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Toxicity and bioaccumulation of lead, cadmium and zinc in Chroococcus minutus and Chlorococcum aegyptiacum.

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ABSTRACT

Studies on toxicity and bioaccumulation of lead, cadmium and zinc in the blue-green alga Chroococcus minutus and the green alga Chlorococcum aegyptiacum were conducted by short-term bioassays using toxicity symptoms, scientific growth rate, chlorophyll a, removal % and bioaccumulation factor parameters. The toxicity symptoms of the studied metals include increasing in crystalline inclusions, aggregation of thylakoid membranes at the sides of the cell and partial disorganization of the cell wall in the case of Chroococcus minutus and a major change in shape of cell wall, damage of chloroplasts, reduction in number of chloroplasts, complete disorganization of the cell components and formation of granules and disintegrated cell wall and cell death in the case of Chlorococcum aegyptiacum. There were significant decreases in the specific growth rate and chlorophyll a contents when the metal concentrations were increased. The removal % and bioaccumulation factor (BCF) of Zn were higher than that of Cd and Pb suggesting that the accumulation potential of the studied two microalgae for Zn was higher than that for Cd and Pb.

Keywords: *Toxicity; Bioaccumulation; Chroococcus minutus; Chlorococcum aegyptiacum*

INTRODUCTION

Heavy metals are among the most dangerous substances in the environment, because of their high level of durability and harmfulness to live organisms. With the rapid industrial development, various wastes containing different metal ions are directly or indirectly discharged into the environment, bringing about serious environmental pollution, and threatening marine life [1]. Industrial effluents may be discharged directly into the sea, or into waterways or sewer but whatever the disposal route, these constitute an important source of contamination of the environment. Many industries discharge the heavy metals lead (Pb) and cadmium (Cd) in their wastewaters. Unlike many other pollutants in the environment, heavy metals are non-biodegradable [2]. Remediation processes for heavy metal-polluted ecosystems are difficult and expensive. Heavy metals can also be accumulated by some organisms through the food chain, eventually posing a serious health risk to inhabitants of an ecosystem, including humans [3, 4]. The bioaccumulation of toxicants, such as heavy metals, by living microorganisms, is generally a good integrative index of exposure and has been extensively used to assess contamination levels of heavy metals in polluted ecosystems [5].

Lead and cadmium are toxic heavy metals and are considered non-essential for living organisms. There is much evidence that algae could accumulate heavy metals in their tissues when grown in polluted waters. Heavy metals

such as Cd, Zn, in wastewaters are hazardous to the environment. These ions may cause toxic and harmful effects to living organisms in water and to the consumers of its [6]. Heavy metal disperses through the various trophic levels of an ecosystem, depending on the bioaccumulation characteristics of the metal of concern. Bioaccumulation occurs when a portion of a metal is retained by an organism. The term bioaccumulation describes an active process in which taking up metal is metabolically controlled. However, heavy metal accumulation, bioavailability, and toxicity in aquatic biota depend essentially on many environmental variables [7]. Many heavy metal ions have a direct influence on various physiological and biochemical processes of microalgae [8].

Microalgae are sensitive indicators of environmental change and, as the basis of most marine and fresh water ecosystems, are widely used in the estimation of hazard development of environmental regulations for metals [9]. There are a remarkable number of investigations demonstrating the toxic effects of heavy metals on different species of algae [10, 11].

The effect of heavy metals on aquatic organisms is currently attracting broad concern, especially in studies related to industrial and anthropogenic pollution. The enrichment of coastal waters with trace metals through sewage and other anthropogenic sources has become a dangerous issue. This circumstance has resulted in considerable studies of the effects of heavy metals on benthic marine algae, especially in coastal areas [12].

As the growth reflects the metabolism of the cell, it has been used as a key indicator of the toxicity of heavy metal ions in microorganisms [13] and it depends on the proper functioning of various metabolic pathways, such as respiration, photosynthesis and nutrient uptake [14]. Growth inhibition in microalgae is related to a number of heavy metal ions bound to the algal cell surface, in some cases, to the amount of intracellular heavy metal ions [15] and to the chemical nature of the heavy metal ions [14]. However, for zinc, growth inhibition may not be related to the intracellular metal concentration, but to extracellular zinc [16]. In fact, the possible mode of toxic action of zinc is related to the plasma membrane, where it may damage the uptake of Ca which is necessary for the Ca-ATPase activity in cell division [17]. Metal uptake by microorganisms has been studied for some years [18, 19]. Research indicates that algae have the ability to accumulate trace metals by bioaccumulation and biosorption. Bioaccumulation is an increasing in the concentrations of a chemical in a microorganism over time, compared to the chemical's concentration in the environment. In this study, the toxicological effects and bioaccumulation of Pb, Cd and Zn cations upon the growth of two microalgae species, *Chroococcus minutus* and *Chlorococcum aegyptiacum*, were thus studied.

MATERIALS AND METHODS

Microalgal materials

Samples of *Chroococcus minutus* and *Chlorococcum aegyptiacum* were kindly supplied by Prof. Dr. Ibraheem, I.B.M. Manager of *Phycological Laboratory, Faculty of Science, Beni-Suef University, Egypt*.

Culture conditions.

Both species of microalgae wine grown in BG-11medium under controlled conditions (28 ± 2ºC, 2200 Lux, 12h/12h light and dark cycle) [20]. The pH of the cultures was 7.0. Algae were exposed to various concentrations of heavy metals (Cd^{2+}, $Pb^{2+}$and Zn^{2+}).

Heavy metal toxicity test

The cultures were supplemented separately with two nominal concentrations of Pb^{2+}(10 and 15 mgL^{-1}), Cd^{2+} (0.5 and 1.0 mgL^{-1}) and Zn^{2+}(10 and 15 mgL^{-1}) (metal sunder experiments) stock solutions were prepared by dissolving their corresponding the salts of $Pb(NO_3)_2$, $Cd(NO_3)_2$ and $Zn(NO_3)_2$(analytical grade from Merck) in deionized water. The heavy metal solutions were introduced with known concentration (Co) into the flasks (each solution contained one metal ion) except one of the flasks that were used as a control. The control solution, containing only nutrient medium and algal biomass. Fresh one microalga species (as living biomass) was added to each flask (4x103 cells ml^{-1}). The initial pH of all solutions was 7.0. The tested concentrations of Pb, Cd, and Zn were different according to levels of their toxicity in a preliminary test (data not included in this paper). There were 3 replicates for each treatment.

a) Toxicity Symptoms

After the exposure of *C. minutus* and *C. aegyptiacum* to Pb, Cd, and Zn, algae were harvested at the end of the test duration (12^{th} day). Toxicity symptoms of treated and control algae were observed under a compound transmission light microscope.

b) Effect on specific growth rate (SGR) and generation time (tg)

In comparing the results of bioassay test, or growth of different microalgae under the same or differ conditions, it is needed to detect growth rates experimentally. Data of cell density are usually gained with a hem cytometer. Growth curves are prepared from data obtained by sampling cultures at intervals (6^{th} and 12^{th} days). Plots of the number of cells against time (in days), and from these curves can be calculated specific growth rate or growth constant (u) and division or generation time (tg). Growth rate (u) is calculated with the following equation:

$$u = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

Where X_1 and X_2 are densities at times t_1 and t_2.

Division time (tg) is calculated with the following equation [21]:

$tg = 0.6931/u$

Effect on Chlorophyll *a* content.

Chlorophyll *a* contents in algae from each treatment were determined after 12 days of experiment by the absorption spectra of algal extract in a spectrophotometer method [22-24]. The microalgal cells were harvested and weighed, thin the chlorophylls were extracted by homogenizing cells twice in a grinder with 80 % acetone. The homogenate was quantitatively transmitted to a 1.5 ml micro centrifuge tube and centrifuged at 3000 r.p.m. for 3 minutes. The filtered extract was placed in the dark and immediately diluted to a 1 ml in a micro centrifuge tube with acetone. The absorbance of the extract was measured at 663 and 645 nm where maximum absorption by chlorophyll a occurred. The instrument was set at zero absorbance with a tube containing acetone before reading was made with the solution of pigments. The concentration of chlorophyll a in mgL^{-1} was calculated by the formula:

Chlorophyll a mgL^{-1} = 12.7 x A 663 – 2.69 x A 645

Heavy metal bioaccumulation.

Percentage of heavy metal removal:

The percentage of metal removal (U %) was calculated according to the following equation [25]:

$$U = \frac{C_o - C_1}{C_o} \times 100$$

Where, C_0 is the initial metal concentration in the solution and C_1 is the remaining concentration of the metal in the medium

Bio concentration factor (BCF)

The BCF was determined for quantifying the metal removal potential of the microalgae. The factor is defined as the ratio of the metal concentration in the dry algal biomass (ug) to the initial concentration of metal in the growth solution (ug) [26]. The BCFs for Pb, Cd, and Zn at the 12^{th} day were determined [27].

Statistical analysis:

Results were tested by one-way Analysis of Variance (ANOVA). ANOVA effects and treatments differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Toxicity symptoms

The toxicity symptoms observed in Pb, Cd and Zn treatments were rather similar. The symptoms appeared on *C. minutus* included increasing in crystalline inclusions, aggregation of thylakoid membranes at the sides of the cell and partial disorganization of the cell wall. On the other hand, the toxicity symptoms appeared on *C. aegyptiacum* included major changes in the shape of the cell wall, damage of chloroplasts, reduction in the number of chloroplasts, complete disorganization of the cell components and formation of granules and disintegrated cell wall and cell death. These symptoms were more severe when the metal concentration was increased. Similar results were obtained from the toxicity symptom study of metal on *Chlorella* [28]. *Chlorella* cells were useful in the characterization of the toxicity of Pb and Cd metals and organic contaminants. The chloroplast is the organelle most affected by metal contamination. The number and size of chloroplasts reduced, and their inner membranes (especially grana) were decreased and swollen. Uptake and excess of metals by plants and algae can initiate a variety of metabolic reactions, finally leading to global phytotoxic responses, e.g., dwarf growth and chlorosis. They are generally considered to affect membrane permeability and to induce cell decompartmentation. An important harmful effect of metals at the cellular level is the alteration of the plasma membrane permeability, leading to leakage of ions like potassium and other solutes [29].

Specific growth rate and generation time

The effects of different concentrations of Pb and Cd and Zn on the specific growth and generation time of *C. minutus* and *C. aegyptiacum* are shown in Table 1 and Figures 1& 2. The specific growth rate of algae exposed to the three metals at every concentration was significantly decreased ($P\leq0.05$) from those of controls. In the case of *C. minutus* the growth rate was reduced to 65, 58.6 and 51 %after exposed to 15 mgL^{-1} Pb^{2+}, 10 mgL^{-1} Pb^{2+} and 1.0 mgL^{-1} Cd^{2+}, respectively. Whereas, in the case of *C. aegyptiacum*, the growth rate was reduced to 75.5, 68 and 60 % after exposed to 15 mgL^{-1} Pb^{2+}, 1.0 mgL^{-1} Cd^{2+} and 10 mgL^{-1} Zn^{2+}, respectively. Regarding the generation time of algae exposed to the three metals at every concentration were significantly increased ($P\leq0.05$) from those of controls. Some workers studied the Pb influence on the specific growth rate of *Lemna gibba*. They found that high Pb concentrations (200-500 mg/L) in the media significantly inhibited the specific growth rate of *L. gibba* [30]. This might be due to the fact that Pb induces the activity of the enzyme peroxidase that is involved in the degradation of indole acetic acid (IAA), the hormone which stimulates growth and multiplication. Several studies have reported on the effects of Cd and algal growth. Other workers studied the effects of Cd, Cu, Zn, Pb and Fe on the green alga *Scenedesmus quadricauda* and found that the toxicity for all the observed parameters increased with the concentration of these metals in the cultivation medium [31]. It was reported that Cd had slight inhibitory effects on algal growth at low concentration (0.05 mg/L), while it severely inhibited algal growth at higher concentrations (>1.0 mg/L) [32]. Other workers found that Cd caused a decrease of the cellular volume, the growth rate and of the level of photosynthetic pigments [33].

Table 1. Specific growth rate (μ) of *Chroococcus minutus* and *Chlorococcum aegyptiacum* exposed to different concentrations (mgL^{-1}) of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days (each value is a mean of three replicates) and generation time (tg) in days.

Metal Conc.		*Chroococcus minutus*		*Chlorococcum aegyptiacum*	
		Specific growth rate (μ)	**Generation Time/ days (tg)**	**Specific growth rate (μ)**	**Generation Time / days (tg)**
Control 0.0		0.29	2.39	0.25	2.77
Pb^{2+} mgL^{-1}	**10**	0.12 ± 0.03	3.30	0.18 ± 0.01	3.65
	15	0.10 ± 0.01	6.93	0.062 ± 0.02	11.18
Cd^{2+} mgL^{-1}	**0.5**	0.23 ±0.01	3.01	0.19 ± 0.01	3.65
	1.0	0.14 ±0.02	4.95	0.08 ± 0.01	8.66
Zn^{2+} mgL^{-1}	**10**	0.24 ± 0.02	2.89	0.20 ± 0.01	3.46
	15	0.18 ± 0.01	3.95	0.10 ± 0.02	6.93

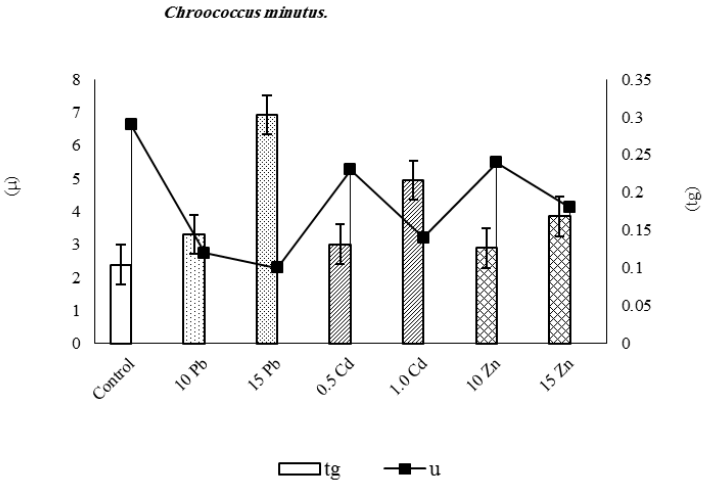

Figure 1. Specific growth rate (μ) of *Chroococcus minutus* exposed to different concentrations (mgL^{-1}) of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days (each value is a mean of three replicates) and generation time (tg) in days.

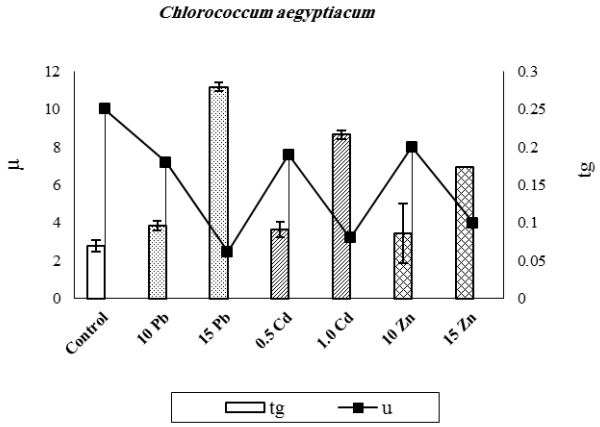

Figure 2. Specific growth rate (μ) of *Chlorococcum aegyptiacum* exposed to different concentrations (mgL^{-1}) of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days (each value is a mean of three replicates) and generation time (tg) in days.

Chlorophyll a contents

The effects of different concentrations of Pb and Cd and Zn on chlorophyll a contents of *C. minutus* and *C. aegyptiacum* at are shown in Table 2 and Figure 3. There were significant decreases ($P \leq 0.05$) of chlorophyll *a* contents in the two studied microalgae when the metal concentration was increased. The lowest chlorophyll *a* contents were found in *C. minutes* exposed to 15 mgL^{-1} of Pb and 1.0 mgL^{-1} of Cd in the case of and *C.aegyptiacum*. According to Figure 3, it is appeared that the toxic effect of heavy metals on the chlorophyll *a* contents of the two algae is in the following arrangement: $Pb^{2+} > Cd^{2+} > Zn^{2+}$. It is well known that Cd can cause disorganization of chloroplasts leading to a reduction of the photosynthetic pigments [33]. Both Cd and Pb were reported to inhibit the biosynthesis of chlorophyll, leading to the decreasing of chlorophyll contents [34]. It was found that the decline in chlorophyll content might be caused by a reduction in the synthesis of chlorophyll, possibly by increasing chlorophyllase activity, by disorders of chloroplast membrane and by inactivation of electron transport in photosystem I [35].

Table 2. The effect of different concentrations of Pb^{2+} and Cd^{2+} and Zn^{2+} (mgL^{-1}) on chlorophyll *a* contents of *Chroococcus minutus* and *Chlorococcum aegyptiacum* after 12 days (each value is a mean of three replicates).

Metal Conc.		***Chroococcus minutus***		***Chlorococcum aegyptiacum***	
		Removal %	**BCF**	**Removal %**	**BCF**
Control 0.0		0.0	0.0	0.0	0.0
Pb^{2+} mgL^{-1}	**10**	52 ± 2.30	5120	22 ± 1.90	2100
	15	40 ± 2.12	3950	16 ± 1.04	1450
Cd^{2+} mgL^{-1}	**0.5**	55 ± 3.02	5460	35 ± 2.08	3400
	1.0	48 ± 1.05	4655	28 ± 2.07	2700
Zn^{2+} mgL^{-1}	**10**	68 ± 3.50	6740	35 ± 3.00	3300
	15	52 ± 3.04	5100	28 ± 1.65	2700

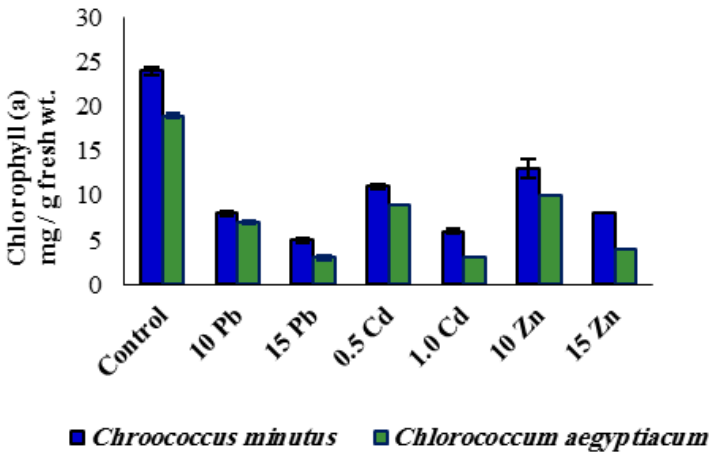

Figure 3. The effect of different concentrations of Pb^{2+} and Cd^{2+} and Zn^{2+} (mgL^{-1}) on chlorophyll *a* contents of *Chroococcus minutus* and *Chlorococcum aegyptiacum* after 12 days (each value is a mean of three replicates).

Heavy metal bioaccumulation.

Percentage of heavy metal removal and Bio concentration factor (BCF).

Table 3 and Figures 4 & 5 showed the removal % of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days by the two studied algal cells as reflected by the percentage removal of the three metals in the culture solutions and their concentration factors. In general, it seemed to be a positive relationship between the two parameters, percentage removal and concentration factors [10]. Higher removal efficiencies and concentration factors were observed with the two microalgae. The above results indicated that *Chroococcus minutes* had higher removal efficiencies of the three metals from the culture solution as compared with that of *Chlorococcum aegyptiacum*. It was suggested that the kinetics of metal uptake may actually comprise of a two-stage mechanism involving initially passive adsorption, which is very rapid and occurs a short time after the microorganisms come into contact with the metal [36]. This is then followed by a slow, possibly active metabolic uptake. The first step involves nonspecific binding of metal to the cell surface, slime layers, extracellular matrices, etc., whereas the second one involves metabolism-dependent intracellular uptake. Exchange of metal cations for constituents on the cell wall has also been proposed as a possible method by which algae could remove metal ions from solution. The data illustrate in Table 3 and Figures 4 & 5 shown a linear removal percentage and concentration factors of the three metals by the two studied microalgal taxa. A similar work was conducted by Vymazal, who concluded that metal uptake was linear over a certain range, but as the quantity of biomass in relation to the available metal is increased, proportional accumulation diminishes hyperbolically as weight dilution effect appear [37]. The bioaccumulation factor (BCF) is a beneficial parameter to estimate the potential of algae for accumulating metals and this value was calculated on a dry weight basis. Similar experiments and similar results have also been reported by Zhu et al., who found that BCFs of water hyacinth were exceedingly high for Cu, Cd, Cr, and Se at low external concentrations [38]. From the view of phytoremediation, a good accumulator should have the ability to concentrate the elements in its tissue, for example, a BCF of more than 1,000 (100-fold compared on a fresh weight basis) [39]. Based on this criterion, the present results showed that *Chroococcus minutus* is a good accumulator of Pb, Cd, and Zn with high BCF values (see Table 3 and Figures 4&5).

Table 3. Percentage of metal removal from the culture media by *Chroococcus minutus* and *Chlorococcum aegyptiacum* exposed to different concentrations (mgL^{-1}) of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days and Bioaccumulation factor (BCF). Each value is a mean of three replicates.

Metal Concentration mgL^{-1}		**Chlorophyll a contents mg g^{-1} fresh weight**	
		Chroococcus minutus	***Chlorococcum aegyptiacum***
Control		24 ± 1.72	19 ± 1.8
Pb^{2+} mgL^{-1}	**10**	16 ± 1.2	12 ± 0.3
	15	3.0 ± 0.1	8.0 ± 0.2
Cd^{2+} mgL^{-1}	**0.5**	17 ± 1.5	14 ± 0.7
	1.0	8.0 ± 0.9	1.0 ± 0.03
Zn^{2+} mgL^{-1}	**5**	19 ± 1.4	17 ± 1.1
	10	14 ± 0.9	4.0 ± 0.2

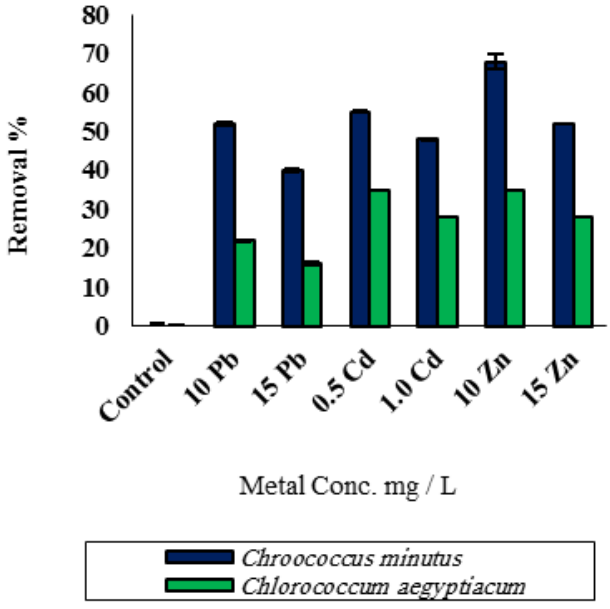

Figure 4. Percentage of metal removal from the culture media by *Chroococcus minutus* and *Chlorococcum aegyptiacum* exposed to different concentrations (mgL^{-1}) of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days. Each value is a mean of three replicates.

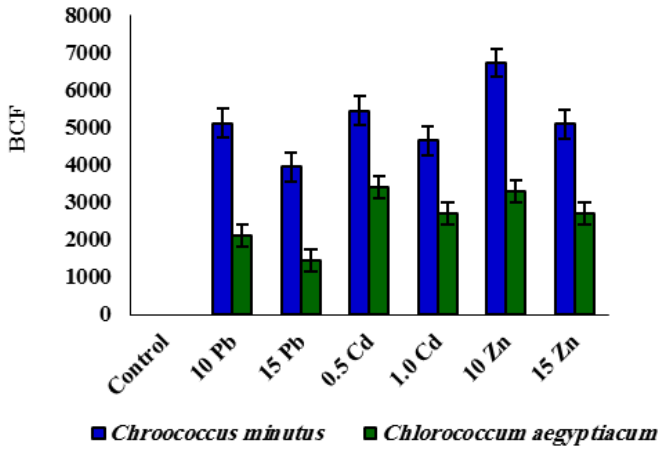

Figure 5. Bioaccumulation factor (BCF) of studied heavy metals achieved by *Chroococcus minutus* and *Chlorococcum aegyptiacum* exposed to different concentrations (mgL^{-1}) of Pb^{2+} and Cd^{2+} and Zn^{2+} after 12 days.

CONCLUSION

In this study, we examined toxicity and bioaccumulation of three heavy metals on *Chroococcus minutus* and *Chlorococcum aegyptiacum*. Lead was the most hazardous chemical to the tested microalgae, followed by cadmium and zinc, respectively. This has proven to be useful in terms of employing these isolated microalgae as a bio indicator for Pb, Cd, and Zn. The inhibitory effects of the used heavy metals depend on the used concentration. However, different organisms have different sensitivities to the same metal and the same organisms may be more or less damaged by different metals. The uptake of an element from the surrounding medium is seldom exactly proportional to the amount present in the medium.

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