Journal of
Chemical Engineering & Process Technology

Molnár et al., J Chem Eng Process Technol 2013, 4:7
DOI: 10.4172/2157-7048.1000172

Research Article Open Access

Comparative Studies on Accumulation of Selected Microelements by *Spirulina Platensis* and *Chlorella Vulgaris* with the Prospects of Functional Food Development

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Abstract

Chlorella vulgaris and Spirulina platensis as plausible basic material of perspective functional foodstuffs were in the focus of the research due to the high content of bioactive compounds, favorable growing feature and pronounced microelement accumulation capabilities.

The emphasis was laid on the investigation of bioaccumulation of four microelements (Fe(III), Cu(II), Zn(II), Mo(VI)) in cases of the two abovementioned alga species. Metal binding aspects of algae in our case was not examined for perspective utilisation in environmental protection, but purposively for revealing ways for possible functional food developments.

Both *Spirulina platensis* and *Chlorella vulgaris* proved to be efficient in terms of metal accumulation in two-week-long experiments. With the exception of molybdenum the bioaccumulation capacity of Chlorella exceeded that of Spirulina. Iron (789.7 ± 102.7mg kg^{-1}) and zinc (378.1 ± 5.5mg kg^{-1}) were accumulated to a large extent by Chlorella vulgaris, while bioaccumulation ability of *Spirulina platensis* proved to be the most significant for iron (676.9 ± 27.6mg kg^{-1}) and molybdenum (5.79 ± 1.1mg kg^{-1}).

The biomass of *Spirulina platensis* increased to greater extent than that of Chlorella after the same incubation time in case of the control media, however metal treatment of the media favoured more pronouncedly the growth of biomass of *Chlorella vulgaris*. The biomass of *Spirulina platensis* was diminished when incubated in metal fortified media, with the exception of iron.

As a consequence it might be established that both studied algae species can be suggested for involvement in further functional food developments, since they may inevitably contribute to microelement supply of humans, or to the prevention of iron deficiency anaemia thanks to their large microelement accumulation capabilities.

Keywords: *Chlorella Vulgaris*; *Spirulina Platensis*; Microelements; Bioaccumulation; Growth of Biomass; Functional food; Iron deficiency anaemia

Introduction

Chlorella and *Spirulina* are of outstanding physiological activity including antioxidative, antiflammatory, antimutagenic, antiviral, cardioprotective effects, immune enhancing and anticancer properties [1-3]. As a consequence, elaborations of food-related applications are in the forefront of interest of recent researches, hence such implications demand thorough scientific arguments. Novelty of this paper might be regarded the fact that majority of previous studies on algae species aimed at revealing their fitoremedial properties and metal sorption activities with regard to utilisation in environmental protection. The prospects of possible functional food applications need to be grounded more extensively by purposive researches on physiological and chemical feature of algae.

The outstanding nutritive value and high content of bioactive components of the algae is already well known as several studies (nutritive biological, human clinical) were focused on the analysis of their important components which have beneficial effects on human health like minerals, proteins, lipids and vitamins [4,5]. The exact and detailed composition and the ratio of organic constituents of selected algae have also been revealed [6]. It was found that *Tetraselmis sp.* and *Nannochloropsis oculata*, cultivated in industrial-scale bioreactors, produced 2.33 and 2.44% w/w lipid (calculated as the sum of fatty acid methyl esters) in dry biomass, respectively [7].

Chlorella sp. and *Nannochloropsis salina* cultivated in a lab-scale open pond stimulating reactor grew well and produced 350-500 mgL^{-1} of biomass containing approximately 40% and 16% of lipids, respectively [8]. Several studies were focused on the biosorption of heavy metals (Pb, Cd, Cu, Zn, Ni) by algae species in order to eliminate toxic elements from the environment [9]. The studied algae were suitable for the elimination of toxic heavy metal levels. Regular analysis and monitoring of the metal content of algae showed the extension of the metal pollution of a given area [10]. Biological materials can bound to cellular surfaces via the process called biosorption or bioaccumulation can take place inside the cell [11,12]. Biosorption of metal ions may be accomplished via complex mechanisms, such as ion exchange, complex formation, electrostatic interactions and precipitation. Correlation was observed between the content of trace elements or other biologically active substances of the growth medium and the composition of the algae biomass [13,14] The capability of metal uptake depends on several factors like the growing circumstances (temperature, pH), the level of available nutrients and microelements, metal concentration of the growth medium, the amount of the alga in the solution (biomass) and the biosorption capacity of the studied species [15].

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Received July 11, 2013; **Accepted** September 03, 2013; **Published** September 05, 2013

Citation: Molnár S, Kiss A, Virág D, Forgó P (2013) Comparative Studies on Accumulation of Selected Microelements by *Spirulina Platensis* and *Chlorella Vulgaris* with the Prospects of Functional Food Development. J Chem Eng Process Technol 4: 190. doi: 10.4172/2157-7048.1000172

Citation: Molnár S, Kiss A, Virág D, Forgó P (2013) Comparative Studies on Accumulation of Selected Microelements by *Spirulina Platensis* and *Chlorella Vulgaris* with the Prospects of Functional Food Development. J Chem Eng Process Technol 4: 172. doi: 10.4172/2157-7048.1000172

By taking advantage of the high bioaccumulation characteristics of algae, their nutritive value can considerably be improved making sensible the concept of plausible functional food applications.

Biosorption capacity of seaweeds from Baltic sea was also studied in order to get information about the accumulated amounts of metals with the prospect of utilisation as feed supplements for livestock [16]. According to our knowledge it might regarded as one of the first attempts to find food or feed related applications for plants suitable for metal accumulation. Our research was carried out with similar objectives.

The majority of nutrition supplements comprise trace elements as inorganic salts but the bioavailability of inorganic forms is not so pronounced. The bioavailability of organic forms is larger and more effective than that of the inorganic forms [17,18]. Application of organic iron complexes signifies a new trend in the clinical practice during the treatment of iron deficiency anaemia [19-22] Studies on dietary application of *Spirulina platensis* [13] related the bioaccumulation of iodine, zinc and selenium. It was observed that the uptaken metals are in organic form in the *Spirulina*. Functional yoghurt has also been produced by the application of this alga with enhanced metal-content.

Several kinds of alga based products appeared on the market as food supplements in the previous decades, but the elaboration of functional, algae containing products started just in the 90's in the European Union [23-25]. The annual growth rate for the sale of functional foods is about 14% at world level, thus these algae containing functional products with enriched metal content would enter an expanding market.

Our objective in this study was to broaden the scope of possible alimentary application of specific algae species (*Chlorella vulgaris* and *Spirulina platensis*) by revealing their bioaccumulation characteristics in terms of 4 important microelements. These examinations should provide a good basis for elaboration of new functional food prototypes based on metal enriched alga biomass. This endeavour might be regarded as the most pronounced novelty and relevance of our study, as formerly environmental protection related utilisations of algae species were in the forefront of researches.

Molybdenum binding capabilities of alga species have not been examined previously, therefore our studies might convey new knowledge to the current state-of-the art of that segment of science.

Most of the previous food analytical studies aim at revealing amino acid, fatty acid and protein profiles of various alga species, while our work focuses on microelement accumulation aspects. In this paper binding efficiency of the studied microelements was studied under various conditions and in different concentrations. Biosorption characteristics of iron has been more thoroughly scrutinised than that of the other microelements, since iron deficiency occurs the most frequently in humans among microelement nutrition disorders.

Materials and Methods

Organisms and applied chemicals

The algae culture of *Spirulina platensis* M2 was obtained from Soley Institute (El Sobrante, California), while wild-type of *Chlorella vulgaris* was obtained from the Plant and Soil Protecting Station (Gödöllő, Hungary). The algae cultures were stored in sterile test-tubes on slant agar at 4oC in dark. All the applied reagents and chemicals (analytical purity) were purchased from Sigma-Aldrich (Darmstadt, Germany), and Merck (Darmstadt, Germany).

Bioaccumulation of metals by *Spirulina platensis* and *Chlorella vulgaris* from growth medium with diverse microelement content

Growing media: The composition of the growth medium in the case of *Spirulina platensis* was in accordance with Zarrouk's medium [26], while for *Chlorella vulgaris* the OECD 201, AAP (US, EPA, 2011) conditions were applied. [27]

Four metals (Fe(III), Cu(II), Zn(II), Mo(VI)) were chosen for experiments. Enhancement of metal content of the growing media was performed separately for each microelement. Five different solutions were prepared in case of each microelement: one control solution and 4 solutions with diverse metal concentrations [28]. The metal content of the control solution (C) was in accordance with the recipe of OECD and Zarrouk guideline, as follows: [$FeCl_3$*$6H_2O$]: 0.16 mgl^{-1}, [$CuCl_2$*$2H_2O$]: 0.012 μgl^{-1}, [ZnCl2]: 0.00327 mgl^{-1}, [Na_2MoO_4*$2H_2O$]: 0.00726 mg l^{-1}. The metal concentration was separately and stepwise enhanced to tenfold of the control's Fe(III), Cu(II), Zn(II) and Mo(VI) content. These solutions were signed as: Fe10X, Cu10X, Zn10X, Mo10X. The metal solutions were prepared from $FeCl_3$ x $6H_2O$, $CuCl_2$ x $2H_2O$, $ZnCl_2$, Na_2MoO_4 x $2H_2O$ (Table 1.).

In order to establish the extent of maximum adsorption (saturation) of iron, and its affect on the biomass production, the iron content was separately and stepwise increased: 5x (C_{Fe5x}=0.165mg l^{-1}), 10x (C_{Fe10x}=0.33mg l^{-1}), 25x (C_{Fe25x}=0.825mg l^{-1}), 50x (C_{Fe50x}=1.65mg l^{-1}) and 100x (C_{Fe100x}=3.3mg l^{-1}), compared to that of the control ($C_{Control}$=0,03.3mg l^{-1}) (OECD 201, Zarrouk's) (Table 1).

Alga suspensions with 10 mgl^{-1} concentration were prepared in Erlenmeyer-flasks (500 ml) by the application of the above described growing media. The experiments were performed in triplicates (n=3) and after the incubation time the whole amount of the gained alga was analysed.

Alga growing apparatus: The alga growing apparatus consists of a horizontal glass surface whereon the Erlenmeyer-flasks were placed. Special lamps (Aqua-Medic Gmbh., Bissendorf, Germany) were applied in order to induce alga growing, constant illumination of 160 μE $m^{-2}s^{-1}$ was provided by 24 W T5 light bulbs. The illuminating periods were set in accordance with the natural conditions by automated switch. Fresh air was pumped into the solutions through plastic tubes in order to avoid the generation of alga film layer on the wall of the flasks. The growing period was 2 weeks; the temperature was 25°C in case of *Chlorella* and 32°C in case of *Spirulina*.

Sample preparation

After the two-week long incubation time the alga solutions were centrifuged for 15 minutes at 6000 rpm than the supernatant was removed and the alga was washed with distilled water (conductivity was lower than 0.055 mS) and centrifuged again. The water was removed from the centrifuge tubes and the alga was dried at 50°C for 48 hours. The amount of the produced dry alga was established gravimetrically.

Growth media	**Fe^{3+}**	**Cu^{2+}**	**Zn^{2+}**	**Mo(VI)**
Control*	33μgl^{-1}	0.004 μgl^{-1}	1.6 μgl^{-1}	2.9 μgl^{-1}
Enhanced media (Fe 10X)	330μgl^{-1}	0.004 μgl^{-1}	1.6 μgl^{-1}	2.9 μgl^{-1}
Enhanced media (Cu 10X)	33μgl^{-1}	0.04 μgl^{-1}	1.6 μgl^{-1}	2.9 μgl^{-1}
Enhanced media (Zn 10X)	33μgl^{-1}	0.004 μgl^{-1}	16 μgl^{-1}	2.9 μgl^{-1}
Enhanced media (Mo 10X)	33μgl^{-1}	0.004 μgl^{-1}	1.6 μgl^{-1}	29μgl^{-1}

*The metal content of the control media in accordance with OECD 201 (Chlorella), Zarrouk media (Spirulina).

Table 1: Applied metal concentration in the growth media.

Prior to establishment of the metal content of the samples the organic matter was decomposed with the application of microwave accelerated reaction system (MARS, CEM Corporation, Metthews, USA) in the presence of 8 mlcc. HNO_3 and 2 ml of cc. H_2O_2. The working conditions of the decomposing system were as follows: 800 W power, 220°C, 30 bars for 45 min.

Sample preparation was performed in triplicates (n=3) from the three parallel growth media with the same metal-composition.

Measurement conditions

The metal content of the samples was measured by atomic absorption spectrometer SpectrAA 50B Varian (Mulgrave Virginia, Australia). During the measurements flame (FAAS) and electrothermal atomization (ET-AAS) were applied (GTA-100, Varian, Mulgrave Virginia, Australia) with deuterium background correction.

For both AAS techniques (flame and electrothermal) Varian SpectrAA hollow cathode lamps were used. For quantitative analysis CertiPUR standard solutions (Merck, Darmstadt, Germany) were applied. The measurement parameters are summarized in Table 2. The obtained results were calculated from the values of three parallel measurements and were expressed in mg kg^{-1} dried alga.

Calculations

Data for the calculations (both EF, WR) gained from the results of measured metal content of 30 samples prepared as follows. Five samples were prepared in triplicates with four different the compositions of the metals (with a control sample) as it is demonstrated in Table 1, so 15 samples were prepared per algae species.

Enrichment factor (EF) was calculated according to the following ratios.

$$EF = \frac{C_E}{C_C}$$

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

Weight ratio (WR) was calculated according to the following

$$WR = \frac{W_E}{W_C}$$

W_E = Weight of dry alga grown in the media with enhanced metal content

W_C = Weight of dry alga grown in the control media

The theoretical maximum of the adsorbing (Fe^{3+}) capacity was calculated from the average iron content (three performed measurements) of the set of six samples by the application of parametrical fit (n=18). The calculations were performed by the application of the following formula:

	Method	Wavelength (nm)	Conc. Range	Dynamic interval
Fe^{3+}	FAAS	248.3	0.1875-3 ppm	0.0039-0.2101
Cu^{2+}	FAAS	324.7	0.125-1 ppm	0.0138-0.1569
Zn^{2+}	FAAS	213.9	0.125-2 ppm	0.0239-0.2735
Mo(VI)	ET-AAS	313.3	0.015-0.045 ppb	0.0099-0.0528

Table 2: Measurement conditions.

Results and Discussion

Comparison of nutritive values of algae's different growing parameters

Food industrial utilization the studied algae should be based on the accurate knowledge on their nutritive characteristics [29]. Therefore their basic nutritive parameters and components were revealed as it is summarized in Table 3.

In comparison with previous results [7,8] it can be stated that the lipid content of the studied samples are in compliance with the global trends. The protein level of *Spirulina* samples are 50% higher than that of *Chlorella*, as it was formerly revealed. All the other examined nutritive parameters have not displayed great alteration when comparing them with previous studies.

Comparison of bioaccumulation capacity of *Spirulina platensis* and *Chlorella vulgaris*

It is of great importance to establish that the actual extent of metal fortification of the alga species in respect of being able to tailor practical application.

Metal-accumulating capacities of *Chlorella vulgaris* and *Spirulina platensis* were compared for 4 distinctive microelements in cases of growing media of 10 times fortified metal concentration. It can be stated that the extent of bioaccumulation of the microelement studied proved to be more pronounced for *Chlorella vulgaris*, with the exception of molybdenum. *Spirulina platensis* accumulated Mo(VI) significantly more efficiently than *Chlorella vulgaris* under the experimental conditions (Figures 1 and 2).

Copper and zinc contents of *Chlorella vulgaris* exhibited great variability when grown in metal fortified media. Ten times more copper and zinc were analysed in *Chlorella* grown in the metal-treated media in comparison with the control medium (Zn 10X: 378.1 mg kg^{-1}, Cu 10X : 103.1 $mgkg^{-1}$ dry biomass). Equal amount of copper was uptaken by *Spirulina* from the control medium and the copper fortified medium (Cu: 10.03 $mgkg^{-1}$, Cu 10X: 10.50 mg kg^{-1} dry biomass). Almost four times more zinc was uptaken by *Spirulina* from the Zn-enriched growth medium than from the control medium (C: 13.1 $mgkg^{-1}$, Zn 10X: 47.2 $mgkg^{-1}$ dry biomass). If the metal accumulation feature is compared of the two species, Chlorella uptakes ten times more zinc and copper than *Spirulina*.

Marked difference was observed in the extent of iron accumulation of the two studied algae grown in the control medium. After two-week-long incubation period the iron concentration of *Spirulina* was 300.5 mg kg-1, while *Chlorella* accumulated five times less metal (66.3 $mgkg^{-1}$ dry biomass). On the contrary, when examining algae grown in iron fortified media the extent of iron uptake of *Chlorella* proved to be more considerable than that of *Spirulina* (*Spirulina* Fe10X: 676.9 $mgkg^{-1}$, Chlorella Fe 10X: 789.7 $mgkg^{-1}$ dry biomass).

		Nutritive Value	
		Alga grown in normal medium	Alga grown in normal medium with 10 times enhanced microelement content
Total Lipid	*Spirulina p.*	9.38 ± 0.38%	8.87 ± 0.28
	Chlorella v.	15.11 ± 1.22	12.34 ± 0.97
Sodium	*Spirulina p.*	1.72 ± 0.05	1.92 ± 0.10
	Chlorella v.	1.11 ± 0.09	1.44 ± 0.71
Total Carbohydrate	*Spirulina p.*	22.88 ± 3.39	19.98 ± 2.88
	Chlorella v.	19.25 ± 2.33	18.23 ± 1.92
Protein	*Spirulina p.*	59.87 ± 3.66	61.22 ± 4.28
	Chlorella v.	41.37 ± 4.55	43.71 ± 3.87

Table 3: Nutritive value of the samples.

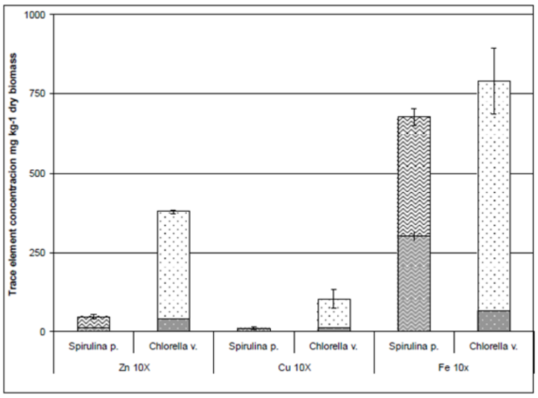

Figure 1: Bioaccumulation of zinc, Cu, iron by Spirulina and Chlorella from growing media with 10 times fortified metal content ■: Content media; □: Growth media with enhanced trace element level.

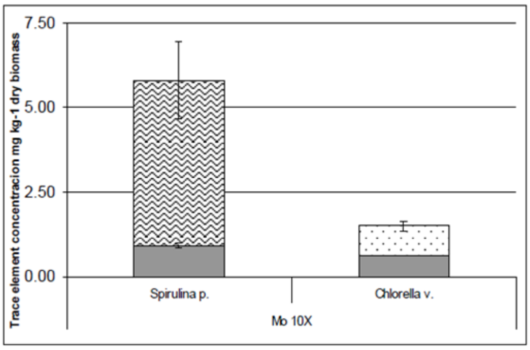

Figure 2: Bioaccumulation of Mo by Spirulina and Chlorella from growing media with 10 times fortified Mo(VI) ■: Content media; □: Growth media with enhanced trace element level.

Extent of bioaccumulation of molybdenum in *Spirulina* was found to be four times larger than in *Chlorella* (*Spirulina* Mo10X: 5.79 mgkg^{-1}, *Chlorella* Mo10X: 1.51 mg kg^{-1} dry biomass).

Comparison of iron accumulation of *Spirulina platensis* and *Chlorella vulgaris*

Figure 3 shows that by enhancement of the iron content of the growing media (5X, 10X, 25X, 50X, 100X) the amount of the accumulated iron in *Spirulina platensis* slowly and continuously increased. The extent of iron accumulation of *Chlorella* was significantly increased when applying sample with 50 times fortified iron concentration. In case of higher concentration of iron less amounts of algae biomass were generated.

When comparing the iron accumulation capacity of the two studied alga species it can be stated that *Spirulina platensis* accumulated greater amount of iron from the control growth medium than *Chlorella vulgaris*. In cases of samples involving growing media with enhanced iron concentration, *Chlorella* adsorbed significantly larger quantity of iron than *Spirulina*.

The calculated theoretical maximum of adsorbing capacity of the biomass was 419.17 mg/100 g dried alga (*Chlorella vulgaris*) and 133.57 mg/100 g dried alga (*Spirulina platensis*), respectively.

Alga mass (biomass) increment

After the two-week-long incubation period the dried biomass of the two studied algae, grown in media with different microelement content, was compared to one another and to their control as well (Figure 4).

The biomass of *Chlorella* grown in the medium with ten times enhanced Zn(II) concentration was approximately three times larger than the biomass of *Chlorella* grown in the control medium (Figure 4). Zinc, molybdenum and copper enrichment of the growing media had adverse effect on the growth of *Spirulina*, as its biomass was significantly lower than the biomass of the control sample after two weeks incubation. Iron fortification of the growing media resulted in a definite increase of the biomass of *Chlorella*, while that of *Spirulina* was not affected. Molybdenum fortification hardly influences the biomass of *Chlorella*, while significantly lower biomass of *Spirulina* was measured than in the control one. The enhanced copper-content of the growing media exerted negative effects on the growth of both studied algae species.

Since our major interest was directed towards the accurate estimation of the extent of biosorption of iron, it was of high importance to reveal its effect on the increase of alga biomass. The biomass of *Spirulina* and *Chlorella* grown in control media were compared to the ones which were grown in media with enhanced iron concentrations (5x, 10x, 25x, 50x) (Figure 5).

It was observed that the amount of biomass of *Chlorella* was found

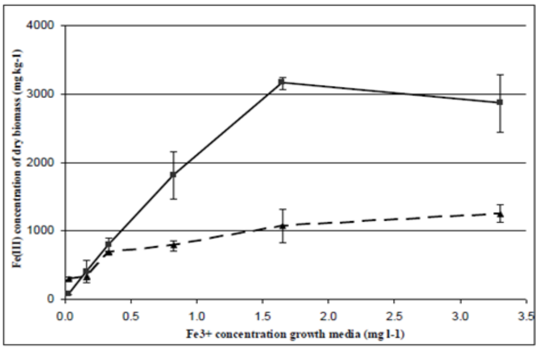

Figure 3: Biosorption of iron (Fe3+) by Spirulina (—▲—) and Chlorella (—■—).

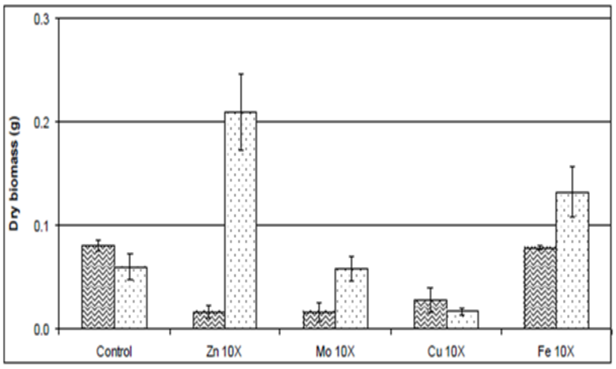

Figure 4: Biomass of spirulina (▨) and Chlorella (□) grown in media with diverse microelement contant after two-week-long incubation.

to be roughly four times larger in comparison with the control sample in case of applying growth medium with 5 times increased Fe (III) content, and three times bigger in case of Fe10X medium. The iron enrichment of the growing media had hardly affected the biomass of *Spirulina* in cases of applying Fe5X and Fe10X growing media; however more significant increase in the iron concentration of the media (Fe25X, Fe50X and Fe100X) led to moderate enhancement in the amount of biomass. On the contrary the biomass of *Chlorella* was dramatically lower at these iron levels (Fe25X, Fe50X and Fe100X).

Therefore the iron can be considered as one of the limiting factors of the growth of *Chlorella* as increase of the biomass reacts sensitively to the alterations in the iron content of the media. Beyond a certain iron concentration further biomass production is hindered. Biomass growth of *Spirulina* is not so adversely affected by the increasing iron concentration of the medium, even though slight biomass enhancement is observed in the previous cases.

Comparison of enrichment factors and weight ratios

The enrichment factor was outstanding in case of *Chlorella* for Fe(III); Cu(II) and Zn(II) as well as in case of *Spirulina* for Mo(VI). In case of *Chlorella* Fe(III) and Zn(II) the weight ratio was also remarkable (Table 4).

Practical application

Both micoalgae species might be suitable as row material of plausible functional food application; however *Chlorella* exhibits more favourable feature in respect of metal binding capability and efficiency, than *Spirulina*. As a consequence, *Chlorella* can be advised for manufacturing use of microalga-based functional food products fortified with distinctive microelements, if microelement enrichment is the major scope of the development. In contrary to the previous arguments, *Spirulina* might be more favoured to *Chlorella* in case of production just Fe-fortified products, as the biomass growth of *Spirulina* proved to be much more pronounced when applying excessively iron-enriched media (Fe25X, Fe50X, Fe100X). In any other cases *Chlorella* displayed more beneficial biomass growing characteristics, however the high reproduction rate and effective biomass production of *Spirulina* make this species also appropriate for involvement in functional food production in a quick, cost-effective and environmentally friendly way.

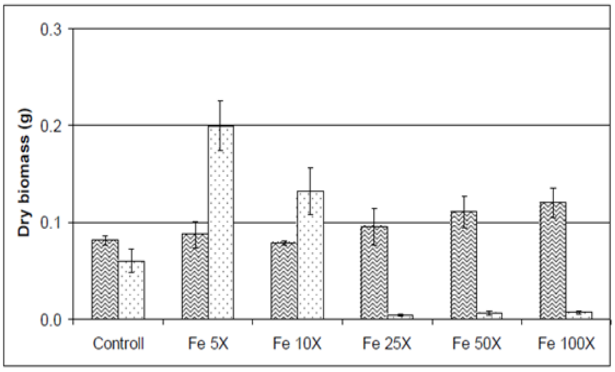

Figure 5: Biomass of spirulina (▨) and Chlorella (□) grown in media with differently enhanced iron content.

	Fe^{3+}		Cu^{2+}		Zn^{2+}		Mo(VI)	
	S.p	*C.v*	*S.p*	*C.v*	*S.p*	*C.v*	*S.p*	*C.v*
Enrichment factor	2.25	11.92	1.05	8.89	3.61	9.14	6.30	2.40
Weight ratio	0.97	2.19	0.35	0.29	0.20	3.49	0.20	0.97

Table 4: Enrichment factor and weight ratio.

In Table 5 the amounts of algae are presented necessary to be consumed in order to ensure 15% of the relevant RDA-s in cases of the studied 4 microelements [26].

Conclusions

It might be concluded that metal enriched microalgae can be considered as highly efficient tools of microelement supply, as it involves considerable amounts of metals in bioavailable forms (organic bonds). Due to the pronounced metal accumulation feature of the two studied species, they are suitable for being involved in possible functional food developments.

If the metal accumulation feature is compared of the two species, *Chlorella* uptakes ten times more zinc and copper than *Spirulina*.

It was found that the extent of bioaccumulation of the studied microelement proved to be significantly more pronounced for *Chlorella* vulgaris than for *Spirulina platensis*, with the exception of molybdenum.

Chlorella displayed more beneficial biomass growth characteristics than *Spirulina platensis* when incubated in media with enhanced microelement content in cases of Zn(II), Mo(VI) and Cu(II).

Iron can be considered as one of the limiting factors of the growth of *Chlorella* as increase of the biomass reacts sensitively to the alterations in the iron content of the media. Biomass growth of *Spirulina* is not so adversely affected by the increasing iron concentration of the medium.

It was established that significantly less amount of algae has to be consumed to comply with the physiological needs of some relevant microelements, if they are incubated in metal fortified media.

The abovementioned results confirm the relevance of development of microelement enriched products based on specifically selected and grown microalga species. The most efficient ways and conditions of metal bioaccumulation have been established laying the foundations for functional food application in the future.

Acknowlegdement

The authors thank the National Office for Research and Technology for financial support of this work (TÁMOP-4.2.2.A-11/1/KONV-2012-0008 and TÁMOP-4.2.3-12/1/KONV-2012-0025).

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		Amount of Algae (g)	
		Alga grown in normal medium	**Alga grown in normal medium with 10 times enhanced microelement content**
Mo(VI)	*Spirulina p.*	40.74	6.47
	Chlorella v.	59.51	24.81
Zu^{2+}	*Spirulina p.*	172.13	47.71
	Chlorella v.	54.37	5.95
Cu^{2+}	*Spirulina p.*	20.94	20.01
	Chlorella v.	18.10	2.04
Fe^{3+}	*Spirulina p.*	6.99	3.10
	Chlorella v.	31.69	2.66

Table 5: Amount of algae necessary for intzake of 15% of RDA of 4 microelements.

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