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Characterization of Omega-3 and Omega-6 Fatty Acid Accumulation in *Chlorococcum novae-angliae* Microalgae Grown under Various Culture Conditions

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ABSTRACT

Chlorococcum novae-angliae is a terrestrial green microalgae species with remarkable potential to synthesize omega-3 (ω-3) and omega-6 (ω-6) fatty acids. In this study, *Chlorococcum novae-angliae* has been subjected to varying growth conditions (light, nitrogen, salinity, and temperature) to investigate the accumulation of ω-3 and ω-6 fatty acids. Among tested growth conditions, eicosapentaenoic acid, α-linoleic acid, γ-linoleic acid, and arachidonic acid were enhanced by nitrogen limitation. Significant increases were observed in concentration of linoleic acid, an essential precursor molecule for the production ω-6 fatty acids under decreased nitrogen concentrations. Despite the lowest biomass growth, monounsaturated fatty acids and docosahexaenoic acid were increased by 14.4% and 8.7% under low light intensities, respectively. Meanwhile, the highest concentrations of palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (18:1cis-9) were also detected under nitrogen limitation. Total accumulation of ω-3 fatty acids was highest in the control group, followed by nitrogen limitation, whereas total ω-6 fatty acid accumulation was highest under nitrogen limitation followed by the control group. Total lowest fatty acid concentrations were obtained under increased salinity while low temperature conditions heavily inhibited cellular growth.

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Introduction

Fatty acids are essential components of cellular membranes composed of long aliphatic carbon chains with carboxylic acid and methyl group at both ends [1, 2]. Long chain polyunsaturated fatty acids (LC-PUFAs), in particular, play considerable roles in important metabolic processes as storage and transport of energy, regulation of gene functions, generation of eicosanoids, lipid peroxidation, acylation of proteins, cell signaling, and inflammatory processes [3, 4]. Many LC-PUFAs also play prophylactic roles against various diseases such as coronary artery, diabetes, cardiac arrhythmias, atherogenesis, and hyperlipidemia [5]. Specifically, ω-3 and ω-6 fatty acids are required for cognitive functions

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and neural network as they maintain cell signaling [6]. A recent study reported that regular intake of ω-3 and ω-6 fatty acids hinders developing major depression and Alzheimer's disease [7]. Among them, linoleic acid (LA) and α-linoleic acid (ALA) were recommended to be included in daily diets of humans as they cannot be synthesized *de novo* due to lack of Δ12 and Δ15 desaturase enzymes [8]. Linoleic acid and ALA are important precursors of ω-3 and ω-6 fatty acids respectively. Alpha-linoleic acid is a key structural component required for biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and produced in mammals in very limited quantities [2]. Alpha-linoleic acid and arachidonic acid (AA) are precursors for prostaglandins, critical immune response mediators such as inflammation, patent vasodilators regulating the body's defense mechanism after injuries and infections [9]. Both EPA and DHA are recommended as dietary supplements for brain and nervous system development, cardiovascular health, visual function, memory and learning and cognitive maintenance and infant development [6]. Impulses generated between the brains cells are significantly affected by DHA [10]. In addition, DHA is one of most substantial components which take place in retina membrane and play role in signaling mechanisms in the eye [11]. Recent studies indicate the presence of the EPA at certain doses in the entire human body promotes inflammation control and enhances the immune system [12]. Substances obtained from EPA also play vital roles in combating heart disease, and chronic and inflammatory processes [10]. EPA and DHA also play a strong role in the body's defense mechanism in reducing inflammation and eliminating reactive oxygen species (ROS) at the cellular level [13]. Meanwhile, LDL (High density lipoprotein) and VLDL (very low density lipoprotein) levels in human blood plasma are considerably related with ω-3 fatty acids that have significant impacts on TAG (triacylglycerol) production [4].

Stearidonic acid (SDA, C18:4n3) and docosapentaenoic acid (DPA, C22:5n3) are other notable ω-3 fatty acids which have been identified as important needs for human well-being in addition to EPA, DHA and ALA [14]. Arachidonic acid and γ-linoleic acid (GLA) are considered to be essential ω-6 fatty acids made from LA [1]. Gamma-linoleic acid is the initial product of a synthesis of ω-6 fatty acids which is further synthesized with DGLA after LA reaches the human body. This fatty acid is essential for the metabolism of ω-6 fatty acids where homo-γ-linolenic acid (DH-GLA) is converted to AA through the action of Δ5

desaturase enzyme [15]. Certain diseases like cancer and diabetes have been reported to have disruptive effects on the conversion of LA to GLA [16]. In fact, some studies suggest that daily intake of GLA may be a possible solution for the circulation of the metabolism of ω-6 fatty acids [17-19].

All these potential health benefits has created a growing market demand for ω-3 and ω-6 fatty acids leading to overfishing and decreased fish stocks [20]. While the demand for fish oil as a source of ω-3 and ω-6 fatty acids increased, some serious issues also emerged in recent years. Unwanted odors and burping of fish oil, bioaccumulation of heavy metals and organic pollutants into fish bodies, diminished fish stocks due to overfishing are some of the key issues. Meanwhile, fish oil cannot to be recommended as the sole source of EPA and DHA due to the possible accumulation of toxic chemicals, heavy metals (i.e. copper, mercury) and other synthetic pollutants (i.e. PCB and dioxin) present in water bodies due to industrial and agricultural discharges and other anthropogenic activities contributing to environmental pollution [21]. Such overarching issues related to fishing and aquaculturing suggest vegan sources of ω-3 and ω-6 fatty acids such as microalgae as sustainable alternative strategies to meet consumer needs in a much broader range [14]. As such, most biotechnology companies have started to prefer microalgae feedstock for large-scale production of EPA and DHA, as they are primary producers of ω-3 and ω-6 fatty acids [22, 23]. As a reliable source of bioactive compounds, microalgae have tremendous capability to produce substantial amounts of EPA and DHA and are regarded as important sources of LC-PUFAs.

Additional benefits of using microalgae feedstock include their faster growth rates and higher production of biomass relative to other omega-rich plants and crops [24]. Microalgal production systems do not compete arable land space for agricultural activities, and freshwater resources are not required if marine or brackish species are preferred [25]. Microalgae also offer the most promising results for conservation benefits as they can be grown with sustainable inputs such as solar light, atmospheric carbon dioxide and recycled nitrogen and phosphorous from pasteurized/pre-treated waste streams.

Microalgae also display exceptional versatility with highly adaptive capacities to complex environmental conditions [26]. However, in order to achieve economical, efficient and sustainable large-scale production for ω-3 and ω-6 fatty acids, microalgal production metrics

need to be optimized. Several cultivation parameters including nutrients (particularly nitrogen and phosphorus), light intensity, temperature, pH, and salinity have considerable impacts on biochemical characteristics of microalgae, and influence biomass growth and ω-3 and ω-6 fatty acid accumulation. Among these parameters, shifts in temperature influence cellular structural components such as membranes and organelles by changing composition of lipids and proteins. Also, temperature changes have strong impacts of enzymatic activities as a result of oscillations in reaction kinetics [27]. Light is another vital parameter for photosynthesis which significantly affects metabolic activities of microalgae [28]. High light intensity is a favorable condition for microalgal growth and production of pigments as it increases photosynthetic activity, however, it might not be favorable for all types of fatty acids. As another important parameter, salt stress could be applied as some studies show that high salt levels in the growth media promotes production of lipid-based pigments, specifically carotenoids. However, increased levels of salt in media reduces microalgal growth [29]. As a macronutrient, nitrogen plays an important role in microalgae cells since accessory pigments such as chlorophylls a and b, vital for photosynthetic efficiency and non-photochemical active pigments such as carotenoids contain nitrogen. In the absence of nitrogen, while protein rich compounds such as chlorophylls a and b are consumed to maintain cell growth and serve as nitrogen pools for vital proteins, lipids and carbohydrates are increased for long term energy storage as a response to nitrogen stress [30]. It should be noted that the effects of these parameters are mostly species/strain specific, and optimal conditions for one species/strain may not always be beneficial for others [3]. Therefore, it is important to understand the roles of these parameters play in different microalgae species' metabolic responses and overall accumulation of desired bioproducts. With respect to lipid and fatty acid metabolism, one of the common strategies is to expose microalgae to different growth conditions by varying temperature, light, salinity, and nutrient levels [27, 28, 31, 32]. This study examined the effects of four varying growth parameters (light, nitrogen, salinity, and temperature) on a terrestrial green microalgae *Chlorococcum novae-angliae* strain SAG 5.85 in an effort to characterize accumulation of ω-3 and ω-6 fatty acids. The rationale for choosing this strain was The Culture Collection of Algae at Georg-August-Universität (SAG, Göttingen, Germany), where the seed culture was obtained, has listed this strain as a potential

oleaginous species in a large scale screening study [33], yet, this is the first study comprehensively investigating fatty acid profile of terrestrial *C. novae-angliae* SAG 5.85 grown in liquid culture medium with varying light, nitrogen, salinity, and temperature. The results shed light on optimal conditions for this green microalgae species to achieve commercial scale ω-3 and ω-6 fatty acids production goals.

Materials and Methods

Microalgal strain selection and culture maintenance

Chlorococcum novae-angliae strain SAG 5.85 was originally isolated from soil samples of semi-desert ecosystem of Cahuilla Reservation (Arizona, USA) and deposited at SAG. Obtained seed cultures were transferred to modified Bold's 3N (MB3N) liquid culture medium [34]. Seed cultures were maintained in 500 mL gas wash bottles with a working volume of 350 mL until they reach mid-exponential growth phase (i.e. 4-5 days) prior to experimental conditions. Cultures were supplied with 0.22 μm filtered air at 0.5 L/min and illuminated with 16000±200 lux light intensity for 14:10 h (light:dark) cycles. Reactor pH levels were maintained at 7.5±0.5. As air diffusers have provided sufficient homogenization, no further mixing has been provided to reactors. Seed cultures reaching mid-exponential phase were harvested at 4500 rpm for five min at 4°C by centrifugation (U-320R, Boeco, Germany) and washed twice with phosphate buffered saline. Concentration of seed culture were determined using a hemocytometer (Neubauer, Isolab, Wertheim, Germany) and were inoculated into experimental reactors at a starting concentration of $3x10^6$ cells/mL.

Experimental set-up and culture growth conditions

All experimental conditions were conducted in a temperature controlled 6L flat panel photobioreactor (PBR) system (Subitec, Germany) (Fig. 1). Reactor pH values were maintained at 7.5-8.0 by pure filtered CO_2 intermittently pumped into the reactors via automated solenoid system. The reactor was supplied with 0.22 μm filtered air at 0.5 L/min. Temperature was kept at 25°C±1 for all experimental conditions except the temperature test condition (17°C±1).

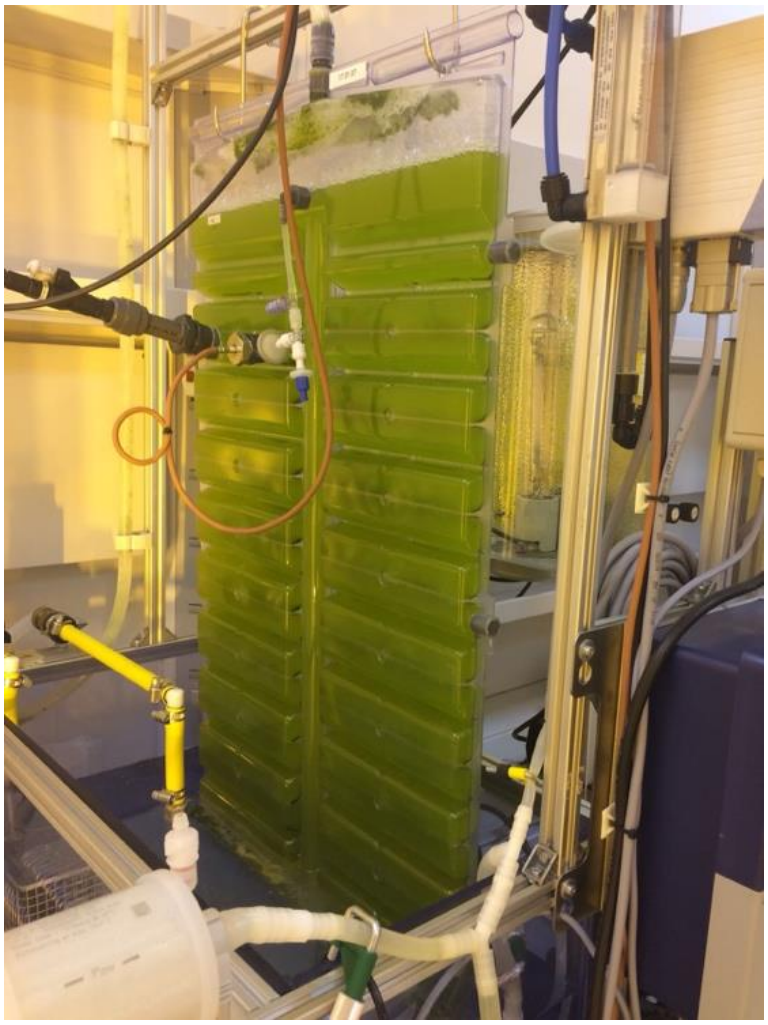

Fig 1 Six-liter capacity photobioreactor systems used for experimental cultures

For nitrogen test, concentration of $NaNO_3$ in MB3N medium were decreased to 1.47 mM (original recipe was 8.82 mM). Light intensity applied to PBR through a sodium lamp was kept at 11100 lux except light test condition where intensity was decreased to 5500 lux. For salinity test, NaCl concentration was increased 2X fold (0.86 mM compared to 0.43 mM in original recipe). All experimental growth conditions and the control group can be seen in Table 1.

Table 1 Culture growth parameters of *Chlorococcum novae-angliae* for each tested condition (**Bold** indicates changed parameter with respect to the control group)

Parameter	Control	Light	Nitrogen	Salinity	Temperature
Light intensity	11100 lux	**5500 lux**	11100 lux	11100 lux	11100 lux
Aeration rate	100 L/min	100 L/min	100 L/min	100 L/min	100 L/min
Temperature	25°C±1	25°C±1	25°C±1	25°C±1	**17°C±1**
$NaNO_3$ conc.	8.82 mM	8.82 mM	**1.47 mM**	8.82 mM	8.82 mM
NaCl conc.	0.43 mM	0.43 mM	0.43 mM	**0.86 mM**	0.43 mM

All experiments were completed in biological triplicates. Optical density measurements were taken at 680 nm wavelength using a spectrophotometer (DR3900, Hach Lange, Manchester, UK) and cell counts were determined every two days using a hemocytometer. Growth rates were compared in exponential phase based on the equation given below.

Growth rate = $\ln(X_1)-\ln(X_0)/N_1-N_2$

where

X_1: Cell number at the end of exponential phase

X_0: Cell number at the beginning of exponential phase

N_1: Number of days at the end of exponential phase

N_2: Number of days at the beginning of exponential phase.

Cultures were harvested by on Day 6 at early stationary phase by centrifugation, washed twice and the biomass pellets were lyophilized at -80°C (Hypercool, Gyrozen, Frankfurt, Germany) and stored at -80ºC ultra-low temperature freezers prior to lipid and fatty acid analyses. An aliquot from each reactor was processed for dry cell weight (DCW) measurements [35].

Lipid extraction

For total lipid extraction a modified protocol was adopted from Breuer et al. and Bligh and Dyer [35, 36]. One mL of extraction solution with 50 mM Tris and 1M NaCl was prepared

at pH 7 and added to 100 mg lyophilized algal biomass. For cell wall disruption and homogenization, acid-washed and autoclaved glass beads were added to extraction vials (for each extraction 0.3 g of 0.1 mm and 0.1 g of 0.5 mm diameter glass beads were used). As internal standard, a mixture of nonadecanoic acid (C19:0) and chloroform: methanol 4:5 (v/v) was prepared at 50 mg/L concentration and processed with each extraction. Samples were homogenized using a bead beater (Minilys, Bertin Technologies, France) for 60 seconds for 8 times with 60 seconds of cooling intervals on ice. Following the homogenization, samples were transferred into 50 mL glass centrifuge tubes with Teflon insert screw caps. A mixture of 2.5 mL de-ionized water (DIW) containing 50 mM Tris and 1 M NaCl was added into glass tubes, vortexed briefly and sonicated for 10 min (Sonorex Super RK 102 H, Bandelin, Germany). Next, tubes were centrifuged at 1200Xg for 5 min. Bottom phases were transferred into new clean glass tubes by glass Pasteur pipettes. Extraction process steps were repeated with chloroform addition to fully recover lipids. Lastly, chloroform was evaporated under N_2 gas stream and the weights of lipid extract tubes were measured. The initial record (tare tube weight) was removed from final measurement providing the amount of total lipids extracted.

Transesterification reactions

Three mL of methanol with 5% sulfuric acid (v/v) were added to dried lipid samples and tightly closed. The samples were incubated in a water bath (Maxturdy-30, Daihan Scientific, Wonju, South Korea) at 70℃ for 3 h and vortexed in every 30 minutes to avoid boiling. Once samples were cooled, 3 mL of DIW water and 3 mL of hexane were added, briefly vortexed and rotated at 15 rpm for 15 min (Wisemix RT-10, Witeg, Wertheim, Germany). Following, samples were centrifuged at 1200Xg for 5 min, and upper 2 mL of organic phase were collected and transferred into clean glass tubes. A washing step was done using 2 mL DIW added to each collected sample. Finally, the tubes were vortexed and centrifuged at 1200Xg for 5 min, transferred to GC vials and stored at -20ºC until analytical measurements.

Analytical measurements and fatty acid methyl ester (FAME) profiling

Identification and quantitation of FAMEs were conducted on a GC system (7820A, Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector (FID). Five µl of FAME samples were injected in splitless mode into HP-88 column (Agilent) with dimensions of 100

m length x 0.25 mm diameter x 0.2 μm film thickness. The temperature program was as follows: initial 140°C hold for 5 min; followed by 4°C/min ramp to 240°C with a 15 min hold. Column flow was set at 1 mL/min and nitrogen was used as carrier gas set at 280°C. Run time for each sample was appx. 45 min. Identification and quantification of individual FAMEs were done using a standard calibration mix (Supelco 37 FAME Mix, Sigma Aldrich, St. Louis, MO, USA) injected at 1 μl using same run method. By comparing peak area of reference standard with peak area of the target, concentrations of each FAME were calculated.

Statistical analysis

All experiments were conducted in biological triplicates and the calculated data values were reported as mean ± standard deviation. Statistical analyses were based on two-tailed paired t-test computed in MS Excel (version 16.42 Microsoft, Redmond, Washington, USA). Results were considered statistically significant only if $P \leq 0.05$.

Results

Culture growth metrics and total lipid content

Growth metrics of *C. novae-angliae* under each experimental condition were monitored and evaluated based on optical density, cell counts, growth rates and dry cell weight (DCW). As presented in Fig. 2, on harvest Day-6, all experimental conditions except salinity test resulted in lower cell counts than the control group, followed by nitrogen and light variations (Fig. 2A). Absorbance values measured under salinity test were slightly higher than the control group (Fig. 2B). Decreased temperature condition completely suspended cellular growth with no increase in optical density and cellular count (Fig. 2). Accordingly, lower temperature exposed cultures were not pursued for further characterization.

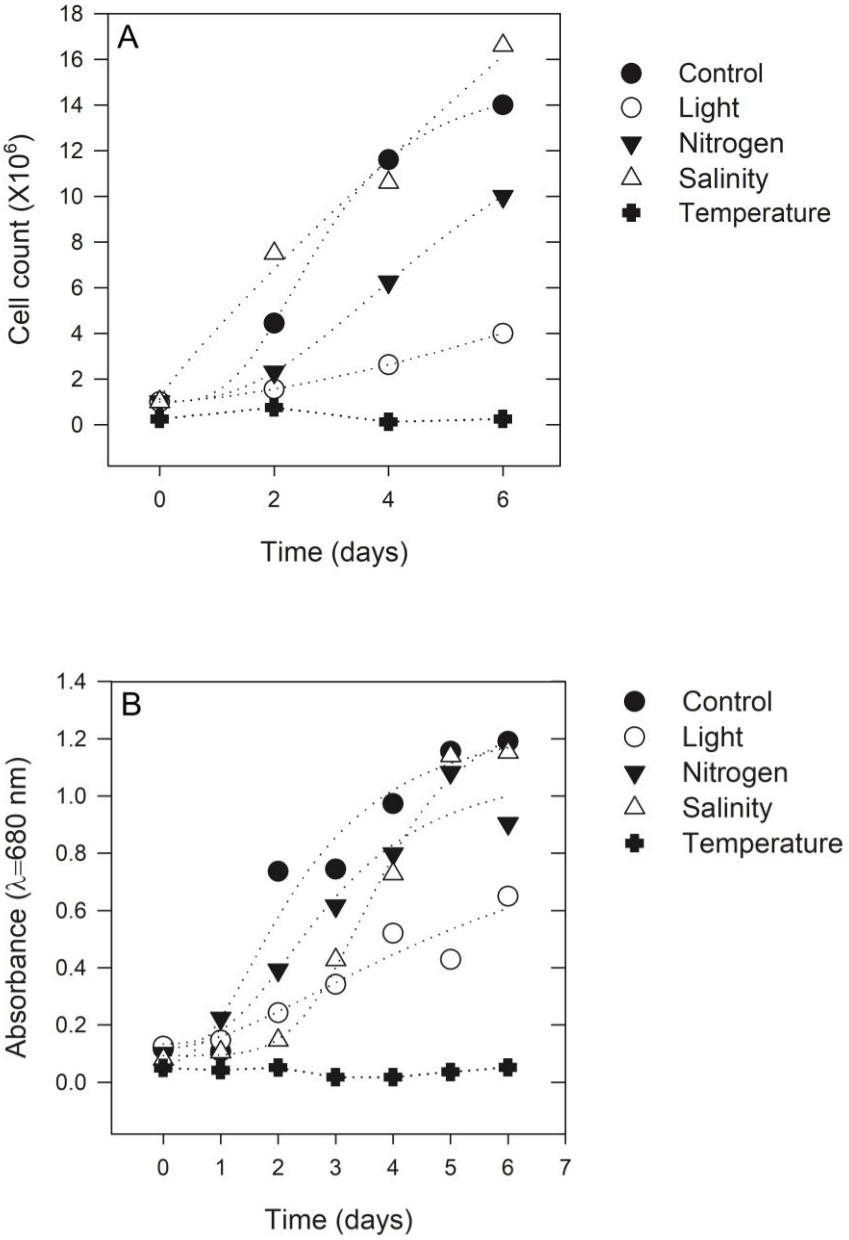

Fig 2 Cell count (A) and optical density (B) measurements of *Chlorococcum novae-angliae* for each tested condition

Growth rates were highest for increased salinity and calculated as 1.32 cells/day followed by the control group (0.61 cells/day), decreased nitrogen (0.45 cells/day), and light (0.23 cells/day) test conditions (Table 2). Average DCW measurements on harvest Day-6 indicated control group had the highest biomass concentration at 0.48 g/L, followed by nitrogen (0.37

g/L), salinity (0.28 g/L) and light test conditions (0.14 g/L) (Table 2). Despite marginally better performance in final cell counts (Fig. 2A), average total lipid content was higher in the control group (21.77%) compared to increased salinity (17.40%) followed by decreased light (16.33%) and nitrogen (13.93%) test conditions (Table 2). Changes in total lipid contents compared to control group were not statistically significant except decreased light condition.

Table 2 Culture growth metrics and total lipid accumulation of *Chlorococcum novae-angliae* under tested conditions

Test condition	Growth rate (cells/day)	Dry cell weight (DCW) (g/L)	Total lipid content (% DCW)
Control	0.61	0.48±0.34	21.77±3.48
Light	0.23	0.14±0.13	16.33±0.71*
Nitrogen	0.45	0.37±0.01	13.93±7.04
Salinity	1.32	0.28±0.06	17.40±2.90
Temperature	-	-	-

*Change is statistically significant ($P \leq 0.05$).

Saturated fatty acid profiles

Palmitic acid (C16:0) and stearic acid (C18:0) were the most dominant saturated fatty acids (SFAs) with highest concentrations among all experimental conditions including the control group (Table 3). The highest total saturated fatty acid (SFA) concentration was observed under decreased nitrogen (700.5 ng/µl) which was 71% higher than the control group where total SFA concentration was 497.81 ng/µl. Cultures exposed to low light intensity resulted in a total saturated fatty acid (SFA) concentration of 366.11 ng/µl, which was 26.4% lower than the control group. Cultures grown in increased salinity accumulated the lowest amount of SFAs at 148.23 ng/µl, 29.78% lower than the control group (Table 3). Changes in total SFA concentrations were statistically significant ($P \leq 0.05$) for all experimental conditions compared to the control group.

Table 3 Saturated fatty acid (SFA) profiles of *Chlorococcum novae-angliae* under tested conditions

	Fatty Acid Concentration (ng/µl)			
Fatty Acid	**Control**	**Light**	**Nitrogen**	**Salinity**
C6:0	1.62±2.8	2.20±3.12	0.47±0.82	1.62±1.23
C8:0	6.04±0.68	3.07±0.9	5.94±2.42	0.51±0.22
C10:0	0.64±0.55	0.33±0.3	0.2±0.02	0.75±0.22
C11:0	0.33±0.05	0.3±0.08	0.07±0.12	0.03±0.05
C12:0	0.74±0.31	0.45±0.18	1.28±0.23	0.17±0.07
C13:0	1.49±1.43	1.84±0.78	0.22±0.03	0.54±0.69
C14:0	38.83±3.46	27.03±9.37	46.45±6.58	7.92±4.64
C15:0	4.55±0.85	3.49±1.25	5.65±0.76	1.25±0.65
C16:0	344.1±20.57	270.45±81.12	492.46±61.26	87.65±45.92
C17:0	17.44±3.35	10.33±4.64	19.87±19.87	3.88±2.09
C18:0	47.28±3	28.87±10.01	78.45±8.11	21.06±10.54
C20:0	13.21±1.97	7.53±1.89	17.52±2.55	3.44±1.63
C21:0	2.72±0.3	1.16±1.11	1.84±1.05	0.75±0.95
C22:0	5.32±1.24	3.53±2.64	6.51±0.81	6.25±5.84
C23:0	9.24±8.88	4.38±3.93	10.88±15.68	3.39±2.02
C24:0	9.24±4.52	1.15±0.44	12.68±19.68	9.02±10.6
Sum of SFAs	**497.81±27.40**	**366.11±103.92**	**700.50±96.3**	**148.23±77.3**

Under decreased nitrogen test condition, palmitic acid and stearic acid levels, which are essential precursors SFAs for the biosynthesis of polyunsaturated fatty acid (PUFA) were increased by 69.88% and 60.26% compared to control group respectively, whereas decreased by 21.41% and 38.94% under decreased light and 74.53% and 55.46%, increased salinity test conditions compared to control group respectively (Table 3).

Monounsaturated fatty acid profiles

Similar to total SFA content, highest total average monounsaturated fatty acid (MUFA) concentration (747.79 ng/µl) were obtained under decreased nitrogen condition, 54% higher than the control group (343.92 ng/µl) (Table 4). Total average MUFA content was measured as 393.61 ng/µl under decreased light condition, slightly higher than the control group

(12.62%), whereas increased salinity condition resulted a major decrease in total MUFA content and measured as 31.46 ng/µl (or 98.86% lower compared to the control group) (Table 4). Changes in total MUFA contents compared to control group were not statistically significant except increased salinity test condition.

Table 4 Monounsaturated fatty acid (MUFA) profiles of *Chlorococcum novae-angliae* under tested conditions

	Fatty Acid Concentration (ng/µl)			
Fatty Acid	**Control**	**Light**	**Nitrogen**	**Salinity**
C14:1	28.53±6.68	18.29±13.39	10.42±2.12*	0.97±0.55*
C15:1	0.38±0.28	0.29±0.05	0.46±0.32	4.17±3.69
C16:1	11.04±1.32	8.32±2.81	27.2±5.24*	3.08±1.74*
C17:1	1.87±0.74	1.42±0.81	2.52±0.32*	0.73±0.34
C18:1n9t	32.73±55.57	11.71±19.28	0.67±0.25	12.46±13.38
C18:1n9c	32.70±5.19	26.60±7.2	136.17±18.89*	4.24±2.14*
C20:1	235.01±403.15	325.84±323.94	566.10±489.5	1.25±1.41*
C24:1	1.67±1.89	1.15±0.71	4.25±1.96	4.56±2.01
Sum of MUFAs	**343.92±377.88**	**393.61±327.42**	**747.79±512.61**	**31.46±13.48***

*Change is statistically significant ($P \leq 0.05$).

Notably, oleic acid (C18:1n9c), an essential ω-9 fatty acid, content was increased more than four fold under decreased nitrogen compared to the control (136,17 ng/µl). Decreased light conditions caused an accumulation of 26.6 ng/µl of oleic acid whereas increased salinity resulted in only 4.24 ng/µl of oleic acid (Table 4).

Polyunsaturated fatty acid profiles

Overall average PUFA accumulation was the highest in the control group (803.55 ng/µl) followed by a slight decrease under decreased nitrogen test condition (741.1 ng/µl) (Table 5). Decreased light condition resulted in average total PUFA concentration (522.59 ng/µl), while the lowest PUFA accumulation was observed in increased salinity conditions (263.88 ng/µl) (Table 5). Changes in total PUFA contents compared to control group were not statistically significant except increased salinity condition.

Table 5 Polyunsaturated fatty acid (PUFA) profiles of *Chlorococcum novae-angliae* under tested conditions

	Fatty Acid Concentration (ng/µl)			
Fatty Acid	**Control**	**Light**	**Nitrogen**	**Salinity**
C18:2n6t	85.08±7.97	53.6±17.74	75.19±10.95	38.54±14.48
C18:2n6c (LA)	222.50±63.13	152.54±70.18	311.25±48.58*	38.33±18.28*
C18:3n6 (GLA)	98.23±20.97	74.2±31.73	137.15±21.41*	23.81±11.84*
C18:3n3 (ALA)	381.09±334.90	191.08±329.27	164.92±285.01	134.18±60.67
C20:2	1.09±0.38	0.61±0.5	0.72±0.24	3.05±2.56
C20:3n6 (DGLA)	1.22±1.07	0.85±0.04	0.73±0.40	0.21±0.06
C20:3n3/C22:1n9	4.46±1.41	7.57±7.24	3.35±0.36	1.91±1.75
C20:4n6 (AA)	1.24±1.14	13.60±9.15	13.95±11.81	6.32±9.30
C22:2	24.97±22.20	3.41±4.88	0.32±0.05	2.35±1.98
C20:5n3 (EPA)	5.58±7.14	17.62±24.15	32.76±23.77	15±23.63
C22:6n3 (DHA)	2.87±2.90	3.12±4.29	0.76±0.35	0.21±0.25
Sum of PUFAs	**803.35±272.857**	**522.59±402.67**	**741.10±222.7**	**263.88±120.51**

*Change is statistically significant ($P \leq 0.05$).

Omega-3 fatty acid profiles

Concentration of essential fatty acid ALA (C18:3n3) was decreased under all tested conditions compared to the control group (381.09 ng/µl) (Table 5). Alpha-linoleic acid concentration under decreased light intensity was 191.08 ng/µl. Under decreased nitrogen condition, the cells also lowered ALA concentration to 164.92 ng/µl. The lowest ALA concentration was seen under increased salinity condition at 134.18 ng/µl. Concentration of EPA (C20:5n3) was the highest under decreased nitrogen condition and accumulated at 32.76 ng/µl, more than five fold higher than the control group. Decreased light and increased salinity test conditions resulted in similar EPA concentrations at 17.62 and 15 ng/µl respectively, while the control group had the lowest EPA content at 5.58 ng/µl (Table 5). The other major ω-3 fatty acid, DHA (C22:6n3), was accumulated in minute amounts in all growth conditions, where decreased light condition had 3.12 ng/µl, 8.7% higher compared to the control group. Decreased nitrogen and increased salinity test conditions caused DHA

accumulation to 0.76 and 0.21 ng/µl, respectively (Table 5). As chromatographic separation of eicosatrienoic acid (ETE) (20:3n3) was not possible from erucic acid (C22:1n9), their accumulation is reported as lump sum, with the highest accumulation under decreased light (7.57 ng/µl), followed by control group (4.46 ng/µl), decreased nitrogen (3.35 ng/µl), and the lowest under increased salinity (1.91 ng/µl) test conditions (Table 5).

Omega-6 fatty acid profiles

Among all tested conditions, concentration of LA (C18:2n6c), an essential ω-6 fatty acid, was positively affected by decreased nitrogen and significantly increased by 39.89% compared to the control group with an overall accumulation of 311.25 ng/µl (Table 5). Decreased light condition also caused 45.86% decrease in LA content compared to the control and calculated as 152.54 ng/µl. Increased salinity resulted in 38.33ng/µl LA accumulation, significantly decreased by 82.77% compared to the control group (Table 5). Cells grown in decreased nitrogen conditions showed significant increase in concentration of GLA (C18:3n6) by 39.62% compared to control group and reported at 137.15ng/µl. However, both decreased light and increased salinity test conditions caused lower GLA content compared to the control group, 74.2 ng/µl (24.46% decrease) and 23.81 ng/µl (75% decrease) respectively (Table 5).

Discussion

Average total lipid contents from *Chlorococcum novae-angliae* obtained in all experimental conditions were between 14-22% in this study. The lipid contents of closest sister species of *C. novae-angliae*, i.e. *Chlorococcum sp.*, were found to be 6-28% in previous studies [37, 38], comparable to our findings. The cells under decreased light test condition (5500 lux compared to 11000 of the control group) caused lower total lipid content and growth rate compared to the control group. Ota et al., reported that the highest growth rate of *Chlorococcum littorale* was observed under 100 µ mol $m^{-2}s^{-1}$ (~7400 lux) [39]. In another study by Rehman and Anal, optimal light intensity was determined as 4340 lux for the growth of *Chlorococcum sp.* TISTR 8583 [40]. In alignment with these studies, lowered light intensity negatively affected cellular growth and suppressed overall lipid accumulation of *C. novae-angliae* in this study. The cells in control reactor grown under 11000 lux provided

highest dry cell weight, total lipid, and reactor productivity which could be interpreted as optimal for the cultivation of *C. novae-angliae* SAG5.85. With respect to FAME profiles, total saturated fatty acid concentrations were decreased under lowered light intensity although Seyfabadi and colleagues found that increased light conditions improve levels of SFAs in *Chlorella vulgaris* [41]. Similarly, all MUFA concentrations were decreased under low light conditions compared to the control except eicosenoic acid (C20:1) which was the only fatty acid increased. This might be due to difficulties in chromatic separation of C20:1 from heneicosanoic acid (C21:0) as evident from higher deviation among samples. Sukenik and coworkers reported that decreasing light intensity during the cultivation of *Nannochloropsis* sp. increased total PUFA content [42], which was not observed for *C. novae-angliae* as the cells under low light intensity decreased total PUFA content compared to the control. The only deviation was in DHA, EPA, and AA contents that were increased under low light intensity. Although Lang and colleagues stated that *C. novae-angliae* had the second highest DHA levels (18.9% of total fatty acids) among SAG strains [33], that was not confirmed by this study.

Second tested growth parameter was decreased nitrogen concentration as one of the most essential nutrients that limits the growth of microalgae while improving the accumulation of lipids. Numerous literature studies suggest that nitrogen limitation increases the accumulation of lipids in microalgae [43-45]. For instance, enhanced lipid accumulation, i.e. 29.59% higher, in *Chlorococcum sp.* TISTR 8583 cells was observed under nitrogen limited conditions, however, DCW was reduced during the cultivation [40]. Li and colleagues examined the effects of nitrogen concentration on lipid accumulation in *Ettlia oleoabundans* (aka *Neochloris oleoabundans*) cells and reported increased lipid accumulation with biomass reduction [30]. In another study, Bona and coworkers reported that nitrogen deplete conditions ($2.9x10^{-4}$ M), enhanced lipid accumulation in *E. oleoabundans* cells [46]. Kiran and colleagues also reported that higher lipid accumulation was observed in *Chlorella sp.* grown under 5 mM $NaNO_3$ (corresponding to one third of nitrogen of original medium) [47]. In this study, both cellular growth and total lipid content of *C. novae-angliae* was lowered under decreased nitrogen test condition compared to the control group. With respect to growth, nitrogen is a critical nutrient as it takes part in the structures of algal chlorophylls,

and amino acids as the key building blocks of proteins. Therefore, decreased nitrogen concentrations lead to inhibited growth rates in *C. novae-angliae*. Meanwhile, contrary to majority of green microalgae species' response, reduction in the concentration of nitrogen also reduced overall accumulation of lipids in this study. However, despite decreased total lipid content, free fatty acid content was the highest under decreased nitrogen. Notably, total SFAs were increased, which was also observed in *E. oleoabundans* [48] and *Nannochloropsis* CCAP 211/78 [49] grown in limited nitrogen. Under decreased nitrogen exposure, total MUFAs of *C. novae-angliae* were also increased compared to the control group. Despite lower than the control group, second highest level of total PUFAs was obtained under decreased nitrogen. In particular, DHA and ALA were decreased while EPA was increased. Contradictive to our findings, Bona and colleagues observed that ALA contents in *E. oleoabundans* cells were increased on the under nitrogen stress [46]. The highest increases in EPA levels were also observed under nitrogen stress in *Nannochloropsis* 211/78 (CCAP) [49]. Meanwhile, Breuer and colleagues reported that LA concentrations were decreased under nitrogen limited conditions in *C. vulgaris*, *C. zofingiensis*, *Dunaliella tertiolecta*, *Isochrysis galbana*, *Nannochloropsis* sp., and *E. oleoabundans* [35].

The third tested growth parameter *C. novae-angliae* cells were exposed was increased salinity, known to impose considerable effect on growth and biochemical composition of microalgae. As osmotic regulation is an important parameter for the survival of microalgal cells, optimal salinity ranges exist for different species [50-52]. In most species, salinity increase usually promotes overall cellular growth up to a certain threshold where salt concentrations start to negatively affect overall homeostasis [53, 54]. In *C. novae-angliae*, doubled salinity levels compared to control group was within optimal levels and had positive impacts on the overall growth. There are several studies showing increased salinity could trigger enhanced lipid production in microalgae [27, 55, 56]. Despite slight decrease compared to the control group, second highest average total lipid accumulation was observed under increased salinity conditions. Rismani and Shariati reported that 200 mM NaCl exposure caused 82% increase in total lipid productivity of *C. vulgaris* [57]. Takagi and Yoshida have shown that *D. tertiolecta* cells increased their lipid accumulation when NaCl in culture media was increased from 0.5 to 1 M [58]. Despite being a freshwater microalgae

species, salt stress adapted *Botryococcus braunii* showed increased growth rate, and lipid content [59], both in compatible with this study. Similarly, Ben-Amotz and colleagues achieved increased lipid content in *B. braunii* exposed to 0.5 M NaCl [60].

Despite relatively higher total lipid content, total SFA accumulation of *C. novae-angliae* was the lowest when exposed to increased salinity compared to the other test conditions and the control group. This was conflicting with total SFAs production enhanced in *Dunaliella* sp. cells when NaCl concentration was increased from 0.4 to 4 M [61]. As *Dunaliella* sp. are robust marine microalgae, it is possible that general adaptive capacity of terrestrial *C. novae-angliae* may not be as strong as *Dunaliella* sp. Total MUFAs were also decreased in increased salinity compared to the control and other test conditions. In literature, Azachi and colleagues showed increased salt stress induced oleic acid production in *B. braunii* and *I. galbana* [60], which was also not the case with *C. novae-angliae*. Similar to other fatty acid fractions, lowest levels of PUFAs content were also observed under increased salinity exposure. Meanwhile, Xu and Beardall observed that NaCl levels led to reductions in total PUFAs content in *Dunaliella* sp., indicating SFA, MUFA and PUFA trends might differ under the same salinity stress exposure [61]. Among PUFA of particular interest, DHA accumulation in *C. novae-angliae* were negatively impacted by increased salinity. Some studies also reported that DHA was below detectable levels in different microalgae species exposed to increased salinity [57, 60]. Moreover, LA and ALA concentration were also the lowest when exposed to increased salinity. This was in agreement with the study by Rao and colleagues where *B. braunii* 572 cells showed decreased LA concentration under increased salinity [59]. In another study, it was also mentioned that increased NaCl concentration might have resulted in decreased fatty acid unsaturation rates [56] as confirmed by decreased concentrations of GLA and DGLA in this study. Although slight increases in average EPA and AA accumulation were observed in *C. novae-angliae* exposed to increased salinity, these results were not statistically significant compared to the control group.

Last tested growth parameter was temperature which was lowered from 25 to 17°C. Temperature is one of the most important parameters that affect membrane fluidity, photosynthetic respiratory actions, and fatty acid composition of microalgae species [27, 62]. Increasing fatty acid unsaturation rate is very common behavior among microalgae in order

to adapt to cold environments as double bonds present in unsaturated fatty acids provide membrane fluidity compared to SFAs. Therefore increases in unsaturation rates are mostly observed at exposure to low temperatures [62]. In a study by Thompson, it was shown that shifting temperature from 30 to 12°C during the cultivation of *D. tertiolecta* has led to increase in unsaturated fatty acid levels by 20% [63]. Sushchik and coworkers reported that increasing temperature from 25 to 30°C during the cultivation of *C. vulgaris* and *B. braunii* decreased fatty acid unsaturation rate [64]. In another study by Thompson and colleagues, fatty acid profiles of eight species were investigated by shifting the cultivation temperature from 25 to 10°C [65]. Such decrease in temperature enhanced PUFAs accumulation in *Chaetoceros calcitrans, C. gracilis, C. simplex, D. tertiolecta, I. galbana, Pavlova lutheri, Phaeodactylum tricornutum* and *Thalassiosira pseudonana* [65]. Aussant and coworkers examined optimal temperature for microalgal growth and lipid production where they suggested 14-20°C for optimal EPA production in *D. salina* [66]. However, in this study the growth of *C. novae-angliae* at 17°C was totally inhibited starting on the very first day of cultivation. For most microalgal species, optimal cultivation temperature usually ranges between 25±5°C [67]. Nevertheless, *C. novae-angliae,* was tested at lower temperature ends to evaluate possible impacts on ω-3 and ω-6 fatty acids. Unfortunately, optical density and cell counts were dramatically decreased in short time while the culture color in PBRs turned into yellowish indicating damaged photosynthetic activity. On harvest Day 6, it was impossible to obtain sufficient amounts of biomass for total lipid and FAME profiling, therefore, temperature stress has been excluded from the study as growth of *C. novae-angliae* cells were not acclimated to 17°C, concluding this strain is not tolerant to temperatures lower than ambient.

Conclusions

Polyunsaturated fatty acids cannot be synthesized in humans and other mammals, yet they have numerous positive health impacts on metabolic processes. Therefore, PUFAs should be taken with daily diets at required concentrations and proper ratios. Main motivation for this study was to examine the effects of varying growth conditions on the accumulation of ω-3 and ω-6 fatty acids in *Chloroccoccum novae-angliae* SAG 5.85. Although original culture

collection indicated this strain as a potential PUFA producer, this study was the first comprehensive evaluation of varying growth conditions with respect to cell growth, total lipid and FAME profile of *C. novae-angliae.* As most common parameters affecting cellular growth and lipid content in green microalgae, light, nitrogen, salinity, and temperature growth conditions were tested. *C. novae-angliae* has been cultivated in triplicate batch PBRs in order to determine optimal conditions for the control and compared stress conditions. One of the most important outcomes was *C. novae-angliae* ω-3 and ω-6 fatty acid was significantly improved under decreased nitrogen conditions, despite lower total lipid content. Different levels and type of nitrogen sources could be applied in order to achieve large scale production goals. Decreased light and increased salinity test conditions did not improve ω-3 and ω-6 fatty acid content in *C. novae-angliae* compared to the control group, concluding that baseline cultivation conditions have better performance with respect to PUFA accumulation in this strain. Lastly, it was also found that lower than ambient temperatures completely halt the growth of *C. novae-angliae* suggesting temperature control in reactor systems are very critical for this particular strain.

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Abbreviations
ALA: alpha-linolenic acid; AA: arachidonic acid; DHA: docosahexaenoic acid; DH-GLA: dihomo-gamma-linolenic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; FAME: fatty acid methyl ester; GLA: gamma-linoleic acid; GC-FID: gas chromatography-flame ionization detector; LA:linoleic acid; MUFA: monounsaturated fatty acids; PBR: photobioreactor; PUFA: polyunsaturated fatty acids, SFA: saturated fatty acid

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