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Effects of heavy metals (Pb^{2+} and Cd^{2+}) on the ultrastructure, growth and pigment contents of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803*

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Abstract The unicellular cyanobacterium *Synechocystis* sp. PCC 6803, a model organism known for its unique combination of highly desirable molecular genetic, physiological and morphological characteristics, was employed in the present study. The species was cultured in BG11 liquid medium contained various initial concentrations of Pb^{2+} and Cd^{2+} (0, 0.5, 1, 2, 4, 6 and 8 mg/L). The experiment was conducted for six days and the metal induced alterations in the ultrastructure, growth and pigment contents were assessed. Alterations in the ultrastructure of the *Synechocystis* sp. PCC 6803 cells became evident with the increased (>4 mg/L Pb^{2+}) metal concentration. The photosynthetic apparatus (thylakoid membranes) were found to be the worst affected. Deteriorated or completely destroyed thylakoid membranes have made large empty spaces in the cell interior. In addition, at the highest concentration (8 mg/L Pb^{2+}), the polyphosphate granules became more prominent both in size and number. Despite the initial slight stimulations (0.2, 3.8 and 6.5% respectively at 0.5, 1 and 2 mg/L Pb^{2+}), both metals inhibited the growth in a dose-dependent manner as incubation progressed. Pigment contents (chlorophyll *a*, β carotene and phycocyanin) were also decreased with increasing metal concentration. Cells exposed to 6 mg/L Pb^{2+}, resulted in 36.56, 37.39 and 29.34% reductions of chlorophyll *a*, β carotene and phycocyanin respectively over the control. Corresponding reductions for the same Cd^{2+}concentrations were 57.83, 48.94 and 56.90%. Lethal concentration (96 h LC_{50}) values (3.47 mg/L Cd^{2+} and 12.11 mg/L Pb^{2+}) indicated that *Synechocystis* sp. PCC 6803 is more vulnerable to Cd^{2+} than Pb^{2+}.

Keyword: growth; pigment contents; *Synechocystis* sp. PCC 6803; ultrastructure

1 INTRODUCTION

Tremendously increasing population and establishment of industries in the urban areas result in the discharge of heavy metals, sewage effluents and organic pollutants into the water bodies. The problem of water pollution is acute in some countries than in others, but is basically the same throughout the world (Javed and Mahmood, 2000). Toxic heavy metals show harmful effects even at very low concentration on the aquatic organisms including plankton, aquatic plants, invertebrates and vertebrates (Atici et al., 2008). Cyanobacteria, a numerous and diverse group of photosynthetic prokaryotes are key contributors to the global photosynthetic productivity. Their response to toxic metal exposure is of great concern due to the fact that bioaccumulation of heavy metals in the aquatic food chain is highly dangerous (Sanita-di-Toppi and Gabbrielli, 1999). Aquatic plants and microorganisms are able to remove metals from water through processes of biosorption and metabolism dependent bioaccumulation (Wang et al., 1998). Microalgae, due to their ubiquitous occurrence in nature and metabolic uptake with continuous growth (Sobhan and Sternberg, 1999), have gained a paramount attention. The cell wall components of microorganisms such as polysaccharides, proteins and lipids offer many functional groups which can bind metal ions (Ari et al., 1999). However, the metal uptake process is complex and dependent upon not only the specific surface properties of the organisms but also cell physiology (Goudey, 1987) and other abiotic factors such as pH and temperature.

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The unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 is basically considered as a model organism which possesses virtually unique combination of highly desirable molecular genetic, physiological and morphological characteristics. This species contains both light-dependent and light-independent chlorophyll biosynthesis pathways (Beale, 1994), enabling the species to utilize culture conditions much easier than most of the other cyanobacteria. The species can grow under many different physiological conditions (i.e., photoauto/mixo/heterotrophically). Having these advantages, *Synechocystis* sp. PCC 6803 sounds a quicker and more convenient evaluator of the toxicity of various chemical pollutants. Furthermore, *Synechocystis* sp. PCC 6803 is the first phototroph to have its complete genomic sequence determined and thus is spontaneously transformable (Vermass, 1996). In ecological point of view, the species is an important component in the aquatic food chain and plays a vital role in the ecology of the fresh water ecosystems. However, only scanty information is available on the effect of heavy metals on growth, morphology and physiology of *Synechocystis* sp. PCC 6803.

Lead and cadmium are frequently found fresh water pollutants and both of them are not known to have biological functions in plants. Cadmium inhibits most of the basic physiological processes in plant cells. Its effects on chlorophyll biosynthesis, photosynthetic carbon assimilation and growth are well documented (Fargasova, 1999). The inhibition capacity of lead on different physiological parameters and photosynthesis in algae has also been studied extensively (Navarro et al., 1997). However, different organisms proved to have different sensitivities to the same metal, while the toxicity of different metals to same organism may also be varied (Fathi, 2002).The present study deals with the toxicity of lead and cadmium to the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803.

2 MATERIALS AND METHODS

2.1 Strain, media and culture procedures

The axenic stock cultures of the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 preserved in our laboratory at Life Science College, Ocean University of China, were used for the experiment. The species was grown in BG11 medium adjusted to pH 7.0 at 25°C. Cultures were gently stirred and illuminated with white light produced by neon tubes at 50 mmol photon m^{-2} s^{-1} with a light/dark cycle of 14:10 h. Seven days old cultures were spun down at 4 000 g for 10 min and the pellets were resuspended in fresh BG11 medium in order to use for the metal treatments.

2.2 Chemicals and analytical methods

The chemicals were of analytical grade and used without further purification. De-ionized water obtained from a Millipore Milli-Q system was used throughout the experiment. Lead nitrate [$Pb(NO_3)_2$] and cadmium chloride [$CdCl_2$] were used as the sources of Pb^{2+} and Cd^{2+} respectively. The stock solutions (1 000 mg/L) were prepared and kept in a refrigerator at 4°C until use. All the working solutions were prepared by properly diluting these stock solutions. Measurements of optical density were performed using a spectrophotometer (UV-2102) and pH of the medium was measured with a pH/ISE meter (model 868). A digital balance (Sartorins, BS 210 S) was used in measuring weights.

2.3 Metal treatments

Toxicity studies were performed separately for the two metals using 150 ml flasks. Each flask was filled with 100 ml of algal suspension, which contained various initial metal concentrations (0, 0.5, 1, 2, 4, 6 and 8 mg/L). Suspensions were continuously homogenized in a rotary shaker at 100 rpm and cultured for 6 days under the conditions as described above. The initial OD_{750nm} was set to 0.1.

2.4 Growth, ultrastructure and pigment contents

The growth of the algae was monitored by recording the optical density at 750 nm. Representative samples were filtered through a 0.22 μm membrane and the cells were dried at 103°C for 2 h to measure the dry weight. A good linear relationship; dry weight (g/L) = 0.357 4 × OD_{750nm} (r=0.992 3) was derived and used to estimate the algal dry weight at relevant OD values. The lethal concentration values (LC_{10}, LC_{25} and LC_{50}) were determined as the parameter of the toxicity. The morphological and ultrastructural observations described in this communication were made on cell material grown for 6 d.

At the end of 6 d, cultures were subjected to analyse pigment contents. Chlorophyll *a* and β carotene were extracted in 90% acetone and assayed according to Bwn-Amotz and Avron (1983). Phycocyanin content of the algal cells was determined spectrophotometrically. The cells were filtered on glass-fiber filter paper (whatman GF/C, 0.45 μm) before being meshed with a glass rod in a

plastic centrifuge tube by adding 10 ml of 0.01 mol/L Na_2HPO_4 solution containing 0.15 mol/L NaCl at pH 10. The meshed sample was sonicated for 4 min and centrifuged at 3 000 rpm for 10 min. The supernatant was transferred into a separate tube and the residue was resuspended in Na_2HPO_4 solution and the extraction was repeated. The supernatants were separated very carefully in order to remove any cell debris. The sample was made up to a known volume and spectrophotometer readings were taken according to (Bennert and Bogorad, 1973). All the procedures were performed under aseptic conditions and the results were based on detection in triplicate of the culture samples.

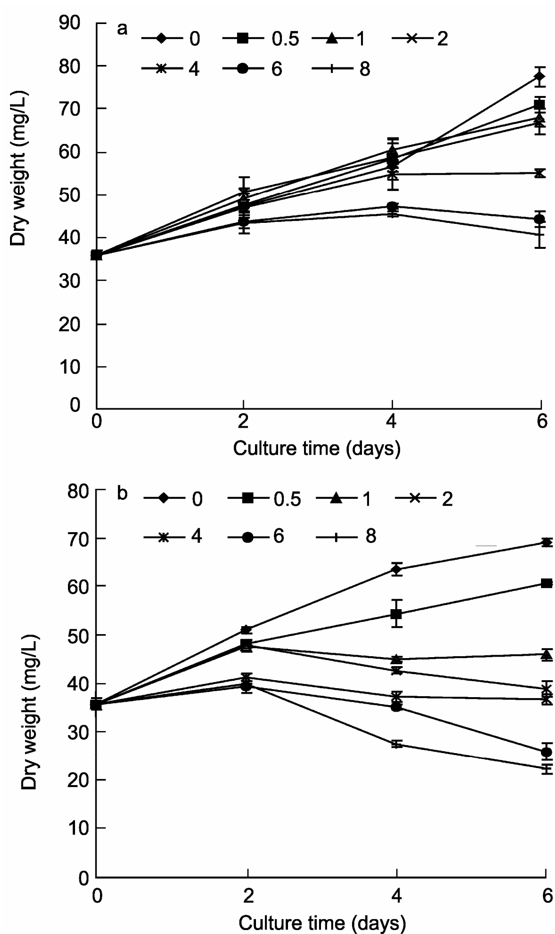

Fig.1 (a) The effects of Pb^{2+} on the growth of *Synechocystis* sp. PCC 6803; (b) The effect of Cd^{2+} on growth of *Synechocystis* sp. PCC 6803

The species was cultured in BG11 liquid medium (pH 7.0) under illumination of 50 mmol photon m^{-2} s^{-1} at 25±1°C. Mean ± SD, n = 3. The growth of the algae was monitored by recording the optical density at 750 nm. Representative samples were filtered through a 0.22 μm membrane and the cells were dried at 103°C for 2 h to measure the dry weight. A good linear relationship; dry weight (g/L) = 0.3574 × OD_{750nm} (r=0.9923) was derived and used to estimate the algal dry weight at relevant OD values.

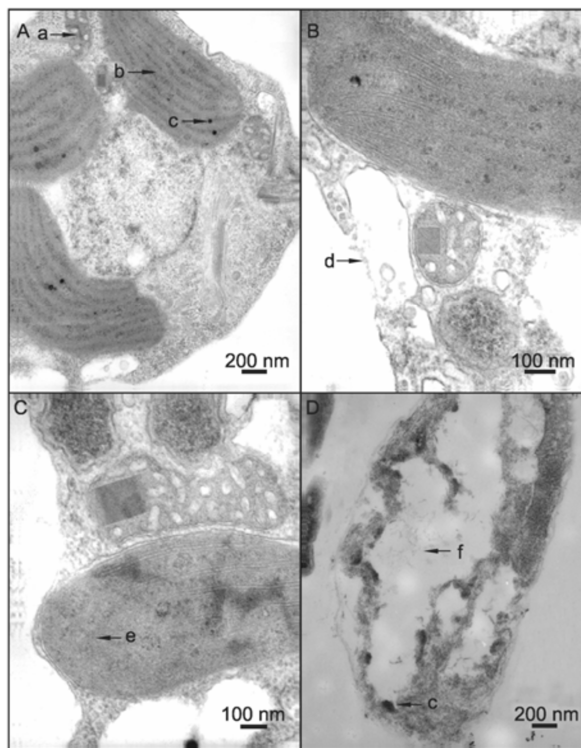

Fig.2 Electron micrographs of the *Synechocystis* sp. PCC 6803 cells grown in BG11 liquid medium (pH 7.0) under illumination of 50 mmol photon m^{-2} s^{-1} at 25±1°C

A. Normal cell grown in Pd^{2+} free BG11 medium; B. Cell grown in BG 11 medium with 4 mg/L Pb^{2+}; C. Cell grown in BG 11 medium with 6 mg/L Pb^{2+}; D. Cell grown in BG 11 medium with 8 mg/L Pb^{2+}

Carboxysomes: a. thylakoid membranes; b. polyphosphate; c. disorganized thylakoid membranes; d. destroyed plasma membrane; e. large empty space; f. Six days old cultures were used for taking micrographs

3 RESULTS

3.1 Growth

The growth of *Synechocystis* sp. PCC 6803 treated with different concentrations of Pb^{2+} and Cd^{2+} is illustrated in figure 1. Despite the low concentrations (0.5, 1, and 2 mg/L of Pb^{2+}) resulted in slight stimulations (0.2, 3.8 and 6.5% respectively) at 2 d, both metals inhibited the growth in a dose-dependent manner as incubation progressed. Compared to the control, inhibitions at the end of 6 d incubation were 8.4, 12.4, 13.9, 29.1, 42.9 and 47.6% respectively for 0.5, 1, 2, 4, 6 and 8 mg/L Pb^{2+}. The corresponding figures for Cd^{2+} were 12.2, 33.6, 43.7, 46.9, 62.5 and 67.9% respectively for 0.5, 1, 2, 4, 6 and 8 mg/L Cd^{2+}.

3.2 Pigment contents

As can be seen in Table 1, pigment contents (chlorophyll a, β carotene and phycocyanin) decreased with increasing metal concentration. In contrast, inhibitions caused by Cd^{2+} were considerably

Table 1 Effect of metals (Pb^{2+} and Cd^{2+}) on pigment contents (chlorophyll *a*, β carotene and phycocyanin) in *Synechocystis* sp. PCC 6803 cultured in BG11 medium (pH 7.0) under 50 mmol photon m^{-2} s^{-1} at 25±1°C (Mean ± S.D., *n* = 3. Six days old cultures were used for determining pigment contents)

Metal	Treatment (mg/L)	Pigment content (mg/L) ± SD		
		Chl *a*	β Car	PC
Pb^{2+}	0	0.425±0.024	0.230±0.015	0.317±0.014
	0.5	0.386±0.012	0.212±0.006	0.278±0.012
	1	0.339±0.007	0.180±0.011	0.267±0.004
	2	0.315±0.006	0.168±0.012	0.250±0.014
	4	0.307±0.013	0.154±0.014	0.236±0.010
	6	0.269±0.010	0.144±0.003	0.224±0.011
	8	0.253±0.011	0.116±0.008	0.185±0.019
Cd^{2+}	0	0.396±0.023	0.188±0.009	0.290±0.019
	0.5	0.355±0.012	0.180±0.021	0.289±0.011
	1	0.330±0.011	0.160±0.007	0.266±0.003
	2	0.269±0.009	0.144±0.011	0.234±0.005
	4	0.244±0.015	0.132±0.007	0.215±0.007
	6	0.167±0.008	0.096±0.008	0.125±0.017
	8	NA[d]	NA[d]	NA[d]

Chl *a*: chlorophyll *a*; β Car: β carotene; PC: phycocyanin; NA: not analyzed

Table 2 Lethal (effective) concentration (mg/L) for Pb^{2+} and Cd^{2+} with *Synechocystis* sp. PCC 6803 cultured in BG11 medium (pH 7.0) under 50 mmol photon m^{-2} s^{-1} at 25±1°C (*n*=3; Associated 95% confidence intervals are given the parentheses)

Metal	Lethal (effective) concentration[e] (mg/L)		
	LC_{10}	LC_{25}	LC_{50}
Pb^{2+}	5.33 (7.46—3.81)	7.87 (11.02—5.62)	12.11 (16.94—8.45)
Cd^{2+}	0.32 (0.79—0.13)	0.99 (2.44—0.40)	3.47 (8.51—1.41)

The lethal (effective) concentration is the concentration of the metal required for relevant inhibition (10, 25 and 50% respectively for LC_{10}, LC_{25} and LC_{50}) of the growth of *Synechocystis* sp. PCC 6803 at 96 h exposure.

higher than those of Pb^{2+}. Cells exposed to 6 mg/L Pb^{2+}, resulted in 36.56, 37.39 and 29.34% inhibitions of chlorophyll *a*, β carotene and phycocyanin respectively. While the inhibitions caused by the same Cd^{2+} concentration were 57.83, 48.94 and 56.90%, respectively for chlorophyll *a*, β carotene and phycocyanin.

3.3 Lethal (effective) concentration

Toxicity measured as the lethal concentration values for the two metals is given in Table 2. After 96 h exposure, the LC_{10}, LC_{25} and LC_{50} values for Pb^{2+} were considerably higher than those of Cd^{2+}.

3.4 Morphology

The ultrastructure of the *Synechocystis* sp. PCC 6803 cells seemed to experience some drastic changes when exposed to 8 mg/L Pb^{2+}. The photosynthetic apparatus (thylakoid membranes) shown to be the worst affected. Deteriorated or completely destroyed thylakoid membranes have made large empty spaces in the cell interior (Fig.2D). When exposed to 6 mg/L Pb^{2+}, thylakoid membranes appeared to be largely disorganized and the regular distance between each other was hard to evident (Fig.2C). In addition, at the highest exposure (8 mg/L Pb^{2+}), the polyphosphate granules became more prominent both in size and number (Fig.2D). Simultaneously to the ultrastructure, gradual changes in the overall shape of the cells could also be observed with the increasing metal exposure. The initial blue-green medium was first changed to yellow-green and then gradually became colorless as increased metal stress.

4 DISCUSSION

In general, changes in cell density, biomass, growth rate, chlorophyll content or absorbance are used in assessing the algal responses to metal exposure. Studies of the effects of Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Fe^{2+} on the green alga *Scenedesmus quadricauda* revealed that the toxicity for all the observed parameters including the growth was increased in a dose-dependent manner (Fargasova,

1999), which is in good agreement with the present results of growth. We observed higher inhibitions in Cd^{2+} treated cultures than those of Pb^{2+} treated; indicating Cd^{2+} is more toxic to *Synechocystis* sp. PCC 6803 than Pb^{2+}. The similar evidence could be found in cyanobacterium, *Anabeana flos-aquae*, which needed just 0.15 mg/L Cd^{2+} to inhibit the growth by 50%, while the species needed 1 mg/L of Pb^{2+} to exhibit the same inhibition (Heng et al., 2004).

Chlorophyll biosynthesis was reported to be inhibited by both Cd^{2+} and Pb^{2+}, leading to the lowered chlorophyll contents (Pahlsson, 1989). Studies with *Cladophora fracta* reported that Pb^{2+} and Cd^{2+} at high concentrations can destroy the chloroplasts (Lamaia et al., 2005). Similarly we observed destroyed and/or disorganized thylakoid membranes in *Synechocystis* sp. PCC 6803 at higher (6 and 8 mg/L Pb^{2+}) exposure. In fact, disorganization of chloroplasts causing damages to photosynthetic pigments is well known for Cd^{2+} toxicity (Leborans and Novillo, 1996). Pd^{2+} is capable of binding to thylakoid membranes, deteriorating their routing functions (Heng et al., 2004). This was confirmed by the drastic changes we observed in the overall ultrastructure of *Synechocystis* sp. PCC 6803 cells in which the surface area of the thylakoids has decreased significantly (Fig.2D). As a consequence, the photosynthetic activity could severely be affected causing growth inhibition or complete death of the cells. According to Atici et al. (2008), Cd affects the photosynthesis and reduces the primary productivity of phytoplankton even at 0.2 mg/L and it affects the community structure of zooplankton too. Biosynthesis of phycocyanin and carotenoid was also proved to be affected by the heavy metals as reported by Atri and Rai (2003). Taking into account all the reports, we confirm here that poor carbon assimilation due to loss of pigments is the major reason for the growth inhibition observed in the present study. In addition, heavy metals could interrupt routing metabolic processes by competing for the protein binding sites, active enzymes and various biological reactive groups, causing poor or no growth.

In order to assess algal responses to toxic metals, the LC_{50} (or EC_{50}) values could effectively be used, despite they are usually preferred for statistical rigor. We observed considerably higher LC_{50} values for Pb^{2+} than those of Cd^{2+} (Table 1). This was obviously different from the findings of Satoha et al. (2005), who reported only marginal differences. Their LC_{50} values (mg/L) respectively for Pb^{2+} and Cd^{2+} were 5.3 and 5.4 for *Synechococcus*; 14.4 and 13.8 for *Heterocapsa* sp. and 10.6 and 9.7 for *Chlorococcum littorale*. However, the exceptionally high (21.4 mg/L Pb^{2+}) LC_{50} value they reported for *Chlorococcum* sp. was a clear indication of possible diverse results as we observed for Pb^{2+}. In addition, our LC_{50} values for Cd^{2+} were within the range (0.05–7.5 mg/L Cd^{2+}) reported by Ismail et al. (2002) who assessed four microalgae species (*Chaetoceros calcitrans*, *Isochrysis galbana, Tetraselmis tetrathele* and *Tetraselmis* sp.) for Cd^{2+} toxicity. Toxicity studies are generally been conducted under different laboratory and experimental conditions with various species. Thus it would hardly evident similar results and the differences in LC_{50} values are unavoidable.

The cells of *Synechocystis* sp. PCC 6803 are basically spherical (Liberton et al., 2006) with the vast majority of the interior part occupied by the thylakoid membranes, however, under Pb^{2+} stressed condition, the spherical shape of the cells has partly or completely changed leaving no specific shape. Thylakoid membranes of *Synechocystis* sp. PCC 6803 cells often maintain a regular distance among each other (Liberton et al., 2006). This could hardly be seen at higher (6 and 8 mg/L Pb^{2+}) exposure, as they were largely disorganized or completely destroyed (Fig.2C and D). The most obvious alteration of the ultrastructure, the vast empty space in the interior is due to apparent loss of thylakoid membranes. This was proved to be common in cyanobacterial response to metal toxicity, as reported by Rangsayatorn et al. (2002) for *Anabaena flos-aquae* exposed to Pb^{2+} and Zn^{2+}. Apart from the thylakoid membranes, the interior contents of a normal *Synechocystis* sp. PCC 6803 cell includes polygonal carboxysomes, cyanophycin granules, glycogen granules and polyphosphate bodies along with a large number of ribosomes. However, at 8 mg/L Pb^{2+}, these components were hardly evident, except polyphosphate granules which increased both in the number and size. Cyanobacteria have the ability to incorporate heavy metals into the polyphosphate granules, which can then act as detoxificants as reported by Jensen et al. (1982). Furthermore, Pettersson et al. (1988) reported that polyphosphate granules offer a significant binding site for many different heavy metals. This ability could lead the bioaccumulation of heavy metals in the food chain causing potential risk to organisms at

the higher levels. However, bioaccumulation of heavy metals in plankton depends on many factors, such as absorptive ability of individual species and season (Atici et al., 2008).

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