Mg, Sn, Cd, Zn and Fe accumulation in unicellular green alga *Chlorella vulgaris* and its effects on growth, content of photosynthetic pigments and protein

A. Saberi[1], M. Taherizadeh[1], N. Amrollahi Biuki[1], L. Fathurrahman[2] and F. Lavajoo[1,*]

[1] Department of Marine Biology, Faculty of Sciences, University of Hormozgan, Bandar Abbas 3995, Iran
[2] East Coast Environmental Research Institute (ESERI), Universiti Sultan Zainal Abidin, Terengganu 21300, Malaysia
* Corresponding author: F.lavajoo@gmail.com
Submission: 7 December 2018 Revised: 10 November 2022 Accepted: 10 November 2022

ABSTRACT

A technique to purify wastewater based on microalgae is an attractive and promising idea for its potential to clean water and as supplementary aquaculture feedstocks. The present study aimed to investigate magnesium (Mg), tin (Sn), cadmium (Cd), zinc (Zn), and iron (Fe) effects in the unicellular green algae *Chlorella vulgaris* as a primary producer and the relationship with the growth, the content of photosynthetic pigments and protein. The ions effects were evaluated by measuring the effect of different ion concentrations on algal growth during a 15-day exposure period. Samples were collected every 3 days over 15 days of the cultivation period to estimate the growth of *C. vulgaris*. Chlorophyll-a (Chl-a) and protein contents of samples were determined on the 15th day of cultivation. Statistical analysis showed that there were significant differences ($P < 0.05$) in the growth and Chl-a content of *C. vulgaris* at different ion concentrations. These could be related to the specific differences in cell metabolism. The highest protein content was found at 5 ppm concentration of Mg (23.03 ± 0.02 μg/mL), Sn (18.82 ± 0.02 μg/mL), Cd (12.52 ± 0.11 μg/mL), Zn (18.99 ± 0.02 μg/mL), and Fe (17.42 ± 0.02 μg/mL) ions. There were significant differences ($P < 0.05$) between the protein content of Mg, Sn, Cd, Zn, and Fe. Growth rate and total Chl-a content (mg/L) were highest at 5 ppm concentration of all ions and the specific growth rate (mg/L), Chl-a, and protein content of *C. vulgaris* were highest at 5 ppm concentration of Mg ions. This study can be a good model for the use of microalgae in the bioremediation of water contaminated with Mg, Sn, Cd, Zn, and Fe.

Keywords: Microalgae, growth, treatment, ion, protein

Thai J. Agric. Sci. (2022) Vol. 55(3): 135−145

INTRODUCTION

Nowadays, heavy metal ions from industrial sectors or agriculture are discharged into aquatic ecosystems and contaminate the total aquatic environment which, not only cause a toxic effect on human, via accumulation in aquatic animals, through the food chain but also affect biodiversity (Lavajoo *et al.,* 2015). Heavy metals such as Cd^{2+} can toxically affect cell physiology. It is frequently considered as a nonessential element for living organisms but Zn^{2+} is a micronutrient for cell growth and metabolism (Leborans and Novillo, 1996; Tukaj *et al.,* 2007). However, Zn^{2+} is an essential element for cell growth but it is also toxic at high concentrations in media (Orús *et al.,* 1991; Miao and Wu, 2006). Dias *et al.* (2002) suggested that heavy metals such as lead, cadmium, mercury, nickel, zinc, aluminum, arsenic, copper, and iron may cause poisoning impacts on the aquatic environment. Instances of the effect of heavy metals on microalgae growth are arrested cell division, inhibited growth rate, restrained enzyme activity, and reduced photosynthesis (Baumann *et al*., 2009; Chen *et al.,* 2009). The response of different microalgae species to the presence of lethal concentrations of heavy metals is varied. Compared to other aquatic organisms in the marine environment, unicellular microalgae exhibit the highest resistance to heavy metals and are highly recommended as bio-indicator for the assessment of marine pollution (Rijstenbeil *et al.,* 1994; Kapkov and Belenikina, 2003; 2007). The unicellular microalgae are photosynthetic organisms with higher efficiency in photosynthesis than terrestrial plants. It utilized light energy and carbon dioxide to produce biomass (Godt *et al.,* 2006; Peters *et al.,* 2013). Various element compositions under different conditions impacted *Chlorella vulgaris* growth stages (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 required for the growth of green algae (Oh-Hama and Miyachi, 1988). Traditional wastewater treatment processes required high operating costs to provide the suitable condition for aerobic bacteria to effectively consume organic components in polluted water. However, microalgae provide an efficient low-cost approach to treat wastewater (Lananan *et al.,* 2014; Nasir *et al.,* 2015). Thus, the current study was carried out to elucidate the influence addition of Mg, Sn, Zn, Cd, and Fe on the growth rate, protein content, and the formation of photosynthetic pigment chlorophyll-a (Chl-a) of *C. vulgaris*. It is an attractive and promising study with the potential to clean water and feedstocks.

MATERIALS AND METHODS

Algal Material and Culture Conditions

The selection of *C. vulgaris* for the current study was based on several considerations such as its existence in most parts of the world, robustness, and suitability for many types of experimental trials, minimal growth conditions, strong tolerability to condition fluctuations, and well-established cross references. The *C. vulgaris* was obtained from the Phytoplankton Culture Laboratory, Institution Persian Gulf and Omani Sea of Hormozgan in Iran. The microalgae were grown in the indoor condition in the N-8 medium based on Vonshak (1986) maintained pH at 7.5 by using H_3PO_4. Stock cultures were incubated in 250 mL conical flasks containing 100 mL of sterilized seawater (35 ppt). The growth chamber was illuminated with cool white fluorescent tube for a 12:12 hours light and dark cycle with the intensity of 100 μmoL $s^{-1}m^{-2}$. The temperature was maintained at 25 ± 2°C.

Analytical Methods

A growth inhibition bioassay for *C. vulgaris* was conducted under laboratory conditions for 15 days. To minimize the metal contamination, all laboratory wares which were in contact with the culture medium were soaked in 1% HNO_3 for 24 hours and rinsed with Milli-Q water. Mg, Zn, Sn, Cd, and Fe solution at a stock concentration of 1,000 mg/L were prepared using analytical grade $MgSO_4$, $ZnCl_2.2H_2O$, $SnCl_2$, $CdCl_2$ and $FeCl_3$ (Dinesh Kumar *et al.,* 2013). About 4.397 g of each salt was dissolved in 1,000 mL of distilled water to make a 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. Culture media with various Mg, Zn, Sn, Cd, and Fe concentrations such as 5, 50, 250, and 500 ppm were prepared by diluting the stock according. The control medium was prepared in the same manner without adding additional elements. The cell growth of algal cultures was 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. Chl-a and protein contents of samples were determined on phase 15th day of cultivation. Chl-a content of the *C. vulgaris* culture was estimated by following the method of Mantoura and Llewellyn (1983). An aliquot of 10 mL of algal culture sample was filtered using a 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 with approximately 2 mL volume was poured over the GF/C filter paper for effective filtration. After filtration, the filtrate was removed and filter paper with algal cells was ground with 90% acetone using a pestle and mortar. The resulting samples were transferred to screw-cap test tubes covered with black cloth and incubated in the refrigerator for 24 hours. After 24 hours of incubation, the contents were ground with 90% acetone and centrifuged at 3,000 rpm for 10 minutes. Then, the clear supernatant was collected, and optical density was measured at different wavelengths such as 630, 645, and 665 nm using a UV-visible spectrophotometer (Shimadzu UV-1800, Japan) for Chl-a determination. Chlorophyll content was calculated according to the extinction coefficients (E) described by Porra *et al*. (1989) as follows:

$$\text{Chl-a } (\mu g/mL) = \frac{12.25 \text{ E630} - \text{E665}}{\text{Sample volume (mL)}}$$

Samples for protein analysis were centrifuged at 1,500 rpm for 10 minutes. The pellet was kept at -20°C until analysis. Total intracellular protein was determined according to the procedure of Bradford (1976) using bovine serum albumin (BSA) as standard; protein extraction (mg L^{-1}) followed the protocol of Rausch (1981).

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's HSD post hoc test was used to test for significant differences among the growth parameters, protein, and Chl-a through the different concentrations.

RESULTS AND DISCUSSION

The effect of Mg, Sn, Cd, Zn, and Fe on the growth of unicellular marine microalgae *C. vulgaris* was successfully elucidated (Table 1).

Table 1 Efficiency comparison of Mg, Sn, Cd, Zn and Fe concentration (5 ppm) on the growth of *C. vulgaris*

Treatments	Relationship	Mean ± standard deviation	P-value
Zn	Mg	0.003 ± 0.0001	0.932
	Fe	0.044 ± 0.0005	0.284
Mg	Zn	-0.003 ± 0.0003	0.932
	Fe	0.041 ± 0.0002	0.323
Fe	Zn	0.044 ± 0.0002	0.284
	Mg	-0.041 ± 0.0004	0.323

Effect of Magnesium, Tin, Cadmium, Zinc and Iron on Protein and Chl-a Contents

In this study, the protein and Chl-a contents of the *C. vulgaris* culture were determined on the final day of the experiment (15th day) for the five elements experimental trials. Notably, the highest protein content was 23.03 ± 0.02, 18.82 ± 0.02, 12.52 ± 0.11, 18.99 ± 0.02, and 17.42 ± 0.02 μg/mL, respectively at 5 ppm concentration of Mg, Sn, Cd, Zn and Fe ($P < 0.05$; Table 2). Results indicated that all five elements significantly affected the content of photosynthetic pigments in *C. vulgaris* (Figure 1). Among the five elements tested, the treatment with Mg showed the highest Chl-a content at 5 ppm concentration than other elements. The lowest Chl-a content was observed in treatments with Sn and Fe at 250 ppm concentration. The Chl-a content decreased with the increase of Cd and Sn concentration. The overall average Chl-a concentration in all treatments was found to be higher in treatments with Mg followed by Zn treatments as shown in Figure 1. Tukey's range test showed significant differences between the Chl-a content of Mg and Zn with Sn and Fe (P < 0.05). Chl-a is the most important pigment in algal cells' photosynthesis to collect solar energy (Van Baalen and O'Donnell, 1978; Takamura *et al.,* 1990). It was also reported that photosynthesis is usually inhibited by heavy metals (Rai *et al.,* 1991). This may be due to the demolition of the photosynthetic membrane, the inhibition of key enzymes of the CO_2-fixation cycle, and the demolition of light-harvesting pigments (Rosko and Rachlin, 1977; Sicko-Goad, 1982; Mallick and Rai, 1989; De Filippis and Pallaghy, 1994; De Filippis and Vincenzini, 1998). In the present study, Cd (II) stress could damage the biosynthesis of chlorophyll in *C. vulgaris*, which is in agreement with the results of Küpper *et al*. (2003), Rai *et al*. (2013), and Çelekli *et al*. (2016). Results from this study also indicated that the increasing growth in the total protein of *C. vulgaris* stimulated by high Mg, Zn, and Fe concentrations was mainly attributed to neutral protein accumulation and increasing growth at 5 ppm concentration. Mg addition led to a slight improvement in total protein content. Sydney *et al*. (2010) showed that regarding the growth stage, cells required constant availability of Mg and N. The high steady-state cell density attained with the addition of Mg at 5 ppm may be due to the fact that this atom plays a major role in photosynthetic activity.

Table 2 Effect of magnesium (Mg), tin (Sn), cadmium (Cd), zinc (Zn) and iron (Fe) on protein content (µg/mL) of *C. vulgaris*

Concentration (ppm)	Mg	Sn	Cd	Zn	Fe
5	23.03 ± 0.02[a]	18.82 ± 0.02[a]	12.52 ± 0.11[a]	18.99 ± 0.02[a]	17.42 ± 0.02[a]
50	19.67 ± 0.10[b]	18.33 ± 0.10[a]	5.10 ± 0.01[b]	18.01 ± 0.10[a]	12.50 ± 0.01[b]
250	19.55 ± 0.01[b]	0.85 ± 0.01[b]	2.20 ± 0.02[c]	12.31 ± 0.10[b]	0.20 ± 0.00[c]
500	18.45 ± 0.10[b]	-	1.60 ± 0.01[c]	12.03 ± 0.02[b]	-

Note: Data are mean ± standard deviation. Values with different letters in the same columns showed differ statistically among themselves (P < 0.05)

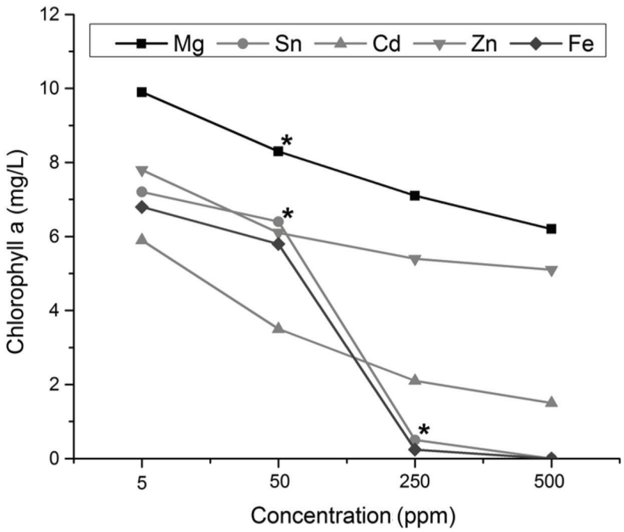

Figure 1 Effect of magnesium (Mg), tin (Sn), cadmium (Cd), zinc (Zn) and iron (Fe) on chlorophyll a content of the *C. vulgaris.* The signs indicate significant differences according to Turkey's range test (P < 0.05)

Effect of Magnesium, Tin, Cadmium, Zinc and Iron on the Growth of the *C. vulgaris*

In all of the laboratory trials, it was found that the highest growth rate of *C. vulgaris* occurred at 5 ppm concentration of Mg (Figure 2A), Zn (Figure 2B), and Fe (Figure 2D). One-way ANOVA analysis showed that there were no significant differences ($P > 0.05$) in the growth of *C. vulgaris* between these three elements.

Effect of magnesium

The optical density of *C. vulgaris* decreased in all concentrations of Mg up to 3 days of incubation. With the additional concentration of Mg and incubation days, the growth of C*. vulgaris* increased drastically (Figure 2A). The highest growth rate was showed in control with 6–9 days of incubation and treatment in the concentration of 5 ppm of Mg with 9–12 days of incubation. The average growth rate (mg/L) for control and treatment in concentration 5 ppm of Mg was respectively 0.44 ± 0.06 and 0.40 ± 0.07 mg/L. For the growth requirement of *C. vulgaris,* the presence of Mg is an important co-factor in cell division and accumulation of cell material. The cell size increased when biomass material was synthesized (Webb, 1949). Shaul (2002) suggested that Mg is a part of photosynthetic Mg-dependent enzymes. The effect of magnesium deficiency on photosynthetic physiology in *C. vulgaris* biomass, protein, chlorophyll a, and chlorophyll b contents decreased in response to magnesium deficiency (Wang *et al.,* 2014).

Effect of zinc

Results showed that *C. vulgaris* were able to tolerate up to 500 ppm concentration of Zn (Figure 2B). Maximum growth in *C. vulgaris* was observed at 5 ppm of Zn concentration with 12–15 days of incubation. The *C. vulgaris* had a good ability to tolerate Zn at the concentration of 250 to 500 ppm. The effect of Zn on *Tetraselmis* sp. showed that it could tolerate up to the concentration of 250 ppm Zn (Dinesh Kumar *et al.,* 2013). Dinesh Kumar *et al*. (2014) revealed that in the presence of up to 250 ppm concentration of Zn, *Tetraselmis* sp. growth increased when the exposure time was increased. In this study, the growth of *C. vulgaris* was negatively influenced by the increasing Zn concentration. However, this microalga could survive at a high Zn concentration of 500 ppm. Lim *et al*. (2006) suggested that in a higher concentration of Zn, the element accumulates onto algal cells thus availability of Zn and its toxicity in culture decreased, resulting in the survival of the cells. This is due to the interaction between the metal ion and the membrane of microalgae cells, and the high-fat layer in the water (Fenchel, 1988; Darmono, 1995). The surface structure of microalgae cells exists of a mosaic of cationic and anionic interchange sites acting as ion exchange (Davies, 1974). These properties are dependent on microalgae species and the concentration of elements impacted the microalgae growth (Gao *et al.,* 2004). Ion stress is an important factor that affects *C. vulgaris* growth. The existence of some algae, such as *C. vulgaris*, in heavy metal-polluted aquatic bodies, may lead us to conclude that these organisms can resist and tolerate metal toxicity. In this study, it was found that *C. vulgaris* was able to survive in high concentrations of Zn. The Zn ions inhibited the growths and nitrogen activity of two species such as *Anabaena* sp. and *Nodularia* sp. Even at a low Zn concentration of 10 ppm, it could completely reduce the growth and nitrogenase activities of all cultures due to the damaged cell membrane which leads to uncontrolled efflux/influx of electrolytes and other vital ions which may be responsible for inhibition of growth (Stratton *et al*., 1979).

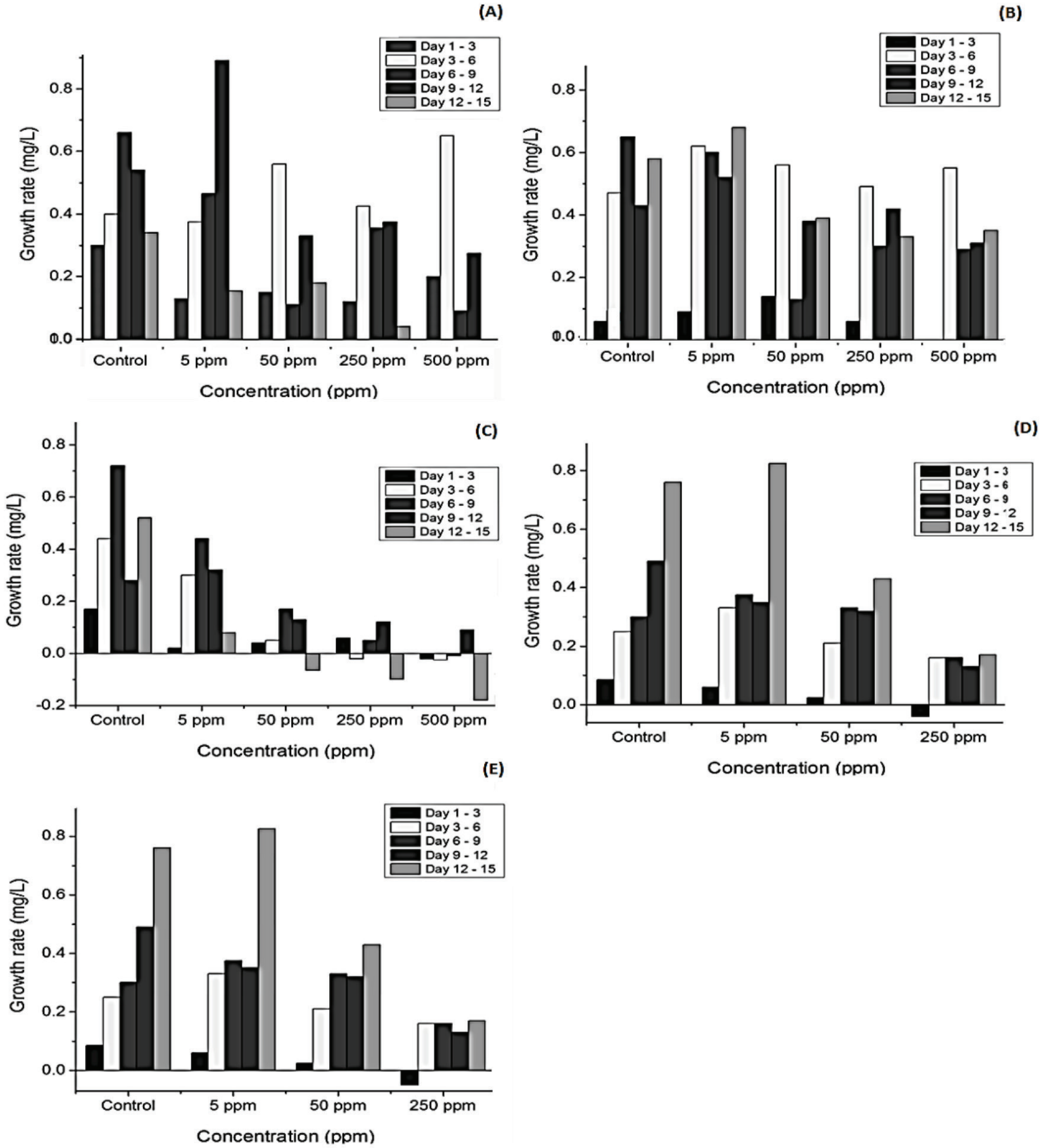

Figure 2 Growth rate of *C. vulgaris* at different magnesium (A), zinc (B), cadmium (C), iron (D) and tin (E) concentrations

Effect of cadmium

The growth rate of *C. vulgaris* decreased with the increase in the concentration of Cd and incubation day (Figure 2C). At 5 ppm of Cd concentration, the growth of *C. vulgaris* continued to day 15. However, at 50 and 250 ppm of Cd concentration the growth of the microalgae decreased (sub-lethal concentration). At 500 ppm of Cd concentration, *C. vulgaris* growth stopped and the highest depletion in growth was shown in 12–15 days (lethal concentration). In this study, time can be an effective parameter in *C. vulgaris* growth in different concentrations of Cd. The researcher showed that the growth and the amount of chlorophyll a and chlorophyll b gradually decreased with increasing Cd over 18 days of exposure (Cheng *et al.,* 2016).

Effect of iron

The growth rate of the *C. vulgaris* cultures decreased with the increase in Fe concentrations. At the concentration of 500 ppm Fe, the growth rate of *C. vulgaris* was zero (lethal concentration). The microalgae cell bleached and died (Figure 2D). In the experiment, it was found that the highest growth of *C. vulgaris* was in the concentration of 5 ppm Fe. It is suggested that the addition of Fe up to 5 ppm could contribute to the increase in the growth of *C. vulgaris.* At 250 ppm Fe concentration, the growth of *C. vulgaris* had sustained to day 15 and then decreased (sub-lethal concentration). At 500 ppm Fe, microalgae cells died. In the absence of Fe, retardation of growth, reduction of photosynthetic activity, and chlorophyll content were reported (Wiesnner, 1962). Estevez ***et al***. (2001) demonstrated that with increasing Fe concentration to 200 ppm, the growth rate of the cultures of *C. vulgaris* decreased which is coincide with our result. It suggests that oxidative stress by the excess of iron may impact cellular growth thus it has a negative effect on microalgae. However, the low concentration of Fe in *C. vulgaris* medium increased the growth rate (Kean *et al.,* 2015). The researcher believed that cell division decreased with increasing Fe concentration and suggested that it realized free radicals by Fe ion (Xiaoling and Jinyao, 2006). According to Iriani *et al*. (2011), protein content (8.34 mg/g dry weight) was highest at the lowest Fe^{3+} concentration (0.35 mg/L).

Effect of tin

The effect of Sn on the growth of the *C. vulgaris* cultures was demonstrated. The growth rate of current microalgae decreased with the increase of the concentration of Sn and incubation day (Figure 2E). The highest growth rate of *C. vulgaris* was observed at the concentration of 5 ppm Sn on day 12–15 of incubation. At 500 ppm concentration of Sn, the growth of *C. vulgaris* stopped and the cells died (lethal concentration). A decrease in the growth can be relatively easily determined and reflects the physiological status of the algal cells (Piovár *et al*., 2011). Heavy metals had adverse effects on the growth of *Scenedesmus quadricauda* (Mohammed and Markert, 2006; Stork *et al*., 2013) and *Spirogyra setiformis* (Çelekli *et al*., 2016) in cultures, the same result was also found in this study, the inhibited growth is mainly under high Sn concentration, the growth of *C. vulgaris* decreased with the increasing Sn concentration.

This study demonstrates that the effects of Mg, Sn, Cd, Zn, and Fe elements on the growth of *C. vulgaris* were dependent on both concentration and exposure time. Previous studies on the impact of five lethal heavy metals on phytoplankton reported that the toxicity elements were in the order of Hg > Cu > Cd > Zn > Pb (Estevez *et al.,* 2001). Hence, the toxicity of elements in the present study could be arranged in the order of Cd > Sn > Fe > Zn.

CONCLUSION

The growth parameter, Chl-a, and total protein contents of the *C. vulgaris* were completely inhibited at 500 ppm Mg, Sn, Cd, and Fe concentrations. Variations in growth conditions influenced the growth and other activities of *C. vulgaris*. Ion stress is an important factor that affects *C. vulgaris* growth. The existence of some algae, such as *C. vulgaris*, in heavy metal-polluted aquatic bodies, may lead us to conclude that these organisms are able to resist and tolerate metal toxicity. In this study, it was found that *C. vulgaris* was able to survive in high concentration of Zn and its growth was negatively influenced by the increasing Zn concentration. However, this microalga could survive at a high Zn concentration of 500 ppm. This may be due to the existence of active intracellular sequestration that prevents exposure to essential cellular components. The *C. vulgaris* had the best optimal performance of growths, Chl-a, and protein content in 5 ppm of Mg, Zn, and Fe stress conditions, making it a suitable candidate for bioaccumulation, biosorption, and food supplement. Further studies will be needed to determine the characteristics of metal sensitivities of other microalgae species.

REFERENCES

Baumann, H.A., L. Morrison and D.B. Stengel. 2009. Metal accumulation and toxicity measured by PAM-chlorophyll fluorescence in seven species of marine macroalgae. Ecotoxicol. Environ. Saf. 72(4): 1063–1075.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Çelekli, A., E. Gültekin and H. Bozkurt. 2016. Morphological and biochemical responses of *Spirogyra setiformis* exposed to cadmium. Clean (Weinh) 44(3): 256–262.

Chen, L., Q.S. Zheng and Z.P. Liu. 2009. Effects of different concentrations of copper ion on the growth and chlorophyll fluorescence characteristics of *Scendesmus obliquus* L. Ecol. Environ. Sci. 18: 1231–1235.

Cheng, J., H. Qiu, Z. Chang, Z. Jiang and W. Yin. 2016. The effect of cadmium on the growth and antioxidant response for freshwater algae *Chlorella vulgaris*. Springerplus 5(1): 1290.

Darmono, O. 1995. Logam Dalam Sistem Biology Makhluk Hidup. UI-Press, Penerbit Universitas Indonesia, Jakarta, Indonesia.

Davies, A.G. 1974. The growth kinetics of *Isochrysis galbana* in cultures containing sub lethal concentrations of mercuric chloride. J. Mar. Biol. Assoc. UK. 54(1): 157–169.

De Filippis, L.F.C. and K. Pallaghy. 1994. Heavy metals: sources and biological effects, pp. 31–77. *In*: L.C. Rai, J.P. Gaur and C.J. Soeder, (Eds), Algaeand Water Pollution. E. Schweizeerbartsche Verlagsbuchhandlung, Science Publisher, Stuttgart, Germany.

De Philippis, R. and M. Vincenzini. 1998. Exocellular polysaccharides from cyanobacteria and their possible applications. FEMS Microbiol. Rev. 22(3): 151–175.

Dias, M.A., I.C.A. Lacerda, P.F. Pimentel, H.F. de Castro and C.A. Rosa. 2002. Removal of heavy metals by an *Aspergillus terreusstrain* immobilized in *polyurethanematrix*. Lett. Appl. Microbiol. 34(1): 46–50.

Dinesh Kumar, S., P. Santhanam, S. Ananth, A. Shenbaga Devi, R. Nandakumar, B. Balaji Prasath, S. Jeyanthi, T. Jayalakshmi and P. Ananthi. 2014. Effect of different dosages of zinc on the growth and biomass in five marine microalgae. Int. J. Fish. Aquac. 6(1): 1–8.

Dinesh Kumar, S., P. Santhanam, T. Jayalakshmi, R. Nandakumar, S. Ananth, A. Shenbaga Devi and B. Balaji Prasath. 2013. Optimization of pH and retention time on the removal of nutrients and heavy metal (zinc) using immobilized marine microalga *Chlorella marina*. J. Biol. Sci. 13(5): 400–405.

Estevez, M.S., G. Malanga and S. Puntarulo. 2001. Iron-dependent oxidative stress in *Chlorella vulgaris*. Plant Sci. 161(1): 9–17.

Fenchel, T. 1988. Marine plankton food chains. Ann. Rev. Ecol. Syst. 19: 19–38.

Gao, K., Y. Ji and J. Tanaka. 2004. Quantitative evaluation of wind effect during emersion on *Porhpyra haitanensis* (Rhodophyta), a farmed species in southern China. Fish. Sci. 70: 710–712.

Godt, J., F. Scheidig, C. Grosse-Siestrup, V. Esche, P. Brandenburg, A. Reich and D.A. Groneberg. 2006. The toxicity of cadmium and resulting hazards for human health. J. Occup. Med. Toxicol. 1: 22.

Iriani, D., O. Suriyaphan and N. Chaiyanate. 2011. Effect of iron concentration on growth, protein content and total phenolic content of *Chlorella* sp. cultured in basal medium. Sains Malays. 40(4): 353–358.

Kapkov, V.I. and O.A. Belenikina. 2003. Biomarkers of pollution of marine ecosystems with heavy metals. Water Ecosyst. Organ. (Moscow) 6: 68–69.

Kapkov, V.I. and O.A. Belenikina. 2007. A study of the resistance of mass marine algae to heavy metals. Moscow Univ. Biol. Sci. Bull. 62(1): 30–33.

Kean, M.A., E. Brons Delgado, B.P. Mensink and M.H.J. Bugter. 2015. Iron chelating agents and their effects on the growth of *Pseudokirchneriella subcapitata*, *Chlorella vulgaris*, *Phaeodactylum tricornutum* and *Spirulina platensis* in comparison to Fe- EDTA. J. Algal Biomass Utln. 6(1): 56–73.

Küpper, H., I. Šetík, E. Šetliková, N. Ferimazova, M. Spiller and F.C. Küpper. 2003. Copper-induced inhibition of photosynthesis: limiting steps of *in vivo* copper chlorophyll formation in *Scenedesmus quadricauda*. Funct. Plant Biol. 30(12): 1187–1196.

Lananan, F., S.H. Abdul Hamid, W.N.S. Din, N. Ali, H. Khatoon, A. Jusoh and A. Endut. 2014. Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing effective microorganism (EM-1) and microalgae (*Chlorella* sp.). Int. Biodeterior. Biodegradation. 95: 127–134.

Lavajoo, F., M. Taherizadeh and M. Dehghani. 2015. The absorption of nitrate and phosphate from urban sewage by blue-green algae (*Spirolina platensis*) (an alternative medium) as application for removing the pollution. J. Appl. Sci. Environ. Manage. 19(3): 353–356.

Leborans, G.F. and A. Novillo. 1996. Toxicity and bioaccumulation of cadmium in *Olisthodiscus luteus* (Raphidophyceae). Water Res. 30(1): 57–62.

Lim, C.Y., Y.H. Yoo, M. Sidharthan, C.W. Ma, I.C. Bang, J.M. Kim, K.S. Lee, N.S. Park and H.W. Shin. 2006. Effects of copper (I) oxide on growth and biochemical compositions of two marine microalgae. J. Environ. Biol. 27(3): 461–466.

Mallick, N. and L.C. Rai. 1989. Response of *Anabaena doliolumto* bimetallic combinations of Cu, Ni and Fe with special reference to sequential addition. J. Appl. Phycol. 1: 301–306.

Mantoura, R.F.C. and C.A. Llewellyn. 1983. The rapid determination of algal chlorophyll and carotenoid and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. Anal. Chim. Acta 151: 297–314.

Miao, X. and Q. Wu. 2006. Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol. 97(6): 841–846.

Mohammed, M.H. and B. Markert. 2006. Toxicity of heavy metals on *Scenedesmus quadricauda* (Turp.) de Brebisson in batch cultures. Environ. Sci. Pollut. Res. Int. 13(2): 98–104.

Nasir, N.M., N.S.A. Bakar, F. Lananan, S.H. Abdul Hamid, S.S. Lam and A. Jusoh. 2015. Treatment of African catfish, *Clarias gariepinus* wastewater utilizing phytoreediation of microalgae, *Chlorella* sp. with *Aspergillus niger* bio-harvesting. Bioresour. Technol. 190: 492–498.

Oh-Hama, T. and S. Miyachi. 1988. Chlorella, pp. 3–26. *In*: M.A. Borowitzka and L.J. Borowitzka, (Eds), Microalgal Biotechnology. Cambridge University Press, Cambridge, UK.

Orús, M.I., E. Marco and F. Martínez. 1991. Suitability of *Chlorella vulgaris* UAM 101 for heterotrophic biomass production. Bioresour. Technol. 38: 179–184.

Peters, K., M. Bundschuh and R.B. Schafer. 2013. Review on the effects of toxicants on freshwater ecosystem functions. Environ. Pollut. 180: 324–329.

Piovár, J., E. Stavrou, J. Kaduková, T. Kimáková and M. Bačkor. 2011. Influence of long-term exposure to copper on the lichen photobiont *Trebouxia erici* and the free-living algae *Scenedesmus quadricauda*. Plant Growth Regul. 63: 81–88.

Porra, R.J., W.A. Thompson and P.E. Kriedemann. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975(3): 384–394.

Rai, L.C., A.K. Singh and N. Mallick. 1991. Studies on photosynthesis, the associated electron transport system and some physiological variables of *Chlorella vulgaris* under heavy metal stress. J. Plant Physiol. 137(4): 419–424.

Rai, U.N., N.K. Singh, A.K. Upadhyay and S. Verma. 2013. Chromate tolerance and accumulation in *Chlorella vulgaris* L.: role of antioxidant enzymes and biochemical changes in detoxification of metals. Bioresour. Technol. 136: 604–609

Rausch, T. 1981. The estimation of micro-algal protein content and its meaning to the evaluation of algal biomass I. Comparison of methods for extracting protein. Hidrobiologia 78: 237–251.

Rijstenbeil, J.W., J.W.M. Derksen, L.J.A. Gerringa, T.C.W. Poortvliet, A. Sandee, M. van den Berg, J. van Drie and J.A. Wijnholds. 1994. Oxidative stress induced by copper: defense and damages in the marine planktonic diatom *Ditylum brightwellii*, grown in continuous cultures with high and low zinc levels. Mar. Biol. 119: 583–590.

Rosko, J.J. and J.W. Rachlin. 1977. The effect of cadmium, copper, mercury, zinc and lead on cell division, growth and chlorophyll a content of the chlorophyte *Chlorella vulgaris*. Bull. Torrey Bot. Club 104(3): 226–233.

Shaul, O. 2002. Magnesium transport and function in plants: the tip of the iceberg. Biometals 15: 309–323.

Sicko-Goad, L. 1982. A morphometric analysis of algal response to low dose, short-term heavy metal exposure. Protoplasma 110: 75–86.

Stork, F., M. Backor, B. Klejdus, J. Hedbavny and J. Kovacik. 2013. Changes of metal-induced toxicity by H_2O_2/NO modulators in *Scenedesmus quadricauda* (Chlorophyceae). Environ. Sci. Pollut. Res. 20: 5502–5511.

Stratton, G.W., A.L. Huber and C.T. Corke. 1979. Effect of mercuric ion on the growth, photosynthesis, and nitrogenase activity of *Anabaena inaequalis*. Appl. Environ. Microbiol. 38(3): 537–543.

Sydney, E.B., W. Sturm, J.C. de Carvalho, V. Thomaz-Soccol, C. Larroche, A. Pandey and C.R. Soccol. 2010. Potential carbon dioxide fixation by industrially important microalgae. Bioresour. Technol. 101(15): 5892–5896.

Takamura, N., F. Kasai and M.M. Watanabe. 1990. Unique response of cyanophyceae to copper. J. Appl. Phycol. 2: 293–296.

Tukaj, Z., A. Bascik-Remisiewicz, T. Skowronski and C. Tukaj. 2007. Cadmium effect on growth, photosynthesis, ultrastructure and phytochelatin content of green microalga *Scenedesmus armatus*: a study at low and elevated CO_2 concentration. Environ. Exp. Bot. 60(3): 291–299.

Van Baalen, C. and R. O'Donnell. 1978. Isolation of a nickel-dependent blue-green alga. J. Gen. Microbiol. 105(2): 351–353.

Vonshak, A. 1986. Laboratory techniques for the cultivation of microalgae, pp. 117–145. *In*: A. Richmond, (Ed), Handbook of Microalgal Mass Culture. CRC Press, Florida, USA.

Wang, M., W.C. Kuo-Dahab, S. Dolan and C. Park. 2014. Kinetics of nutrient removal and expression of extracellular polymeric substances of the microalgae, *Chlorella* sp. and *Micractinium* sp., in wastewater treatment. Bioresour. Technol. 154: 131–137.

Webb, M. 1949. The influence of magnesium on cell division. 2. The effect of magnesium on the growth and cell division of various bacterial species in complex media. J. Gen. Microbiol. 3: 410.

Wetherell, D.F. 1961. Culture of fresh water algae in enriched natural sea water. Physiol. Plant. 14(1): 1–6.

Wiesnner, W. 1962. Inorganic micronutrients, pp. 267–286. *In*: R.A. Lewin, (Ed), Physiology and Biochemistry of Algae. Academic Press, New York, USA.

Xiaoling, Y. and G. Jinyao. 2006. Regulation of Fe growth and material accumulation of *Dunaliella salina*. Chinese Agricultural Science Bulletin 22(10): 476–476.