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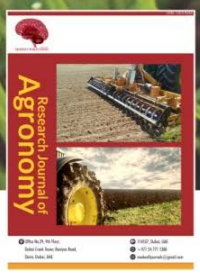

Effect of Different Concentration of Mg, Sn, Zn, Cd and Fe on the Growth of *Chlorella vulgaris*

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Key words: Heavy metal, *Chlorella vulgaris*, growth, chlorophyll a, concentration

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Abstract: The impact of different concentrations (5, 50, 250, 500 ppm) of the Magnesium (Mg), tin (Sn), Cadmium (Cd), Iron (Fe) and Zinc (Zn) on the growth of *Chlorella vulgaris* was studied during 15d exposure experiments. The results showed that the effects of these five metals on the growth of *Chlorella vulgaris* were dependent on both concentration and exposure time. It was found that 50 ppm treatments of Sn, Cd and Fe significantly inhibited the growth of *Chlorella vulgaris* and the effect became weaker and reached to lethal stages with an increase in concentration. It has a good ability in treatments with up to 50 ppm of Mg and Zn. Among the five elements tested, the treatment with Mg showed maximum chlorophyll 'a' (9. 91±1.2 μg L^{-1}) content than other of the elements. Therefore the lowest chlorophyll 'a' of 0.5±0.12 and 0.24± 0.12 μg L^{-1} were noticed in treatments with Sn and Fe at 250 ppm, respectively. From the all above results it could be understood that *C. vulgaris* can resist in low concentration of Sn, Cd and Fe and high concentration of Mg and Zn in aquatic ecosystems. Thus, it can infer that use to bioremediation of pollution water.

INTRODUCTION

Nowadays, heavy metal ions from industrial sectors or agriculture discharged to aquatic ecosystems and contaminated total aquatic environment which not only cause toxic effect on human (by accumulation in aquatic animals) through food chain but also affect biodiversity (Benemann, 1997). Heavy metals such as Cd^{2+} can toxic effect on cell physiology. It is frequently considered as a nonessential element for living organisms (Leborans and Novillo, 1996; Tukaj *et al*., 2007) but Zn^{2+} is a micronutrient for cell growth and metabolism. However, Zn^{2+} is an essential element for cell growth but it is also toxic at high concentrations in media (Miao and Wu, 2006; Orus *et al*., 1991; Munoz and Guieysse, 2006). Dias *et al*. (2002) suggested that heavy metals such as lead, cadmium, mercury, nickel, zinc, aluminum, arsenic, copper and iron may cause poisoning impact on aquatic environment. The effect of heavy metals on microalgae growth were arrested cell division, inhibited growth rate, restrained enzyme activity and reduced photosynthesis (Chen *et al*., 2009; Baumann *et al*., 2009). The responses of different species to the occurrence of lethal concentrations of heavy metals are varied. Between

different organisms in marine environment, unicellular microalgae resistant to heavy metals and they may recommend as bio indicators for the assessment of marine pollution (Kapkov and Belenikina, 2003, 2007; Rijstenbeil *et al*., 1994). The unicellular microalgae are photosynthetic organisms with higher efficiency photosynthetic than plants. They use light energy and carbon dioxide for the production of biomass (Peters *et al*., 2013; Godt *et al*., 2006). There are various element compositions under different conditions that impacted on stages of growth *Chlorella vulgaris* (Oh-Hama and Miyachi, 1988; Baumann *et al*., 2009). Different elements such as: N, P, K, Mg, Ca, S, Fe, Cu, Mn and Zn are needed for the growth of green algae (Oh-Hama and Miyachi, 1988). Traditional wastewater treatment process is costly to provide suitable condition to aerobic bacteria for consume the organic components in pollution water whereas microalgae provide an efficient way for decrease wastewater (Oswald *et al*., 1953). In this study, toxic effect of different concentration of Mg, Sn, Zn, Cd and Fe on the growth and the formation of photosynthetic pigment (chlorophyll 'a') of *Chlorella vulgaris* were observed.

MATERIALS AND METHODS

Algal material and culture conditions: The election of *Chlorella vulgaris* for this study was based on several considerations such as: It existence all over the world. It is a suitable material for toxicity tests. Have simple growth conditions. Have a strong tolerability. Previous studies for comparison are accessible. The *Chlorella vulgaris* was obtained from the Phytoplankton Culture Laboratory, of institution Persian Gulf and Omani Sea of Hormozgan in Iran. The microalgae were grown in the N-8 media (Vonshak, 1986) at pH 7.5 which was adjusted using H_3PO_4 (Table 1 and 2). Stock cultures were incubated in 250 mL conical flasks containing 100 mL of sterilized seawater under a photon irradiance of approximately 7500 lux (mixing of white and red lighting) in 12:8 h light and dark cycle and temperature were maintained at 25±2°C.

Toxicity analysis: Growth inhibition bioassay (Table 1) for *C. vulgaris* was conducted under laboratory conditions for fifteen days. To minimize the metal contamination, all laboratory wares contact with the culture medium were soaked in 1% HNO_3 for 24 h and rinsed with Milli-Q water. Magnesium, Zinc, Tin, cadmium and iron solution at a stock concentration of 1000 mg L^{-1} were prepared using analytical grade $MgSO_4$, $ZnCl_2.2H_2O$, $SnCl_2$, $CdCl_2$ and $FeCl_3$ (Kumar *et al*., 2013). About 4.397 g of each salt were dissolved in 1000 mL of distilled water to make stock solution. From the stock solution, 0.1 mL was taken and dissolved in 100 mL of distilled water to make 1 ppm of each solution. The culture media with various Mg, Zinc, Sn, Cd and Fe concentrations such as 5, 50, 250, 500 ppm were prepared by diluting the stock according to Kumar *et al*. (2013). Control medium was prepared in the same manner without adding the toxicant. The cell growth of algal cultures were monitored carefully at regular intervals such as 3, 6, 9, 12 and 15 days of incubation by measuring the optical density of algal suspension at 540 nm (Wetherell, 1961). Triplicates were maintained for all the treatments and control. Also determines of lethal dosage of the microalgae *Chlorella vulgaris* using different concentration of Mg, Sn, Zn, Cd and Fe. Measurement of growth was made using a spectrophotometer at 540 nm to optical density for *Chlorella vulgaris*.

At the end of experiment chlorophyll 'a' content of the *Chlorella vulgaris* culture was estimated by following the method of Mantoura and Llewellyn (1983) (Mantoura and Llewellyn, 1983). As brief 10 mL of algal culture sample was filtered using Millipore filtering system fitted with a 4.5 cm diameter GF/C filter paper by applying low suction. Before filtering the sample, a thin bed of magnesium carbonate (2 mL) was poured over the GF/C filter paper for effective filtration. After filtration, the filtrate was removed and filter paper with algal cells ground with 90% of acetone using mortar and pestle. The resulting samples were transferred to screw cap test tubes covered with black cloth and incubated in the refrigerator

Table 1: The protocol for *Chlorella vulgaris* growth inhibition bioassay

Task	Condition
Test type	Static
Temperature	25±2°C
Light quality	Mixing of white and red lighting
Light intensity	7500 lux
Illumination	12:8 h light and dark cycle
Conical flask size	250 mL
Culture solution volume	100 mL
Age of test organisms	Every 3 days
Number of replicate	3
Mixing rate	Constant aeration
Test duration	15 days
pH	7.5
Test endpoint	Growth inhibition
Culture medium	N-8
Type of cultivation	indoor

Table 2: Composition of macronutrients in N-8 media (Vonshak, 1986)

Compound	N-8 (mg L^{-1})
KNO_3	1000
KH_2PO_4	740
$Na_2HPO_4.2H_2O$	260
$CaCl_2.2H_2O$	13
Fe EDTA	10
$MgSO_4.7H_2O$	50
Micronutrients	N-8 (g L^{-1}) 1 mL
$Al_2(SO_4)3.18H_2O$	3.58
$MnCl_2.4H_2O$	12.98
$CuSO_4.5H_2O$	1.83
$ZnSO_4.7H_2O$	3.2

for 24 h. After 24 h incubation, the contents were ground with 90% of acetone and centrifuged at 3000 rpm for 10 min. Then the clear supernatant was collected and optical density was measured at different wavelengths such as 630, 645 and 665 nm using UV-visible Spectrophotometer (1800 Shimadzu UV) for chlorophyll 'a' estimation.

Statical analysis: The experimental data were subjected to statistical analysis using SPSS Software v. 16.0 (USA) via. Analysis of Variance (ANOVA) and Excell software.

RESULTS AND DISCUSSION

Effect of Magnesium on the growth of the *C. vulgaris* cultures: In the present investigation, the effect of Magnesium, Tin, Cadmium, Zinc and Iron on the growth of unicellular marine microalgae *Chlorella vulgaris* were evaluated. The result indicates that the optical density *Chlorella vulgaris* decreased in all the concentration of Mg up to 3 days of incubation after that with additional the concentration of the Mg and incubation days, the growth of *C. vulgaris* increased drastically (Fig. 1). However, OD were lower than the blank in all cases (average blank OD was 1.69) when concentration of Mg increased. The highest OD showed in concentration of 50 ppm of Mg in 15days (average OD = 1.22). Additionally, one-way ANOVA indicated that were not significantly ($p>0.05$) different from each Mg concentrations in *C.vulgaris* (Table 3). The effect of magnesium is of two aspects: cell multiplication: synthesis of cell material (Webb, 1949). In *C. vulgaris* different levels of magnesium concentration are important factors on cell division and accumulation of cell material. The cell size increased when material synthesized (Webb, 1949). Webb (1949) reported magnesium is a controlling factor in the division of bacteria.

Effect of Tin on the growth of the *C. vulgaris* cultures: Effect of Tin on the growth of the *C. vulgaris* cultures demonstrated that the growth of C. *vulgaris* decreased drastically (Fig. 2). The initial OD was 0.28 and it decreased when the concentration of the Sn and incubation days increased and in concentration 500 ppm of Sn it became zero. According to, one-way ANOVA

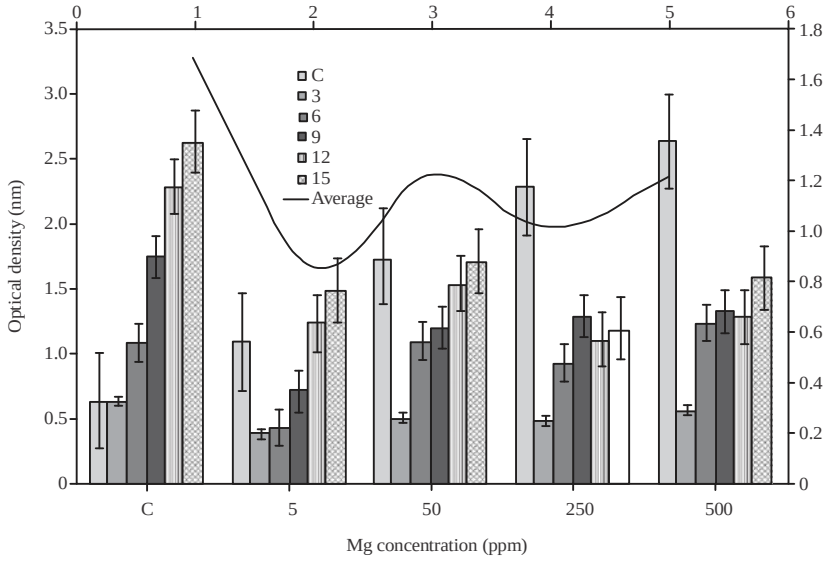

Fig. 1: Optical density a *C. vulgaris* thought time at various Mg concentrations. Results are expressed as means; error bars represent standard error

Table 3: One-way-ANOVA on the biomass of *Chlorella vulgaris* exposed to different metal concentrations

Treatments	p-values	Average test	Average 5 ppm	Average 50 ppm	Average 250 ppm	Average 500 ppm
Mg	0.240	1.69.0	0.866	1.226	1.152	1.249
Sn	0.020	1.015	1.057	0.805	0.465	0.000
Zn	0.800	1.384	1.560	1.166	1.136	1.068
Cd	0.001	1.534	1.008	0.559	0.433	0.308
Fe	0.008	0.945	1.097	0.917	0.445	0.000

Underline showed the lethal dosage

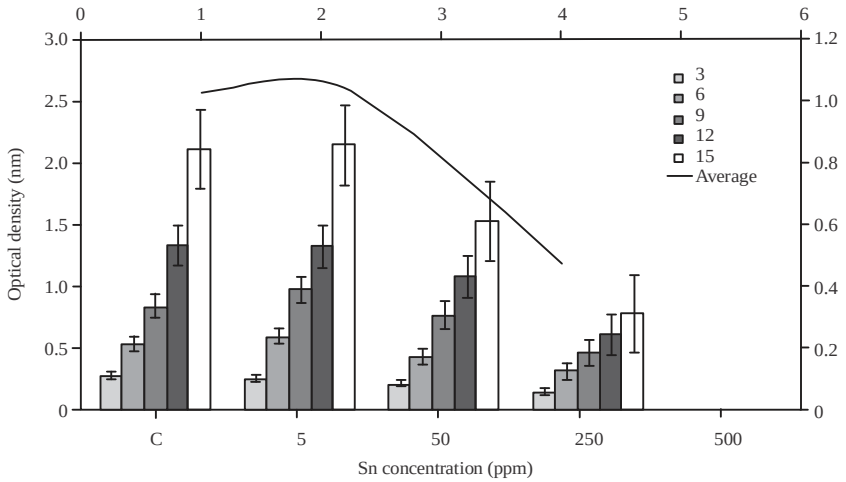

Fig. 2: Optical density a *C. vulgaris* thought time at various Sn concentrations. Results are expressed as means; error bars represent standard error

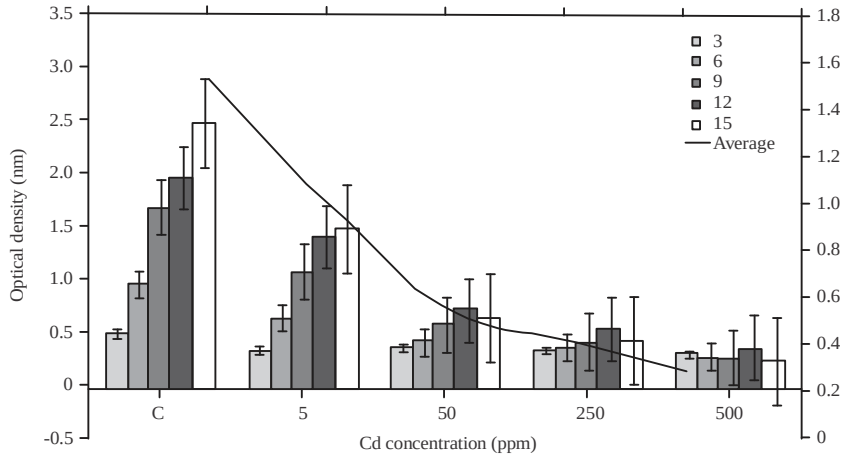

Fig. 3: Optical density a *C. vulgaris* thought time at various Cd concentrations. Results are expressed as means; error bars represent standard error

indicated that were significantly ($p<0.05$) different from each Sn concentrations in *C. vulgaris* (Table 3). The effects of a number of inorganic and organic tin compounds on pure cultures of green and blue-green algae and natural phytoplankton demonstrated that the toxicity of organic tin compounds were generally more to growth of the algae than inorganic tin compounds (Wong *et al*., 1982).

Effect of Cadmium on the growth of the *C. vulgaris* cultures: The optical density results of *C. vulgaris* in different concentration of Cd, revealed that it was decreased when, the concentration of the Cd and incubation days increased (Fig. 3). One-way, ANOVA analysis was performed on the data pertaining to Cd different concentration was significantly ($p<0.05$) different from each Cd concentrations in *C. vulgaris* (Table 3).

Effect of Zinc on the growth of the *C. vulgaris* cultures: The result indicates that *Chlorella vulgaris* were able to tolerate up to the concentration to 500 ppm zinc (Fig. 4 and 5). One-way, ANOVA indicated that were not significantly ($p>0.05$) different from each Zn concentrations in *C. vulgaris*. The result indicates that *Chlorella vulgaris* optical density was found to increase from the initial cell density after 3 days of incubation (Table 3). *C. vulgaris* had good ability to tolerate up to the concentration 250-500 ppm zinc. One-way, ANOVA indicated that were not significantly ($p>0.05$) different

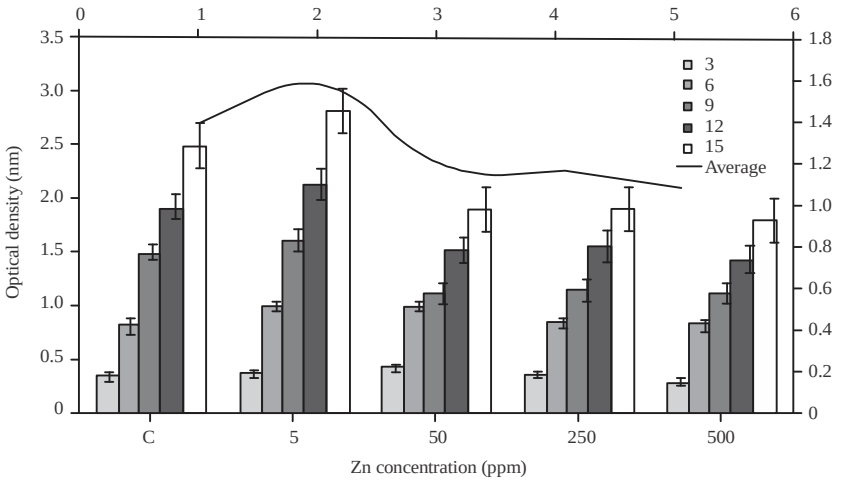

Fig. 4: Optical density a *C. vulgaris* thought time at various Zn concentrations. Results are expressed as means; error bars represent standard error

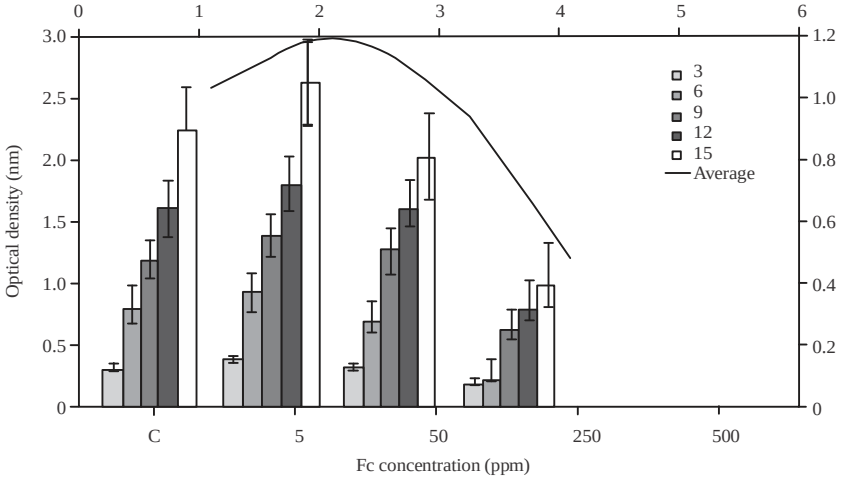

Fig. 5: Optical density a *C. vulgaris* thought time at various Fe concentrations. Results are expressed as means; error bars represent standard error

from each zinc concentrations in *C. vulgaris*. In certain microalgae species such as *Thalassiosira weissflogii* in a low zinc concentration, cadmium stimulates it growth because in this condition cadmium can substitute zinc in specific macromolecules (Price and Morel, 1990). The impact of cadmium and zinc on growth in number of microalgae is inhibitory also severity of inhibition was dependent on the concentration of these elements and the type of microalgae species (Tukaj *et al*., 2007; Knauer *et al*., 1997). However, Lee and Morel (1995) suggested that in zinc-limited media in certain microalgae existence of low concentration of cadmium can enhancement growth of algae and it can act as a nutrient in certain microalgae. The effect of zinc onto Tetraselmis sp showed that they can able to tolerate up to the concentration of 250 ppm zinc (Kumar *et al*., 2013). Kumar *et al*. (2013, 2014) revealed that in presence up to 250 ppm concentration of zinc metal, *Tetraselmis* sp. growth increased when the exposure time was increased. Lim *et al*. (2006) suggested that in higher concentration of zinc, it accumulates onto algal cells thus availability zinc and its toxicity in culture decreased. It result is the survival of cells. This event happened for the reason that interaction between metal ion and the membrane of microalgae cell (fat layer) in the water is high (Fhencel, 1988). The surface of microalgae cells exists of a mosaic of cationic and anionic interchange sites acting as ion exchange (Davies, 1974). These properties dependent on microalgae species and concentration of elements that impacted on microalgae

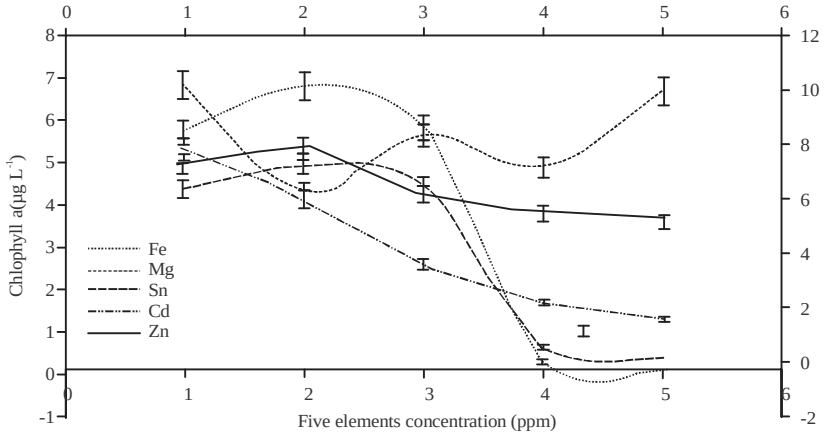

Fig. 6: Effect of five elements on the chlorophyll 'a' content of the C. *vulgaris*

growth (Gao *et al*., 2004). The responses *I. galbana* to heavy metals were indifferent over an incubation period (Veroy *et al*., 1980; Fisher *et al*., 1984) and the variability of metal accumulation in algae associated to the competition between the metals to bind with such polysaccharides.

Effect of Iron on the growth of the *C. vulgaris* cultures: Effect of Fe on the growth of the *C. vulgaris* cultures demonstrated that with increasing Iron concentration the growth of *C. vulgaris* decreased drastically and in concentration 500 ppm of Fe the OD was zero (Fig. 5). One-way, ANOVA indicated that were significantly ($p<0.05$) different from each Fe concentrations in *C. vulgaris* (Table 3). In the absence of iron, retardation of growth, reduction of photosynthetic activity and chlorophyll content is observed (Wiesnner, 1962). Estevez *et al*. (2001) demonstrated that with increasing iron concentration to 200 µM, the growth rate of the cultures of *C. vulgaris* decreased. It suggests that oxidative stress by excess of iron may impact on cellular growth thus it have a negative effect to microalgae (Estevez *et al*., 2001).

Previous studies in impact of five lethal heavy metals on phytoplankton is demonstrated that the toxicity of Hg>Cu>Cd>Zn >Pb (Eichenberger, 1993). Aspect of elements toxicity of five elements in our study can arrange in the following order: Sn>Fe>Cd>Zn>Mg. Two-way ANOVA analysis was performed between concentration and time; for the all treatments had a significant effect ($p<0.001$) on biomass growth, thus, it can be inferred that the effects of these five metals on the growth of *Chlorella vulgaris* were dependent on both concentration and exposure time.

Effect of Magnesium, Tin, Cadmium, Zinc and Iron in chlorophyll 'a' concentration: In this study, chlorophyll 'a' content of the *C. vulgaris* culture was estimated on the final day of experiment (15th day) for five elements treated cultures as well as for the control. The results estimated that the all of five elements significantly affects the chlorophyll 'a' concentration in all the tested of *Chlorella vulgaris*. Among the five elements tested, the treatment with Mg showed maximum chlorophyll 'a' (9. 91±1.2 µg L^{-1}) content than other of the elements. Therefore the lowest chlorophyll 'a' of 0.5±0.12 µg L^{-1} and 0.24±0.12 µg L^{-1} were noticed in treatments with Sn and Fe at 250 ppm respectively. The overall average chlorophyll 'a' concentration in all the treatments with five elements concentration was recorded to be higher in treatments with Mg (5.12±0.55 µg L^{-1}) followed by Zn treatments (7.2±0.10 µg L^{-1}) as shown in Fig. 6.

CONCLUSION

The present study provides clear evidence that the addition of five elements in different concentration have a various impact in the growth of *C. vulgaris* due to the differences in their resistances. From the results, we conclude that. *C vulgaris* having the good ability in treatments with up to 50 ppm of Mg and Zn but it reached to lethal stage in treatments with up to 50ppm of Sn, Cd and Fe. In general, Sn and Fe were the most harmful metals among the five heavy metals investigated. Although all of five elements had differential influences on the growth and photosynthesis, heavy metals in this study with different concentrations can decrease the algal growth rate, which indicates that the presence of these metals should be attention to it. From the all above results it could be understood that *C. vulgaris* can resist in low concentration of Sn, Cd and Fe and high concentration of Mg and Zn in aquatic ecosystems. Thus, it can infer that use to bioremediation of pollution water.

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