	0.4925± 0.00034	0.5284± 0.00024	0.4384± 0.00028
0.5	0.4063± 0.00021	0.3815± 0.00028	0.4843± 0.00026	0.3755± 0.00028	0.6315± 0.00024	0.3814± 0.00024
1.0	0.3425± 0.00029	0.2185± 0.00020	0.3944± 0.00028	0.3147± 0.00021	0.4852± 0.00017	0.2985± 0.00032
2.0	0.2116± 0.00024	0.1872± 0.00010	0.3183± 0.00455	0.2613± 0.00016	0.2785± 0.00020	0.2104± 0.00012
3.0	0.0626± 0.00021	0.1256± 0.00024	0.2114± 0.00017	0.2255± 0.00016	0.2175± 0.00024	0.1827± 0.00016
4.0	0.0073± 0.00024	0.0625± 0.00029	0.1875± 0.00033	0.1625± 0.00034	0.0684± 0.00024	0.1135± 0.00032
5.0	0.0046± 0.00017	0.0064± 0.00024	0.1214± 0.00026	0.0924± 0.00024	0.0076± 0.00020	0.0873± 0.00020

Data are mean ± S.D. of three replicates per treatment.

Table 2 exhibits the effects of different metal concentrations on total protein contents of algae. The data expresses that the total protein content was inhibited 50% (IC_{50}) at 2.0 mg/L, 1.0 mg/L,3.0 mg/L, 3.0 mg/L, 3.0 mg/L and 2.0 mg/L concentrations of Cr, Cu, Fe, Mn, Se and Zn resp. Similar results were found, during the study of effect of Zn and Cd on *Chroococcus mintus* (Battah, 2010). At lower concentrations (0.01 to 0.1 ppm) of Ni increased the protein content in *P. stratiotes* leaves, which decreased at higher concentrations (1.0 and 10.0 ppm) (Vajpayee *et al.*, 2000). It was also reported that, such a reduction in the protein content could be due to effect on nitrate reductase activity. The protein levels of *E. crassipes* treated with different concentrations of heavy metals, decreased gradually as the metal concentration increased. The poor protein formation could be related to the disruption of nitrogen metabolism by the high doses of the metals (Odjeba and Fasidi, 2006).

Table. 3: Effects of metal concentrations on total carbohydrate content of *Anabaena ambigua*

Conc. (mg/L)	Chromium	Copper	Iron	Manganese	Selenium	Zinc
Control	0.0564± 0.00024	0.0565± 0.00034	0.0565± 0.00024	0.0564± 0.00028	0.0563± 0.00024	0.0566± 0.00024
0.1	0.0524± 0.00033	0.0615± 0.00024	0.0834± 0.00020	0.0614± 0.00028	0.0765± 0.00024	0.0683± 0.00024
0.5	0.0325± 0.00016	0.0525± 0.00024	0.0615± 0.00024	0.0594± 0.00026	0.0624± 0.00029	0.0415± 0.00020
1.0	0.0233± 0.00020	0.0394± 0.00028	0.0544± 0.00029	0.0493± 0.00024	0.0415± 0.00024	0.0355± 0.00024
2.0	0.0155± 0.00024	0.0285± 0.00020	0.0345± 0.00020	0.0406± 0.00029	0.0324± 0.00024	0.0292± 0.00049
3.0	0.0094± 0.00034	0.0124± 0.00024	0.0294± 0.00028	0.0305± 0.00024	0.0265± 0.00028	0.0204± 0.00024
4.0	0.0083± 0.00012	0.0065± 0.00020	0.0094± 0.00020	0.0285± 0.00028	0.0495± 0.00024	0.0145± 0.00029
5.0	0.0064± 0.00028	0.0024± 0.00028	0.0065± 0.00020	0.0194± 0.00020	0.0465± 0.00021	0.0095± 0.00032

Data are mean ± S.D. of three replicates per treatment.

In this research work, it was cleared that lower concentrations of metals, showed stimulatory effects on carbohydrate content while inhibitory effects at higher concentrations of these metals in culture medium. In table 3 the effects of different manganese concentrations on carbohydrate contents of algae was shown. The data expresses that the total carbohydrate content was inhibited 50% (IC_{50}) at 1.0 mg/L, 2.0 mg/L,3.0 mg/L, 4.0 mg/L, 3.0 mg/L and 2.0 mg/L concentrations of Cr, Cu, Fe, Mn, Se and Zn resp.

Our study coincide with the report mentioning that there is increase in the carbohydrate content of *Spirodela polyrhiza* at 0.1 ppm of Cd by 30.03% after 12 days of exposure (Rolli *et al.*, 2010). The metals are inducers for PC synthesis and have important role in detoxification and hence at lower concentration, carbohydrate content increases (Bhattacharya and Choudhary, 1995). But at higher concentrations the heavy metals damages the photosynthetic apparatus, mainly harvesting complex II (Krupa, 1998) and photosynthesis I and II (Hasan *et al.*, 2009). Our results also supported by other researcher who reported the total carbohydrate content of *Chlorella* cultures grown for seven days under various concentrations of cobalt, copper and zinc (Afkar *et al.*, 2010). The total carbohydrate content of the tested alga also declined in manner dependent on the metal concentration in the culture medium. Alga *Cylindrothica fusiformis* produce carbohydrate as a defense mechanism against copper toxicity in stationary phase when cells are exposed to 0.05 mg/L of copper (Torres *et al.*, 1998).

Table. 4: Effects of metal concentrations on total starch content of *Anabaena ambigua*

Conc. (mg/L)	Chromium	Copper	Iron	Manganese	Selenium	Zinc
Control	0.3281± 0.00455	0.3315± 0.00024	0.3315± 0.00024	0.3315± 0.00024	0.3314± 0.00028	0.3316± 0.00029
0.1	0.3944± 0.00024	0.3914± 0.00020	0.3834± 0.00016	0.3904± 0.00028	0.3424± 0.00020	0.3223± 0.00020
0.5	0.3215± 0.00024	0.3085± 0.00024	0.3425± 0.00026	0.3573± 0.00020	0.3167± 0.00017	0.2484± 0.00028
1.0	0.1684± 0.00034	0.2374± 0.00028	0.3218± 0.04697	0.3215± 0.00028	0.2814± 0.00024	0.1944± 0.00024
2.0	0.1483± 0.00024	0.1534± 0.00020	0.1994± 0.00024	0.1975± 0.00020	0.2155± 0.00024	0.1615± 0.00029
3.0	0.0754± 0.00024	0.0874± 0.00020	0.1595± 0.00029	0.1625± 0.00029	0.1645± 0.00024	0.1267± 0.00016
4.0	0.0125± 0.00028	0.0231± 0.00024	0.1124± 0.00028	0.1295± 0.00033	0.0714± 0.00028	0.0984± 0.00020
5.0	0.0075± 0.00016	0.0044± 0.00024	0.0715± 0.00032	0.0944± 0.00020	0.0184± 0.00024	0.0724± 0.00024

Data are mean ± S.D. of three replicates per treatment.

The data regarding the starch contents shows significant changes resulting from exposure of different metals. Here Cr shows most toxic effects than other studied metals. The data of present study given in table 4 showed the effects of different metal concentrations on total starch contents of algae. The data expresses that the total starch was inhibited 50% (IC_{50}) at 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 3.0 mg/L, 3.0 mg/L and 2.0 mg/L concentrations of Cr, Cu, Fe, Mn, Se and Zn resp.

All these metals show inhibitory effects on starch at higher concentration. Such decrease in starch contents could be due to the interference with photosystems and in inhibition of the Calvin cycle enzymes (Clijsters *et al.*, 1999). Our findings were also supported by several authors, who submitted twenty day old *cucumber* plants to copper stress for five days, to observe the effect of copper on starch content (Badr *et al.*, 2004). In their investigation they found that, the starch concentration measured in first and second leaves of the stressed plants increased significantly as compared to controls. In control plants, starch content was 6 mg/g of dry weight in first leaves and it was over 4 mg/g of dry weight in second leaves. While in the plants, the starch content was around 16 mg/g of dry weight in first leaves and around 10 mg/g of dry weight in second leaves which are treated with 4 µg of copper chloride/g of sand/day. The response of *Lemna minor* to the pollutants from common effluent treatment plant containing copper, chromium, nickel, zinc and iron. In control set, the starch content was found to be around 24 mg/g of fresh weight and in 25, 50, and 100% of waste water treated samples the starch content was around 0.2, 0.25, 0.05 mg/g of fresh weight respectively (Singh *et al.*, 2008).

Table. 5: Effects of metal concentrations on total free amino acids content of *Anabaena ambigua*

Conc. (mg/L)	Chromium	Copper	Iron	Manganese	Selenium	Zinc
Control	0.3605± 0.00029	0.3604± 0.00024	0.3404± 0.01414	0.3606± 0.00020	0.3603± 0.00020	0.3605± 0.00029
0.1	0.3945± 0.00020	0.3905± 0.00024	0.4125± 0.00024	0.3815± 0.00028	0.3914± 0.00028	0.3784± 0.00028
0.5	0.2924± 0.00020	0.2914± 0.00020	0.3835± 0.00033	0.3975± 0.00024	0.3785± 0.00028	0.2804± 0.00024
1.0	0.2145± 0.00024	0.1846± 0.00029	0.3165± 0.00028	0.2864± 0.00024	0.2964± 0.00028	0.2354± 0.00020
2.0	0.1804± 0.00029	0.1164± 0.00033	0.2864± 0.00024	0.2125± 0.00028	0.2176± 0.00024	0.1905± 0.00028
3.0	0.0764± 0.00020	0.0525± 0.00020	0.2424± 0.00029	0.1765± 0.00028	0.1794± 0.00024	0.1405± 0.00016
4.0	0.0224± 0.00024	0.0174± 0.00020	0.1854± 0.00020	0.1525± 0.00029	0.0715± 0.00024	0.0985± 0.00033
5.0	0.0064± 0.00024	0.0055± 0.00032	0.1054± 0.00028	0.1145± 0.00028	0.0025± 0.00024	0.0435± 0.00033

Data are mean ± S.D. of three replicates per treatment.

The data regarding the free amino acid contents shows significant changes resulting from treatments of metals. Here Cu shows most toxic effects than other metals. The data of present study given in table 5 showed the effects of different metal concentrations on total free amino acid contents of algae. The data expresses that the total free amino acid was inhibited 50% (IC_{50}) at 2.0 mg/L, 1.0 mg/L, 4.0 mg/L, 3.0 mg/L, 3.0 mg/L and 2.0 mg/L concentrations of Cr, Cu, Fe, Mn, Se and Zn resp.

Our results showed the effects of different metal concentrations on free amino acid contents of algae. The obtained data expresses that the metals showed stimulatory effects on amino acid content at lower concentrations. Zinc at lower concentrations increases total amino acid contents and decreased as the metal concentration increases (Omar, 2002). Generally the stimulation in amino acid content in response to metals concentration may be due to the suppressed protein biosynthesis encouraged free amino acid accumulation or may be due to some counteracting mechanism against heavy metal toxicity (El-Sheekh *et al.*, 2003; Osman *et al.*, 2004; Fathi *et al.*, 2005).

Conclusion

In the present study it was found that in toxicity study, most of the metals exhibited stimulatory effects on chlorophyll, protein, starch, carbohydrate and amino acids up to 0.5 mg/L of their initial concentration. But after this concentration they exhibited inhibitory effects on photosynthetic parameters.

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