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A N I N T E R N A T I O N A L J O U R N A L

Effect of nutrients on total lipid content and fatty acids profile of *Scenedesmus obliquus*

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ABSTRACT

The effect of nutrients on the total lipid content and fatty acid profile of Scenedesmus obliquus isolated from the south coast of the Caspian Sea was evaluated. The nutritional compositions of the media impacted the growth rate and biomass of S. obliquus that ranged from 0.175 day^{-1} to 0.209 day^{-1}and 0.92 gr·l^{-1} to 1.79 gr·l^{-1}, respectively. The alga grew better in the medium which was characterized by higher levels of sodium and trace elements such as Fe, Mn, Mo, and Co and poor in N and P as compared with the other media. The highest level of the total lipid (32%) and the highest values of saturated fatty acids, in particular palmitic acid also were positively correlated with these nutrients. Peaks in polyunsaturated fatty acids (43.7 %), especially α-linolenic acid (28.4%) were related to N and P, but its correlation with K and Mg was more evident. The most important factors correlated with high amount of monounsaturated fatty acids were also N and P, followed by K and Mg to a lesser extent. This study demonstrated that the same algal strain may be a source of different amount of fatty acids, depending on the composition of the culture medium.

Key words: monounsaturated fatty acids; polyunsaturated fatty acids; Canonical correspondence analysis; green algae; Caspian Sea

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INTRODUCTION

Many microalgae contain considerable amounts of polar and nonpolar or neutral lipids that play important roles in different consumers [1-4]. Among them, polyunsaturated fatty acids (PUFAs) especially eicosapentaenoic (EPA), docosahexaenoic (DHA), and arachidonic acids (AA) are the most valuable to create significant physiological and biochemical changes in a body, including reducing risk of cardiovascular diseases [5-9].

Definitely, the total lipid content may vary noticeably among individual species or strains within and between taxonomic groups [7]. In recent years, species of green algae are often considered as a source of essential fatty acids that is necessary not only for improving the organism nutrition, but also for production of biodiesel [10-12]. However, this may not be because green algae naturally contain considerably more lipids than other algal taxa, but rather because many green algae are ubiquitous in diverse natural habitats, can easily be isolated, and generally grow faster than species from other taxonomic groups under laboratory conditions [13]. Among green algae, Scenedesmaceae family is one of the most popular food sources in experiments with herbivorous zooplankton [14], and also a potential source of lipids that constitute up to 47% DW [15], particularly PUFAs group, including oleic, linoleic, palmitic and alpha-linolenic acids [16]. *Scenedesmus* Meyen is a freshwater and marine genus with 433 known species worldwide [17], of which 27 species has been reported in aquatic ecosystems of Iran [18].

In nature, the cytoplasmic oil droplets are accumulated under stressful environmental conditions, such as high temperature, light intensity and rise in salinity [19-22]. In culture, nutritional composition of the medium has more effects on the total lipid content and fatty acid profile, among which nitrogen, silicon and phosphorus have greater roles [20-24], followed by iron [25-28]. In 1987, the lipid content and fatty acid composition of *Scenedesmus obliquus* was determined [29], but at a later time, the values differed within the species [30-32]. Therefore, the present study focused on whether the nutritional composition could affect the total lipid content and fatty acid profile of *S. obliquus* that was isolated from the Caspian Sea.

MATERIALS AND METHODS

The alga strain and growth conditions

Sampling was carried out in the Nour shore of the Caspian Sea shore near the Department of Marine Biology of Tarbiat Modares University (36° 35' 22"N, 52° 02' 05"E) by a plankton net with a mesh size of 55 and the Ruthner's bathometer in 2012. *S. obliquus* was isolated and purified using the agar plates and repeated liquid culture. To obtain a liter of suspension, *S. obliquus* strain from the stock culture was inoculated into the sterile medium close by the flame, until the transmission coefficient (T) got to 92-93% [33]. The alga was grown in the growth chamber of Binder model maintaining the light intensity of 60 µE. m^{-2} s^{-1} for 0:24 h a dark/light cycle and temperature of 25 ±0.5 ºC for 31 days. Three media, viz. Trenkenshu, Tamiya and Guillard were used to grow the alga (Table 1).

Table 1 – Nutritional composition of three culture media [33-35].

Guillard			**Tamiya**			**Trenkenshu**	
Name solution	Compound	Required amount (gr l^{-1})	Name solution	Compound	Required amount (gr l^{-1})	Compound	Required amount (gr l^{-1})

Main solution	NaNO3	0.075	**Main solution**	KNO_3	5	$NaNO_3$	1.8
	NaH_2PO_4	0.005		KH_2PO_4 $3H_2O$	1.25	NaH_2PO_4 $2H_2O$	0.3
	$2H_2O$	0.03		$MgSO_4$ $7H_2O$	2.5	Na_2 EDTA	0.037
	Na_2SiO_3 $9H_2O$			Na_2 EDTA	0.037	$FeC_6H_5O_7$ 7 H_2O	0.042
Vitamins		0.0005		$FeSO_4$ $7H_2O$	0.009	$MnCl_2$ $4H_2O$	0.008
	Biotin	0.00001		TES	1ml	$Co(NO_3)_2$ $6H_2O$	0.00625
	Thiamine	0.0005				$(NH_4)_6Mo_7O_{24}$	0.00183
	B12					$4H_2O$	0.00238
						$K_2Cr_2(SO_4)_2$	0.00058
		1000ml		Filtrated	1000ml	$24H_2O$	1000ml
	Filtrated seawater			seawater		TiO_2 Filtrated seawater	
Trace element solution (TES)	Na_2 EDTA	0.00436	**Trace element solution (TES)**	H_3BO_3	2.86		
	$FeCl_3$ $6H_2O$	0.0032		$MnCl_2$ $4H_2O$	1.81		
	$MnCl_2$ $4H_2O$	0.0002		$ZnSO_4$ $7H_2O$	0.222		
	$CuSO_4$ 5 H_2O	0.00001		NH_4VO_3	0.023		
	ZnSO4 $7H_2O$	0.00002		MoO_3	0.015 or		
	$CoCl_2$ $6H_2O$	0.00001		$(NH_4)_6Mo_7O_{24}$	0.023		
	Na_2MoO_4	0.000000		$4H_2O$			
	$2H_2O$	6		Distilled water	1000 ml		

Assessment of the microalgae growth

Cell number was daily counted with a Neubauer hemocytometer using a light microscope in triplicate for each sample and cell density was measured according to the following formula [34]:

Cell density per milliliter =Total cell counts × 10^4× Dilution factor

The growth of alga also was monitored by determining the absorbance using a UV-Vis spectrophotometer at 750 nm [33]. The biomass values were obtained by the calibration curve of absorbance versus dry weight biomass concentration.

The specific growth rate (GR) of cultured microalgae was calculated by the following equation:

GR = ln (X1- X2)/t2 – t1

Where,

X1 = Biomass concentration at the end of selected time interval,

X2 = Biomass concentration at the beginning of selected time interval,

t2 –t1 = Elapsed time between selected time in the day.

The stationary phase was reached when the absorbance values stabilized, proceeding then to the biomass harvest.

Total lipid and fatty acid analysis

The total lipid was estimated from dry cells of *S. obliquus* collected in the stationary phase (it was for each medium on the different days) according to the protocol of Bligh and Dyer [36]. Before the lipid extraction, the samples of 0.05±0.001 gr DW were fairly soaked in 4 ml of distilled water and were homogenized with a manual homogenizer for one minute. The algal samples were extracted with chloroform: methanol mixture (2:1 v/v) and kept for 5 minutes at room temperature. After a five-minute rest, they were dissolved in a methanol-chloroform-water mixture (MCWM) (3/3/1, v/v/v) and were homogenized; the piston was washed every time with 3.5 ml

of MCWM. The resulting mixture was incubated for 15 minutes at room temperature, then 5 ml of chloroform was added and the mixture was given 15 minutes rest for lipid extraction. For appearing two phases in the samples, 5 milliliter of distilled water was added and the samples were heavily shaken. The lipid fractions were separated in a clean pre-weighed vial (first wt) and the solvent was evaporated using a rotary evaporator. The weight of the vial was again recorded (second wt). Total lipid was calculated by subtracting first wt from second wt. The final organic phase was dried under nitrogen.
Preparation of fatty acid methyl ester from the total lipid was performed according to Radwan [37]. All analyses for identification of fatty acid contents were performed using gas chromatography (Varian CP-3800 model).

Statistical analysis
All experiments were repeated three times independently, and the data were recorded as the mean. The statistical analyses were performed using the software Statistical Program for Social Sciences 17.0. A one-way ANOVA and Duncan test were used to evaluate the differences among the treatments. Canonical-correlation analysis (CCA) and Pearson's coefficient were used to determine relationships among the nutritive composition of a culture medium and amount of total lipid content and fatty acid profile.

RESULTS

Nutrient availability had a significant impact on growth of the microalga and broad effects on its lipid and fatty acid composition. The effect of the nutritional compounds as three investigated media on the growth of was recorded by way of cell count (cell number l^{-1}) and optical density (OD) every day, both of which showed quite similar curves (Figures 1 and 2). The algal cell growth was greatly affected in Trenkenshu and Tamyia media. In Trenkenshu medium, the maximum cell count (17×10^6 cell·ml^{-1}) and OD (1.26 at A_{750}) were recorded on the 25th day. The lowest cell number was recorded in Guillard medium (max. 14×10^6 cell·ml^{-1} on the 30th day). In Trenkenshu medium, the lag phase lasted less than in the other media that points to more quickly culture adaption. The exponential phase started at 0.2 OD_{750} and continued to 1.2 OD_{750}. The longest onset of the stationary phase was observed in Guillard medium on 31th day. The nutritional compositions of the media had markedly impacted the growth rate and biomass of *S. obliquus* (Figures 3 and 4). The slowest growth rate (0.175 day^{-1}) and biomass value (0.92 gr·l^{-1}) were recorded in Guillard medium, while the maximum growth rate(0.209 day^{-1}) and biomass(1.79 gr·l^{-1}) were observed in Trenkenshu medium.

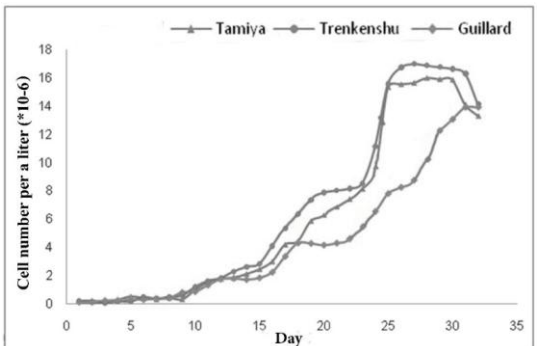

Figure 1 – Cell number dynamics of *S. obliquus* in three culture media

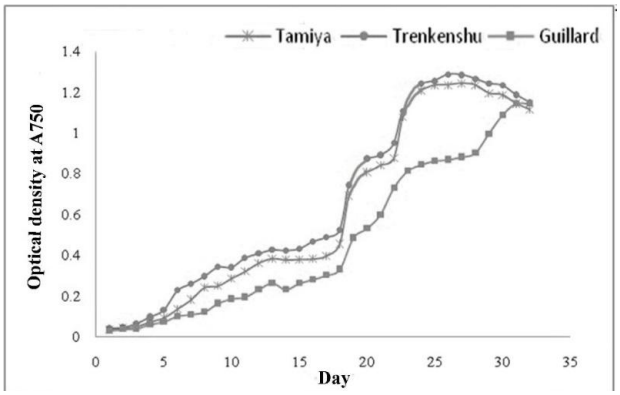

Figure 2 – Optical density dynamics of *S. obliquus* growth in three culture media

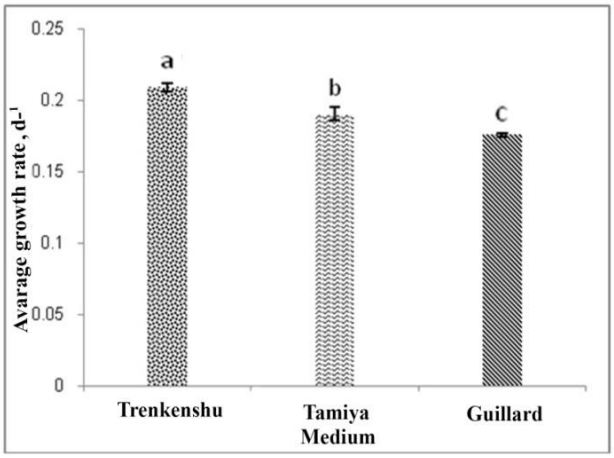

Figure 3 – Specific growth rate of *S. obliquus* in three culture media

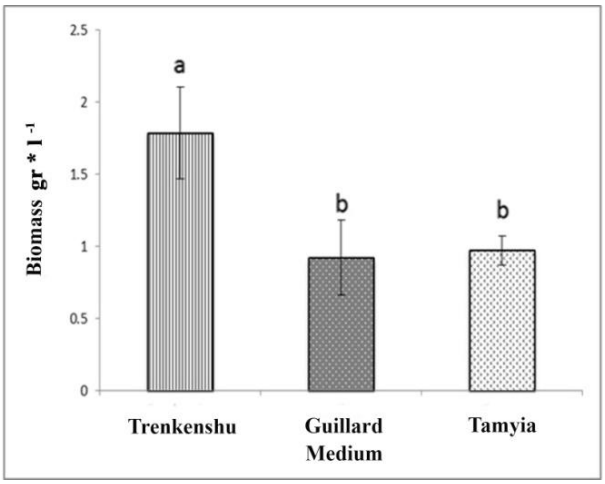

Figure 4 – Biomass of *S. obliquus* culture in three culture media

The highest total lipid content (32% DW) was observed inTrenkenshu medium (Table 2), which was significantly ($p<0.05$) higher than Tamiya (24% DW) and Guillard (21%) media, but no significant difference $p>0.05$) was observed between the latter two media. The highest level of SFAs (45.7%) was observed in Trenkenshu medium which was significantly different from the other two media ($p<0.05$), while the highest level of MUFAs (47.6%) was recorded in Guillard medium which was significantly different from the other two media ($p<0.05$). The highest percentage of PUFAs (43.7%) was recorded in Tamyia medium, which was significantly different

from the other two media ($p<0.05$).The fatty acids profile of *S. obliquus* also varied according to medium (Table 2). Being the most common saturated fatty acid in living organisms, palmitic acid (C16:0) was also the main SFA in all the three media. Elaidic acid, the trans isomer of oleic acid (C18:1 n-9 trans), was the most important in MUFAs ranging 11.8-39.2. Moreover, cetoleic acid (C22:1n11) showed quite high value (11.8%) in Trenkenshu medium. Among PUFAs, ALA was the dominant fatty acid, for which Tamyia medium was a better source. It is interesting to note that lauric and arachidic acids with a 12- and 20-carbon atom chain, respectively, were only quantified in Trenkenshu medium.

Table 2 – Fatty acid profile of *S. obliquus* in three investigated media. Data are given as mg g^{-1} of dry weight

Fatty acid*	**Medium**		
	Trenkenshu	**Guillard**	**Tamiya**
SFA			
C12:0	4.0	-	-
C14:0	1.6	1.7	4.7
C15:0	2.7	1.5	4.2
C16:0	26.7	21.2	14.0
C17:0	-	0.2	-
C18:0	7.9	1.3	9.3
C20:0	2.8	-	-
Sum SFAs	**45.7**	**25.9**	**32.2**
MUFA			
C16:1n7	2	2.8	3.3
C17:1	3.3	4.4	3.7
C18:1n9 trans	12.1	39.2	11.8
C18:1n9 cis	-	0.1	2.6
C22:1n11	11.8	1.1	-
Sum MUFAs	**29.2**	**47.6**	**21.4**
PUFA			
C18:2n6 trans	8.3	5.5	9.3
C18:2n6 cis	-	10.2	1.8
C18:3n3	16.7	7.4	28.4
C18:3n6	-	2.4	4.2
Sum PUFAs	**25.0**	**25.5**	**43.7**
Total FA	**99.9**	**99.0**	**97.3**
Total lipid**	**32.3**	**21.0**	**24.3**
SFA/MUFA/PUFA***	**1.8**/1.2/1	1/**1.9**/1	1.5/1/**2**

*Fatty acid as a percentage of the total fatty acids mixture
**Total lipids as a percentage of dry weight
***SFA/MUFA/PUFA ratio of saturated fatty acids to monounsaturated and polyunsaturated fatty acids

Based on the CCA, eigenvalues of axes 1 ($\lambda = 0.463$) and 2 ($\lambda = 0.063$) explained 88.0% and 12.0% of the relation between fatty acids and nutrient data, respectively (Fig. 5, Table 3). The ranks of the nutrition variables contributing to this model were (1) sodium, cobalt and molybdenum, (2) potassium and magnesium, (3) manganese, (4) iron, (5) PUFA, C14:0, C18:1n9c, (6) C15:0, C16:0, C18:3n3, (7) C18:3n6 and C16:1n7.

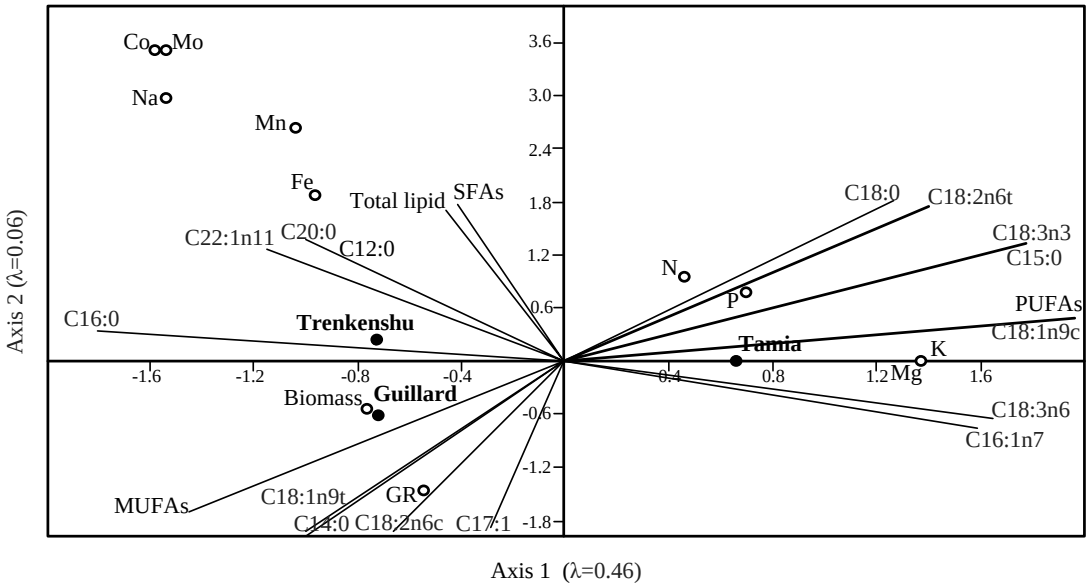

Figure 5 – Canonical correspondence analysis (CCA) biplots showing the relationships between media, nutrients growth parameters, total lipid and fatty acids.

Table 3 – Correlations of nutrients, growth parameters and fatty acids with axes from reference site data. **λ** represents eigenvalue for each axes.

Variables	**Axes 1 λ=0.46**	**Axes 2 λ=0,06**
GR	-0.55	-1.49
Biomass	-0.76	-0.56
N	0.46	0.95
P	0.70	0.77
Na	**-1.54**	2.97
K	**1.37**	0.01
Mg	**1.37**	0.01
Fe	**-0.96**	1.87
Mn	**-1.04**	2.64
Mo	**-1.55**	3.51
Co	**-1.58**	3.52
SFA	-0.21	0.89
PUFA	**0.99**	0.25
MUFA	-0.73	-0.86
C12:0	-0.50	0.70
C14:0	**0.99**	0.24
C15:0	**0.89**	0.69
C16:0	**-0.90**	0.17
C17:0	-0.50	-0.97
C18:0	0.63	0.91
C20:0	-0.50	0.70
C16:1n7	0.79	-0.37
C17:1	-0.15	-0.99
C18:1n9t	-0.50	-0.97
C18:1n9c	**0.99**	0.24
C22:1n11	-0.57	0.64
C18:2n6t	0.70	0.87
C18:2n6c	-0.35	-0.99
C18:3n3	**0.90**	0.67
C18:3n6	0.82	-0.33

GR: growth rate; significant coefficients shown in bold.

The total lipid and SFA were correlated with sodium, potassium, magnesium, manganese, molybdenum and cobalt while MUFA and PUFA had positive correlation with nitrogen and phosphorus (Fig. 5, Table 3). Furthermore, PUFA depended strongly on potassium and magnesium. The main fatty acids such as C16:0 and C18:1n9t were correlated with potassium, magnesium, sodium and nitrogen, phosphorus, respectively. ALA (C18:3n3) was associated strongly with nitrogen, phosphorus and less with potassium and magnesium.

DISCUSSION

The quantity and quality of fatty acids content as well as the entire biochemical composition varies in response to environmental conditions [8,20,24,25,38,39]. Particularly, under unfavorable environmental or stress conditions for growth, such as salinity, temperature, pH, and nutrient levels, many algae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids to control intracellular stress [13,40]. Finding the appropriate conditions to stimulate the synthesis of different fatty acids of interest is crucial for developing an efficient biological production process.

The present research showed significant differences in total lipid and fatty acid profile for the same strain of *S. obliquus* cultivated in three different media (Fig. 5 and Table 3).

Table 4 – Results from Pearson's correlation between total lipid, SFA, PUFA, MUFA and nutritional variables

Fatty acid	Nutrients										
	GR	**B**	**N^-**	**P^-**	**Na^+**	**K^+**	**Mg^+**	**Fe^+**	**Mn^+**	**Mo^+**	**Co^+**
SFA	**0.99***	**0.97**	0.22	0.08	**0.94**	-0.21	-0.21	**0.99**	**0.99**	**0.96**	**0.96**
PUFA	-0.09	-0.48	**0.90**	**0.95**	-0,55	**1.00**	**1.00**	-0.35	-0.35	-0.51	-0.52
MUFA	**-0.63**	-0.28	**0.95**	**0.90**	-0.20	**-0.73**	**-0.73**	-0.41	-0.41	-0.23	-0.23
Total lipid	**0.99**	**0.97**	0.20	0.50	**0.95**	-0,23	-0.23	**0.99**	**0.99**	**0.95**	**0.95**
C12:0	**0.90**	0.99	-0.09	-0.23	**1.00**	-0.50	-0.50	**0.98**	**0.98**	**1.00**	**1.00**
C14:0	-0.10	-0.48	**0.89**	**0.95**	-0.55	**1.00**	**1.00**	-0.35	-0.35	-0.52	-0.53
C15:0	0.38	-0.10	**1.00**	**0.98**	-0.09	**0.89**	**0.89**	0.13	0.13	-0.06	-0.07
C16:0	0.50	**0.80**	-0.64	-0.75	**0.84**	**-0.90**	**-0.90**	0.70	0.70	**0.82**	**0.83**
C17:0	-0.83	-0.54	**-0.82**	-0.72	-0.47	-0.50	-0.50	-0.66	-0.66	-0.51	-0.50
C18:0	0.73	0.40	**0.90**	**0.83**	0.32	0.64	0.64	0.52	0.53	0.36	0.35
C20:0	**0.90**	**0.99**	-0.09	-0.24	**1.00**	-0.50	-0.50	**0.98**	**0.98**	**1.00**	**1.00**
C16:1n7	-0.66	-0.90	0.46	0.58	-0.93	0.79	0.79	-0.83	-0.83	-0.92	-0.93
C17:1	**-0.97**	-0.80	-0.55	-0.42	-0.75	-0.15	-0.15	**-0.88**	**-0.88**	-0.78	-0.78
C18:1n9t	**-0.82**	-0.53	**-0.82**	**-0.73**	-0.46	-0.50	-0.50	-0.64	-0.65	-0.49	-0.49
C18:1n9c	-0.10	-0.48	**0.89**	**0.95**	-0.55	**0.99**	**0.99**	-0.35	-0.35	-0.52	-0.53
C22:1n11	**0.85**	**0.99**	-0.17	-0.31	0.99	-0.57	-0.57	**0.96**	**0.96**	**0.99**	**1.00**
C18:2n6t	0.66	0.31	**0.93**	**0.87**	0.23	0.70	0.70	0.44	0.45	.027	0.26
C18:2n6c	**-0.91**	-0.67	-0.71	-0.59	-0.61	-0.35	-0.35	-0.77	-0.77	-0.64	-0.64
C18:3n3	0.38	-0.02	**1.00**	**0.98**	-0.09	**0.89**	**0.89**	0.13	0.13	-0.06	-0.07
C18:3n6	-0.62	**-0.88**	0.51	0.62	**-0.91**	-0.82	-0.82	**-0.80**	**-0.80**	**-0.90**	**-0.91**

*negative and positive correlating values are shown in bold

The fatty acids content as well as the ratio between unsaturated and saturated fatty acids is an important parameter for determination of algae value. SFAs were found to increase by increasing the iron amount in *Nannochloropsis oculata* [20]. In *Dunaliella tertiolecta* and *Stephanodiscus minutulus*, changes of the SFA's values were directly related to changes in the amount of nitrogen [24].In the present study, SFAs showed significantly positive correlationship with manganese and iron first of

all, then sodium which were sufficiently present in Trenkenshu medium (Table 3 and Figure 5).In addition, trace elements such as molybdenum and cobalt are the important nutritional variables for obtaining high SFAs and total lipid. Dou et al. [27] also found that the addition of Fe^{3+}, Zn^{2+}, Mn^{2+}, Mo^{6+}, and EDTA can increase the lipid productivity. *S. obliquus* cultivated in Trenkenshu medium can be used for biofuel because quite high content of palmitic acid obtained in this medium is desirable for good quality biodiesel [12]. Furthermore, palmitic, lauric and myristic acids are not good substances in nutrition of living organisms because they are responsible for raising bad cholesterol levels in blood serum [41]. According to Napolitano et al. [42], SFAs may play a double role: as a store of saturated fatty acids to be used as a source of energy and as a store of PUFAs required for phospholipid synthesis to various membrane structures or to be integrated in several metabolic processes.

PUFAs are one of the most nutritionally important and essential fatty acids because they are key nutrients in animal nutrition, and the most algae are rich in these acids [43]. Nitrogen and phosphate are two important macronutrients for phospholipids because nitrogen and phosphorus starvation shifts the lipid metabolism from membrane lipid synthesis to neutral lipid storage [39,44-46]. Results of the present study also showed clearly that peaks in PUFAs are related to nitrogen and phosphorus, but its correlation with potassium and magnesium was more evident. In Tamiya medium, the most dominant fatty acid ALA also was related to nitrogen, phosphorus, potassium and magnesium. The ratio between PUFA and SFA content observed in Tamiya medium can be considered as nutritional value of *S. obliquus* cultivated in this medium because the higher value of P/S index means a smaller deposition of lipids in the body [47].

There are reports that amount of MUFAs goes up due to the reduction of nitrogen in the culture medium [20,23]. The potential relationship between phosphorus and amount of MUFAs have beenshown intwo microalgae *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* [48] and the yellow-green alga, *Monodus subterraneus* [49], in which reduced phosphorus in medium had impacted MUFAs. By analyzing the changes in fatty acids composition of several algal species at different concentrations of nitrogen and silica, Shifrin and Chisholm [50] showed increase in MUFAs as the result of nitrogen limitation. In our experiment, the most important factors correlated with high amount of MUFAs were also nitrogen and phosphorus, followed by potassium and magnesium to a lesser extent. Limitation of these parameters caused growth delay and an accumulation of fatty acids forming MUFAs especially elaidic acid.

CONCLUSIONS

Based on the results of the present study, it can be concluded that the same algal strain may be a source of different fatty acids, depending on the nutritive composition of the culture medium. Sodium, iron, cobalt and molybdenum impacted on the growth rate, SFAs in particular palmitic acid, and, as the result, the total lipid content in the green alga *S. obliquus.* The highest amount of PUFAs and oleic acid were related to potassium and magnesium and less nitrogen and phosphorus. The highest amount of MUFAs, especially ALA were obtained at limitation of nitrogen and phosphorus caused growth delay and an accumulation of fatty acids forming MUFAs especially elaidic acid. Thus, the knowledge about stimulation of the synthesis of different fatty acids may be used for algal cultivation with the different purposes.

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