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EVALUATION OF ALGAE FARMING USING THE *Chlorella* BIOASSAY

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ABSTRACT

Algae are gaining attention for their application in aquaculture as a highly sustainable source of useful products. As microalgae have a significant role in primary production in aquatic ecosystems and are the basis of many food chains, it is important to understand the processes that provide them with better survival in a toxicant-polluted environment. In this study the *Chlorella* bioassay was evaluated: (1) as a potential method for algae farming, (2) as a method for testing advantages or disadvantages of symbiotic association, including two species of aposymbiotic algae, i.e. endosymbiotic algae isolated from green hydra *Mychonastes homosphaera* (Skuja) Kalina and Punčochářová and *Desmodesmus subspicatus* (Chodat) Hegewald and Schmidt) and two related free-living algal species (*Chlorella kessleri* Fott and Novak. [K&H, 1992] and *C. vulgaris* Beij. [K&H, 1992]), (3) through algal bioindicator responses related to comparative toxicity and ecotoxicological pollution of iron, and (4) by using algal bioindicators for microscopical and morphometrical application in environmental stress. Increasing concentrations of iron led to cell changes (dry dotted clusters of dying cells, intensive green wet bubbles representing a mucous structure, area, diameter and length), deformations (empty cells, aberrant divisions, irregular coenobia, tetrads and transitional forms) and ultrastructural changes (chloroplasts and nuclei). All modifications were more pronounced in aposymbiotic algae, suggesting a lower degree of adaptation to iron toxicity than their free-living relatives. A free-living species *C. kessleri* showed the best ability to survive in given unfavorable environmental conditions. High statistical significance was noticed in the cell division parameter, underlining the hormetic effect of increasing the biomass in free-living algal species. This increasing of the cell divisions at the specific concentration of iron demonstrated that the *Chlorella* bioassay may represent a useful tool for evaluating the growth of different microalgal species, and has a prospective application in a comparative study of algae farming.

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INTRODUCTION

Symbiosis is ubiquitous in terrestrial, freshwater and marine communities. It played a key role in the emergence of major life forms on Earth and in the generation of biological diversity (Moran, 2006). Iron is ubiquitous in the living systems, but it is also a potential toxin (Ponka, 2000). Only the appropriate iron concentration ensures the undisturbed growth and metabolism of freshwater organisms. Due to its dual toxic limit, increasing or decreasing of iron concentrations may cause lethal or sublethal effects. In spite of being an ubiquitous metal in the Earth's crust, iron concentration in water is quite low due to poor solubility (Xing and Liu, 2011) and the anthropogenic source of wastewaters is concurrently the largest source of iron in freshwater (Chen et al., 2014, Jones et al., 2017). Iron has a significant impact on the growth of microalgae and algal species can be used in the remediation of contaminated areas due to their ability to absorb metals (Dwivedi et al., 2008). Microalgae are significant as primary producers in aquatic ecosystems and are the basis of many food chains, presenting potentially important bioindicators of aquatic pollution (Torres et al., 2008). Changes in phytoplankton appearance and biomass can point out the altered environmental and ecological conditions (Špoljar et al., 2018). Within this research the *Chlorella* bioassay was evaluated: (1) as a potential method for farming of free-living and endosymbiotic algae previously isolated from green hydra (tested algae were aposymbiotic), in order to answer the question if it could present a useful tool for evaluating the growth of different microalgal species, and if it could have a prospective application in a comparative study of algae farming, (2) as a method to point out advantages or disadvantages of symbiotic association, by using two species of aposymbiotic algae, i.e. endosymbiotic algae isolated from green hydra (*Mychonastes homosphaera* (Skuja) Kalina and Punčochář ová and *Desmodesmus subspicatus* (Chodat) Hegewald and Schmidt) and two related free-living algal species (*Chlorella kessleri* Fott and Novak. [K&H, 1992] and *C. vulgaris* Beij. [K&H, 1992]), (3) through algal bioindicator responses related to comparative toxicity and ecotoxicological pollution of iron, and (4) by using algal bioindicators for microscopical and morphometrical application in environmental stress. Algae farming is an important branch of aquaculture, fish farming and pharmaceutical industry (Gouveia et al., 1996, Marauyma et al., 1997, Odo et al. 2015, Stramarkou et al., 2017). Algae are gaining attention for their application in aquaculture as a highly sustainable source of useful products, being recognized as an important renewable source of bioactive lipids with a high proportion of polyunsaturated fatty acids (Priyadarshani and Rath, 2012). As fish do not synthesize long chain omega-3 fatty acids in significant quantities, they rather acquire them through their diet by eating zooplankton that fed on marine algae, the primary producers in the marine food chains, which are also the primary synthesizers of omega-3 fatty acids (Barclay et al., 1994). Herewith the *Chlorella* bioassay is considered an applicable method for algae farming, as well as for ecotoxicological research and morphometrical analyses related to pollution and environmental stress.

MATERIALS AND METHODS

In this study, the following series of four photoautotrophic unicellular algae were used: endosymbiotic algae isolated from green hydra (*Hydra viridissima* Pallas, 1766), species *M. homosphaera* (Skuja) Kalina and Punčochář ová (CZ10) and *D. subspicatus* (Chodat) Hegewald and Schmidt (CZ43) (both belong to *C. zagrebiensis* group Kovac. & Jelen.) (Kovačević et al., 2010a) as well as their free-living relatives, species *C. kessleri* Fott and Novak. [K&H 1992] strain LARGE/1 (A) and *C. vulgaris* Beij. [K&H 1992] strain SAG 211 - 11b (CV). Composition of medium used for algal growth was: 2 g agar, 100 mg KNO_3, 1 mL $MgSO_4x7\ H_2O$, 1 mL of K_2HPO_4 and 0.1 ml $FeCl_3$, and 100 ml of distilled water. Green algae were maintained *in vitro* on the sterile stock agar (Pratt, 1941, Horvatić et al., 2000). Algae were cultivated in test tubes 16 cm long, 15 mm in diameter, in a climate chamber under sterile conditions at 24°C and constant light intensity of 80 mmol/m^2 (fluorescent lamps Osram L36W/20, Cool/WHITE/2850 lm Osram, Berlin, Germany). Opposite to the round-cell algal species *C. kessleri*, *C. vulgaris* and *M. homosphaera*, species *D. subspicatus* shows ellipsoid-cell structure (Fig. 1a) (Kovačević et al., 2010b).

The *Chlorella* bioassay, a microbiological method using iron dextran, was performed onto the specified series of test organisms in three concentrations of iron (1, 400 and 1400 mg/L Fe) and compared with the control samples (Kovačević et al., 2016). Instead of distilled water, equivalent amount of iron solution in agar medium was added, as the base principle of *Chlorella* bioassay (Kovačević et al., 2008). The duration of the test was 21 days and the experiment was performed in replicates. Six glass tubes per concentration and control sample for each of the four species of algae were used (altogether 192 glass tubes). On the third day of the *Chlorella* bioassay, two tubes from each species were used for microscopical studies and four tubes were used for macroscopical observations (growth, dry dotted clusters and mucous wet bubbles) during the remaining time of the experiment. Viability, morphological changes, mortality, shape of the cells, level of damage, changes of chloroplasts and nuclei, intensity of green color, regular and irregular cell formations were monitored. Morphometrical analysis included the measurement of cell area, diameter, length, number of coenobia, tetrads, transitional forms, cell divisions and empty cells on a sample of 200 cells and a chloroplast and nucleus area on a sample of 50 cells per

species and concentration, as well as for the controls, in five fields of vision. Morphometrical analysis was performed by light microscope Nikon Eclipse E600, software Lucia G DXM1200, version 4.80. Micrographs were recorded by digital camera Nikon DXM1200.

Statistical analysis was provided by STATISTICA 12.0 (StatSoft, Inc., USA). Normality of the data was tested by Shapiro–Wilk W test. Homogeneity of variance for each variable was tested by Levene's test. Basic statistical parameters were assessed by basic statistical method. The possible difference in the mean values of cell diameter, cell area, chloroplast area, nuclei area, cell length among the groups (negative control and the four algal species treated with 1, 400 and 1400 mg/L Fe) was assessed by one-way ANOVA followed by Newman-Keuls post hoc comparison test. Prior to the tests, all data were normalized by log-transformation. The same tests were applied for the testing of the difference of the percentage of empty cells and cell divisions. Statistical significance was set to $p<0.05$.

RESULTS

Macroscopical evaluation using the Chlorella bioassay

Macroscopical observations comprised the appearance of dry dotted clusters of dying cells, green mucous structures of wet bubbles and intensity of green color of all algal cultures. The control groups of all four algal species throughout the experiment grew unrestrictedly. The round-cell algal species *C. kessleri*, *C. vulgaris* and *M. homosphaera* appeared in light to dark green color. Only ellipsoid-cell algal species *D. subspicatus* showed specific dried growth and olive-green to yellow color, and during the experiment it gradually became more yellow in color. In *C. vulgaris*, drying of cells occurred in 84% of tubes with higher concentrations of iron, and in species *M. homosphaera* in 50% of tubes with the highest concentration of iron at the beginning of the experiment, while in species *C. kessleri* drying occurred later with the middle concentration of iron, in 50% of the tubes. The dried cells were visible as a formation of white dotted clusters. The area around the dotted clusters occasionally seemed to be wet and intensively green, forming mucous wet bubbles representing the viable cells. In species *D. subspicatus*, dried cells were observed in up to 100% of tubes with higher concentrations of iron, and no wet bubbles occurred (Fig. 1b).

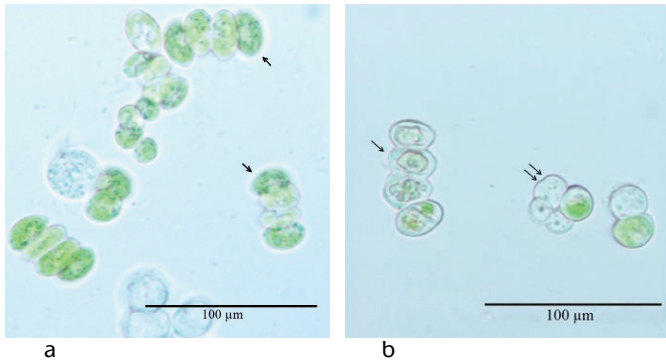

Fig 1. *D. subspicatus*: a) control sample with regular cup-like chloroplasts (arrow), and b) treated with 1400 mg/L

Microscopical analysis

Microscopical analysis comprised morphology (deformations, reduced chloroplasts, irregular coenobia, tetrads and transitional forms) (Fig. 1) and morphometry (cell diameter, length and area, chloroplast and nucleus area, empty cells, aberrant divisions) (Figs. 2-4, Tables 1 and 2) of all four used algal species. Cells of *C. kessleri* in the control group were of regular round shape with regular cup-like chloroplasts. Divisions of up to five cells and a small number of ellipsoid-shaped cells with a dotted structure of chloroplasts were noticed. In concentration 1 mg/L of iron, a small number of elongated cells were observed and plastids were damaged. In concentration 400 mg/L of iron, a larger number of giant cells were observed and particular chloroplasts had a dotted structure. Algae treated with 1400 mg/L of iron appeared in elliptic shape with few giant cells, and chloroplasts appeared swollen with a dotted pattern. Both regular and irregular divisions of up to five cells were noticed. *C. vulgaris* in the control group and in concentration 1 mg/L of iron had a regular round shape and chloroplasts had a regular cup-like form. The divisions of up to five cells were visible. Algal cells treated with 400 mg/L of iron were elongated and divisions of up to three cells were observed. In concentration of 1400 mg/L of iron, a large number of large cells with an empty lumen appeared. Chloroplasts appeared to be dot-patterned and irregular divisions of up to five cells were observed. *M. homosphaera* cells in the control were round-shaped with a proper form of chloroplasts. Divisions of up to six cells were observed. Algae treated with 1 mg/L of iron had dot-patterned chloroplasts. Divisions of up to five cells were observed with particular cells larger in comparison to the control. In concentration 400 mg/L of iron, chloroplasts were deformed. Giant algal cells were observed in the group treated with 1400 mg/L of iron, with an empty lumen inside the cells and with reduced chloroplasts. A reduced number of divisions was noticed. *D. subspicatus* grew in the formation of coenobia of mostly four cells (cells were connected and lined up by grouping together after the cell divisions occurred, but they did not separate after the divisions) (Fig. 1a). Regular and irregular tetrads (forms which consist of four cells connected within an imaginary square) and transitional forms (stadia of differently shaped cell formations with the morphological appearance between regular coenobia and tetrads) were also noticed. Chloroplasts were damaged. Particular algal cells treated with 1 mg/L of iron lost the intense green color

and divisions of up to six cells were noticed within coenobia divisions. In concentration 400 mg/L of iron, coenobia were arranged in a zig-zag pattern. Chloroplasts within the cells were reduced, deleted, fragmented and dot-patterned and therefore not suitable for morphometrical analysis. Dead cells and a larger number of irregular cell divisions were observed. In concentration of 1400 mg/L of iron, huge cells and cells of irregular shape were noticed. A large number of tetrads and irregular coenobia in a zig-zag line were observed. Within the same coenobium and the same tetrad both viable and dying cells were visible (Fig. 1b).

The main trends of the morphometrical analysis showed the following: a major reduction of cell diameter was present in species *C. vulgaris* (up to 0.25 µm) and the overall constant increase in cell diameter was present in species *M. homosphaera* (up to 0.59 µm). Analyzed parameters showed significant difference (one-way ANOVA, $p<0.05$; Fig. 2). In species *D. subspicatus* high increase in cell length in the highest concentration of iron was noticed, with all the values showing significant difference compared to the highest concentration of iron (one-way ANOVA, $p<0.05$; Fig. 2). By increasing the iron concentration all four species showed changes in cell area. Overall, the highest cell area changes occurred in both aposymbiotic algae, *M. homosphaera* and *D. subspicatus* (oscillations in 6.05 and 18.99 µm^2, respectively; Fig. 3a). Changes in chloroplast area were irregular in all species and showed no significant pattern. The overall constant increase in chloroplast area was present in species *M. homosphaera* (one-way ANOVA, $p>0.05$; Fig. 4a). Changes in nucleus area showed no significant pattern. The highest values were noted in species *C. vulgaris* and *D. subspicatus* (oscillations in 0.98 and 1.80 µm^2, respectively), and significance was noticed only in the groups compared to the highest concentration of iron (one-way ANOVA, $p<0.05$) (Fig. 4b).

The highest number of empty cells was present in species *D. subspicatus* (40%) treated with 1400 mg/L of iron (Fig. 1b). The vastly increased number of cell divisions was present in the free-living species, *C. kessleri* (7%) and *C. vulgaris* (11%),

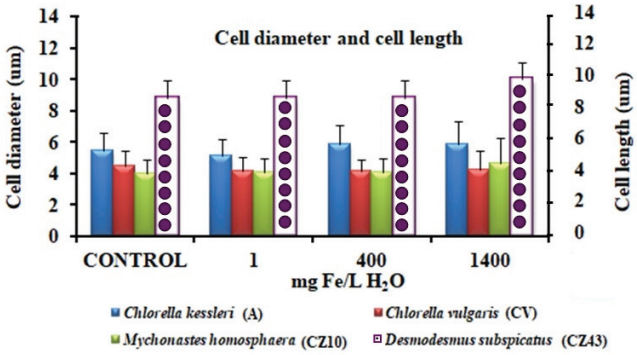

Fig 2. Cell diameter changes in species *C. vulgaris*, *C. kessleri* and *M. homosphaera*; cell length changes (patterned) in species *D. subspicatus* shown on the secondary axis.

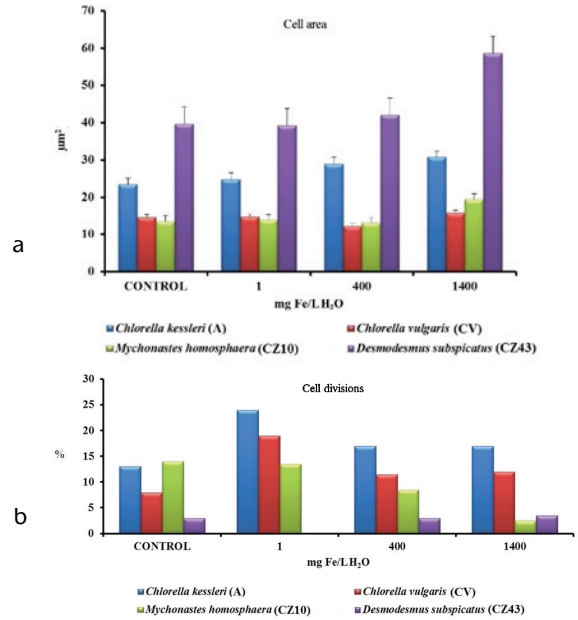

Fig 3. Changes in**:** a) cell area in species *C. vulgaris*, *C. kessleri*, *M. homosphaera* and *D. subspicatus*, and b) cell divisions in species *C. vulgaris*, *C. kessleri*, *M. homosphaera* and *D. subspicatus*.

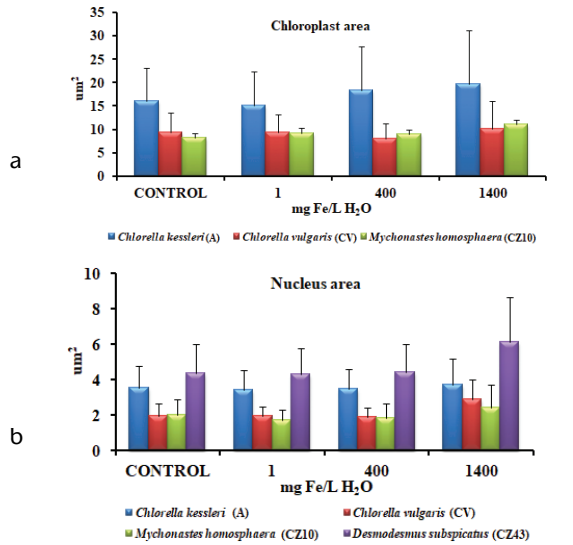

Fig 4. Changes in: a) chloroplast area in species *C. vulgaris*, *C. kessleri* and *M. homosphaera*, and b) nucleus area in species *C. vulgaris*, *C. kessleri*, *M. homosphaera* and *D. subspicatus*.

showing the highest increase of divisions in concentration 1 mg/L of iron. In all the concentrations of iron these algae showed an increasing trend. Both aposymbiotic species, *M. homosphaera* and *D. subspicatus*, showed a lower number of cell divisions or no divisions (Figs. 3b). As the concentration of iron increased, the number of ceonobia was lower and the number of tetrads and transitional forms was higher in species *D. subspicatus*. Statistically significant difference was present between the measured values for both empty cells and cell division parameters (one-way ANOVA, $p<0.04$; Tables 1 and 2).

Table 1. The results of one-way ANOVA for the difference in the percentage of empty cells in four different algal species

Species	Concentration (mg/L)	Control	1 mg/L	400 mg/L
Chlorella kessleri	1	0.000275*		
	400	0.024289*	0.000291*	
	1400	0.000292*	0.000291*	0.000275*
C. vulgaris	1	0.000292*		
	400	0.000325*	0.000275*	
	1400	0.000275*	0.000674*	0.000291*
Mychonastes homosphaera	1	0.001737*		
	400	0.002325*	0.051386	
	1400	0.004820*	0.024289*	1.000000
Desmodesmus subspicatus	1	0.000293*		
	400	0.000275*	0.000292*	
	1400	0.000291*	0.000275*	0.000291*

Table 2. The results of one-way ANOVA for the difference in the percentage of cell divisions in four different algal species

Species	Concentration (mg/L)	Control	1 mg/L	400 mg/L
Chlorella kessleri	1	0.000291*		
	400	0.000292*	0.000275*	
	1400	0.000276*	0.000291*	1.000000
C. vulgaris	1	0.000291*		
	400	0.000293*	0.000275*	
	1400	0.000276*	0.000291*	0.024289*
Mychonastes homosphaera	1	0.024289*		
	400	0.000275*	0.000291*	
	1400	0.000291*	0.000275*	0.000291*
Desmodesmus subspicatus	1	0.000281*		
	400	1.000000	0.000293*	
	1400	0.015238*	0.000294*	0.032545*

DISCUSSION

In this study, for the first record the comparative effect of iron as ubiquitous pollutant was researched on the series of four unicellular photoautotrophic algal species: two species of aposymbiotic algae previously isolated from green hydra and two species of their non-symbiotic free-living algal relatives. Thus, this experiment showed the *Chlorella* bioassay as a suitable method in algae farming, as well as in evaluation of evolutionary aspects of endosymbiotic and free-living algal species that can be used as bioindicators for further microscopical and morphometrical application in environmental stress.

Four algal species cultivated *in vitro* were slowly losing their shiny reflection, most likely due to drying of the cultures and depletion of nutrients from the culture medium. Macroscopic observations suggested that iron seemed to be destructive to chloroplasts in the species *D. subspicatus* due to observed green-to-yellow color changes. Drying of the cells indicated by white dotted clusters was observed already at the beginning of the experiment in species *C. vulgaris*, *M. homosphaera* and *D. subspicatus,* while in the species *C. kessleri* drying occurred later indicating better adaptation to the changed environmental conditions. Isolated aposymbiotic algae showed trends of intensive drying, with total drying present

in species *D. subspicatus*, proving them to be generally the least adapted to unfavorable environmental conditions. In the free-living species at the lowest concentration (1 mg/L) drying was not observed, but on the other hand the higher biomass production was observed instead, proving the enhanced growth of the free-living algal species by this specific concentration of iron. Swollen mucous wet bubbles unevenly distributed across the cultures were observed, presenting viable cells and a possible form of ejection of iron from the algal cell. Places with bubbles had intensive green color, although the culture around the bubbles was covered by dry white dots of non-viable cells. Algae possibly formed a wet "protective balloon" as a defensive mechanism for survival through a period. Possible due to lack of these capabilities, species *D. subspicatus* showed the highest sensitivity to iron. A certain concentration of iron leads to an increase in biomass in species *C. vulgaris* and this is referred to as the optimum concentration of iron, while the deficiency and excess of iron leads to a reduction of biomass (Barghbani et al., 2012). Some toxicants influence the inhibition of growth and photosynthesis and affect plastids, which could be observed by the changes in green color (Abrous et al., 1998, Ponka, 2000). There are discussions about the difficulties of growing symbiotic algae outside their hosts because endosymbionts transfer part of their genetic material to the nuclear genome of *Hydra* (Habetha et al., 2003). Research of various toxicants on symbiotic and aposymbiotic hydra report that aposymbiotic hydra shows a lower limit of sensitivity to adverse conditions in comparison to endosymbiotic or non-symbiotic hydras (Karntanut and Pascoe, 2005). It is known that hydra possess detoxifying mechanisms where the toxin is removed from the body with the help of mucus (Kovačević et al., 2009). Metal ions in a solution bind passively onto algal cells and this occurs mainly in functional groups present on algal cell walls. Maximum capacity of bioapsorption of iron for algal species is higher than for other metals such as manganese and nickel (Muzenda et al., 2011). Iron uptake is strictly required for phytoplankton development since the photosynthetic apparatus contains numerous loci for iron. Ferrous chelates can undergo several reactions that might lead to the formation of an initiator of lipid peroxidation. The decomposition of hydroperoxides formed during membrane peroxidation leads to the generation of lipid radicals (Estevez et al., 2001, Carlsen et al., 2005).

Microscopic observations showed that all four species of algae exerted a more extent deformations in the proportion of cells than their plastids and had the largest area and the diameter at the highest concentration of iron, as a possible consequence of oxidative stress that leads lipid membranes under the influence of catalytic reactions that result in an increase of the proportion of cells (Estevez et al., 2001). This increase of the proportion of cells at the highest concentration of iron was accompanied by the increase in the chloroplast area for all the species of algae, and chloroplasts became swollen and dot-patterned. Chloroplasts might have lost their liquid content and the compression of chloroplasts led to the dot pattern. The loss of liquid content is possible because of intensive lipid peroxidation in biological membranes, which leads to the loss of fluidity and a decrease of the membrane potential value, increase of permeability toward H^+ and other ions, as well as possibility of cell rupture and releasing of its contents (Štefan et al., 2007). The same as the cell and chloroplast area, nucleus area followed the same trend of area increasing at the maximum concentration of iron. In general, by increasing the concentration of iron, cells became dry and cell viability is thus reduced. The largest number of empty dead cells with no ability of photosynthesis was present in species *D. subspicatus* at the highest concentration of iron, which underlined this aposymbiotic species as the most susceptible. In the free-living species, *C. kessleri* and *C. vulgaris*, the number of cell divisions highly increased at the concentration of 1 mg/L of iron in comparison with the number of divisions in the control group. Further increasing in the concentrations of iron resulted in incomplete and deformed divisions suggesting that, despite the attempts of division, at higher concentrations iron disrupts the ability to reproduce. Obviously, a specific amount of iron in the nutrient medium is desirable in order to enhance the growth of the algal cultures. The optimal amount of iron that should be added to the medium in order to stimulate the growth of the free-living species *C. kessleri* and *C. vulgaris* is proposed to be 1 mg/L. This specific concentration of iron pointed out that in both free-living algal species a hormetic effect of increased biomass occurred (Stebbing, 1988). In species *D. subspicatus*, increasing of iron concentration reduced their ability to form proper coenobia of four cells, resulting in the increasing number of tetrads and transitional forms. Deformations of cells were observed, i.e. increasing the number of cells and disproportionately sized cells within the same coenobium. It can be assumed that iron is a trigger to oxidative stress, it affects cell growth and can exert a negative impact on the biology of the organisms that are found in their environmental setting (Estevez et al., 2001, Lei et al., 2016).

In conclusion, the *Chlorella* bioassay showed to be a method whose application may enhance current methods of determining the toxicity of iron in aquatic ecosystems. A free-living species *C. kessleri* showed the best adaptation for survival in a given unfavorable environmental conditions. Aposymbiotic species *M. homosphaera* showed better adaptation to these conditions than *D. subspicatus*, showing overall less damage. The *Chlorella* bioassay proved to be a useful method in evolutionary and ecotoxicological research and could successfully be used in comparative algae farming. Therefore, this quick, simple, indicative, non-expensive and applicative toxicity test is proposed also upon green algae

farming. Further applications might also include the *Chlorella* bioassay in evaluation of the quality and toxicological status of aquatic (micro-) environments in overall aquaculture and fish farming, providing simple and quick detection of preliminary effects of environmental pollutants upon green algae in *vitro*. Already after a short period macroscopical and microscopical changes upon algal bioindicators can be distinguished, including sublethal, lethal and observed effect concentration of different xenobiotics with different mechanisms of action (Kovačević et al., 2008). As algal cultivation using the *Chlorella* bioassay increases, it is more likely that new applications would be found.

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SAŽETAK

VREDNOVANJE UZGOJA ALGI KORIŠTENJEM METODE *CHLORELLA* TEST

Zbog svoje primjene u akvakulturi, alge dobivaju sve više pozornosti kao održiv izvor korisnih produkata. Budući da mikroalge imaju važnu ulogu kao primarni proizvođači u vodenim ekosustavima i osnova su mnogih prehrambenih lanaca, važno je razumjeti procese koji im omogućuju bolje preživljavanje u toksičnom okolišu. Cilj ovog rada je evaluirati metodu *Chlorella* testa: (1) kao potencijalnu metodu za uzgoj algi, (2) kao metodu za procjenu prednosti i nedostataka života u simbiotskoj asocijaciji, uključujući dvije vrste aposimbiotskih algi (endosimbiotske alge prethodno izolirane iz zelene hidre) (*Mychonastes homosphaera* (Skuja) Kalina i Punčochář ová i *Desmodesmus subspicatus* (Chodat) Hegewald i Schmidt te dvije vrste algi kao njihovih slobodnoživućih srodnika *Chlorella kessleri* Fott i Novak. [K&H, 1992] i *C. vulgaris* Beij. [K&H, 1992]), (3) s obzirom na odgovore algalnih bioindikatora povezane s komparativnom toksičnošću i ekotoksikološkim onečišćenjem željezom, i (4) putem korištenja algalnih bioindikatora za mikroskopijsku i morfometrijsku primjenu u okolišnom stresu. Povišenjem koncentracije željeza pojavile su promjene na stanicama (nakupine suhih umirućih stanica, mukozne strukture intenzivno zelenih mjehurića vlage, površina, promjer i duljina), deformacije (prazne stanice, nepotpune diobe, nepravilni cenobiji, tetrade i prijelazni oblici) i ultrastrukturne promjene (kloroplasti i jezgre). Sve promjene su bile izraženije u aposimbiotskih algi, što ukazuje na niži stupanj prilagođenosti na toksičnost željeza od njihovih slobodnoživućih srodnika. Slobodnoživuća vrsta *C. kessleri* se pokazala najprilagođenijom vrstom. Uočena je visoka statistička značajnost razlike u parametru stanične diobe, što ističe hormestički učinak povećanja biomase u slobodnoživućih vrsta algi. Ovo povećanje biomase pri specifičnoj koncentraciji željeza ukazuje na činjenicu da se *Chlorella* test može uspješno koristiti za vrednovanje rasta različitih mikroalgalnih vrsta i pokazuje perspektivnu primjenu u komparativnim studijama uzgoja različitih vrsta algi.

Ključne riječi: endosimbioza, zelena hidra, *in vitro*, morfometrija, ekotoksikologija, željezo

REFERENCES

Abrous, O., Benhassaine Kesri, G., Tremolieres, A., Mazijak, P. (1998): Effect of norflurazon on lipid metabolism in soya seedlings. Photochemistry, 49, 979-985.

Barclay, W.R., Meager, K.M., Abril J.R. (1994): Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. Journal of Applied Phycology, 6, 2, 123-129.

Barghbani, R., Rezaei, K., Javanshir, A. (2012): Investigating the Effects of Several Parameters on the Growth of *Chlorella vulgaris* Using Taguchi's Experimental Approach. International Journal of Biotechnology for Wellness Industries, 1, 128-133.

Carlsen, C.U., Moller, K.S., Skibsted, L.H. (2005): Heme-iron in lipid oxidation. Coordination Chemistry Reviews, 249, 3-4, 485-498.

Chen, J.B., Busigny, V., Gaillardet, J., Louvat, P, Wang, Y.N. (2014): Iron isotopes in the Seine River (France): Natural versus anthropogenic sources. Geochimica et Cosmochimica Acta, 128, 128-143.

Dwivedi, S., Srivastava, S., Mishra, S., Dixit, B., Kumar, A., Tripathi, R.D. (2008): Screening of native plants and algae growing on fly-ash affected areas near National Thermal Power Corporation, Tanda, Uttar Pradesh, India for accumulation of toxic heavy metals. Journal of Hazardous Materials, 158, 2-3, 359-65.

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

Gouveia, L., Gomes, E., Empis, J. (1996): Potential use of a microalga *(Chlorella vulgaris)* in the pigmentation of rainbow trout *(Oncorhynchus mykiss)* muscle, Zeitschrift für Lebensmittel-Untersuchung und Forschung, 202, 1, 75-79.

Habetha, M., Anton-Erxleben, F., Neumann, K., Bosch, T.C. (2003): The *Hydra viridis/Chlorella* symbiosis. Growth and sexual differentiation in polyps without symbionts. Zoology, 106, 2, 101-108.

Horvatić, J., Vidaković Cifrek, Ž., Regula, I. (2000): Trophic level, bioproduction and toxicity of the water of lake Sakadaš (Nature park Kopački rit, Croatia). Limnological Reports, 33, 89-94.

Jones, D.S., Lapakko, K.A., Wenz, Z.J., Olson, M. C., Roepke, E.W., Sadowsky, M.J., Novak, P.J., Bailey, J.V. (2017): Novel microbial assemblages dominate weathered sulfide-bearing rock from copper-nickel deposits in the Duluth complex, Minnesota, USA. Applied and Environmental Microbiology, 83, 16, e00909-17.

Karntanut, W., Pascoe, D. (2005): Effects of removing symbiotic green algae on the response of *Hydra viridissima* (Pallas 1776) to metals. Ecotoxicology and Environmental Safety, 60, 3, 301-305.

Kovačević, G., Franjević, D., Jelenčić, B., Kalafatić, M. (2010a): Isolation and Cultivation of Endosymbiotic Algae from Green Hydra and Phylogenetic Analysis of 18S rDNA Sequences. Folia biologica (Kraków), 58, 1-2, 135-43.

Kovačević, G., Radić, S., Jelenčić, B., Kalafatić, M., Posilović, H., Pevalek-Kozlina, B. (2010b) Morphological features and isoenzyme characterization of endosymbiotic algae from green hydra. Plant Systematics and Evolution, 284, 1-2, 33-39.

Kovačević, G., Gregorović, G., Matijević, A., Kalafatić, M. (2016): Toxic effects of iron on green and brown hydra. Current Science, 110, 4, 502-504.

Kovačević, G., Jelenčić, B., Kalafatić, M., Ljubešić, N. (2008): Chlorella test. Periodicum biologorum, 110, 4, 373-374.

Kovačević, G., Kalafatić, M., Horvatin, K. (2009): Aluminium deposition in Hydras. Folia biologica (Kraków), 57, 3-4, 139-142.

Lei, C., Zhang, L., Yang, K., Zhu, L., Lin, D. (2016): Toxicity of iron-based nanoparticles to green algae: Effects of particle size, crystal phase, oxidation state and environmental aging. Environmental Pollution, 218, 505-512.

Maruyama, I., Nakao, T., Shigeno, I., Ando, Y., Hiarayama, K. (1997): Application of unicellular algae *Chlorella vulgaris* for the mass-culture of marine rotifer *Brachionus*. Hydrobiologia, 358, 133-138.

Moran, N. A. (2006): Symbiosis. Current Biology, 16, 20, 866-871.

Muzenda, E., Matambo, T.S., Glasser, D., Hildebrandt, D., Zimba, J., Diale, P.P. (2011): Biosorption of heavy metals contaminating the wonderfonteinspruit catchment area using *Desmodesmus sp.* International Journal of Materials and Metallurgical Engineering, 5, 4, 384-393.

Odo, G.E., Agwu, J.E., Iyaji, F.O., Madu, J.C., Ossai, N.I., Allison, L.N. (2015): Mass production of rotifer (*Branchionus calyciflorus*) for aquaculture in south-eastern Nigeria. International Journal of Fisheries and Aquaculture, 7, 9, 151–159.

Ponka, P. (2000): Iron metabolism: Physiology and pathophysiology. Journal of Trace Elements in Experimental Medicine, 13, 1, 73-83.

Pratt, R. (1941): Studies on *Chlorella vulgaris* IV. American Journal of Botany, 28, 6, 492–497.

Priyadarshani, I., Rath, B. (2012): Commercial and industrial applications of micro algae – A review. Journal of Algal Biomass Utilization, 3, 4, 89-100.

Stebbing, A. (1982): Hormesis – The stimulation of growth by low levels of inhibitors. Science of the Total Environment, 22, 3, 213-234.

Stramarkou, M., Papadaki, S., Kyriakopoulou, K., Krokida, M. (2017): Effect of drying and extraction conditions on the recovery of bioactive compounds from *Chlorella vulgaris*. Journal of Applied Phycology, 29, 6, 2947-2960.

Špoljar, M., Dražina, T., Lajtner, L., Kovačević, G., Pestić, A., Matijašec, D., Tomljanović, T. (2018): Impact of water level fluctuation in the shaping of zooplankton assemblage in a shallow lake. Croatian Journal of Fisheries, 76, 1, 27-34.

Štefan, L., Tepšić, T., Zavidić, T., Urukalo, M., Tota, D, Domitrović, R. (2007): Lipidna peroksidacija – uzroci i posljedice. Medicina Fluminensis 43, 2, 84-93.

Torres, M.A., Barros, M.P., Campos, S.S.G., Pinto, E., Rajamani, S., Sayre, R.T., Colepicolo, P. (2008): Biochemical biomarkers in algae and marine pollution: A review. Ecotoxicology and Environmental Safety, 71, 1, 1-15.

Xing, W., Liu, G. (2011): Iron biogeochemistry and its environmental impacts in freshwater lakes. Fresenius Environmental Bulletin, 20, 6, 1339-1345.