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Translocation and *de novo* synthesis of eicosapentaenoic acid (EPA) during nitrogen starvation in *Nannochloropsis gaditana*

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ABSTRACT

The microalga *Nannochloropsis gaditana* is known for accumulating fatty acids, including the commercially interesting eicosapentaenoic acid (EPA) within the polar lipids (PL) and neutral lipids (NL). During microalgal growth EPA is mainly present in the PL. Upon nitrogen starvation *N. gaditana* accumulates large amounts of TAG in lipid bodies. The neutral lipid fraction will mainly consist of triacylglycerol (TAG). When expressed per total cell dry weight, the NL-localized EPA increased while the PL-localized EPA decreased, suggesting that EPA is translocated from the PL into the NL lipids during nitrogen starvation. Here, we elucidated the origin of EPA in NL of *N. gaditana* by firstly growing this microalga under optimal growth conditions with $^{13}CO_2$ as the sole carbon source followed by nitrogen starvation with $^{12}CO_2$ as the sole carbon source. By measuring both ^{12}C and ^{13}C fatty acid isotope species in time, the *de novo* synthesized fatty acids and the already present fatty acids can be distinguished. For the first time, we proved that actual translocation of EPA from the PL into the NL occurs during nitrogen starvation of *N. gaditana*. Next to being translocated, EPA was synthesized *de novo* in both PL and NL during nitrogen starvation. EPA was made by carbon reshuffling within the cell as well. EPA was the main fatty acid translocated, suggesting that the enzyme responsible for fatty acid translocation has a high specificity for EPA.

1. Introduction

Microalgae naturally produce lipids and can serve as a promising alternative lipid source for the production of food, feed and chemicals [1,2]. *Nannochloropsis gaditana* is a promising marine microalgae species due to its ability to accumulate large amounts of triacylglycerol (TAG) including the high value omega-3 fatty acid eicosapentaenoic acid (EPA) during nitrogen starvation.

In photosynthetic organisms (*e.g.* microalgae and plants), fatty acids are present in different fractions and location; the polar lipids (PL) are present in membranes and the neutral lipids (triacylglycerol or TAGs) in lipid globules. During growth polyunsaturated fatty acids (PUFAs) like EPA are mainly located in the PL. Nitrogen starvation is known to induce TAG accumulation in lipid bodies in the cytoplasm but also degradation of photosynthetic membranes [3]. During nitrogen starvation many fatty acids are synthesized *de novo* [3,4]. Next to that, fatty acids can also be translocated between lipid fractions. PUFAs are hypothesized to be translocated from the PL to the TAG fraction, since during nitrogen starvation its content decreases in the PL and increases in TAGs [3,5,6]. Several functions have been proposed for this TAG accumulation during nitrogen starvation. One of them is to serve as storage of membrane lipid components which later, when cultivation conditions become more favorable, can again be made available for the construction of PUFA-rich chloroplast membranes [7]. Using radiolabeling it has been shown that in *Parietochloris incisa* the PUFA arachidonic acid (C20:4) present in TAGs was used for construction of chloroplast membrane lipids upon nitrogen recovery [8]. Pulse-chase labeling in *Chlamydomonas reinhardtii* showed galactoglycerolipid as pool for TAG during nitrogen starvation [9].

The PUFA accumulation into TAG is species and growth phase dependent [10]. For different *Nannochloropsis* species, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Pavlova lutheri* EPA accumulation into TAG takes place in the stationary phase [5,6,10,11]. In *T. pseudonana* and *P. lutheris* also docosahexaenoic acid (DHA, C22:6) accumulated into TAG [10]. In *Nannochloropsis* the EPA accumulation in TAG correlated with the degradation of EPA-containing membrane

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glycerolipids [3,12]. For *Nannochloropsis oceanica* EPA was supplied to other lipids from phosphatidylethanolamine (PE) and diacylglyceryl-*N*-trimethylhomoserine (DGTS) [13]. In these studies, the overall change in fatty acid accumulation in the cell was measured. Based on the fatty acid mass balances within the different lipid fractions, it was hypothesized that PUFAs were transferred from the PL fraction into TAG upon nitrogen starvation. However, in this way it is not possible to distinguish the proposed translocation of intact free fatty acids between the different lipid fractions from the alternative possibility of *de novo* fatty acid synthesis in one lipid fraction and simultaneous degradation in another fraction. The actual transfer of intact fatty acids released from (chloroplastic) polar membrane lipids into TAGs upon nitrogen starvation has never been proven for *Nannochloropsis*. Detailed knowledge on the mechanisms of carbon partitioning during nitrogen starvation is still lacking [11,14]. Previous studies with radiolabeling of *Nannochloropsis* sp. did show that the label increased in TAG and decrease in monogalactosyldiacylglycerol (MGDG), mainly due to the turnover of lipid headgroup and glycerol backbone [15]. For the microalgae *Coccomyxa subellipsoidea* isotopic labeling showed that fatty acids were *de novo* synthesized and that acyl chain were transferred from the membrane lipids in TAG [16].

The present study focusses on a better understanding of fatty acid partitioning between NL and PL during nitrogen starvation, with special interest in EPA, using ^{13}C isotopic labeling of the microalgae *Nannochloropsis gaditana*. *De novo* synthesis, degradation and translocation of fatty acids between NL and PL were elucidated in this work.

2. Materials and methods

2.1. Microalga, growth medium and pre-cultivation conditions

The microalgae *Nannochloropsis gaditana* CCFM-01 (Microalgae Collection of Fitoplancton Marino S.L., CCFM) was used. Pre-cultures were maintained in 250 mL Erlenmeyer flasks containing 100 mL culture, in an orbital shaker incubator (100 rpm) with approximately 100 μmol m^{-2} s^{-1} continuous light and 2.5% CO_2 enriched air. The growth medium was based on [17] and contained: NaCl 445 mM; KNO_3 33.6 mM; Na_2SO_4 3.5 mM; $MgSO_4{\cdot}7H_2O$ 3 mM; $CaCl_2{\cdot}2H_2O$ 2.5 mM; K_2HPO_4 2.5 mM; NaFeEDTA 28 μM; $Na_2EDTA{\cdot}2H_2O$ 80 μM; $MnCl_2{\cdot}4H2O$ 19 μM; $ZnSO_4{\cdot}7H_2O$ 4 μM; $CoCl_2{\cdot}6H_2O$ 1.2 μM; $CuSO_4{\cdot}5H_2O$ 1.3 μM; $Na_2MoO_4{\cdot}2H_2O$ 0.1 μM; Biotin 0.1 μM; vitamin B1 3.3 μM; and vitamin B12 0.1 μM. The pH was adjusted with NaOH to pH 7.5. During pre-cultivation and maintenance of the cultures in Erlenmeyer flasks 100 mM 4-(2-hydroxyethyl)piperazin-1-ethanesulfonic acid (HEPES) was added to the culture medium. In nitrogen free medium KNO_3 was replaced by 33.5 mM KCl to keep equal osmolarity.

During growth phase 6 g $NaH^{13}CO_3$ (Sigma Aldrich) dissolved in 100 mL growth medium was added to the photobioreactor as carbon source at days 0, 3 and 6. The growth medium was filter sterilized (0.22 μm filtered, minisart, Sartorius Stedim) and flushed with nitrogen gas to remove any air left. At the start of the nitrogen starvation phase 0.84 g L^{-1} $NaH^{12}CO_3$ was added to the nitrogen free medium.

2.2. Experimental approach

In this research, ^{13}C isotopic labeling was used to study the lipid metabolism in *N. gaditana* and be able to distinguish between *de novo* synthesis, translocation and carbon reshuffling during nitrogen starvation. Fatty acid accumulation in the PL and NL fractions in nitrogen starved *N. gaditana* was followed using ^{13}C isotope labeling. First, *N. gaditana* was cultivated with ^{13}C-bicarbonate as sole carbon source in a closed photobioreactor to ensure that all carbon in the biomass consists of ^{13}C at the start of the starvation period. Secondly, the nitrogen starvation phase is started and the carbon source is changed to $^{12}CO_2$. The biomass concentration and fatty acid profiles in the NL and PL fractions are measured over time during nitrogen starvation. The fatty acids were quantified as their fatty acid methyl esters (FAMEs) using gas chromatography coupled to flame ionization detection (GC-FID). Liquid chromatography coupled to high resolution mass spectrometry (LC-MS) was used to enable distinction between ^{13}C and ^{12}C isotopes. The experiment was performed twice as biological duplicate.

2.3. Photobioreactor setup for the growth phase

The experiment was performed in a batch-operated, heat-sterilized airlift-loop flat panel photobioreactor with a light path of 20.7 mm, 1.8 L working volume and 0.08 m^2 surface area (Labfors 5, Infors HT, Switzerland, 2010). During the growth phase the photobioreactor was mixed by recirculation of nitrogen gas in an almost gas tight photobioreactor to prevent $^{12}CO_2$ and oxygen from entering the reactor and the loss of ^{13}C from the reactor (Fig. S1). The air that could still enter the reactor was estimated to be less than 800 mL air per day based on the off-gas measurements after gas recirculation with a mass spectrometer (PRIMA δB process MS, Thermo Scientific, USA), which was acceptable due to the low CO_2 concentration in air. To prevent oxygen inhibition by oxygen accumulation, we increased the gas volume by including a 10 L gas tank within the gas recirculation setup. During the growth phase the gas was recirculated with a membrane pump, placed inside this gas tank to prevent gas exchange with the air, because membrane pumps always tend to draw some ambient air (KNF membrane vacuum pump, type NMS 020B). The nitrogen gas recirculation flow supplied for mixing the culture was approximately 1.0–1.5 L min^{-1}. Prior to starting the experiment, the culture medium in the reactor was flushed with nitrogen gas to remove any remaining oxygen and $^{12}CO_2$. Similar growth was observed for cultures in reactors with and without gas recirculation. The pH was controlled at 7.5 by on-demand addition of 0.9 M sulphuric acid (5% v:v). The culture temperature was maintained at 26 °C by water recirculation through the water jacket in contact with the reactor cultivation chamber. The culture was illuminated on the culture side of the reactor by 260 LED light with a warm white spectrum (450–620 nm). During the growth phase the outgoing light intensity was kept at around 30 μmol m^{-2} s^{-1} by daily increasing the incident light intensity up to 636 μmol m^2 s^{-1} on the day before nitrogen starvation was started. The added $NaH^{13}CO_3$ was used as sole carbon source during the growth phase. The reactor was inoculated at low unlabeled biomass concentration (0.03 g L^{-1}) to ensure that more than 98% of cells consisted of ^{13}C when the nitrogen starvation phase was started.

2.4. Photobioreactor setup for nitrogen starvation phase

After the growth phase the nitrogen starvation was started by centrifuging the biomass (800*g* for 15 min), washing with nitrogen-free growth medium and re-suspending the culture with nitrogen-free growth medium to remove the remaining nitrate and ^{13}C. The nitrogen starvation phase was started at a biomass concentration of 1.3 ± 0.1 g L^{-1} for the two biological duplicates. During the nitrogen starvation phase, a similar reactor setup to the one described above was used, except that the gas was not recirculated and 2% $^{12}CO_2$ in nitrogen gas was used as carbon source. The incident light intensity was kept continuously at 636 μmol m^2 s^{-1} during this phase.

2.5. Offline measurements

Culture growth was assessed by measuring dry weight of the culture in triplicate according to Kliphuis et al. 2012 with the exception that ammonium formate (0.5 M) was used to dilute the sample and wash the filter [18]. The optical density was measured in duplicate at wavelengths 750, 680 and 480 nm with UV-VIS-spectrophotometer (DR 6000, Hach Lange, Germany, light path 10 mm). Cell concentration and volume were measured using the Multisizer III (Beckman Coulter Inc., USA) using a 50 μm aperture tube. Samples were diluted in ISOTON II

diluent. Cell concentration was only measured during one of the duplicate experiments. The average dry weight-specific optical cross section (α_c in $m^2\ g^{-1}$) was measured and calculated according to de Mooij et al. 2015 using the absorbance from 400 to 750 nm with a steps size of 1 nm. The absorbance was measured in UV-VIS/double-beam spectrophotometer (Shimadzu UV-2600, Japan, light path: 2 mm) equipped with integrating sphere (ISR-2600) for samples diluted to an optical density 750 nm of 0.3–0.5 [19]. The dry weight-specific optical cross section was calculated using the measured dry weight concentration ($g\ L^{-1}$). The photosystem II (*PSII*) maximum quantum yield (F_v/F_m) was measured at 455 nm with AquaPen-C AP-C 100 (Photon Systems Instruments, Czech Republic) after 15 min dark adaptation at room temperature.

2.6. Biomass samples

Biomass samples for fatty acid analysis were taken at day 0, 1, 2, 3, 5, 7, 10 and 14 from the moment of nitrogen starvation. The biomass samples (approximately 60 mL) were centrifuged for 5 min at 4255*g* (Beckman coulter, Allegra X-30R centrifuge) and washed twice with ammonium formate (0.5 M). The biomass pellets were stored at −20 °C and lyophilized. The measured dry weight was corrected for the dry weight of the reddish supernatant left after centrifugation, as this was washed away before biomass composition analysis.

2.7. Fatty acid analysis

The fatty acids within the neutral lipids (NL) and polar lipids (PL) were quantified with GC-FID according to [17,20]. In short, lipids were extracted from 6 to 7 mg lyophilized biomass with chloroform: methanol (1:1.25, v:v) mixture with 237 μg mL^{-1} tripentadecanoin (T4257, Sigma Aldrich) and 170 μg mL^{-1} 1,2-dipentadecanoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (840446P, Avanti Polar Lipids Inc.) as internal standard for the NL and the PL fraction, respectively. The NL lipids were separated from the PL on SPE silica gel cartridge (Sep-Pak Vac 6cc, Waters). NL were eluted with a solution of hexane:diethylether (7:1, v:v) and the PL were eluted with a solution of methanol:acetone:hexane (2:2:1, v:v:v). The separated fractions were divided for analysis by GC-FID and LC-MS before organic solvents were evaporated.

For analysis on the GC-FID the fatty acids were saponified and methylated in methanol with 5% H_2SO_4 at 70 °C for 3 h. The fatty acids methyl esters (FAMEs) obtained were extracted with hexane and quantified on the GC-FID (Agilent 7890) [17]. The TAG and PL fatty acid contents were calculated by summing all fatty acids measured per mg of biomass in each lipid fraction.

For LC-MS analysis the extracted lipids were saponified without subsequent methylation [21]. In short, the separated lipids were suspended in 200 μL of methanol with 6% KOH in MQ (4:1, v:v), incubated for 2 h at 60 °C and cooled to room temperature. 100 μL saturated NaCl was then added and the mixture acidified by adding 50 μL of 29% HCl. The samples were thoroughly mixed and centrifuged for 30 s at 20238*g* (Eppendorf centrifuge 5424). The free fatty acids were extracted three times with 200 μL chloroform: heptane (1:4, v:v), pooled and washed with 200 μL water. The fatty acid fraction was separated and dried by evaporation of the solvent and stored at −20 °C until use. Before analysis the dried fatty acids were dissolved in ethanol with 0.1% butylhydroxytoluene and sonicated for 5 min and centrifuged. The samples were analyzed by LC-Orbitrap FTMS.

2.8. LC-MS analysis

Fatty acids present in the saponified lipid fractions were detected by high resolution LC-MS using an Acquity UPLC (Waters, Milford, MA, USA) connected to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). A Waters Acquity HSS T3 column (1.8 μm particles; 2.1 × 150 mm) at 55 °C and a gradient of 20% acetonitrile in water containing 10 mM ammoniumacetate and 0.1% formic acid (eluent A) and 10% acetonitrile in isopropanol containing 10 mM ammoniumacetate and 0.1% formic acid (eluent B) were used to separate the compounds. The solvent gradient started with an increase from 35 to 70% B in 3 min, then from 70 to 85% B in 6 min and finally from 85 to 90% B in 13 min followed by a washing step at 90% B for another 5 min. The flow rate was 200 μL/min. Masses were recorded at a resolution of 60,000 in negative electrospray ionization mode and in the *m/z* range of 105–1400. Mass calibration and other settings of the MS were as described by Hooft et al. [22].

Raw LC-MS data were processed using MetAlign software [23] for noise estimation, peak picking and alignment of mass features across samples, resulting in a large table (peak list) with the relative intensities, expressed as peak heights, of nearly 7500 mass features in each sample. Mass features corresponding to the molecular ions, $[M-H]^-$, of both fully ^{12}C and fully ^{13}C isoforms of fatty acids of interest, as well as all possible partially labeled combinations thereof, were identified based on their calculated exact masses, allowing a maximum mass deviation of 3 ppm. The relative intensities of these molecular ion peaks were used for further data analysis.

Using the correlation of each sample between the peak intensities of the fatty acids detected by LC-MS and the quantified fatty acids of the GC-FID, the relative LCMS-peak intensities were converted into fatty acid concentrations.

2.9. Calculation time-averaged NL yield on light

The time-averaged NL yield on light ($Y_{NL/ph}$ in $g_{NL}\ mol_{ph}^{-1}$) was calculated as described by Remmers et al. [5]. The amount of NL produced over a period was divided by the amount of light supplied in that period. The light necessary for inoculum production was included in the light supplied, and was calculated from the biomass concentration at the start of nitrogen starvation and a theoretical biomass yield on light of 1 $g_x\ mol_{ph}^{-1}$ [5].

3. Results and discussion

The fatty acid accumulation in the NL and PL fraction was studied in *Nannochloropsis gaditana* during nitrogen starvation. A distinction between *de novo* synthesis, translocation and carbon reshuffling was made by measuring ^{12}C and ^{13}C fatty acids. The start of nitrogen starvation was considered as time 0. All figures represent the average of two independent biological photobioreactor experiments. All values mentioned in the text give the average of two independent biological replicates with their absolute deviation from the average.

3.1. Biomass production and photosystem activity

The nitrogen starvation started at a biomass concentration of 1.3 ± 0.1 $g\ L^{-1}$. The biomass concentration rapidly increased during the first 5 days up to 4.2 ± 0.02 $g\ L^{-1}$ and stabilized at 4.5 ± 0.3 $g\ L^{-1}$ at day 14 (Fig. 1A). During this period the cells number increased 3-fold from 2.2×10^8 to 6.6×10^8 cells per mL (Fig. 1B), indication that on average the cells divided 1.5 times after nitrogen starvation until day 5. Pal et al., 2011 also observed that cells of *Nannochloropsis* sp. divide at least once during nitrogen starvation [24]. The average cell diameter increased linearly from 3.3 to 3.6 μm with increasing nitrogen starvation time (Fig. 1B).

The dry weight-specific optical cross section rapidly decreased during the first 5 days of nitrogen starvation from 176 ± 19 to 29 ± 1.7 $m^2\ kg^{-1}$ (Fig. 1C). The *PSII* maximum quantum yield (F_v/F_m) decreased from 0.67 to 0.40 (Fig. 1D), indicating a reduced photosystem II activity. *PSII* maximum quantum yield is known to decrease during abiotic stress [25]. Our findings are similar to a decrease from 0.60 to 0.49 found by Simionato et al. 2013 during 7 days of nitrogen

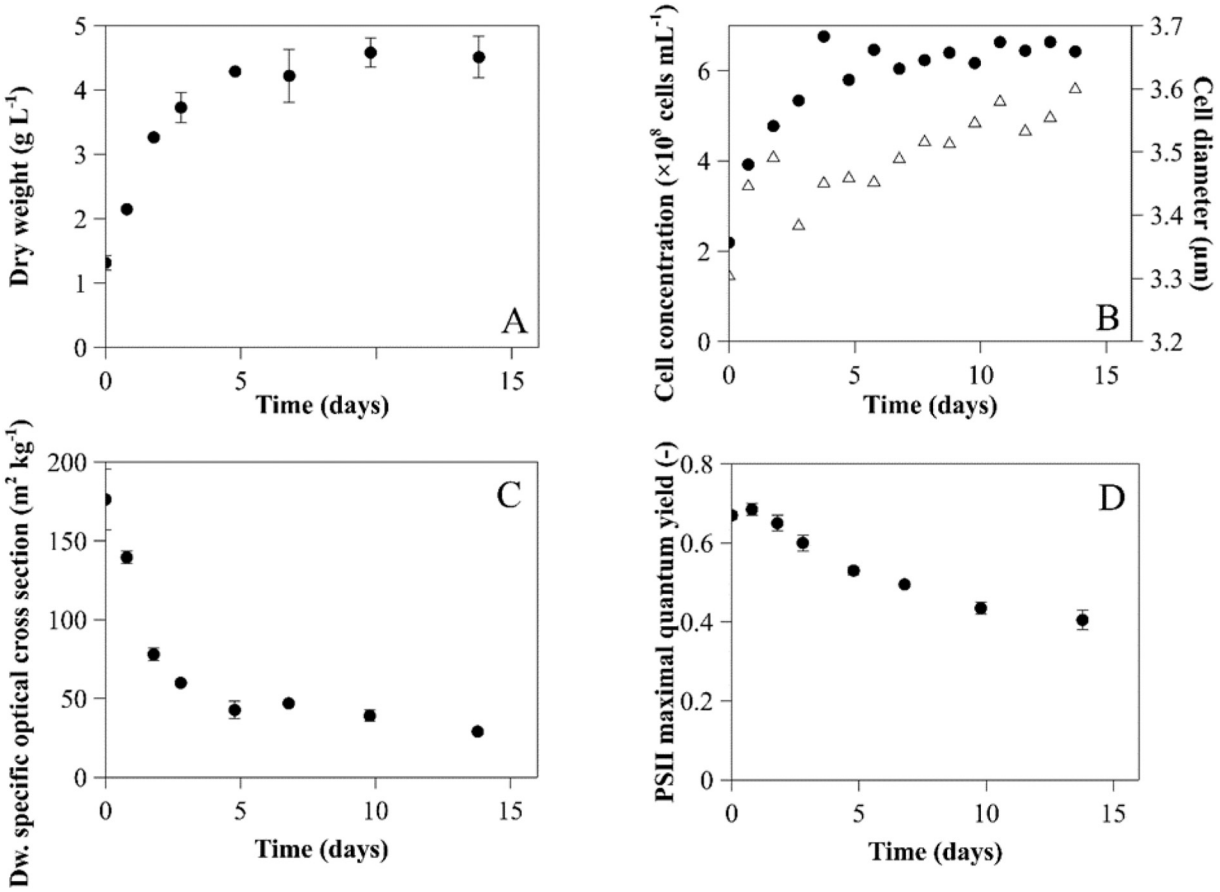

Fig. 1. Average dry weight concentration ($g\,L^{-1}$) (A), cell concentration ($cells\,mL^{-1}$) (circles) and diameter (μm) (triangles) from one of the duplicate experiments (B), average dry weight-specific optical cross section ($m^2\,kg^{-1}$) (C) and average *PSII* maximal quantum yield (D) measured over time from the moment of nitrogen starvation. Error bars in A, C and D represent the minimum and maximum values of two biological reactor experiments ($n = 2$).

starvation [3]. The decrease in *PSII* maximum quantum yield, dry weight-specific optical cross section and reduction in biomass productivity all point to a decrease in photosynthetic activity during nitrogen starvation.

3.2. Fatty acids analysis using GC-FID

Neutral lipids (NL) were separated from the polar lipid fraction (PL) and the fatty acid composition of both fractions were analyzed (as their FAMEs) during nitrogen starvation. The PL fraction will mainly consist of membrane lipids. The NL and PL fractions were calculated as the sum of each fatty acids in that fraction.

The total fatty acid content increased from 18 to 42% of the biomass dry weight in 14 days of nitrogen starvation (Fig. 2A). This increase was mainly caused by the increase of the NL fraction. The NL concentration in the culture also rapidly increased 21-fold due to the increase in both NL content and biomass concentration (Fig. 2B). The maximal time-averaged TAG yield on light ($0.14 \pm 0.0004\ g_{TAG}\,mol_{ph}^{-1}$, or $0.18 \pm 0.004\ g_{TAG}\,mol_{ph}^{-1}$ when not corrected for the energy necessary for inoculum production) was achieved after 1 day of nitrogen starvation (Fig. 2C). The PL content decreased within the first 3 days of nitrogen starvation from 0.11 ± 0.01 to $0.04 \pm 0.01\ g\,g_{dw}^{-1}$. At day 14 of nitrogen starvation 90% of all fatty acids were present in the TAG fraction, similar to what was found for *Nannochloropsis oculata* [10].

During nitrogen starvation the total cell EPA content expressed per total dry weight decreased from 0.06 ± 0.01 to $0.04 \pm 0.01\ g_{epa}\,g_{dw}^{-1}$ while EPA present in the NL fraction increased from 0.01 ± 0.001 to $0.03 \pm 0.001\ g_{epa}\,g_{dw}^{-1}$. At the same time, EPA present expressed per total dry weight in the PL fraction decreased from 0.05 ± 0.006 to $0.01 \pm 0.004\ g_{epa}\,g_{dw}^{-1}$ (Fig. 2E). In this study the total EPA content before nitrogen starvation was thus slightly higher compared to 4.3% found in *N. gaditana* and 4.2–4.9% in *Nannochloropsis* sp. [6,26].

In addition to the decrease in the algal EPA content upon nitrogen starvation, the EPA concentration in the bioreactor also changes due to the change in biomass concentration: from 82.9 ± 15.7 to $163.3 \pm 33.2\ mg\,L^{-1}$. This increase thus represents *de novo* synthesis of EPA of $80.2\ mg\,L^{-1}$ in total. The EPA concentration expressed per reactor volume, present in the NL fraction increased from 11.4 ± 1.7 to $113.6 \pm 10.9\ mg\,L^{-1}$, while in the PL it decreased from 71.5 ± 14.1 to $49.6 \pm 22.3\ mg\,L^{-1}$ (Fig. 2F). From this data it was estimated that up to ~22% of the EPA could have been translocated from PL to NL.

At the start of nitrogen starvation, $13.8 \pm 0.6\%$ of all EPA was present in the NLs and it increased to $71 \pm 7.8\%$ at day 14 of nitrogen starvation (Fig. 2D). Similar results have been reported for *N. oculata*, in which 8% of EPA was present in TAG during the exponential phase and increased to 68% in the stationary phase [10].

The total EPA in the culture increased during nitrogen starvation, indicating *de novo* synthesis of EPA. Nevertheless, the absolute increase in EPA present in NL is larger than the absolute increase of EPA in the culture and therefore it could be hypothesized that EPA was partially transferred from the PL into NL.

3.3. Specific analysis of ^{12}C and ^{13}C fatty acids using accurate mass LC-MS

By just measuring the fatty acid contents, no distinction can be made between possible *de novo* synthesis, degradation and

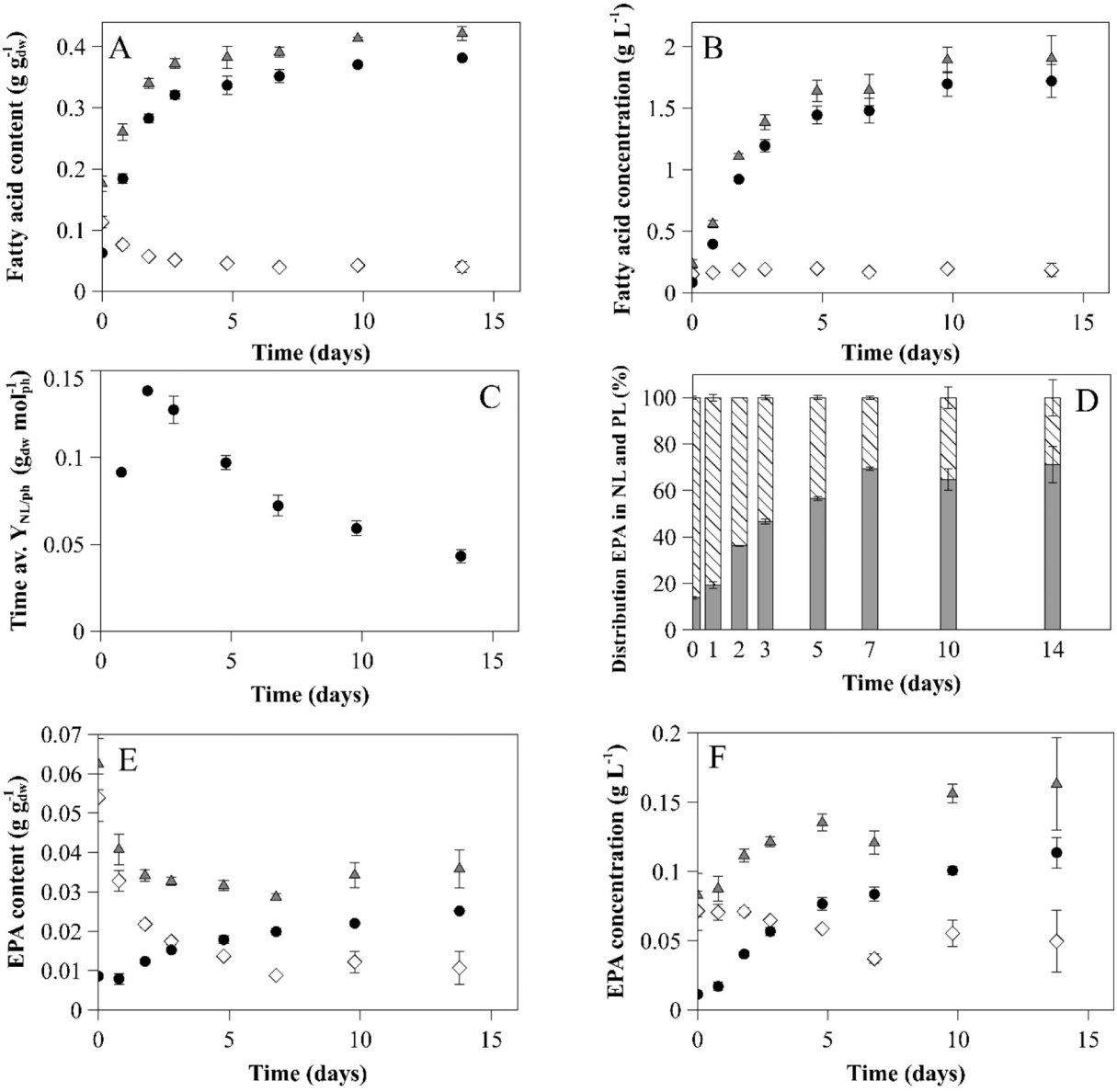

Fig. 2. Average fatty acid content (g g_{dw}^{-1}) (A) and fatty acid concentration (g L^{-1}) (B) in NL (circles), PL (diamonds) and total lipids (triangles; NL + PL). The average time-averaged TAG yield on light (g_{TAG} mol_{ph}^{-1}) (C). Average distribution of total EPA over the NL (grey bars) and PL (diagonal stripes) (D). The average content (g g_{dw}^{-1}) (E) and concentration (g L^{-1}) (F) of EPA in both NL (circles), PL (diamonds) and total lipids (triangles). All from the start of nitrogen starvation. The error bars represent the minimum and maximum values of two biological photobioreactor experiments ($n = 2$).

translocation of fatty acids between the NL and PL fractions. Therefore, the $^{13}C/^{12}C$ labeling patterns of the fatty acids were analyzed. Because ^{13}C was the sole carbon source during the growth phase, virtually all microalgal carbon consisted of ^{13}C at the moment of nitrogen starvation (t0). 79% of all EPA at start of nitrogen starvation consisted only of ^{13}C. During subsequent nitrogen starvation ^{12}C was used as carbon source. Consequently, all *de novo* synthesized fatty acids will consist of ^{12}C while fatty acids originating from the growth phase will fully consist of ^{13}C. Fatty acids consisting of a mixture of ^{12}C and ^{13}C are synthesized by reshuffling ^{13}C-carbon available in the cell at the onset of nitrogen starvation combined with ^{12}C-carbon that is subsequently fixed *de novo*. The recycled ^{13}C-carbon can originate from existing fatty acids but also from other biomass components and free $^{13}CO_2$ present in the cell prior to starvation.

At the onset of nitrogen starvation, the largest part of all fatty acids consisted only of ^{13}C. Fig. 3 shows the ^{13}C, ^{12}C and $^{12}C/^{13}C$ fatty acid concentrations of the four most abundant fatty acids, eicosapentaenoic acid (C20:5), palmitic acid (C16:0), palmitoleic acid (C16:1) and oleic acid (C18:1) in *Nannochloropsis gaditana* during nitrogen starvation. Upon subsequent nitrogen starvation, palmitic acid, palmitoleic acid and oleic acid in the NL fraction were mainly produced by *de novo* synthesis from ^{12}C sources only (carbon chains consisting of ^{12}C only) and *de novo* synthesis combined with ^{13}C-carbon reshuffling (carbon chain consisting of a mix of ^{12}C and ^{13}C). The fatty acid in the PL only showed a slight increase, originating from *de novo* synthesis (only ^{12}C) and *de novo* synthesis combined with ^{13}C-carbon reshuffling (mix of ^{12}C and ^{13}C).

EPA consisting of only ^{13}C decreased in the PL fraction and increased in the NL fraction (Fig. 3). The absolute EPA decrease in the PL fraction was 0.043 g L^{-1} and the absolute increase in the NL fraction was 0.026 g L^{-1}. This result provides evidence for translocation of part of the complete EPA molecules from PL to NL, since it is unlikely that all 20 carbon atoms of EPA were completely made from recycled ^{13}C-carbon. Of all the EPA present in NL after 14 days of nitrogen starvation, 23% originated of translocation from PL to NL. This is the first time showing actual translocation of intact EPA from PL into NL in

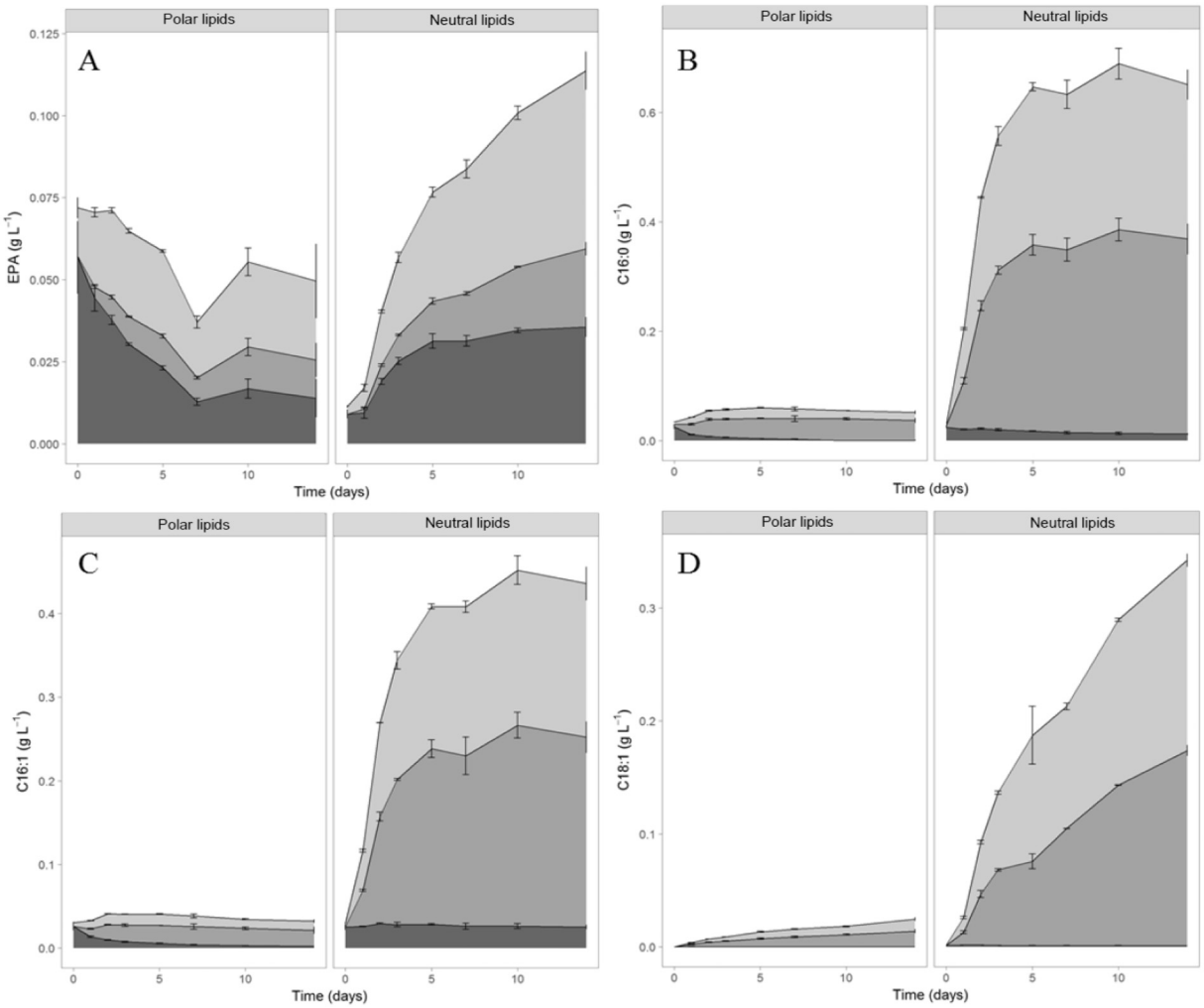

Fig. 3. Average fatty acids (mg L^{-1}) consisting of only ^{13}C (dark grey), only ^{12}C (grey) and a mix of ^{12}C and ^{13}C (light grey) at different times during nitrogen starvation in PL and NL. Eicosapentaenoic acid (C20:5) (A), palmitic acid (C16:0) (B), palmitoleic acid (C16:1) (C) and oleic acid (C18:1) (D). Error bars represent the minimum and maximum values of two biological experiments (n = 2).

microalgae. In addition to translocation, in both PL and NL fractions EPA was *de novo* synthesized from both ^{13}C-carbon recycled within the nitrogen starved cell and *de novo* synthesis from ^{12}C-carbon. In the NL fraction, after 14 days of nitrogen starvation 21% of all EPA was completely made *de novo* from ^{12}C-carbon, 46% was made from ^{13}C-carbon recycled within the cell together with *de novo* ^{12}C-carbon, 23% originated from translocation of intact ^{13}C-EPA and 10% was already present at the start of nitrogen starvation. One enzyme which could be responsible for the EPA transfer into TAG is phospholipid:diacylglycerol acyltransferase (PDAT). This gene has been identified in the genome of *Nannochloropsis gaditana* [27] and it would be interesting to study its transcriptional and protein levels during nitrogen starvation. It is interesting to note that mainly EPA was translocated, relative to the other fatty acid species, suggesting that the enzyme responsible for the translocation has a relative high affinity for EPA as a substrate.

4. Conclusions

Using ^{13}C isotope labeling, we proved that the vast majority of fatty acids accumulated in the neutral lipids (NL) are made *de novo* during nitrogen starvation. Preexisting fatty acid (*e.g.* palmitic acid, palmitoleic acid) in the polar lipids (PL) were degraded but not translocated to the neutral lipids during nitrogen starvation. In contrast, preexisting EPA in the PL was both degraded and translocated into NL at comparable rates. Conclusive evidence was provided that a modest but significant proportion of intact EPA carbon chains were translocated from the PL into the NL upon nitrogen starvation. This indicates that the enzyme responsible for translocation of intact fatty acids has a high affinity towards EPA relative to other fatty acids. Next to translocation, EPA present in the NL was also synthesized both *de novo* and from carbon reshuffling within the cell. Future research will focus on elucidating the biochemical pathways active in fatty acid translocation and *de novo* synthesis, in order to gain better insight into the regulation of EPA accumulation in nitrogen starved microalgae. This knowledge can potentially be used to produce EPA-rich NL from *Nannochloropsis*.

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Author contributions

JHJ, PPL, RHW and MJB conceived the research and designed the experiments. JHJ performed the experiments, analysed and interpreted the data. RHCV did the LCMS analysis and together with JHJ analysed and interpret these data. JHJ wrote the original draft. PPL, RHW and MJB supervised. All authors edited and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of authors' agreement

All authors agreed to the authorship and submission of the manuscript to Algal research for peer review.

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