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Bioremoval capacity of three heavy metals by some microalgae species (Egyptian Isolates)

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Three fresh water microalgal isolates [*Phormidium ambiguum* (Cyanobacterium), *Pseudochlorococcum typicum* and *Scenedesmus quadricauda* var *quadrispina* (Chlorophyta)] were tested for tolerance and removal of mercury (Hg^{2+}), lead (Pb^{2+}) and cadmium (Cd^{2+}) in aqueous solutions as a single metal species at conc. 5–100 mg / L under controled laboratory conditions. The obtained results showed that Hg^{2+} was the most toxic of the three metal ions to the test algae even at low concentration (< 20 mg/L). While lower concentration of Pb^{2+} and Cd^{2+} (5–20 mg / L) enhanced the algal growth (chlorophyll a and protein), elevated concentrations (40–100 mg / L) were inhibitory to the growth. The results also revealed that *Ph. ambiguum* was the most sensitive alga to the three metal ions even at lower concentrations (5 and 10 mg / L) while *P. typicum* and *S. quadricauda* were more tolerant to high metal concentrations up to 100 mg / L. The bioremoval of heavy metal ions (Hg^{2+}, Pb^{2+} and Cd^{2+}) by *P. typicum* from aqueous solution showed that the highest percentage of metal bioremoval occurred in the first 30 min of contact recording 97% (Hg^{2+}), 86% (Cd^{2+}) and 70% (Pb^{2+}). Transmission electron microscopy (TEM) was used to study the interaction between heavy metal ions and *P. typicum* cells. At ultrastructural level, an electron dense layers were detected on the algal cell surfaces when exposed to Cd, Hg, and Pb. At the same time, dark spherical electron dense bodies were accumulated in the vacuoles of the algal cells exposed to Pb. Excessive accumulation of starch around the pyrenoids were recorded as well as deteriorations of the algal cell organelles exposed to the three metal ions

Introduction

Heavy metals are elements having atomic weights between 63.5 and 200.6, and a specific gravity greater than 5.0. Living organisms require trace amounts of some heavy metals, including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium, and zinc. Excessive levels of essential metals, however, can be detrimental to the organism. Non-essential heavy metals of particular concern to surface water systems are cadmium, chromium, mercury, lead, arsenic, and antimony. Heavy metals which are relatively abundant in the Earth's crust and frequently used in industrial processes or agriculture are toxic to humans. These can make significant alterations to the biochemical cycles of living bodies.[1]

The presence of heavy metals in water and wastewater is increasing due to the industrial development-disposal in the sewerage or in the water bodies. Cadmium, Mercury and Lead, are the big three heavy metals posing the greatest hazard to human health, in addition to As, Be, and Cr which are known to be carcinogenic. It can create serious damage to the aquatic life because they are accumulated through the trophic chain and produce toxic effect and teratogenic changes in plants, animals and human beings. They also remain in the sediments and are slowly released into the final receptor water.[2]

Uptake and accumulation of heavy metals by crop plants[3,4] represents the main entry pathway for potentially health-threatening toxic metals into human and animal food of major concerne are the metalloid arsenic (As), Selenium (Se) and metals, cadmium (Cd), mercury (Hg) and lead (Pb).[5] Many investigations were directed toward the use of aquatic macrophytes (Eichhornia, Azolla, Salvinia, Lemna,…) in metal ion bioremoval.[6,7]

The accumulation of metals by algae, bacteria, fungi and yeast has been extensively studied in the last two decades. Of the microorganism studied, algae are gaining increasing attention, due to the fact that algae, particularly marine algae, are a rich source in the oceanic environment, relatively cheap to process and able to accumulate high metal content.[2]

Although adsorption on the cell surface is the dominant mechanism both surface adsorption and internal diffusion are involved in the uptake of metals by algae.[8,9] Biosorption occurs by both metabolically and non-metabolically mediated processes.

Conventional physicochemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation, and sorption for heavy metal removal from waste streams are not cost effective and hence biological approach has been considered as an alternative remediation for heavy metal contamination. Recently microbial systems like fungus, bacteria and algae have been successfully used as adsorbing agents for

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removal of heavy metals Microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal resistant.[10]

Metal concentrations absorbed by algae (macro and microalgae) were influenced by many environmental variables. It was strongly pH dependent and the presence of co-cations generally reduced the uptake of the target cations by algae.

Metal removing capability was both metal and alga-specific; certain algae (Chlorella, Scenedesmus, Hydrodictyon) performed better over all than the remaining strains. Certain algal species remove > 90% of at least one metal and their relative performance varied according to the metal being investigated.

This investigation was focused on the tolerance of the three microalgae; *Phormidium ambiguum* (Cyanobacterium), *Pseudochlorococcum typicum* and *Scenedesmus quadricauda* var quadrispina (Chlorophyta) to heavy metals treatments. The biosorption and bioaccumulation of heavy metal ions by *P. typicum* (considered high tolerant species) during short period of contact (24 h) and TEM examination of the heavy metals-stressed algal cells to detect the metal ion incorporation into algal cell wall and / or in the cytoplasm.

Results and Discussion

Heavy metals tolerance. According to Stokes[19] algae appearing in polluted sites are considered to be either metal-tolerant or metal-resistant species. Several green algal species are tolerant or resistant to Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+}.[20-23] Bioremoval is defined as the accumulation and concentration of pollutants from aqueous solutions by the use of biological material, thus allowing the recovery and / or environmentally acceptable disposal of the pollutants.[24,25]

The biosorption of heavy metal ions by microorganisms has often been observed to occur in two stages; an initial passive and rapid uptake (lasting less than 30 min) due to surface adsorption on the cell wall components (e.g: carboxyl, amine, hydroxyl, phosphate, sulfate groups,–etc) and subsequent active and slow uptake (extended more than one month) due to membrane transport of metal ions to the cytoplasm of the cell[26–28] they reported that red alga *Mastocarpus stellatus* attained over 50% of the total biomass cadmium uptake within 2 min of contact and over 90% in the first 9 min. The obtained results in this investigation (**Fig. 1** and **2**) revealed that, Hg^{2+} seemed to exert high toxicity to the three algal species even at its lower concentration used (5 μg/ml). *Phormidium ambiguum* was the most sensitive species followed by *Pseudochlorococcum typicum* and *Scenedesmus quadricauda* which tolerate higher metal concentrations.

The data in **Figure 1** and **2** illustrated that, the three algae tolerated the toxicity of Pb^{2+} even at higher concentrations (80–100 μg/ml), moreover the lower concentration of Pb^{2+} (5–10 μg/ml) induced a pronounced stimulation of chlorophyll "a" and protein which was much more observed in Scenedesmus and Pseudochlorococcum. But in case of Phormidium, the lower concentrations of Pb^{2+} (5–10 μg/ml) were stimulatory to chlorophyll "a" synthesis and slightly inhibitory to protein synthesis at the same time.

On the other hand, Hg^{2+} showed a strong inhibition of chlorophyll "a" biosynthesis even at the lower concentrations (5–10 μg/ml) and a complete destruction of the algal cell at concentration above 20 μg/ml (**Fig. 1**). This effect seemly to be more pronounced in Phormidium followed by Pseudochlorococcum and *Scenedesmus* whatever the concentration of Hg^{2+}. This means that the efficiency of the photosynthetic apparatus seemed to be less affected by Pb^{2+} and severely altered by Hg^{2+}. Cadmium toxicity was mostly intermediate (between that of Hg^{2+} and Pb^{2+}), it exhibited stimulatory effect to the algal growth (chlorophyll "a" and protein contents) at lower concentrations (5–20 μg/ml) in case of Pseudochlorococcum and Scenedesmus, while in Phormidium, the enhancement effect was only restricted to concentration of 5 and 10 μg/ml.

This might be linked with the synthesis of carbohydrates (the most building material) and consequently the growth and survival of the three algae under investigation. This was confirmed by the data of proteins, where the trend in the accumulation of protein went parallel in most cases with the data of the photosynthetic pigment (Chlorophyll a). This means that the efficiency of photosynthetic apparatus and the production of carbohydrates were closely associated with nitrogen-metabolism. This leads us to conclude that the regulation between carbohydrate and N-metabolism was associated with the heavy metal tolerance whereas the toxicity of these metabolic inhibitors disturbed both components (carbohydrates and proteins), [c.f. the stimulatory effect of Pb^{2+} in Chl. a pigment synthesis and consequently protein].

In case of Scenedesmus cells treated with lower Hg^{2+} concentration (5–10 μg/ml), the chlorophyll "a" content was strongly destroyed while the protein content was markedly elevated (**Figs. 1 and 2**). This means that the correlation between the efficiency of the photosynthetic apparatus and the manufacture of organic matter is not necessarily linked. Thus the high protein content in 5 μg/ml Hg-treated Scenedesmus, which accompanied with the considerable reduction in growth means that this organism transmitted most of manufactured protein from a state of growth to a state of survival (the increased protein might be used in the production of phytochelatin as a defense mechanism).

The obtained results in this investigation concerning the tolerance of *P. typicum* and *S. quadricauda* (green algae) and the sensitivity of *Ph. Ambiguum* to the tested heavy metal ions (Hg, Cd and Pb) were in agreement with the results reported by Foster, 1982 and Stokes, 1983 concerning the tolerance and resistance of green algal species to heavy metal ions (as Cu, Cd, Pb and Zn). Also the results in the present study were in accordance with those of Takamura et al.,[29] who reported that cyanobactera were found to be sensitive to Cu, Cd, Pb and Zn whether or not isolated from polluted sites. While green algal species tended to have high tolerance even in isolates from unpolluted sites. Moreover, the high and moderate tolerance of *P. typicum* and *S. quadricauda* in this investigation to Pb and Cd went parallel with the finding of Liu et al.,[30] where *Chlorella vulgaris* could tolerate concentration of 100 μg/ml Pb^{2+} while it could die in 30 μg / ml Cd^{2+} solution.

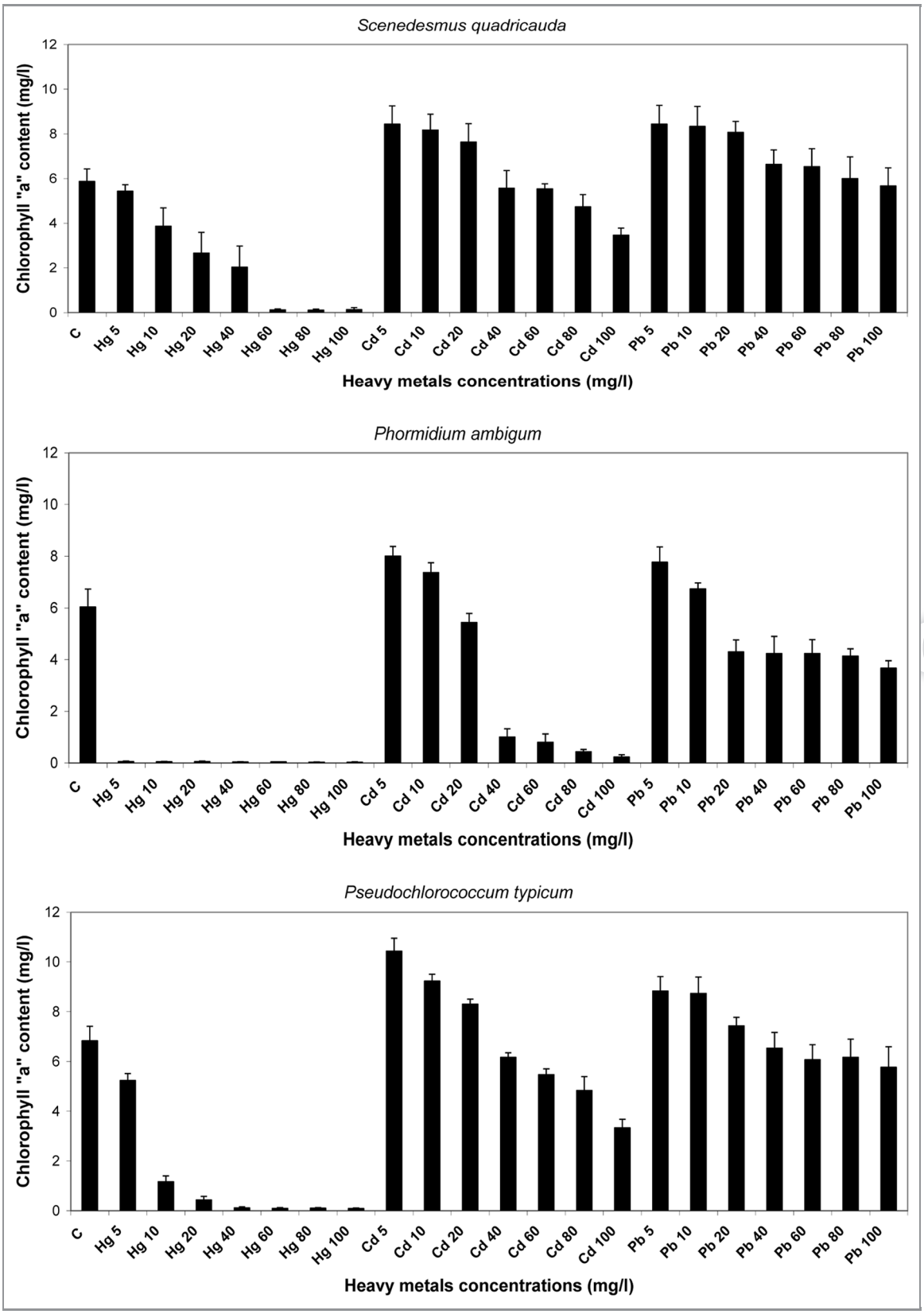

Figure 1. Effect of heavy metals on growth of *Scenedesmus quadricauda* var quadrispina, *Phormidium ambiguum* and *Pseudochlorococcum typicum* after 21 d expressed as mg chlorophyll "a" /ml. (C) represents algal treatment without heavy metals (Error bars represent Means ± standard errors for three independent experiments).

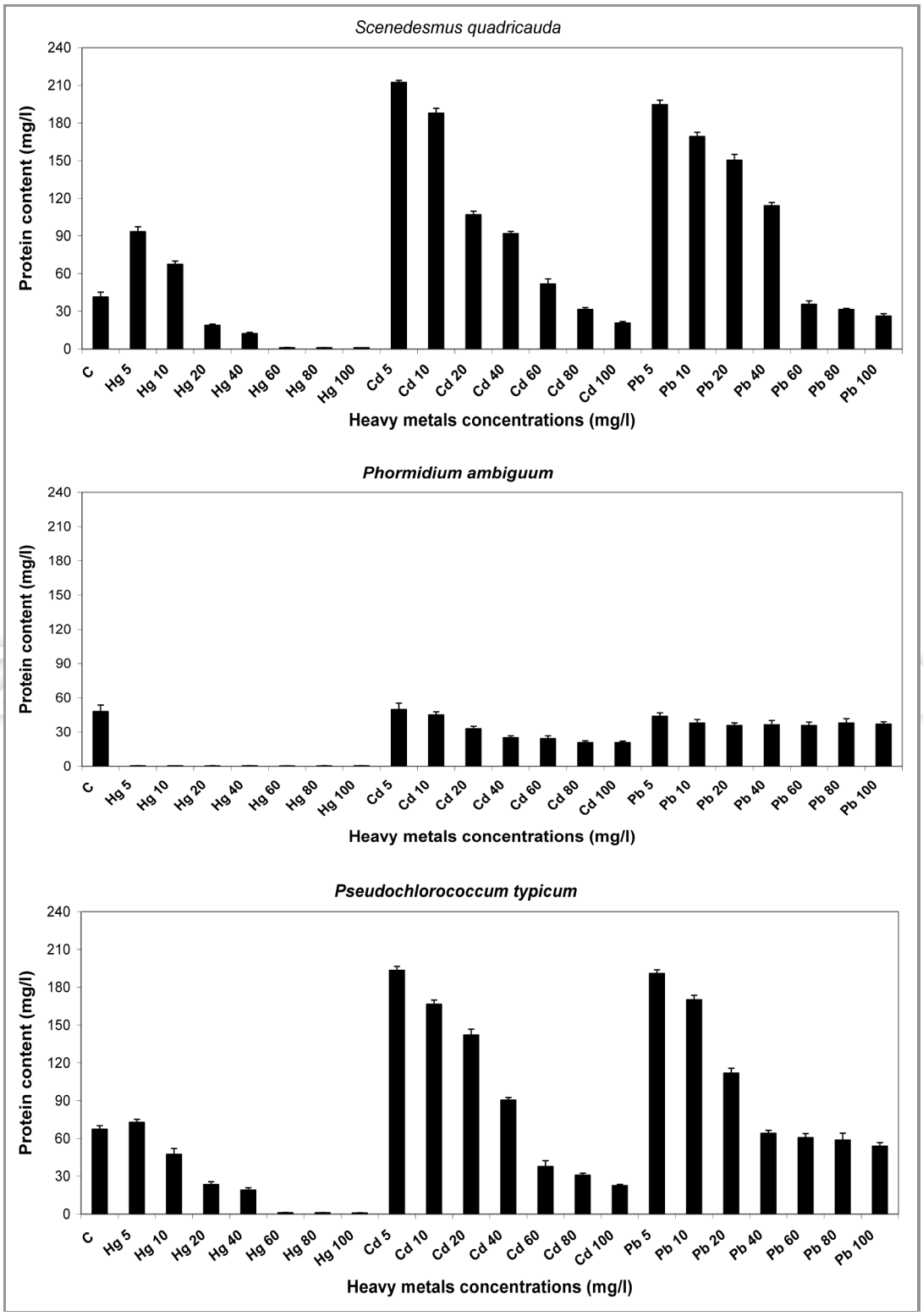

Figure 2. Effect of heavy metals on growth of *Scenedesmus quadricauda* var quadrispina, *Phormidium ambiguum* and *Pseudochlorococcum typicum* after 21 d expressed as mg protein /ml. (C) represents algal treatment without heavy metals (Error bars represent means ± standard errors for three independent experiments).

Table 1. Removal capacity (mg g-1) and Removal efficiency (%) of *Pseudochlorococcum typicum* cells for three heavy metals removal at different times

Time (hrs)	Metal ions removal					
	Hg^{2+}		Cd^{2+}		Pb^{2+}	
	RC[a] (mg g^{-1})	RE[b] (%)	RC[a] (mg g^{-1})	RE[b] (%)	RC[a] (mg g^{-1})	RE[b] (%)
0	0.0	0.0	0.0	0.0	0.0	0.0
0.5`	15.08	97.75	6.26	86.24	5.11	70.06
2	15.06	97.58	5.77	79.74	4.75	66.11
8	14.71	95.34	2.94	39.68	3.56	49.35
24	15.13	98.17	5.48	75.59	4.49	61.76

a RC: Removal capacity; b RE: Removal efficiency. RC = *V* (Ci – Ct) /m; RE (%) = Ci – Ct / Ci x 100. Where V is the volume of solution, Ci the initial concentration of metals, Ct the equilibrium concentration of metals and m the mass of biosorbent added.

Heavy metals biosorption. Biosorption has always been reported as a promising method to treat various kinds of pollutants. A microalga is one of the most important biosorbents. **Table 1** showed the biosorption of heavy metals (Mercuric, cadmium and lead) by green microalga *P. typicum*. The obtained results showed that *P. typicum* had high capacity for bioremoval of Mercuric more than Cadmium and Lead.

The tolerant green microalga *P. typicum*, showed a high efficiency of heavy metal (Hg^{2+}, Cd^{2+} and Pb^{2+}) biosorption. The maximum removal of metal ions occurred during the first 30 min of contact recording 86% for Cd^{2+}, 70% for Pb^{2+} and 97% of Hg^{2+} (**Fig. 3**). By increasing the exposure time (to 24 h), the percentage of Cd^{2+} and Pb^{2+} removal decreases gradually till equilibrium establishment between the percentage of metal ions removal by algal cells and the concentration of the heavy metal ions in external solution. After the equilibrium period, the metal ions sorbed by the algal biomass did not significantly changed with time in case of Cd^{2+} and Pb^{2+} while in case of Hg^{2+}, the percentage of removal stay more or less unchanged during the 24 h of contact.

These biosorptive activities may be due to the algal contents of phycocolloide, Sulfate, phosphorus and nitrogen in algal cell. However, the obtained results indicated that the maximum bioremoval capacity (For Hg, Cd and Pb) was occurred after 30 min., of the experimental duration which was decreased progressively during 24 h of contact with the heavy metals. These may be due to the equilibarium between inside and outside the algal cell or due to break the bonds between the algal cell and metals by some microorganism e.g., fungi and bacteria which grown during experiment. The results are in agreement with those obtained by Awadalla and Pesic.[31] In the present investigation, the high metal bioremoval efficiencies of *P. typicum* (during short exposure period) were in concomitant with the high removal capacities of different heavy metal ions (including Hg^{2+}, Cd^{2+} and Pb^{2+}) by various chlorophycean algal species during short periods (15–30 min) of contact.[24,32-34]

Ultrastructural changes due to heavy metals exposure. Transmission electron microscopy (TEM) was used in this study to demonstrate the ultrastructural changes in *P. typicum* cells treated with Hg^{2+}, Cd^{2+} and Pb^{2+} compared with the normal untreated cells (control). The morphological features (shape and size) of the algal cells as seen in (**Fig. 4A**) remained unchanged after heavy metal treatments (**Fig. 4B, C and D**) while some changes and alterations were observed outside (on the cell

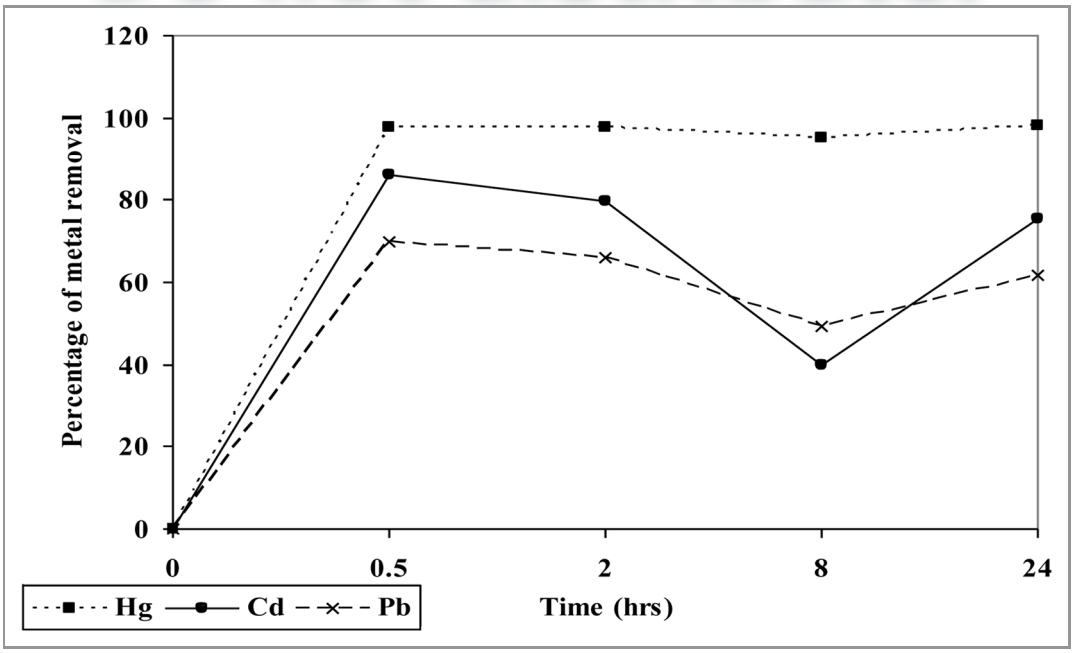

Figure 3. Percentage of heavy metals removal from solution by using *Pseudochlorococcum typicum* cells. The data shown are for an initial Cd^{2+}, Pb^{2+}, and Hg^{2+} concentrations of 3.29, 3.31, and 6.98 μg/ml, respectively.

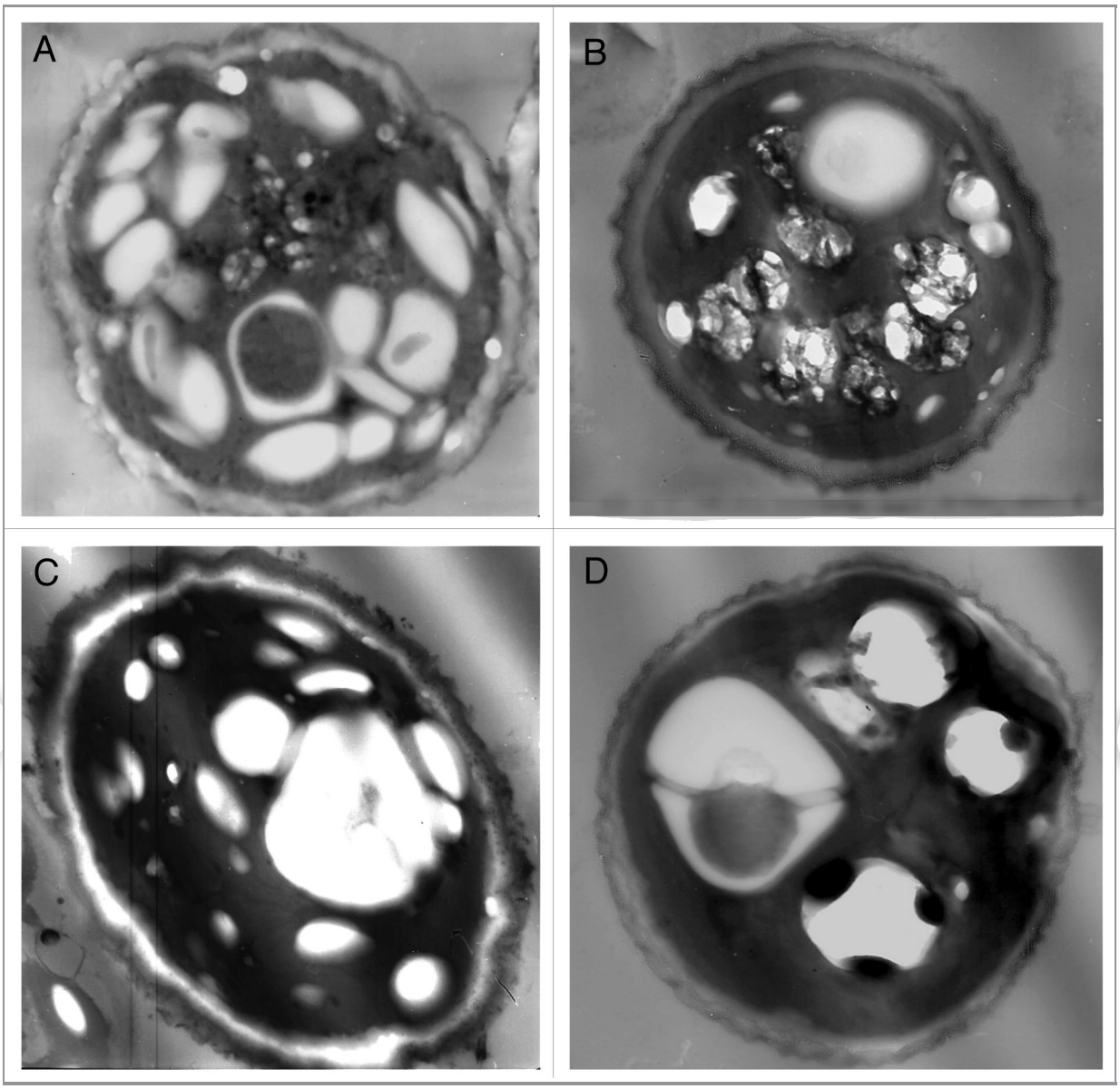

Figure 4. Transmission Electron Micrographs of cells of *Pseudochlorococcum typicum*. (A) Algal cells cultured in standard medium and (B), (C), (D) Algal cells cultured for 48 h in nutritive medium supplemented with 10 μg/ml of $HgCl_2$, $CdCl_2$, and $Pb(NO_3)_2$, respectively. Arrows indicate a dark layer on the cell wall surfaces in (B), (C), (D) but in (D) dark precipitates was shown. The algal cell organelles were badly damaged in (B). The magnification used was 12500 xs.

surfaces) and inside the cell (ultrastructure inclusions and organelles).

An electron dense layer on the cell surfaces of all treated cells (**Fig. 4B, C and D**) as well as an accumulation of starch around the pyrenoids was detected. In case of Pb-treated cells (**Fig. 4D**) spherical electron dense bodies were noticed within the cells. A clear deterioration of cell organelles were obviously recorded in Hg and Cd- treated cells (**Fig. 4B and C**) more than in Pb-treated ones (**Fig. 4D**) in the order Hg > Cd > Pb. The observed electron dense layer on the algal cell surfaces after heavy metal treatments could represent the biosorbed (adsorbed) metal ions binded with different functional groups on algal cell surfaces which was considered as a protective mechanism for limiting most of the toxic ions.[27] The percentage of metal ion adsorbed fraction and insoluble fractions increased with metal concentration.[35] The accumulation of starch grains in the heavy metal treated *P. typicum* cells might act as energy reserve to the cell after the deterioration of organelles especially chloroplast, pyrenoid and mitochondria, which coincided with the results reported by Wong et al.,[18] in *chlorella fusca* .

The bioaccumulation of spherical electron dense bodies inside the Pb-treated *P. typicum* cells in this study was in accordance with similar granules observed in different heavy metal treated microalgal cells.[36] These metal deposition inside the vacuoles or cytoplasm was a mechanism contributed to the heavy metal tolerance by minimizing as possible the cytoplasmic metal

concentrations by binding or complexing the metal ions with phytochelatin or in the form of metallo-sulfur, metallo-iron or metallo-phosphate complexes in the cytosol and carrying them into the vacuoles where the acidic pH displace the metal, allowing the peptide to return to the cytosol. In the vacuole the metal would sequestered by organic acids usually present in high concentration in the vacuoles.[37] This was performed as a cellular protection or detoxification mechanisms.[36,38] The most notable structural alteration in *P. typicum* cells treated with heavy metals ions (especially Hg and Cd) was the chloroplasts which appeared to be the primary target of metal contamination, also pyrenoids, mitochondria, nucleus, golgi bodies, lipids and cell membranes which have all been reported to be affected by metals with various test algal species and they are the same organelles damaged by herbicides, pesticides,….[18]

Materials and Methods

1. Algal Cultures. The algal species used in this study were isolated from River Nile and Ain Helwan Spring,[11] identified (according to Bourelly, 12 and 13) and maintained as pure unialgal isolates on nutritive media (Bold's basal medium[14] for green algae and BG_{11}[15] for Cyanobacteria) and incubated at temperature 20 ± 1°C, light intensity of 30 µE / m^2 / s, photo-period 16–8 h and regularly subcultured until use. The same conditions were used in tolerance and bioremoval experiments but with using shaking (130 M / min) and the culture media were lacking EDTA. The algal species used are:

a- *Phormidium ambiguum* Gomont (Ain Helwan)
b- *Pseudochlorococcum typicum* Archibald (Ain Helwan)
c- *Scenedesmus quadricauda* var quadrispina (Chod.) G. M. Smith (River Nile)

2. Heavy metal concentrations. Stock solutions of the heavy metals $CdCl_2$, $Pb(NO_3)_2$ and $HgCl_2$ (500 mg/100 ml) were prepared, from which concentrations 0, 5, 10, 20, 40, 60, 80 and 100 µg / ml were used in case of algal tolerance experiments, and in biosorption experiment concentration of 10 µg/ml of heavy metals was used.

3. Measurements of algal growth. Algal tolerance to different heavy metal concentrations was achieved by the determination of algal growth as

a. Chlorophyll a. Chlorophyll content was determined according to Metzner et al.,[16] where, 3g of fresh sample was ground in a mortar together with acetone and calcium carbonate. Pigment content in the filtered extract were determined by the absorbance at 663, 645 and 450 nm in a 1cm quartz cell against a blank of 80% aqueous acetone.

b. Protein content. Protein content in different algae were determined spectrophotometrically at 650 nm, using Folin-Ciocalteau reagent according to Lowry et al.[17] Standard curve of protein using bovine serum albumin (20–200 µg / ml) was performed.

4. Heavy metal removal (biosorption). The microalga *Pseudochlorococcum typicum* was used in the experiment of heavy metal removal using the algal concentrations 4.52 µg chl a / ml. The metal concentration used was 10 µg / ml and the exposure time was 0, ½, 2, 8 and 24 h. pH was adjusted to 7.0 and incubation was performed at the previous mentioned conditions. At the end of each exposure time, decantation was performed and the supernatant was used for the determination of heavy metal removal using Perkin EL Mer 3300 Atomic Absorption Spectroscopy (using hydride system) for Hg^{2+} determination (at Water, Soil and Environment Research Institute, Agriculture Centers, Ministry of Agriculture). While Cd^{2+} and Pb^{2+} were determined using Unicam 989 AA Spectometer-Solaar (at the Principal Central Laboratory, Faculty of Agriculture, Cairo University). The uptake of metal ions were determined using changes in the metal concentration in the test medium during the exposure period expressed as percentage removal; (R1 – R2) / R1 X 100 where, R1; control concentration and R2; concentration of heavy metal after each exposure period.

5. Bioaccumulation and Electron microscopy examination. At the end of the bioremoval experiments, algal pellets were harvested by centrifugation (1000 rpm) and prepared for TEM (Ziess-EM 10) examination using the method described by Wong et al.,[18] for the detection of heavy metal ions biosorbed and / or bioaccumulated by the microalga. This was performed at the Central Lab. Services, National Research Center.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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