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Nitrogen Deprivation in *Fremyella diplosiphon* Augments Lipid Production without Affecting Growth

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Abstract: Metabolic products such as lipids and proteins produced in cyanobacteria represent an excellent source of biomass and do not compete with agricultural land use unlike soybean and corn. Given their potential use as novel materials for biodiesel production, we aimed to explore the effect of cultivation period and nitrogen concentration on the growth rate and lipid content of *Fremyella diplosiphon*, a model cyanobacterium. In this study, *F. diplosiphon* grown in BG11/HEPES medium supplemented with 1.5 g L^{-1} sodium nitrate ($NaNO_3$) for 7, 10, 15, and 20 days were compared to the untreated control in media amended with 0.25, 0.5, and 1.0 g L^{-1} $NaNO_3$. Cultures were inoculated in liquid media and grown under continuous fluorescent light in an orbital incubator shaker, and extracted lipids subjected to gravimetric analysis and gas chromatography-mass spectroscopy to determine the best culture conditions for lipid production. Our results demonstrated that a reduction in nitrogen concentration had no significant effect on the growth rate across all cultivation periods; however, the accumulation of total lipid content was significantly influenced by nitrogen concentration. A maximum lipid production (40%) with no reduction in growth was observed in 10-day old cultures in a BG11/HEPES medium supplemented with 1.0 g L^{-1} $NaNO_3$. Fatty acid methyl ester composition of transesterified lipids demonstrated high amounts of methyl palmitate (50–70%) followed by methyl octadecenoate (17–30%) in the accumulated lipids at all treatments. Trace quantities of methyl dodecanoate, methyl hexadecanoate, methyl octadecanoate, and methyl octadecadienoate (1–8%) were also observed in all tested samples, indicating that nitrogen deprivation in culture media increases lipid production without affecting growth.

Keywords: biomass; biodiesel; culture; cyanobacteria; growth; lipid; nitrogen

1. Introduction

Adopting fossil fuels as the chief energy source has resulted in the surplus emissions of CO_2 and other greenhouse gases leading to global climate change. Simultaneously, worldwide nonrenewable energy resource supplies are dwindling, while energy demand is increasing day-by-day [1]. Exhaustion of fossil fuel reserves, increased oil prices, and rising levels of greenhouse gases have driven worldwide interest in renewable energy as an alternative to fossil fuels. Biofuels are renewable green fuels, which have driven interest in methods to maximize production and attracted researchers to meet the growing demand for fuel [2]. Their production offers an opportunity to develop an alternative for fossil fuels

while also assisting rural economies [3–6]. In addition, harmful emissions of carbon monoxide and hydrocarbons can be mitigated, which can decrease greenhouse effects and improve environmental sustainability [7,8]. Most recently, research efforts have been aimed at identifying suitable strains of algae/cyanobacteria which can provide greater energy yields to displace conventional fuels.

According to the 2019 World Gas and Renewables Review, over 2.6 million barrels/day of biofuels were produced worldwide in 2018. The world's biodiesel supply has exponentially increased from 3.9 billion liters in 2005 to 18.1 billion liters in 2010, and is expected to reach 41.4 billion liters in 2025 [9]. As a result of this growing demand for renewable biodiesel, many researchers have attempted to produce biodiesel from sources such as rapeseed, soybean, peanut, and vegetable oils [10,11]. However, these first-generation fuels are not practical for commercial production since they compete for water and arable lands [12]. The price of biodiesel remains a major hindrance for commercial production, primarily as a result of the high feed cost of vegetable oils [13], thus we are in dire need to identify other new sources. In recent years, microalgae and cyanobacteria have emerged as one of the most promising sources of biodiesel and gained great importance as its high lipid content can serve as raw material for biofuel production [2,14]. These organisms have higher photosynthetic efficiency (10–100 times higher than for plants) and faster growth rate compared to any other energy crop [15–18]. With a short and non-seasonal life-cycle, these microbes can thrive without the use of agrochemicals. Additionally, cyanobacteria are more efficient and can be cultivated in marginal lands and used wastewater, thus minimizing competition with food crops [1]. Thus, mass cultivation of cyanobacteria as a biofuel feedstock is being evaluated worldwide, especially since these organisms thrive in the presence of basic nutrients, such as water, CO_2, mineral salts, and light [19]. Cyanobacteria are also easily subjected to genetic modification allowing the generation of high-value products such as lipids and proteins [20–23]. In spite of the advantages that these organisms provide, generating significant amounts of bioenergy at a plausible scale to impact the energy economy and the return of investment based on capital input are potential bottlenecks faced. In particular, a key challenge to achieving viability is the cultivation and harvesting steps, which is critical in maximizing yield while minimizing inputs such as light and nutrients. For commercial production of biodiesel from cyanobacteria, factors such as optimized harvesting, oil extraction, and conversion to fuel processes is imperative. Ideal strains need to be selected and optimized for biomass production, and the fatty acid composition analyzed [24]. Unless we reach a high lipid production per gallon per acre from the selected strains, economic viability would be very hard to achieve. Major breakthroughs in this area can be advanced via the induction of lipid biosynthesis by environmental stresses [25–27]. Cyanobacterial strains generate varying quantities of carbohydrates, lipids, and proteins; choice species can adjust their metabolism through basic changes to the composition of growth media. A significant increase in lipid content has been reported in several species grown in nitrogen-deficient environments. Factors such as phosphorous, nitrogen, and iron levels in the medium, salt stress, radiation, acidity, heavy metals, temperature, light intensity, and irradiance [28–32] have been reported to impact the lipid content. Under optimal growth, significant quantities of biomass are produced, but with limited lipid abundance, while species with high lipid levels are typically slow-growing. This indicates an inverse relationship between lipid content and nitrate concentration [7]. Several reports suggest that lipids tend to accumulate in nitrogen-deficient conditions [7,10,24].

High-lipid yield is a major prerequisite for commercial biodiesel production. *Fremyella diplosiphon* is a model organism for studying photosynthetic pathways and exhibits extreme regulation of phycoerythrin and phycocyanin using a process known as complementary chromatic adaptation. Furthermore, its fast generation time and potential to grow in a wide range of light including shaded light, make this organism an ideal candidate for large-scale cultivation while reducing capital input. However, it is crucial to establish stable culture conditions for achieving high lipid yield in scaled-up generation of biofuels from *F. diplosiphon*, while reducing capital input [33]. Prior efforts to overexpress genes using electroporation-mediated transformation in this strain has resulted in salt tolerant [34] and high-lipid producing strains [35], as well as the identification of fatty acid methyl esters (FAMEs)

that prove its efficacy as a biodiesel agent [36]. In this study, we investigated the growth response and lipid yield of *F. diplosiphon* when subjected to varying levels of sodium nitrate ($NaNO_3$) in the culture medium. We also examined the best culture conditions for maximal lipid production, and analyzed extracted lipids by gravimetric and gas chromatographic methods to determine the impact of nitrogen deprivation on biodiesel quality.

2. Materials and Methods

2.1. Cyanobacterial Strain and Culture Conditions

A short filamentous *F. diplosiphon* strain (SF33) obtained from Dr. Beronda Montgomery at Michigan State University, capable of growth in both red and green light, was used in this study. Actively growing cells from 3–6 day old cultures were inoculated from plates into sterilized 250 mL flasks containing 150 mL of BG11/HEPES buffer, and grown under continuous shaking at 170 rpm and permanent 30 µmol/m^2/s white light at a temperature of 28 °C in an Innova 44R incubator shaker series (Eppendorf). The BG11 medium (hereafter referred to as BG11/HEPES) was composed of stock cultures containing Na_2Mg EDTA, ferric ammonium citrate, citric acid, $MgSO_4.7H_2O$, $K_2HPO_4.3H_2O$, Na_2CO_3, H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, and 1.5 g of $NaNO_3$.

2.2. Growth of F. diplosiphon in Varying Sodium Nitrate Concentrations

Actively growing cultures were transferred into 500 mL flasks containing 300 mL BGll/HEPES media amended with 0.25, 0.5, 1.0, and 1.5 g of $NaNO_3$ and grown for a period of 7, 10, 15, and 20 days. Cultures grown in 1.5 g/L $NaNO_3$, which is the standard amount in the BG11 media served as the control. Inoculated cultures were grown in culture conditions as mentioned above, and optical densities at 750 nm measured at 48-h intervals throughout the course of the experiment. Three replicated treatments were maintained and the experiment repeated once.

2.3. Lipid Accumulation in F. diplosiphon Grown in Varying Levels of Sodium Nitrate

F. diplosiphon cultures were grown in BGll/HEPES media amended with varying nitrate levels as mentioned in Section 2.1. Cells were centrifuged using a Beckman-Coulter Avani-J25I with a JA 25.50 rotor, lyophilized overnight, and sonicated in 5 mL chloroform:methanol (2:1) for 30 s. Lipids were extracted using a 2:1 chloroform:methanol mixture according to the method of Folch et al. [37]. The mixture was agitated for 15–20 min in an orbital shaker after dispersion at room temperature and the homogenate centrifuged to recover the liquid phase. The solvent was washed with 0.2 volumes (1 for 5 ml) of distilled H_2O, vortexed briefly, and centrifuged at 2000 rpm to separate the two phases. The lower phase was transferred to a pre-weighed vial and the interface was rinsed twice using methanol:water (1:1) without mixing the whole preparation. The lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator after centrifugation and siphoning. The dried flasks were weighed to establish the total lipid content, which was determined by the conventional gravimetric method [38].

2.4. Simultaneous Transesterification and Lipid Extraction

To investigate the effect of nitrate deprivation on the *F. diplosiphon* fatty acid profile, cultures were grown under conditions mentioned above and extracted lipids were subjected to one-step direct transesterification in a multimode commercial scientific reaction microwave (CEM Corp, Matthews, NC, USA) as described by Tabatabai et al. [36].

2.5. Gas Chromatography-Mass Spectrometry in F. diplosiphon Grown in Varying Sodium Nitrate Levels

The fatty acid composition of *F. diplosiphon* transesterified product was determined using Shimadzu GC17A/QP5050A gas chromatography-mass spectrometry (GC-MS) according to the method described

by Rosenberg et al. [39] and Tabatabai et al. [36]. Peaks were identified by comparing mass spectra to the lipid Web Archive of FAME mass spectra. Three biological replicates of each sample were analyzed.

2.6. Statistical Analysis

All measurements included triplicate treatments. The mean and standard deviation of the data values were calculated using MS-Excel. The effect of treatments was determined by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test conducted to determine the statistical significance of the differences between means of various treatments.

3. Results and Discussion

3.1. Effect of Nitrogen Deprivation on F. diplosiphon Growth

Optimization of culture conditions for maximizing lipid yield is critical to enhance lipid production, and is influenced by various interconnected factors such as growth rate, biomass, and lipid content. The deprivation of essential nutrients including phosphorus, sulfur, nitrogen, and potassium has been reported to result in significant alterations of cellular growth and compositions in algae [40,41]. As an essential macronutrient, nitrogen significantly impacts growth and total lipid content, and is vital for metabolism and development [42]. Exposure to nitrogen starvation has been reported to enhance lipid levels in various microalgal and cyanobacterial species. Reduction in cellular thylakoid levels, acyl hydrolase activation, and phospholipid hydrolysis stimulation have been reported to enhance the intracellular fatty acid abundance [43–47]. Our results revealed that the growth rate was not significantly affected by varying concentrations of $NaNO_3$ (Figure 1), suggesting that partial nitrogen deprivation is not detrimental to *F. diplosiphon* growth. By contrast, a previous study in *Botrycoccus sp.*, *Scenedesmus obliquus*, and *Chlorella pyrenoidosa* revealed that the organism exhibited a significant reduction in growth when subjected to nitrogen starvation [7,28,48,49].

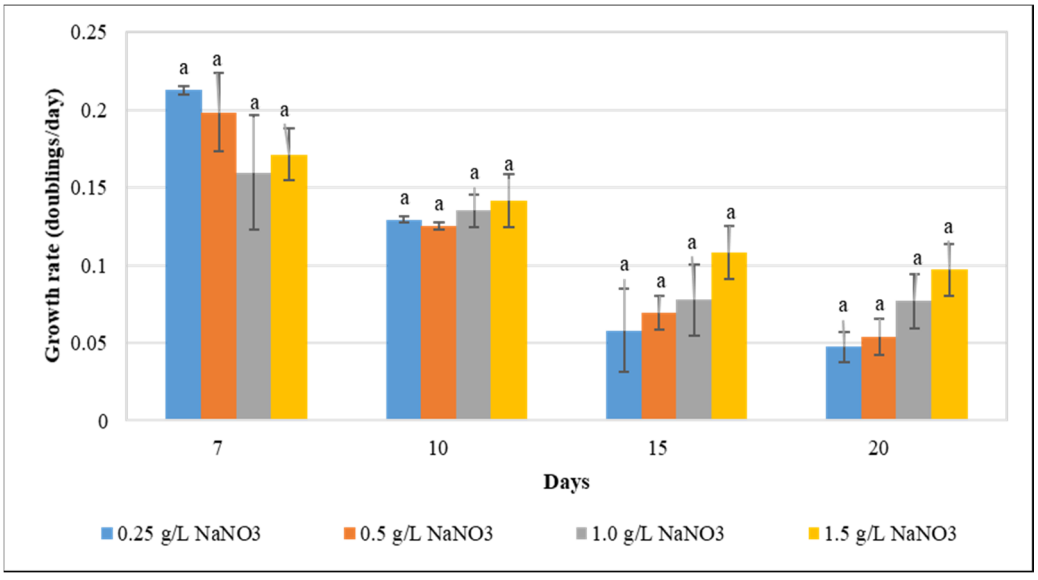


Figure 1. The impact of varying sodium nitrate ($NaNO_3$) concentrations (0.25, 0.5, 1.0, and 1.5 g L^{-1}) on *Fremyella diplosiphon* growth rate over a period of 7, 10, 15, and 20 days. The average growth rate (± standard error) for three biological replicates for each treatment is shown. Different letters above bars indicate significance among treatment means ($p < 0.05$).

The unaffected growth of *F. diplosiphon* under nitrogen-deprived conditions may also be due to their diazotrophic nature which makes nitrogen available for proper growth by nitrogen fixation. A possible reason for this might be that nitrogen pools could have been consumed to support the cell

growth after nitrogen exhaustion [49]. This explanation also finds support from the study of Li et al. [50], who reported that in nitrogen deficient conditions chlorophyll provides the nitrogen required to sustain *Chlorella* growth. Furthermore, our results find support from the work of Suen et al. [51], who reported the unaltered growth of microalgal species *Nannochloropsis* in nitrogen deprived conditions. Future studies will be aimed towards investigating the effects of even more drastic reductions in nitrogen supply in *F. diplosiphon*.

3.2. Effect of Nitrogen Deficiency on F. diplosiphon Total Lipid Content

While *F. diplosiphon* growth was unaffected in varying levels of nitrate concentrations, a reduction in nitrate levels correlated to a significant increase in total lipid content. We observed maximum lipid production in 1.0 g L^{-1} $NaNO_3$ concentrations on 7 and 10-day-old cultures, indicating that lipid yield in *F. diplosiphon* can be enhanced under these conditions (Figure 2). These results are in accordance to a report by Stephenson et al. [52], where an increase in lipid content was reported in nitrogen-deprived *Chlorella vulgaris* cultures. Similar results were also observed in the microalgae *Scenedesmus* sp., where the lipid content was enhanced six-fold after exposure to nitrogen starvation for 21 days [53]. High-lipid accumulation in a nitrogen-deprived medium was also reported in studies by Stuart et al. [54] and Becker et al. [55]. Accumulation of lipid content under nitrogen deprivation in *F. diplosiphon* as observed in our study, is comparable to the accumulated lipid content of *Synechocystis* spp., *Oscillatoria* spp., *Lyngbya semiplena*, *Limicolaria martensiana*, *Calothrix* spp., and other algae [56]. Further, our results support the findings of Wahlen et al. [57], who demonstrated that microalgae could be better sources for biodiesel production.

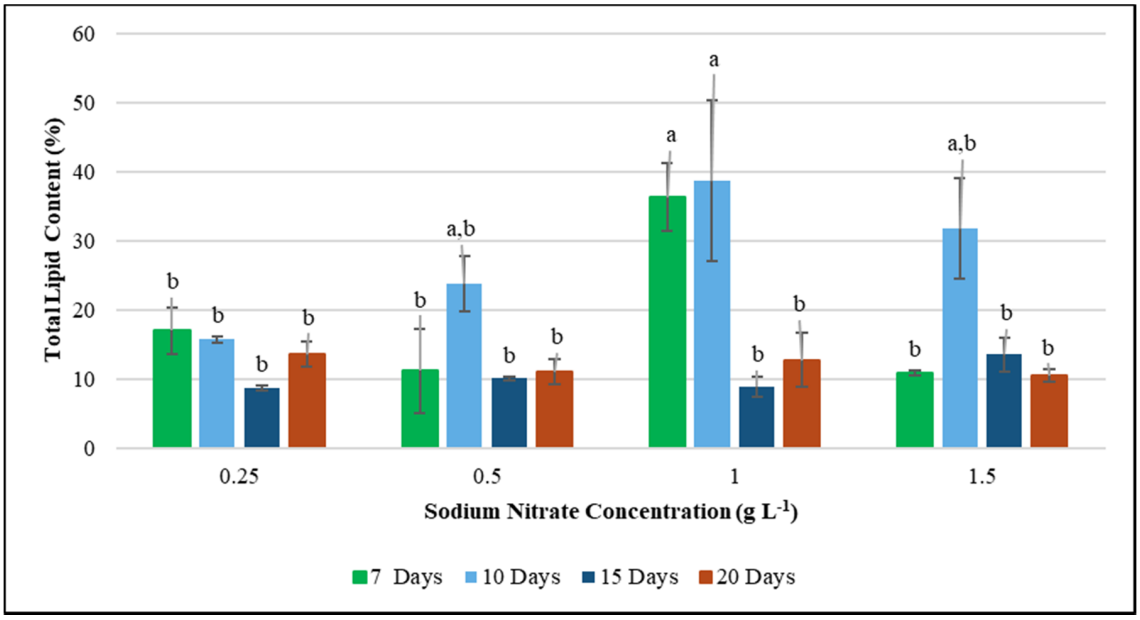


Figure 2. Effect of total lipid production in *Fremyella diplosiphon* grown in nitrogen concentrations of 0.25, 0.5, 1.0, and 1.5 g L^{-1} sodium nitrate over a period of 7, 10, 15, and 20 days. The average % lipid content of total cellular dry weight (±standard error) for three biological replicates of each treatment is shown. Different letters above bars indicate significance among treatment means ($p < 0.05$).

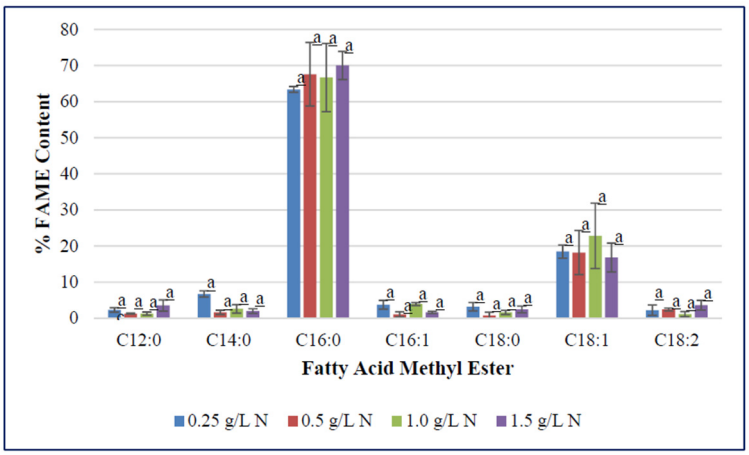
3.3. Effect of Nitrogen Deficiecny on F. diplosiphon Fatty acid Profile

To determine the impact of nitrogen starvation on high-value fatty acid profile in *F. diplosiphon*, cultures were grown in BG11/HEPES amended with 0.25, 0.5, 1.0, and 1.5 g L^{-1} $NaNO_3$ and subjected to FAME analysis. The determination of FAME composition using GC-MS showed a high abundance of methyl palmitate (50–70%) followed by methyl octadecenoate (17–30%), trace amounts of methyl dodecanoate, methyl hexadecanoate, methyl octadecanoate, and methyl octadecadienoate (1–8%) in all

samples tested (Table 1; Figure 3). Saturated fatty acids such as methyl dodecanoate (12:0), methyl myristate (14:0), methyl palmitate (16:0), and methyl octadecanoate (18:0) attributed for 60–80% of the total FAMEs. Unsaturated fatty acids were present in smaller quantities relative to the saturated fatty acids. Approximately 20–40% of extracted FAMEs were unsaturated, and were mainly comprised of methyl hexadecanoate (16:1), methyl octadecenoate (18:1), and methyl octadecadienoate (18:2). The FAME components, methyl palmitate (C16:0), methyl hexadecanoate (16:1), octadecanoate (18:0), octadecenoate (C18:1), methyl octadecadienoate (C18:2), and methyl hexadecanoate (16:1) were the major components observed in FAME analyses. These results are in accordance to the FAME profile reported in *Chlorella minutissima* [58]. In addition, prior studies in our laboratory have reported approximately 25% FAME in total transesterified lipids in wild type *F. diplosiphon*, while up to 70% FAME were present in genetically modified strains [36]. A high ratio of saturated fatty acids in the lipid profile was observed across all treatments, which is a desirable trait for a prospective biofuel agent [59]. We observed methyl palmitate (C16:0) to be the major constituent, which is similar to FAME profiles reported in the microalgae *Chlorella pyrenoidosa* and *C. vulgaris* [60–62]. Interestingly, the level of methyl palmitate decreased over time in cultures grown in 0.5 and 1.0 g L^{-1} $NaNO_3$ (Figure 4) indicating that exhaustion of nitrogen supply could have altered the fatty acid composition of *F. diplosiphon.* These results indicate a direct correlation between the depletion of nitrate in the medium to the fatty acids produced. By contrast, an increase in unsaturated FAME species such as methyl hexadecanoate (16:1), methyl octadecenoate (18:1), and methyl octadecadienoate (18:2) was observed over time, suggesting that lipid profiles of nitrogen-starved cultures contain a higher proportion of unsaturated fatty acids. The presence of saturated fatty acid in lipid profile is valuable for biofuel production but its high melting point could result in fuel gelling in colder climates. On the contrary, the low melting point of unsaturated fatty acids is necessary for biofuel generation, and aids their endurance in cold weather [63]. In the present study, FAME profiling of *F. diplosiphon* indicated the presence of methyl octadecadienoate (C18:2), which is reported to provide oxidative stability [35]. Further, the presence of both hexadecanoate (16:1) and methyl myristate (14:0) in the lipid profile of *F. diplosiphon* is beneficial as hexadecanoate (16:1) improves the oxidative stability [63] and methyl myristate (14:0), a shorter chain fatty acid improves NOx emissions [64]. Thus, carbon chain lengths of fatty acid as well as the degree of unsaturation are both key properties for biodiesel quality [65]. Wang et al. [66] demonstrated that enhanced saturated fatty acid abundance in the marine protist, *Schizochytrium* sp. PKU#Mn4, met the ASTM6751 standards. Tabatabai et al. [36] reported that the high abundance of saturated FAMEs, while beneficial with regards to cetane number and oxidative stability, resulted in high pour and cloud points. This suggests that use of the resultant biofuel from *F. diplosiphon* in a blend with conventional fuels and additives would the most viable approach. Our findings indicate that nitrogen deprivation at 1.0 g L^{-1} $NaNO_3$ significantly enhances lipid yield in *F. diplosiphon*, while achieving a desired saturated:unsaturated fatty acid ratio for biofuel production. Since nitrogen is a major nutrient for chlorophyll production and other proteins, deprivation of this essential macronutrient could inhibit structural and physiological components of photosynthesis [67]. In a report on the changes in lipid composition in *Chlorella* sp. and *Nannochloropsis* sp. during nitrogen starvation, the comprehensive fatty acid composition of polar lipids was unaffected, indicating that maintenance of a particular fatty acid profile in each compartment allows its continued function, despite a significant decrease lipid quantity [68].

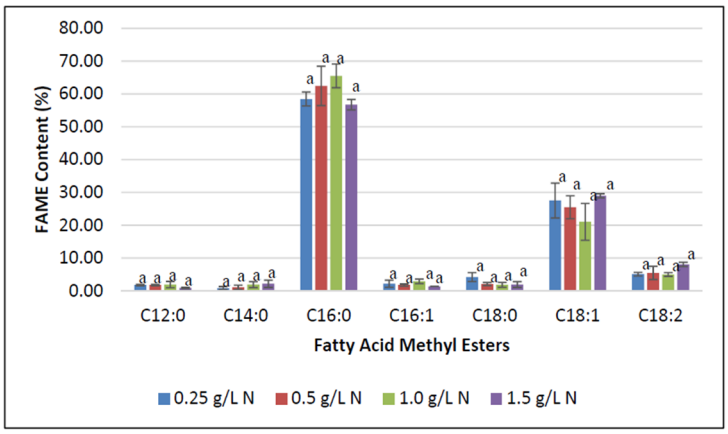
Table 1. Fatty acid methyl ester (FAME) composition in *Fremyella diplosiphon* grown over 7, 10, 15, and 20 days in media containing 0.25, 0.5, 1.0, 1.5 g L^{-1} sodium nitrate ($NaNO_3$) based on relative abundance of each component (% total extractable FAMEs ± SE).

7-Day	**$NaNO_3$ Concentration**			
Fatty Acid Methyl Esters	**0.25 g L^{-1}**	**0.5 g L^{-1}**	**1.0 g L^{-1}**	**1.5 g L^{-1}**
methyl dodecanoate (12:0)	2.28 ± 0.618	1.25 ± 0.136	1.25 ± 0.439	3.53 ± 1.55
methyl myristate (14:0)	6.7 ± 0.861	1.61 ± 0.599	2.59 ± 1.199	1.97 ± 0.651
methyl palmitate (16:0)	63.38 ± 0.809	67.59 ± 8.78	66.67 ± 9.46	70.03 ± 3.90
methyl hexadecanoate (16:1)	3.72 ± 1.21	1.06 ± 0.726	3.92 ± 0.415	1.62 ± 0.305
methyl octadecanoate (18:0)	3.21 ± 1.45	0.75 ± 0.370	1.60 ± 0.693	2.45 ± 1.35
methyl octadecenoate (18:1)	18.46 ± 1.82	18.19 ± 6.11	22.81 ± 9.04	16.80 ± 3.98
methyl octadecadienoate (18:2)	2.23 ± 1.16	2.41 ± 0.916	1.15 ± 0.567	3.60 ± 0.940
10-Day				
methyl dodecanoate (12:0)	1.78 ± 0.204	1.76 ± 0.224	1.90 ± 0.969	0.86 ± 0.193
methyl myristate (14:0)	0.84 ± 0.444	1.07 ± 0.662	1.90 ± 0.910	2.14 ± 1.11
methyl palmitate (16:0)	58.45 ± 2.13	62.45 ± 5.99	65.49 ± 3.63	56.71 ± 1.59
methyl hexadecanoate (16:1)	2.17 ± 1.01	1.75 ± 0.332	2.90 ± 0.693	1.36 ± 0.047
methyl octadecanoate (18:0)	4.19 ± 1.35	2.07 ± 0.464	1.82 ± 0.754	1.92 ± 0.872
methyl octadecenoate (18:1)	27.51 ± 5.30	25.47 ± 3.52	21.02 ± 5.60	28.97 ± 0.623
methyl octadecadienoate (18:2)	5.06 ± 0.539	5.44 ± 2.02	4.98 ± 0.544	8.04 ± 0.640
15-Day				
methyl dodecanoate (12:0)	1.44 ± 0.400	1.39 ± 0.278	1.55 ± 0.086	1.94 ± 0.202
methyl myristate (14:0)	0.76 ± 0.374	0.50 ± 0.117	0.63 ± 0.063	0.86 ± 0.108
methyl palmitate (16:0)	57.39 ± 5.25	62.14 ± 1.97	62.29 ± 0.523	65.80 ± 1.30
methyl hexadecanoate (16:1)	2.74 ± 0.957	7.46 ± 2.34	4.46 ± 2.52	4.92 ± 2.19
methyl octadecanoate (18:0)	1.75 ± 1.40	1.50 ± 0.931	2.44 ± 0.638	1.27 ± 0.170
methyl octadecenoate (18:1)	28.19 ± 5.135	21.15 ± 2.83	20.02 ± 1.61	18.62 ± 1.95
methyl octadecadienoate (18:2)	7.73 ± 0.815	5.85 ± 1.83	8.61 ± 0.924	6.59 ± 0.213
20-Day				
methyl dodecanoate (12:0)	2.20 ± 1.61	2.83 ± 2.30	2.13 ± 0.958	2.41 ± 0.496
methyl myristate (14:0)	3.56 ± 2.45	8.13 ± 1.19	3.34 ± 1.65	3.69 ± 2.82
methyl palmitate (16:0)	60.71 ± 6.23	50.85 ± 6.11	50.25 ± 11.9	64.58 ± 4.13
methyl hexadecanoate (16:1)	2.24 ± 1.34	3.11 ± 1.65	4.94 ± 2.71	1.60 ± 0.472
methyl octadecanoate (18:0)	1.90 ± 0.923	3.60 ± 1.41	4.07 ± 1.41	2.31 ± 1.27
methyl octadecenoate (18:1)	25.03 ± 6.71	27.79 ± 4.91	30.88 ± 9.21	23.18 ± 5.26
methyl octadecadienoate (18:2)	4.36 ± 0.860	3.69 ± 2.54	4.39 ± 1.33	2.24 ± 1.07



(**A**)

Figure 3. *Cont.*



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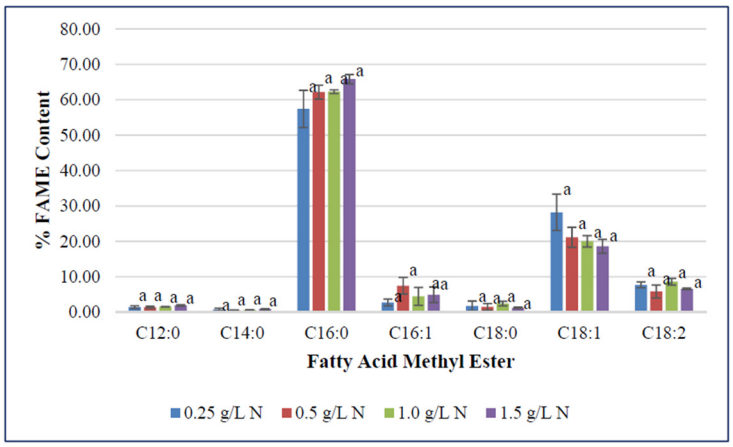

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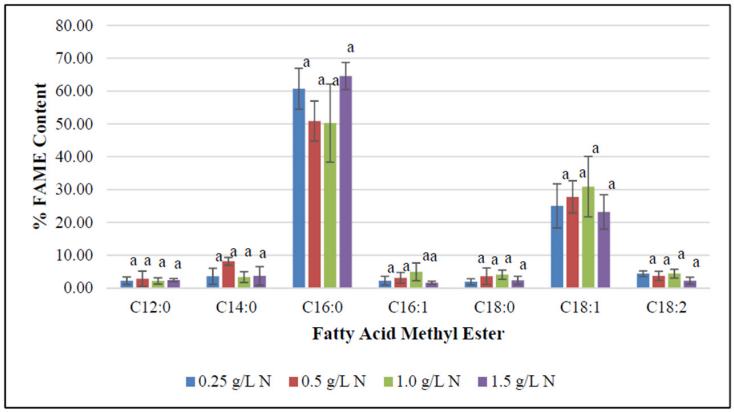

(**D**)

Figure 3. Fatty methyl ester composition (FAME) of *Fremyella diplosiphon* grown over (**A**) 7, (**B**) 10, (**C**) 15, and (**D**) 20 days in media containing 0.25, 0.5, 1.0, 1.5 g L^{-1} sodium nitrate ($NaNO_3$). Bars represent the average % relative content of specific FAME species (± standard error) for three biological replicates of each nitrate treatment. Different letters above bars indicate significant differences in FAME species abundance between varying nitrate treatments ($p < 0.05$).

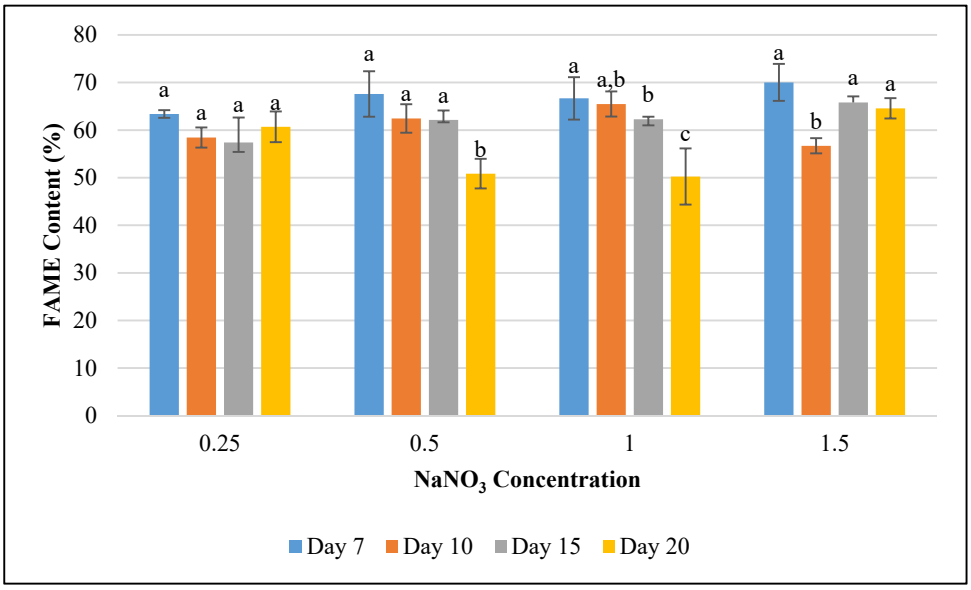

Figure 4. Methyl palmitate relative abundance in *Fremyella diplosiphon* grown over 7, 10, 15, and 20 days in media containing 0.25, 0.5, 1.0, 1.5 g L^{-1} sodium nitrate ($NaNO_3$). Bars represent the average % relative content of specific fatty acid methyl esters (± standard error) for three biological replicates of each nitrate treatment. Different letters above bars indicate significant differences in the methyl palmitate content between varying time periods ($p < 0.05$).

4. Conclusions

In the present study, we investigated the effect of nitrogen deprivation on growth rate, lipid yield, and FAME composition in *F. diplosiphon* to identify optimal conditions that enhance lipid accumulation. Maximum lipid productivity was observed under moderately nitrogen-limited conditions (i.e., 1.0 gL^{-1} sodium nitrate) on the 10th day, without the growth rate being hindered. In addition, we identified and quantified the FAME profile in transesterified *F. diplosiphon* lipids, which is a prerequisite to evaluate biofuel physical and chemical properties. These findings suggest that manipulating nitrogen input during *F. diplosiphon* cultivation could enhance lipid production, thus increasing its potential viability as a source of renewable biofuel. Results of this study could lead to a more optimal photobioreactor design for large scale *F. diplosiphon* cultivation, for the development of natural bio-products across various applications, from fuel to food and cosmetics.

Author Contributions: V.S., B.T., and A.K.S. designed and conceived the study; B.T. and A.A. performed the experiments and analyzed the data; B.T., A.K.S., P.K.S., and V.S. interpreted the data and drafted the manuscript. All authors gave critical comments for the important intellectual content in the article. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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