Carbon partitioning and lipid remodeling during phosphorus and nitrogen starvation in the marine microalga *Diacronema lutheri* (Haptophyta)

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Abstract :

The domesticated marine microalga *Diacronema lutheri* is of great interest for producing various highly valuable molecules like lipids, particularly long-chain polyunsaturated fatty acids (LC-PUFA). In this study, we investigated the impact of phosphorus (P) and nitrogen (N) starvation on growth, carbon fixation (photosynthetic activity) and partitioning, and membrane lipid remodeling in this alga during batch culture. Our results show that the photosynthetic machinery was similarly affected by P and N stress. Under N starvation, we observed a much lower photosynthetic rate and biomass productivity. The degradation and re-use of cellular N-containing compounds contributed to triacylglycerol (TAG) accumulation. On the other hand, P-starved cells maintained pigment content and a carbon partitioning pattern more similar to the control, ensuring a high biomass. Betaine lipids constitute the major compounds of non-plastidial membranes, which are rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. Under P and N starvations, EPA was transferred from the recycling of membrane polar lipids, most likely contributing to TAG accumulation.

Keywords : carbon partitioning, *Diacronema lutheri*, nitrogen, phosphorus, polyunsaturated fatty acid

Abbreviations:

FA, fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; TAG, triacylglycerol; PL, phospholipid; GL, glycolipid; BL, betaine lipid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, Phosphatidylglycerol; DPG, diphosphatidylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; DGCC, diacylglycerol-3-carboxymethyl-choline; DGTA, diacylglycerol hydroxymethyl-N,N,N-trimethyl- β -alanine; DGGA, diacylglycerylglucuronide; FAME, fatty acid methyl ester; μ , specific growth rate; F_0 , minimum Chl fluorescence yield; F_m , maximum fluorescence yield; F_v/F_m , the maximum quantum yield; Φ_{II} , PSII operating efficiency; NPQ, non-photochemical quenching; SIMPER, similarity percentage analysis; ANOSIM, one-way analysis of similarities.

Introduction

In the last decade, microalgae have been cultured around the world for different applications in aquaculture, food-processing, biofuel production, cosmetics, and pharmaceuticals (Cadoret et al. 2008, Hu et al. 2008, Mimouni et al. 2012, Koller et al. 2014). They are a promising source for the production of a number of highly valuable compounds, such as pigments, antioxidants and lipids, particularly long-chain polyunsaturated fatty acids (LC-PUFA).

The Haptophyte *Diacronema lutheri*, previously named *Pavlova lutheri*, is a marine microalga widely used as a feed in aquaculture hatcheries (Brown et al. 1997, Ponis et al. 2008). This species is rich in omega-3 LC-PUFAs, notably eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA, C22:6n-3) acids (Brown et al. 1993, Meireles et al. 2003), which are known for their potential health benefits (Doughman et al. 2007, Mimouni et al. 2012). In microalgae, LC-PUFAs are mainly present in polar lipids constituting the membranes, whereas *D. lutheri* is able to partition omega-3 LC-PUFAs into triacylglycerols (TAG; Tonon et al. 2002, Guihéneuf and Stengel 2013). Moreover, *D. lutheri* is also a high producer of phytosterols, which are well known for their ability to reduce low density lipoprotein cholesterol (Ahmed et al. 2015). A previous study showed that lipid extracts from *D. lutheri* can effectively inhibit lipopolysaccharide-stimulated inflammatory pathway in human macrophages, suggesting an anti-inflammatory effect in the prevention of chronic inflammation-linked metabolic illnesses such as cardiovascular diseases (Robertson et al. 2015).

Previous studies have shown that physicochemical factors, such as irradiance, temperature or carbon source, play an important role in the growth and biochemical composition of *Diacronema lutheri* (Tatsuzawa and Takizawa 1995, Carvalho and Malcata 2003, Carvalho et al. 2009, Guihéneuf et al. 2009, 2011, Guedes et al. 2010, Guihéneuf and Stengel 2017). Moreover, nutrient limitation can directly or indirectly affect the fixation (photosynthesis), partitioning and storage of carbon in the cells, thus the molecules of interest (Carvalho and Malcata 2000, Carvalho et al. 2006, Guschina and Harwood 2009, Zhu et al. 2016).

Nitrogen (N) and phosphorus (P) are the most limiting or co-limiting macro-elements in natural conditions (Moore et al. 2013). Both elements are involved in the composition of essential molecules for all organisms, and closely connected to carbon metabolism (Geider and La Roche 2002). The influence of N on the reorientation of carbon flux has been intensively studied, and N has

been considered as the main nutrient triggering the accumulation of neutral lipids (Sharma et al. 2012, Guihéneuf and Stengel 2013). Indeed, the degradation of nitrogenous cellular compounds such as proteins, amino acids and chlorophylls, could offer both reducing agents and carbon skeletons for the *de novo* synthesis of fatty acids (Ge et al. 2014). Furthermore, many studies indicate that thylakoid membrane degradation results in TAG synthesis and that plasma membrane remodeling contributes substantially to the accumulation of neutral lipids (Simionato et al. 2013, Yang et al. 2013, Martin et al. 2014, Tan et al. 2016). Nevertheless, N limitation in microalgae is often accompanied by a decrease in photosynthetic capacity due to reduction of structural components (Young and Beardall 2003); as a consequence, lipid productivity rapidly decreases because of the lowering of energy intake (Klok et al. 2013, Benvenuti et al. 2015).

In contrast to N limitation, the effect of P limitation in microalgae is mostly species-specific. Some studies show that the integrity of the thylakoid membranes is largely maintained (Iwai et al. 2014), and photosynthetic parameters are not modified in the absence of P (Kamalanathan et al. 2015). The influence of P limitation on the reorientation of carbon flux and cellular energy has been studied in some species (Falkowski and Raven 2007, Lin et al. 2016, Brembu et al. 2017). P limitation triggers TAG increase in several model algae such as *Chlamydomonas reinhardtii* (Iwai et al. 2014), *Phaeodactylum tricornutum* (Alipanah et al. 2018, Abida et al. 2015, Huang et al. 2019a), *Nannochloropsis oceanic* (Mühlroth et al. 2017) and *Tisochrysis lutea* (Huang et al. 2019b). Some microalgae demonstrate a specific process of polar lipid remodeling, characterized by a substitution of phospholipids (PL) with glycolipids (GL) and/or betaine lipids (BL; Khozin-Goldberg and Cohen 2006, Van Mooy et al. 2009, Martin et al. 2011, Lu et al. 2013, Iwai et al. 2014, Abida et al. 2015, Shemi et al. 2016, Cañavate et al. 2017a, Mühlroth et al. 2017, Alipanah et al. 2018, Hunter et al. 2018, Huang et al. 2019a). Conversely, in some other microalgae with intrinsically low PL levels, the BL content is often independent of P availability (e.g., *T. lutea*, *Isochrysis galbana* and *Diacronema vlkianum*; Cañavate et al. 2017a,b, Huang et al. 2019b).

To our knowledge, the plasma membrane of *Diacronema lutheri* does not contain PL (Eichenberger and Gribi 1997), which could mean it requires little P and is adapted to low P conditions (Cañavate et al. 2017a,b). Carvalho et al. (2006) compared the effects of N and P on the production of biomass, EPA and DHA by *D. lutheri*. These authors suggested a trend between

decreasing N concentration and increasing yield of EPA at high P level, while the effect of P limitation is remained unclear.

In this study we focused on the effect of P stress in *Diacronema lutheri* on carbon fixation and orientation, and lipid remodeling, particularly with respect to fatty acid partitioning. For this purpose, the impact of P starvation was compared with N-starvation and non-stressing media. Photosynthetic activity and different biochemical responses of *D. lutheri* were measured during batch cultivation under continuous light.

Materials and methods

Microalgal strain and growth conditions

Axenic *Diacronema lutheri* CCAP 931/6 was obtained from the Culture Centre of Algae and Protozoa (Oban, Scotland) and maintained in natural seawater (Saint-Malo, France) enriched with Walne solution (Walne 1966). Several precautions were taken during the preparation of the inocula: Microalgae were pre-cultivated in Walne-enriched seawater until the exponential growth phase was reached; cells were then transferred into natural seawater for 3 d. This 3-d period was necessary to lower the cellular P and N quotas and to minimize dissolved inorganic N and P inputs in the subsequent cultures.

Pre-cultivated cells were inoculated with an initial density of 10^5 cells $\cdot$ mL⁻¹ in 1.5 L of medium prepared with seawater enriched with original Walne (control) or modified Walne (P-limited or N-limited) solutions. The modified or non-modified Walne solution provided nutrient concentrations of 1176 μ M NO₃⁻ and 128 μ M PO₄³⁻ (N:P = 9.18) in the control medium, 500 μ M NO₃⁻ and 4 μ M PO₄³⁻ (N:P = 125) in the P-limited medium, and 125 μ M NO₃⁻ and 125 μ M PO₄³⁻, (N:P = 1) in the N-limited medium. These concentrations were chosen according to Bougaran (2014). Culture was conducted in batch mode at $16 \pm 2^\circ\text{C}$, under continuous fluorescent light (Osram 54W cool daylight) with a photon flux density of 120 μ mol photons $\cdot$ m⁻² $\cdot$ s⁻¹ (Guihéneuf, 2008) and sterile-filtered air bubbling over a total period of 11 days. Three biological replicates of each of the above treatments were studied. Cell density was determined on samples fixed with Lugol (iodine solution) using optical microscopy, assessed with a Neubauer hemocytometer twice a day. Specific

growth rate (μ) was calculated according to the equation: $\mu = (\ln N_{t2} - \ln N_{t1}) / (t2 - t1)$, where N_{t2} and N_{t1} are the number of cells on two subsequent culture days ($t2$ and $t1$).

Nutrient analyses

Defined culture volumes containing about 30×10^6 cells were filtered on GF/C glass fiber filters (Whatman, Fisher Scientific, Illkirch, France) and residual PO_4^{3-} (including HPO_4^{2-} and H_2PO_4^-) and NO_3^- concentrations in the media were measured in the filtrates. PO_4^{3-} was estimated by visible spectrophotometry (Murphy and Riley 1958), and NO_3^- was determined by ultraviolet spectrophotometry (American Public Health Association 1992). Cells on filters were washed twice with 10 mL 0.516 M NaCl, dried overnight at 60°C, and stored at -20°C. Levels of particulate C and N in cells were determined with a FLASH 2000 NC analyzer (Thermo Fisher Scientific, Villebon-sur-Yvette, France).

Photosynthesis and chlorophyll *a* fluorescence yield measurements

Photosynthesis was measured by the rate of oxygen evolution (Nguyen-Deroche et al. 2012), using a luminescent oxygen indicator (PyroScience FireSting O_2 , Aachen, Germany). Net photosynthesis was recorded under an actinic irradiance of $120 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Intralux 4000-1, Schlieren, Switzerland) at 16°C.

Chlorophyll *a* fluorescence yield measurements were taken as described by Roháček et al. (2014) at 16°C, using a FMS1-modulated fluorimeter (Hansatech, Cergy, France). After a dark-adaptation period (15 min), the minimum Chl fluorescence yield (F_0) was recorded under a weak modulated light ($0.1 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Next, a saturation pulse (more than $1200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) was applied to determine maximum fluorescence yield (F_m). The maximum quantum yield (F_v/F_m) was calculated according to the equation: $F_v/F_m = (F_m - F_0) / F_m$. PSII operating efficiency (Φ_{II}), photochemical and non-photochemical quenching (NPQ) were determined following a 7-min non-saturating white actinic irradiation ($120 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) according to Roháček et al. (2008, 2014). For quenching components analysis, a higher actinic irradiance ($1000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) was applied, and dark relaxation of Chl fluorescence yield was recorded (Roháček et al. 2014).

Protein, carbohydrate, neutral lipid, and pigments assays

For proximate protein and carbohydrate assays, about 30×10^6 cells were harvested by centrifugation (3000 g, 10 min), washed with 0.516 M NaCl, resuspended in 2 mL ultrapure water and homogenized. The homogenate was centrifuged at 4°C, 16100g for 20 min, protein and carbohydrate dosages were then measured on the same cell lysate. Soluble protein content was quantified using the Bradford method (Bradford 1976), and soluble carbohydrate content by the phenol-sulfuric acid method (Dubois et al. 1951).

Cellular neutral lipid accumulation was estimated by Nile red (Sigma-Aldrich, Saint-Quentin-Fallavier, France) fluorescence. Briefly, 3 mL of culture containing about 3×10^6 cells were stained directly with 3 µL Nile red solution (1 mg · mL⁻¹ in acetone). The fluorescence was measured at excitation/emission wavelengths of 530/580 nm respectively (Perkin Elmer LS-55, Villebon-sur-Yvette, France).

The pigments Chl *a*, Chl *c*, and fucoxanthin were extracted from the cell pellet with 100% acetone and quantified spectrophotometrically (Perkin Elmer Lambda-25, Villebon-sur-Yvette, France) as described in Heydarizadeh et al. (2017).

Lipid extraction and identification and fatty acid analysis

For lipid analysis, about 300×10^6 cells were collected at days 4, 7, and 11 on a GF/C glass fiber filter (Whatman, Fisher Scientific, Illkirch, France). Total lipids were extracted in a chloroform/methanol/0.28% NaCl (1/2/0.2, v/v/v) mixture according to Bligh and Dyer (1959). The solvent was then removed by evaporating under partial vacuum using a rotary evaporator (Heidolph 94200, Fisher Scientific, Illkirch, France), and the dry extracts were dissolved in 2 mL pure chloroform and stored at -20°C under nitrogen (N₂). Total lipid content was quantified by carbonization (Marsh and Weinstein 1966) using triacylglycerol (TAG) as a standard (Sigma Aldrich, Saint-Quentin-Fallavier, France).

Lipid extracts from about 7.5×10^6 cells were separated using thin layer chromatography (TLC) on 20 × 20 cm silica gel 60 plates (Merck, Fontenay-sous-Bois, France). Three separation solvents were successively used: (1) methyl acetate/ isopropanol/ chloroform/ methanol/ 0.25% KCl

(25/25/25/10/9, v/v/v/v/v), (2) methyl acetate/ isopropanol/ chloroform/ methanol/ 0.25% KCl (5/5/5/2/1, v/v/v/v/v) and (3) hexane/ diethyl ether/ glacial acetic acid (40/10/1, v/v/v). Lipid spots were visualized under UV light (366nm) after spraying with primuline (Sigma-Aldrich, Saint-Quentin-Fallavier, France; White et al. 1998). Quantification was made with a digit image system (Versadoc 3000 Bio-Rad, Marnes-la-Coquette, France) combined with the Quantity One program (Bio-Rad, Hercules, CA, USA) and a range of lipid standards. For phospholipids, plates were sprayed with Zinzadze reagent (Molybdenum Blue Spray, Sigma-Aldrich, Saint-Quentin-Fallavier, France; Dittmer and Lester 1964). All the lipid standards used for TLC analyses were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France) or Advanti Polar Lipids (Alabaster, AL, USA).

Lipid fractions were separated using a column of 500 mg silica gel 60 (normal-phase, 0.063–0.200 mm, Merck, Fontenay-sous-Bois, France). The column was preconditioned with pure chloroform and subsequently loaded with concentrated crude lipid extract. Neutral lipids were eluted using 20 mL chloroform/acetone (9/1, v/v), glycolipids with 4 mL chloroform/ methanol (5/2, v/v) and 6 mL acetone/ methanol (9/2, v/v), and finally phospholipids and betaine lipids with 20 mL methanol/ H₂O (9/1, v/v). The fractions were evaporated to dryness and dissolved in 2 mL pure chloroform for gas-chromatography analyses.

Fatty acid methyl esters (FAMES) from each lipid fraction were obtained according to Slover and Lanza (1979). FAMES were analyzed by a Focus gas-chromatograph (Thermo Electron Corporation, Les Ulis, France) equipped with a capillary column CP Sil-88 25 m × 0.25 mm (Varian, Les Ulis, France), coupled with flame-ionization detection. Nitrogen was used as the mobile phase with a constant flow rate of 1 mL/min. Injection was performed in the split mode at 250°C. The analyses were carried out from 120°C to 220°C: 120°C for 4 min, 120–220°C with 6°C · min⁻¹, and held at 220°C for 5 min. FAMES were identified from authentic FAME standards (Sigma-Aldrich, Saint-Quentin-Fallavier, France), analyzed with the Azur V4 program (Datlys, Les Ulis, France), and expressed as molar percent of total fatty acids.

Statistical analysis

The normalization of concentration data to cell C made it possible to follow carbon partitioning between cell components. The data were analyzed statistically using a one-way repeated measures

ANOVA followed by a Fisher's LSD post-hoc test (level of significance: $p \leq 0.05$). Unless otherwise specified, the SIGMAPLOT software package was used for all statistical calculations. All the results were expressed as means $\pm$ standard errors.

Principal components analyses (PCA) were carried out using R (Multivariate exploratory data analysis and data mining, version 1.41; Husson et al. 2018) to study and visualize correlations between variables. The data were scaled to unit variance. Hierarchical clustering was performed using the Ward's criterion on the PCA results.

Membrane lipid and fatty acid (FA) data were analyzed with a similarity percentage analysis (SIMPER), performed on the relative percentage data to demonstrate the dissimilarity in membrane lipid and FA composition. SIMPER identified the compounds that contributed the most to the variance between tested conditions. The membrane lipids and FAs, which together contributed up to >80% of the dissimilarities, were selected to identify the differences between culture conditions. Data on the relative percentage were logarithmically ($\log[x+1]$) transformed and converted into a Bray-Curtis similarity matrix. A one-way analysis of similarities (ANOSIM) was used to statistically test the overall difference by calculating a global R statistic that weights the differences between groups of sampling units. An R value close to 1 indicates high separation of the clusters, while a value of 0 represents the null hypothesis (Chapman and Underwood 1999). SIMPER and ANOSIM analyses were performed using R (Community Ecology Package, version 2.4-6) (Oksanen et al. 2018).

Results and Discussion

Cell growth

Cell growth of *Diaconema lutheri* was monitored over 11 days in batch cultures. The P-stressed (P-) culture showed a similar cell density evolution to the control (NP) and N-stressed (N-) cultures until day 6. The specific growth rate reached its maximum ($\mu_{\max} = 0.8\sim 0.9 \cdot \text{d}^{-1}$) on day 4 under the three cultivation conditions, without any significant difference (Fig. 1a).

The N-starved cells entered stationary phase on day 7, after about 2 days of NO_3^- deprivation (NO_3^- concentration below the detection limit) in the medium, with a cell density of $3 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ (Fig. 1b). The P-starved cells reached stationary phase on day 10~11, after about 6 days of PO_4^{3-} deprivation (PO_4^{3-} concentration below the detection limit) in the medium, with a cell density of

8×10^6 cells $\cdot$ mL⁻¹ (Fig. 1c). These results corroborate the findings of Carvalho et al. (2006), suggesting a good adaptation to P scarcity in this species. Despite the high N:P enrichment ratio used (N:P = 125), it was noted that the P-limited medium also became deprived of NO₃⁻ about 2 days before the cells entered stationary phase. With regard to the level of P and N depletion, their duration and the cell density, we could not distinguish whether it was P or N starvation that caused cell division to stop. Therefore, these data will not be used as a basis for drawing conclusions about P starvation, but are included for context. In the control culture, NO₃⁻ deprivation was observed around day 9, while PO₄³⁻ remained available (Fig. 1d).

According to the growth rate and residual PO₄³⁻ and NO₃⁻ concentrations in culture media, days 4 (exponential growth phase), 7 (2~3 days after P or N depletion in limited cultures) and 11 (6~7 days after P or N depletion in limited cultures) were chosen for comparisons in the subsequent experiments.

The growth rate of microalgae depends on the quota of the limiting element according to Droop's model (Droop 1973). In the present study, we compared the cellular quota of N (N/C, normalized to cell C) in the three culture conditions (Fig. 1e). The same increase in N quota supporting cell division and growth was observed during the exponential growth phase under the three culture conditions. After N depletion in the medium (day 5), the N quota was greatly reduced in N-starved cultures and remained very low during the stationary phase. In P-starved cells, from day 6 until day 8, the N quota was also reduced compared with control cells, despite ambient N still being available. This result suggests that N assimilation ability was restricted by P limitation, as previously described (Pahlow and Oschlies 2009, Bougaran et al. 2010): the low N assimilation could be related to the need for ATP to achieve active N uptake. Following the depletion of NO₃⁻ in the medium (after day 8), the N quota quickly dropped to the same level as in N-limited conditions when the cells entered stationary phase (Fig. 1e), suggesting a potential N deficiency. Conversely, in the control culture, the N quota remained high whereas the growth rate was reduced from day 4 onwards. Based on earlier information, and given the high cell density, the reduced growth rate might be due to light or CO₂ limitation that could decrease photosynthesis and subsequently carbon metabolism (Brembu et al. 2017).

Photosynthetic activity and pigment content

The maximum quantum yield (F_v/F_m), which quantifies the maximum photochemical efficiency of PSII (Roháček et al. 2008), was roughly constant (around 0.71) in control culture until NO_3^- deprivation occurred in the medium (day 9). F_v/F_m decreased significantly in N- and P-starved cultures ($F_v/F_m = 0.64$ and 0.65 on day 7, respectively) (Fig. 2a), suggesting a reduced photosynthetic capacity of PSII reaction centers under both N and P stress. Similar F_v/F_m decreases in response to N or P deficiency were also reported in other species (Liu et al. 2011, Abida et al. 2015, Alipanah et al. 2018), which might be strongly related to the reorganization of thylakoid membranes.

Similarly, PSII operating efficiency (Φ_{II}) remained high in control cells until NO_3^- deprivation in the medium. Φ_{II} decreased dramatically in N- and P-starved cultures from day 4 onwards, and much lower in P-starved cultures (Fig. 2b). This decline in Φ_{II} might be due to delayed electron transfer modulated by the rate of CO_2 fixation (Chylla and Whitmarsh 1989, Wykoff et al. 1998), particularly because of the depletion of phosphorylated intermediates (Brooks 1986, Jacob and Lawlor 1993).

Non-photochemical quenching (NPQ) is an important mechanism that allows dissipation of excess light energy as heat, thus maintaining the balance between the photon energy absorbed and energy utilized (Müller et al. 2001). In the present study, NPQ started to increase in both N- and P-stressed cultures from day 4 onwards (Fig. 2c). The increase of NPQ might be related to xanthophyll de-epoxidation through the xanthophyll cycle (Roháček et al. 2014). The lower values in P-starved cultures compared with N- could be due to the more persistent growth, which largely consumes light energy and thus reduces energy imbalance at the cellular level. According to Roháček et al. (2014), complementary measurements of fluorescence parameters were performed during the relaxation period. A significant increase of the slow component of the dark-relaxation kinetics was observed in both P- and N-starved cultures (Fig S1 in the Supporting Information), suggesting an enhanced photoinhibition of PSII.

Overall, Chl *a* fluorescence parameters show that the photosynthetic machinery was similarly affected by P and N starvations in *D. lutheri*. However, cellular pigment contents, normalized to cell C, evolved differently depending on the type of nutrient stress (Fig. 3, a-c). Pigment contents were strongly reduced in N-starved cells after NO_3^- deprivation in the medium, as N is required for the

biosynthesis of chlorophyll (Ge et al. 2014). This decline of the light-harvesting pigments could lead directly to a lower photosynthetic rate (Fig. 3d) and biomass productivity. Nonetheless, an increase in the ratio of fucoxanthin/chlorophyll was observed (Fig. 3c), similar to results obtained in a previous study (Huang et al. 2019b). Fucoxanthin is also an effective fluorescence quencher that could prevent or reduce potential oxidative damage to the photosynthetic apparatus (Young and Britton 1990, Di Valentin et al. 2012).

In stark contrast, the pigment contents continued increasing in P-starved cells until NO_3^- deprivation in the medium (day 8). The pigments might be less sensitive to P starvation. They were still lower compared with control cells on day 7, which might be due to the lack of ATP and NADPH needed for pigment synthesis (Roopnarain et al. 2014). Maintenance of pigment content levels could play an important role in maintaining a moderate photosynthetic rate in P-starved cells (Fig. 3d), which would ensure continued growth.

Carbon partitioning

Cellular soluble proteins, carbohydrates, and lipids were measured and expressed on the basis of cellular C quota. Principal components analysis (PCA) ordination of samples from different culture stages, using the major classes of cellular compounds as descriptor variables, reflected the carbon partitioning according to the nutrient stress. Fig. 4a shows the projection of the variables into the plane spanned by the first two principal components. The first principal component (Dim1) accounted for 71.6% of total variation and positively correlated to content of soluble proteins, carbohydrates and polar lipids: (i.e., phospholipids [PL], betaine lipids [BL] and glycolipids [GL]), while negatively correlated with the neutral lipid content (mainly TAG) as well as total lipid content. The hierarchical clustering allowed two main clusters to be discriminated, well separated on the first two principal components (Fig. 4b). The largest cluster included the samples from the control (NP) cultures, 4-d and 7-d P-stressed cultures and 4-d N-stressed cultures. The second cluster included 11-day P-starved cultures, 7-d and 11-d N-starved cultures. The clusters were mainly characterized by GL ($\eta^2 = 0.78$, $p = 9.75\text{e-}10$), neutral lipid ($\eta^2 = 0.76$, $p = 2.75\text{e-}9$) and soluble protein ($\eta^2 = 0.66$, $p = 2.44\text{e-}7$) contents, followed by PL ($\eta^2 = 0.55$, $p = 1.08\text{e-}5$) and BL ($\eta^2 = 0.37$, $p = 8.26\text{e-}4$). The clusters were highly related to growth phase and nutrient status (χ^2 test, $p = 2.03\text{e-}7$), while less related to the type of

nutrient stress (χ^2 test, $p = 1.11 \times 10^{-2}$). Thus, in the following, we refer to cluster 1 as “replete or early stress” and cluster 2 as “starvation”.

In the replete or early stress cluster, the flow of carbon and energy were directed towards a high growth rate. One of the main carbon sinks was proteins, which are essential for cell growth. In the starvation cluster, on the contrary, cell division eventually halted, and the synthesis of proteins was strongly inhibited (Fig. 5a). The rate of protein loss was greater than the rate of cell division (data not shown), suggesting protein degradation. This reduced protein content under N-starved conditions has also been reported in earlier studies (Harrison et al. 1990, Brown et al. 1993, Da Costa et al. 2017). The products of amino acid decarboxylation could provide both the reducing power (NADH) and the precursor of acetyl-CoA for further fatty acid biosynthesis (Ge et al. 2014).

The content of soluble carbohydrates was only weakly related to the clusters (Fig. 5b). Given the low content measured, the soluble carbohydrates (especially chrysolaminarin) do not appear to be the major reservoir of carbon in this species. However, carbohydrate and fatty acid synthesis could still compete for the use of precursors (Wang et al. 2014). Orientation of the carbon flux towards the synthesis of soluble carbohydrates was strongly inhibited under N deficiency (in 11-day P-starved cultures and 11-day N-starved cultures) in the starvation cluster. The high loss rate of carbohydrates under N starvation suggested that carbohydrates could be partially used and converted into neutral lipids (Garnier et al. 2014).

TAG have been reported as the major lipid constituents in *Diatronema lutheri*, representing about 40-56% of the total lipids (Tatsuzawa and Takizawa 1995, Eichenberger and Gribi 1997, Meireles et al. 2003). In our study, TAG and membrane polar lipids behaved differently between the two clusters. Overall, in the replete or early stress cluster the cellular TAG content remained low (Fig. 5c), whereas actively dividing cells synthesized considerable amounts of polar lipids to generate membranes for daughter cells (Fig. 5, d-f). On day 7, PL content in P-starved cells was notably lower than in control cells (Fig. 5d), suggesting an inhibition of their formation. P would thereby be available for other essential cellular processes and ensure cell growth in low-P conditions (Raven 2013, Lin et al. 2016). Otherwise, the TAG content was much higher in P-starved cells compared with control cells on day 7 (Fig. 5c), suggesting TAG accumulation. Indeed, TAG production rate reached

a high level after about 3 days of P depletion in the medium, while ambient N was still available (Fig S2 in the Supporting Information).

In the starvation cluster, membrane polar lipid content was much lower (Fig. 5, d-f). The rate of GL loss was greater than the rate of cell division (data not shown), suggesting their degradation and inter-conversion. Thus, the fatty acid recycling from these membrane lipids could constitute an important source for TAG assembly (Guarnieri et al. 2011, Simionato et al. 2013, Martin et al. 2014, Garnier et al. 2016). The partitioning of carbon was reoriented towards neutral lipid accumulation. TAG content was more than twice that found in the replete or early stress cluster (Fig. 5c). This high accumulation of neutral lipids can be interpreted as a means of channeling excess carbon, energy and reductive power (NADPH) during the metabolic imbalance, when the relative growth rate is weak or null (Lacour et al. 2012, Valenzuela et al. 2012).

Lipid remodeling

Not only did nutrient stress affect the lipid content, but they might also modify the lipid composition, as reported in other species (Khozin-Goldberg and Cohen 2006, Li et al. 2014, Abida et al. 2015, Shemi et al. 2016, Cañavate et al. 2017a,b, Mühlroth et al. 2017, Huang et al. 2019a,b). In the present study, we analyzed lipid profiles to examine the lipid remodeling modes in *Diacronema lutheri* in response to P or N deprivation.

The two BLs found by TLC were diacylglycerol-3-carboxymethyl-choline (DGCC) and diacylglycerol hydroxymethyl-N,N,N-trimethyl- β -alanine (DGTA; Kato et al. 1995, Eichenberger and Gribo 1997). Phosphatidylglycerol (PG) was only detected in small quantities, and the absence of phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS) was confirmed by our results (Eichenberger and Gribo 1997, Meireles et al. 2003). With our current one-dimensional TLC, we did not separate diphosphatidylglycerols (DPG), which might be co-eluted with PG. Five different GLs were separated: monogalactosyldiacylglycerol (MGDG), digalactosyl-diacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) as the main compounds. One unidentified GL (GLx1) was assumed to be an acylated sterol glucoside as reported in previous studies (Meireles et al. 2003, Guihéneuf et al. 2015). In addition, one unidentified lipid was assumed to be

diacylglycerylglucuronide (DGGA) (Eichenberger and Gribi 1997), detected in a very small quantity, whereas further identification is still necessary. (Fig S3 in the Supporting Information).

BL constitute the major compounds of non-plastidial membranes in *Diacronema lutheri*, with DGCC being the main molecular species, accounting for 63–78% of the total extraplastidial lipids in all experimental conditions (Fig. 6a). The non-plastidial membrane composition was less affected by nutrient starvation. On day 7, it showed 3.7% dissimilarity under N starvation and 2.7% dissimilarity under P starvation, with no difference ($R = 0.0741$, $p = 0.002$ in N- and $R = -0.1111$, $p = 0.006$ in P-, respectively) compared with the control.

In the BL fraction, the major fatty acids (FA) were palmitic (C16:0), oleic (C18:1n-9), eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA, C22:6n-3) acids, accounting for 84–90% under all experimental conditions (Fig. 7a). The last two LC-PUFAs represented large proportions. Tatsuzawa and Takizawa (1995) have suggested that EPA and DHA are at maximum levels in BL, and play a major role in acclimation to low temperatures. In the present study, the FA composition of BL varied little under nutritional starvation conditions on day 7 compared with controls ($R = 0.1852$, $p = 0.003$ in N-; $R = -0.03704$, $p = 0.005$ in P-). However, there was a tendency for a decrease in the relative proportion of EPA, especially under N starvation, which might be related to the transfer of EPA between the lipid fractions (Guihéneuf and Stengel 2017). Eichenberger and Gribi (1997) studied the incorporation of [$1\text{-}^{14}\text{C}$] oleate into lipids of *D. lutheri* and found that DGCC is involved in the transfer of FA from cytoplasm to chloroplasts. Indeed, DGCC is considered to be a primary acyl carrier for other lipids in *D. lutheri* (Kato et al. 1995), whereas DGTA is a final acceptor of C22 fatty acids (Eichenberger and Gribi 1997). Our results showed that C18:1n-9 and DHA did not follow the same tendency of EPA, suggesting that these acyl groups might be transferred independently (Eichenberger and Gribi 1997).

In chloroplasts, thylakoid membranes are mainly composed by MGDG, DGDG, SQDG, and PG (Li-Beisson et al. 2010). MGDG and DGDG are uncharged GL, constituting most of the thylakoid lipid bilayer (Demé et al. 2014), while SQDG may be related to the maintenance of an anionic charge on the surface of the thylakoid membrane (Kobayashi et al. 2016). PG is the main PL in chloroplasts and constitutes an essential component in PSII centers (Boudière et al. 2014, Li-Beisson et al. 2016). Even though the GL content was highly reduced in the second cluster due to N deficiency as

mentioned above, the polar lipid composition seemed to be little affected (Fig. 6b). On day 7, the thylakoid membrane composition of N-starved cells showed 5.5% dissimilarity with no difference ($R = 0.1481$, $p = 0.004$) compared with the control. The maintenance of lipid composition in thylakoid membranes might play an important role in homeostasis under N shortage despite the massive degradation. Following P deprivation, thylakoid membrane composition was relatively more affected (6.6% dissimilarity with a moderated overall difference, $R = 0.6296$, $p = 0.001$) compared with the control (Fig. 6b). This increased overall difference was mainly due to the decrease in PG proportion and increase in SQDG proportion. Similar results have also been reported in previous studies in other species (Abida et al. 2015, Shemi et al. 2016, Cañavate et al. 2017b, Hunter et al. 2018, Huang et al. 2019a). The increase of SQDG could partly compensate the lack of PG in plastids (Jouhet et al. 2009), although there was no significant difference in SQDG content compared with control cells on day 7 (data not shown). Otherwise, MGDG/DGDG ratio was reduced in both P and N-starved cells compared with control cells on day 7. The reduced MGDG/DGDG ratio often signifies impaired photosynthesis, reflecting senescence of thylakoid membranes (Boudière et al. 2014, Abida et al. 2015, Huang et al. 2019a).

The FA composition of GL in *Diacronema lutheri* on day 7 is given in Fig. 7b. The C16:0, palmitoleic acid (C16:1n-7), C18:1n-9, and EPA accounted for 70–76% of total FA in GL under all experimental conditions. According to the literature, the major FA of MGDG and DGDG are EPA and stearidonic acid (C18:4n-3), whereas SQDG mainly contains C16:0 and myristic acid (C14:0; Kalisch et al. 2016). DHA was particularly present in DGGA, but difficult to detect in MGDG, DGDG or SQDG (Kalisch et al. 2016).

The FA composition of GL was little affected by N deficiency: 7% dissimilarity with no difference ($R = -0.03704$, $p = 0.005$) compared with the control on day 7. Nevertheless, the proportions of EPA and C18:4n-3 tended to decrease. These two FA could contribute to the constitution of other lipid fractions (Guihéneuf and Stengel 2017). Following P deprivation, on the one hand, the proportions of LC-PUFAs such as EPA and C18:4n-3 tended to decrease; on the other hand, the proportions of the newly synthesized FA species: C14:0, C16:0 and C16:1n-7 tended to increase. Given the continuous growth, GL synthesis in daughter cells might be strongly affected by

de novo FA synthesis in chloroplasts. The FA composition showed 11% dissimilarity with a large overall difference ($R = 0.8148$, $p = 0.001$), compared with control one on day 7.

The most notable changes in FA composition upon nutritional starvation occurred in the fraction of neutral lipids. The major FA of neutral lipids were C16:0, C16:1n-7, C18:1n-9 and linoleic acid (C18:2n-6), accounting for 70–78% under all experimental conditions (Fig. 7c). The proportions of EPA and DHA remained low in this fraction. P and N starvations on day 7 caused 36% and 45% dissimilarity respectively, with a strong overall difference (ANOSIM $R = 1$, $p = 0.001$) compared with the control. The main changes in FA profile were associated with C14:0, C16:0 and C16:1n-7 increase and concomitant C18:1n-9 and C18:2n-6 decrease. C14:0, C16:0 and C16:1n-7 are normally newly synthesized FA species. Their increase suggests that the accumulation of TAG was mainly from the *de novo* FA synthesis pathway (Simionato et al. 2013, Martin et al. 2014, Abida et al. 2015, Huang et al. 2019a). However, the decrease of C18 proportions suggested a relatively low FA elongation outside chloroplasts (Guihéneuf et al. 2011). Otherwise, the proportion of EPA in neutral lipids increased 2.1 and 2.9-fold on day 7 under P- and N-starved conditions, respectively, which might mainly arise from the recycling of membrane polar lipids as mentioned before. Conversely, the proportion of DHA remained relatively stable. This result suggests that the accumulation of EPA and DHA in neutral lipids could be regulated by two different mechanisms (Boelen et al. 2017).

Overall, omega-3 LC-PUFAs content in neutral lipids was significantly higher under P and N starvations, and the omega-6/ omega-3 ratio decreased. Such a low ratio in neutral lipids in *Diacronema lutheri* makes it a good candidate for use as a dietary supplement with health benefits.

In this study, the biochemical and physiological changes triggered by N or P stress were observed under continuous light. This light regime generally achieves high biomass productivity (Jacob-Lopes et al. 2009) and is suited for biotechnological purposes. However, under natural conditions the photoperiodic light can modulate and trigger different physiological and biochemical responses. Under these natural conditions, the interpretation of our results may not be straightforward.

Conclusions

N starvation seems to be a severe stress for *Diacronema lutheri*, triggering arrest of cell division, much lower photosynthetic rate and reduced biomass productivity. The degradation and inter-

conversion of essential cellular compounds contributed to TAG accumulation. The photosynthetic machinery was also hampered by P starvation. Conversely, maintaining the pigment content maintained a moderate photosynthetic rate in P-starved cells. The flows of carbon and energy were still mainly towards high growth rate.

The present study produced an overall lipid profile of *Diacronema lutheri* at different growth phases and under P and N stress. BL constitute the major compounds of non-plastidial membranes in *D. lutheri*. PL were reduced under both P- and N-starvations and so might not be involved in the regulation of P storage. However, a specific increase of SQDG proportion was observed in thylakoid membranes following P deprivation, which could partly compensate the lack of PG in plastids.

In *Diacronema lutheri*, EPA and DHA are mainly associated with BL. Under P and N starvations, a transfer of EPA from the recycling of membrane polar lipids was presumed, most likely contributing to the accumulation of TAG. Indeed, the proportion of EPA in the fraction of neutral lipids increased under both P- and N-starved conditions. This characteristic makes *D. lutheri* a good candidate for further investigation of low-cost, sustainable and environmentally friendly production-extraction systems for LC-PUFAs.

In our study, the stationary phase under P starvation was accompanied by NO_3^- depletion, leading to very similar responses to N starvation. To better understand the impact of P starvation in *Diacronema lutheri*, it would be necessary to test a much higher N:P ratio. Future work should address the characterization of lipid synthesis and degradation and the trafficking of FA involved in lipid class reshuffling. A search for genes involved in lipid metabolism in this species should also be undertaken.

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Fig. 1. Growth of *Diacronema lutheri* under control (NP), P-stressed (P-) or N-stressed (N-) conditions over 11 days in batch cultures. (Mean $\pm$ S.D., $n = 3$). (a) Semi-log plot of growth curves (cell density $\cdot \text{mL}^{-1}$) and specific growth rate (d^{-1}), (b) Dissolved NO_3^- concentration (mM) and PO_4^{3-} concentration (μM) in N-stressed cultures, (c) Dissolved NO_3^- concentration (mM) and PO_4^{3-} concentration (μM) in P-stressed cultures, (d) Dissolved NO_3^- concentration (mM) and PO_4^{3-} concentration (μM) in NP cultures, (e) Cellular quota of N (N/C) ($\text{mol} \cdot \text{mol}^{-1}$), the arrows and the dash-dotted line indicate the beginnings of stationary phases and the corresponding N quota levels, respectively.

Fig. 2. Chlorophyll *a* fluorescence parameters of *Diacronema lutheri* grown under control (NP), P-stressed (P-) or N-stressed (N-) conditions. (Mean $\pm$ S.D., $n = 3$). (a) Maximum photochemical efficiency of PSII (F_v/F_m), (b) Effective photochemical efficiency of PSII (Φ_{II}) under $160 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, (c) Non-photochemical quenching (NPQ) under $160 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

Fig. 3. Cellular pigment contents and net photosynthesis rate of *Diacronema lutheri* grown under control (NP), P-stressed (P-) or N-stressed (N-) conditions. Different letters indicate significant ($p < 0.05$) differences (Mean $\pm$ S.D., $n = 3$). (a) Chl *a* content per unit cell carbon ($\text{mg} \cdot \text{g}^{-1}$), (b) The ratio of Chl *c*/Chl *a* ($\text{mol} \cdot \text{mol}^{-1}$), (c) The ratio of Fucoxanthin/Chl *a* ($\text{mol} \cdot \text{mol}^{-1}$), (d) Net photosynthesis rate per unit cell carbon ($\mu\text{mol O}_2 \cdot \text{h}^{-1} \cdot \text{mg C}^{-1}$).

Fig. 4. Principal components analysis (PCA) using the major classes of cellular compounds (per unit cell carbon) as descriptor variables on days 4, 7 and 11 under control (NP), P-stressed (P-) or N-stressed (N-) conditions. (a) Variable factor map, (b) Individual factor map; samples were separated into 2 main clusters. In the right: “replete or early stress” cluster. In the left: “starvation” cluster.

Fig. 5. Proximate biochemical composition of *Diacronema lutheri* grown under control (NP), P-stressed (P-) or N-stressed (N-) conditions, on days 4, 7 and 11 of culture. Different letters indicate

significant ($p < 0.05$) differences (Mean $\pm$ S.D., $n = 3$). (a) Soluble protein content per unit cell carbon ($\text{g} \cdot \text{g}^{-1}$), (b) Soluble carbohydrate content per unit cell carbon ($\text{g} \cdot \text{g}^{-1}$), (c) Neutral lipid content measured as fluorescence of microalgal cells stained with Nile red per unit cell carbon ($\mu\text{g C}^{-1}$), (d) Phospholipid content per unit cell carbon ($\text{mg} \cdot \text{g}^{-1}$), (e) Glycolipid content per unit cell carbon ($\text{mg} \cdot \text{g}^{-1}$), (f) Betaine lipid content per unit cell carbon ($\text{mg} \cdot \text{g}^{-1}$).

Fig. 6. Lipid composition (expressed in mass %) in non-plastidial and plastidial membranes of *Diacronema lutheri* grown under control (NP), N-stressed (N-) or P-stressed (P-) conditions on days 4, 7 and 11 of culture. Different letters indicate significant ($p < 0.05$) differences (Mean $\pm$ S.D., $n = 3$). (a) Composition of major classes of lipids in non-plastidial membranes (DGCC, diacylglycerol-3-carboxymethylcholine; DGTA, diacylglycerylhydroxymethyltrimethyl- β -alanine), (b) Composition of major classes of lipids in plastidial membranes (MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyl-diacylglycerol; PG, phosphatidylglycerol).

Fig. 7. Fatty acid composition (expressed in mol %) in three main fractions of lipids extracted from *Diacronema lutheri* grown under control (NP), N-stressed (N-) or P-stressed (P-) conditions on day 7 of culture. Different letters indicate significant ($p < 0.05$) differences (Mean $\pm$ S.D., $n = 3$). (a). Fatty acid composition in phospho- and betaine lipids, (b). Fatty acid composition in glycolipids, (c). Fatty acid composition in neutral lipids. Fatty acids: C14:0, myristic acid; C16:0, palmitic acid; C16:1n-7, palmitoleic acid; C16:2n-6, hexadecadienoic acid; C18:0, stearic acid; C18:1n-9, oleic acid; C18:2n-6, linoleic acid; C18:3n-3, α -linolenic acid; C18:4n-3, stearidonic acid; C20:2n-6, eicosadienoic acid; C20:4n-6, arachidonic acid; C20:5n-3, eicosapentaenoic acid; C22:4n-6, docosatetraenoic acid; C22:5n-3, docosapentaenoic acid; C22:6n-3, docosahexaenoic acid.

Fig. S1. Slow Chl *a* fluorescence induction kinetics of *Diacronema lutheri* grown under (a-1) control (NP), (b-1) P-stressed (P-) or (c-1) N-stressed (N-) conditions on day 7 of culture, measured under a high actinic irradiance ($1000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and their respective qN relaxation kinetics after switching off the actinic light ((a-2) control (NP) ; (b-2) P-stressed (P-); (c-2) N-stressed (N-)). Three individual components of non-photochemical quenching (qN) of variable Chl fluorescence: qN_f (fast component, s timescale), qN_i (intermediate component, min timescale) and qN_s (slow component, h timescale) were studied according to Roháček et al. (2014). $F_v''(t)$ fit resulted from the sum of the three component qN_f, qN_s et qN_i. (d) Proportions of qN_f, qN_i and qN_s in qN.

Fig. S2. Neutral lipid production rate (d^{-1}) of *Diacronema lutheri* grown under control (NP), P-stressed (P-) or N- stressed (N-) conditions. (Mean $\pm$ S.D., $n = 3$). Neutral lipid production rate was calculated according to the equation: $\mu_{\text{NL}} = (\ln \text{NL}_{t2} - \ln \text{NL}_{t1}) / (t2-t1)$, where NL_{t2} and NL_{t1} are the neutral lipid content expressed as intensity of Nile Red fluorescence per unit cell carbon ($\mu\text{g C}^{-1}$) on two subsequent culture days ($t2$ and $t1$).

Fig. S3. Thin-layer chromatography (TLC) plate of a sample (Sp, right) of *Diacronema lutheri* and standards (Std, left). On both sides of the graphic, the spectrum of bands was read after spraying with primuline at 530nm. TAG, triacylglycerols; FFA, free fatty acids; MGDG, monogalactosyldiacylglycerol; GLx1, 2, unidentified glycolipids 1 and 2; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyl-diacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; DGTS, diacylglyceryltrimethylhomoserine; DGTA, diacylglycerylhydroxymethyltrimethyl- β -alanine; DGCC, diacylglycerol-3-carboxymethylcholine; UK, unidentified lipid.

a

b

c

d

e

a **b** **c**

a

b

c

d

a

b

a

Lipid distribution in non-plastidial membranes

Days of culture and lipid class

b

Lipid distribution in plastidial membranes

Days of culture and lipid class