Int. J. Aquat. Biol. (2023) 11(2): 159-169
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Effects of different physical shocks and sampling time on lipid content and fatty acids composition of *Nannochloropsis oculata*

Farzaneh Noori*, Saeid Vahdat

Artemia and Aquaculture Research Institute, Urmia University, Urmia, Iran.

Abstract: The microalga *Nannochloropsis oculata* is marine widely used in aquaculture systems as an essential source of protein, lipid, and polyunsaturated fatty acids. Day/night pH fluctuations driven by photosynthesis and respiration create an environment that exhibits changing pH ranges. The aim of this study was to find whether physical shocks could change the complete profile of nutrients (lipid, fatty acid, carbohydrate, chlorophylls, and proteins) in *N. oculata*. The algae were cultivated in 32 flasks of ten-liter for biomass production for 12 days using Guillard medium (f/2). The cells were reared at 0.5 molar salinity (29 ppt), under 3500 lux light intensity with a 12L:12D photoperiod and 21ºC temperature. After 12 days, when the cell density reached its stationary phase, they were centrifuged. The pellets were then re-suspended in fresh seawater thoroughly and transferred into thirty-two containers with 10-liter volumes, including eight treatments with four replicates. The algae in treatments 3, 4, and 8 were subjected to salinity (88 ppt), starvation, and pH (11) shocks, respectively and treatments 2, 5, 6, and 7 were subjected to salinity + pH + starvation, pH + starvation, salinity + pH and salinity + starvation shocks, respectively. The biochemical composition of *N. oculata* demonstrated that T3 at the end of the dark period, and T1 at the end of the light period, possessed significantly higher (51.51%), and lower (24.9%) lipid content, respectively. According to the results, EPA under pH shock, linoleic acid under pH + salinity + starvation shock, and DHA and omega-3 under pH + salinity shock at the end of the dark period revealed significant differences with the control group. The saturated fatty acids showed significantly higher value in the control group during the dark period. The monounsaturated fatty acids increased significantly under pH shock at the end of the light period on day 18. Based on the results, the best treatment to obtain more lipid production in *N. oculata* was using six-day salinity shock and harvesting algae at the end of the dark period, and for more EPA synthesis in *N. oculata,* pH shock for six days and harvesting the algae at the end of dark period is recommended.

Article history:
Received 15 December 2022
Accepted 11 March 2023
Available online 25 April 2023

Keywords:
Sampling time
Fatty acid
Physical shocks
Biochemical composition

Introduction

Some species of microalgae synthesize very long-chain fatty acids (carbon chains 20+ in length), including eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) (Bigogno et al., 2002; Guiheneuf et al., 2013). These omega-3 (ω-3) fatty acids are essential components of high-quality fish diets (Muhlroth et al., 2013). Fatty acids are the building blocks of lipids, but they are not distributed equally amongst different lipid classes. Algal polar lipids are located in structural and functional cell membranes, while neutral lipid triacylglycerols (TAGs) function as storage molecules (Hu et al., 2008).

The microalga *Nannochloropsis* sp. is a Eustigmatophyte widely used in marine aquaculture systems as an important source of vitamins, pigments, and PUFAs (Sukenik, 1991; Sukenik et al., 1993; Pal et al., 2011). This microalga has been widely studied due to its high PUFA content. As found in many other species of algae, nutrient depletion significantly increased FA accumulation in *Nannochloropsis*. Other studies have reported that *Nannochloropsis salina* reaches a total FA accumulation of 37.5% lipid/DW when arriving at the stationary phase and could grow well at 34 ppt

*Correspondence: Farzaneh Noori
E-mail: f.noori@urmia.ac.ir

DOI: http//doi.org/10.22034/ijab.v11i2.1809
DOR: https://dorl.net/dor/20.1001.1.23830956.2023.11.2.10.2

salinity (Bartley et al., 2013). Similarly, *Nannochloropsis gadiata* grows at low irradiance demonstrating accumulation of up to 50% lipid/DW after a nutrient deprivation phase (Bartley et al., 2013). Doan and Obbard (2011, 2012) reported a total lipid production of 50 and 55% of DW in *Nannochloropsis* sp. using cell sorting and ethyl methanesulfonate, respectively. *Nannochloropsis* is a genus of robust, oleaginous microalgae that synthesizes EPA during balanced growth and is a promising candidate for commercial applications (Kilian et al., 2011; Radakovits et al., 2012; Li et al., 2014; Sharma et al., 2015).

Research demonstrated that subjecting the culture to a temporary change of environmental factors such as pH can decrease unwanted organisms and improve final culture densities. Day/night pH fluctuations driven by photosynthesis and respiration create an environment that exhibits changing pH ranges (Richmond and Becker, 1986). Further, pH may affect the lipid composition (Griffiths et al., 2009, Rodolfi et al., 2009, Moheimani, 2013). Nitrogen and phosphorus starvation shifts the lipid metabolism from membrane lipid synthesis to neutral lipid storage. This, in turn, increases the total lipid content of green algae (Hu, 2004). Higher salinity increases the algae's lipid content (Fabregas et al., 1984; Zhila et al., 2011). Besides the pigment contents, other algal cell components such as carbohydrates and proteins can also be influenced by the light regime (Renaud et al., 1991). The aim of this study was to find out how different physical shocks, alone or in combination, can influence the synthesis of different nutrients such as lipids, fatty acids, carbohydrates, chlorophylls, and proteins by *Nannochloropsis oculata*.

Materials and Methods

Nannochloropsis oculata was obtained from Artemia and Aquaculture Research Institute (AARI), Urmia University, Iran. The algae were cultivated in ten-liter conical flasks for biomass production for 12 days using Guillard medium (f/2)[1] (Guillard, 1973). The cells were reared at 0.5 molar salinity (29 ppt), 3500 lux light intensity with a 12L: 12D photoperiod and 21ºC temperature. After 12 days when the cell density reached its stationary phase, they were centrifuged (3500 rpm, 5 min). The pellets were collected and then re-suspended thoroughly, equally divided (0.2713 $g.L^{-1}$ DW), and transferred into thirty-two 10-liter containers, including eight treatments and four replicates. Then the *Nannochloropsis* algae were exposed to different shocks for six days. Treatments 3, 4, and 8 were subjected to salinity of 88 ppt, nutrient starvation, and pH (11) shocks, respectively and treatments 2, 5, 6, and 7 were subjected to salinity + pH + starvation, pH + starvation, salinity + pH, and salinity + starvation shocks, respectively. However, the control group (T1) was cultured under normal conditions. Each container was aerated continuously at a rate of 758 $mL.min^{-1}$. The samples were collected every three days and at the end of light and dark periods.

Chlorophyll a, b and total carotene analysis: Fifty mL algae were frozen under -20°C and then defrost (for breaking down of cell wall), filtered with S&X filter (1 µm), and weighed. The pigments were extracted with 96% methanol (50 $ml.g^{-1}$ algae). The samples were homogenized for one minute at 1000 rpm. The homogeneous mixture was filtered and centrifuged for 10 min at 2500 rpm. Supernatants were separated and the absorbance at 666, 653 and 470 nm was recorded using the microplate reader synergy HT. The following formula was used to calculate chlorophyll and total carotene:

$C_a = 15.65A_{666} - 7.340A_{653}$

$C_b = 27.05A_{653} - 11.21A_{666}$

$C_c = 1000A_{470} - 2.860C_a - 129.2C_b / 245$

Where Ca = chlorophyll a, Cb= chlorophyll b, and Cc= total carotene in terms of micrograms per gram of wet weight (Dereet al., 1998).

Lipid and ash contents analysis: Laboratory methods were used to measure lipid content by ether extraction (AOAC, 2005). For this purpose, a certain amount of samples was weighted and held for 7 hours on soxhlet within diethyl ether (98%). To calculate the ash content samples were weighed and placed in an electric furnace for 6 hours at 550°C

(AOAC, 2005).

Fatty acid profiles: The fatty acid composition of *Nannochloropsis* algae was determined based on Miquel and Browse (1992). In brief, 200 mg of algae was heated to 80°C in 1 ml of a mixed solution of H_2SO_4 2.5% and methanol 98% (1:40, v/v) for one hour in a Teflon lined screw cap glass tube. The mixture was cooled at room temperature and then 500 µl of hexane and 1.5 mL of NaCl 0.9% (w/v) were mixed and added to the samples. The samples were centrifuged for 10 min at 4000 rpm and supernatant (1 µl) was injected to Gas chromatograph (GC) to determine the fatty acid profiles (Miquel and Browse,1992).

Protein content: The protein content was measured according to Slocombe et al. (2013). Five mg of dried algae was mixed with 0.2 ml trichloroacetic acid (TCA) 24% (w/v). The mixture was incubated for 15 min at 95°C and cooled at room temperature. The homogenate was centrifuged at 15000 g for 20 min at 4°C and the supernatant was discarded. The pellets were re-suspended in 0.5 mL Lowry reagent and were incubated for 3 hours at 55°C. The samples were cooled at room temperature and centrifuged at 15000 g for 20 min. The supernatant was placed for 30 min in Lowry reagent and absorption was read at 600 nm (Slocombe et al., 2013). The standard curve was prepared using bovine serum albumin (BSA) as standard.

Total Carbohydrate content: The carbohydrate content of the samples was measured by adding 5 ml of 2.5 N HCl to 100 mg of algae. The samples were hydrolyzed to simple sugars by keeping them in a boiling water bath for three hours and then cooled at room temperature. The sample was neutralized by solid Na_2CO_3 until the effervescence ceases and then the volume was made to 100 mL with distilled water and centrifuged at 5000 rpm for 5 minutes. To 0.5 ml of supernatant, 0.5 mL of distilled water and 4 mL of 0.2% anthrone reagent were added and the mixture was heated for 8 min in boiling water bath. The sample cooled rapidly and the absorbance was read at 630 nm. The glucose standard solution was prepared at a concentration of 100 $\mu.mL^{-1}$ (Hedge and Hofreiter, 1962).

Statistical analysis: The normality of all data was checked by the Kolmogorov-Smirnov test. Mean (±SD) and factorial multivariate were used. One-Way ANOVA, ANCOVA (Bonferroni test) and Post-hoc Tukey analysis were used at 5% (P<0.05). Calculated data and drawing charts were done by SPSS (version 22) and Excel (2013 version), respectively.

Results

Biochemical compositions and some fatty acid profiles of *Nannochloropsis* cultivated in f/2 medium on day 12 under laboratory conditions are shown in Table 1. The results (Fig. 1) showed that the protein content was affected by salinity and pH shock (T6) at the end of the dark period showing the lowest value, while the highest protein content was found in T4 at the end of the dark period on day 18 (P<0.05). Lipid content was significantly influenced by the dark period and significantly higher values were observed in T7 and T3 (P<0.05) and the lowest value was achieved in T1 (4.68%) at the end of the dark period on day 18 (P<0.05). The result of ash reveals a significantly higher value at the end of the light period in starvation + salinity shock (T7) on day 15 (P<0.05). Carbohydrate content was influenced significantly by starvation + salinity (T7) and salinity (T3) shocks at the end of the dark period and exhibited the highest and lowest values, respectively (P<0.05).

The protein content was significantly decreased at the end of the light and dark period in T6 on day 18 and increased in T4 at the end of the dark period on day 18 (P<0.05). Moreover, at the end of the dark period on day 18, T3 showed a significantly higher lipid content (51.51%) (P<0.05). Carbohydrate content in T7 at the end of the dark period on day 18 showed the highest values (P<0.05). The chlorophyll a and b on day 18 and at the end of the dark photoperiod under pH + starvation + salinity shock revealed highly significant (P<0.05).

The pH + starvation + salinity shock at the end of the dark period on day 18 increased total carotene

Table 1. Bio-chemical and fatty acid compositions of *Nannochloropsis oculata* cultivated in f/2 medium on day 12 under standard conditions.

Day 12	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)	Chlorophyll a (µg.g^{-1} fw)	Chlorophyll b (µg.g^{-1} fw)	Total carotenoid (µg.g^{-1} fw)
End of Light	33.24±0.25	18.62±0.15	35.65±0.24	12.01±0.20	35.62±0.14	8.15±0.31	126.24±1.01
End of Dark	30.12±0.08	19.31±1.11	37.99±1.01	12.08±0.35	34.21±0.14	7.16±0.44	124.21±0.14

Day 12	ARA	DHA	EPA	Linoleic acid	Linolenic acid	SFA	MUFA	PUFA	Sum of ω3	Sum of ω6
End of Light	ND	ND	10.35±0.62	8.55±0.65	3.68±0.54	41.35±0.35	37.15±0.31	21.35±1.21	14.03±1.24	8.55±0.65
End of Dark	ND	ND	10.95±0.35	8.84±0.24	3.71±0.84	41.95±1.21	37.45±1.32	21.42±1.67	14.66±0.71	8.84±0.24

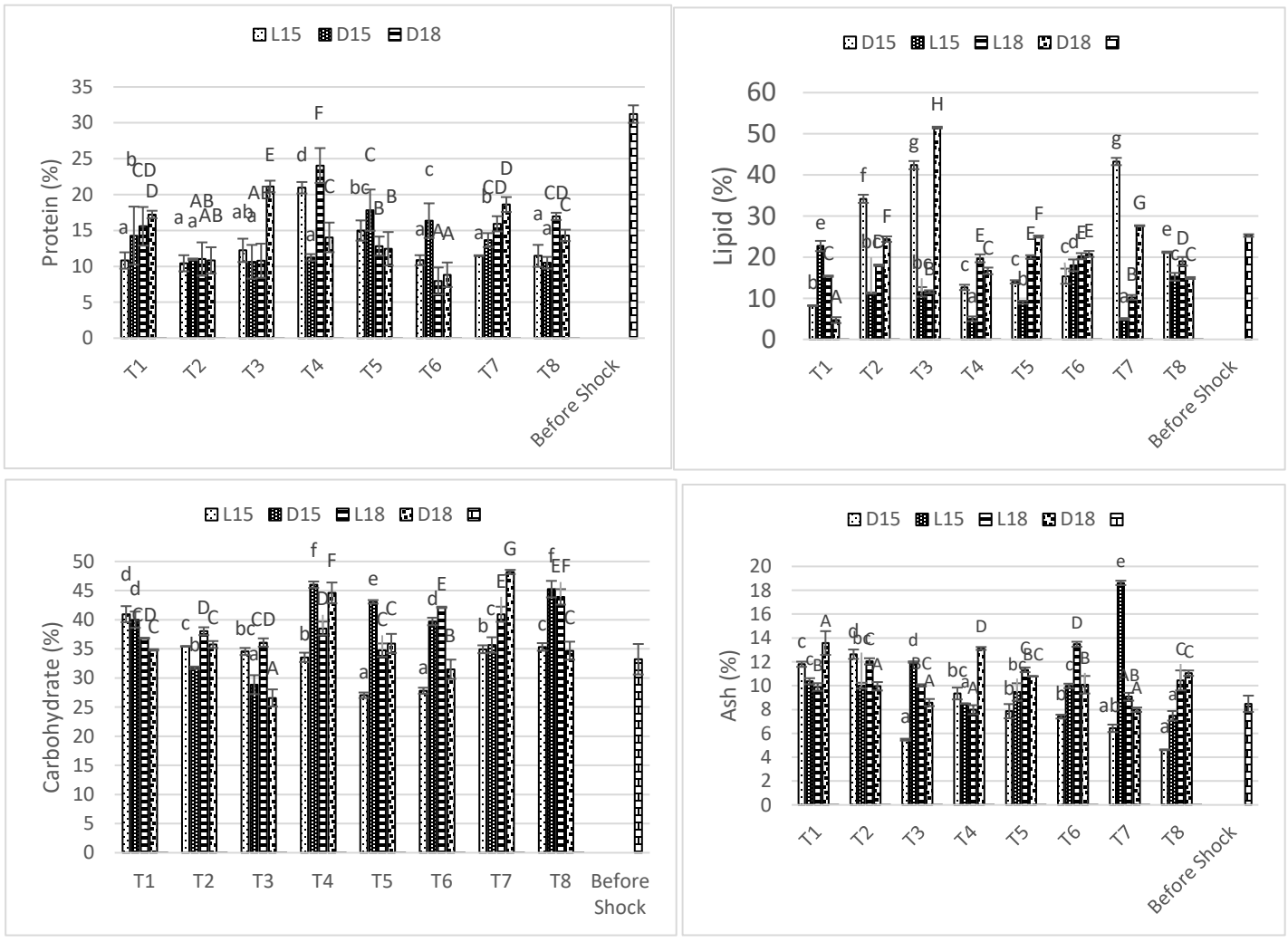

Figure 1. Mean (±SE) lipid, carbohydrate, ash and protein contents of *Nannochloropsis oculata* cultivated in f/2 medium on day 15 and 18 under different shocks (P<0.05). a, b, c and … indicate the significance differences on day 15 (dark and light) and A, B, C and … indicate the significance differences on day 18 (dark and light).

level significantly (P<0.05) (Fig. 2). The results on day 18 showed pH + starvation + salinity shock at the end of dark period made chlorophyll a and b and total carotene to increased significantly (P<0.05) (Fig. 2). Based on the results, EPA in pH, DHA, and omega-3 in pH + salinity, and MUFA under starvation + salinity treatments at the end of the dark period on day 15 demonstrated significant

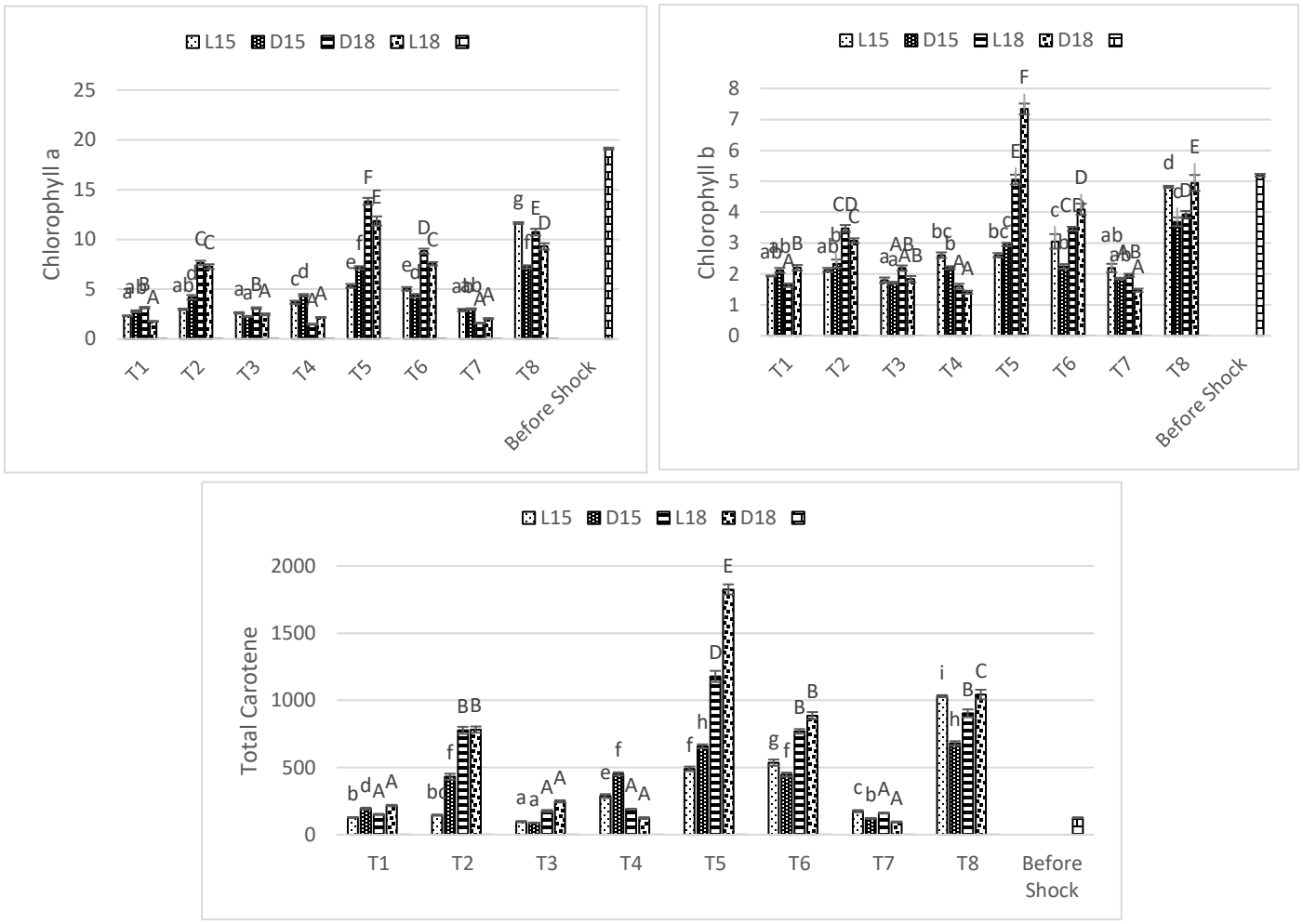

Figure 2. Mean (±SE) Chlorophyll a, b and total carotenoids contents of *Nannochloropsis oculata* cultivated in f/2 medium on day 15 and 18 under different shocks (P<0.05). a, b, c and … indicate the significance differences on day 15 (dark and light) and A,B,C and … indicate the significance differences on day 18 (dark and light).

differences compared to other treatments (P<0.05). Furthermore, Linoleic acid (or omega-6) in T2 (pH + salinity + starvation) at the end of the dark period showed a significantly higher value compared to other treatments (P<0.05). At the end of the dark period, in T1, SFAs were increased significantly (P<0.05). Moreover, the PUFAs values under T8 and at the end of the dark period on day 15 increased significantly (P<0.05). Linolenic acid in pH + salinity at the end of the light period on day 15 showed a significant difference between all groups (P<0.05) (Table 2).

According to the result obtained omega-6 and Linoleic acid in T4 (starvation), EPA in T4, and SFAs in T1 at the end of the dark period revealed significantly higher values than other treatments (P<0.05). The DHA in T2, PUFAs in T7, and linolenic acid in T3 at the end of the light period on day 18 showed significantly higher amounts (P<0.05). The MUFAs in T8 at the end of the light period exhibited significantly higher than other treatments (P<0.05). Moreover, the total omega-3 at the end of the dark period on day 18, in T8 was significantly higher than other treatments (P<0.05) (Table 3).

Discussion

Microalgae can accumulate considerable quantities of carbohydrates, proteins, and/or lipids (Ho et al., 2012, 2013). Changes in microalgae biochemical composition are likely to occur as a result of variations in pH (Khalil et al., 2010), temperature (Roleda et al., 2013), light, salinity (Ruangsomboon et al., 2013), and metal contents (Sun et al., 2014).

Table 2. Mean (±SE) some fatty acid compositions of *Nannochloropsis oculata* cultivated in f/2 medium on day 15 under different shocks. The data shown represent the mean values of three repetitions.

Sampling Time	Conditions (Shock)	ARA	DHA	EPA	Linoleic acid	Linolenic acid	SFA	MUFA	PUFA	Sum of ω3	Sum of ω6
End of dark (Day 15)	T1 (Control)	ND	0.22±0.03^{a}	7.08±0.35a	ND	25.85±1.70gh	34.69±0.65f	13.49±3.69a	33.15±1.75a	33.15±1.75a	ND
	T2 (pH+Salinity+Starvation)	ND	ND	33.47±1.48h	6.55±1.41	4.13±0.26a	22.66±0.13cde	16.79±1.88ab	44.14±3.16bc	37.60±1.74ab	6.55±1.41
	T3 (Salinity)	ND	ND	31.44±0.68gh	ND	7.13±0.82b	19.82±0.32bcd	34.92±3.43fg	38.57±1.50ab	38.57±1.50ab	ND
	T4 (Starvation)	ND	ND	12.26±1.78abc	ND	24.89±1.44fgh	23.78±1.36cdef	25.68±2.75cde	37.15±0.33ab	37.15±0.33ab	ND
	T5 (pH+Starvation)	ND	ND	24.29±1.08efg	ND	13.25±0.53c	24.01±0.39cdef	31.80±0.32efg	37.54±1.62ab	37.54±1.62ab	ND
	T6 (Starvation+Salinity)	ND	ND	20.83±1.02def	ND	16.49±1.32cd	17.72±0.55abc	38.82±1.02g	37.32±0.30ab	37.32±0.30ab	ND
	T7 (PH+Salinity)	ND	4.75±0.23b	9.74±3.56ab	ND	19.92±1.76def	25.50±1.12def	30.81±0.09def	34.42±1.56ab	34.42±1.56ab	ND
	T 8(pH)	ND	ND	47.20±0.50i	ND	15.89±0.84cd	14.89±1.63ab	20.94±0.94abc	63.09±0.33e	63.09±0.33e	ND
End of light (Day 15)	T1 (Control)	ND	ND	16.29±0.57bcd	ND	18.49±0.50cde	27.52±3.08g	22.64±0.22bcd	34.78±0.07ab	34.78±0.07ab	ND
	T2 (pH+Salinity+Starvation)	ND	ND	43.47±4.21i	ND	5.37±1.48ab	21.19±0.30cde	22.79±2.73bcd	48.83±2.73cd	48.83±2.73cd	ND
	T3 (Salinity)	ND	ND	55.34±3.09k	ND	ND	20.56±3.68bcd	17.15±2.39ab	55.34±3.09de	55.34±3.09de	ND
	T4 (Starvation)	ND	ND	15.48±1.51bcd	ND	20.82±2.03defg	30.44±2.97ef	32.18±3.14efg	36.29±3.54ab	36.29±3.54ab	ND
	T5 (pH+Starvation)	ND	ND	27.44±2.12fgh	ND	16.09±3.18cd	23.01±0.77cde	26.11±1.22cde	43.52±5.30bc	43.52±5.30bc	ND
	T6 (Starvation+Salinity)	ND	ND	15.00±0.24bcd	ND	23.36±0.14efg	22.85±0.85cde	25.57±0.44cde	38.37±0.39ab	38.37±0.39ab	ND
	T7 (pH+Salinity)	ND	ND	21.69±1.78def	ND	28.28±2.32h	19.27±1.58bcd	29.00±2.38cdef	49.97±4.11cd	49.97±4.11cd	ND
	T 8(pH)	ND	ND	17.48±0.10cde	ND	19.17±0.39def	13.01±2.24a	30.02±0.54def	36.65±0.28ab	36.65±0.28ab	ND

Table 3. Mean (±SE) some fatty acid compositions of *Nannochloropsis oculata* cultivated in f/2 medium on day 18 under different shocks. The data shown represent the mean values of three repetitions.

Sampling Time	Conditions (Shock)	ARA	DHA	EPA	Linoleic acid	Linolenic acid	SFA	MUFA	PUFA	Sum of ω3	Sum of ω6
End of dark (Day 18)	T1 (Control)	ND	5.74±0.72^{b}	17.15±2.32ab	ND	6.19±1.12cd	37.44±4.02de	30.97±0.47b	22.58±1.66bcd	30.97±0.47b	ND
	T2 (pH+Salinity+Starvation)	ND	ND	17.88±5.05ab	ND	15.09±3.54gh	10.82±2.46a	32.97±1.50b	27.76±3.19cde	32.97±1.50bc	ND
	T3 (Salinity)	ND	ND	30.81±0.38de	ND	4.12±0.65bc	43.49±2.33e	34.94±0.27bc	15.14±0.85a	34.94±0.27bcd	ND
	T4 (Starvation)	ND	ND	44.36±1.18f	4.73±0.92c	ND	12.44±0.35a	49.08±2.10de	27.86±0.72cde	44.36±1.18ef	4.73±0.93^{c}
	T5 (pH+Starvation)	ND	ND	39.37±0.89ef	ND	9.22±0.25de	12.42±1.08a	48.59±0.63d	27.43±0.49cde	48.59±0.63f	ND
	T6 (Starvation+Salinity)	ND	ND	28.31±1.42cd	ND	7.63±0.68cd	23.48±1.75bc	39.04±2.65bc	21.05±3.53bcd	39.04±2.65cde	ND
	T7 (pH+Salinity)	ND	ND	18.96±0.43abc	ND	12.46±0.80fg	20.58±1.29b	31.42±0.36b	44.40±2.02f	31.42±0.36bc	ND
	T 8(pH)	ND	ND	63.46±1.24h	ND	1.64±0.73ab	11.57±0.09a	65.09±0.51f	17.95±0.08ab	65.09±0.51h	ND
End of light (Day 18)	T1 (Control)	ND	3.82±0.98^{a}	15.70±1.61a	ND	2.82±0.08ab	52.07±0.14f	22.34±2.51a	14.88±1.21a	22.34±2.51a	ND
	T2 (pH+Salinity+Starvation)	ND	7.63±0.58^{c}	34.09±2.77de	ND	2.52±0.49ab	36.19±1.09d	42.35±2.68cd	15.03±0.28a	42.35±2.68def	ND
	T3 (Salinity)	ND	ND	17.66±1.40gh	ND	17.64±1.49h	26.60±0.21bc	35.31±0.09bc	27.80±0.07cde	35.31±0.09^{bcd}	ND
	T4 (Starvation)	ND	ND	54.32±5.48g	ND	2.79±0.01ab	20.78±1.96b	57.10±5.48ef	21.25±3.58bcd	57.10±5.48^{g}	ND
	T5 (pH+Starvation)	ND	ND	19.05±2.62abc	ND	13.52±1.32fg	27.10±0.50bc	32.56±1.29b	28.47±3.04de	32.56±1.29^{bc}	ND
	T6 (Starvation+Salinity)	ND	ND	48.23±0.04fg	2.66±0.16b	8.77±0.37de	8.92±1.20a	59.72±0.48f	29.00±0.95de	57.05±0.32^{g}	2.66±0.16^{b}
	T7 (pH+Salinity)	ND	ND	25.22±0.60^{bcd}	ND	ND	28.11±0.59c	32.17±0.46b	35.74±6.58ef	32.17±0.46^{bc}	ND
	T 8(pH)	ND	ND	30.74±1.70de	1.09±0.04a	1.60±0.54a	40.13±1.52de	33.44±2.29b	18.78±1.86bc	32.34±2.25^{bc}	1.09±0.04^{a}

Regarding the effects of starvation, studies demonstrated that N and/or P limitation in growth media causes metabolism alterations that induce lipid accumulation (Liang et al., 2013; Li et al., 2014). Despite several studies made in an attempt to understand the potential effects of N and/or P limitations on microalgae biochemical composition changes, the phenomena seem to be species-dependent (Shifrin and Chisholm, 1981).

Nitrogen and phosphorus starvation shifts the lipid metabolism from membrane lipid synthesis to neutral lipid storage. This, in turn, increases the total lipid content of green algae (Hu, 2004). Major effects of nitrogen deficiency in algal culture include the enhanced biosynthesis and accumulation of lipids (Thompson, 1996, Shifrin and Chisholm, 1981; Converti et al., 2009; Demirbas, 2010) and triglycerides (Takagi et al., 1999; Stephenson et al., 2010) with a concomitant reduction in protein content (Fogg, 1956; Morris et al., 1974; Kilham et al., 1997; Heraud et al., 2005), resulting in a higher lipid/protein ratio (Converti et al., 2009). In this study, a high alkaline pH + starvation + high salinity increased carbohydrate and decreased lipid and protein contents of algae, however high salinity in combination with any other shock caused higher lipid production, especially on day 18. This could be explained that under salinity conditions the cell would release nitrogen from the photosynthetic pigments and utilize the same for the metabolic processes. Higher salinity increases the algae lipid content (Fabregas et al., 1984; Zhila et al., 2011). *Dunaliella*, a marine alga, exhibited an increase in saturated and monounsaturated fatty acids with an increase in NaCl concentration from 0.4 to 4 M (Xu and Beardall, 1997). In another study with *Dunaliella tertiolecta*, an increase in intracellular lipids (60 to 67%) and triglyceride concentration (40 to 56%) with an increase in NaCl concentration from 0.5 (freshwater concentration) to 1.0 M was observed (Takagi and Karseno, 2006).

In this work, when the *N. oculate* biomass was cultured under normal conditions and transferred to high salinity, they showed different levels of lipid accumulation in the cell. Among all conditions and sampling time, the most favorable treatment to achieve high lipid content per cell was at the end of the dark period on day 18. *Nannochloropsis oculata*, showed a profound increase in lipid content under salinity shock at the end of the dark period on day 18 (51.51%). The lipid accumulation is enhanced by a combination of salinity shock. This inference is achieved on the bases of the results obtained from treatment 3 (end of light, day 15, and end of dark, day 18), where a significant increase in the cellular lipid content of *N. oculate* was observed under salinity shock individually. In a study conducted by Ben-Amotz, the lipid content of *Botryococcus braunii* cultured in 0.50 M NaCl was higher compared to media without the addition of NaCl, but protein, carbohydrates, and pigments levels were lower (Ben-Amotz et al., 1985). Another study with the same alga reported a decrease in protein content with unchanged carbohydrate and lipid content with an increase in salinity (Vazquez-Duhalt et al., 1991). Research on *Tetraselmis suecica* also reported a reduction in protein content per cell of up to 20% with an increase in salinity (Fabregas et al., 1984), but protein in all shocks decreased to less than 25%.

Variations in salinity also influence several biochemical and physiological mechanisms such as lipid production and growth which are essential in marine organisms (Fava and Martnini, 1988). In the current study, the treatments of 2 (salinity + pH + starvation), 7 (salinity + starvation), and 8 (pH) on day 15 at the end of the light period showed the highest lipid level, while the other treatments revealed different action, that it would be due to different physiological mechanisms in a specific algal. Lipids in general (as a percentage of particulate organic matter or as absolute amounts) and particularly fatty acids have been targeted as important variables determining the food quality of algae (Ahlgren et al., 1990; Coutteau and Sorgeloos, 1997; Weers and Gulati, 1997a).

The characteristic types of fatty acids produced by diatoms, green algae and/or cryptomonads have been studied (Piorreck et al., 1984; Cranwell et al.,

1989; Ahlgren et al., 1990). The general pattern observed is that green algae rarely produce fatty acids in excess of eighteen carbons, while diatoms and cryptophytes make many long-chained (18C) polyunsaturated fatty acids (PUFAs). Ahlgren et al. (1990) showed that the long-chained PUFAs produced by cryptophytes enhance reproduction in zooplankton, including *Daphnia*. Many studies of algal biochemistry have been undertaken in the past on cells that were either growing at the maximal rate (μmax) or at the stationary phase. Growth rates of algae measured in lakes are frequently found to be at some intermediate growth rate (Lehman and Sandgren, 1985; Sommer, 1989), rather than at stationary phase or μmax. Algae grown in continuous or semi-continuous culture have reduced variation in physiological conditions, can be easily replicated, and are more representative of the in-situ condition. Immediate effects of phosphorus limitation include a reduction in the synthesis and regeneration of substrates in the Calvin-Benson cycle and a consequential reduction in the rate of light utilization required for carbon fixation (Reitan Barsanti and Gualtieri, 2005). Phosphate limitation also reduces the synthesis of n-3 PUFA (Reitan Barsanti and Gualtieri, 2005). Salinity and pH (T6) shocks together on day 15 and combination of pH and salinity and starvation (T2) shocks on day 18 induced DHA production in *N. oculata* at the end of dark period, However, pH shock alone did influence the cell EPA level to more than 47% of FAME on day 15 and 63% of FAME on day 18 at the end of the dark period.

Conclusion

In conclusion based on the results for high lipid production (more than 51% of whole-body weight), it is recommended to keep the *N. oculate* in salinity shock for 6 days (day 18) and harvest at the end of dark period. Our data indicate that high DHA (7.63% of FAME) is obtained at the end of the dark period on day 18 and EPA level (up to 63% of FAME) is improved under pH shock at the end of light period on day 18 (incubate under shock condition for 6 days). While to obtain high level of total carotene (more than 1825 μg/g FW), it is recommended to apply pH + starvation + salinity shock and harvest the algae at the end of the dark period on day 18 (6 days shock). Finally, in this research, the highest value of carbohydrates was produced under pH + salinity + starvation shock at the end of the dark period on day 18 (6 days shock).

References

Ahlgren G., Lundstedt L., Brett M., Forsberg C. (1990). Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. Journal of Plankton Research, 12(4): 809-818.

AOAC. (Association of Official Analytical Chemists). (2005). Official methods of analysis of official analytical chemists international (16th ed.), Arlington, VA, USA: Association of Official Analytical Chemists.

Bartley M.L., Boeing W.J., Corcoran A.A., Holguin F.O., Schaub T. (2013). Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. Biomass and Bioenergy, 54: 83-88 .

Ben-Amotz A., Tornabene T.G., Thomas W.H. (1985). Chemical profile of selected species of microalgae with emphasis on lipids. Journal of Phycology, 21(1): 72-81 .

Bigogno C., Khozin-Goldberg I., Boussiba S., Vonshak A., Cohen Z. (2002). Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid. Phytochemistry, 60(5): 497-503.

Converti A., Casazza A.A., Ortiz E.Y., Perego P., del Borghi M. (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chemical Engineering and Processing, 48(6): 1146-1151.

Coutteau P., Sorgeloos P. (1997). Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. Freshwater Biology, 38(3): 501-512.

Cranwell P.A., Creighton M.E., Jaworski G.H.M. (1989). Lipids of four species of freshwater chrysophytes. Phytochemistry, 27(4): 1053-1059.

Demirbas A. (2010). Use of algae as biofuel sources.

Energy Conversion and Management 51(2): 2738-2749 .

Dere S., Gunes T., Sivaci R. (1998). Spectrophotometric determination of chlorophyll - a, b and total carotenoid contents of some algae species using different solvents. Turkish Journal of Botany, 22(1): 13-17.

Doan T.T.Y., Obbard J.P. (2011). Enhanced lipid production in *Nannochloropsis* sp. using fluorescence-activated cell sorting. GCB Bioenerergy, 3(3): 264-270 .

Doan T.T.Y., Obbard J.P. (2012). Enhanced intracellular lipid in *Nannochloropsis* sp. via random mutagenesis and flow cytometric cell sorting. Algal Research, 1(1): 17-21.

Fabregas J., Abalde J., Herrero C., Cabezas B., Veiga M. (1984). Growth of the marine microalga *Tetraselmis suecica* in batch cultures with different salinities and nutrient concentrations. Aquaculture, 42(3-4): 207-215.

Fava G., Martnini E. (1988). Effect of inbreeding and salinity on quantitative characters and asymmetry of *Tisbe holothuriae* (Humes). Hydrobiologia, 167: 463-467.

Fogg G. (1956). Photosynthesis and formation of fats in a diatom. Annals of Botany 20(2): 265-285.

Griffiths M.J., Harrison S.L. (2009). Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology, 21: 493-507.

Guiheneuf F., Stengel D.B. (2013). LC-PUFA-Enriched Oil Production by Microalgae: Accumulation of Lipid and Triacylglycerols Containing n-3 LC-PUFA is Triggered by Nitrogen Limitation and Inorganic Carbon Availability in the Marine *Haptophyte Pavlova lutheri*. Marine Drugs, 11(11): 4246-4266.

Guillard R.R.L. (1973). Division rates. In: J.R. Stein (Ed.), Handbook of Physiological Methods: Culture Methods and Growth Measurements. Cambridge University Press Cambridge, pp: 289-312.

Hedge J.E., Hofreiter B.T. (1962). Carbohydrate Chemistry, 17. In: R.L. Whistler, J.N. Be Miller (Eds.). Methods in carbohydrate chemistry. Academic Press, New York. pp: 17-22.

Heraud P., Wood B.R., Tobin M.J., Beardall J., McNaughton D. (2005). Mapping of nutrient-induced biochemical changes in living algal cells using synchrotron infrared microspectroscopy. FEMS Microbiology Letter, 249(2): 219-225.

Ho S.H., Chen C.Y., Chang J.S. (2012). Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresource Technology, 113: 244-252.

Ho S.H., Huang S.W., Chen C.Y., Hasunuma T., Kondo A., Chang J.S. (2013). Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E. Bioresource Technology, 135: 157-165.

Hu Q. (2004). Environmental Effects on Cell Composition. In Handbook of Microalgal Culture: Biotechnology and Applied Phycology; Richmond, A., Ed.; Blackwell: Oxford, UK, pp: 83-93.

Hu Q., Sommerfeld M., Jarvis E., Ghirardi M., Posewitz M., Seibert M., Darzins A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant Journal, 54(4): 621–639.

Khalil Z.I., Asker M.M.S., El-Sayed S., Kobbia I.A. (2010). Effect of pH on growth and biochemical responses of *Dunaliella bardawil* and *Chlorella ellipsoidea*. World Journal of Microbiology and Biotechnology, 26: 1225-1231.

Kilham S., Kreeger D., Goulden C., Lynn S. (1997). Effects of nutrient limitation on biochemical constituents of Ankistrodesmus falcatus. Freshwater Biology, 38(3): 591-596.

Kilian O., Benemann C.S., Niyogi K.K., Vick B. (2011). High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. Proceedings of the National Academy of Sciences of the United States of America, 108(52): 21265-21269.

Lehman J.T., Sandgren C.D. (1985). Species specific rates of growth and grazing loss among freshwater algae. Limnology and Oceanography, 30(1): 34-46.

Li J., Han D., Wang D., Ning K., Jia J., Wei L. (2014). Choreography of Transcriptomes and Lipidomes of *Nan-nochloropsis* Reveals the Mechanisms of Oil Synthesis in Microalgae. Plant Cell, 26(4): 1645-1665.

Li Y., Han F., Xu H., Mu J., Chen D., Feng B., Zeng H. (2014). Potential lipid accumulation and growth characteristic of the green alga *Chlorella* with combination cultivation mode of nitrogen (N) and phosphorus (P). Bioresource Technology, 174: 24-32.

Liang K., Zhang Q., Gu M., Cong W. (2013). Effect of phosphorus on lipid accumulation in freshwater microalga *Chlorella* sp. Journal of Applied

Phycology, 25: 311-318.

Miquel M., Browse J. (1992). Arabidopsis mutants deficient in polyunsaturated fatty acid synthesis: Biochemical and genetic characterization of a plant oleoyl-phosphatidylcholine desaturase. Journal of Biology Chemistry, 267(3): 1502-1509.

Moheimani N.R. (2013). Inorganic carbon and pH effect on growth and lipid productivity of *Tetraselmis suecica* and *Chlorella* sp. (Chlorophyta) grown outdoor in bag photobioreactors. Journal of Applied Phycology, 25: 387-398.

Morris I., Glover H., Yentsch C. (1974). Products of photosynthesis by marine phytoplankton: The effect of environmental factors on the relative rates of protein synthesis. Marine Biology, 27: 1-9.

Mu¨hlroth A., Li K., Røkke G., Winge P., Olsen Y., Hohmann-Marriott M.F. (2013). Pathways of Lipid Metabolism in Marine Algae, Co-Expression Network, Bottlenecks and Candidate Genes for Enhanced Production of EPA and DHA in Species of *Chromista.* Marine Drugs, 11(11): 4662-4697.

Pal D., Khozin-Goldberg I., Cohen Z., Boussiba S. (2011). The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. Applied Microbiology and Biotechnology, 1-13 .

Piorreck C.C., Baasch K.H., Pohl P. (1984). Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry, 23(2): 207-216.

Radakovits R., Jinkerson R.E., Fuerstenberg S.I., Tae H., Settlage R.E., Boore J.L. (2012). Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. Nature Communication, 3(1): 686.

Reitan Barsanti L., Gualtieri P. (2005). Algae: Anatomy, Biochemistry, and Biotechnology, 1st ed.; CRC Press: Boca Raton, FL, USA. 344 p.

Renaud S., Parry D., Thinh L.V., Kuo C., Padovan A. (1991). Sammy, N. Effect of light intensity on the proximate biochemical and fatty acid composition of *Isochrysis* sp. and *Nannochloropsis oculata* for use in tropical aquaculture. Journal of Applied Phycology, 3: 43-53.

Richmond A., Becker E.W. (1986). Technological aspects of mass cultivation—a general outline. In: A. Richmond (ed.). CRC handbook of microalgal mass culture. CRC, Boca Raton. pp: 245–264.

Rodolfi L., Chini Zittelli G., Bassi N., Padovani G., Biondi N., Bonini G., Tredici M.R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology Bioenergy, 102(1): 100-112.

Roleda M.Y., Slocombe S.P., Leakey R.J.G., Day J.G., Bell E.M., Stanley M.S. (2013). Effects of temperature and nutrient regimes on biomass and lipid production by six oleaginous microalgae in batch culture employing a two-phase cultivation strategy. Bioresource Technology, 129: 439-449.

Ruangsomboon S., Ganmanee M., Choochote S. (2013). Effects of different nitrogen, phosphorus, and iron concentrations and salinity on lipid production in newly isolated strain of the tropical green microalga, *Scenedesmus dimorphus* KMITL. Journal of Applied Phycology, 25: 867-874.

Sharma K., Schenk P.M. (2015). Rapid induction of omega-3 fatty acids (EPA) in *Nannochloropsis* sp. by UV-C radiation. Biotechnology and Bioengineering, 112(6): 1243-1249.

Shifrin N.S., Chisholm S.W. (1981). Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate and light-dark cycles. Journal of Phycology, 17: 374-384.

Slocombe S.P., Ross M., Thomas N., McNeill S., Stanley M.S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. Bioresource Technology, 129: 51-57.

Sommer U. (1989). Nutrient status and nutrient competition in a shallow, hypertrophic lake. Limnology and Oceanography, 34(7): 1162-1173.

Stephenson A.L., Dennis J.S., Howe C.J., Scott S.A., Smith A.G. (2010). Influence of nitrogen-limitation regime on the production by *Chlorella vulgaris* of lipids for biodiesel feedstocks. Biofuels, 1(1): 47-58 .

Sukenik A. (1991). Ecophysiological considerations in the optimization of eicosapentaenoic acid production by *Nannochloropsis* sp. (Eustigmatophyceae). Bioresource Technology, 35(3): 263-269 .

Sukenik A., Zmora O., Carmeli Y. (1993). Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II. *Nannochloropsis* sp. Aquaculture, 117(3-4): 313-326 .

Sun X., Cao Y., Xu H., Liu Y., Sun J., Qiao D., Cao Y. (2014). Effect of nitrogen-starvation, light intensity and iron on triacylglyceride/carbohydrate production

and fatty acid profile of *Neochloris oleoabundans* HK-129 by a two-stage process. Bioresource Technology, 155: 204-212.

Takagi M., Karseno Y.T. (2006). Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunalliela* cells. Bioscience Bioenergy, 101(3): 223-226.

Takagi M., Watanabe K., Yamaberi K., Yoshida T. (2000). Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of *Nannochloris* sp. UTEX LB1999. Applied Microbiology and Biotechnology, 54: 112-117.

Thompson G.A. Jr. (1996). Lipids and membrane function in green algae. Biochimica et Biophysica Acta, 1302(1): 17-45.

Vazquez-Duhalt R., Arredondo-Vega B.O. (1991). Haloadaptation of the green alga *Botryococcus braunii* (race A). Phytochemistry, 30(9): 2919-2925.

Weers P.M.M., Gulati R.M. (1997a). Effect of addition of polyunsaturated fatty acids to the diet on growth and fecundity of *Daphnia galeata*. Freshwater Biology, 38(3): 721-729.

Xu X.Q., Beardall J. (1997). Effect of salinity on fatty acid composition of a green microalga from an antarctic hypersaline lake. Phytochemistry, 45(4): 655-658 .

Zhila N.O., Kalacheva G.S., Volova T.G. (2011). Effect of salinity on the biochemical composition of the alga *Botryococcus braunii* Kütz IPPAS H-252. Journal of Applied Phycology, 23: 47-52.