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Effects of microelements (Fe, Cu, Zn) on growth and pigment contents of *Arthrospira* (*Spirulina*) *platensis*

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Abstract

A laboratory experiment was conducted to assess the bioaccumulation of microelements Fe^{2+}, Cu^{2+}, Zn^{2+} and their effects on the growth and pigment contents of *Spirulina platensis* in Zarrouk's media. The mentioned metals concentrations enhanced separately to tenfold of the Zarrouk's content. The results indicated no differences in the dry weights for the different medias (p>0.05). A rapid increase in optical density values has been observed in tenfold of Cu^{+2} concentration giving the maximum optical density values (0.498± 0.234 mg L^{-1}). In contrast, a gradual increase rate in the optical density has been observed at all concentration of treatments. Enrichment factor (EF) improved with increasing the metal concentration of treatments. The maximum EF value has been observed at 13.54 in Zn^{+2} concentrations. At all concentrations, the maximum production of chlorophyll A (1.213±0.514 mg L^{-1}) occurred in 7th day of incubation. The highest total carotenoid concentration was recorded in *S. platensis* treated with Cu^{+2} (0.0042± 0.0004 mg L^{-1}) after 14 days. The phycobiliproteins decreased in all of the treatments. Results suggested that *S. platensis* would be important part of in functional food developments.

Keywords: Growth, Bioaccumulation, Microelements, Pigments, *Spirulina platensis*.

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Introduction

Arthrospira (*Spirulina*) *platensis* is a photosynthetic filamentous, helical shaped, multicellular, and green-blue microalga that grows vigorously in strong sunshine under high temperatures and alkaline conditions (Sanchez *et al*., 2003; Habib *et al*., 2008). *Spirulina* sp. has a high content of protein (up to 70%), along with high amounts of essential fatty acids, essential amino acids, minerals (such as iron, copper, zinc), vitamins (especially B12), antioxidant pigments (i.e. phycobiliproteins, carotenoids, and chlorophyllp-A) and polysaccharides (Belay *et al*., 1993; Vonshak, 1997). The commercial production of *Spirulina* sp. has gained worldwide attention as a human food supplements, animal feed (terrestrial, fresh water and marine) and pharmaceuticals. In aquaculture, *Spirulina* sp. is using as an additive to improve growth, coloration and probiotic agent (Ramakrishnan *et al*., 2008; Ghaeni *et al*., 2011; Ansarifard *et al*., 2018). The growth of microalgae and the composition of the biomass produced depend on many factors such as nutrient availability, temperature, and light (Sabzi *et al*., 2018; Hadizadeh *et al*., 2019).

Trace metals such as iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), copper (Cu) and nickel (Ni) are essential components which required by microalgae for various metabolic functions and growth (Bruland *et al*., 1991; Rastar *et al*., 2018). It has been reported that iron promotes the growth of cyanobacteria and increase photosynthesis and nitrogen fixation (Rueter and Petersen, 1987). Microalgae utilize zinc as an enzyme cofactor; and, the higher concentrations are toxic to most aquatic life (Omar, 2002). Szabolcs *et al*. (2013) investigated on four microelements such as Fe^{+2}, Cu^{+2}, Zn^{+2} and Mo^{+2} on S. platensis and Chlorella vulgaris and proved to enhanced bioaccumulation ability and biomass. Arunakumara *et al*. (2008) results showed that bioaccumulation of Pb^{2+} at low concentrations (5 μg ml^{-1}), cause stimulate its growth slightly, although the chlorophyll α and β carotene were decreased. Balaji *et al*. (2014) investigated the effects of zinc and nickel on growth of different *Spirulina* sp. strains which significantly affected by the concentration of selected metals in the culture medium. Maximum growth has exhibited by *S. maxima* at 0.01mM zinc (6.9 mg L^{-1}) and 0.01mM nickel (17 mg L^{-1}). Deniz *et al*. (2011) reported that growth and chlorophyll A contents in *Spirulina* sp. decreased at most exposure levels to copper and sodium chloride, with increase in carotenoid pigment. Okmen *et al*., (2011) reported that lower zinc concentration (2.5 mg L^{-1}) were stimulated the biomass, chlorophyll A, total carbohydrate and protein content in *Anabaena* sp. GO1 and *Gloeothece* GO9 cyanobacteria. Zinc trace element in the 10 mg L^{-1} and more concentrations were decreased the biomass and other parameters except for *Anabaena* sp. GO2 species. Most of the previous studies have focused on the remediation of heavy metals by microalgae species to eliminate toxic

elements (Romera *et al*., 2007). The objective of this study was to examine the accumulation of selected important microelements by *S. platensis* and its effects on growth and biopigments accumulation.

Materials and methods

Microorganism and applied chemicals

S. platensis was obtained from the Research laboratory, Agriculture and Natural Resources of Ahwaz Islamic Azad University, Ahwaz, Iran. All the applied reagents and chemicals have been obtained from either Merck and/or Sigma-Aldrich companies.

Culture medium

The composition of the growth media in the case of *spirulina platensis* was in accordance with Zarrouk's medium (Zarrouk, 1996). Three metals (Fe^{+2}, Cu^{+2} and Zn^{+2}) were chosen for experiments with concentration enhanced separately to tenfold of the Zarrouk's media (control) (OECD, 2011). Four different solutions have been prepared with control and three others with diverse metal concentrations. The metal content of the control was in accordance with the Zarrouk guideline, as [$FeSO_4.7H_2O$]: 0.01 g L^{-1}, [$ZnSO_4.4H_2O$]: 0.222 g L^{-1}, [$CuSO_4.5H_2O$]: 0.079 g L^{-1}. The metal solutions have prepared from $FeSO_4.7H_2O$, $ZnSO_4.4H_2O$, $CuSO_4.5H_2O$ (Table 1). All experiments have been performed in triplicates (n=3).

Table 1: Applied metal concentrations in the growth media.

Growth media	FeSO4.7H2O	Fe^{2+}	ZnSO4.4H2O	Zn2+	CuSO4.5H2O	Cu2+
control*	0.01g L^{-1}	0.002g L^{-1}	0.222g L^{-1}	0.050mg L^{-1}	0.079g L^{-1}	0.020mg L^{-1}
Enhanced media(Fe 10fold)	0.1	0.02	0.222	0.050	0.079	0.020
Enhanced media(Zn 10fold)	0.01	0.002	2.22	0.50	0.079	0.020
Enhanced media(Cu 10fold)	0.01	0.002	0.222	0.050	0.79	0.20

*Control is accordance with Zarrouk media.

Culture conditions and growth

The alga growing apparatus consists of a horizontal glass surface where on the Erlenmeyer flasks had been placed. Erlenmeyer flasks of 1000 ml capacity have prepared containing 100 ml *S. platensis* (10%) with initial optical density 0.019 (Biomass concentration of 0.002 g L^{-1} dry weight) and 200 ml Zarrouk media (Zarrouk, 1966) at temperature 32 ˚C, pH 8.7, salinity 20 ppt with an illumination of 2500 lux light intensity, with a light/dark cycle of 12/12 h. Fresh air was pumped into the solution through plastic tubes in order to avoid the generation of alga film layer on the wall of the flasks for a period of 14 days.

Growth of microalgae

Biomass concentration was determined every day by measuring the optical density at 560 nm to produce a standard curve. Standard curve has been

subsequently used to calculate the biomass of individual samples based on their optical density (Gupta *et al*., 2006; Rastar *et al*., 2018).

Determination of dry weight

The dry weight of biomass has been determined by filtration of sample (15 ml) through dried Whatman filter (pore size 0.42 µm) with carefully up to 0.0001 g level. The sample is being filtered and dried at 80˚C for 4 h and cooled in desiccators after it has been washed up twice with distilled water (Olguin *et al*., 2001; Sabzi *et al*., 2018).

Bioaccumulation analysis

To estimate the iron, copper and zinc metals, the samples have been separated from the solution by centrifugation MICRO 22R model manufactured by Hettich of Germany. One gram of the wet sample more accurately weigh scales Sartryvs 124S model. They were transferred to crucible and then in electric furnaces BATEC PC 21 model with ashing process has performed at 550 °C. The contents dissolved in 3 ml 1:1 nitric acid solution and then reach to 10 ml of distilled water in volumetric flask after cooling. The solutions of iron, copper and zinc ions have been prepared from standard stock solutions (1000 mg L^{-1}) in the concentration range of 0.1 to 10 mg of iron, 0.1 to 15 mg per liter for copper and 0.1 to 1.5 mg per liter for zinc have analyzed by flame atomic absorption spectrometer PG-990 model. Results have been calculated from the values of three parallel measurements and were expressed in mg kg^{-1} dried alga (Forstner and Muller, 1974).

EF calculation

Data for the calculation EF gained from the results of measured metal content of 15 samples prepared as follows: Five samples were prepared in triplicates with four different the compositions of the metals (with a control sample).

Enrichment factor (EF) has calculated according to the following ratios (Szabolcs *et al*., 2013):

$EF= C_E/C_C$

C_E =Microelement concentration of dry alga grown in the media with enhanced metal content

C_C=Microelement concentration of dry alga grown in the control media

Biopigments estimation

Chlorophyll content has determined by centrifugation for 10 minute at 4000 rpm. Chlorophyll A (Ch-A) was extracted by using 5 ml 90% acetone and placed the tube in dark for 24 hour. Samples have centrifuged after extraction at 5000 rpm for 15 minute and collect the supernatant. Read the absorbance at 630 nm, 645 nm, and 665nm against 90% acetone as blank by using UV/VIS spectrophotometer and concentration of Chl-a A was calculated using the below formula:

C=11.6 A665– 1.31 A645– 0.14 A630

The concentration of Chl-A in a given volume of culture can be determined by below formula:

Chl-A-(mg L^{-1}) $=\frac{C \times Ve}{Vc}$

C=Value obtained from above equation

Ve=Volume of extract (ml)

Vc=Volume of culture (liters)

Phycobiliproteins estimation

Amount of 5 ml cyanobacterial cell suspension was taken and subjected to centrifugation at 4000 rpm for 10 minutes. Phycobiliproteins have extracted in 5 ml of phosphate at pH 6.7, 0.05 M by 3 times repeated freezing and thawing. Freeze thawed samples subjected to Centrifugation at 1000 rpm for 15 minutes. The final extract has been measured at 562 nm, 615 nm, and 652 nm against phosphate buffer as blank.

The concentration of phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) have calculated by using the formula (Bennett and Bogorad, 1971):

$$PC=\frac{A615-0.474(A652)}{5.34}$$

$$APC=\frac{A652-0.208(A615)}{5.09}$$

$$PE=\frac{A562-2.41(PC)-0.849(APC)}{9.62}$$

The concentration of phycobiliprotein in a total volume of culture can be determined as follows:

$$\text{Phycobiliprotein (mg ml}^{-1}) =\frac{C\times Ve}{Vc}$$

C=Value of PC, APC and PE obtained from above equations

Ve=Volume of extract (ml)

Vc=Volume of culture (ml)

Carotenoids estimation

Harvested biomass has been homogenized in homogenizer with 5 ml, 90% acetone and centrifuged the sample at 5000 rpm for 15 minutes. The carotenoids in samples were determined spectrophotometrically at 450 nm by using the fallowing calculation formula (Jensen, 1978):

$$C=\frac{A450\times V\times f\times 10}{2500}$$

C=Total amount of Cart (mg ml-1)

V=Volume of extract (ml)

f=Dilution factor

Statistical analysis

Data analyzed statistically using one way analysis of variance (ANOVA) using SPSS version 18. Duncan's multiple range test was used to compare differences among treatment means at ($p\leq0.05$) level (Duncan, 1955).

Results

Growth analysis

The effect of different microelement contents in media on the growth of S. platensis was evaluated daily during 14 days by determination of dry weights (g L-1) (Fig. 1 and Table 2).

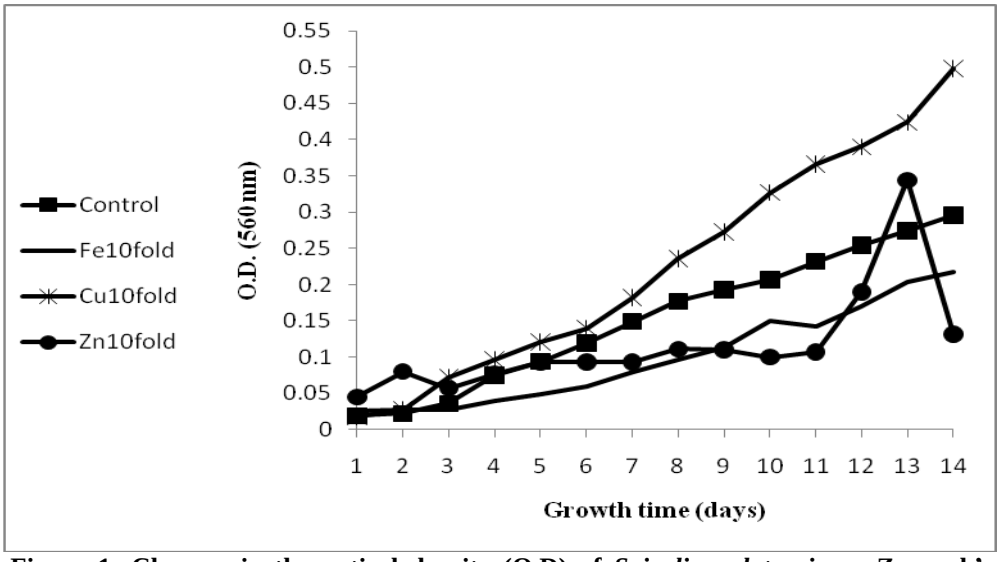


Figure 1: Changes in the optical density (O.D) of *Spirulina platensis* on Zarrouk's media as a control, and growth mediums with enhanced trace element level.

Table 2: Dry weight and OD (Optical density) of *Spirulina platensis* cultivated in Zarrouk's medium as a control and growth medias with enhanced trace element level.

Parameter	Day	Control	Fe10fold	Cu10fold	Zn10fold
Dry weight ($g\ L^{-1}$)	14	1.55 ± 0.02^{a}	1.57 ± 0.03^{a}	1.54 ± 0.03^{a}	1.57 ± 0.01^{a}
OD	1	0.196 ± 0.009^{a}	0.026 ± 0.005^{a}	0.018 ± 0.005^{a}	0.045 ± 0.025^{a}
OD	2	0.022 ± 0.005^{a}	0.028 ± 0.007^{a}	0.027 ± 0.009^{a}	0.080 ± 0.092^{a}
OD	3	0.036 ± 0.011^{ab}	0.028 ± 0.017^{a}	0.072 ± 0.019^{b}	0.057 ± 0.019^{ab}
OD	4	0.075 ± 0.010^{b}	0.039 ± 0.014^{a}	0.096 ± 0.029^{b}	0.076 ± 0.004^{b}
OD	5	0.094 ± 0.011^{b}	0.049 ± 0.013^{a}	0.121 ± 0.032^{b}	0.093 ± 0.010^{b}
OD	7	0.149 ± 0.037^{ab}	0.080 ± 0.019^{b}	0.182 ± 0.053^{b}	0.093 ± 0.036^{a}
OD	8	0.177 ± 0.044^{ab}	0.096 ± 0.026^{a}	0.236 ± 0.075^{b}	0.111 ± 0.057^{a}
OD	9	0.193 ± 0.054^{ab}	0.113 ± 0.045^{a}	0.273 ± 0.104^{b}	0.110 ± 0.063^{a}
OD	10	0.207 ± 0.052^{ab}	0.150 ± 0.043^{a}	0.327 ± 0.113^{b}	0.100 ± 0.058^{a}
OD	11	0.232 ± 0.067^{ab}	0.143 ± 0.083^{a}	0.366 ± 0.132^{b}	0.107 ± 0.071^{a}
OD	13	0.274 ± 0.106^{a}	0.203 ± 0.116^{a}	0.424 ± 0.223^{a}	0.344 ± 0.139^{a}
OD	14	0.296 ± 0.115^{ab}	0.217 ± 0.124^{ab}	0.498 ± 0.234^{b}	0.132 ± 0.089^{a}

Within rows, means followed by the different letters are significantly different at $p\leq0.05$ as determined by the Duncan's test.

Results indicated no significant differences in the dry weights for the different medium, although the values of dry weight in iron and zinc treatments were higher than the control treatment the (Table 2). Results showed that there are no significant differences in OD values between control and other treatments after 14^{th} day of culture (Table 2). Similar to these results, the optical density increased by 13 days, but the growth of *S. platensis* started to decline after incubation in the medium with ten times enhanced Zn^{+2} concentration. The growth curves showed no lag phase for the Zarrouk, s and other medium. A rapid increase in OD values has observed in the media with ten times enhanced Cu+2 concentration with maximum 0.498 ± 0.234 mg L^{-1} on the 14th day ($p<0.05$). In contrast, a gradual different rate of increase in the OD has measured at all concentration (Fig. 1).

Bioaccumulation analysis

The extent of bioaccumulation and EF of iron, copper and zinc by *Spirulina* sp. grown in media with diverse microelement content were measured (Table 3).

Table 3: Bioaccumulation and EF of iron, copper and zinc by *Spirulina* sp. grown in media with diverse microelement content after 14 days of incubation.

Treatment	Bioaccumulation of ions by *Spirulina* (mg kg^{-1})		EF
	Alga grown in normal medium	Alga grown in normal medium with 10 times enhanced microelement content	
Fe^{2+}	668±25.53[a]	4465±39.68[b]	6.68
Cu^{2+}	81.66±5.50[a]	394±7.54[b]	4.82
Zn^{2+}	52±4.00[a]	704.33±8.14[b]	13.54

Within rows, means followed by the different letters are significantly different at $p \leq 0.05$ as determined by the Duncan's test.

Pigment contents

Chlorophyll-A and Carotenoids

The maximum production of chlorophyll occurred in 7th days of incubation at all concentration of treatments with maximum chlorophyll-A values (1.21±0.30 mg L^{-1}) in ten times enhanced Cu concentration. Although the concentration of chlorophyll accumulation in *S. platensis* grown in different media with maximum values of chlorophyll contents in copper treatment by 14th days of culture (Fig. 2).

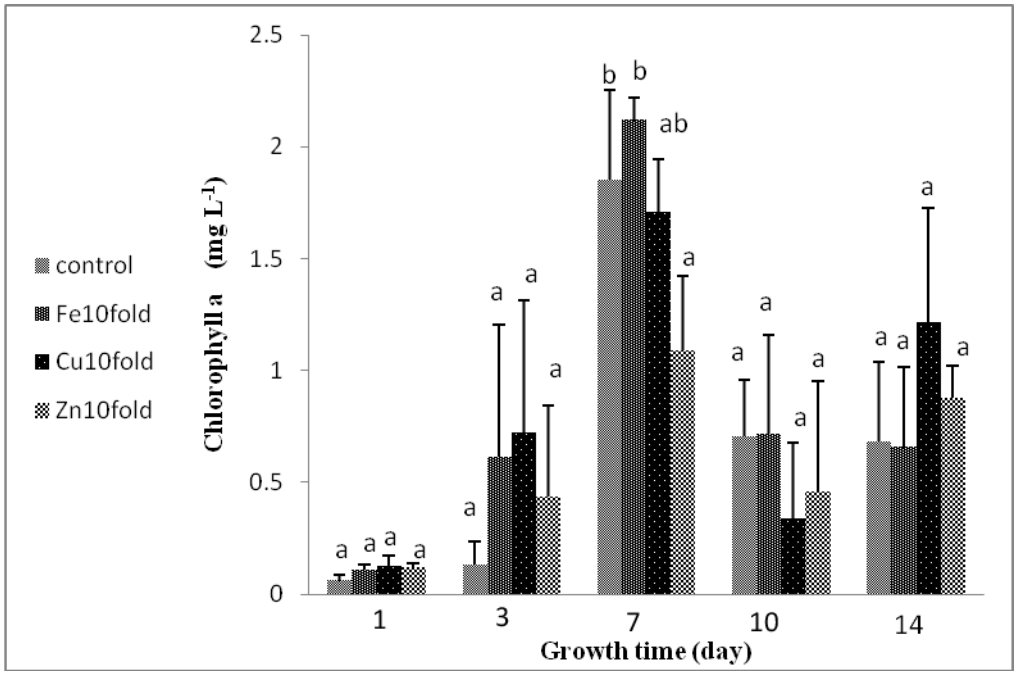


Figure 2: Changes in the chlorophyll A content of *Spirulina platensis* on Zarrouk's media (control) and growth medias with enhanced trace element level ($p \leq 0.05$).

Fig. 3, shows that the highest total carotenoid concentration was recorded in *S. platensis* treated with Cu^{2+} (0.0042±0/00 mg L^{-1}) after 14 day of

inoculation. There were no significant differences in concentration of carotenoids between control and other treatments in all experimental days.

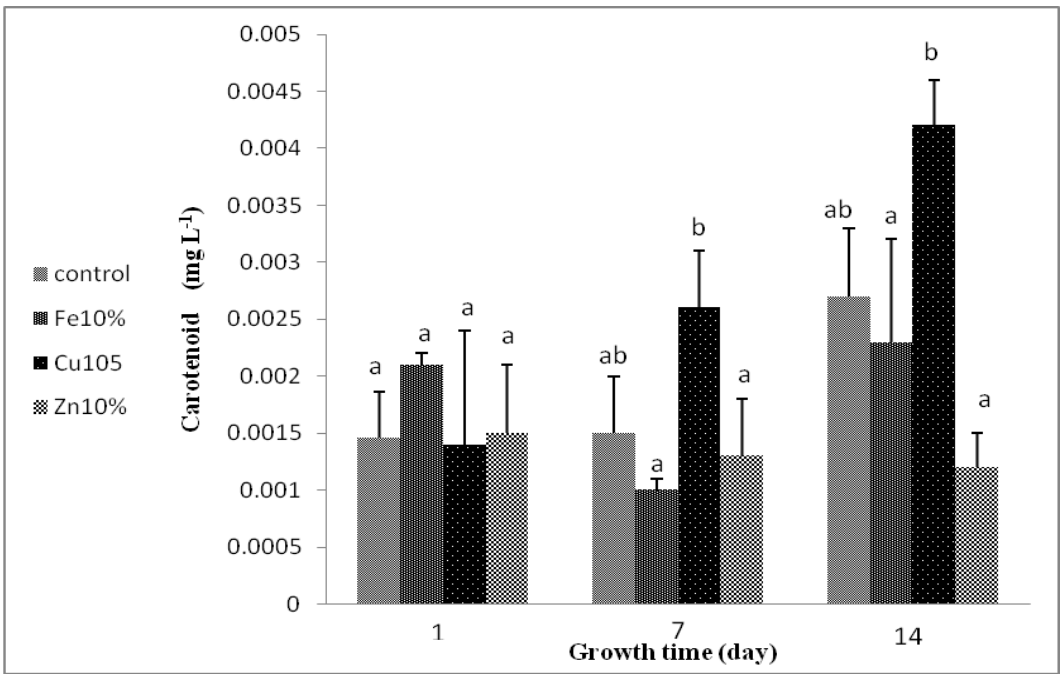
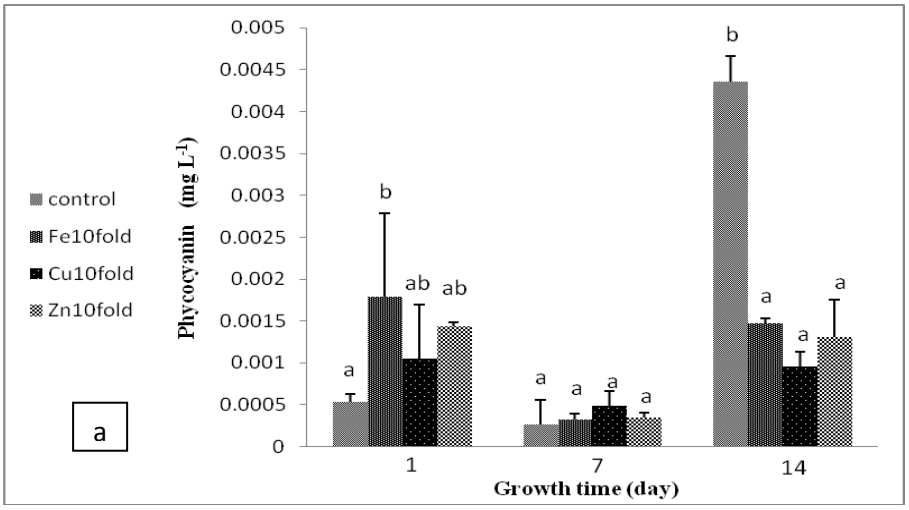


Figure 3: Changes in the carotenoid content of *Spirulina platensis* on Zarrouk's media (control) and growth medias with enhanced trace element level ($p \leq 0.05$).

Phycobiliproteins

Fig. 4 (a, b and c), shows the concentration phycobiliproteins accumulation in Zarrouk's (control) and growth medias with enhanced trace element level. There was a significant difference in phycobiliproteins content for the different medias as compared to control ($p<0.05$). It has observed that an increase of ions concentration caused reduction of phycobiliproteins of *S. platensis*.



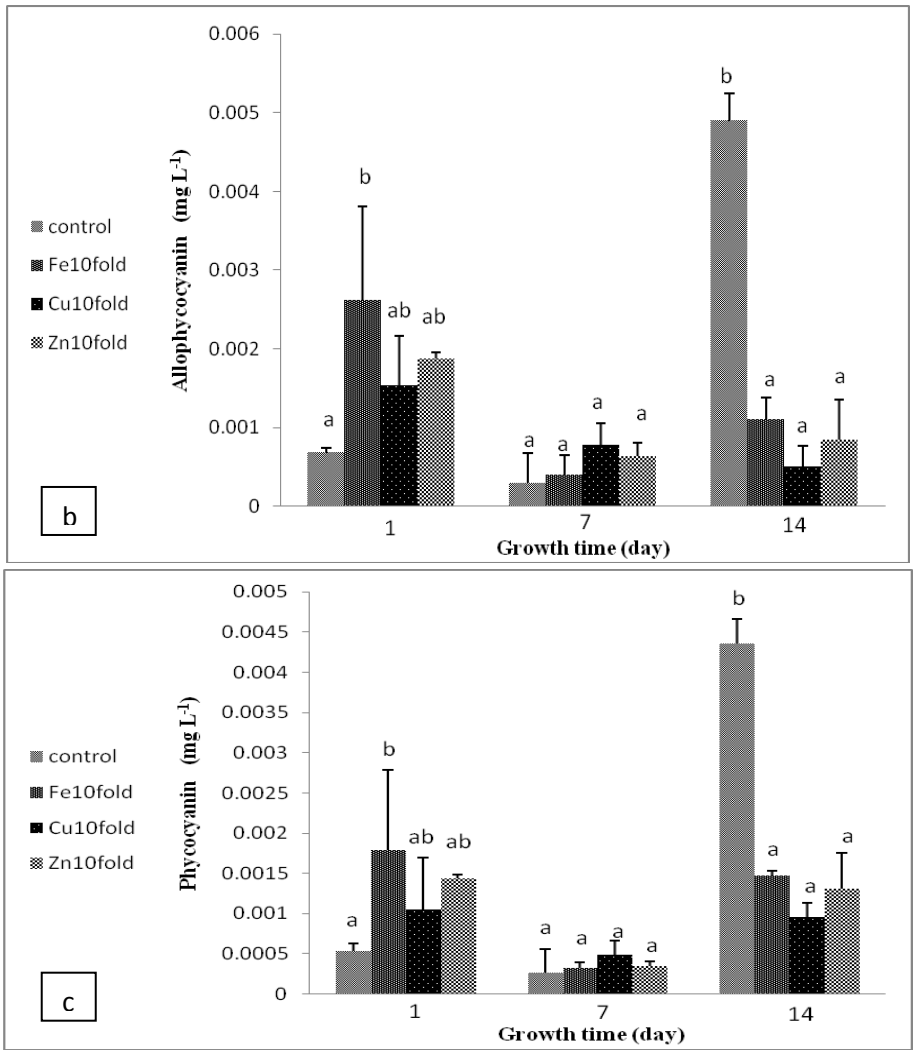


Figure 4: (a,b,c). Changes in phycocyanin, allophycocyanin and phycoerythrin content of *Spirulina platensis* on Zarrouk's (control) and growth media with enhanced trace element level ($p \leq 0.05$).

Discussion

Nutrition concentration is one of the key factors that controls growth and dry weight of microalgae (Vonshak and Richmond, 1988; Faintuch *et al*., 1991). The results in adverse with previous published data have showed that lower biomass of *Spirulina* sp. was measured in case of the ten times enhanced Zn^{+2} concentration (Szabolcs *et al.,* 2013). Biomass growth of *Spirulina* sp. is not so adversely affected by the increasing iron concentration of the media, even though slight biomass enhancement has observed in the previous cases. This conclusion is in agreement with Dou *et al.* (2013) who has found that dry weight of microalgae decreased firstly and then up gradually with the increase of concentration of Fe^{3+}, Zn^{2+}, and EDTA. The increase of Zn^{2+}concentration would high the efficiency of photosynthesis in microalgae (Yamasaki *et al.,* 2012).

Results of this experiment showed that media culture supplemented with zinc has declined after 13 days of incubation. This conclusion is in agreement with Gok and Esra (2009) who suggested, Zn^{2+} is not a constituent

of the enzyme but necessary for its synthesis. Bascik Remisiewicz *et al.* (2009) reported that, Zn promote growth rate, since it is a main metabolic requirement for microalgae where it acts as an important enzyme cofactor. Omar (2002) reported that, very low zinc concentration improved growth of *Scenedesmus obliquus* and *S. quardricauda* but low zinc concentrations (i.e. 1.5, 4.5 and 8.0 µg L^{-1}) inhibit growth of *S. quadricauda*. However, higher concentrations support toxicity (20.6-37.7% growth inhibition) as well as longer exposure period.

Copper is a micronutrient required by microalgae growth and plays an important role as an enzymatic cofactor and electron carrier in the photosynthetic and respiratory processes (Andrade *et al.,* 2004). This experiment in confirmed with Estevez *et al.* (2001) and Abd El Baky *et al*. (2012) showed that green microalgae with addition of iron concentration cause increases biomass, with lower considered limiting for algal growth. Our experiment with Soeprobowati and Hariyati (2014) findings showed that the growth of *Spirulina* at 1 mg L^{-1} concentration increased until 11 day. In adverse with Dou *et al.* (2013) results showed that *Spirulina* sp. treated with Cu^{2+} causes the decrease of this ion with little effects on the growth density. Rueter and Petersen (1987) and Kilulya *et al*. (2015) reported that iron promotes the growth of cyanobacteria in natural waters. Results of this experiment have been showed that photosynthesis of microalgae depended on Fe^{3+}, which is an important part of nitrate and nitrite reductase. The possibility that excess iron ion could be responsible for the decrease in alga growth by inducing oxidative stress (Wells *et al*., 1994; Wilhelm and Trick, 1994; Boyer and Brand, 1998; Davey and Geider, 2001; Estevez *et al*., 2001).

Regarding to reduction OD in zinc treatment after 13th day of culture, its uptake in different growth stage is changed. The maximum biomass values occurred in experiment with ten times enhanced Cu^{+2} concentration with the largest slope value being 0.498±0.234 ($p \leq 0.05$) which is in agreement with views of Mihova and Godjevargova (2000) and Pavasant *et al*. (2006) who reported low concentration of Cu and Zn even stimulate the growth and the activity of the metabolic processes. Sunda and Guillard (1976) reported that copper toxicity generally due to the presence of free copper ions in the water. At the same time, the result reflected that, Cu ion supports growth only within the lowest concentration (5 µg Cu L^{-1}) as shown in this study. Wong *et al*. (1979) and Mosleh and Mofeed (2014) explain that, presence of Cu^{+2} in the growth media by low concentration could enhance the peroxidase activity, which involved in IAA (Indole Acetic Acid) degradation, a hormone widely known by its ability for stimulating growth.

As agreed by this research, Szabolcs *et al.* (2013) reported that amount of iron, copper and zinc uptake and EF by *S. platensis* were higher than the control treatments. Similarly, Mane and Bhosle (2012) reported the highest percent bioaccumulation by *Spirulina* sp. for Fe

(98.93%), Cu (81.2%) and Zn (79%) respectively at 5 mg L^{-1} initial metal concentration.

In similar tests with Szabolcs *et al*. (2013), reports, the difference uptake in iron, copper, zinc ions uptake and EF by the algae *Spirulina* sp. might be related to absorption capacity. The capability of metal uptake depends on several factors like the growing condition such as temperature and pH circumstances and the level of available nutrients and microelements, metal concentration, the amount of the alga in the solution (biomass) and the absorption capacity of microalgae (Lovley, 2000).

Concerning the influence of medium type on carotenoids concentrations, it has found that similar trend of total chlorophyll content profile. The highest total carotenoid concentration has been recorded in experiment with ten times enhanced Cu10 concentration media (0.0042±0.0004 mg L^{-1}) after 14 days of inoculation. This may indicate a strong relation between both chlorophyll and carotenoids contents. Such correlation could be attributed to that the carotenoids protect chlorophyll molecules against photo destruction and oxidation by molecular oxygen (Krinsky, 1979). Similarly, Vonshak (1997) reported that there was a positive correlation between chlorophyll and carotenoids content of *S. platensis*. Collen *et al*. (2003) and Pinto *et al*. (2011) observed similar results in *Gracilaria tenuistipitata* exposed to copper and cadmium, with the increase of lutein and b-carotene.

Our results demonstrated that phycobiliprotein levels, including APC, PC, and PE, decreased in *S. platensis* after iron, zinc and copper treatments. These molecules absorb solar energy, transferring it to the reaction center of photosystem II, where chlorophyll A is excited by the flow of electrons (Gantt, 1981). According to Xia *et al.* (2004), a high concentration of copper altered phycobilisome structure, and these changes resulted in a decline of absorbed light energy, thus inhibiting photosynthesis. We found a decrease in phycobiliprotein levels, similar to the findings of Xia *et al.* (2004), who studied the red macroalgae *Gracilaria lemaneiformis* cultivated with copper during 4 four days. This indicates that iron, zinc and copper strongly inhibited the accumulation of phycobiliproteins. Similarly, Gouveia *et al.*, (2013) reported the amounts of phycobiliproteins decreased in *Gracilaria domingensis* treated with lead and copper. In addition, it could be due to its peripheral position in phycobilisomes on the thylakoid membrane (Gantt, 1981) and attributable to its sensitivity to metals (Kiran and Thanasekaran, 2011).

In the results from the measurement, growth of *S. platensis* has the highest uptake ability and high metals accumulation in their cells. By conducting this research, it was determined that *S. platensis* is the suitable species for enrichment. More studies in this field with regard to its high usage need, by its industrial culture in Iran it could be necessary in

different industries especially in food industry.

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