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Effects of iron and zinc concentrations on growth performance and biochemical composition of *Haematococcus pluvialis*: a comparison between nanoparticles and their corresponding metals bulks

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Abstract

Microalgae communities play a significant role in natural environment and also in many commercial industries. Hence, a number of techniques are being used to enhance the algal biomass and by-products. In this study, *Haematococcus pluvialis* standard growth medium was manipulated by different levels of iron and zinc nanoparticles (NPs) and compared with the corresponding metals bulks forms to assess the density and some biochemical components. *H. pluvialis* was grown with 4.98 mg/L $FeSO_4$ and 8.82 mg/L $ZnSO_4$ (T1), 2.49 mg/L Fe NPs and 4.41 mg/L Zn NPs (T2) and 4.98 mg/L Fe NPs and 8.82 mg/L Zn NPs (T3) for 14 days. Also, the standard growth medium with 2.49 mg/L FeSO4 and 4.41 mg/L $ZnSO_4$mg/l was included as a control group. The highest algal density was measured in T2 (4.52×10^5 cell/mL) ($p<0.05$). Also, the highest content of total chlorophyll (7.59 µg/mL) and astaxanthin (7.29 mg/L) contents were obtained in T2 ($p<0.05$). The treated growth medium with two-fold concentration of Fe and Zn NPs showed the highest metal accumulation in the algal cells (0.65 mg/l Zn and 5.25 mg/l Fe). Results showed that Fe and Zn in nano forms at the standard level (T2) were significantly improved the growth performance and biosynthesis of chlorophyll and astaxanthin contents in *H. pluvialis*.

Keywords: *Haematococcus pluvialis*, nanoparticle, astaxanthin, growth, Zn, Fe.

Introduction

Recently, nanoparticles (NPs) are the most important products in nanotechnology and have received core of attention in many fields including medicine, pharmaceutics, cosmetics, food, therapeutics, biosensors and environmental remediation (Dunphy Guzman *et al.,* 2006; Dastjerdi and Montazer, 2010; Comotto *et al.,* 2014). Also, size and surface area of NPs play a crucial role in biological systems due to easily intrude in organism cells (Verma and Stellacci, 2010). Meanwhile, many studies have been reported that the iron nanoparticles (Fe NPs) mainly employed for reducing environmental pollution in soils and water (Lin *et al.,* 2008; Gui *et al.,* 2012).

Fe is the third limiting nutrient for plants and all aerobic organisms. Moreover, it is necessary in numerous enzymatic reactions and photosynthetic electron transport chains (Raven *et al.,* 1999; Estevez *et al.,* 2001; La Fontaine *et al.,* 2002). However, the bioavailability of Fe salts in environment are limited due to natural oxidation and transformation to ferric form which has extremely low solubility in water (Schmidt, 1993; Sunda *et al.,* 2005). In contrast, Fe NPs has better stability, bioavailability, faster dissolved rates and higher permeability into the microorganism cytosol (Van Hoecke *et al.,* 2008). Fe NPs are used as an effective agent for reducing chlorinated organic contaminants in soil or groundwater in line with green and eco-friendly concepts (Becerra *et al.,* 2007; Hoag *et al.,* 2009). Growth rate of some microalgae species including *Desmodesmus subspicatus*, *Dunaliella salina*, *Parachlorella kessleri* and *Raphidocelis subcapitata* was enhanced by fortifying the standard growth media with 5.1 mg/L Fe NPs (Pádrová *et al.*, 2015). In addition, Pribyl *et al.* (2012) demonstrated that using Fe NPs in *P. kessleri* culture in accordance with the standard growth medium requirement, lead to significant increase in the lipid accumulation from 27.1% to 30.9%.

Zinc (Zn) is Known as an essential trace element in all organisms from bacteria to human population. Zn also plays structural, catalytic and co-catalytic roles in over than 300 enzyme, including nucleic acid metabolism and protein synthesis (Marschner, 1986; Andreini *et al.,* 2006). Although, many studies have focused on the toxic effects of Zn NPs on different microalgae species (Aravantinou *et al.,* 2015; Neale *et al.,* 2015; Tang *et al.,* 2015). However, a few studies showed the potential application of Zn NPs in microalgae culture (Thema *et al.,* 2015; Rajiv *et al*., 2013).

Haematococcus pluvialis (Chlorophyceae, Volvocales) is a unicellular green flagellated microalga which mainly found in small, transient and freshwater bodies. Among many microalgae species (e.g. *Chlorella sp*., *Chlorococcum sp*. and *Scenedesmus sp.*) that are known as a potential source of natural pigments, *H. pluvialis* is proposed as the best natural source of astaxanthin pigment (up to 4% of dry basis) (Zhekisheva *et al.,* 2005). This microalgae becomes spherical, immobile and forms a lot of large red aplanospores in the absent of chlorophyll synthesis in harsh conditions (Boussiba *et al.,* 1999, Wayama *et al.,* 2013; Boussiba, 2000). At the same time, the vegetative cells begin to synthesis the astaxantin cysts (Kobayashi *et al.,* 1997, Li *et al.,* 2010). In

this regard, many studies have been performed on manipulating of *H. pluvialis* standard growth medium nutrients to obtain higher algal astaxanthin production efficiency. For instance, the microalgae cells can produce a large amount of astaxanthin pigment upon the stress conditions (Sarada *et al.,* 2002), light intensity regimes (Jeon *et al.,* 2006), nitrogen and phosphorus starvation (Boussiba and Vonshak, 1991; Boussiba *et al.,* 1999).
This study aimed to estimate density, metals accumulations, chlorophyll *a* and astaxanthin contents of *H. pluvialis* in the manipulated standard growth mediaum by different levels of Fe and Zn nanoparticle and compared with the corresponding metals bulks for 14 days.

Materials and methods
H. pluvialis initial pure stock was obtain from SAG Culture Collection (SAG 19-a, Universität Göttingen, Göttingen, Germany) and cultivated on autotrophic Bold's Basal medium (BBM) (Bischoff and Bold 1963) which was modified to contain two different levels and sources of Fe and Zn for 14 days. $FeSO_4$ and $ZnSO_4$ (Merck) was considered as Fe and Zn salt sources. Also, NPs of the metal oxides were purchased from Sigma-Aldrich with the mean particle size specified by the manufacturer being 25-50 nm and 50–70 nm for Fe_3O_4 and ZnO NPs, respectively. Three concentrations of the metals with two different resources were 4.98 mg/L $FeSO_4$ and 8.82 mg/L $ZnSO_4$ (T1), 2.49 mg/L Fe NPs and 4.41 mg/L Zn NPs (T2) and 4.98 mg/L Fe NPs and 8.82 mg/L Zn NPs (T3). Also, control group (Tc) was contained 2.49 mg/L $FeSO_4$ and 4.41 mg/L ZnSO4 according to nutrient requirement of BBM growth medium. Cultures were grown with 10 mL inoculum of the algae (0.5× 10^7 cells/mL) on a platform shaker in sterilized baffled flasks at 25±1 °C with 2.42 kilo lux of cool-white fluorescent light and 12-hour intervals photoperiods. Also, continuous filtered aeration was included during the trial.

Algal density
Total cell density was determined by hemocytometer according to Arnon (1949) method.
Chlorophyll measurement
Chlorophyll *a* (Chl) content of the grown algae was measured by centrifuging at 2500 ×g for 5 min and extracting of the algal cells with 80% acetone overnight. Then, the absorbance of the supernatants were measured at 645 and 663 nm using a Varian Cary 50 UV–vis spectrophotometer (Varian Co., US) and calculated using the following equation (Zhang *et al.,* 2008):
$Chl\ content\ (mg/L) = (8.02 \times OD_{663}) + (20.21 \times 0OD_{645})$

Astaxanthin content
The extractable astaxanthin content was measured as free and total astaxanthin levels according to Okagbue and Lewis, (1983) methods with some modifications. The algal cells were mixed with 90% (v/v) acetone for 1 h and centrifuged at 3000 ×g for 10 min at 4°C. The supernatants were collected and measured at 444 nm to calculate free astaxanthin content. The precipitates were mixed with 90% (v/v) acetone for 1 h, then destroied by ultrasonic bath system (Wise clean, Germany). Then, the samples were again centrifuged for 10 min at 3000 ×g and the supernatants were subjected to read at 444 nm to determine total amount of astaxanthin content (Kobayashi *et al.,* 1997). All steps were carried out under the dim light. Extractability of astaxanthin content was measured using the below equation (Kobayashi *et al.,* 1997):
Astaxanthin Extractability (%) = Free astaxanthin/Total astaxanthin × 100

Metals concentration in the algal cells
The bioaccumulation of Fe and Zn were calculated by an Atomic absorption spectrophotometer (Varian SpectrAA 200, Victoria, Australia) using Hollow cathode lamp after microwave sample digestion at 510 and 214 nm, respectively. In summary, the algal dry matter was acquired by filtration of the treatments through a Whatman GF/C paper prior to dry at 60°C for 42 h in a FD-115 oven (Binder, Tutlingen, Germany). Then, 0.5 g (dry basis) of the algal cells were microwave-digested in PTFE (polytetrafluoroethylene) vessel with 4 mL of nitric acid (65%) and 1 mL of distilled water at 80°C for 10 min. Afterward, The aqueous solution was poured and kept at 4C° in stoppered polyethylene bottles prior to further analysis.

Statistical analysis
Values represent the mean of assays performed in triplicate ± SD. Statistical analyses were conducted in SPSS 20.0 for Windows (SPSS, Inc., Chicago, USA). The one-way ANOVA was used to verify significance of differences between means, using Duncan's multiple range test at the significance level of 5%.

Results and discussion

Biomass production

Algal cells density of *H. pluvialis* in the supplemented growth media by different levels of Fe and Zn NPs and the corresponding metals bulks is shown in Fig. 1. The algal density was significantly ($p<0.05$) decreased by increasing in the concentration of both bulks and NPs metals, however the highest value was obtained in T2 which represents NPs metals at standard concentration according to the nutrient requirement of BBM growth medium ($p<0.05$).

The potential application of metal-based NPs has been recently attracted an enormous attention due to their unique properties (Wang *et al.,* 2014). Our results revealed that the highest concentration of Fe and Zn in both forms of NPs and bulks inhibited the algae density compared to the control group. It was demonstrated that the NPs at the highest concentrations can adhere to algal cell surfaces, restrict the light accessibility and finally reduce the photosynthesis process (Hund -Rinke and Markus, 2006; Kwok *et al.,* 2010). Also, Sibi *et al.* (2017) showed that specific growth rate and biomass density of *Chlorella vulgaris* were decreased by increasing in Cu-NPs, Pb-NPs, Zn NPs and Mg-NPs concentrations in the growth media. Furthermore, a number of studies reported that *Chlorella sp*. growth performances were decreased by increasing in magnetic Fe oxide NPs concentration due to agglomeration of the metal-based NPs with algae cells (Long *et al.,* 2012; Toh *et al.,* 2014). In addition, some metal-based NPs like Fe NPs have the potential toxicity by generating reactive form of oxygen species (ROS) which is induced oxidative stress. The production of ROS is believed to induced oxidative damage to the microorganism cell walls and DNA (Imlay and Linn 1988; Keenan *et al.,* 2009; Li *et al.,* 2009; Wu *et al.,* 2014). However, some recent studies showed that the positive effects of metal-based NPs on different plants and algae species at appropriate concentrations. For instance, Hong *et al.* (2005) showed that growth performances of spinach (*Spinacia oleracea*) was promoted with TiO_2 NPs at 0.25% by stimulating the photosynthesis. Moreover, Kadar *et al.* (2012) revealed that growth rate of three microalgae (*Pavlova lutheri, Isochrysis galbana and Tetraselmis suecica*) increased by adding Zn NPs at 1.17×10^{-5} M concentration. Also, Meilin *et al.* (2017) indicated that adding of Fe_2O_3 NPs at 20 mg/L in the growth medium of *Scenedesmus obliquus* enhanced the cell density as well as chlorophyll content. This result also showed that metal-based NPs can enhance the cell density of *H. pluvialis* at the standard concentrations according to the nutrient requirement of BBM growth medium.

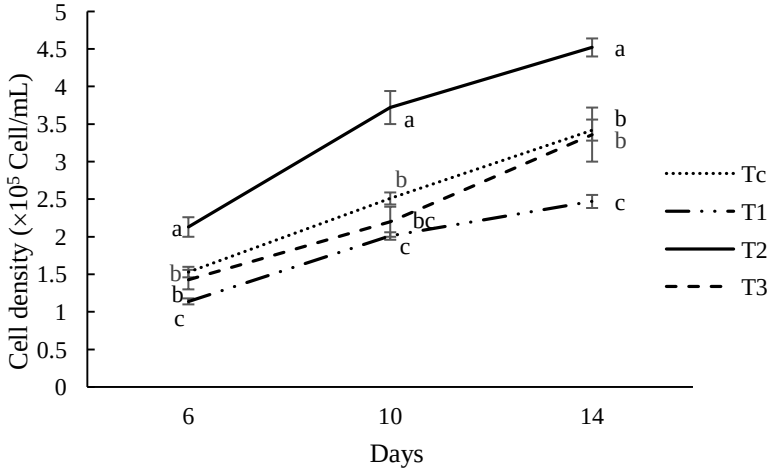

Fig. 1 Different levels of Fe and Zn in the forms of NPs and bulks on *H. pluvialis* density. Data with different superscript letters are significantly different (n=3, p<0.05).

Chlorophyll a content

The chlorophyll *a* content of the grown algae using different levels of iron and zinc metals in different forms of NPs and bulks is shown in Fig 2. The highest chlorophyll *a* content was observed in T2 which was treated by the metals NPs at 2.49 mg/L Fe NPs and 4.41 mg/L Zn NPs concentration ($p<0.05$). It has revealed that using of metal-based NPs instead of metals salts in algae culture can stimulate and enhance the growth rate, biomass, cellular pigment, and other bioactive compounds of the microorganism (Sibi *et al.,* 2017). However, double fold concentrations of Fe and Zn NPs were probably toxic to *H. pluvialis* and inhibited the chlorophyll *a* synthesis.

The main limiting factor in using of metal-based NPs in microorganism culture is ROS generation and aggregation process (Yan *et al.,* 2011). The same results were observed in supplemented growth medium of *Chlorella vulgaris* with 1 g/L $MgSo^4$ NPs (Sarma *et al.,* 2014). Morgalev *et al.* (2017) showed that adding of 0.1 mg/L Zn NPs in *Chlorella vulgaris* growth medium resulted in the chlorophyll *a* and *b* enhancement. Furthermore,

it has been proven that lower concentrations of Cu (20-40 mg/L) and Se (0.07-0.2 mg/L) nanocarboxylates in *Chlorella vulgaris* growth media can increase the chlorophyll contents (Mykhaylenko and Zolotareva, 2017).

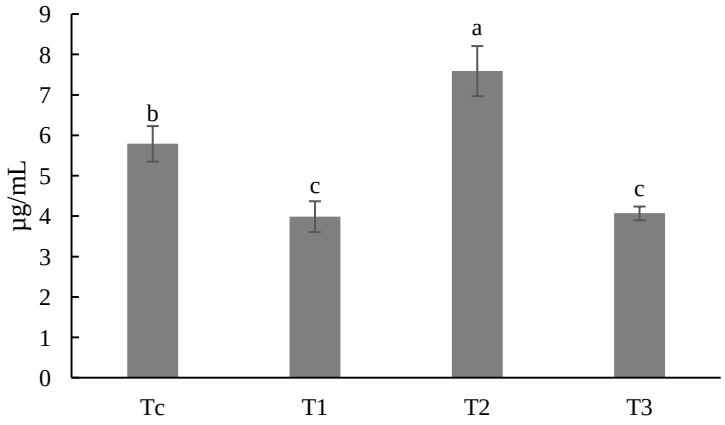

Fig. 2 Different levels of Fe and Zn in the forms of NPs and bulks on chlorophyll *a* content of *H. pluvialis*. Data with different superscript letters are significantly different (n=3, p<0.05).

Astaxanthin synthesis

As shown in Fig. 5, the highest content of astaxanthin content (7.29 mg/L) was obtained in T2 compared to other treatments ($p<0.05$).

Algal pigments such as Chlorophylls, carotenoids and phycobilins absorb light in the process of photosynthesis. Astaxanthin pigment is a secondary metabolite product and synthesized by *H. pluvialis* against stress conditions such as high light, salinity, and/or nutrient depletion (Gao *et al.,* 2012). Also, metal-based NPs can induce oxidative stress, improve algal growth and promote the production of secondary metabolites compounds (Kang *et al.,* 2014). Many investigations showed that free ferrous (Fe^{2+}) represents a hazard of fenton reaction process and generates ROS which are augmented synthesis of astaxanthin in *H. pluvialis* (Yu *et al.,* 2015; Harker *et al.,* 1996; Shah *et al.,* 2016). Ma and Chen (2001) observed that using of 0.1 M H_2O_2 (as a ROS agent) enhanced astaxanthin formation in *Chlorococcum sp*.

In the current study, the production of astaxanthin pigment was significantly ($p<0.05$) decreased in the treated growth medium with double fold concentration of the both bulk and nano metals compared to control group. The toxicity of metal-based NPs to microalgae at higher concentration is mainly due to ROS (Lapresta-Fernández *et al.,* 2012). It has been reported that ROS can damage microorganisms by creating many holes into the cellular membrane (Sondi and Salopek-Sondi, 2004; Brayner *et al.,* 2006).

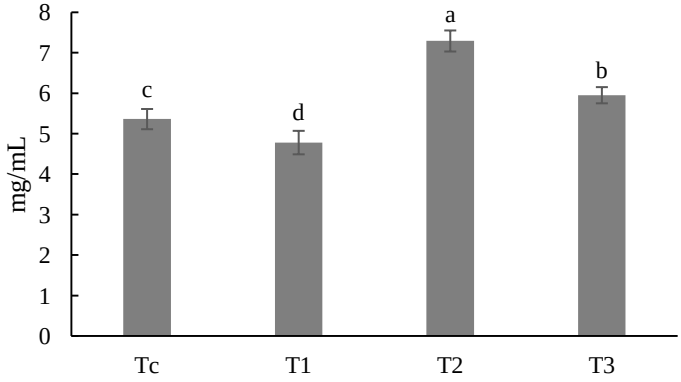

Fig. 3 Different levels of Fe and Zn in the forms of NPs and bulks on astaxanthin accumulation of *H. pluvialis*. Data with different superscript letters are significantly different (n=3, p<0.05).

Bioaccumulation of the metals

The accumulation of Fe and Zn in *H. pluvialis* cells are illustrated in Fig. 3. The treated growth medium with the metals NPs showed the lowest Zn and Fe accumulation compared to other treatments ($p<0.05$).

Bioaccumulation of metals in microalgae may be posed a major threat for the organism health and consumer safety. Accumulated of heavy metals in some microalgae (e.g. *Chlorella, Chlamydomonas, Scenedesmus* and *Pseudokirchneriella*) caused lower growth rate and even death due to dwindled competition between the metal

ions and H^+ at the cell surface (Franklin *et al.,* 2000; De Schamphelaere *et al.,* 2003). Toxicity of metal-based NPs in microalgae is highly depending on initial metals concentrations, size, shape, chemical composition, charge, and surface structures (Oberdörster 2005).

Algal cell structures are mainly consisting of cellulose and semipermeable membrane which are allowing small molecules to pass and enter into the cell (Navarro *et al.,* 2008). As a result, only NPs and molecules with the smaller size than the largest cellular pores can pass through the algae cell membrane and reach the plasma. In this study, the highest metals accumulation was measured in manipulating growth medium with two-fold concentration of Fe and Zn NPs (T4). Some studies showed that higher content of metals in an aquatic ecosystem may be increased in absorption of the metals by microorganisms (Sun *et al.,* 2007; Zhang *et al.,* 2007; Wick *et al.,* 2007).

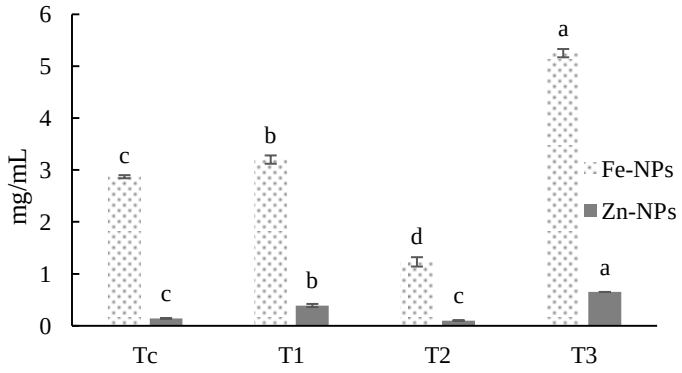

Fig. 4 Different levels of Fe and Zn in the forms of NPs and bulks on the metal accumulation of *H. pluvialis*. Data with different superscript letters are significantly different (n=3, p<0.05).

In this study, results showed the positive influence of Fe and Zn nanoparticles in accordance with their standard concentration of BBM medium. Biomass, astaxanthin and chlorophyll *a* contents of *H. pluvialis* were improved in treated growth medium with 2.49 mg/L Fe NPs and 4.41 mg/L Zn NPs. However, higher concentrations of the metals NPs probably have toxic effects, but further trials are needed.

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