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Toxicological Response of the Green Alga *Chlorella vulgaris*, to Some Heavy Metals

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Abstract: Problems statement: The disturbance of aquatic ecosystems provoked by heavy metals pollution from industrial and domestic sources, has as consequence the loss of biological diversity, as well as increased bioaccumulation and magnification of toxicants in the food chain. The aim of this study was to evaluate the effect of some heavy metals on some physiological activities of *Chlorella vulgaris* beyerinck with special references to metal bioaccumulation. **Approach:** *Chlorella vulgaris* Beyerinck was isolated from Al-Asfar Lake, Al-Hassa, Saudi Arabia. A standard initial inoculum of the isolated algae was inoculated to culture flasks. The culture flasks were supplied with various concentrations of Cobalt, Copper and Zinc ranging from 10^{-6}-10^{-9} M. At the end of the incubation period cultures were filtered and washed several times by distilled water for measurements the various experimental parameters. **Results:** The data show that the lower doses of the three tested metals had stimulatory effect in biomass yield of *Chlorella vulgaris*, whereas the higher doses were inhibitory depending on the type of the metal. The inhibitory effect of copper to the growth parameters of *Chlorella vulgaris* was more pronounced than other two tested metals. The total protein content, total carbohydrate and the total free amino acids of the tested green alga *Chlorella vulgaris* gradually decreased in a manner dependent on the metal concentration in the medium. On the other hand, bioaccumulation of cobalt, copper and zinc by *Chlorella vulgaris* cells were parallel to increasing the concentrations in the culture medium. **Conclusion:** The inhibitory and stimulatory effects of either of the used 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 uptake of an element from the surrounding medium is seldom exactly proportional to the amount present in the water.

Key words: Bioaccumulation, *Chlorella vulgaris*, heavy metals, metabolism

INTRODUCTION

Many pollutants like pesticides, oil hydrocarbons, heavy metals as well as thermal and radioactive pollution can get into aquatic environments after direct or indirect release from industries, agriculture and households (Fathi *et al*., 2008). As an important group of these various chemical substances, heavy metals may be deposited into all ecosystems (Mutlak *et al*., 1979). The disturbance of aquatic ecosystems provoked by heavy metals pollution from industrial and domestic sources, has as consequence the loss of biological diversity, as well as increased bioaccumulation and magnification of toxicants in the food chain (Pena-Castro *et al*., 2004).

Microalgae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for metals (Levy *et al*., 2007). There are a remarkable number of investigations demonstrating the toxic effects of heavy metals on different species of algae (El-Sheekh *et al*., 2003; Osman *et al*., 2004; Fathi *et al*., 2005; Akira *et al*., 2005; Muwafq and Bernd, 2006; Anne *et al*., 2006).

Heavy metal constitutes a stress factor for many microbes where there concentrations increased as a consequence of a wide range of agricultural and industrial activities. De Filippis and Pallaghy (1994) although some of these metals are required for cell growth, the high concentrations of all metals exert toxic effects on metabolic machinery of algae. The phenomenon of the growth of algae subjected to heavy metals stress was clarified (Fathi and Falkner, 1997; Fathi, 2002; 2005; Fathi *et al*., 2008). The aim of this study was to evaluate the effect of some heavy metals

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on some physiological activities of *Chlorella vulgaris* Beyerinck with special references to metal bioaccumulation.

MATERIALS AND METHODS

Organism and culture condition: *Chlorella vulgaris* Beyerinck was isolated from Al-Asfar Lake, Al-Hassa, Saudi Arabia. Isolation and purification was made by dilution and plating technique. The alga was grown in 250 mL flasks containing 100 mL Kuhl (1962) medium and incubated in an illuminated incubator (Precision, USA) at 22°C and irradiance at 150 μmol m^{-2} sec^{-1}, provided by cool white fluorescent lamps set on 14:10 h photoperiod. All cultures were shaken twice daily to prevent cells from clumping.

Sterile technique determinations of pigment content: Chlorophyll a and b content were estimated in acetone extract according to Jeffrey and Humphery (1975). The content of the pigments fractions (μg chl mL^{-1} algal suspension) was then calculated under consideration of the dilution factors.

Algal counting: Cell number was determined using a Hematocytometer Chamber. Hemacytometer 0.1 mm deep, having improved Naubauer ruling was used. One drop of the algal suspension was pipetted on the slide, covered and left for two minutes for algal settling. The mean counts of three replicates were taken into consideration and the data were given as cell mL^{-1} algal suspension.

Calculation of growth rate: The growth rate of the algal growth was calculated by the following equation (Nichol's, 1973):

$$K\ (day) = [3.322 / (t_2-t_1)]. (Log\ N_2 / N_1)$$

Determination of dry weight: A definite volume (100 mL) of algal suspension was filtered through weighted glass fiber (Schleicher and Schull, Germany). The cells, after being precipitated on the filter study, were washed twice with distilled water and dried overnight in an oven at 105°C. Data were given as μg mL^{-1} algal suspension.

Treatment: A standard initial inoculum of the isolated algae was inoculated to culture flasks (500 mL each) that contained 200 mL of sterile nutrient medium (Kuhl's medium). The culture flasks were supplied with various concentrations of Cobalt, Copper and Zinc ranging from 10^{-6}-10^{-9} M. At the end of the incubation period (7 days) cultures were filtered and washed several times by distilled water. At least three replicates for each sample and controls were used.

Biochemical analysis: The enthrone method (Roe, 1955) was applied for total carbohydrate estimation using fresh material and glucose as a standard. Total amino acid content was determined according to Moore and Stein (1948). Total protein was measured according to Lowery *et al.* (1951)

Metal uptake: For the analysis of metal contents, the cultures were centrifuged to harvest the algal mass (50 mL). The algal pellet was washed with 2 mM EDFA for 10 min. to remove surface-bound metal. After centrifugation the pellet was digested 5 mL mixture containing HNO_3 (70%), H_2O_2 (30%) and deionized water in 1:1:3 ratio (Bates *et al*., 1982). After digestion the samples were analyzed for metal content with a Perkin-Elmer atomic absorption spectrophotometer.

Calculation of bioaccumulation factor: The bioaccumulation factor defined by (Brooks and Rumsby, 1965) is the ratio of concentration of an element in dry plant biomass and in the water.

Statistics: 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

It is well known that algal cells exposed to heavy metals may suffer serious morphological and biochemical alterations (Rocchetta *et al*., 2006). The results of this investigation show that the inhibitory and stimulatory effects of either of the used heavy metals depend on concentration.

The bioassay results as illustrated in Fig. 1 showed clear differences in pigments content (Chlorophyll a and b) of algal cells between control and treated ones when algae were exposed to different concentrations of the three tested metals. The pigments content gradually increased in the culture supplemented by concentration 10^{-9} M during exposure periods, whereas other concentrations (10^{-8}-10^{-6} M) cause a clear reduction in the pigments content of *Chlorella vulgaris.* The same effect was observed with respect to growth rate as another indicator of algal growth as shown in Fig. 2. The growth rates decreased in respect of increasing metals concentration more than 10^{-9} M. Generally, the

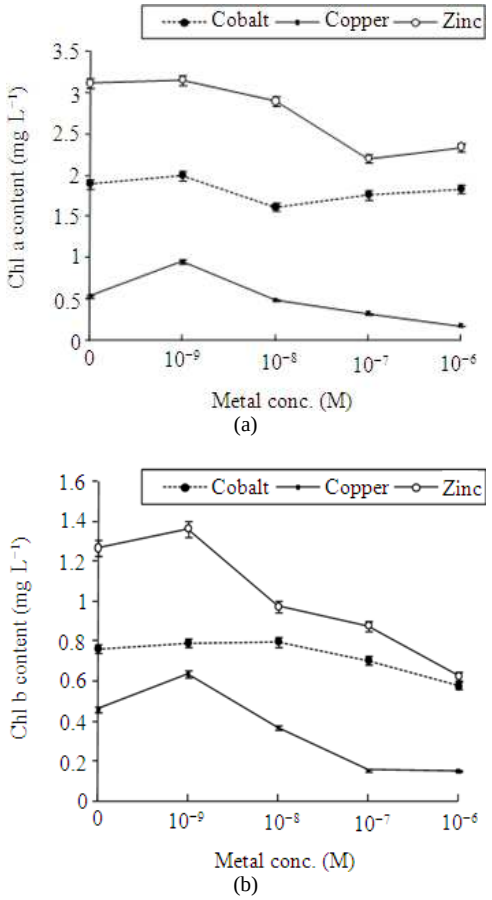

Fig. 1: Effect of various concentrations of cobalt, copper and zinc on pigments content (Chl a and b) of *Chlorella vulgaris* after 7 days incubation period. Vertical bars indicate SE, n = 3

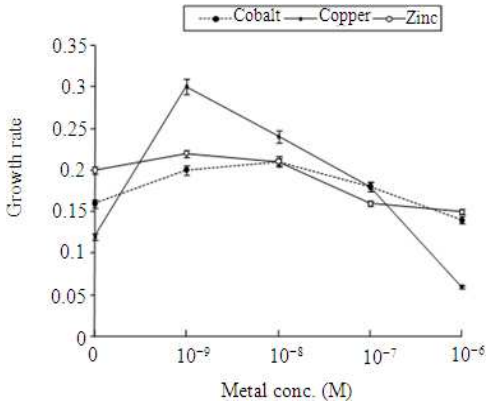

Fig. 2: Effect of various concentrations of cobalt, copper and zinc on growth rate of *Chlorella vulgaris* after 7 days incubation period. Vertical bars indicate SE, n = 3

results shows that the low dose (10^{-9} M) of the three tested metals had stimulatory effect in biomass yield of *Chlorella vulgaris*, whereas the higher doses were inhibitory depending on the type of the metal. The total cell death occurred at concentrations 10^{-5} M of copper and at 10^{-4} M of cobalt and zinc (data not shown). The results also show that the inhibitory effect of copper on the growth rate and pigments content is more pronounced than for the other two tested metals two tested metals. These findings are in agreement with several previously published data (El-Sheekh *et al*., 2003; Osman *et al*., 2004; Fathi *et al*., 2005; Muwafq and Bernd, 2006; Romera *et al*., 2007; Cecilia *et al*., 2007; Deng *et al*., 2007).

Regards to the stimulatory or inhibitory effect of cobalt showed on this investigation, the present results are in agreement with those obtained by El-Naggar *et al*. (1999) who found that a lower Co^{2+} concentration (0.01 ppm) stimulated growth of *Nostoc muscorum*, while it showed a non-significant effect on *Calothrix fusca* growth. However, higher Co^{2+} concentrations were inhibitory for both organisms. On the other hand, growth promotion at low Co^{2+} concentrations may be due to Co^{2+} substitution for Zn^{2+} in some metalloenzymes *in vitro* and *in vivo* (El-Sheekh *et al*., 2003; Osman *et al*., 2004). Moreover, high concentrations of cobalt were inhibitory for pigment content of tested alga. These results are in agreement with those obtained by El-Naggar *et al*. (1999) who reported inhibition of chlorophyll biosynthesis as a result of Co^{2+} treatment. The mechanism proposed for this inhibition is the replacement of magnesium in the chlorophyll molecule. In this regard, De Filippis *et al*. (1981) reported that reduction of chlorophyll a content is a common symptom of heavy metals toxicity.

The stimulatory effect of copper recorded in this study with lower concentrations (10^{-9} M) can be accounted for either as a result of algal requirement of this element in metabolic processes or explained by production of some organic compound which decreases metal toxicity. Albergoni *et al*. (1980) and Rijstenbil *et al*. (1998) suggested that some of the algae capable to produce metal binding compounds there from get the ability to bind and sequester copper ions in the cytoplasm and reduce toxicity. On the other hand, the toxic effect of copper at higher concentrations (10^{-7} and 10^{-6} M) detected in the present study may be due to the oxidative potential of copper (II) that causes reduction of chlorophyll and decrease of oxygen evolution rates and depletion of ATP by inhibition of

enzymes (nitrate reductase and alkaline phosphatase) which are involved in nitrate and ammonia cellular metabolism (Muwafq and Bernd, 2006).

The inhibitory effect of the zinc on the studied growth parameters at higher doses depended on their concentration in the culture medium. Fisher and Jones (1981), who reported that low Zn^{2+} levels enhanced the total chlorophyll in *Asterionella japonica*. Prassad and Prassad (1987) found that heavy metals inhibit the enzymes that are responsible for the chlorophyll (che) synthesis. De Filippis and Pallaghy (1994) reported that toxicity of Zn results from their binding to SH groups and disruption of enzyme structure (Omar, 2002). On the other hand, Zn does not directly accelerate the formation of reactive oxygen species due to its redox inertness and it, therefore, exerted comparatively less stress on the test organism (Tripathi and Gaur, 2006).

The data of Fig. 3 show that the total protein content of the green alga *Chlorella vulgaris* gradually decreased in a manner dependent on the metal concentration in the medium. The data also shows that all the three metals affected negatively the total protein content at higher doses. On the other hand, the supplementation of copper and zinc by concentration 10^{-9} M increases the total protein content as compared to the control. However, no marked change in total protein content occurred in cells of *Chlorella vulgaris* which exposed to cobalt. It could be suggested that accumulation of protein at low heavy metal concentrations may be one of the ways through which the algae can abolish their toxic effects, or to increase respiration leading to the utilization of carbohydrate in favor of protein accumulation (Osman *et al*., 2004). Whereas, the suppression of protein accumulation may be attributed to shortage of carbon skeleton results from low photosynthetic rate. Such results are in accordance with those of Fathi *et al*. (2000). However, some authors (Osman *et al*., 2004; Tripathi and Gaur, 2006; Barbara and Michael, 1994) reported that the toxic action of heavy metals on the enzymatic reactions responsible for protein biosynthesis.

The total carbohydrates content of *Chlorella* cultures grown 7 days under various concentrations of cobalt, copper and zinc were also determined. Carbohydrate content of the tested alga also declined in manner dependent on the metal concentration exist in the medium, but the inhibitory effect of the three tested metals were not pronounced as on protein content. The results obtained in Fig. 4 shows that the three tested metals initiated the total carbohydrates accumulation at the culture supplemented by 10^{-8} M. It is worth to mention that high concentrations of the three tested metals did not reduce the total amount of carbohydrates. The increased total carbohydrate accumulation following metals was accompanied by decrease in Fathi *et al*. (2005) reported that the higher doses severely attenuate chlorophyll synthesis coupled with severe drop in protein resulting in increased carbohydrates. From another point of view, Torres *et al*. (1998) demonstrated that algae *Cylindrothica fusiformis* produce carbohydrate as a defense mechanism against copper toxicity in stationary phase when cells are exposed to 0.5 mg Cu L^{-1}.

Figure 5 clearly shows that the total free amino acids of *Chlorella vulgaris* gradually increased with increasing metals concentration. The most pronounced stimulation was detected at the culture supplemented with 10^{-7} M copper in comparison to the other tested metals. However, increasing copper concentration more than 10^{-7} M, the total amount of free amino acids partially reduced. On the other hand, cobalt and zinc also stimulated the biosynthesis of the total free amino acids, but the stimulatory effect is less than that obtained with copper. Omar (2002) showed that zinc at low concentrations increased total amino acid contents, however, decreased it at high concentrations. Generally, the accumulation of amino acids in response to metals concentrations may lead to the assumption that suppressed protein biosynthesis encouraged free amino acids accumulation, or may be due to some counteracting chelating mechanism against heavy metals toxicity (El-Sheekh *et al*., 2003; Osman *et al*., 2004; Fathi *et al*., 2005).

Algae are known to be able to accumulate heavy metals. They are able to eliminate metal ions from aquatic solutions in a short time by biosorption in uncomplicated systems, without any problems of toxicity. It is an important biochemical function of algae in the shaping of proper ecological relationships and interactions between organisms in the aquatic environment (Wilde and Benemann, 1993; Sandau *et al*., 1996; Bajguz, 2000).

The data of Fig. 6 performed that accumulation of cobalt, copper and zinc by *Chlorella vulgaris* cells were parallel to increasing the concentrations in the culture medium. Also, it can be seen that the tested alga *Chlorella vulgaris* accumulated an appreciable amounts of copper more than other that observed with cobalt and zinc. However, no significant difference was observed between each of cobalt and Zinc. It is known that the uptake of an element from the surrounding medium is seldom exactly proportional to the amount present in the water. The data of Fig. 7 shows that the bioaccumulation factors (the ratio of concentration of an element in dry biomass and in the surrounding medium) of the three tested metals were parallel also to

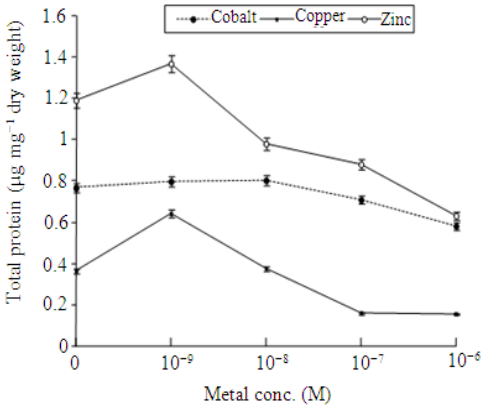

Fig. 3: Effect of various concentrations of cobalt, copper and zinc on total protein content (µg mg^{-1} dry weight) of *Chlorella vulgaris* after 7 days incubation period

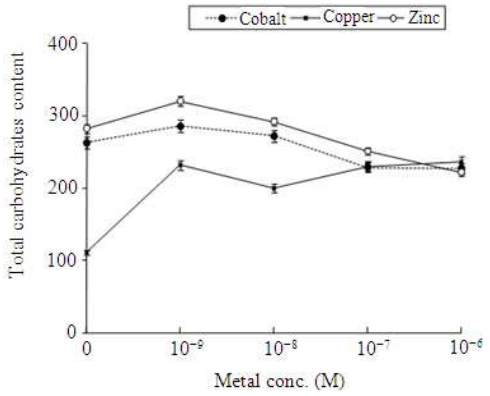

Fig. 4: Effect of various concentrations of cobalt, copper and zinc on total carbohydrates content (µg mg^{-1} dry weight) of *Chlorella vulgaris* after 7 days incubation period. Vertical bars indicate SE, n = 3

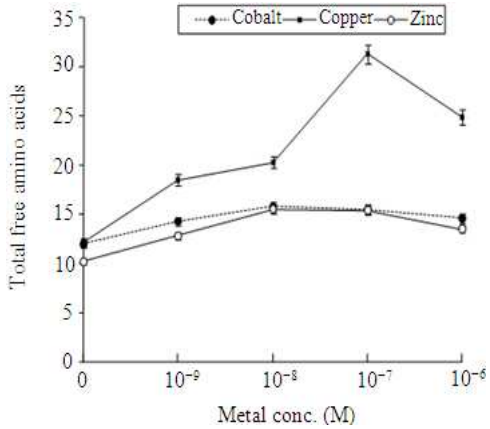

Fig. 5: Effect of various concentrations of cobalt, copper and zinc on total free amino acids (µg mg^{-1} dry weight) of *Chlorella vulgaris* after 7 days incubation period. Vertical bars indicate SE, n = 3

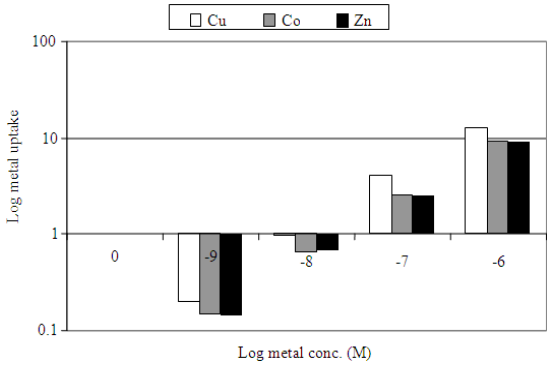

Fig. 6: Bioaccumulation of cobalt, copper and zinc (µg mg^{-1} dry mass) by *Chlorella vulgaris* after seven days growth period

increasing the concentrations in the culture medium. However, the bioacuumulation factor of copper is always higher than that of cobalt and zinc in all treatments. Metal accumulation by *Chlorella vulgaris* were shown to be in an order of $Cu^{2+}>Co^{2+}>Zn^{2+}$.

The ability of microalgae to accumulate metals from aqueous solution is well-documented (Fathi and Falkner, 1997; Fathi *et al*., 2000; Giusti, 2001) as well as the possibility of using microbial biomass to remove metals from effluents (Macaskie, 1991; Hamdy, 2000). Algae take metals up both passively and actively. Some, such as Pb and Sr, may be passively adsorbed by charged polysaccharides in cell wall and intracellular matrix (El-Sheekh *et al*., 2003; Osman *et al*., 2004; Fathi *et al*., 2000; 2005). Other metals (e.g., Zn, Cd) are taken up actively against large intracellular concentration gradients. On the other hand, Barbara and Michael (1994) reported that the phenomenon of metal accumulation by microbial cells is quite complex, two principal mechanisms to adsorption on to the surface of the cell and a slower, active uptake into the cytoplasm. As passive biosorption mainly depends on binding to functional surface ligands the cell wall structure is most important for rapid metal ion uptake. Hamdy (2000) reported that metal uptake dependent on the type of biosorbant, with different accumulation affinities towards the tested elements and the amount of metal uptake increased steeply by increasing the weight of the biomass. Fathi *et al*. (2005) reported that the uptake of an element from the surrounding medium is seldom exactly proportional to the amount present in the water.

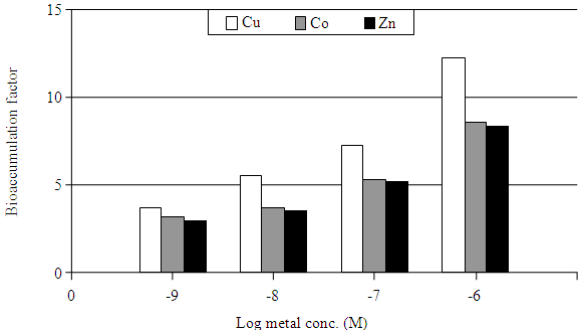

Fig. 7: Bioaccumulation factor of cobalt, copper and zinc by *Chlorella vulgaris* after seven days growth period

CONCLUSION

The inhibitory and stimulatory effects of either of the used 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 uptake of an element from the surrounding medium is seldom exactly proportional to the amount present in the water. Different organisms, however, have different sensitivities to the same metal and the same organisms may be more or less damaged by different metals.

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