Influence of long term nitrogen limitation on lipid, protein and pigment production of *Euglena gracilis* in photoheterotrophic cultures

**Marika Tossavainen** Corresp., 1, **Usman Ilyass** 1,2, **Velimatti Ollilainen** 3, **Kalle Valkonen** 1,4, **Anne Ojala** 1,5,6, **Martin Romantschuk** 1

1 Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, University of Helsinki, Lahti, Finland
2 Oy Soya Ab / Jalofoods, Tammisaari, Finland
3 Department of Food and Nutrition Sciences, University of Helsinki, Helsinki, Finland
4 Kyrö Distillery Company, Isokyrö, Finland
5 Faculty of Agriculture and Forestry, Institute of Atmospheric and Earth System Research (INAR)/Forest Sciences, University of Helsinki, Helsinki, Finland
6 Faculty of Biological and environmental Sciences, Helsinki Institute of Sustainability Science (HELSUS), University of Helsinki, Lahti, Finland

Corresponding Author: Marika Tossavainen
Email address: marika.tossavainen@helsinki.fi

Nitrogen limitation is considered a good strategy for enhancement of algal lipid production while conversely N repletion has been shown to result in biomass rich in proteins. In this study the influence of long-term N limitation on *Euglena gracilis* fatty acid (FA), protein, chlorophyll *a*, and carotenoid concentrations was studied in N limited cultures. Biomass composition was analyzed from three time points from N starved late stationary phase cultures, exposed to three different initial N concentrations in the growth medium. Total lipid content increased under N limitation in ageing cultures, but the low N content and prolonged cultivation time resulted in the formation of a high proportion of saturated FAs (SAFAs). Furthermore, growth as well as the production of proteins, chlorophyll *a* and carotenoids were enhanced in higher N concentrations and metabolism of these cellular components stayed stable during the stationary growth phase. Our findings showed that a higher N availability and a shorter cultivation time is a good strategy for efficient *E. gracilis* biomass production, regardless of whether the produced biomass is intended for maximal recovery of polyunsaturated FAs (PUFAs), proteins, or photosynthetic pigments. Additionally, we showed an increase of neoxanthin, β-carotene, and diadinoxanthin as a response to higher N availability.
Influence of long term nitrogen limitation on lipid, protein and pigment production of *Euglena gracilis* in photoheterotrophic cultures

Marika Tossavainen¹, Usman Ilyass¹,², Velimatti Ollilainen³, Kalle Valkonen¹,⁴, Anne Ojala¹,⁵,⁶, Martin Romantschuk¹

¹Faculty of Biological and Environmental Sciences, Ecosystems and Environment Research Programme, University of Helsinki, Lahti, Finland

²Oy Soya Ab / Jalofoods, Tammisaari, Finland

³Department of Food and Nutrition Sciences, University of Helsinki, Helsinki, Finland

⁴Kyrö Distillery Company, Isokyrö, Finland

⁵Faculty of Agriculture and Forestry, Institute of Atmospheric and Earth System Research (INAR)/Forest Sciences, University of Helsinki, Helsinki, Finland

⁶Faculty of Biological and Environmental Sciences, Helsinki Institute of Sustainability Science (HELSUS), University of Helsinki, Lahti, Finland

Corresponding author:

Marika Tossavainen¹

Email address: marika.tossavainen@helsinki.fi
Abstract

Nitrogen limitation is considered a good strategy for enhancement of algal lipid production while conversely N repletion has been shown to result in biomass rich in proteins. In this study the influence of long-term N limitation on *Euglena gracilis* fatty acid (FA), protein, chlorophyll *a*, and carotenoid concentrations was studied in N limited cultures. Biomass composition was analyzed from three time points from N starved late stationary phase cultures, exposed to three different initial N concentrations in the growth medium. Total lipid content increased under N limitation in ageing cultures, but the low N content and prolonged cultivation time resulted in the formation of a high proportion of saturated FAs (SAFAs). Furthermore, growth as well as the production of proteins, chlorophyll *a* and carotenoids were enhanced in higher N concentrations and metabolism of these cellular components stayed stable during the stationary growth phase. Our findings showed that a higher N availability and a shorter cultivation time is a good strategy for efficient *E. gracilis* biomass production, regardless of whether the produced biomass is intended for maximal recovery of polyunsaturated FAs (PUFAs), proteins, or photosynthetic pigments. Additionally, we showed an increase of neoxanthin, β-carotene, and diadinoxanthin as a response to higher N availability.
1. Introduction

Microalgae are useful organisms in the field of biotechnology, and the main interest is currently in the production of high value substances for human and animal nutrition, cosmetics, and pharmaceuticals (Pulz & Gross, 2004; Spolaore et al., 2006). As primary producers of long chain polyunsaturated fatty acids (LC-PUFAs) microalgae are an ecological alternative for fish oils in food, food supplements, and aquaculture feed (Pulz & Gross, 2004; Harwood & Gushina, 2009; Van Hoestenberghe et al., 2016) and provide a protein source with an amino acid composition comparable to other plant based proteins (Becker, 2007). Algal pigments are used as colorants, antioxidants, and vitamin A precursors in cosmetics and nutritional products (Spolaore et al., 2006; Vílchez et al., 2011), and anti-inflammatory, antitumor, and antibacterial properties of chlorophylls and carotenoids have been reported (Vílchez et al., 2011; da Silva Ferreira & Sant’Anna, 2017). The potential of several microalgal strains for these applications is widely studied, but only a few strains are utilized commercially. For example, *Crypthecodinium* is a producer of LC-PUFA docosahexaenoic acid (DHA) (Pulz & Gross, 2004), *Chlorella* and *Spirulina* are protein-rich strains used in human nutrition, *Dunaliella salina* is a natural source of β-carotene and *Haematococcus fluvialis* is used for astaxanthin production for aquaculture feed (Spolaore et al., 2006).

Algal growth and biomass composition is regulated by environmental factors, such as light, temperature and availability of essential nutrients and carbon (C). Growth is enhanced under optimal light and temperature and with abundant nutrients and C. Generally, in nutrient replete growth conditions, cellular C is mostly allocated to formation of nitrogen (N) containing macromolecules, especially proteins, and nucleic acids, but also chlorophylls, amino acids, and betaine glycine (Geider & La Roche, 2002). However, variation in N allocation between cellular
protein (59.3-96.8% of Total N) and non-protein N containing compounds is broad, and the
importance of non-protein compounds tends to be greater during the exponential growth phase
than in aging cultures (Lourenço et al., 1998). In addition, C partitioning between starch and
lipids has been shown to be regulated by N availability. In N replete conditions, C is allocated to
starch synthesis whereas lipid synthesis is inhibited and vice versa (Wang et al., 2015). In N
limited growth, cellular C flow turns into non-N containing compounds, especially neutral lipids
(NL), carbohydrates, and carotenoids (Geider & La Roche, 2002). Cellular phosphorus (P) is
mostly allocated to RNA, DNA and phospholipids (PLs) (Geider & La Roche, 2002). Within the
lipid fraction, growth phase and the availability of C, N, and P also influence cellular fatty acid
(FA) composition. Generally, under optimal growth conditions, cellular lipids mainly consist of
membrane lipids such as PLs, which are rich in polyunsaturated FAs (PUFAs) and long chain
polyunsaturated FAs (LC-PUFAs) (Hodgson et al., 1991). Synthesis of saturated and
monounsaturated FAs (SAFAs and MUFAs), typical in storage lipids, is enhanced during the
stationary growth phase under nutrient depletion (Hodgson et al., 1991).

Among the microalgae, *Euglena gracilis* is known as a producer of PUFAs (Schwarzhans et al.,
2015), proteins (Becker, 2007), vitamins B, C, and E (Baker et al., 1981; Takeyama et al.,
1997), chlorophylls *a* and *b*, several types of carotenoid pigments (Takaichi, 2011), and the
carbohydrate paramylon (β-1,3-glucan) (Santek et al., 2009). A decrease in cellular PUFA
content in stationary growth phase cells in comparison to exponential growth phase cells in N
deprived, photoheterotrophic *E. gracilis* cultures has been shown (García-Ferris et al., 1996) and
the protein content has been proven to decrease under N limitation (Regnault, Piton & Calvayrac,
1990). A decrease in chlorophyll and total carotenoid production by *Euglena gracilis* was
observed during a short term exposure to N deprivation (García-Ferris et al., 1996).
So far, production of particular cellular components of *E. gracilis* as a response to environmental factors have mostly been studied separately, and in many studies the biochemical composition is analyzed only at single time points (Hulanicka, Erwin & Bloch, 1964; Rocchetta et al., 2006). However, earlier studies have observed a transition in cellular biochemical composition between the exponential and stationary growth phases (Regnault et al., 1995). To date, the most thorough study of FA formation during the growth of *E. gracilis* under photoheterotrophic and heterotrophic conditions with different concentrations of glucose (C source) and proteose peptone (N source) is provided in the study by Schwarzhans et al. (2015). However, Schwarzhans et al. (2015) did not analyze the cellular nutritional status of *E. gracilis*, and it is thus unclear whether C or N limited the growth.

For utilization of microalgae biomass as such or for maximizing production of specific compounds it is important to determine the influence of growth conditions on biomass composition. Since N limitation is often considered the most critical factor regulating cellular metabolism, this study aimed to measure the time-dependent influence of long-term N deprivation on growth and biomass composition of *E. gracilis* in photoheterotrophic growth conditions and to investigate the influence of N limitation on cellular metabolism during the stationary phase. Biomass N and C accumulation, total lipid, protein, chlorophyll *a*, and carotenoid contents as well as the FA profile of *E. gracilis* were analyzed at three time points of late stationary phase. Main carotenoids were identified and quantified at the end of the cultivation. Our first hypothesis was that higher N content in the growth medium boosts N and C uptake and thus the growth of *E. gracilis*, and that exposure to N limitation increases lipid production and decreases protein production. Second, we hypothesized that the proportion of PUFAs is higher in biomass grown under high initial N concentrations and it decreases as a
function of time. Third, higher N concentrations were hypothesized to favor chlorophyll \( a \) production while carotenoid production was assumed to be independent of N concentration since the carotenoids, acting in light harvest and photoprotection, do not contain N (Christaki et al., 2012).

2. Material and Methods

Strain, medium and culturing methods

*E. gracilis* (CCAP 1224/5Z) was cultivated in autoclaved (120 °C, 2 bar, 1 h) modified Hutner medium (Takeyama et al., 1997) with the following modifications; L-Glutamic acid was not used, the amount of glucose was reduced to 5 g L\(^{-1}\), \((\text{NH}_4\text{})_2\text{SO}_4\) was added as an extra N source, \(\text{CaCO}_3\) was replaced with \(\text{CaCl}_2\) (0.2 g L\(^{-1}\)), and the amount of vitamin B\(_{12}\) was doubled from the original to 0.02 mg L\(^{-1}\). Three different N levels were used in the experiments: 0.0, 0.2 and 0.5 g L\(^{-1}\) of \((\text{NH}_4^+)\)_2\text{SO}_4\) (hereafter called low N=LN, medium N=MN and high N=HN treatments).

The composition of the cultivation medium and the modified trace element solution are given in table 1. The initial ammonium nitrogen (\(\text{NH}_4^+\)-N) concentrations in the treatments were 42.5, and 84.9 and 148.5 mg L\(^{-1}\), respectively. N limitation, rather than P or C, during the stationary growth phase, was confirmed by using high P containing medium and both glucose and CO\(_2\) as a C source.

Culturing with three replicates was done in a growth chamber (SANYO growth cabinet MLR-350; 294L, SANYO Electric Co. Ltd, Japan) in 2 L borosilicate bottles with a cultivation volume of 1.6 L. 10 mL of algal seed culture with DW 2.7 g L\(^{-1}\) was used as an inoculant.

Cultivation bottles were equipped with aeration, degassing and harvesting pipes. The light and
dark cycle was 16:8, light intensity 170 µmol m\(^{-2}\) s\(^{-1}\) (Li-Cor 190R Quantum Sensor and LI-1400 Light Sensor Logger, Li-Cor, Lincoln, Nebraska, USA), and temperature 25 °C. Cultures were fed with 2% CO\(_2\) (99.8%) in moist air (0.5 L min\(^{-1}\)) during the light period. For mixing the supplied gas, compressed air (10 L min\(^{-1}\), Hailea 318 air compressor) and CO\(_2\) (0.2 L min\(^{-1}\)) were pumped via flasks half filled with distilled H\(_2\)O, and from the gaseous phase of the flask, the mixture of moist air and CO\(_2\) was supplied to the cultivation bottles trough PTFE membrane filters (Acro®37 TF Vent).

**Sampling and growth determination**

Biomass growth was followed as DW, with samples taken twice a week. DW was determined as described by Tredici & Zitteli (1998) from samples filtered onto pre-dried (105 °C, overnight) glass fiber filters (GF/C 47, Whatman). Specific growth rates (µ, d\(^{-1}\)) during the exponential growth phase (days 0-5) were calculated using the equation µ=\(\frac{\text{Ln} (\text{DW}_1 / \text{DW}_0)}{t_1 - t_0}\), where \(\text{DW}_0\) and \(\text{DW}_1\) are the biomass DWs at the beginning \((t_0)\) and end \((t_1)\) of the exponential growth.

The supernatants were collected for analysis of NH\(_4\)-N in the cultivation medium. Exponential growth ceased after five days and samples for C, N, FA, and total carotenoid and chlorophyll \(a\) analysis were taken from the late stationary growth phase on cultivation days 14, 16 and 19 and for HPLC analysis of carotenoid pigments on cultivation day 19. Biomass was collected on day 19 by centrifugation (Kendro Laboratory Products, Multifuge 1S-R, Germany) (3000 rpm, 4 °C, 15 min). Algal pellets for C and N analyses were stored at -20 °C and for lipid and pigment analysis at -70 °C. Before analysis, biomass pellets were freeze-dried (B. Braun Biotech International - Christy® Alpha 1-4, Germany). Approximately 100 mg of dried biomass was used for C and N analyses and lipid extraction, and 40 mg for pigment extraction.
Analytical methods

$\text{NH}_4^+-\text{N}$ in the growth medium was analyzed using Hach Lange Kits (Hach Lange, Germany) and a DR 2800TM spectrophotometer (Hach Lange, Germany). C and N content of the biomass was analyzed with a Leco CNS-2000 analyzer (Leco Corporation, St Joseph, MI, USA). Lipids were extracted according to the method described by Parrish (1999) and modified by Natunen et al. (2017). Methylation was carried out with the modified method (Natunen et al., 2017) of Christie & Han (2010). FAs were analyzed with GC–MS (GC/MS-QP 2010 Ultra SYSTEM, Shimadzu, USA) equipped with an autosampler (AOC-20 s, Shimadzu, Japan) and the operating software (GCMS solution, Version 2.6) using a DB-23 capillary column (Agilent Technologies). The temperature program for GC-MS analysis was set as described earlier (Natunen et al., 2017; Tossavainen et al., 2017). FAs were identified using retention times and mass spectra of FAs in FAME standard solution (Supelco™ 37 Component FAME Mix, Sigma). For quantification, a quantitative FAME standard mix was prepared in four concentrations and standard curves were made for each FAME. FAs were quantified with internal standard method using deuterated octadecanoic acid (C18:0-d3) (Larodan Fine Chemicals, Sweden) as an internal standard (Natunen et al., 2017). Total FA (TFA) content was calculated as a sum of quantified FAs.

Biomass protein content was calculated by multiplying cellular N content by 4.78, which is the average conversion factor for microalgae (Lourenço et al., 2004).

Pigments were extracted by accelerated solvent extraction (ASE-350, Dionex) (110 °C, 20min) using acetone as an extraction solvent. After extraction, acetone was evaporated under nitrogen flow, and samples were then dissolved in 10 mL of MeOH for HPLC analysis. For total carotenoid and chlorophyll analysis, 1 mL of MeOH extract was evaporated, and the pigments were dissolved in EtOH and filtered (PTFE syringe filter, 0.2 µm, VWR International) before
analysis. Total carotenoid and chlorophyll \( a \) concentrations were measured spectrophotometrically (UV-Vis spectrophotometer, UV-2401PC, Shimadzu) using wave lengths 450 nm for carotenoids and 665 nm for chlorophyll \( a \). Total carotenoid concentration was calculated using Beer-Lamberts law and a specific absorption coefficient of 2620 (A\(1\%\) cm\(^{-1}\)) for \( \beta \)-carotene in ethanol (Rodriguez-Amaya, 2001). Chlorophyll \( a \) concentration was calculated using absorption coefficient 84 (L g\(^{-1}\) cm\(^{-1}\)) and the standard protocol (SFS 5772).

For HPLC analysis of carotenoid pigments, two separate standard mixes with five (first mix) and four (second mix) concentrations for identification and quantification were prepared. The first contained fucoxanthin, neoxanthin, astaxanthin, zeaxanthin, cantaxanthin, \( \beta \)-carotene (Sigma-Aldrich Chemie GmbH, Germany) and lutein (CaroteNature GmbH, Switzerland) (Fig. S1), and the second included violaxanthin, diadinoxanthin, diatoxanthin, alloxanthin, myxoxantophyll and echinenone (DHI, Denmark) (Fig. S2). Trans-\( \beta \)-apo-8`-carotenal (Sigma-Aldrich Chemie GmbH, Germany) was added both to the standard mix and to the samples to confirm stability of retention times. Carotenoids were analyzed from MeOH extracts with HPLC (Prominence liquid chromatograph, LC-20AT, Shimadzu) using YMC carotenoid C30 column (250 x 4.6 mml.D.) (YMC, America, INC.), and the detection wave length 450 nm (Prominence UV/Vis detector, Shimadzu). Essentially the analysis was performed as described in the column manufacturer’s instructions: Elution solvent A for MeOH:MTBE:H\(_2\)O was prepared according to instructions (81:15:4 vol), and elution solvent B was slightly modified (16:80:4 vol). The flow rate was 1 ml min\(^{-1}\) and the running time in analysis (100% A to 100% B) was 55 min. Concentrations were quantified using the external standard method. All the extraction and preparation steps were carried out in dim light to avoid deterioration of pigments.
A high definition mass spectrometer (Synapt G2-Si Q-Tof, Waters, Milford, MS, USA) equipped with APCI interface in positive mode was used to characterize diadinoxanthin in *E. gracilis* samples. The instrument settings were as following: mass range 50-2000amu, corona current 7µA, probe and source temperatures 400 and 120°C, desolvation gas 880L/h, trap collision energy 30V. For accurate mass measurement the instrument was calibrated with a mixture of sodium iodide and Ultramark standard material. Proper mass calibration was considered to be <3ppm. Leucine enkephaline ([M+H]⁺ = 556.2766 amu, Waters, Milford, MS, USA) served as a lock mass calibrant.

Statistics

Influence of time and N treatment on N and C accumulation, growth (biomass DW), protein, TFA, PUFA, MUFA, chlorophyll *a*, and carotenoid, contents were analyzed using repeated measures ANOVA. Tukey’s test was used as a post hoc –test. Because of heterogeneity in variances (Levene statistics, test of homogeneity of variances), statistics for SAFA and LC-PUFA contents were performed with the non-parametric Friedman’s test. A significance level of *P*<0.05 was used in all tests. Results from non-parametric tests were Bonferroni corrected. All statistical analyses were done with SPSS (Version 24, IBM USA).

3. Results

Growth and C: N ratio

N availability regulated biomass growth of *E. gracilis*. Higher N content in the cultivation medium boosted growth, and differences in biomass production between LN, MN and HN.
treatments started to appear on day 5 when exponential growth ceased (Fig 1). Growth rates
during the exponential growth phase were 0.88±0.01, 0.91±0.01 and 0.97±0.02 d\(^{-1}\) in LN, MN
and HN treatments, respectively. A slow increase in biomass DW continued until the end of
cultivation in all cultures, but the increase was significant (P<0.05) only in MN and HN cultures.
Biomass yield was highest in HN and lowest in LN cultivation (P<0.05) at all three sampling
points when the samples for biochemical analysis were taken. At the end of the cultivation,
biomass yields in HN, MN and LN treatments were 4.5, 4.1 and 2.9 g L\(^{-1}\), respectively.
In all cultures, NH\(_4\)-N was rapidly removed from the cultivation medium, apparently as a result
of uptake by the algae (Fig. 2). NH\(_4\)-N was taken up almost completely after five cultivation days
in LN (91.1\%) and MN (97.1\%) treatments and on day seven in HN (98.2\%) treatment.
The proportions of N and C in stationary phase biomass reflected the amount of added NH\(_4\)-N in
the medium, i.e., the highest initial NH\(_4\)-N addition (HN treatment) resulted in higher biomass N
and C concentrations (P<0.05), whereas there was no significant difference between MN and LN
treatments (P>0.05). In all cultures biomass N content decreased and C content increased
slightly, but not significantly (P>0.05) between days 14 and 19, when the cultures had reached
the stationary phase (Table 2). The proportion of N and C in DW after 14, 16 and 19 days of
cultivation were 2.0-2.1 and 45.5-47.8\% in LN, 2.3-2.5\% and 47.0-47.9\% in MN and 3.7-3.9\%
and 47.9-48.8\% in HN treatments, respectively (Table 2). Based on the molar C: N ratio in algal
biomass (under optimal growth conditions C: N ratio = 6.6; Redfield, 1958; Geider & La Roche,
2002) all the cultures were N limited at the time samples for C: N analysis were taken. In all
cultures the C: N ratio increased towards the end of cultivation. In the LN treatment, the C: N
ratio was 25 on day 14 and increased to 28 in the last samples. In MN and HN treatments, C: N
ratios were 22 and 14 on the first sampling day and increased to 24 and 16, respectively, towards the end of the cultivation (Table 2).

Biochemical composition

In all N-treatments, TFA content of *E. gracilis* increased towards the end of the cultivation (P<0.05) (Fig. 3). Although TFA contents were always highest in the late growth phase on day 19, i.e., 121, 123 and 99.5 mg g⁻¹ (12.1, 12.3 and 9.95 % of DW) in LN, MN and HN treatments, respectively, the differences between cultures were not statistically significant (P>0.05).

Culture age, as well as N-treatment, influenced the degree of FA unsaturation. Generally, the pro-longed cultivation time resulted in higher SAFA and lowered PUFA contents. The SAFA content increased in MN and HN treatments (P<0.05) and the PUFA content decreased in MN treatment (P<0.05) (Table 3). The MUFA and LC-PUFA contents remained stable (P>0.05) in all cultures until the end of the cultivation (Table 3). Influence of different N treatments on proportions of SAFAs and LC-PUFAs were significant only when comparing HN and LN treatments. The contents of SAFAs were lower (44.4-52.9%) and LC-PUFAs higher (26.1-29.3) in HN treatment than in LN treatment (SAFAs 68.1-71.5% and LC-PUFAs 18.2-19.2%) (P<0.05), whereas the differences were not significant between LN and MN or MN and HN treatments (P>0.05) (Table 3). The PUFA content was highest in HN treatments (13.4-18.3%), and MUFA content was lower in the cultures from LN treatment (6.2-6.5%) than in other cultures (P<0.05). The main FAs in all cultures were C14:0 and C16:0 SAFAs. The most abundant PUFA in all cultures was α-linolenic acid (ALA) and the most abundant LC-PUFAs were arachidonic acid (ARA) and eicosapentaenoic acid (EPA) (Table 3). In addition, *E. gracilis* is known to produce C16:4 PUFA (Shibata et al. 2018; Tossavainen et al. 2018), but since it was
not included in our standards, it was excluded from the analysis. The nutritional status of cultures
clearly influenced FA metabolism. The high overall C: N ratio in LN treatment and the increase
in C: N ratio in all cultures during the experiment resulted in a higher content of C14:0 and a
lowered C16:0 content. Lower C: N ratio favored the synthesis of PUFAs and LC-PUFAs,
especially ALA, ARA, and EPA.

In each N-treatment biomass protein content was stable (P>0.05) during the stationary phase, but
in the HN treatment the content was higher than in other cultures (P<0.05). Protein contents in
HN treatment varied between 175.1-185.6 mg g\(^{-1}\) (17.51-18.56 % of DW) whereas the protein
contents in LN and MN treatments were 100.2-101.9 mg g\(^{-1}\) (10.02-10.19 % of DW) and 109.9-
120.9 mg g\(^{-1}\) (10.99-12.09 % of DW) (Fig. 4).

In all treatments, the biomass total carotenoid and chlorophyll \(a\) concentrations were stable
during the stationary phase (Fig. 5a, b) (P>0.05). Chlorophyll \(a\) and total carotenoid
concentrations were higher in HN treatment (6554-8250 and 2975-3109 \(\mu g \, g^{-1}\) than in MN
(3595-4638 and 1734-1960 \(\mu g \, g^{-1}\) or LN (2493-3260 and 873-1337 \(\mu g \, g^{-1}\) treatments (P<0.05)
(Fig. 5a, b). Diadinoxanthin (Fig. S2), \(\beta\)-carotene and neoxanthin (Fig. S1) were identified as the
most abundant carotenoid pigments on the last cultivation day. Concentrations of identified
carotenoids were always lowest in LN treatment and highest in HN treatment (Fig. 6).

Diadinoxanthin was eluted as a front part of the double peak (Rt = 12.1min) containing the
carotenoid (m/z 583) and a green pigment (m/z 909). UV-VIS (277, 422, 445 (max), 476nm) and
mass spectra data ([M+H]\(^+\) = 583.4125 amu, 3.6ppm error, and fragment ions (m/z 565, 547,
221) confirmed the presence of diadinoxanthin (Young & Britton 2012). Due to this co-elution
of diadinoxanthin with a green pigment, diadinoxanthin amounts were considered only as
suggestive values.
4. Discussion

As hypothesized, the higher N addition in HN treatment boosted N and C uptake and resulted in a higher biomass yield of *E. gracilis*. Exponential growth in all cultures ceased after five days, whereas the NH$_4$-N was exhausted from the medium on day five in LN and MN treatments and on day seven in the HN treatment. This indicates that growth was N limited in LN and MN cultures on day five but in the thicker HN culture, the primary reason for growth slowing down was probably light limitation and decreased photosynthetic activity. However, the high C: N ratio in stationary phase cultures shows that all cultures were N limited in late stationary phase; this was seen already on day 14 when the first samples for C: N analysis were taken. The slow assimilation of C to biomass in stationary phase cultures was not significant (P>0.05). Biomass DW increased significantly only in MN and HN treatments between days 14 and 19 (P<0.05). Sufficient amount of NH$_4$-N in HN culture resulted also in higher C and N contents (P<0.05). Thus, the differences in biomass production between cultures can be explained by influence of initial NH$_4$-N concentrations on cellular metabolism. Continued C assimilation and growth under short-term exposure to N starvation has been shown earlier in cultures of *Isochrysis zhangjiangensis*, whereas long-term N deprivation restricted both assimilation and growth as a response to decreased photosynthetic activity (Wang et al., 2015). Deterioration of photosynthetic pigments in N deprived cells has been shown earlier (Wang et al., 2015; da Silva Ferreira & Sant’Anna, 2017) and indications of this effect was also seen in this study. Additionally, C uptake of *E. gracilis* from organic substrates (Ogbonna, Ichige & Tanaka, 2002) allows C accumulation also under conditions restricting photosynthesis, which makes growth under N deprivation less sensitive to chlorophyll degradation.
TFAs were the only cellular compounds, which quantitatively were influenced by prolonged incubation (P<0.05), whereas the differences between treatments were not significant (P>0.05). This result indicates that for TFA production of *E. gracilis*, culture age is more significant than N concentration and that under long-term N limitation, and C replete conditions, C flow turns to formation of non-N containing FAs. C allocation to lipid synthesis in C rich and N deplete conditions has also been shown earlier (Wang et al. 2015). Clear enhancement of lipid production in aging cultures has also been observed earlier (Schwarzhans et al., 2015), and here this enhancement took place regardless of initial N concentrations. Additionally, as shown earlier, significant enhancement of FA content occurs after the transition from exponential growth phase to the stationary phase (Regnault et al., 1995). In addition, the availability of C is essential for FA synthesis, and in C and N limited stationary phase cultures of *E. gracilis*, C deficiency results in lower FA content than under C replete conditions, regardless of N availability (Regnault et al., 1995). However, under heterotrophic growth conditions, paramylon formation is enhanced, whereas lipid production is improved in photoheterotrophic growth (Schwarzhans et al., 2015). Earlier, high glucose content has been shown to shift metabolism of *E. gracilis* towards heterotrophy (Schwarzhans et al., 2015). Thus, photoheterotrophy in the low glucose medium used in this study can be assumed to be a good strategy for lipid production in N depleted growth conditions. The C availability for lipid formation is ensured under N limited conditions which restricts chlorophyll formation and thus inhibits photosynthetic C assimilation.

Algal FA composition defines the usefulness of the produced lipids for different applications. Following earlier studies (Regnault et al., 1995; Schwarzhans et al., 2015), our results showed that high N concentration and short cultivation time results in an FA composition of good nutritional quality since generally the LC-PUFA and PUFA contents were high and SAFA
contents were low. The influence of time for FA saturation degree was significant (P<0.05) only for PUFAs in MN and SAFAs in MN and HN treatment. This indicates that FA metabolism is more sensitive to initial N concentrations than to the length of time under N deprivation. LC-PUFAs and PUFAs are typically structural compounds in cell membranes and their proportion in total FAs is relatively high in optimal growth conditions (Hodgson et al., 1991). Thus, it can be assumed, that relative proportion of membrane lipids in cultures grown in HN conditions was higher than in other treatments.

Also, the high content of C14:0 SAFA in LN and MN treatments emphasizes the importance of nutritional status. C14:0 is more injurious to humans than other SAFAs, since it more efficiently elevates the blood LDL cholesterol, thus increasing the risk of cardiovascular diseases (Dubois et al., 2007). A slight increase of C14:0 and decrease in C16:0 SAFA contents were seen here during the growth in all cultures. A similar trend of simultaneous lowering of C16:0 content and increase of C14:0 content has been shown earlier in aging cultures (Schwarzhans et al., 2015).

Low N and high organic C content in the growth medium induces wax ester synthesis in E. gracilis cells (Regnault et al., 1995) and the high content of SAFAs in LN treatment indicates the same. Harvesting in the late stationary phase ensures high biomass content and TFA yield.

However, a cultivation of E. gracilis under N depletion results in an undesirable FA profile with low PUFA and high SAFA content, and is thus not a recommended method for production of lipids for food or feed applications. Furthermore, from the seventh day onwards, the biomass in the HN treatment was much higher than in LN treatment. The higher biomass compensates the lower TFA content and results in similar TFA yields, but with less saturated FAs. By knowing this, we can simplify the production process of E. gracilis based PUFAs and LC-PUFAs; maximum yield is achieved faster allowing a short harvesting cycle. With the proposed method a
long N starvation period is not needed for maximizing the yield thereby saving costs in large scale production. Results also indicate that for the optimal LC-PUFA and PUFA production, maximizing biomass production of *E. gracilis* instead of FA content is more important.

As assumed, higher N availability resulted in higher protein content (P<0.05), indicating that N augmentation is a good strategy for production of protein-rich biomass for food or feed applications. However, the protein content in our cultures was low in comparison to earlier reported protein contents of 31-61% in *E. gracilis*. In general, protein content in algal biomass can vary between 6-63% (Becker, 2007). Since N is the key component in proteins, the low protein content of our cultures was a consequence of N limitation. A general response to lower cellular N content and higher C: N ratio in biomass is a decrease in protein as well as non-protein N compounds, as has been shown for several marine microalgae (Lourenço et al., 2004). A drastic decrease in cellular protein content of *E. gracilis* immediately after exposure to N depletion was demonstrated by Regnault, Piton & Calvayrac (1990).

Chlorophyll *a* concentration increased as a response to HN treatment (P<0.05), a phenomenon shown earlier (Regnault, Piton & Calvayrac, 1990; Lourenço et al., 2004). Declining chlorophyll concentrations are typical of stationary phase algal cultures (Lourenço et al., 2004), and since N is a structural component in chlorophyll (da Silva Ferreira & Sant’ Anna, 2017), this decline is probably at least partly a consequence of nutrient limitation. Chlorophyll degradation under N depletion has been observed in cultures of *E. gracilis* and *Isochrysis zhangjiangensis* (García-Ferris et al., 1996; Wang et al., 2015), but our results reveal that chlorophyll *a* concentrations are stable during the stationary phase (P<0.05). Thus, we assume that degradation of chlorophyll at the end of the exponential growth phase and its stability during the late stationary growth phase might be a response to the switch from autotrophic to photoheterotrophic growth in conditions
where chlorophyll formation and photosynthetic activity is inhibited. The influence of changing light intensities on increased chlorophyll \( a \) synthesis in HN treatment cannot, however, be completely excluded, since light limitation has been shown to boost chlorophyll synthesis (Geider, Macintyre & Kana, 1997).

Against our hypothesis, in the HN treatment, the total carotenoid concentrations also increased (\( P<0.05 \)). Structurally, carotenoids are N free compounds, and the observed increase under HN conditions may be related to the need for N in the formation of pigment-protein complexes in the thylakoid membrane (Takaichi, 2011). Alternatively, production of light harvesting primary carotenoids was enhanced in the dense culture. The primary carotenoids have functions in photosynthesis, as light harvesting or photoprotecting pigments, whereas the secondary carotenoids are metabolized under stress conditions (Christaki et al., 2012), i.e., during nutrient deficiency or high light intensity (Grung & Liaaen-Jensen, 1993). However, response to N as well as P limitation seems to be specific for different carotenoid pigments. For example, the content of the secondary carotenoid astaxanthin in \( \textit{Haematococcus pluvialis} \) increased under N and P limitation, whereas the concentrations of primary carotenoids lutein and \( \beta \)-carotene, and the secondary carotenoid cantaxanthin decreased (Boussiba et al., 1999). The major carotenoids identified here (neoxanthin, diadinoxanthin and \( \beta \)-carotene), have been classified as primary carotenoids of \( \textit{Euglena sanguinea} \) (Grung & Liaaen-Jensen, 1993).

We could confirm diadinoxanthin as the most abundant carotenoid pigment in \( \textit{E. gracilis} \), which is following earlier findings (Brandt & Wilhelm, 1990; Schagerl, Pichler & Donabaum, 2003; Kato et al., 2017). Diatoxanthin is a minor carotenoid in \( \textit{E. gracilis} \) (Schagerl, Pichler & Donabaum 2003; Takaichi, 2011; Kato et al., 2017), but it was not present in our cultures, whereas \( \beta \)-carotene was shown here and earlier to be one of the major carotenoids of the species.
(Goodwin & Jamikorn, 1954; Heelis et al., 1979; Takaichi, 2011). However, both diadinoxanthin and diatoxanthin are pigments synthesized in the so-called diadinoxanthin cycle, and in diatoms this cycle has importance in photoprotection (Lepetit et al. 2010). In high light conditions, diadinoxanthin is de-epoxidized to diatoxanthin, and in low light intensity, diatoxanthin is epoxidized back to diadinoxanthin (Lepetit et al. 2010). This might explain the lack of diatoxanthin in our dense cultures. The stability of total carotenoid content during the stationary phase (P>0.05) shows that in *E. gracilis*, carotenoids are not degraded when the growth conditions change unfavorable for photosynthesis. Thus, a rapid transformation of stored diadinoxanthin to diatoxanthin can provide an excellent photoprotection system when cells are exposed to high light intensity. Lutein has also been identified as an abundant carotenoid in *E. gracilis* (Goodwin & Jamikorn, 1954), but this was later claimed to be incorrect, and the corresponding pigment was first identified as antheraxanthin (Krinsky & Goldsmith, 1960) and later as diadinoxanthin (Heelis et al., 1979).

Our study is the first one to reveal the influence of N limitation on carotenoid composition of *E. gracilis* on the late stationary growth phase. The response to N availability was similar for all quantified carotenoids, which is in line with earlier studies showing that concentrations of β-carotene in *Haematococcus pluvialis* (Boussiba et al., 1999) and diadinoxanthin in *Heterocapsa* sp. (Latasa & Berdalet, 1994) cultures decrease under N limitation. Knowledge of carotenoid production in *E. gracilis* is still insufficient and contradictory, and in future, the differences between genotypes or response of pigment biosynthesis to different growth conditions should be clarified. Presently, diadinoxanthin is not utilized in biotechnology. The diadinoxanthin cycle pigments are important in photoprotection and singlet oxygen scavenging in algae, and
antioxidative properties and the potential applications of diadinoxanthin need further
investigations.

Conclusion

This study showed that long term N limitation is not a good strategy to boost lipid production of
*E. gracilis* for nutritional use. Long cultivation time and strict N limitation results in higher TFA
concentrations but poor FA composition with low PUFA and LC-PUFA concentrations and high
SAFA content. Greater availability of N results in higher protein, chlorophyll *a*, and carotenoid
concentrations. Thus, N availability is critical for the maximal production of PUFAs, LC-
PUFAs, proteins and pigments and long-term N limitation is not a recommended method for
production of *E. gracilis* biomass for nutritional purposes.

Acknowledgements

We thank our laboratory personnel Riikka Koivula and Santeri Savolainen, for helping in the
laboratory work. We also thank John Allen, who checked the language of the manuscript.

References

Baker ER, McLaughlin JJA, Hutner SH, DeAngelis B, Feingold S, Frank O, Baker H. 1981.
Water-soluble vitamins in cells and spent culture supernatants of *Poteriochromonas stipitata,*
Euglena gracilis, and Tetrahymena thermophila. Archives of Microbiology 129: 310-313 DOI: 10.1007/BF00414703.

Becker EW. 2007. Micro-algae as a source of protein. Biotechnology Advances 25: 207-210 DOI: 10.1016/j.biotechadv.2006.11.002.

Boussiba S, Bing W, Yuan J, Zarka A, Chen F. 1999. Changes in pigments profile in the green alga Haematococcus pluvialis exposed to environmental stresses. Biotechnology Letters 21: 601-604 DOI: 10.1023/A:1005507514694.

Brandt P, Wilhelm C. 1990. The light-harvesting system of Euglena gracilis during the cell-cycle. Planta 180: 293-296 DOI: 10.1007/BF00194010.

Christaki E, Bonos E, Giannenas I, Florou-Paneri P. 2012. Functional properties of carotenoids originating from algae. Journal of the Science of Food and Agriculture 93: 5-11 DOI: 10.1002/jsfa.5902.

Christie WW, Han X. 2010. Preparation of derivates of fatty acids. In: Lipid Analysis, Isolation, Separation, Identification and Lipidomic Analysis. The Oily Press, 145-158.

da Silva Ferreira V, Sant’Anna C. 2017. Impact of culture conditions on the chlorophyll content of microalgae for biotechnological applications. World Journal of Microbiology and Biotechnology 33:20. DOI: 10.1007/s11274-016-2181-6.

Dubois V, Breton S, Linder M, Fanni J, Parmentier M. 2007. Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. European Journal of Lipid Science and Technology 109 710-732. DOI: 10.1002/ejlt.200700040.
García-Ferris, C., de los Rios, A., Ascaso, C., & Moreno, J. (1996). Correlated biochemical and ultrastructural changes in nitrogen-starved *Euglena gracilis*. *Journal of Phycology* 32: 953-963. DOI: 10.1111/j.0022-3646.1996.00953.x.

Geider, R., & La Roche, J. (2002). Redfield revisited: variability of C:N:P in marine microalgae and its biochemical basis. *European Journal of Phycology* 37: 1-17. DOI: 10.1017/S0967026201003456.

Geider, J., Macintyre, H. L., & Kana, T. (1997). Dynamic model of phytoplankton growth and acclimation: responses of the balanced growth rate and the chlorophyll a:carbon ratio to light, nutrient-limitation and temperature. *Marine Ecology Progress Series* 148: 187-200. DOI: 10.3354/meps148187.

Goodwin, T., & Jamikorn, M. (1954). Studies in carotenogenesis. Some observations on carotenoid synthesis in 2 varieties of *Euglena gracilis*. *Journal of Protozoology* 1: 216-219. DOI: 10.1111/j.1550-7408.1954.tb00820.x.

Grung, M., Liaaen-Jensen, S. (1993). Algal carotenoids 52; secondary carotenoids of algae 3; carotenoids in a natural bloom of *Euglena sanguinea*. *Biochemical Systematics and Ecology* 21: 757-763. DOI: 10.1016/0305-1978(93)90088-9.

Harwood, J. L., & Guschina, I. A. (2009). The versatility of algae and their lipid metabolism. *Biochimie* 91: 679-684. DOI: 10.1016/j.biochi.2008.11.004.

Heelis, D., Kernick, W., Phillips, G., & Davies, K. (1979). Separation and identification of the carotenoid pigments of stigmata isolated from light-grown cells of *Euglena gracilis* strain Z. *Archives in Microbiology* 121: 207-211. DOI: 10.1007/BF00425057.
Hodgson PA, Henderson JR, Sargent JR, Leftley JW. 1991. Patterns of variation in the lipid class and fatty acid composition of *Nannochloropsis oculata* (Eustigmatophyceae) during batch culture. *Journal of Applied Phycology* 3: 169-181 DOI: 10.1007/BF00425057.

Hulanicka D, Erwin J, Bloch K. 1964. Lipid Metabolism of *Euglena gracilis*. *The Journal of Biological Chemistry* 239: 2778-2787.

Kato S, Soshino M, Takaichi S, Ishikawa T, Nagata N, Asahina M, Shinomura T. 2017. Suppression of the phytoene synthase gene (EgcrtB) alters carotenoid content and intracellular structure of *Euglena gracilis*. *BMC Plant Biology* 17: 125 DOI: 10.1186/s12870-017-1066-7.

Krinsky NI, Goldsmith TH. 1960. The carotenoids of the flagellated alga, *Euglena gracilis*. *Archives of Biochemistry and Biophysics* 91: 271-279 DOI: 10.1016/0003-9861(60)90501-4.

Latasa M, Berdalet E. 1994. Effect of nitrogen or phosphorus starvation on pigment composition of cultured *Heterocapsa* sp. *Journal of Plankton Research* 16: 83-94 DOI: 10.1093/plankt/16.1.83.

Lourenço SO, Barbarino E, Lavín PL, Lanfer Marquez UM, Aidar E. 2004. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. *European Journal of Phycology* 39: 17-32 DOI: 10.1080/0967026032000157156.

Lourenço SO, Barbarino E, Lanfer Marquez UM, Aidar E. 1998. Distribution of intracellular nitrogen in marine microalgae: Basis for the calculation specific nitrogen-to-protein conversion factors. *Journal of Phycology* 34: 798-811 DOI: 10.1046/j.1529-8817.1998.340798.x.

Lepetit B, Volke D, Gilbert M, Wilhelm C, Goss R. 2010. Evidence for the existence of one antenna-associated, lipid-dissolved and two protein-bound pools of diadinoxanthin cycle pigments in diatoms. *Plant Physiology* 154: 1905-1920. DOI: 10.1104/pp.110.166454.
Natunen K, Seppälä J, Koivula R, Pellinen J. 2017. Monitoring cell-specific neutral lipid accumulation in *Phaeodactylum tricornutum* (Bacillariophyceae) with Nile Red staining – a new method for FlowCAM. *Journal of Phycology* 53: 396-404 DOI: 10.1111/jpy.12504.

Ogbonna J, Ichige E, Tanaka H. 2002. Interactions between photoautotrophic and heterotrophic metabolism in photoheterotrophic cultures of *Euglena gracilis*. *Applied Microbiology and Biotechnology* 58: 532-538 DOI: 10.1007/s00253-001-0901-8.

Parrish CC. 1999. Determination of total lipid, lipid classes, and fatty acids in aquatic samples. In: Arts MT, Wainman, BC, ed. *Lipids in freshwater ecosystems*. Springer-Verlag, 4-20.

Pulz O, Gross W. 2004. Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology* 65: 635-48 DOI: 10.1007/s00253-004-1647-x.

Redfield AC. 1958. The biological control of chemical factors in the environment. *American Scientist* 46: 205-221.

Regnault A, Chervin D, Chammai A, Piton F, Calvayrac R, Mazliak P. 1995. Lipid composition of *Euglena gracilis* in relation to carbon-nitrogen balance. *Phytochemistry*. 40: 725-733 DOI: 10.1016/0031-9422(95)00268-C.

Regnault A, Piton F, Calvayrac R. 1990. Growth, proteins and chlorophyll in *Euglena* adapted to various C/N balances. *Phytochemistry*. 29: 3711-3715 DOI: 10.1016/0031-9422(90)85318-A.

Rocchetta I, Mazzuca M, Conforti V, Ruiz L, Balzaretti V, Ríos de Molina Mdel C. 2006. Effect of chromium on the fatty acid composition of two strains of *Euglena gracilis*. *Environmental Pollution* 141: 353-358 DOI: 10.1016/j.envpol.2005.08.035.

Rodriguez-Amaya DB. 2011. *A guide to carotenoid analysis in foods*. ILSI Press, 64p.
Santek B, Felski M, Friehs K, Lotz M, Flaschel E. 2009. Production of paramylon, a β-1,3-glucan, by heterotrophic cultivation of *Euglena gracilis* on a synthetic medium. *Engineering in Life Sciences* 9: 23-28 DOI: 10.1002/elsc.200700032.

Schagerl M, Pichler C, Donabaum K. 2003. Patterns of major photosynthetic pigments in freshwater algae. 2. Dinophyta, Euglenophyta, Chlorophyceae and Charales. *Annales de Limnologie – International Journal of Limnology* 39: 49-62 DOI: 10.1051/limn/2003005.

Schwarzhans J, Cholewa D, Grimm P, Beshay U, Risse J, Friehs K, Flaschel E. 2015. Dependency of the fatty acid composition of *Euglena gracilis* on growth phase and culture conditions. *Journal of Applied Phycology* 27: 1389-1399 DOI: 10.1007/s10811-014-0458-4.

Shibata S, Arimura S, Ishikawa T, Awai K. 2018. Alterations of membrane lipid content correlated with chloroplast and mitochondria development in *Euglena gracilis*. *Frontiers in Plant Science* 9: 370 DOI: 10.3389/fpls.2018.00370.

Spolaore P, Joannis-Cassan C, Duran E, Isambert A. 2006. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* 101: 87-96 DOI: doi.org/10.1263/jbb.101.87.

Takaichi S. 2011. Carotenoids in algae: Distributions, biosyntheses and functions. *Marine Drugs* 9: 1101-1118 DOI: 10.3390/md9061101.

Takeyama H, Kanamaru A, Yoshino Y, Kakuta H, Kawamura Y, Matsunaga T. 1997. Production of antioxidant vitamins, β-carotene, vitamin C, and vitamin E, by two-step culture of *Euglena gracilis* Z. *Biotechnology and Bioengineering* 53: 185-190 DOI: 10.1002/(SICI)1097-0290(19970120)53:2<185::AID-BIT8>3.0.CO;2-K.
Tossavainen M, Lahti K, Edelmann M, Eskola R, Lampi A-M, Piironen V, Korvonen P, Ojala A, Romantschuk M. 2018. Integrated utilization of microalgae cultured in aquaculture wastewater: wastewater treatment and production of valuable fatty acids and tocopherols. *Journal of Applied Phycology* 1-11 DOI: 10.1007/s10811-018-1689-6.

Tossavainen M, Nykänen A, Valkonen K, Ojala A, Kostia S, Romantschuk M. 2017. Culturing of *Selenastrum* on diluted composting fluids; conversion of waste to valuable algal biomass in presence of bacteria. *Bioresource Technology* 238: 205-213 DOI: 10.1016/j.biortech.2017.04.013.

Tredici MR, Zittelli GC. 1998. Efficiency of sunlight utilization: Tubular versus flat photobioreactors. *Biotechnology and Bioengineering* 57: 187-197 DOI: 10.1002/(SICI)1097-0290(19980120)57:2<187::AID-BIT7>3.0.CO;2-J.

Van Hoestenberghe S, Fransman C, Luyten T, Vermeulen D, Roelants I, Buysens S, Goddeeris BM. 2016. *Schizochytrium* as a replacement for fish oil in a fishmeal free diet for jade perch, *Scortum barcoo* (McCulloch & Waite). *Aquaculture Research* 47: 1747-1760 DOI: 10.1111/are.12631.

Vílchez C, Forján E, Cuaresma M, Bédmar F, Garbayo I, Vega JM. 2011. Marine carotenoids: Biological functions and commercial applications. *Marine Drugs.* 9: 319-333 DOI: 10.3390/md9030319.

Wang H, Meng Y, Cao X, Ai J, Zhou J, Xue S, Wang W. 2015. Coordinated response of photosynthesis, carbon assimilation, and triacylglycerol accumulation to nitrogen starvation in the marine microalgae *Isochrysis zhangjiangensis* (Haptophyta). *Bioresource Technology* 177: 282-288 DOI: 10.1016/j.biortech.2014.11.028.
Young A, Britton G. 2012. *Carotenoids in photosynthesis*. Springer Science & Business Media, 498p.
Figure 1

Biomass growth (DW) in *E. gracilis* cultures.

LN=Low N (○), MN=medium N (□), HN=high N (Δ). (Mean ± SE, n=3, error bars are not visible). Differences in biomass DW were statistically significant (p<0.05) in different N treatments.
Figure 2

NH$_4$-N removal in *E. gracilis* cultures grown under different initial N concentrations.

LN=Low N (○), MN=medium N (□), HN=high N (Δ). (Mean ± SE, n=3, smallest error bars are not visible).
Figure 3

TFA contents in different N treatments in the late stationary phase cultures on days 14, 16 and 19.

LN=Low N (grey column), MN=medium N (white column), HN=high N (black column). (Mean ± SE, n=3). Statistically significant (P<0.05) differences in TFA contents (% of DW) during the cultivation are shown (a=day 14, b=day 16, c=day 19). Differences in LN, MN and HN treatments were not statistically significant (P>0.05).
Figure 4

Protein contents in different N treatments in the late stationary phase cultures on days 14, 16 and 19.

LN=Low N (grey column), MN=medium N (white column), HN=high N (black column). (Mean ± SE, n=3). Statistically significant (P<0.05) differences on days 14, 16 and 19 in different N treatments are shown *significantly higher protein content (% of DW).
Figure 5

Chlorophyll a and total carotenoid concentrations in the late stationary phase *E. gracilis* cultures on days 14, 16 and 19.

(A) Chlorophyll a and (B) total carotenoid concentrations (µg g⁻¹) in the late stationary phase cultures on days 14, 16 and 19. LN=Low N (grey column), MN=medium N (white column), HN=high N (black column). (Mean ± SE, n=3). Statistically significant (P<0.05) differences on days 14, 16 and 19 in different N treatments are shown. *significantly higher chlorophyll a or carotenoid content.
Figure 6

Concentrations of neoxanthin, diadinoxanthin and β-carotene in different N treatments at the end of the cultivation.

LN=Low N (grey column), MN=medium N (white column), HN=high N (black column). (Mean ± SE, n=3).
Table 1 (on next page)

Composition of culture medium
| Reagent                        | (g L\(^{-1}\)) |
|-------------------------------|----------------|
| Glucose                       | 5              |
| \((NH_4)_2\) SO\(_4\)        | 0.0-0.5\(^a\) |
| KH\(_2\) PO\(_4\)            | 0.4            |
| \((NH_4)_2\) HPO\(_4\)       | 0.2            |
| MgSO\(_4\) 7H\(_2\)O         | 0.5            |
| CaCl\(_2\)                    | 0.2            |
| H\(_3\)BO\(_3\)              | 0.0144         |
| Vitamin B1                    | 0.0025         |
| Vitamin B12                   | 0.00002        |

\(^a\)Trace element stock solution (in g 100 mL\(^{-1}\) MQ-water)

| Reagent                        | (g 100 mL\(^{-1}\) MQ-water) |
|-------------------------------|-------------------------------|
| ZnSO\(_4\) 7H\(_2\)O          | 4.4                           |
| MnSO\(_4\) H\(_2\)O           | 1.16                          |
| Na MoO\(_4\) 2\(\times\)H\(_2\)O | 0.3                           |
| CuSO\(_4\) 5H\(_2\)O          | 0.32                          |
| CoCl\(_2\) 6H\(_2\)O          | 0.28                          |

\(^b\)Fe-solution (in g 100 mL\(^{-1}\) M\(\Omega\) water)

| Reagent                        | (g 100 mL\(^{-1}\) M\(\Omega\) water) |
|-------------------------------|---------------------------------------|
| \((NH_4)_2\)SO\(_4\) Fe (SO\(_4\))\(_2\)\(\times\)6H\(_2\)O | 1.14 |
| EDTA                           | 1.0                                   |

\(^a\)0.0, 0.2 and 0.5 g L\(^{-1}\) in low N (LN), medium N (MN) and high N (HN) treatments

\(^b\)\(^c\)1 mL of stock solutions was added to 1L of base medium
Table 2 (on next page)

Proportions of C and N and molar C:N ratio in the biomass under different N treatments in the late stationary phase cultures on cultivation days 14, 16 and 19.

Values for C and N are mean ± SE (n=3). LN=Low N, MN=medium N, HN=high N.
|      | LN            | MN            | HN            | LN            | MN            | HN            | LN            | MN            | HN            |
|------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| C (%)| 45.45±0.56    | 47.02±0.49    | 47.90±0.36    | 46.74±0.64    | 47.53±0.13    | 48.63±0.21    | 47.81±0.50    | 47.88±0.34    | 48.81±0.65    |
| N (%)| 2.1±0.11      | 2.53±0.13     | 3.88±0.13     | 2.07±0.08     | 2.43±0.08     | 3.81±0.11     | 1.98±0.08     | 2.3±0.12      | 3.66±0.11     |
| C:N  | 25            | 22            | 14            | 26            | 23            | 15            | 28            | 24            | 16            |
Table 3 (on next page)

Percentage of FAMEs and sum of SAFAs, MUFAs, PUFAs and LC-PUFAs in the late stationary phase cultures on days 14, 16 and 19.

Values are mean ± SE (n=3).
| FAME       | day 14          | day 16          | day 19          |
|------------|-----------------|-----------------|-----------------|
|            | LN (%)          | MN (%)          | HN (%)          | LN (%)          | MN (%)          | HN (%)          | LN (%)          | MN (%)          | HN (%)          |
| C12:0      | 2.4 ± 0.4       | 1.4 ± 0.1       | 0.9 ± 0.0       | 2.5 ± 0.1       | 2.1 ± 0.3       | 2.1 ± 0.2       | 2.1 ± 0.3       | 2.2 ± 0.1       | 1.9 ± 0.5       |
| C13:0      | 12.4 ± 1.0      | 5.7 ± 0.8       | 3.7 ± 0.1       | 13.4 ± 0.3      | 6.0 ± 0.9       | 5.4 ± 1.8       | 14.9 ± 0.7      | 7.7 ± 0.6       | 7.2 ± 2.5       |
| C14:0      | 28.0 ± 0.6      | 21.5 ± 0.9      | 14.8 ± 0.2      | 28.9 ± 0.6      | 23.6 ± 0.3      | 19.2 ± 2.3      | 30.7 ± 0.1      | 25.2 ± 0.0      | 21.0 ± 3.3      |
| C15:0      | 7.1 ± 0.1       | 3.2 ± 0.4       | 2.4 ± 0.2       | 7.7 ± 0.1       | 3.1 ± 0.4       | 2.5 ± 0.6       | 8.7 ± 0.1       | 3.5 ± 0.5       | 3.3 ± 0.7       |
| C16:0      | 17.3 ± 0.3      | 21.3 ± 0.5      | 20.9 ± 0.1      | 15.8 ± 0.2      | 20.9 ± 0.7      | 19.8 ± 1.3      | 14.3 ± 0.3      | 19.4 ± 0.6      | 18.7 ± 2.1      |
| C16:1      | 4.0 ± 0.1       | 4.8 ± 0.2       | 4.0 ± 0.1       | 3.9 ± 0.1       | 5.3 ± 0.2       | 4.4 ± 0.3       | 3.9 ± 0.1       | 5.2 ± 0.3       | 4.4 ± 0.5       |
| C18:0      | 0.9 ± 0.1       | 0.9 ± 0.1       | 1.7 ± 0.7       | 0.9 ± 0.0       | 1.0 ± 0.1       | 1.0 ± 0.0       | 0.8 ± 0.0       | 1.2 ± 0.4       | 0.9 ± 0.0       |
| C18:1(n-9c)| 2.5 ± 0.2       | 3.3 ± 0.3       | 3.9 ± 0.8       | 2.3 ± 0.2       | 3.2 ± 0.3       | 3.0 ± 0.1       | 2.2 ± 0.0       | 3.6 ± 0.5       | 3.1 ± 0.0       |
| C18:2(n-6c)| 2.9 ± 0.2       | 4.5 ± 0.2       | 6.6 ± 0.5       | 2.3 ± 0.2       | 3.8 ± 0.0       | 5.8 ± 1.0       | 1.9 ± 0.2       | 3.3 ± 0.1       | 5.1 ± 1.1       |
| C18:3(n-3)| 3.5 ± 0.3       | 6.4 ± 0.3       | 11.7 ± 1.7      | 3.0 ± 0.5       | 5.2 ± 0.5       | 9.9 ± 1.9       | 2.2 ± 0.3       | 3.9 ± 0.5       | 8.3 ± 2.0       |
| C20:2      | 2.7 ± 0.1       | 3.5 ± 0.2       | 3.1 ± 0.1       | 2.5 ± 0.1       | 3.2 ± 0.2       | 2.6 ± 0.0       | 2.4 ± 0.0       | 3.1 ± 0.1       | 2.6 ± 0.1       |
| C20:3(n-6)| 0.7 ± 0.1       | 1.0 ± 0.1       | 1.0 ± 0.1       | 0.7 ± 0.1       | 0.9 ± 0.1       | 0.8 ± 0.1       | 0.7 ± 0.1       | 0.9 ± 0.1       | 0.8 ± 0.1       |
| C20:4(n-6)| 5.7 ± 0.7       | 9.0 ± 0.4       | 10.4 ± 0.1      | 5.8 ± 0.3       | 8.6 ± 0.2       | 9.5 ± 0.4       | 5.5 ± 0.2       | 8.0 ± 0.1       | 8.5 ± 0.5       |
| C20:3(n-3)| 1.0 ± 0.0       | 1.6 ± 0.1       | 1.6 ± 0.0       | 1.0 ± 0.0       | 1.5 ± 0.1       | 1.6 ± 0.0       | 0.9 ± 0.0       | 1.4 ± 0.1       | 1.8 ± 0.1       |
| C20:5(n-3)| 6.1 ± 0.4       | 8.2 ± 0.4       | 9.1 ± 0.0       | 6.5 ± 0.3       | 8.2 ± 0.3       | 8.8 ± 0.2       | 6.2 ± 0.2       | 8.0 ± 0.2       | 9.1 ± 0.5       |
| C22:6(n-3)| 2.6 ± 0.2       | 3.6 ± 0.2       | 4.1 ± 0.1       | 2.7 ± 0.1       | 3.2 ± 0.2       | 3.4 ± 0.2       | 2.5 ± 0.1       | 3.0 ± 0.1       | 3.3 ± 0.3       |
| SAFA       | 68.1 ± 1.7      | 54.0 ± 1.4      | 44.4 ± 1.3      | 69.2 ± 1.0      | 56.8 ± 1.0      | 50.0 ± 3.8      | 71.5 ± 0.6      | 59.7 ± 0.3      | 52.9 ± 4.9      |
| MUFA       | 6.5 ± 0.3       | 8.2 ± 0.3       | 8.0 ± 0.7       | 6.2 ± 0.1       | 8.5 ± 0.4       | 7.5 ± 0.2       | 6.2 ± 0.1       | 8.8 ± 0.3       | 7.6 ± 0.5       |
| PUFA       | 6.4 ± 0.4       | 11.0 ± 0.4      | 18.3 ± 2.2      | 5.4 ± 0.7       | 9.1 ± 0.4       | 15.7 ± 2.9      | 4.1 ± 0.5       | 7.2 ± 0.6       | 13.4 ± 3.1      |
| LC-PUFA    | 19.0 ± 1.5      | 26.8 ± 1.4      | 29.3 ± 0.2      | 19.2 ± 0.4      | 25.6 ± 0.9      | 26.8 ± 0.7      | 18.2 ± 0.2      | 24.4 ± 0.2      | 26.1 ± 1.4      |