Microalgal lipid production: A comparative analysis of *Nannochloropsis* and *Microchloropsis* strains

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Abstract

The oleaginous genera *Nannochloropsis* and *Microchloropsis* are recognized for their lipid accumulation capacity. Microalgal lipid accumulation is triggered by nitrogen starvation, negatively affecting photosynthesis and growth. Moreover, light and temperature play pivotal roles in microalgal physiology, lipid accumulation and composition. This study focuses on comparing the responses of eight microalgal strains from *Nannochloropsis (N. oceanica* Necton*, N. oceanica* IMET1*, Nannochloropsis.* sp*.* CCAP211/78*, N. oculata,* and *N. limnetica)* and *Microchloropsis (M. gaditana* CCFM01, *M. gaditana* CCMP526, and *M. salina)* to light, temperature, and nitrogen availability. Biomass, lipid content and productivities were monitored under different light intensities (150 (LL) and 600 µmol photons $m^{-2} s^{-1}$ (HL)) and temperatures (15, 25, 30℃) under nitrogen (N-) starvation and replete conditions. Under N-starvation and HL, *N.* sp. exhibited the highest lipid content (59%) and productivity (0.069 g L^{-1} day^{-1}), while *N. oculata* had the lowest lipid content (37.5%) and productivity (0.037 g L^{-1} day^{-1}) among the eight strains. Notably, *M. gaditana* CCFM01 achieved the highest EPA content (4.7%), contrasting with *N. oceanica* IMET1 lowest EPA content (2.9%) under 150 µmol photons $m^{-2} s^{-1}$ and N-repletion. The response to temperature fluctuations under LL was strain-dependent. *Microchloropsis salina* and *M. gaditana* CCFM01 demonstrated the highest and lowest lipid productivities (0.069 g L^{-1} day^{-1} and 0.022 g L^{-1} day^{-1}, respectively) at 15℃ under N-starvation. Moreover, significant EPA accumulation across various strains was observed in *N. oculata* (5.7%) under N-repletion at 15°C, surpassing *M. gaditana* CCFM01 by 40%. Ultimately, the physiological responses to cultivation conditions vary markedly among microalgal strains, even within the same genus or species. This knowledge is essential for selecting suitable strains for the efficient microalgal lipid production industry.

Keywords Microalgae · Eustigmatophyceae · *Nannochloropsis* · *Microchloropsis* · Lipid · TAG · Nitrogen starvation · Light intensity · Temperature

Introduction

Microalgae are of interest because their potential to produce oils and a diverse array of high-value compounds, including unsaturated fatty acids, vitamins, pigments, carotenoids, polysaccharides, minerals, and other fine chemicals (Schulze et al. 2019; Abo-Shady et al. 2023) for an extensive range of applications, from biofuels to nutraceuticals (Abdel-Karim et al. 2020; Gheda et al. 2021). Their ability to thrive under adverse conditions without needing fertile land makes them suitable for sustainable cultivation without competing with food production (Sivagurulingam et al. 2022). Moreover, many microalgae can achieve high cell densities in marine or brackish water, significantly reducing water consumption and enhancing sustainability (Park and Lee 2016). They also require specific yet cost-effective growth conditions, allowing for rapid growth rates under high-light conditions.

However, there are challenges in microalgae lipid production and cultivation that hinder market introduction and expansion. These challenges encompass strain selection, strain improvement, the high cost of extracting essential components from algal biomass, and the adverse

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impacts of climate variations on microalgae production. Overcoming these hurdles will command technological advancements and scientific innovation in producing and processing microalgae-based lipids (Aratboni et al. 2019; Hanifzadeh and Nabati 2019).

The lipid content in microalgae typically ranges from 20 to 50% of the cell's dry weight under normal conditions (Gui et al. 2021) and under specific conditions it can reach up to 80% (Chisti 2007; Gui et al. 2021), comprising diverse molecules that vary in function from cellular structures to energy storage. Triacylglycerols (TAGs) are ideal for biofuel production and polyunsaturated fatty acids (PUFAs) like (eicosapentaenoic acid, EPA), which are commercially used in nutraceuticals production and aquaculture. Numerous strategies have been exploited to produce these lipids (El Shafay et al. 2021; Elshobary et al. 2022). These strategies encompass controlling nutrient availability (carbon, nitrogen and phosphorus), adjusting environmental conditions (pH, salinity, light intensity, and temperature) (Sibi et al. 2016; Gaignard et al. 2021; Morales et al. 2021), and implementing genetic and metabolic engineering approaches (Li-Beisson et al. 2015; Hulatt et al. 2020).

One of the widely adopted strategies for stimulating lipid accumulation, especially TAGs (energy storage) in microalgae, is nitrogen limitation. Nitrogen deficiency hampers microalgal growth and causes an energy imbalance by accumulating photosynthetic reductant power, leading to photooxidative damage of photosystems I and II (Juergens et al. 2015). To counteract this, TAG biosynthesis is stimulated to act as an alternative sink for the excess reductant power to protect the photosynthetic apparatus and enhance lipid accumulation functioning as energy reserves for cell survival (Klok et al. 2013; Karima et al. 2018; Maltsev et al. 2023). This approach has been shown to yield the highest lipid production in various species, including the genera *Nannochloropsis* and *Microchloropsis* (Converti et al. 2009; Jia et al. 2015; Huete-Ortega et al. 2018).

The choice of the most productive and resilient species for a given application is contingent upon their response to essential environmental factors like light and temperature (Sibi et al. 2016; Gaignard et al. 2021; Morales et al. 2021). Light is critical, impacting microalgae's photosynthetic and metabolic pathways. High-light intensities have enhanced lipid accumulation, influencing the equilibrium between the storage lipids and structural membrane (Nadzir et al. 2018; Metsoviti et al. 2020; Nzayisenga et al. 2020). It imposes a variation in the allocation of lipid classes, changes in fatty acid profile, and the increased percentage of shorter fatty acids associated with the formation of TAGs (Maltsev et al. 2021; Chin et al. 2023). The stress caused by higher light intensity under nitrogen deprivation further exacerbates the energy imbalance, leading to even greater TAG production

(Solovchenko et al. 2014; El Shafay et al. 2021; Chin et al. 2023).

After light, temperature is another critical cultivation parameter that significantly influences all microalgal metabolic processes, growth, and biological composition, with the optimal temperature for a particular strain profoundly impacting culture productivity (Borowitzka 2016). Variability in culture environmental conditions, such as daily and seasonal temperature fluctuations, can lead to growth rate fluctuations and microalgae lipid accumulation (Sheng et al. 2011; Wei et al. 2015; Ippoliti et al. 2016). Deviations from the optimal temperatures can disrupt the metabolic balance by reducing the enzymatic processes associated with carbon and nitrogen assimilation, impact microalgal growth, affect membrane integrity, and alter the fatty acid profiles, particularly reducing unsaturated fatty acids which are vital for maintaining cellular membrane fluidity (Wei et al. 2015; Barati et al. 2019; Ferrer-Ledo et al. 2023).

The genera *Nannochloropsis* and *Microchloropsis* (formerly *Nannochloropsis,* Fawley et al. 2014) are some of the most attractive oleaginous microalgae for lipid production. Under stress conditions, they can produce storage carbohydrates (a β-1,3-glucan) and TAG as intracellular energy storage compounds (Morales et al. 2021). These genera comprise many different marine species (*N. oceanica, N. oculata, N. granulata, M. gaditana* and *M. salina*) and the freshwater *N. limnetica,* which notably accumulates over 60-70% TAG of its dry biomass under nitrogen starvation, which is drastically higher than the content of higher plants and other microalgae genera (Ma et al. 2014). In addition, they are natural sources of high-value PUFAs such as omega-3 fatty acids, including (EPA), which are commercially used in nutraceutical production and in aquaculture (Ryu et al. 2020). Moreover, these species have proven suitable for outdoor cultivation, offering versatile genetic platforms for the desired application (Naduthodi et al. 2021).

Nevertheless, significant variations have been reported between the different species and strains of *Nannochloropsis* and *Microchloropsis* in growth, lipid content, and lipid productivity, indicating the need for tailored cultivation strategies. For instance, Ma et al. (2014) observed that among 9 different strains of *Nannochloropsis* and *Microchloropsis*, *N. oceanica* IMET1 exhibited the fastest specific growth rate, lipid and TAG production. Moreover, *N.* sp. showed the highest fatty acid content and biomass concentration among the tested species (Benvenuti et al. 2014). Yet, it is worth noting that none of these studies were conducted under the same cultivation conditions, making direct species-to-species comparisons challenging. Additionally, they focused on how light and temperature conditions impact growth under nitrogen-replete conditions, leaving limited information on their influence on lipid accumulation during nitrogen starvation.

In this study, we explore the effects of light intensity, temperature, and their interaction with nitrogen availability on biomass and lipid production for eight strains from the genera *Nannochloropsis* (*N. oceanica* Necton, *N. oceanica* IMET1, *N.* sp. CCAP211/78, *N. oculata,* and *N. limnetica*) and *Microchloropsis* (*M. gaditana* CCFM01, *M. gaditana* CCMP526, *M. salina*).

Materials and methods

Algal strains, growth medium, and pre-cultivation conditions

Nannochloropsis oceanica Necton (was provided by NEC-TON, SA (Olhão, Portugal)), *Nannochloropsis oceanica* IMET1 was a kind gift from Prof. Jian Xu (Qingdao Institute for Bioenergy and Bioprocess Technology, Chinese Academy of Sciences). The other strains were from various culture collections: *Microchloropsis gaditana* CCFM-01 (Microalgae Collection of Fitoplancton Marino S.L., CCFM, Spain), *Microchloropsis gaditana* CCMP526 and *Nannochloropsis limnetica* (Culture Collection of National Center for Marine Algae and Microbiota, USA), *Nannochloropsis* sp. CCAP211/78 (Culture Collection of Algae and Protozoa, UK)*, Nannochloropsis oculata* SAG38.85 *and Microchloropsis salina* SAG40.85 (Department Experimental Phycology and Culture Collection of Algae (EPSAG), Göttingen University, Germany.

The marine strains were cultivated in an artificial seawater (ASW) medium prepared based on Breuer et al. (2013a). Nutrients were supplied in excess; nitrogen and phosphorous were added to support a biomass concentration of 4 g L^{-1}, assuming a 10% and 1% w/w biomass content of nitrogen and phosphorous, respectively. This was done to prevent nutrient limitations. In starvation conditions, there was no nitrogen in the medium, and only the amount of phosphate was adjusted to prevent any limitation.

The complete artificial seawater medium with modified Nutribloom per liter contained final concentrations of NaCl 418.14 mM; Na_2SO_4 13.33 mM; $CaCl_2 \cdot 2H_2O$ 5.42 mM; K_2SO_4 5.47 mM; $MgCl_2 \cdot 6H_2O$ 48.54 mM; $NaNO_3$ 42.82 mM; KH_2PO_4 1.03 mM; $ZnSO_4 \cdot 7H_2O$ 0.44 mM; $MnCl_2 \cdot 4H_2O$ 3.6 mM; $Na_2MoO_4 \cdot 2H_2O$ 0.126 mM; $CoCl_2 \cdot 6H_2O$ 0.2 mM; $CuSO_4 \cdot 5H_2O$ 0.196 mM; $Na_2EDTA \cdot 2H_2O$ 0.372 mM; $MgSO_4 \cdot 7H_2O$ 0.292 mM; $FeCl_3 \cdot 6H_2O$ 0.108 mM; and 20 mM HEPES (4-(2-hydroxy-ethyl) piperazine-1-ethanesulfonic acid). The pH of the medium was adjusted with 1 M NaOH to pH 7.5. For ASW (N-starvation), no $NaNO_3$ was added.

Nannochloropsis limnetica was cultivated in BG-11 freshwater medium (Das and Kundu 2011), designed according to the chemical composition per liter with final concentration of Na_2EDTA 0.269 mM; ferric ammonium citrate 0.16 mM; citric acid.$1H_2O$ 0.168 mM; $CaCl_2 \cdot 2H_2O$ 0.956 mM; $MgSO_4 \cdot 7H_2O$; 0.986 mM; K_2HPO4 0.646 mM; H_3BO_3 1.872 mM; $MnCl_2 \cdot 4H_2O$ 0.325 mM; $ZnSO_4 \cdot 7H_2O$ 0.030 mM; $CuSO_4 \cdot 5H_2O$ 0.024 mM; $CoCl_2 \cdot 6H2O$ 0.017 mM; $NaMoO_4 \cdot 2H_2O$ 0.103 mM; Na_2CO_3 0.189 mM; $NaNO_3$ 17.65 mM, the pH of the growth medium was adjusted with 1M HCl to pH 7.5. For BG-11(N-starvation) no $NaNO_3$ was added. The media were directly filtered and sterilized by Sartobran Capsule 0.2 μm into the Erlenmeyer flasks.

All strains were cultivated in duplicate with an initial optical density (OD) of 0.5 at 750 nm under identical controlled conditions in 250 mL Erlenmeyer flasks (150 mL of culture) in an orbital shaker incubator (Multitron, Infors HT, Switzerland) (100 rpm) with approximately 150 μmol photons $m^{-2} s^{-1}$ continuous light supplied by fluorescent lamps (TL-D Reflex 36W/840, Philips) at 25 °C and 2% CO_2 enriched air. The cultures were kept under a day/night cycle light regime (12:12 h).

Experimental approach

a. The effect of light intensity

The duplicate flasks were pooled. Then, cells were resuspended in 50 mL Erlenmeyer flasks (25 mL of culture) of either N-replete or N-starvation medium at an OD_{750} of 0.5. The moment of resuspension was considered the beginning of the experiment (t=0). Finally, triplicate flasks were incubated for 12 days under identical controlled conditions in Algem HT24 from Algenuity on an orbital shaker incubator (Multitron, Infors HT, Switzerland) with 100 rpm at 25 °C and 2 % CO_2 enriched air and two different light intensities of approximately 150 and 600 μmol photons $m^{-2} s^{-1}$. The cultures were kept under a day/night cycle light regime (12:12 h).

b. The effect of temperature

The duplicate flasks were pooled. Then, cells were resuspended in 50 mL Erlenmeyer flasks (25 mL of culture) of either N-replete or N-starvation medium at an OD_{750} of 0.5. The moment of resuspension was considered the beginning of the experiment (t=0). Finally, triplicate flasks were incubated for 12 days under identical controlled conditions in Algem HT24 from Algenuity on an orbital shaker incubator (Multitron, Infors HT, Switzerland) with 100 rpm at 150 μmol photons $m^{-2} s^{-1}$ and 2 % CO_2 enriched air and three different temperature degrees, approximately 15, 25, and 30 °C. The cultures were kept under a sinus day/night cycle light regime (12:12 h).

Offline culture measurements

Samples were taken from all flasks at days 0, 2, 4, 6, 8, 10, and 12 for offline optical density (OD), cell concentration, and quantum yield (QY) measurements. Biomass concentration, dry weight (DW), and fatty acid analysis were measured at the experiments' beginning and end.

The optical density (OD) was measured at 750 nm using a UV-VIS spectrophotometer (Hack Lange DR-6000, light path 10 mm).

Cell concentration was measured with the mutlisizer III (Beckman Coulter) using a 50 µm aperture tube and after diluting the sample 100 times in ISOTON II diluent.

The photosystem II (PSII) maximum quantum yield (F_v/F_m) was measured after 20 min of dark adaptation at room temperature (AquaPen-C 100, PSI, Czech Republic; excitation light 455 nm, saturating light pulse: 3000 µmol photons $m^{-2}s^{-1}$). The F_v/F_m ratio was calculated according to Benvenuti et al. (2016).

Biomass dry weight (Cx, $g\ L^{-1}$) was measured from the moment of nitrogen starvation (t=0) as described by Breuer et al. (2013b).

Biomass volumetric production rate P_x was calculated according to (Eq. (2)), where C_{xt} and C_{x0} correspond to the dry weight measured for day t and day 0, respectively, and T is the time of the experiment (12 days):

$$P_x(g\ L^{-1}day^{-1}) = \frac{C_{xt} - C_{x0}}{T}$$

Nitrogen presence was monitored using the nitrate test (colorimetric, 10-500 mg L^{-1} (NO_3^-), for use with MQuant StripScan App). Although nitrogen was not directly measured, the nitrate levels served as an indicator of its presence. Samples were analyzed every 2 days to ensure consistent monitoring of nitrogen availability throughout the experiment.

Fatty acid analysis: The lipid composition of the different strains was measured at the experiment's beginning and end. Biomass samples were centrifuged, washed two times with 0.5M ammonium formate, and stored at -20 °C until lyophilization. Biomass fatty acid content (FFA, g g^{-1}) and profile were determined by mechanical cell disruptions, solvent-based lipid extraction, and trans-esterification of fatty acids to fatty acid methyl esters (FAMEs). Lipids were extracted, separated into triacylglycerol (TAG) and polar (PL) fractions, and quantified with GC-FID as reported by Breuer et al. (2013a). Briefly, more than 10 mg (Tables 1S and 2S, show the exact amounts) of lyophilized biomass was disrupted with a beater. Lipids were extracted with chloroform:methanol (1:1.25, v:v) containing the internal standards for TAG and PL fractions, 170 µg mL^{-1} of tripentadecanoin (9:0) and 170 µg mL^{-1} of

1,2-dipentadecanoyl-*sn*-glycero-3-[phosphorrac-(1-glycerol)] (sodium salt) (15:0) respectively. Different elution solvents separated TAG and PL fatty acids, hexane:diethyl ether (7:1, v:v) and methanol:acetone:hexane (2:2:1, v:v), respectively, in a SPE silica gel column (Sep-Pak Vac 6cc, Waters), which were evaporated after that. Then, fatty acids were saponified and methylated in methanol with 5% H_2SO_4 at 100 °C for 1 h. The fatty acid methyl esters (FAMEs) were extracted with hexane and quantified on the GC-FID (Agilent 7890). The TAG and PL fatty acid contents were calculated by summing all fatty acids measured per mg of biomass in each lipid fraction.

$$P_{FA}(g\ L^{-1}day^{-1}) = \frac{LC_t \times C_{Xt} - LC_0 \times C_{X0}}{T}$$

where P_{FA}= lipid productivity, LC_t= final lipid content after 12 days of cultivation, C_{xt}= final biomass of the microalgae in the lipid-producing phase after 12 days of cultivation, LC_0= initial lipid content at zero time, C_{x0}= initial biomass in the lipid-producing phase at zero time, T= the cultivation time.

Statistical analysis

Statistical analysis was performed using one-way ANOVA and two-way ANOVA followed by comparing means in cross-tabulation and the Bonferroni t-test (IBM SPSS Statistics 28 software)., All results are expressed as mean ± standard deviation (SD) (n=3) at a significance level of $P<0.05$.

Results and discussion

Nannochloropsis and *Microchloropsis* are relevant in microalgal research and biotechnological applications due to their capability to produce high contents of triacylglycerols (TAGs) and essential omega-3 polyunsaturated acids like eicosapentaenoic (EPA Rodolfi et al. 2009; Benvenuti et al. 2014). Strains of *Nannochloropsis* and *Microchloropsis* can thrive in marine and freshwater (*N. limnetica*) habitats. This adaptability is critical for cultivating these algae in seawater, which does not compete with the freshwater resources needed for agriculture, increasing its sustainability (Converti et al. 2009; Simionato et al. 2013).

In this study, we systematically evaluated eight *Nannochloropsis* and *Microchloropsis* strains under varied abiotic stressors, including nitrogen availability, light intensity, and temperature. This comprehensive approach provides a systematic and robust comparative analysis showing not only species-specific responses but also strain-specific

responses and new insights into optimizing microalgal lipid production.

The effect of the different light intensities and nitrogen availability on

a. Photosynthetic efficiency.

When screening microalgae for lipid productivity it is essential to know the photosynthetic capacity under stress conditions. Maximum quantum yield (F_v/F_m) values around 0.6–0.7 are expected for healthy cells, whereas lower values are observed if cells have been exposed to biotic or abiotic stress (Young and Beardall 2003; Simionato et al. 2013). In our study F_v/F_m of cultures cultivated in nitrogen (N)-replete conditions did not change over time in low light (LL) (Fig. 1A). However, the quantum yield decreased in the first two days when the cells were exposed to high light (HL) and then increased to the typical values (Fig. 1C). This confirmed the findings of Benvenuti et al. (2014) where PSII maximum quantum yield is independent of growth irradiance as long as nutrients are not limiting and cells are fully photoacclimated.

On the contrary, N-starvation resulted in a significant decrease in quantum yield for all strains (Fig. 1, with a substantial decline in F_v/F_m by the end of the 12-day cultivation period in N-starved cultures under LL. Notably, cells grown in HL exhibited an even greater decrease than those in LL. The lowest F_v/F_m values were observed in *N. oceanica* IMET1 and Necton, at 0.23 and 0.28, respectively, while *N. limnetica* showed the highest F_v/F_m of 0.54 at HL. This may be attributed to the fact that, out of the eight strains studied, only *N. limnetica* was cultured in freshwater. The ability of freshwater *N. limnetica* to maintain its F_v/F_m under N-starvation likely results from its evolutionary adaptation to freshwater environments, involving efficient light harvesting, nutrient utilization and specialized physiological and metabolic mechanisms for coping with nutrient fluctuations, unlike the marine strains (Ma et al. 2014).

b. Lipid content.

Figure 2 shows variations in the total lipid content (%) during the cultivation of different strains under varying light intensities and nitrogen availability conditions. Our findings reveal that all cells exhibited a modest increase in lipid content under high light intensities under N-repletion conditions. Notably, there were no substantial variations in lipid content among the different strains. Among all the species

Fig. 1 PSII maximum quantum yield of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) or nitrogen starvation conditions (N-) under low light intensity 150 µmol photons m^{-2} s^{-1} (**A** and **B**) and high light intensity 600 µmol photons m^{-2} s^{-1} (**C** and **D**)

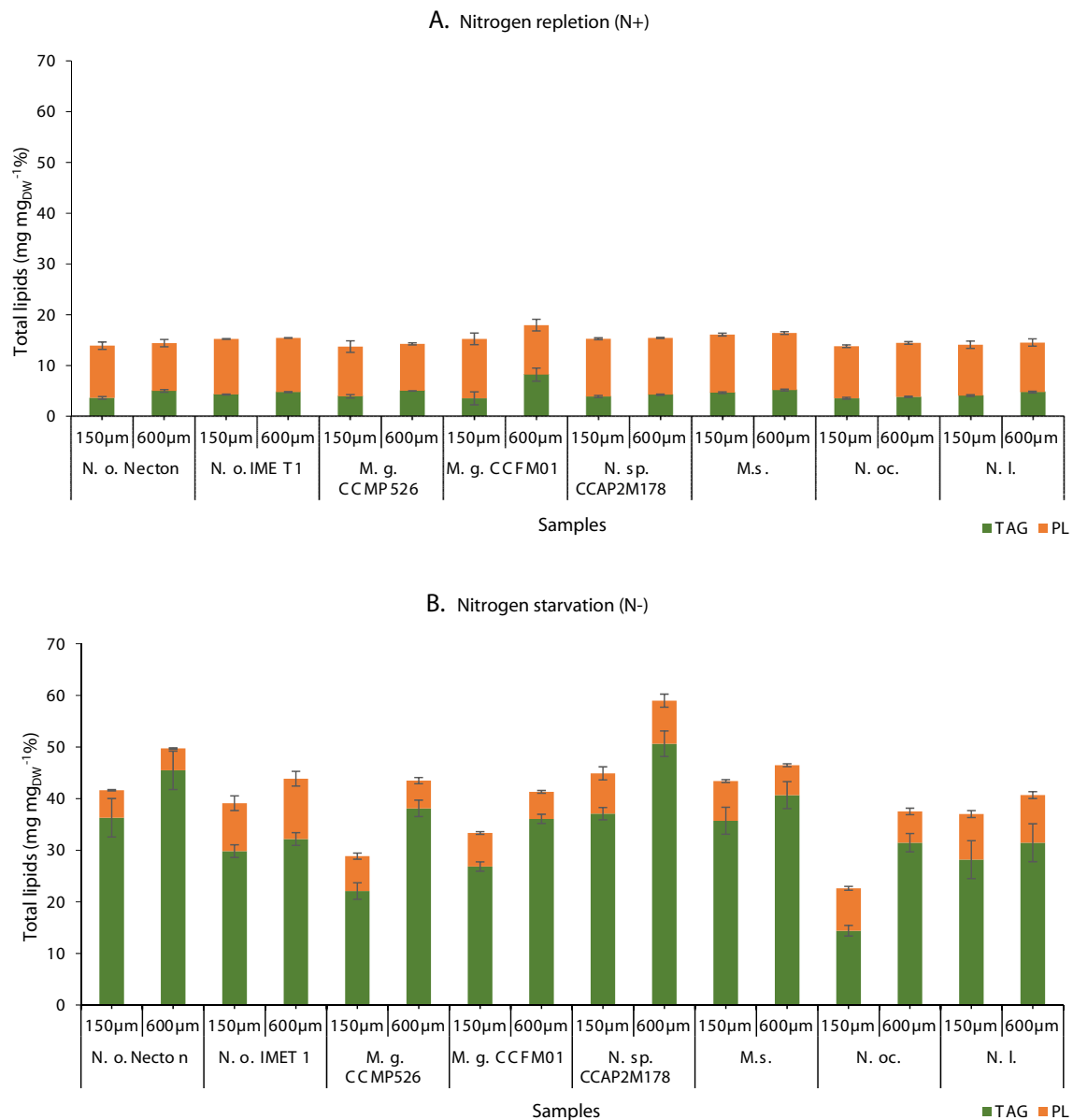A. Nitrogen repletion (N+)

B. Nitrogen starvation (N-)
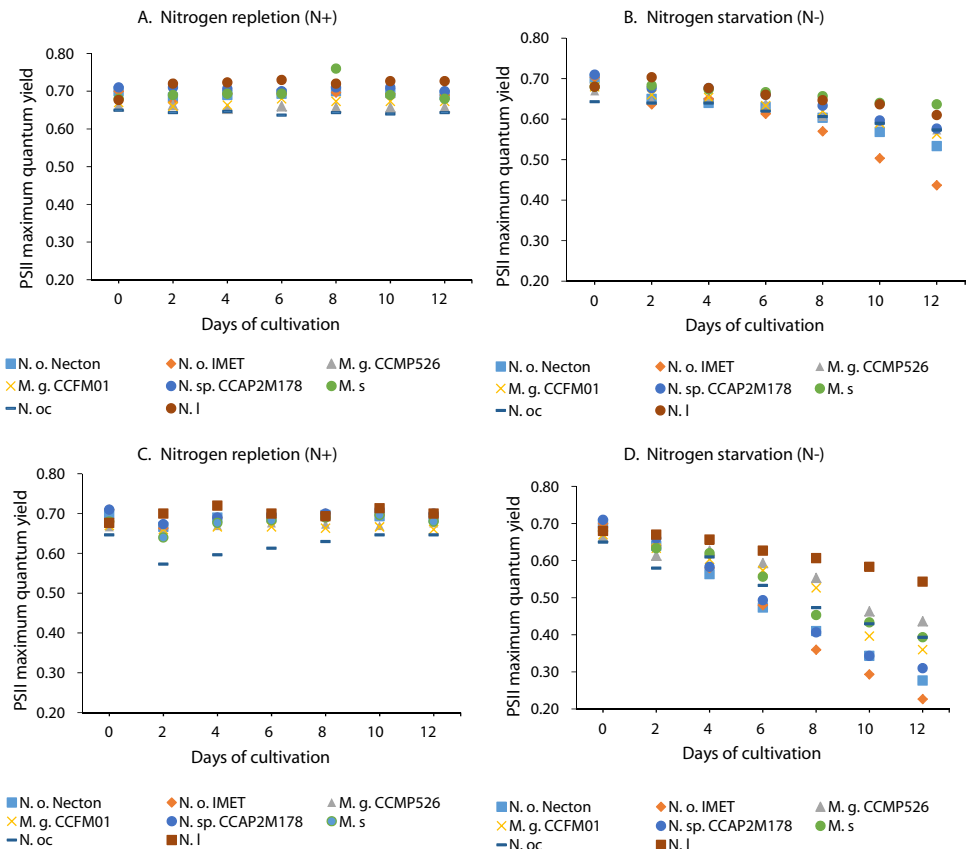
Fig. 2 Lipid content (mg mg_{DW}^{-1}%) in the triacylglycerol (TAG) and polar (PL) fractions of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) **(A)** and nitrogen starvation (N-) **(B)** conditions under different light intensities 150 and 600 μmol photons m^{-2} s^{-1}. Error bars represent the standard deviation of the mean (n=3)

examined, *M. gaditana* CCFM01 displayed the highest lipid content, reaching 18.0% at HL (Fig. 2B).

N-starvation, regardless of light intensity, led to notable variations in lipid content among the different strains. There was a substantial boost in the total lipid content (%) across all strains under N-starvation, particularly at a light intensity of 600 μmol photons m^{-2} s^{-1} compared to 150 μmol photons m^{-2} s^{-1}. For instance, *N. sp* exhibited the highest lipid content under LL conditions, reaching 44.9%, while *N. oculata* had the lowest lipid content (22.6%). HL-induced lipid accumulation for all strains, with *N. sp.* having a peak lipid concentration of 59.0%,

30% higher than in *M. gaditana* CCFM01, making *N.* sp. highly suitable for various applications where high lipid yield is essential. Various factors, including environmental stimuli, genetic heterogeneity, developmental stages, acclimation responses, and nutrient fluxes, influence lipid content in *Nannochloropsis* and *Microchloropsis* species. In our case the strains under investigation originated from different geographic locations and are experiencing diverse cultivation conditions, resulting in genetic variations (Wacker et al. 2016; Shi et al. 2017). This variation was not confined to inter-generic comparisons but was also evident within individual species, potentially explaining

the observed significant lipid content differences among strains (Mishra et al. 2019).

c. TAG and EPA contents.

There were no significant differences in the TAG content (expressed as %) between the strains under both light regimes when cultivated in the N-replete medium (Fig 3). Under N-repletion conditions (Fig. 3A), TAG content was not significantly different among the evaluated algal strains. *Microchloropsis gaditana* CCFM01 had a TAG content of 8.2% under HL. Contrastingly, N-starvation prompted a pronounced rise in TAG synthesis in all strains (Fig. 3B). However, the response to N-starvation significantly varied among strains, regardless of light intensity. It is essential to emphasize that increased light intensity stimulated the cells to accumulate more TAG. Under HL, *N.* sp. exhibited the maximal TAG concentration of 59.6%, a value substantially higher than the 31.5% in *N. oculata*. Furthermore, under LL conditions, *N.* sp. achieved a TAG content of 37.1%, eclipsing the lowest recorded value by *N. oculata* by a substantial margin of 61.7%. This increase in intracellular lipids when microalgae are subjected to high light intensity may be attributed to the oxidative stress induced by photocatalysts. Moreover, the observed differences in TAG accumulation among the strains under various conditions underscore the complex interplay between environmental factors, cellular responses, and intracellular lipid dynamics. Furthermore, the TAG content in different microalgal species increases with higher light intensity and nitrogen starvation, while membrane lipids decrease under these conditions (Pal et al.

2011; He et al. 2015; Nogueira et al. 2015; Shi et al. 2017). These findings underscore the pivotal role of light intensity in modulating lipid composition and productivity in various microalgae species, offering valuable insights for applications in biotechnology and biofuel production. Nonetheless, it is essential to note that this phenomenon is not consistent across all microalgae species, as only some species exhibit oleaginous characteristics similar to those in the studied strains. For instance, it has been reported that low-light conditions favoured TAG production in *Rhodomonas* sp. (Oostlander et al. 2020) and *Phaeodactylum tricornutum* (Remmers et al. 2017). This variability underscores the complexity of microalgal lipid metabolism, which is influenced by biomass cultivation and mode of cultivation.

Cells can swiftly undergo ultrastructural modifications in response to changes in irradiance (Ma et al. 2016). In the present study EPA content (%) was negatively correlated with light intensity and nitrogen starvation. Under LL conditions (Fig. 4A.) all strains exhibited elevated levels of EPA with the highest value observed in N-replete *M. gaditana* CCFM01 cells (4.69%), followed by *N. oculata* (4.11%), which is significantly greater than the lowest value recorded by *N. oceanica* IMET1 (2.9%). This suggests an adaptive response to maximize light absorption and utilization under LL. Our findings indicate that *M. gaditana* CCFM01 and *N. oculata* cells are well-suited for nutraceutical applications where EPA is desired. These results are correlated with the quantum yield values, which were high at low light intensity. Higher quantum yield is associated with increased relative chloroplast volume, higher photosynthetic unit density, and a greater number
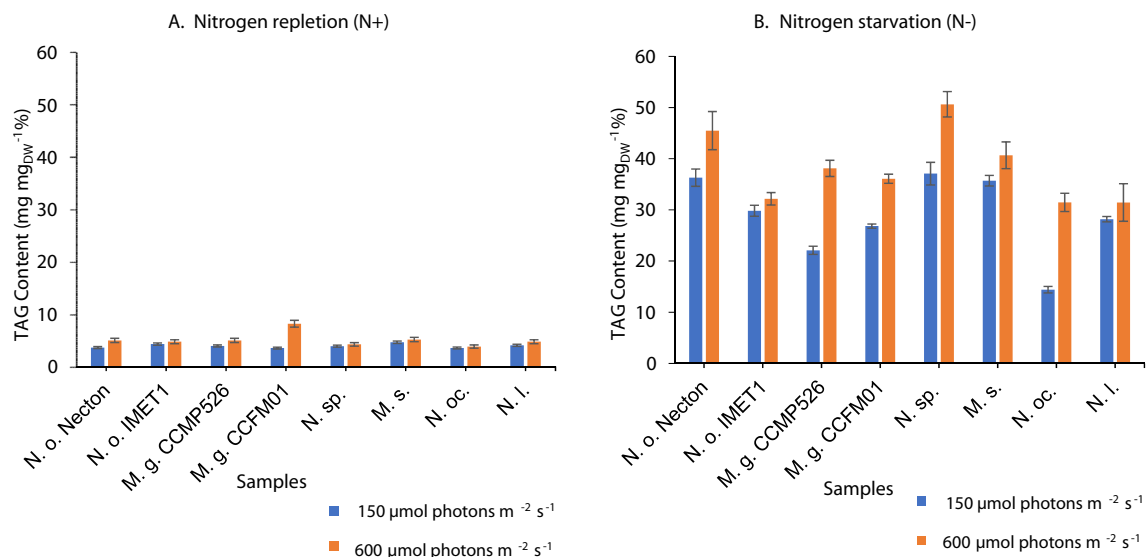

Fig. 3 TAG content (mg mg_{DW}^{-1}%) of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) **(A)** and nitrogen starvation (N-) **(B)** conditions under different light intensi-

ties 150 and 600 µmol photons m^{-2} s^{-1}. Error bars represent the standard deviation of the mean (n=3)
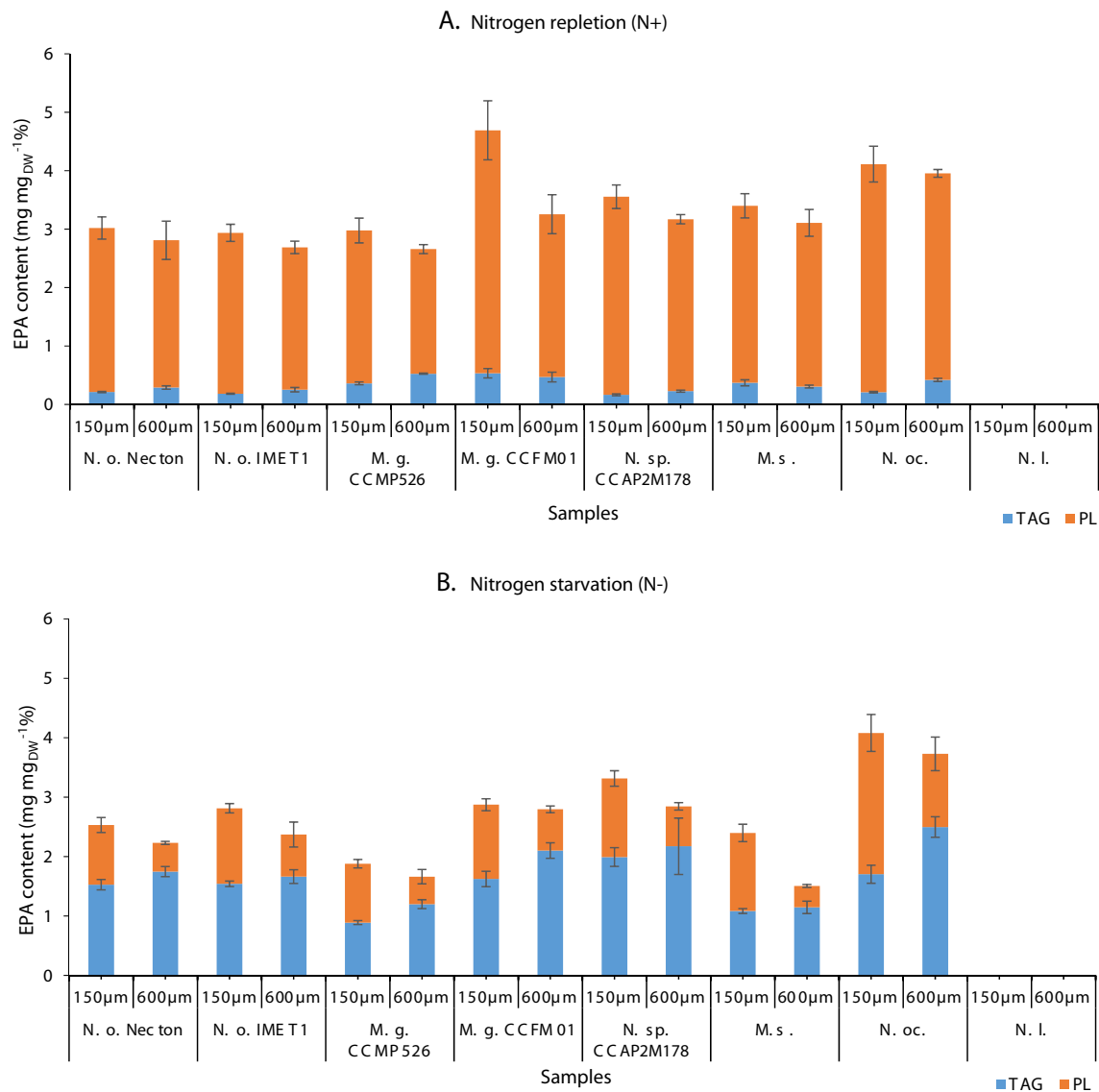
Fig. 4 EPA content (mg mg$_{DW}$$^{-1}$%) in the triacylglycerol (TAG) and polar (PL) fractions of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) **(A)** and nitrogen starvation (N-) **(B)** conditions under different light intensities 150 and 600 µmol photons m^{-2} s^{-1}. Error bars represent the standard deviation of the mean (n=3)

of thylakoid stacks per chloroplast, which means more efficient photosynthesis and potentially higher EPA (Sukenik et al. 1989). Conversely, cells grown under higher light (Fig. 4B) displayed a marked reduction in EPA percentage, particularly under N-starvation. *Microchloropsis salina* had the lowest EPA content at 1.51%. In this scenario, the chloroplast volume decreased, leading to a concurrent reduction in EPA and increased neutral lipid oil droplets, TAG explicitly, within the cells (Sukenik et al. 1989). Our findings align with prior research, reinforcing that high light conditions or severe nitrogen limitation typically substantially reduce the proportion of polyunsaturated fatty acids, particularly EPA. This correlation has

been documented in studies of *Nannochloropsis* sp. (Pal et al. 2011) and *Phaeodactylum tricornutum* (Mishra et al. 2019).

d. Fatty acid profile.

Figure 5 illustrates the impact of nitrogen availability and light conditions on the fatty acid profiles of the 8 strains examined in our study. Under N-repletion conditions (Fig. 5 A and C), the predominant fatty acids in all strains were C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:1 (oleic acid), and C20:5 (eicosapentaenoic acid), except for *N. limnetica*. This freshwater strain displayed a distinct fatty acid
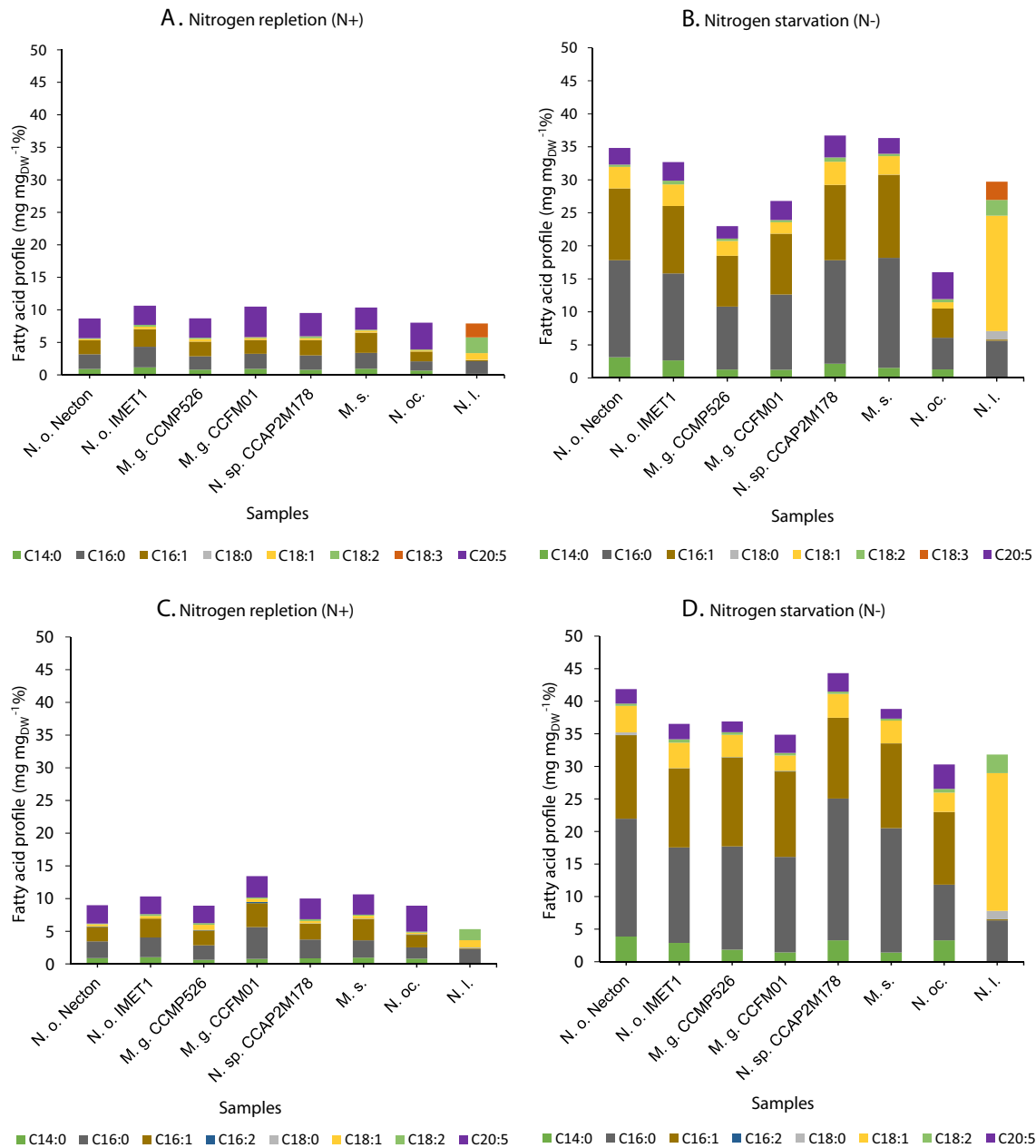
Fig. 5 Fatty acid profile (mg mg$_{DW}^{-1}$%) of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) or nitrogen starvation (N-) conditions under low light intensity 150 μmol photons m^{-2} s^{-1} **(A & B)** and high light intensity 600 μmol photons m^{-2} s^{-1} **(C & D)**

profile compared to the marine species, with C18:1 as the primary fatty acid and the absence of C20:5. In response to N-starvation, the proportions of these fatty acids exhibited a significant increase across the strains, except for EPA (C20:5), which displayed the opposite trend. Depending on the species, under low light (Fig. 5B) the marine strains demonstrated percentages ranging from 14% to 38% for C16:0 and 0.4% to 28% for C16:1 as a proportion of the total fatty acids when subjected to N-starvation. With higher light intensity (Fig. 5D), the C16 group represented

the dominant percentages of the total fatty acids, ranging from 15 to 41% for C16:0 and 0.4 to 31% for C16:1. In the freshwater strain *N. limnetica*, under N-starvation and high light intensity, accumulated more than 50% of the total fatty acids as C18:1, with this proportion slightly declining when exposed to lower light intensity. These values are in accordance with several studies done on different microalgae species (Pal et al. 2011; Wagenen et al. 2012; Mishra et al. 2019; Conceição et al. 2020; Nzayisenga et al. 2020). It has been suggested that at a high light intensity, microalgal cells

use excess energy to produce storage lipids, mainly SFA and MUFA, which are usually involved in the formation of thylakoid membranes. The decrease in PUFA, especially EPA, would be due to its role in synthesizing structural components (Wacker et al. 2016).

These observations underscore the significant influence of both nitrogen and light on the fatty acid composition of microalgae, emphasizing the intricate interplay between these factors and the resulting variations in fatty acid profiles.

e. Biomass and lipid productivity.

Lipid productivity is a paramount factor in determining the economic viability of microalgae-based processes, as demonstrated in Tables 1 and 2. There was a positive correlation between increased light intensity and enhanced

biomass productivity, with *N.* sp. and *M. salina* achieving the highest average biomass productivity, at 0.290 ± 0.016 and 0.222 ± 0.015 g L^{-1} day^{-1}, respectively, under HL and N-replete conditions (Table 2). These findings align with Ma et al. (2014) who also found *M. salina* to have the highest biomass productivity among the nine tested strains, with a value of 0.363 ± 0.009 g L^{-1} day^{-1}. Nevertheless, it is essential to note that biomass yield was approximately 50% lower in N-starved conditions than in N-replete media, irrespective of the light regime.

In addition to biomass productivity, we assessed lipid productivity in our study. Although the highest biomass productivity was observed under N-repletion, the highest lipid yields were consistently observed in N-starved cells, and this effect was notably accentuated under HL (Tables 1 and 2). There were statistically significant differences in lipids productivity between the strains, regardless of the conditions.

Table 1 Biomass productivity (P_x) g L^{-1} day^{-1} and lipid content (LC) mg mg$_{DW}$$^{-1}$% and Lipid productivity (P_{FA}) g L^{-1} day^{-1} of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) and nitrogen starvation (N-) conditions under low light intensity 150 μmol photons m^{-2} s^{-1}

	P_x		LC		P_{FA}	
	N+	N-	N+	N-	N+	N-
N. oceanica Necton	$0.115_a \pm 0.011$	$0.053_a \pm 0.008$	$13.91_a \pm 0.40$	$41.63_{a,d} \pm 1.54$	$0.015_{a,c} \pm 0.001$	$0.033_a \pm 0.007$
N. oceanica IMET1	$0.063_{b,c} \pm 0.005$	$0.020_b \pm 0.008$	$15.22_{a,b} \pm 0.31$	$39.12_{a,g} \pm 0.78$	$0.009_{b,d} \pm 0.018$	$0.018_b \pm 0.003$
M. gaditana CCMP526	$0.058b \pm 0.013$	$0.023_b \pm 0.008$	$13.72_a \pm 0.37$	$28.87_b \pm 0.88$	$0.006_b \pm 0.002$	$0.014_b \pm 0.001$
M. gaditana CCFM01	$0.095_{a,c,d} \pm 0.012$	$0.070_{a,c} \pm 0.008$	$15.25_a \pm 1.68$	$33.34_c \pm 0.29$	$0.015_{a,c} \pm 0.003$	$0.032_a \pm 0.001$
N. sp. CCAP2M178	$0.111_{a,d} \pm 0.006$	$0.077_{a,c} \pm 0.005$	$15.27_{a,b} \pm 0.29$	$44.90_d \pm 2.70$	$0.016_a \pm 0.001$	$0.043_a \pm 0.007$
M. salina	$0.100_{a,d} \pm 0.002$	$0.076_{a,c} \pm 0.010$	$16.10_b \pm 0.39$	$43.41_{d,e} \pm 1.35$	$0.016_{a,c} \pm 0.001$	$0.042_a \pm 0.004$
N. oculata	$0.080_{b,d,e} \pm 0.017$	$0.062_a \pm 0.027$	$13.79_a \pm 0.43$	$22.62_f \pm 0.97$	$0.010_{b,c,d} \pm 0.003$	$0.017_b \pm 0.002$
N. limnetica	$0.104_{a,e} \pm 0.016$	$0.093_c \pm 0.006$	$14.093_{a,b} \pm 0.365$	$37.013_{c,g} \pm 1.078$	$0.013_{a,d} \pm 0.002$	$0.041_a \pm 0.002$

Values in the same column day not sharing the same subscript are significantly different at $p < 0.05$ in the two-sided test of equality for column means. Tests assume equal variances. The values are means $\pm$ the standard deviation SD (n=3)

Table 2 Biomass productivity (P_x) g L^{-1} day^{-1} and lipid content (LC) mg mg$_{DW}$$^{-1}$% and Lipid productivity (P_{FA}) g L^{-1} day^{-1} of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) and nitrogen starvation (N-) conditions under high light intensity 600 μmol photons m^{-2} s^{-1}

	P_x		LC		P_{FA}	
	N+	N-	N+	N-	N+	N-
N. oceanica Necton	$0.190_{a,c,f} \pm 0.026$	$0.084_{a,d,f,g} \pm 0.009$	$14.41_a \pm 0.88$	$49.73_a \pm 3.81$	$0.026_{a,b} \pm 0.005$	$0.053_{a,c,e} \pm 0.006$
N. oceanica IMET1	$0.180_{a,c,f} \pm 0.014$	$0.046_{a,b} \pm 0.014$	$15.43_{a,c} \pm 0.10$	$43.86_{a,b,d} \pm 0.27$	$0.027_{a,c} \pm 0.002$	$0.037_{a,b} \pm 0.007$
M. gaditana CCMP526	$0.130_{a,b} \pm 0.020$	$0.067_{a,c,d} \pm 0.017$	$14.26_a \pm 0.16$	$43.54_{a,b,d} \pm 1.01$	$0.017_b \pm 0.003$	$0.045_{a,d,e,f} \pm 0.006$
M. gaditana CCFM01	$0.194_{c,f} \pm 0.039$	$0.105_{d,f,g,h} \pm 0.003$	$17.97_b \pm 1.22$	$41.31_{b,d} \pm 0.99$	$0.036_{c,d} \pm 0.005$	$0.056_{a,c,e} \pm 0.001$
N. sp. CCAP2M178	$0.290_d \pm 0.016$	$0.096_{d,e,f,g,h} \pm 0.006$	$15.44_{a,c} \pm 0.37$	$58.997_c \pm 2.45$	$0.044_d \pm 0.003$	$0.069_c \pm 0.004$
M. salina	$0.222_{c,e} \pm 0.015$	$0.111_{f,h} \pm 0.011$	$16.39_{b,c} \pm 0.13$	$46.48_{a,b} \pm 2.64$	$0.036_{c,d,e} \pm 0.003$	$0.062_{c,d} \pm 0.007$
N. oculata	$0.174_{a,c,f} \pm 0.004$	$0.072_{b,c,g} \pm 0.022$	$14.44_a \pm 0.39$	$37.52_d \pm 2.17$	$0.024_{a,b} \pm 0.002$	$0.037_{b,e} \pm 0.008$
N. limnetica	$0.155_{b,f} \pm 0.008$	$0.125_h \pm 0.008$	$14.52_a \pm 0.67$	$40.69_{b,d,e} \pm 4.07$	$0.021_{a,b} \pm 0.002$	$0.059_{c,f} \pm 0.008$

Values in the same column not sharing the same subscript are significantly different at $p < 0.05$ in the two-sided test of equality for column means. Tests assume equal variances. The values are means $\pm$ standard deviation SD (n=3)

The maximum daily average lipid productivity was achieved in N-starved cultures exposed to HL with *N. sp.*, yielding 0.069 ± 0.004 g L^{-1} day^{-1}. *Microchloropsis salina* closely followed with a lipid productivity of 0.062 ± 0.007 g L^{-1} day^{-1} (Table 2). Conversely, *N. oculata* exhibited the lowest lipid productivity under the same conditions, with 0.037 ± 0.007 g L^{-1} day^{-1}. These variations in lipid productivity are primarily attributed to differences in biomass productivity between the strains since lipid productivity correlates strongly with biomass productivity. However, under N-starvation stress, although biomass productivity decreases, the lipid content increases as a survival mechanism, redirecting metabolic pathways to store energy in lipids. This shift can result in higher lipid productivity even with reduced biomass production, mainly if the increase in lipid content compensates for the drop in biomass (Huete-Ortega et al. 2018; Chin et al. 2023). These observations align with earlier research conducted on various *Nannochloropsis* and *Microchloropsis* species under light or nitrogen starvation stress, as well as in combination (Pal et al. 2011; Su et al. 2011; Wagenen et al. 2012; Fakhry and El Maghraby 2015). This detailed comparative data is crucial for selecting the robust strain for industrial lipid production.

Effect of temperature and nitrogen availability

Cultivation temperature is a critical parameter in microalgae cultivation. It influences various aspects of algal growth, as it affects chemical reaction rates, diffusion rates, membrane permeability, respiratory and photosynthetic electron transport, and enzyme activities, ultimately shaping the growth rate of algal populations. Furthermore, when selecting a microalgal strain for lipid production, it is essential to consider its lipid amount, fatty acid types, and biomass yield at different temperatures. Strains that grow well across various temperatures are beneficial, as they ensure steady production all year round, aiding in sustainable and dependable output.

In the second part of this study, we studied the effect of temperatures 15, 25, and 30℃ and nitrogen availability on biomass and lipid productivity at low light (LL) intensity 150 µmol photons m^{-2} s^{-1}.

Photosynthetic efficiency Temperature and nutrient availability significantly influence the microalgal metabolism, photosynthetic efficiency, growth, and biomass composition. The time-course variations of the photosynthetic activity (represented by F_v/F_m) at various temperatures are shown in Fig. 6 (A, B and C) under N-replete and Fig. 6 (D, E and F) under N-starvation conditions. The data reveal a consistent pattern in photosynthetic efficiency across different temperatures over 12 days under N-replete conditions, with minimal variations ranging from 0.63 to 0.73, depending on the species. This stability is noteworthy as it suggests the remarkable resilience of these species to temperature fluctuations, underlining their adaptability to a range of conditions (Fakhry and El Maghraby 2015; Ferrer-Ledo et al. 2023; Gao et al. 2023). However, *N. oculata* under N-replete conditionswas a notable exception a, where a sharp decrease in photosynthetic efficiency was evident, dropping to 0.26 at 30 ℃. This abrupt decline highlights the specific sensitivity of *N. oculata* to higher temperatures when nutrients are plentiful (Hung 2017).

Interestingly, the different species exhibited a slight decrease in photosynthetic efficiencies across varying temperatures under N-starvation conditions compared to N-replete conditions. Notably, *N. oculata* and *N. limnetica* displayed the lowest values at 30 and 15 ℃, with an efficiency of 0.25 for each. These results underline the dependence of microalgal photosynthetic efficiency on nutrient levels, and they can adjust to low nutrients but with less efficient photosynthesis (Ranglová et al. 2019; Peng et al. 2020; Ferrer-Ledo et al. 2023; Gao et al. 2023). Moreover, when grown at temperatures outside their ideal range, their photosynthesis suffers, likely due to enzyme issues. High temperatures affect enzymes crucial for growth, disrupt cell processes, and lower protein production, impairing photosynthesis, particularly the part that generates oxygen. This leads to less efficient energy transfer within the cells and increased stress, further reducing photosynthesis and overall cell health (He et al. 2018; Ferrer-Ledo et al. 2023).

Lipid content Temperature is a crucial environmental factor that needs thorough study because it dramatically affects the lipid makeup of photosynthetic organisms. Changes in temperature and lack of nitrogen impact the biomass, photosynthetic efficiency, and especially the lipid content in cells by altering chemical reaction rates and cell component stability (Sonmez et al. 2016). The effect of temperature on lipid content varies not only between different algal species but also among strains within the same species. The total lipid content of these strains varied significantly at different culture temperatures. In N-replete conditions (Fig. 7A) the various strains exhibited uniformity in lipid content across various temperatures with one notable exception. *Nannochlorpsis oculata* emerged as the outlier in this context, exhibiting the highest lipid content at 30 ℃, registering an impressive 31.8%, followed by *N. sp.* (22.3%), while *N. limnetica* had the lowest value (11.3%) at this temperature. Similarly, Wei et al. (2015); Hung (2017) found that in the presence of nitrogen, the highest lipid content in *N . oculata* was at 30°C, which was higher than its optimal growth temperature (25°C). Meanwhile, under N-starvation conditions (Fig. 7B), the lipid content doubled, depending on the strain, compared to N-repleted conditions. In this scenario, the decrease in the temperature from 25℃ to 15℃ led to an increase in the lipid content, and *M. gaditana* CCMP526 recorded a 45.6% lipid
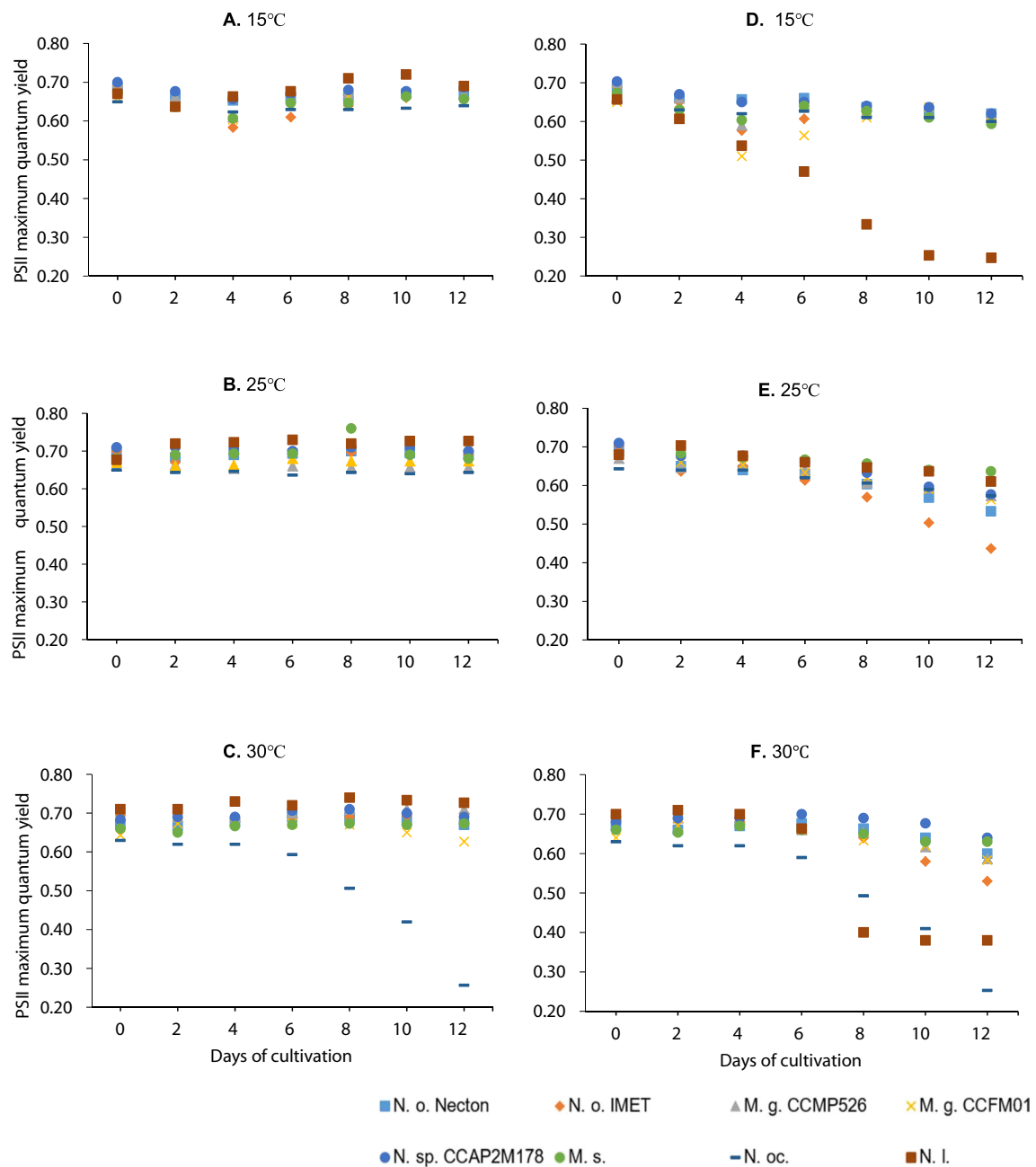
Fig. 6 PSII maximum quantum yield of different *Nannochloropsis* and *Microchloropsis* species cultivated under different temperatures 15, 25, and 30℃ cultivated in nitrogen repletion (N+) **(A, B, and C)** and nitrogen starvation (N-) **(D, E, and F)** conditions

content at 15℃, which is twice as much as the lowest yielding strain, *N. limnetica*. These findings underscore the intricate relationship between temperature, nutrient availability, and strain-specific responses, illuminating the potential for tailored strategies to optimize lipid production in diverse microalgal species and strains. In addition, there is a wealth of literature documenting the impact of temperature on lipid accumulation in microalgae. Some reports have revealed a detrimental effect of temperature increases on various strains and their tendency to accumulate more lipids under

low temperatures (e.g., Converti et al. 2009; Wagenen et al. 2012; Chaisutyakorn et al. 2018; Peng et al. 2020; Ferrer-Ledo et al. 2023). Conversely, other studies have demonstrated an increase in the lipid content of various *Nannochloropsis* species with rising temperatures, which is linked to the pivotal role of total lipid or increased reliance on lipids as a storage product (Fakhry and El Maghraby 2015; Malakootian et al. 2016). While various studies report different optimal temperatures for lipid accumulation in microalgae it is clear that the impact of temperature is complex and varies

Fig. 7 Lipid content (mg mg$_{DW}$$^{-1}$%) of different *Nannochloropsis* and *Microchloropsis* species cultivated under different temperatures 15, 25, and 30℃ cultivated in nitrogen repletion (N+) **(A)** and nitrogen starvation (N-) **(B)** conditions. Error bars represent the standard deviation of the mean (n=3)

A. Nitrogen repletion (N+)

B. Nitrogen starvation (N-)
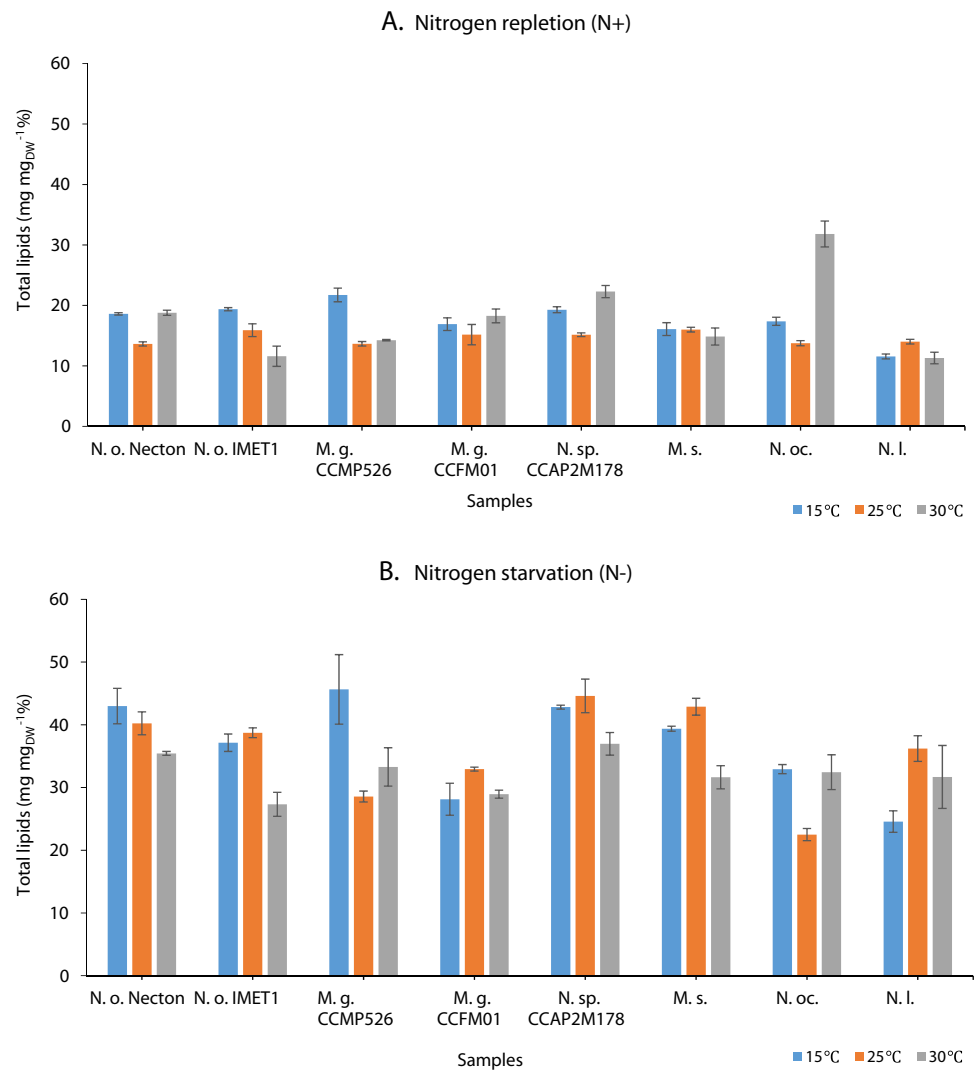
by species and even by strains in the same species. This variability could be due to the different temperature ranges used in these studies. Furthermore, the specific effects of temperature on lipid accumulation depend on the algal species or strain and their origin, with the exact mechanisms still unclear. However, it is generally observed that there is a negative correlation between lipid content and growth rate in microalgae under stress.

TAG and EPA contents It has been observed in most microalgae that polar lipid content increases with decreasing temperature, whereas increased temperature leads to a higher accumulation of nonpolar lipids (TAG) (Renaud et al. 2002).

On the one hand, typically, when microalgae face stressful conditions, such as high irradiance, elevated temperatures and nitrogen deficiency, they tend to accumulate lipids, primarily in the form of TAG. Conversely, under ideal growth conditions, their lipid production remains relatively low (Wijffels and Barbosa 2010). Our findings demonstrate

that under nitrogen repletion conditions Fig. 8A, all strains exhibited a similar trend in TAG accumulation, which was more favored at low temperatures, with the one notable exception of *N. oculata*. This strain had the highest TAG content of 17.6% at 30℃. This result can be attributed to the high stress experienced by *N. oculata* at the higher temperature, which in turn restricted its growth potential, ultimately resulting in elevated TAG levels.

In contrast, *N. limnetica* displayed the lowest TAG accumulation of only 2% at the same temperature, . In addition, under N-starvation (Fig. 8B) the TAG content in all strains doubled. However, the dynamics differed from those observed under N-repleted conditions. Surprisingly, 25℃ emerged as the optimal temperature for TAG accumulation in most strains, with *N. sp* and *M. salina* recording the highest contents of 36.9% and 35.3%, respectively, surpassing the lowest recorded value by *N. oculata* by 61%. Previous research on *N. oceanica* Necton has demonstrated it tends to accumulate higher levels of (TAG) in response to stress

Fig. 8 TAG content (mg mg$_{DW}$$^{-1}$%) of different *Nannochloropsis* and *Microchloropsis* species cultivated under different temperatures 15, 25, and 30℃ cultivated in nitrogen repletion (N+) **(A)** and nitrogen starvation (N-) **(B)** conditions. Error bars represent the standard deviation of the mean (n=3)


A. Nitrogen repletion (N+)

B. Nitrogen starvation (N-)
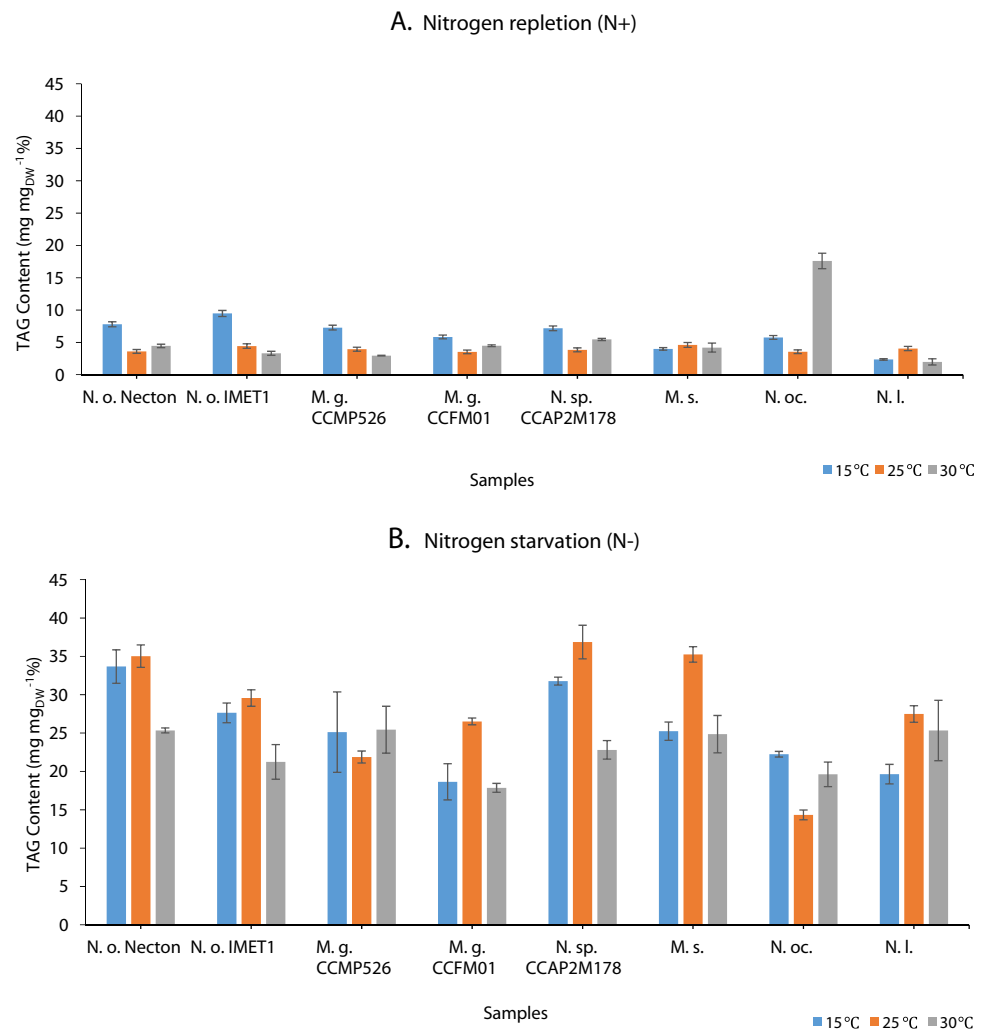
induced by low and high temperatures. This accumulation of TAG is believed to be part of a protective cellular mechanism aimed at safely dissipating excess carbon, energy, and electrons during unfavourable conditions. The exposure to low and high temperatures results in the build-up of reducing equivalents, which, if not effectively managed, can lead to the accumulation of reactive oxygen species. Thus, the synthesis of TAG serves as a vital complementary safety quenching mechanism (Ferrer-Ledo et al. 2023).

Nannochloropsis and *Microchloropsis* species hold significant industrial appeal due to their remarkable ability to accumulate high levels of eicosapentaenoic acid (EPA) compared to other microalgae-producing species. However, it is worth noting that the economic viability hinges on the current low productivity within photoautotrophic systems. Researchers have explored lowering temperatures as a strategic approach to elevate both EPA contents and productivity despite the potential growth impediments (Hoffmann et al. 2010). Figure 9A and B show the total EPA content and the distribution between polar and neutral lipids. The data reveal

significant differences in total EPA accumulation among the various strains at different temperatures. Notably, it becomes evident that cells tend to accumulate more EPA outside the optimal growth temperature range.

Interestingly, the strains exhibit enhanced EPA accumulation at lower temperatures. Under N-repletion (Fig. 9A), at 15°C, *N. oculata* and *M. gaditana* CCMP526 significantly accumulated EPA across various temperatures, registering 5.7% and 5.1%, respectively. In contrast, *M. gaditana* CCFM01 accumulated 40% lower than *N. oculata* under the same conditions. These levels declined when the strains were subjected to nitrogen starvation (Fig. 9B); *N. oculata* achieved its peak EPA content of 5.7% at 15°C. In contrast, *M. gaditana* CCFM01 accumulated less than half the EPA amount of the highest recording strain under the same conditions. This decrease can be attributed to the reduction in EPA content in the polar fraction and the increase in the TAG fraction in response to nitrogen starvation stress. Moreover, the EPA content within lipid classes displayed temperature-dependent variations, suggesting a dynamic

Fig. 9 EPA content (mg mg$_{DW}^{-1}$%) in the triacylglycerol (TAG) and polar (PL) fractions of different *Nannochloropsis* and *Microchloropsis* species cultivated under different temperatures 15, 25, and 30°C cultivated in nitrogen repletion (N+) **(A)** and nitrogen starvation (N-) **(B)** conditions. Error bars represent the standard deviation of the mean (n=3)

A. Nitrogen repletion (N+)

B. Nitrogen starvation (N-)
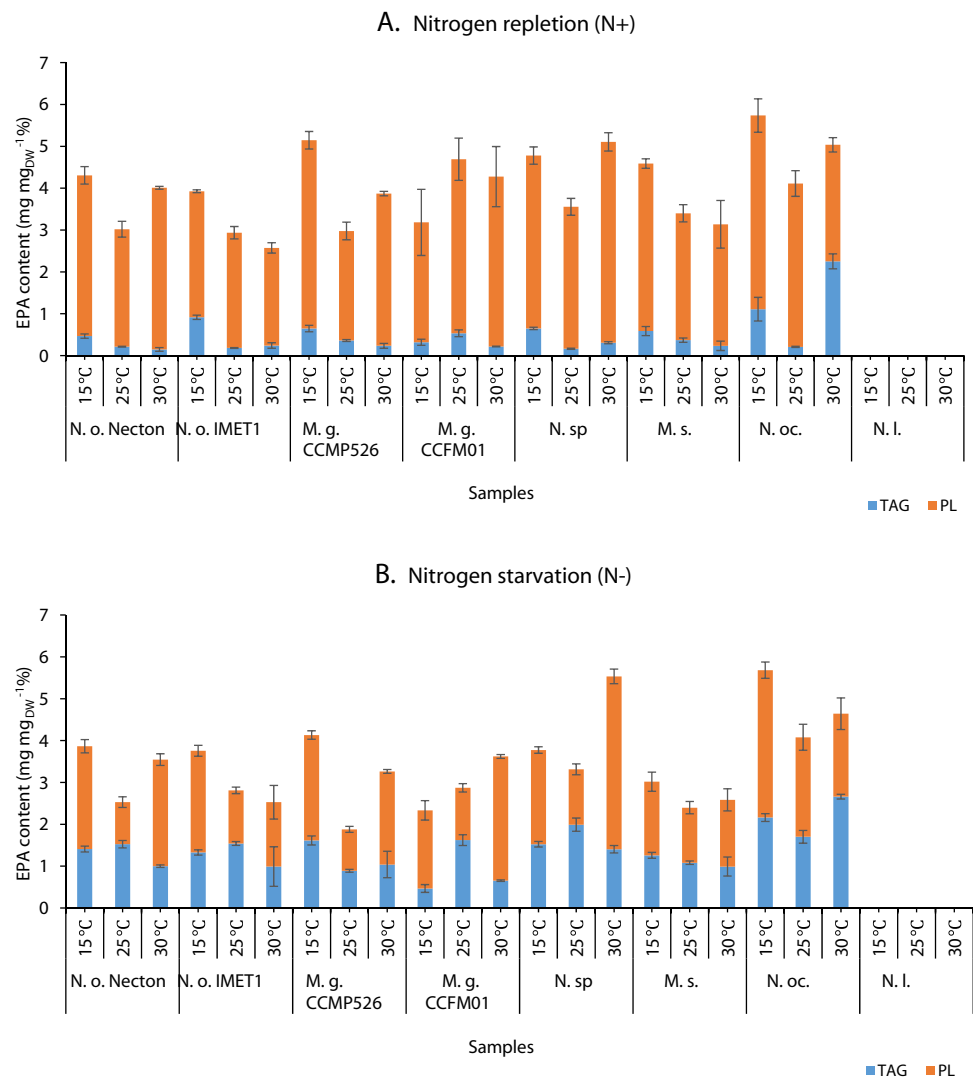
mobilization of EPA and a precise regulation of its synthesis and degradation processes (Ferrer-Ledo et al. 2023). Furthermore, it is noteworthy that the freshwater strain *N. limnetica*, under various cultivation conditions, did not exhibit significant EPA accumulation. Consistent with our findings, numerous studies have explored the impact of temperature on EPA content across various species of *Nannochloropsis* and *Microchloropsis*. These studies have consistently demonstrated a notable rise in EPA content in response to lower temperatures (Hoffmann et al. 2010; Mitra et al. 2015; Wei et al. 2015; Aussant et al. 2018; Willette et al. 2018; Sá et al. 2020).

Fatty acid profile As mentioned earlier, the fatty acid composition is distinctly influenced by temperature fluctuations and this influence varies from strain to strain. Among all the strains under nitrogen repletion conditions (Fig. 7S (A, B, and C)) the prevailing fatty acids were C:14, C:16:0, C16:1 and C20:5. However, there was a significant change when

the strains were exposed to nitrogen starvation (Fig. 7S (D, E, and F)). Under N-starvation, there was substantial increase in C:14, C:16:0, C16:1, and C18:0 in all strains except for *N. limnetica*, which featured C16:0 and C18:0 as the primary fatty acids. This shift can be attributed to the culture's adaptation under N-starvation, where the content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) significantly rises while polyunsaturated fatty acids (PUFAs) decrease (Fig. 8SB). Additionally, under these conditions, the composition of the fatty acids also varied with temperature fluctuations (Fig. 7S). Notably, most strains exhibited a decrease in the amounts of C16:1, C18:1, and C20:5, accompanied by an increase in C16:0 and C18:0 at 30°C. This may be because higher temperatures (25°C and 30°C) favour SFA synthesis, potentially as a mechanism to maintain cell membrane integrity under thermal stress. In contrast, lower temperatures (15°C) promote the synthesis of MUFAs and PUFAs, which are essential for membrane fluidity and function under cooler conditions (Fig. 8SB). The

strain-specific responses to temperature changes underline the importance of selecting appropriate strains for specific environmental conditions to maximize lipid productivity.

While the impact of temperature on lipid content displays significant variation, the effects of temperature on the fatty acid profiles of microalgae exhibit remarkable consistency and have been extensively studied. Temperature-induced changes in FA composition play a pivotal role in modulating the physical properties of membranes, thereby facilitating the maintenance of essential metabolic functions under different temperatures, including photosynthesis, ion permeability, and respiration (Sheng et al. 2011; Peng et al. 2020; Gao et al. 2023).

In accordance with our results, numerous findings have consistently revealed that microalgae tend to increase their relative concentration of unsaturated FAs when exposed to lower temperatures (Aussant et al. 2018; Ferrer-Ledo et al.

2023). This response is attributed to the need for maintaining membrane fluidity, particularly thylakoid membranes, protecting the photosynthetic machinery from photoinhibition and ensuring the functionality of critical cellular processes (Chaisutyakorn et al. 2018). Conversely, at elevated temperatures, there is a propensity for increased saturated fatty acid (SFA) content, a strategy employed to preserve the integrity of microalgal cell membranes (Converti et al. 2009; Wagenen et al. 2012; Wei et al. 2015). The knowledge of the effect of temperature on fatty acid profiles can guide the targeted production of specific fatty acids, benefiting the biofuel and nutraceutical industries.

Biomass and lipid productivity Among the foremost challenges in commercial microalgae utilization, two pivotal issues stand out: optimizing cell concentrations and enhancing the desired components' productivity. Tables 3 and 4

Table 3 Biomass productivity (P_x) g L^{-1} day^{-1} and lipid productivity (P_{FA}) g L^{-1} day^{-1} of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen repletion (N+) conditions at different temperature degrees 15, 25, and 30℃

	15℃		25℃		30℃	
	P_x	P_{FA}	P_x	P_{FA}	P_x	P_{FA}
N. oceanica Necton	$0.099_{a,A}\pm0.011$	$0.019_{a,d,e,A}\pm0.002$	$0.116_{a,A}\pm0.011$	$0.014_{a,B}\pm0.001$	$0.152_{a,B}\pm0.008$	$0.029_{a,c,C}\pm0.002$
N. oceanica IMET1	$0.124_{a,b,A}\pm0.013$	$0.025_{a,b,A}\pm0.003$	$0.063_{b,c,B}\pm0.005$	$0.010_{b,c,B}\pm0.001$	$0.081_{b,B}\pm0.011$	$0.007_{b,B}\pm0.002$
M. gaditana CCMP526	$0.124_{a,b,A}\pm0.009$	$0.029_{a,c,A}\pm0.004$	$0.059_{b,B}\pm0.013$	$0.006_{b,B}\pm0.002$	$0.061_{b,B}\pm0.024$	$0.007_{b,B}\pm0.003$
M. gaditana CCFM01	$0.079_{a,A}\pm0.004$	$0.015_{d,A}\pm0.001$	$0.095_{a,c,d,A}\pm0.012$	$0.015_{a,b,A}\pm0.003$	$0.143_{a,c,B}\pm0.026$	$0.028_{a,c,B}\pm0.003$
N. sp. CCAP2M178	$0.096_{a,A}\pm0.012$	$0.019_{a,d,e,A}\pm0.003$	$0.111_{a,d,A}\pm0.006$	$0.016_{a,A}\pm0.001$	$0.152_{a,B}\pm0.017$	$0.035_{a,B}\pm0.005$
M. salina	$0.156_{b,A}\pm0.008$	$0.025_{b,c,e,A}\pm0.003$	$0.101_{a,d,B}\pm0.002$	$0.016_{a,B}\pm0.000$	$0.066_{b,C}\pm0.016$	$0.009_{b,C}\pm0.001$
N. oculata	$0.104_{a,c,A}\pm0.032$	$0.019_{a,d,e,A,B}\pm0.005$	$0.080_{b,d,e,A}\pm0.018$	$0.010_{a,b,A}\pm0.003$	$0.053_{b,A}\pm0.005$	$0.024_{c,B}\pm0.002$
N. limnetica	$0.148_{b,c,A}\pm0.016$	$0.015_{d,f,A}\pm0.002$	$0.104_{a,e,B}\pm0.015$	$0.013_{a,c,A}\pm0.002$	$0.093_{b,c,B}\pm0.015$	$0.008_{b,B}\pm0.001$

Values in the same column not sharing the same small subscript and values in the same row and subtable not sharing the same capital subscript are significantly different at $p<0.05$ in the two-sided test of equality for column means. Tests assume equal variances. The values are means $\pm$ standard deviation SD (n=3)

Table 4 Biomass productivity (P_x) g L^{-1} day^{-1} and lipid productivity (P_{FA}) g L^{-1} day^{-1} of different *Nannochloropsis* and *Microchloropsis* species cultivated in nitrogen starvation (N-) conditions at different temperature degrees 15, 25, and 30℃

	15℃		25℃		30℃	
	P_x	P_{FA}	P_x	P_{FA}	P_x	P_{FA}
N. oceanica Necton	$0.072_{a,a,A}\pm0.010$	$0.042_{a,a,A,B}\pm0.003$	$0.053_{a,A}\pm0.008$	$0.032_{a,A}\pm0.007$	$0.131_{a,B}\pm0.014$	$0.055_{a,B}\pm0.002$
N. oceanica IMET1	$0.115_{b,A}\pm0.005$	$0.052_{b,A}\pm0.002$	$0.021_{b,B}\pm0.008$	$0.017_{b,B}\pm0.002$	$0.043_{b,d,C}\pm0.005$	$0.017_{b,B}\pm0.001$
M. gaditana CCMP526	$0.106_{b,A}\pm0.009$	$0.063_{c,A}\pm0.005$	$0.023_{b,B}\pm0.008$	$0.014_{b,B}\pm0.001$	$0.031_{b,B}\pm0.015$	$0.019_{b,B}\pm0.007$
M. gaditana CCFM01	$0.056_{a,A}\pm0.000$	$0.022_{d,A}\pm0.003$	$0.070_{a,c,A}\pm0.008$	$0.032_{a,B}\pm0.001$	$0.098_{c,C}\pm0.004$	$0.035_{c,d,e,B}\pm0.002$
N. sp. CCAP2M178	$0.064_{a,a,A}\pm0.013$	$0.035_{a,e,A}\pm0.004$	$0.077_{a,c,A}\pm0.005$	$0.043_{a,A}\pm0.007$	$0.107_{a,c,B}\pm0.005$	$0.045_{a,c,A}\pm0.004$
M. salina	$0.0.144_{c,A}\pm0.007$	$0.069_{c,A}\pm0.002$	$0.076_{a,c,B}\pm0.010$	$0.042_{a,B}\pm0.004$	$0.051_{b,d,C}\pm0.008$	$0.021_{b,C}\pm0.000$
N. oculata	$0.070_{a,A}\pm0.007$	$0.031_{d,e,A}\pm0.001$	$0.062_{a,A}\pm0.018$	$0.017_{b,B}\pm0.002$	$0.053_{b,d,A}\pm0.007$	$0.025_{b,d,A,B}\pm0.005$
N. limnetica	$0.127_{b,A}\pm0.008$	$0.034_{a,f,A,B}\pm0.003$	$0.093_{c,B}\pm0.006$	$0.040_{a,A}\pm0.004$	$0.058_{d,C\pm}0.006$	$0.024_{b,e,B}\pm0.006$

Values in the same column not sharing the same small subscript and values in the same row and subtable not sharing the same capital subscript are significantly different at $p<0.05$ in the two-sided test of equality for column means. Tests assume equal variances. The values are means $\pm$ the standard deviation SD (n=3)

present data on biomass and lipid productivity of different *Nannochloropsis* and *Microchloropsis* species cultivated at different temperatures under N-repletion (Table 3) and N-starvation (Table 4). The data indicate a notable variance in the impact of temperature on biomass and lipid productivity across different strains. A key observation is that, for most strains 15°C was the optimal temperature for maximizing biomass and lipid productivity. Under N-repletion, among the different strains, *M. salina* stood out, exhibiting the highest biomass productivity at 15°C across all tested temperatures (0.156 g L^{-1} day^{-1}), outperforming *M. gaditana* CCFM01, which recorded the lowest rate (0.079 g L^{-1} day^{-1}). These results contradict the cell concentration data (Fig. 4S where at 25°C under N-repletion the highest cell concentration was reached by *N. oceanica* Necton and *M. salina* with 338.67 and 286.76 ×10^6 cells mL^{-1}, respectively. This can be attributed to the differences in cell size between strains or different genera under specific conditions influencing biomass yields. For instance, at 15°C, slower metabolic rates might result in larger cell sizes as the duration of cell growth phases extends before division occurs. This can increase biomass per cell, enhancing overall biomass productivity if the cells accumulate more internal components (like lipids, carbohydrates, and proteins) before dividing. At 25°C, the higher temperature may accelerate metabolic processes and cell division rates without necessarily increasing the size of each cell (Huerlimann et al. 2010) resulting in higher cell concentrations, as cells divide more frequently. However, each cell may be smaller in size or less dense in biomass content, leading to lower overall biomass productivity compared to the scenario at 15°C. In addition, biomass productivity, typically measured as the dry weight per volume of culture per unit time, is a function of cell size and number (Borowitzka 2016). Larger, fewer cells might yield higher biomass productivity due to greater internal component accumulation. In contrast, cultures with smaller, more numerous cells might show elevated cell concentrations with proportionately less biomass per cell (Renaud et al. 2002).

In terms of lipid productivity, both *M. salina* and *M. gaditana* CCMP526 achieved peak lipid productivity at 15°C under N-starvation, with rates of 0.069 and 0.063 g L^{-1} day^{-1}, respectively surpassing *M. gaditana* CCFM01 by approximately 70%. This suggests that *M. salina* could be optimal for cooler climate operations focusing on lipid production. Our findings align with numerous studies highlighting the impact of temperature on lipid accumulation in microalgae. For instance, in the case of different species of *Nannochloropsis*, the peak fatty acid productivity was observed at low temperatures, underscoring the influential role of temperature (Hoffmann et al. 2010; Malakootian et al. 2016; Chaisutyakorn et al. 2018). However, it is noteworthy that our results contrast with the commonly observed pattern, as they observed that low temperatures had a relatively minor

impact, whereas high temperatures maximized lipid production (Ho et al. 2014; Han et al. 2016; Peng et al. 2020), thus providing new insights into the temperature-lipid productivity relationship.

The study's novelty lies in its comparative approach, which reveals significant genus and strain-specific response differences and the interactions of multiple environmental stressors such as light intensity, temperature, and nitrogen availability. The research identifies optimal growth and lipid production conditions for specific strains, demonstrating notable variability even within the same genus. These findings highlight the importance of considering multiple stressors simultaneously to develop effective strategies for maximizing lipid yield in microalgae. The knowledge gained can drive advancements in microalgal biotechnology, leading to more cost-effective and sustainable production methods.

Conclusion

This comprehensive study systematically evaluated eight oleaginous microalgal strains of *Nannochloropsis* and *Microchloropsis* for their growth and lipid accumulation abilities under various abiotic stressors, including nitrogen limitation and light and temperature variations, contributing significantly to existing knowledge. The results demonstrated significant variations in response to these environmental conditions among the strains. A substantial increase in lipid production was noted under HL and N-starvation conditions, albeit with considerable variability across different strains. Notably, *N.* sp. emerged as the top performer in lipid productivity (0.069 g L^{-1} day^{-1}), while *N. oculata* recorded the lowest (0.037 g L^{-1} day^{-1}). In addition, under N-repletion at LL conditions, *M. gaditana* CCFM01 exhibited the highest EPA content (4.7%), contrasting with *N. oceanica* IMET1's lowest EPA content (2.9%).

The study also revealed that the optimal temperature for growth and lipid production and the response to temperature varied significantly between species, not only from different genera but also within the same genus and even among different strains of the same species, likely due to differences in their native environments. Among the strains tested, the highest lipid productivity was achieved by *M. salina*, registering 0.069 g L^{-1} day^{-1}, surpassing *M. gaditana* CCFM01 by approximately 70% at 15℃ under N-starvation conditions. Moreover, N-starvation and temperature fluctuations markedly influenced the composition of fatty acids. SFAs and MUFAs significantly rose, while PUFAs in response to N-starvation. In addition, as the culture temperature decreased, SFAs significantly reduced, while the content of MUFAs and PUFAs, primarily EPA, increased. In this regard, *N. oculata* had the highest EPA accumulation,

reaching 5.7% at 15 °C under N-repletion, which is substantially higher by 40% compared to *M. gaditana* CCFM01.

Despite the study limitations, including the monitoring of nitrogen presence, and the study's limited duration of 12 days, this study underscores the potential of stressors as promising tools in algal photobiotechnology, enabling the production of specific lipid classes and emphasizing the significance of acquiring knowledge the physiology and responses of various species, even within the same genus. This knowledge is a clear guide for selecting the robust strain with optimal operation conditions for specific applications, which is critical for developing efficient and sustainable microalgae-based industrial lipid production.

Future research can build on our findings by exploring the genetic and metabolic pathways responsible for the observed strain-specific responses using omics technologies. Additionally, longer-term studies are needed to assess the sustainability of lipid production in continuous or semicontinuous systems. Addressing the economic and practical aspects of scaling up these processes through techno-economic analyses and pilot-scale experiments will also be crucial for developing an efficient and sustainable microalgal lipid-production industry.

Declarations

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