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RESEARCH PAPER

Ammonium Nutrition Induces Triacylglycerol, β-carotene, and Lutein Production in *Dunaliella tertiolecta* Butcher

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Abstract

Distinct impacts of ammonium nutrition for simultaneous production of triacylglycerol, lutein, and β-carotene were evaluated in *Dunaliella tertiolecta*. A physiologically realistic growth system was employed to compare metabolite production and growth characteristics of nitrogen deprived, ammonium- and nitrate supplied *D. tertiolecta*. The nitrogen dependent changes in *D. tertiolecta* were reflected as a dramatic decrease in growth related to decreased photosynthetic activity, reduced pigmentation and increased triacylglycerol production as observed in nitrogen deprived microalgae. On the other hand, ammonium nutrition caused a time-dependent increase of neutral lipids, triacylglycerols, chlorophylls and carotenoids, lutein and β -carotene in particular. Finally, ammonium nutrition improved FAME profile of *D. tertiolecta* for biodiesel production via an exclusive impact on increased saturation.

Introduction

Light, water, and mineral nutrients are of vital importance for microalgal growth. Amongst mineral nutrients, nitrogen is required in large amounts which represent 7-20% of microalgae dry weight depending on the physiological state of microalgae (Qiang, 2013). Availability of nitrogen is a major limiting factor for microalgal growth as it is a structural component of amino-acids, nucleic acids and a variety of secondary metabolites Nitrogen is commonly available to photosynthetic organisms in oxidized (mainly nitrate) and reduced (mainly ammonium) forms (Patterson, Cakmak, Cooper, Lager, Rasmusson, & Escobar, 2010). Microalgae prefer direct uptake of inorganic nitrogen forms and immediately incorporate inorganic nitrogen into amino acids and recycle it in order to meet physiological needs (Qiang, 2013).

There is a large volume of literature information on the production of neutral lipid and carotenoids from microalgae. In the general sense, when a stress condition arises, microalgae tend to store photosynthetic energy in the form of lipids and carbohydrates at the expense of reduced growth rate (Juneja, Ceballos, & Murthy, 2013). Nitrogen limitation or starvation of microalgae has been employed as an effective strategy to induce production of lipids (Cakmak, Olmez, Cakmak, Menemen, & Tekinay, 2014), carbohydrates (Dean, Sigee, Estrada, & Pittman, 2010) and carotenoids (Markou & Nerantzis, 2013) along with a simultaneous reduction in total protein content. Miscellaneous impacts of nitrogen deficiency include a decrease in chlorophyll content, carbon dioxide fixation, and oxygen evolution (Peccia, Haznedaroglu, Gutierrez, & Zimmerman, 2013).Ammonium nutrition was reported to increase the amino-acid content of

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Chlorella pyrenoidosa at the expense of sugar phosphates (Holm-Hansen, Nishida, Moses, & Calvin, 1959). Nitrate and ammonium nutrition were reported to induce sudden changes in Chl-a fluorescence in nitrogen-deprived *D. tertiolecta* (Young & Beardall, 2003). Time-based fluctuations of growth *D. tertiolecta* were analyzed as a response to ammonium and nitrate (Chen, Tang, Ma, Holland, Ng, & Salley, 2011). They reported that high nitrate concentrations do not affect growth but even low concentrations of ammonium may cause considerable decreases in growth of *D. tertiolecta*. More recently,Chlorophytes were defined as the most resistant class of algae to high ammonium concentrations (Collos & Harrison, 2014).

The physiological effects of nitrate supply on *D. tertiolecta* have been well documented (Peccia *et al*., 2013); however, little is known about ammonium nutrition and its differential effects on neutral lipid and carotenoids production in *D. tertiolecta*. This paper evaluates the ammonium-induced simultaneous production of triacylglycerol, β-carotene, and lutein from *D. tertiolecta*. The rationale behind employing *D. tertiolecta* for this study is that *D. tertiolecta* has high tolerance to elevated salinity, motile, simple to cultivate, favorable for massive outdoor cultivation, rich carotenoid composition, and lastly it has relatively high oil content with high PUFA composition which is not favorable for use as biodiesel (Byrd, Burkholder, & Zimba, 2017). Moreover, *D. tertiolecta* does not possess rigid cell wall which facilitates the extraction process that makes this microalga a promising source for production of biodiesel and value-added products such as carotenoids (Rizwan, Mujtaba, & Lee, 2017).

Materials and Methods

Strain and Culturing Conditions

The microalga *D. tertiolecta* was isolated from Meke Crater Lake a volcanic saline lake located in Konya Province of Turkey (37°41'7"N 33°38'28"E) in July 2013. The strain was identified based on morphological characteristics (Borowitzka & Borowitzka 1988) and genomic information. The strain has been cultured and maintained in Istanbul Medeniyet University Microalgae Culture Collection, Istanbul Medeniyet University, Istanbul, Turkey. The species was identified using a sequence analysis of the 18S rRNA gene. For this aim, the genomic DNA fragment was amplified by PCR, sequenced, and analyzed according to (Hoham, Bonome, Martin, & Leebens-Mack, 2002). DNA amplification from genomic DNA containing a partial 18S ribosomal RNA region was performed by PCR using the following primers: Forward: 5'-ATTGGAGGGCAAGTCTGGT-3' and Reverse: 5'- ACTAAGAACGGCCATGCAC-3'. Same primers were used for Sanger sequencing. Sequence comparison of the 18S rRNA genes between *D. tertiolecta* and related species was performed using the NCBI databases with BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit-graphical biological sequence editor v7.0.9.

For experimentation, *D. tertiolecta* was grown in the 200 mL medium in 500 mL flasks under the continuous light intensity of 150 μmol photons $m^2.s^{-1}$ as measured at the external glass wall of the flasks(light source, Philips® TL-D 18W/840 recyclable cool daylight) in a temperature-controlled orbital shaker (Sartorius, Certomat BS-T) with a 120 rpm speed under 25°C temperature. Modified Johnson's medium (Johnson, Johnson, MacElroy, Speer, & Bruff, 1968) with 20% NaCl concentration and 1000 mg/L KNO_3^- as nitrogen source was used as a control. In order to apply ammonium as an inorganic nitrogen source, 500 mg/L NH_4Cl was replaced with KNO_3. Lastly, KNO_3^- was replaced with KCl for N deprivation of *D. tertiolecta*. In order to determine the moderately high concentration of NH_4^+ for growth, *D. tertiolecta* was incubated in modified Johnson's medium with 25, 50, 125, 250, 500, 1000 mg/L NH_4Cl as a nitrogen source. As shown in (Figure 1a), 500 mg/L NH_4Cl was found as the moderate-high concentration causing an approximately 42% inhibition of cell growth at the end of 25 days of incubation. Thus, an ammonium-supplied group in this study is defined as *D. tertiolecta* grown in modified Johnson's medium with 500 mg/L NH_4Cl as an inorganic nitrogen source.

Starting cell density was approximately $1x10^4$ cells/mL for all groups. For experimentation, cells from the stock cultures were centrifuged at 900 *g* for 3 min, and the pellets were washed two times by using N-free Johnson's medium. The pellets were then re-suspended in respective medium and the cells were grown under constant light exposure on a rotary shaker with 120 rpm speed and incubated as stated above. Initial pH values in all media were set to 7.5 in advance of microalgae inoculation, media pH values were followed during experiment period. Microalgae from at least three biological replicates of each experimental groups were cultivated under defined conditions for an experimental period of 25 days. Growth response of *D. tertiolecta* was followed during 25 days by calculating cell numbers with a hemocytometer using Lugol solution (Sigma) to immobilize microalgae. For experimental analysis, microalgae were harvested on 1st, 3rd, 6th, 10th and 15th days of incubation.

Oxygen Evolution

Net oxygen evolution activity of *D. tertiolecta* was measured using a Clark-type oxygen electrode system (HansatechOxytherm, Hansatech Ins. Ltd., Norfolk, U.K.) as done by (Kaliamurthi, Selvaraj, Cakmak, & Cakmak, 2016) . 2 mL of cell culture with adjusted absorbance value of (A_{680} = 2.0) with respective solutions was inoculated into the reaction vessels and continuously stirred at 25°C. Cells were first adapted to

dark conditions for 2 min, and then oxygen evolution rate was measured under dark and illuminated conditions. Each process was recorded for 5 min. The intensity of the illumination in the vessel was 480 µmol photons $m^2.s^{-1}$. Net oxygen evolution was calculated by subtracting consumed oxygen rate from the light-saturated rate of oxygen evolution.

Quantification of Total Chlorophyll, Carotenoids, and HPLC Analysis of Lutein and β-carotene

Spectrophotometric quantification of total chlorophyll and carotenoids was performed as described by (Jeffrey, & Humphrey, 1975). Forchromatographic analysis of β-carotene and Lutein (Sigma-Aldrich, USA), the lyophilized microalgae samples were extracted with ethanol (1 mL) and mixed by vortexing. Samples were developed by using 40 kHz ultrasonicbath with a power of 200 W and then placed in an ultrasonicbath at 40^0C for 2 h. Then samples were transferred into the vialsforchromatographic analysis.

Quantification of luteinand β-carotene of *D. tertiolecta* was determined as described by(Chagas, Rios, Jarenkow, Marcilio, Ayub, & Rech, 2015) with modifications. The Standard calibration curvesfor β-carotene and lutein were obtained by using Standard stock solution sprepared in Methyltert-butylether (MTBE):acetonitrile (50:50, v/v) with 0.5, 1, 2, 4, or 8 µg/mLfor β-carotene, and 2, 4, 8, 16 or 32 µg/mLforlutein. An Agilent 1200 HPLC-DAD system (Waldbronn, Germany) equipped with a vacuum degasser, binary pump, autosampler, and a diode-array detector was used to determine the carotenoids in the extracts. The column used was C18 column (ZorbaxEclipseplus) 100 mm × 2.1 mm and 1.8 µm particle size (AgilentTech, Böblingen, Germany). The elution flow rate was set at a constant 200 mL/min at 25°C. The mobile phase was acetonitrile / methanol / MTBE, starting with a ratio of 20:75:5 reaching 10:90:10 in 12 min, 0:90:10 in 25 min, 0:75:25 in 40 min, and finally 0:50:50 in 60 min.

Fourier Transform Infrared Spectroscopy (FT-IR) Measurement of TAGs

Approximately 50 mg of wet biomass was obtained by means of absorbance calculation (A_{680}=4) and centrifugation at 2000 *g* for 3 min under 4°C. Then samples were vacuum-dried at 40°C for 1 hour and dried algae sample was pelleted and placed on sampler module. Infrared spectra of the sampler were recorded over a wavenumber range of 4,000 to 400 cm^{-1} with 128 scans on a Fourier transform infrared spectroscopy (PerkinElmer-L160000A, USA) equippedwith an ATR module. The bands were assigned to specific molecular groups on the basis of biochemical standards and published studies as previously described (Mairet, Bernard, Masci, Lacour, & Sciandra, 2011). FT-IR peak values attributed to ester group (C=O) vibration of triglycerides (1744 cm^{-1}) and amide I absorption (1652 cm^{-1}) was used for calculation of changes in triglyceride levels. The deviation of FT-IR spectrum level of amide I band was not more than 14% in any group so amide I band was taken as reference for FT-IR spectra normalization and ratio determination. Relative TAG content was determined by calculating the ratio of TAG band to the amide I band as described before (Dean *C.*, 2010). For each time point, ratios of the TAG to Amide I of control samples were arbitrarily assigned to a value of 1; thus, increases in relative TAG content of N-starved and ammonium-supplied *D. tertiolecta* was displayed as “-fold increase” in the ratio of the TAG to Amide I band.

Fluorescence Quantification and Imaging of Neutral Lipids

Spectrofluorometric quantification of neutral lipids was achieved by using fluorescent Nile Red staining method as described by (Elsey, Jameson, Raleigh, & Cooney, 2007). Relative fluorescence intensity of Nile Red staining was quantified on a fluorescence spectrometer (Schimadzu RF-6000, Japan) using 530 nm excitation and 570 nm emission wavelengths.

For fluorescence imaging of neutral lipid bodies, cells were stained with Nile Red (5 µg/mL final concentration; Invitrogen) by incubating on a shaker under dark conditions for 15 min. Images were acquired using a Zeiss AXIO Imager M2 fluorescence microscope system. For imaging, TXRED ($560nm_{ex}$-$630nm_{em}$) filter was used to image lipid droplets in Nile red-stained cells.

Fatty Acid Methyl Ester (FAME) Quantitation

FAME quantitation was performed as suggested by (Praveenkumar, Shameera, Mahalakshmi, Akbarsha, & Thajuddin, 2012). A 300µl extraction buffer (MeOH containing 2% H_2SO_4 v/v) was added to approximately 20 mg lyophilized algal sample including 30 µg nonadecanoic acid (Sigma-Aldrich, USA) as an internal standard. Samples were incubated for 2h at 80°C and 750 rpm. Then, samples were cooled down to room temperature, supplemented with 300 µl NaCl and 300 µl hexane, vortexed, centrifuged and put in a rack under room temperature for phase formation. Then, the hexane layer on upper phase was transferred into a glass insert vial for Gas Chromatography (Thermo, TRACE 1310) analysis. One microliter of each sample was injected into FAMEWAX column (Restek, USA) (30m x 32mm ID x 25µm film thickness). The temperature program was set to initial 120°C with 10 min hold; 5°C increment up to 230°C with a 5 min hold. Column flow was adjusted to 22.2 mL/min. The instrument condition was as follows: carrier gas

nitrogen; FID set to 260°C, and a split ratio of 10:1. Each sample was analyzed in triplicates, and FAME identification was done by comparison with standard certificate, Supelco FAME mix C8-C24 (Bellefonte, PA, USA).

Statistical Analysis

Each experiment was repeated twice with three biological replicates. Thus, final data in this article are the mean values of at least three separate samples collected at two different times (n=6). Means of averages with standard errors are presented throughout the manuscript and data evaluation was done by using t-tests (two tails, pair type) with the significance criteria of 0.05 to assess the significance between different groups evaluated for the same time point.

Results and Discussion

Inorganic Nitrogen Dependent Changes in Growth

Ammonium-supplied group is determined as *D. tertiolecta* grown in modified Johnson's medium with 500 mg/L NH_4Cl as an inorganic nitrogen source which caused around 42% decrease at the end of 25 days of incubation (Figure 1a). Approximately 50% inhibition of growth of*D. tertiolecta* was reported when the ammonium concentration of the growth medium was adjusted to 500 mg/L (Gutierrez, Kwan, Zimmerman, & Peccia, 2016). The proposed nitrate concentration in Johnson's medium (which is 1000 mg/L) and half concentration of nitrate (500 mg/L) were also compared. No significant differences were observed on growth when the KNO_3 concentration was 500 mg/L or 1000 mg/L. (Chen *et al*., 2011) also did not report any significant change in growth of *D. tertiolecta* when they reduced nitrate concentration by half. On the other hand, when applied as a nitrogen source, 500mg NH_4Cl introduces approximately 130.8 mg N while 1000mg KNO_3 introduce approximately 138.6 mg N to the medium. So the N content of KNO_3 and half amount of NH_4Cl are close to each other. Thus, we applied 1000mg/L KNO_3 concentration for the controlled group and 500mg/L concentration of NH_4Cl was used for the ammonium-supplied group.

Ammonium supply and nitrogen deprivation caused significant decreases in growth of *D. tertiolecta* (Figure 1b). Ammonium-supplied and N-deprived microalgae entered stationary phase on the 10th day of incubation with a value of 670x10^4 and 550x10^4 cells respectively. On the other hand, control group microalgae entered stationary phase on the 15th day with 1150x10^4 cells and ended up with 1270x10^4 cells at the end of 25 days of the incubation period (Figure 1b). The decrease in cell growth as a response to N-deprivation is a common response of all eukaryotic microalgae (Sharma, Schuhmann, & Schenk, 2012), and likewise, inhibition of growth by high ammonium concentration was reported before (Collos & Harrison, 2014).

In order to see whether the decrease in growth is a cause or a result of a change in pH, the cells were incubated in the control medium with different initial pH levels (pH=4, 6, 7.5 or 10) where nitrate was used as nitrogen source. Surprisingly, exponential growth phase of *D. tertiolecta* was longer, and a higher cell amount was recorded at the end of 25 days of incubation when the initial pH was acidic, mainly 4 or 6 when compared with controlled or alkali medium (Figure 1c). On the other hand, the change in pH was directly associated with deceleration of the growth in N-deprived and ammonium-supplied *D. tertiolecta*. To check pH changes in the medium as a response to a change in inorganic nitrogen source, initial pH value was set to 7.5 for all media and changes in pH during 25 days of growth were followed. The increase of pH value for first 3 days of incubation was observed as a common response to microalgal growth in all groups (Figure 1d). The pH value was recorded as 8.9, 8.75, and 7.8 in controlled, N-deprived, and ammonium-supplied microalgae growth media on the 3rd day of incubation. The pH level of growth medium was stable in control group after the 3rd day and ended up with a pH value of 8.95 at the end of 25 days of incubation. The pH value of N-free medium decreased after the 3rd day and lasted with a value of 7.1 which was slightly lower than starting pH value of 7.5. On the other hand, there was a gradual decrease in pH value of ammonium-supplied medium after the 3rd day of incubation. The pH was recorded as 6.4 on the 6th day and ended up with a value of 3.95 at the end of 25 days of the incubation period (Figure 1d). Decreased pH values as a response to ammonium nutrition of microalgae were reported before (Goldman, Dennett, & Riley, 1982). The decrease in pH during growth of algae on ammonium based growth medium was reported to stem from the release of H^+ ions to the medium and an increase of pH in nitrate based growth medium was due to the release of OH^- ions (Goldman *et al*., 1982). Thus, it can be concluded that decrease in growth accompanied by the simultaneous decrease of medium pH level might be a result of ammonium nutrition but not the main cause of deceleration of the growth rate of *D. tertiolecta*. Supportively, internal ammonium levels resulting from ammonium uptake was reported as the source of oxidative stress in *Dunaliellasalina* (Giordano, 1997) and *Chlamydomonasreinhardtii* (Giordano, Norici, Forssen, Eriksson, & Raven, 2003).

Oxygen Evolution and Chlorophyll Content

Net oxygen evolution rate of *D. tertiolecta* increased approximately 21% and 49% in N-deprived

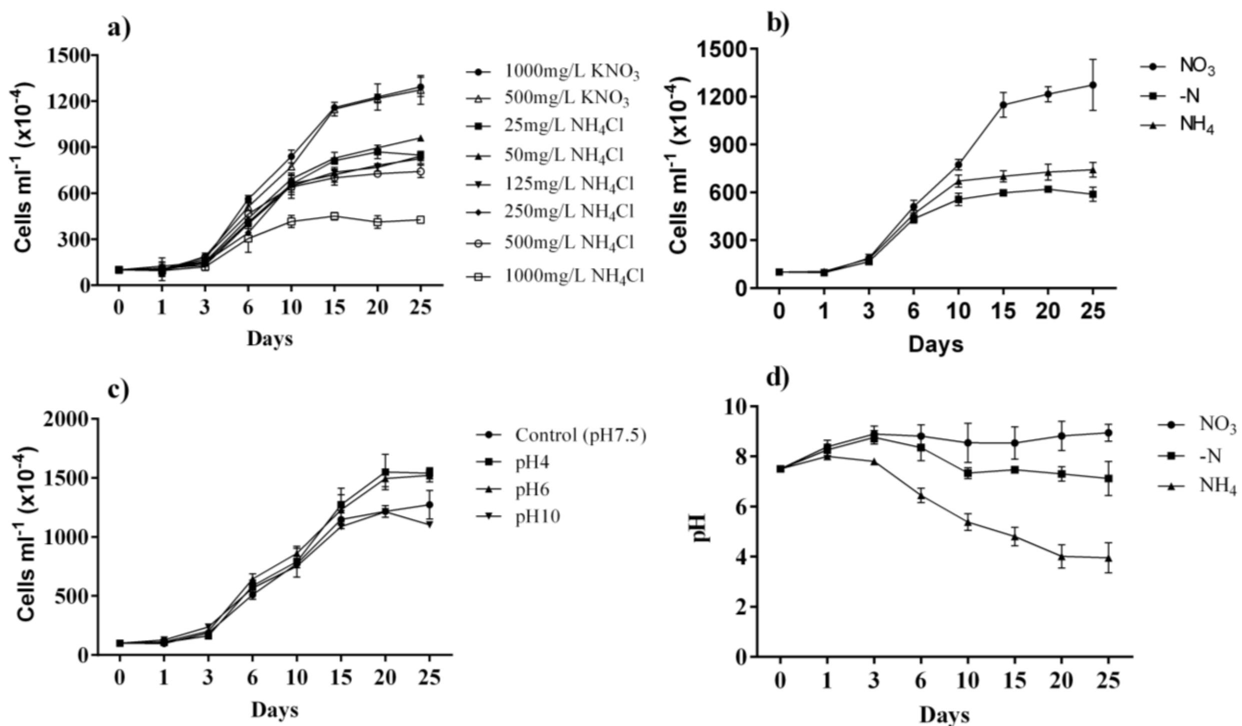

Figure 1. Growth analysis under different conditions. *D. tertiolecta* was first incubated in Johnson's medium with different ammonium concentrations (a) to find out moderately high ammonium concentration. The strain was incubated in Johnson's medium with 500mg/L NO_3, NH_4 or N-free conditions (b). Change in pH was recorded (c), and impact of initial pH was analyzed (d) during 25 days of incubation. For all data sets, each point represents the mean (±SD) of at least six replicate culture flasks.

and ammonium-supplied microalgae during first 3 days of incubation (Figure 2a). Increased photosynthetic oxygen evolution activity of *D. tertiolecta* was reported as a response to a mild salt stress while a progressive inhibition was highlighted when the salt concentration was high (Goyal, 2007). In this study, following 3 days of increased oxygen evolution activity, a gradual decrease in oxygen evolution ratio was followed on 6th and 10th days of incubation and ended up with approximately 56% and 27% decrease in N-deprived and ammonium-supplied *D. tertiolecta* at the end of 15 days of the incubation period (Figure 2a). It seems that not only the level but also the duration of the nutrient stress is effective on dynamic adjustment of anabolic and catabolic reactions in *D. tertiolecta*.

Total chlorophyll content of N-deprived *D. tertiolecta* showed a time-dependent gradual decrease while chlorophyll a/b ratio did not show remarkable variations during 15 days of experimental period (Figure 2b). A time-dependent decrease in total chlorophyll content was reported as an autophagy response of *D. tertiolecta* which would induce accumulation of lipids and carbohydrates (Tan, Lin, Shen, & Lee, 2016). On the other hand, ammonium nutrition did not cause a change in chlorophyll content and Chl a/b ratio for first 6 days but total chlorophyll content of *D. tertiolecta* increased while Chl*a/b* ratio decreased after the 6th days of incubation (Figure 2c). Inverse proportion of chl*a* to increasing ammonium concentration in *D. tertiolecta* was reported previously (Fabregas, Abalde, & Herrero, 1989).

Evaluation of Carotenoid Production

The total carotenoid content of N-deprived *D. tertiolecta* did not show any significant changes during 15 days of incubation (Figure 3a). Supportively, (Kim, Liu, Lee, Hong, Cho, Lee, Lee & Choi, 2013) reported an only slight decrease in total carotenoid content and productivity of *D. tertiolecta* as a response to nitrogen deprivation. Likewise, (Yilancioglu, Cokol, Pastirmaci, Erman, & Cetiner, 2014) showed that total carotenoid content of *D.salina* was not affected by nitrogen limitation. On the other hand, ammonium nutrition triggered long-term increases in the total carotenoid content of *D. tertiolecta*. There was no significant change in carotenoid content for first 6 days but it increased and ended up with 21% higher carotenoid content at the end of 15 days of the incubation period (Figure 3a). This increase might be related to the simultaneous increase of total chlorophyll content or ammonium stress itself that causes a cellular redox imbalance. Because carotenoids are not only vital players of photosynthesis as light harvesters and photo-protectors but also they display very high antioxidant properties (Markou & Nerantzis, 2013). Thus, change in the level of two main primary carotenoids found in *D. tertiolecta*, β-carotene and lutein levels, were measured.

The β-carotene content of *D. tertiolecta* increased for first 6 days, reached the maximum level of 19% increase on the 6th day of incubation under N-

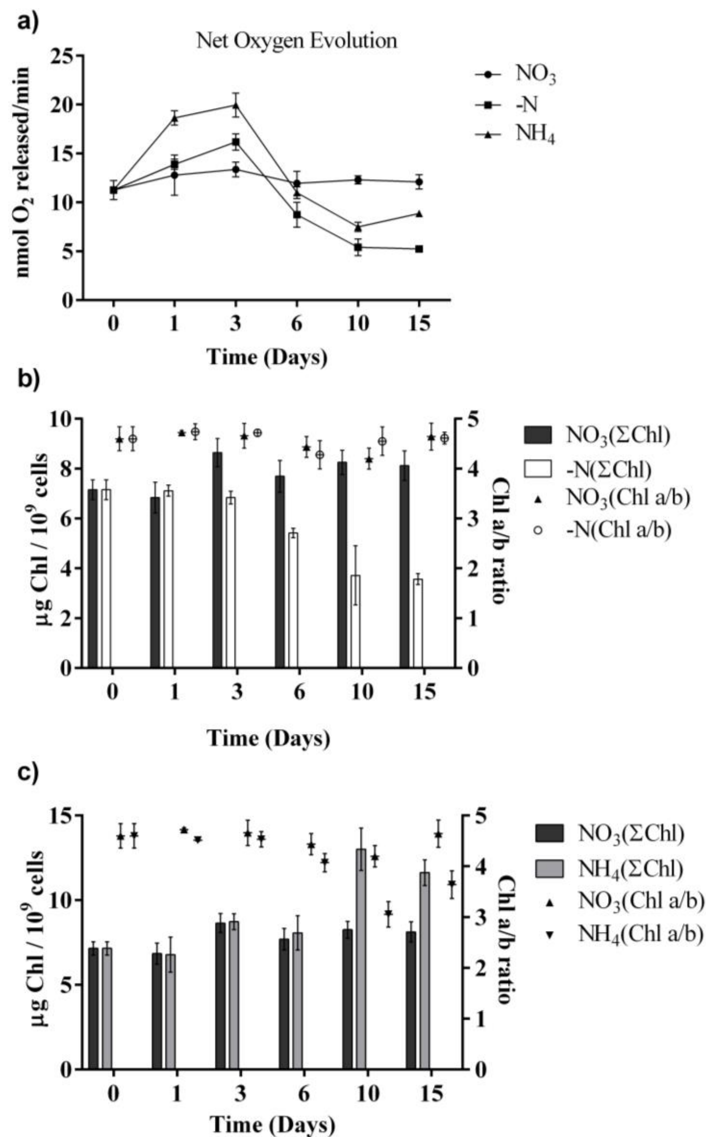

Figure 2. Changes in net oxygen evolution (a), total chlorophyll vs Chl a/b ratio of N-deprived (b) and ammonium supplied (c) *D. tertiolecta* against nitrate supplied controls. For all data sets, each point represents the mean (±SD) of at least six replicate culture flasks. NO_3, nitrate as a nitrogen source for microalgae, NH_4, ammonium as a nitrogen source for microalgae, -N, no nitrogen source for microalgae.

deprivation; however, there was no significant change recorded afterward (Figure 3b). On the other hand, the β-carotene content of *D. tertiolecta* showed a dramatical increase of approximately 40% on the first day of ammonium nutrition and finally, a 21% increased β-carotene level was recorded at the end of 15 days of the incubation period (Figure 3b). *D.salina* is the most pronounced microalgae species for β-carotene production. Nutrient starvation, particularly nitrogen, sulfur, and phosphorus starvation, was reported to increase β-carotene production in *D. salina* (Phadwal & Singh, 2003). This study shows that this is the case for *D. tertiolecta* as well. Additionally increased salinity and light stress were reported to induce β-carotene production in *D. salina* (Coesel, Baumgartner, Teles, Ramos, Henriques, Cancela, & Varela, 2008). Increased β-carotene content may be related to the level and duration of the oxidative stress in *Dunaliella* strains. It was previously reported that nitrogen source would differentiate β-carotene production targets of *D. tertiolecta* and nitrite reduced β -carotene level while it did not change in response to ammonium or urea supplementation (Abalde, Fabregas, & Herrero, 1991). However, the current study shows that there is a clear increase of β -carotene level as a response to ammonium nutrition. This difference is probably because of the concentration of ammonium applied. They used low concentration while the moderate-high concentration of ammonium was employed in this study.

There was a significant decrease in lutein content of *D. tertiolecta* grown under N-deprivation (Figure 3c).

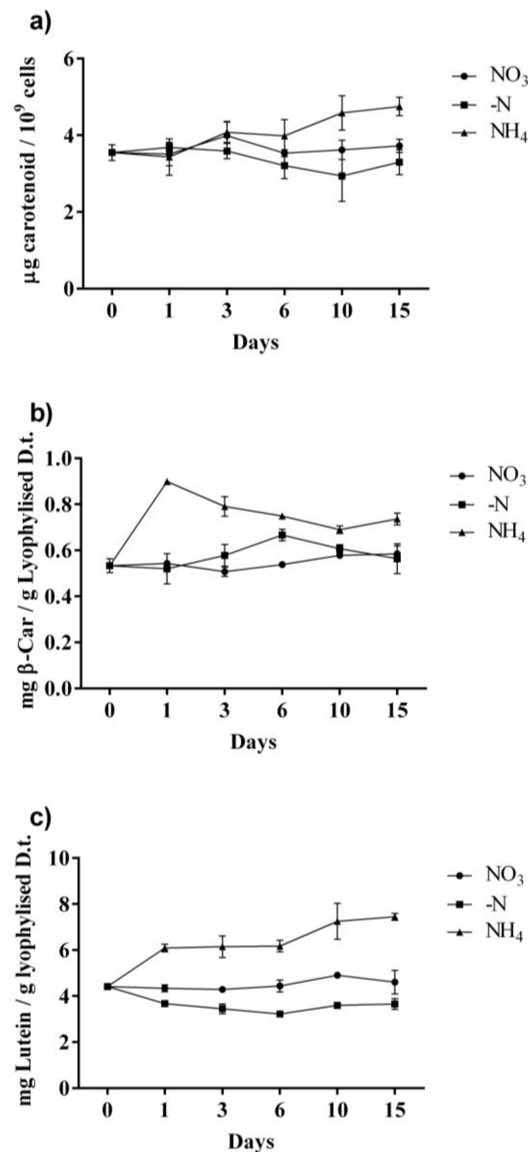

Figure 3. Changes in total carotenoids (a), β-carotene (b), and lutein (c) concentrations in N-deprived, ammonium or nitrate supplied *D. tertiolecta*. For all data sets, each point represents the mean (±SD) of at least six replicate culture flasks. NO_3, nitrate as a nitrogen source for microalgae, NH_4, ammonium as a nitrogen source for microalgae, -N, no nitrogen source for microalgae.

Lutein content decreased about 18% on the first day, it was found lower than the control group at each time point and approximately 26% decrease of lutein content was recorded on the 15th day of N-deprivation. Conversely, there was a rapid increase in lutein content of ammonium-supplied *D. tertiolecta* starting from the first day of the experiment (Figure 3c). The increase in lutein content was 28% on the first day and 38% increase was recorded at the end of 15 days of growth. Strikingly, increase in β-carotene level was most pronounced on first days of ammonium nutrition while this is not the case for lutein production. There was a continuous increase in lutein production as a response to ammonium nutrition. As a major carotenoid, lutein is mainly involved in harvesting blue light and take action as a hydrogen donor to save photosystems against oxidative stress (Markou & Nerantzis, 2013). Thus, lutein production is expected to increase in response to oxidative stress. On the other hand, depending on the duration or type of the stress, microalgae may face irreparable damage that could lead cell death (Fu, Paglia, Magnusdottir, Steinarsdottir, Gudmundsson, Palsson, Andresson, & Brynjolfsson, 2014). Results of this study show that ammonium nutrition stimulates lutein production while it is degraded in response to N-deprivation in *D. tertiolecta* (Figure 3b, 3c). Lutein production by *Muriellopsis*sp. was reported to decrease in response to nitrogen deprivation (Del Campo, Moreno, Rodriguez, Vargas, Rivas, & Guerrero, 2000). Amongst microalgae, *Muriellopsis*sp. (Del Campo *et al*., 2000), *Scenedesmus*sp. (Sanchez, Fernandez, Acien, Rueda,

Perez-Parra, & Molina, 2008), *Chlorella* sp. (Wei, Chen, Chen, Zhang, Liu, & Zhang, 2008), and *Dunaliella*sp. (Fu *et al*., 2014) were reported as potential lutein producers in the literature. Most recently, *D. tertiolecta* with the lutein content representing around 40% (mass fraction) of its total carotenoids was reported as 2-40 times higher than in other important food sources of lutein (e.g. parsley, carrot, red pepper and broccoli) which would be an excellent source of lutein that could be commercially exploited by the food and pharmaceutical industries (Diprat, Menegol, Boelter, Zmozinski, Rodrigues Vale, Rodrigues, & R, R., 2017). Our data suggest that *D. tertiolecta* can be exploited as both β-carotene and lutein source when ammonium is supplied as a nitrogen source.

Dissection of Triacylglycerol Production

It is well known that nitrogen limitation or deprivation stimulates lipid droplet formation in a variety of microalgae and *D. tertiolecta* is not an exception to this fact (Sharma, *et al*., 2012). In this study, N-deprivation and ammonium nutrition induced neutral lipid production in *D. tertiolecta* as suggested by fluorescent Nile Red staining measurements (Figure 4a). Approximately 22% increase in neutral lipid content of *D. tertiolecta* on day 6 was found statistically significant and gradual increase of neutral lipid content reached up to 234% at the end of 15 days of incubation under N-deprivation. On the other hand, ammonium nutrition caused an increase in neutral lipid content of *D. tertiolecta* up to 20% on the 10th day and ended up with 43% increase at the end of 15 days of incubation period. It seems that not only the nitrogen availability but the source is an important factor that induces lipid accumulation in *D. tertiolecta*.

Following spectrofluorometric detection of neutral lipid content, FT-IR measurement was performed for detection of changes in triacylglycerol (TAG) level of *D. tertiolecta* in response to inorganic nitrogen form and availability. As suggested by FT-IR results, TAG levels of *D. tertiolecta* showed a gradual increase in response to N-deprivation and ammonium nutrition (Figure 4b). Nitrogen deprivation stimulated

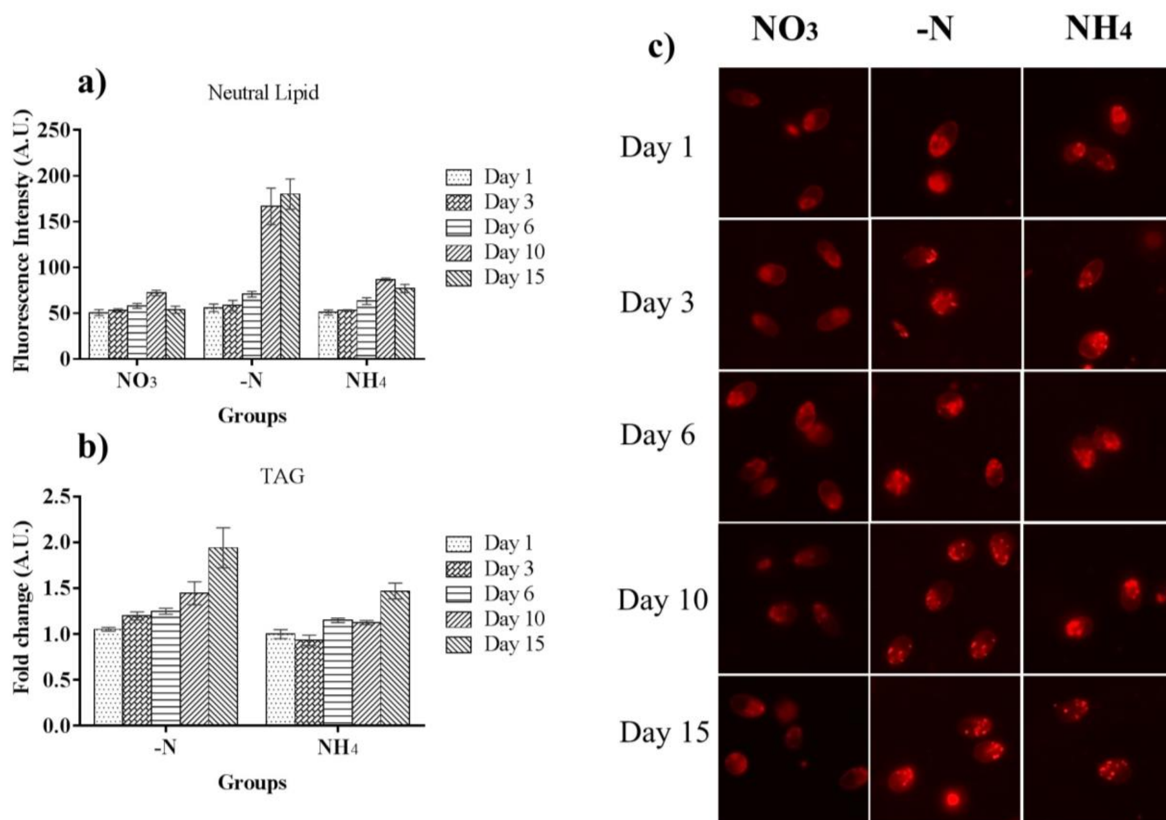

Figure 4. Changes in neutral lipid content (a), TAG: amide I ratio (b), and representative fluorescent microscopy imaging of N-deprived, ammonium or nitrate supplied *D. tertiolecta*. Neutral lipid content of the cells was measured by fluorescence measurement following Nile Red staining. For the evaluation of TAG: amide I ratios, each FTIR spectrum was separately acquired from randomly selected groups of cells. Ratios of the TAG to amide I of control samples were arbitrarily assigned a value of 1, independently for each time point. Thus, comparisons of changes between different time points are not possible in this representation. For imaging, TXRED ($560nm_{ex}$-$630nm_{em}$) filter was used to image lipid droplets in Nile red-stained cells. For the graphs, each point represents the mean (±SD) of at least six replicate culture flasks. NO_3, nitrate as a nitrogen source for microalgae, NH_4, ammonium as a nitrogen source for microalgae, -N, no nitrogen source for microalgae.

TAG production up to 1.95 fold by the end of 15 days of incubation. Besides, ammonium nutrition induced a 1.15 fold increase in relative TAG content on the 6th day and ended up with 1.47 fold increase at the end of 15 days of incubation. It seems that the neutral lipids in *D. tertiolecta* are mainly composed of TAGs. Supportively, (Rabbani, Beyer, Von Lintig, Hugueney, & Kleinig, 1998) reported that TAGs are the main component of neutral lipid bodies in *Dunaliellabardawil*.More recently, (Pick & Avidan, 2017) reported that polar lipids and starch made by photosynthetic carbon assimilation at the early stages of N deprivation might be utilized for the synthesis of TAG in *D. tertiolecta*.

In this study, FT-IR results were in accordance with data obtained by spectrofluorometric measurements showing that N-deprivation and, to a lesser extent, ammonium nutrition induces a time-dependent increase of neutral lipid and TAG production in *D. tertiolecta*. Lastly, cytoplasmic lipid body formation was followed by fluorescence imaging of Nile-Red staining of *D. tertiolecta* (Figure 4c). Corresponding to the regular morphology of *D. tertiolecta*, ellipsoidal cells were gradually replaced with more spherical cell morphologies with aggregated lipid body formation in response to N-deprivation. On the other hand, ammonium nutrition did not induce a morphological change, yet time-dependent cytoplasmic lipid body formation was clearly visible (Figure 4c). Change in cellular morphology is probably related to the level of lipid body formation in *D. tertiolecta*. Increased cellular biovolume and spherical cell morphology were associated with the density and volume of neutral lipid bodies in another flagellated microalga *Chlamydomonasreinhardtii* (Cakmak, Angun, Demiray, Ozkan, Elibol, & Tekinay, 2012).

FAME Composition

FAME profile of lipids is of central importance for biodiesel production as density, lubricity, cetane number, viscosity, NOx emissions, the heat of combustion, and oxidative stability of resulting biodiesel is mainly dependent on FAME profile (Francisco, Neves, Jacob-Lopes, & Franco, 2010). When unsaturated fatty acids (UFAs) concentration is high, the NOx emission ratio increases as Polyunsaturated fatty acids (PUFAs) are prone to oxidation (Saraf & Thomas, 2007). On the other hand, esterification of saturated fatty acids (SFA) results with lower cetane number and increased the stability of produced biodiesel as SFAs are more resistant to auto-oxidation (Sharma *et al*., 2012). Hence, the saturation level of lipids is expected to be high for use in biodiesel production (Praveenkumar *et al*., 2012). In the present study, results showed that ammonium nutrition improves FAME profile of *D. tertiolecta* for biodiesel production via an exclusive impact on increased saturation and a concomitant reduction of PUFA level.

For a comparative evaluation of FAME profiles of experimental groups in a logarithmic growth phase and linear growth phase, extracted lipids on 6th and 15th days of nitrate-fed, ammonium-supplied or N-deprived *D. tertiolecta* was analyzed for their FAME composition (Table 1). Nitrogen deprivation of *D. tertiolecta* caused slight changes in the ratio of some specific fatty acids; however, it did not cause an overall manipulation of fatty acids composition for first 6 days while only slight, but statistically significant ($P<0.05$) increase was observed in total SFA content at the end of 15 days of incubation. This result is in concert with a recent publication where researchers made a comparative lipidomic profiling of two *D. tertiolecta* strains and they reported a minor increase in SFA content when both strains were grown under nitrate-deficient conditions (Kim, Ahn, Lim, Hong, Cho, Lee, Lee, & Choi, 2015).

FAME analysis showed that ammonium nutrition caused a dramatic increase in saturation level of fatty acids in *D. tertiolecta*. There was an approximately 1.55

Table 1. FAME (C16-C18) composition of microalgal lipids extracted on 6th and 15th days of incubation. For all data sets, each point represents the mean (±SD) of at least six replicate culture flasks. NO_3, nitrate as a nitrogen source for microalgae, NH_4, ammonium as a nitrogen source for microalgae, -N, no nitrogen source for microalgae

FAMEs	C16-C18 FAME composition (%)					
	Day 6			Day 15		
	NO3	NH4	-N	NO3	NH4	-N
C16:0	25,6±2,6	36,4±1,9	28,9±1,5	24,1±1,4	42,6±3,1	31,3±2,1
C16:1	0,7±0,1	9,8±0,7	1,1±0,2	0,8±0,2	11,9±0,8	1,4±0,1
C16:2	1,2±0,1	1,3±0,1	1,3±0,2	1,3±0,5	1,4±0,2	1,2±0,1
C16:3	0,7±0,1	0,5±0,1	1,1±0,2	1,3±0,1	0,9±0,1	1,0±0,1
C18:0	2,8±0,4	7,8±0,2	1,6±0,1	2,9±0,1	11,7±0,7	2,0±0,1
C18:1	4,6±0,2	7,7±0,4	3,8±0,6	3,7±0,2	6,9±0,1	3,5±0,2
C18:2	9,2±2,1	11,9±0,5	10,1±1,4	12,1±1,1	13,4±1,4	11,3±0,4
C18:3	55,2±2,6	24,6±1,1	51,9±1,7	53,8±1,9	11,2±1,2	48,3±3,1
Σ SFA	28,4±3,1	44,2±2,2	30,7±1,7	27±1,6	54,3±3,8	33,3±2,3
Σ MUFA	5,3±0,3	17,5±1,1	4,94±0,8	4,5±0,4	18,8±0,5	4,9±0,3
Σ PUFA	66,3±4,9	38,3±1,8	64,4±3,5	68,5±3,6	26,9±2,9	61,8±3,7

fold increase in SFA ratio on the 6th day of ammonium nutrition, and the increase was detected as 2 fold at the end of 15 days of incubation. Moreover, MUFA content increased over 3.3 and 4.1 fold in ammonium-supplied *D. tertiolecta* on the 6th and 15th day of incubation. Strikingly, overall PUFA content decreased over 42% and 61% on 6th and 15th days of ammonium nutrition. Most recently, (Gutierrez *et al*., 2016) also reported that high ammonium concentration induces an aggressive increase in saturation level of lipids in *D. tertiolecta* while it is not the case for *Neochloris oleoabundans, Chlorella sorokiniana,* and *Nannochloropsis occulata*. In this study, the decrease in overall PUFA content mainly stemmed from decreased C18:3 (linolenic acid) content. According to European standard EN 14214 requirements (European standard EN 14214, 2008) the level of C18:3 (linolenic acid) in FAME fuel is supposed to be lower than 12%. The linolenic acid ratio was 57% and 48.3% in nitrate-fed and N-deprived *D. tertiolecta* while it was detected as 11.2% when ammonium was supplied as a nitrogen source during 15 days of experiment period. Lastly, SFA+MUFA to PUFA ratio of ammonium-supplied *D. tertiolecta* meet the European standard EN 14214 requirements (European standard EN 14214, 2008).

In conclusion, this study reports that ammonium nutrition can be employed as an effective approach for induction of major carotenoids and triacylglycerol production in *D. tertiolecta*. Short term ammonium nutrition favor β -carotene and lutein production while triacylglycerol production may necessitate longer incubation periods. Lastly, ammonium nutrition improves FAME profile of *D. tertiolecta* for biodiesel production via an exclusive impact on increased saturation and a concomitant reduction of PUFA.

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