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Biochemical modulation of stress related compounds in lead resistant cyanobacteria in short and long term duration

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Abstract
Two Lead resistant cyanobacterial taxa, *Lyngbya majuscula* and *Spirulina subsalsa* isolated from Lead contaminated area, were exposed to sub-lethal dose of lead (5 mg L^{-1}) for short and long term duration to understand the biochemical modulation in stress related compounds in metal exposure. Both the taxa showed gradual increase in Pb (II) accumulation with time (5-30 mg/g), where *Spirulina* showed ~6 times more accumulation compared to that of *Lyngbya*. The growth in terms of chlorophyll content at this particular concentration remained almost unchanged in *Lyngbya* up to 7 days of exposure followed by growth suppression, but *Spirulina* showed no significant variation in metal exposure during the experimental tenure. The toxicity level, as indicated by total ROS content was determined in terms of malondialdehyde production and maximum value was obtained after long term exposure for both the strains. Upregulation with variation in stress enzyme activities like catalase, ascorbate peroxidase and super oxide dismutase along with proline level were observed within 1 to 24 h of metal exposure giving protection to the metal exposed cell against oxidative stress. But in long term exposure, high rate of carotene accumulation and reduction in cellular glutathione pool may provide protection to oxidative damage of metal treated cyanobacteria.

Keywords: Cyanobacteria, Lead, *Lyngbya majuscule*, *Spirulina subsalsa,* Stress enzymes, ROS

1. Introduction
Cyanobacteria, the most primitive plants on earth surface are quite resistant to metal stress as they appeared 2.7 billion years ago, when the earth was full of metals. They have metal accumulating capacity in one hand and are capable to produce different metabolites and metal chelating compounds like, ascorbate, proline, glutathione and other enzymatic antioxidants including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), therefore giving resistance. Among other naturally occurring toxic heavy metals, lead (Pb) contamination in soil and water pose a serious problem for agriculture and aquaculture farms worldwide. Lead is one of the heavy metals that impart toxicity to organisms even at very low concentration resulting in oxidative stress, thus producing high level of ROS that damages cell and its components (Ruley *et al*. 2004) [12]. Metal induced antioxidative defense mechanism is quite active in cyanobacteria and other (Zutshi *et al*. 2008) [16].
In our background work, we had surveyed the extensive area of south east India and observed that the entire area is highly contaminated by lead (0.008-12.2ppm) and the flora is represented mainly by different cyanobacteria and eukaryotic algae (Chakraborty *et al*. 2011) [5]. From the contaminated area two cyanobacterial taxa *Lyngbya majuscula* and *Spirulina subsalsa* were isolated and taken as experimental materials. The present study is aimed to understand lead induced antioxidative defense mechanism in short and long term exposure to sublethal dose of lead employing the cyanobacterial taxa *Lyngbya* and *Spirulina*.

2. Materials and Methods
Lyngbya and *Spirulina*, isolated from Pb contaminated area of south east India were cultured in artificial sea water medium (ASN III) at 20 ˚C in 16/8 hour light/dark cycle under cool fluorescent light (intensity 20-30µ mol photons m^{-2} s^{-1}). Measured amount of $Pb(NO_3)_2$ was dissolved in 1 L doubled distilled water to prepare 5 mg L^{-1} Pb (II) solution. For each set of

experiment, ~0.5 g (FW) of cyanobacterial biomass from exponential growth phase was exposed to 100 ml of 5 mg L^{-1} Pb (II) solution at pH 6 for 28 days as this condition showed maximum accumulation of lead in our early study (Chakraborty *et al*. 2011) [5]. The exposure solution contained NaCl, $NaNO_3$ and stock metal solution in a required ratio. All the experiments were done in triplicate. Lead content of the algal tissue collected at different time intervals (1 h, 3 h, 24 h and 28 days) was measured using Flame Atomic absorption spectrophotometer (AA-575 Varian Techtron). In an attempt to quantify the amount of surface adsorbed Pb, metal exposed biomass was treated with 0.1 M EDTA solution for 30 min for removing the surface bound metal, then the biomass was analyzed again. Among different solvents used like, HCl, HNO3, thiourea, distilled water, 0.1 M EDTA solutions was found to be the best desorbing reagent.

Chlorophyll and carotenoids were measured from the cyanobacterial biomass employing standard methods (Arnon 1949; Sadashivam and Manickam1996) [2, 13]. Oxidative stress was measured as lipid peroxidation by quantification of malondialdehyde (MDA) production in the cells in terms of total content of 2-thiobarbituric acid reactive substances (Heath and Packer 1968). Activity of the stress enzymes like, APX, SOD and CAT were also estimated following standard methods (Sadashivam and Manickam1996; Beauchamp and Fridovich 1971; Nakano and Asada 1981) [13, 4, 9]. Proline and reduced glutathione content of treated and control biomass were also determined (Bates *et al*. 1973; Ellman 1959) [3, 6]. All the experiments were done in triplicates. For statistical tests SPSS 11.3 software was used ($p \leq 0.05$).

3. Results and Discussion

Both the cyanobacteria showed Pb accumulation by surface adsorption as well as active uptake at pH 6. From Fig.1, it was evident that *Lyngbya* biomass showed quick absorption of Pb in one hour exposure followed by a gradual increase up to 28 days whereas, *Spirulina* showed a slow and steady increase in the accumulation up to 24 h reaching to its maximum after 28 days' treatment. Quick absorption by *Lyngbya* was mainly due to the thick polysaccharide investment surrounding *L. majuscula* filaments which allowed higher Pb adsorption. Although after 28 days of exposure, *Spirulina* accumulated ~3.5 times more lead (31mg g^{-1}) in comparison to *Lyngbya* (8.4 mg g^{-1}) of which almost 75% was surface adsorption. From EDTA (0.1M) washed filaments, retention test was done and it was found that 41.6% and 25.8% of Pb was retained within *Lyngbya* and *Spirulina* filaments respectively. Therefore, it can be concluded that *Lyngbya* accumulated more Pb by active uptake at intracellular level, whereas *Spirulina* showed more adsorption than uptake.

We had already reported that *Lyngbya majuscula* filaments enmeshed in a glass wool packed in a column removed 95.8% of the Pb from a 5 mg L-1 Pb solution compared to free and dead biomass which removed 64 and 33.6% Pb respectively (Chakraborty *et al*. 2011) [5]. Growth pattern of the experimental taxa under Pb exposure was determined in terms of total chlorophyll content as expressed in Fig. 2. The chlorophyll content reached a plateau in lead treated *Spirulina* irrespective of time of exposure, whereas in case of *Lyngbya* it was observed to be decreased from 7 days onwards in metal exposure (Fig. 2).

In the present study a significant increase in lipid peroxidation was observed (above 5 times) in metal exposed cyanobacteria from 3 to 24 h onwards up to 28 days (Table 1 and 2). Lipid peroxidation which is regarded as an indicator of oxidative damage, involves oxidative degradation of polyunsaturated fatty acyl residues of membranes leading to the formation of lipid peroxidation products such as MDA (Singh *et al*. 2007a) [14].

Changes in stress enzymes are represented in Table 1 and 2. Upregulation in catalase activity (5 – 7 times) was observed as short term effect (3h) for *Lyngbya* (1737.34 □M. min-1.mg-1 of protein; p= 0.001) but as long term effect for *Spirulina* (2719 □M.min-1.mg-1 protein, p=0.0038) after 7 days. Beyond this the activity was almost stopped.On the other hand SOD activity in longer period of exposure (Table 1 and 2). Lipid peroxidation in Pb treated cyanobacteria were found to be inversely proportional to catalase activity, as expected. Increased catalase activity was found to give protection against lipid peroxidation up to 3 h in *Lyngbya* and up to 7 days in *Spirulina*. Progressive increase in catalase production was also found in Pb^{2+} stressed pea root cells (Malecka *et al*, 2001) [8]. Stress condition causes a depletion of catalase activity during long term probably by inhibiting enzyme synthesis, change in assembly of enzyme sub units, reducing the rate of protein turnover or by over accumulation of H_2O_2 (Qureshi *et al*. 2007) [11].

In *Lyngbya* ~3.5 folds increase in SOD activity was observed in 24 h treatment ($p = 0.001$) but in *Spirulina* the SOD activity was comparatively less with a little increase with time. In both the taxa SOD activity was almost nil after 21 days of exposure (Table 1 and 2). Significant upregulation of SOD in short term treatment indicates its protective role against oxidative stress and peroxidation in *Lyngbya*. An increase in APX activity was observed in metal stress condition in both the strains within 1-24 hrs exposure followed by a drastic decrease. Comparatively the activity was more in *Spirulina* than that of *Lyngbya* (Table 1 and 2). In *Spirulina* ~3.4 fold increase was recorded after 24 h of treatment (0.345 $mM.min^{-1}$ mg^{-1} of protein, p= 0.001), whereas a ~2 fold increase was observed in case of *Lyngbya* (0.08 $mM.min^{-1}$ mg^{-1} of protein, p= 0.002). The combined activation of SOD and APX is indicative of the tight coupling between $O_2^{\cdot-}$, H_2O_2 and ascorbate. The enhanced APX strongly suggests the oxidation of ascorbate by APX in both the genera during short term exposure. Total reduced glutathione (GSH) content was found to be increased up to 3 h for *Spirulina* and 3 days for *Lyngbya* followed by a gradual decrease in long term exposure for both the cases (Table 1 and 2). The downward trend in GSH content displayed were congruent with the general consumption of cellular GSH in scavenging reactive species and free radicals produced in metal stressed condition. GSH consumption can also be seen as a sign of enhanced activity of H_2O_2 removing ascorbate oxidase (APX) system. There are also reports of decreased reduced glutathione pool in Pb^{2+} exposed *Gonyaulax polyedra* (Okamoto *et al*. 2001) [10]. Reduction in GSH can also be explained by an inhibition of GSH synthesis. However no direct evidence for inhibition of the enzymes involved in GSH synthesis by algae has been reported. Alternatively it could be explained by the channeling of GSH to phytochelatins which are glutathione polymers known by their metal chelating properties and by ROS scavenging activity. Phytochelatin showed scavenging activity of ROS higher than GSH (Tsuji *et al*. 2002) [15].

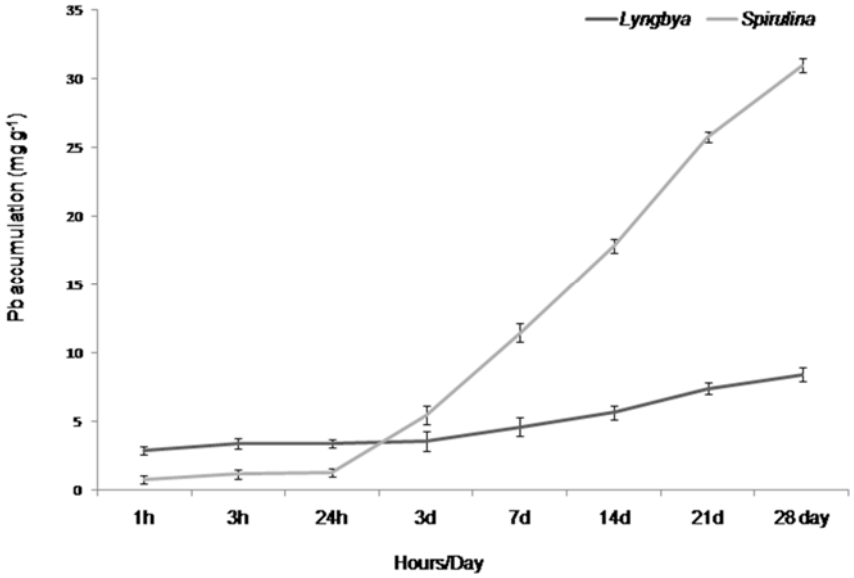

Fig 1: Accumulation of Pb (II) in cyanobacterial biomass with function of time. *Sfig*

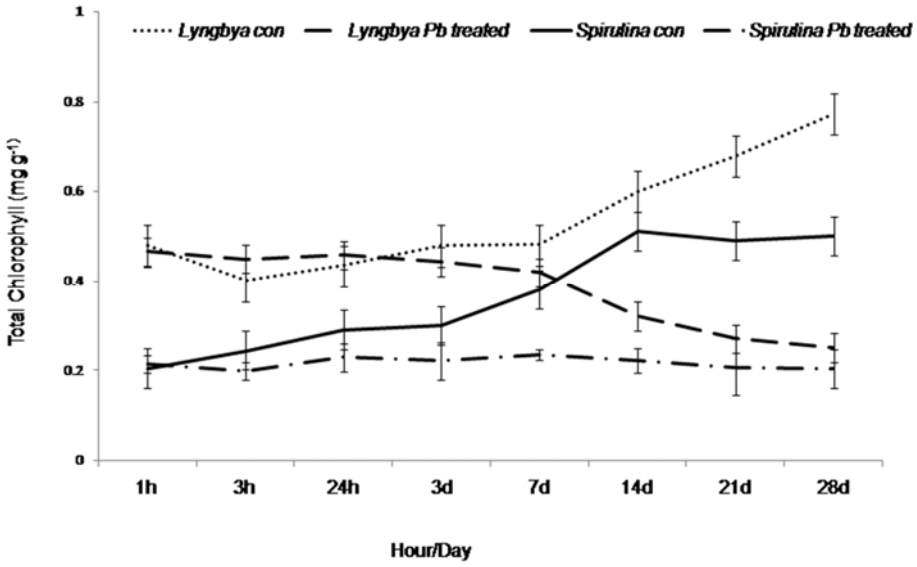

Fig 2: *pirulina* accumulated more (31mg g^{-1}) in comparison to *Lyngbya* (8.4 mg g^{-1}) after 28 days of exposure to Pb (II) solution.

Table 1: Associated changes in biochemical parameters of gold exposed *Lyngbya* with function of time

Table 1		Time							
		1h	**3h**	**24h**	**3d**	**7d**	**14d**	**21d**	**28d**
MDA (mMg^{-1})	Control	2.01±0.035	1.61±0.02	1.63±0.02	1.87±0.008	1.73±0.02	2.33±0.05	2.63±0.02	3.99±0.05
	5 mgL^{-1} Pb	1.96±0.011	1.67±0.036	4.4±0.077	5.27±0.046	5.76±0.03	5.79±0.05	5.51±0.01	6.01±0.04
CAT (µM min^{-1} mg protein^{-1})	Control	250.81±15	255±12	241±11	280±5	367.7±13	460.5±14	550.2±9	760.3±16
	5 mgL^{-1} Pb	601.5±11	1737.3±61	846.3±27	312.9±29	194.7±31	180.9±21	174.6±15	156.4±11
IC_{50} of SOD (mg protein)$^{-1}$	Control	0.1±0.0011	0.12±0.001	0.08±0.001	0.17±0.01	0.2±0.012	0.23±0.012	0.26±0.01	0.36±0.016
	5 ppm Pb	0.127 ±0.0025	0.181 ±0.0026	0.372 ±0.0027	0.153 ±0.0026	0.12 ±0.003	0.101 ±0.004	0.011 ±0.001	0
APX (mM min^{-1} mg protein $^{-1}$)	Control	0.041 ±0.0025	0.041 ±0.002	0.042 ±0.0022	0.045 ±0.003	0.052 ±0.002	0.058 ±0.005	0.063 ±0.004	0.056 ±0.003
	5 mgL^{-1} Pb	0.048 ±0.0011	0.035 ±0.002	0.08 ±0.003	0.021 ±0.002	0.011 ±0.003	0.002 ±0.0001	0	0
Glutathione (nM g^{-1})	Control	0.042 ±0.0015	0.040 ±0.0015	0.0422 ±0.001	0.045 ±0.0012	0.055 ±0.0011	0.07 ±0.0013	0.05 ±0.0015	0.075 ±0.0026
	5 mgL^{-1} Pb	0.04 ±0.0011	0.0812 ±0.002	0.114 ±0.0017	0.128 ±0.0026	0.058 ±0.0011	0.0558 ±0.002	0.048 ±0.001	0.034 ±0.0011

Proline (mM g^{-1})	Control	43±1.5	44±1.2	43±2.0	47±1.7	51.2±2.6	55.2±2.8	68.4±3.1	88±4.2
	5 mgL^{-1} Pb	65±1.1	87±2.6	42±2.7	41±2.65	36±3.11	34±4.1	34±1.5	32±1.1
Carotenoids (mg g^{-1})	Control	0.48±0.025	0.478±0.0	0.49±0.0	0.52±0.0	0.65±0.0	0.79±0.0	0.99±0.0	0.76±0.0
	5 mgL^{-1} Pb	0.49 ±0.011	0.48 ±0.026	0.45 ±0.027	0.27 ±0.026	0.9 ±0.05	2.53 ±0.041	2.96 ±0.015	3.25 ±0.071

Table 2: Associated changes in biochemical parameters of gold exposed *Spirulina* with function of time

Table 2		**Time**							
		1h	**3h**	**24h**	**3d**	**7d**	**14d**	**21d**	**28d**
MDA (mMg^{-1})	Control	0	0.61±0.01	0.63±0.015	0.87±0.02	1.73±0.03	1.8±0.05	2.5±0.05	3.59±0.04
	5 mgL^{-1} Pb	0.193 ±0.002	1.99±0.032	2.05±0.077	2.02±0.046	2.39±0.03	7.79±0.05	9.51±0.01	9.9±0.04
CAT (µM min^{-1} mg $protein^{-1}$)	Control	323.1±15	345±12	310±11	350±5	360±13	430.5±14	670±9	693±16
	5 mgL^{-1} Pb	751.5±11	1372±61	1403 ±27	1903.5±29	2719.7±31	250±21	207.6±15	140.2±11
IC_{50} of SOD (mg protein)$^{-1}$	Control	0.02 ±0.002	0.024 ±0.001	0.028 ±0.001	0.044 ±0.01	0.056 ±0.01	0.063 ±0.012	1.26 ±0.06	2.37 ±0.06
	5 ppm Pb	0.025 ±0.002	0.037 ±0.003	0.058 ±0.004	0.041 ±0.002	0.025 ±0.0015	0.011 ±0.006	0	0
APX (mM min^{-1} mg $protein^{-1}$)	Control	0.106 ±0.002	0.041 ±0.002	0.042 ±0.0022	0.045 ±0.003	0.052 ±0.002	0.058 ±0.005	0.063 ±0.004	0.056 ±0.003
	5 mgL^{-1} Pb	0.129 ±0.002	0.295 ±0.003	0.345 ±0.004	0.12 ±0.001	0.051 ±0.002	0.019 ±0.006	0	0
Glutathione (nM g^{-1})	Control	0.091 ±0.0025	0.096 ±0.001	0.13 ±0.001	0.145 ±0.001	0.16 ±0.0015	0.27 ±0.002	0.35 ±0.001	0.75 ±0.002
	5 mgL^{-1} Pb	0.104 ±0.002	0.136 ±0.001	0.085 ±0.0012	0.081 ±0.0024	0.049 ±0.0011	0.039 ±0.001	0.035 ±0.002	0.027 ±0.002
Proline (mM g^{-1})	Control	49±2	46±1.5	47±2	52±1.5	71.5±2.8	85.2±2.7	85.4±3	98±5.2
	5 mgL^{-1} Pb	98±2	147±2.2	215±2.8	187±3.65	147±2.11	67±2.6	43±1.5	41±2.1
Carotenoids (mg g^{-1})	Control	0.47±0.025	0.48±0.02	0.495±0.024	0.55±0.015	0.68±0.01	0.8±0.012	1.19±0.011	1.76±0.01
	5 mgL^{-1} Pb	0.38 ±0.011	0.25 ±0.026	0.32 ±0.027	0.47 ±0.026	0.48 ±0.05	0.72 ±0.041	2.00 ±0.015	2.5 ±0.071

Highly significant (p=0.001) proline content was recorded in treated biomass of *Spirulina* up to 7 days of treatment being maximum at 24 h (220.00 □mol proline g^{-1} fresh weight). In contrast *Lyngbya* showed 2 fold increase in proline production (90.455 □mol proline g^{-1} fresh weight, p= 0.001) than that of control biomass at 3 h of metal exposure (Table 1 and 2). Proline accumulates rapidly under unfavorable conditions and is responsible for acting as osmoprotectant thereby protecting the enzymes and stabilizing the cytosolic acidity (Alia and Sharadhi 1991) [1]. There are also reports of considerable proline accumulation in *Hapalosiphon*, in Pb exposure (Zutshi *et al*. 2008) [16]. Therefore accumulation of high amount of proline in *Lyngbya* and *Spirulina* in the present study suggests its protective role against Pb stress during different exposure time. Carotenoid content was increased from 7 days onwards reaching its maximum value at 28th day of exposure which is almost 5 to 7 fold higher (p= 0.001) than that observed in control filaments (Table 1 and 2). Significant increase in carotenoids in long exposure indicated secondary carotenogenesis in Pb stress condition. Our result suggests that carotenoids being essential components of algae which participate in light harvesting process protect the photosynthetic apparatus from photo oxidative damage under long term exposure to Pb.

Form the present study, it can be concluded that in short term exposure stress enzymes together with increased level of proline gave the protection against oxidative damage, whereas in chronic exposure, cellular glutathione and carotenoids are responsible for metal resistance. The significant depletion of stress enzymes in long exposure may be due to the toxic effect of lead.

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