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Selecting for a high lipid accumulating microalgae culture by dual growth limitation in a continuous bioreactor

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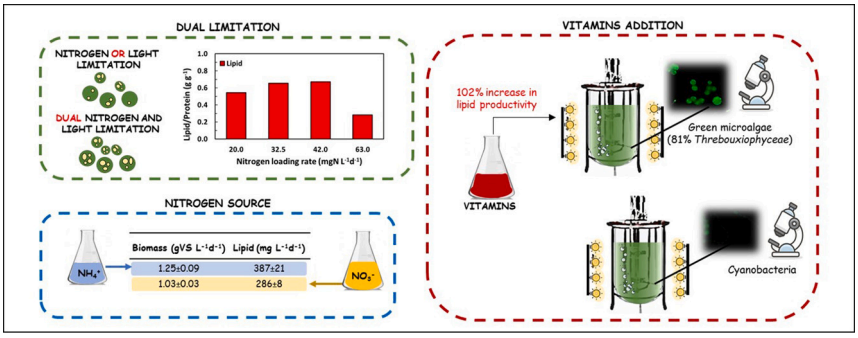
HIGHLIGHTS

- Production of lipids by microalgal enrichment culture
- Dual growth limitation (light/nitrogen) and vitamins select for lipid accumulation
- Ammonium preferred nitrogen source
- Enrichment culture dominated by *Trebouxiophyceae*

GRAPHICAL ABSTRACT



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ABSTRACT

A dual-growth-limited continuous operated bioreactor (chemostat) was used to enhance lipid accumulation in an enrichment culture of microalgae. The light intensity and nitrogen concentration where both limiting factors resulting in high lipid accumulation in the mixed culture. Both conditions of light and nitrogen excess and deficiency were tested. Strategies to selectively enrich for a phototrophic lipid-storing community, based on the use of different nitrogen sources (ammonium vs. nitrate) and vitamin B supplementation in the growth medium, were evaluated. The dual limitation of both nitrogen and light enhanced the accumulation of storage compounds. Ammoniacal nitrogen was the preferred nitrogen source. Vitamin B supplementation led to a doubling of the lipid productivity. The availability of vitamins played a key role in selecting an efficient lipid-storing community, primarily consisting of *Trebouxiophyceae* (with an 82 % relative abundance among eukaryotic microorganisms). The obtained lipid volumetric productivity (387 mg L^{-1} d^{-1}) was among the highest reported in literature for microalgae bioreactors. Lipid production by the microalgae enrichment surpassed the efficiencies reported for continuous microalgae pure cultures, highlighting the benefits of mixed-culture photo-biotechnologies for fuels and food ingredients in the circular economy.

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1. Introduction

The global challenge of reducing dependence on fossil fuels and combating climate change through the use of clean and renewable energy sources is a widely debated topic. Currently, in the European Union (EU), transportation and energy sectors are the two major anthropogenic sources of greenhouse gas emissions (Mandl et al., 2020). Biofuels are a promising alternative for decarbonizing transportation and increasing energy sustainability. Biofuels are mainly produced from biomass and contribute to lower combustion emissions per equivalent power output, compared to fossil fuels (Ebadian et al., 2020). The most common biofuels are biodiesel and bioethanol, which can replace diesel and gasoline, respectively, with little or none modifications of existing vehicle engines (Mata et al., 2010).

Among the various options for producing biofuels, third generation biorefining systems using microalgae have significant potential (Behera et al., 2021). The main advantages of microalgae-derived biofuels include: the high growth rates, the ease of cultivation and the possibility of coupling microalgae cultivation with CO_2 sequestration, and/or wastewater treatment. Microalgae cultivation is 20 times more productive per hectare compared to biofuel crops and does not compete with food production (Rawat et al., 2013). Moreover, via the photosynthesis process, microalgae sequestrate CO_2 and convert it into high-energy organic compounds (Mooij et al., 2013). Finally, It is possible to utilize the nitrogen and phosphorous present in waste streams as nutrients for the growth of microalgae, thereby removing pollutants from the water (Mata et al., 2010).

The possibility of producing biofuel from microalgae relies on their capacity of accumulating value-added organic compounds, including starch and lipid, which are precursors of bioethanol and biodiesel. Lipid production, in particular, is more favourable than starch production as lipid have a higher energy density (Li et al., 2015).

In addition, microalgal lipids have gained significant attention for their potential utilization in food applications. These lipids, extracted from microalgae, are rich in essential fatty acids, such as omega-3 and omega-6 (Zhou et al., 2022). The presence of these beneficial fatty acids makes microalgal lipids highly suitable for enhancing the nutritional properties of food products. Microalgal lipids can also be used as natural food colorants and flavor enhancers, providing a sustainable alternative to synthetic additives. Their potential as a source of functional ingredients, such as emulsifiers and antioxidants, further expands their applicability in various food formulations (Bumbac et al., 2023).

As a result, the induction of lipid accumulation in microalgae has been heavily researched in recent years. When grown under optimal physiological conditions, large amounts of microalgae biomass are produced, with relatively low lipid content. However, when exposed to stress conditions, microalgae switch their biosynthetic pathways from biomass generation towards lipid accumulation (Sharma et al., 2012). To this aim, different stress conditions have been tested to increase lipid accumulation, such as non-optimal light irradiance, nutrition starvation, non-optimal culture pH and temperature, and other non-optimal operating conditions (Behera et al., 2021; Sibi et al., 2016). Nitrogen starvation has been reported as the most successful technique inducing lipid accumulation. Nitrogen is the most growth-limiting factor for eukaryotic microalgae and it is relatively easy to induce nitrogen starvation. Moreover, while the response to other stress factors is strain specific, all the microalgae species studied so far seem to increase lipid production under nitrogen stress (Sharma et al., 2012), which makes this approach particularly suitable for mixed culture cultivations.

Nitrogen starvation for lipid accumulation by microalgae is usually performed in two-step batch processes. In the first step, algae are grown under optimal conditions to increase the photosynthetic activity and the biomass concentration. In the second step, lipid accumulation is induced by arresting the growth using nitrogen deprivation (Kandilian et al., 2019). Using this strategy, high lipid content can be reached after prolonged nitrogen starvation (Hu et al., 2008). During the initial period of the second step, carbohydrate polymers are the main by-products. Successively, lipid production occurs due to de novo fatty acid synthesis or due to the conversion of the sugar polymers (Mooij, 2016). However, the exposition to unfavourable conditions for relatively long time dramatically limits the biomass growth. Consequently, the maximum lipid productivity is reached within the first days of cultivation, when the biomass concentration is high and the cellular lipid content is still low (Klok et al., 2013).

As a consequence, continuous cultivation of microalgae in a chemostat mixed-culture offers several advantages such as stable productivity and better process control compared to batch regime (Seo et al., 2017). This allows for the study and optimization of operational conditions to achieve the highest lipid productivity (Wen et al., 2014). A trade-off between microalgae growth and storage metabolites production should be carefully analyzed to optimize the nutrient manipulation strategy. Maintaining photosynthetic activity in a prolonged period of nitrogen starvation could result in improved lipid productivity, which contributes to improved process economics (Remmers et al., 2017).

Selective enrichment of the desired microalgae culture is paramount for overcoming the major bottlenecks hindering the commercial production of bioproducts derived from microalgae. Pure cultures of microalgae require aseptic inflows and carefully controlled conditions, resulting in high investments and operating costs. Conversely, open mixed cultures do not require sterilization of the inflow wastewater and confer more stability and robustness to the process (Kleerebezem and van Loosdrecht, 2007). The use of enrichment cultures is based on the selection of a specific phenotype rather than a specific strain. Through this methodology, the necessary metabolic capabilities and the corresponding microbial population can be efficiently enriched from a natural environment (Kleerebezem and van Loosdrecht, 2007).

In this study, a chemostat enrichment system was investigated to select for lipid accumulating microalgae. To apply a strategy enhancing storage compounds productivity, we hypothesized that a dual-growth-limited (DGL) chemostat can efficiently induce lipid accumulation in microalgae while also promoting biomass growth. Light intensity and nitrogen concentration were adopted as the limiting factors to induce lipid accumulation. Specifically, a transient zone where both nitrogen and light were limiting, by manipulating the nitrogen to light ratio was found. The approach was chosen inspired by previous research on polyhydroxyalkanoates (PHA) production. Egli and Quayle (Egli and Quayle, 1986) have studied the effect of the carbon (C) to nitrogen (N) ratio on polyhydroxybutyrate (PHB) accumulation by a yeast in chemostat systems. They identified three different growth zones: i) a C limitation zone at low C/N ratios, ii) a N limitation zone at high C/N ratios, and iii) a transient zone, in which both nutrients were completely consumed. The existence of this dual nutrient limitation growth zone relies on the ability of microorganisms to adjust their cellular composition to different nutrient limitation conditions. PHB accumulation improved under such conditions, and has been later confirmed by other studies that focused on dual limitations of C and N (Durner et al., 2000; Poblete-Castro et al., 2012) or C and P (Cavaillé et al., 2016). A medium manipulation to stronger enrich for a phototrophic, lipid-storing community was evaluated. In particular, the medium was supplied with a vitamin trace solution, as in previous batch or pure culture studies, it has been shown to significantly increase lipid concentration (Fazeli Danesh et al., 2018). The objective was to assess the effectiveness of adding synthetic B vitamins as a preliminary approach, to investigate whether utilizing vitamin-rich wastewater could enhance process productivity, especially in the context of dual growth limitation within a continuous mixed culture system. Finally, the impact of the nitrogen source in the medium on lipid accumulation was tested. Overall, this research aimed to explore innovative methods for generating lipids in open enrichment cultures.

2. Materials and methods

2.1. Inoculum and initial enrichment of microalgae in batch mode

The initial mixed culture was obtained from samples collected from different water bodies in Delft (Netherlands). This combined inoculum was seeded at a volumetric ratio of 20 % (percentage of inoculum in the medium) and in a 500 mL batch reactor. The reactor was a borosilicate glass open bottle, magnetically stirred at 250 r.p.m. and continuously flushed with air. The bottle was continuously illuminated using external LED to enrich microalgae for almost 30 days.

A modified f/2 medium was used. The f/2 medium is a seawater medium in which the concentration of the original formulation "f Medium" has been reduced by half (Guillard and Ryther, 1962). The composition was the following (mg L^{-1}): NH_4Cl (477.0); $MgSO_4$.$7H_2O$ (394.4); KCl (18.2); K_2HPO_4 (156.8); $CaCl_2.2H_2O$ (22.0); $FeCl_3.6H_2O$ (38.0); NaEDTA.$2H_2O$ (150.5); H_3BO_3 (46.4); $NaSiO_3.9H_2O$ (85.3); allylthiourea (70); in addition, 1 mL L^{-1} trace element solution and 1 mL L^{-1} vitamin trace solution were supplied to the medium. The final trace elements concentrations in the medium were (mg L^{-1}): $ZnSO_4.7H_2O$ (2.20); $MnSO_4$ (4.32); $CuSO_4$.$5H_2O$ (1.57); $CoCl_2.6H_2O$ (1.61); $Na_2MoO_4.2H_2O$ (2.18). The final vitamin concentrations were (mg L^{-1}): vitamin B_1 (1.00); vitamin B_7 (0.49); vitamin B_{12} (0.53); vitamin B_5 (4.38). Carbon was provided by flushing CO_2, as reported in the next paragraph.

2.2. Photobioreactor set up and operation in continuous mode

The enriched inoculum was used to start up a 3 L photobioreactor (Applikon Biotechnology, the Netherlands) operated at a working volume of 2 L. The medium composition was the same used in the enrichment phase. A volume of 200 mL of enriched inoculum was centrifuged and wet pellets were harvested to inoculate the photobioreactor.

Continuous illumination at a constant value of 500 μmol m^{-2} s^{-1} was provided by external LED strips, controlled via a Photo Biosym (Designinnova, India). The light availability level was regulated by the concentration of microalgae biomass, as reported in the next paragraph. The reactor was aerated with CO_2 (6 % *v*/v in air) at a flow rate of 500 mL min^{-1}, using a mass flow controller (Brooks Instruments, Ede, the Netherlands). The off-gas stream was dried by cooling at 4 °C and analyzed for CO_2 and O_2 percentages by a Rosemount NGA off-gas analyzer (Emerson, USA). The speed of the stirrer was set to 150 rpm. pH was maintained at 7.5 by additions of HCl or NaOH at 1 mol L^{-1}, and temperature at 30 °C with a TetratecHT 300-W heater (Tetra, Germany). A Biocontroller ADI 1030 (Applikon, The Netherlands) continuously measured the pH. A Masterflex pump (Cole-Parmer, Vernon Hills, IL, USA) with two heads was used at inflow and outflow rates of 0.69 mL min^{-1} to provide the constant dilution rate of 0.5 d^{-1} and hydraulic retention time (HRT) of 2 d. A simplified scheme of the PBR is reported in Fig. 1.

The photobioreactor was first operated in batch mode until the optical density of the mixed liquor at 680 nm (OD_{680}) reached a level above 1.8 absorbance units (Klok et al., 2013). Successively, the feeding mode was switched to continuous regime. All experiments described hereafter were conducted in the bioreactor under non-sterile conditions in order to address the efficiencies of open microalgae mixed cultures. All mentioned conditions were set according to previous studies on mixed microalgae culture (Fazeli Danesh et al., 2018; Mooij, 2016).

2.3. Experimental tests

To test the effect of the DGL, four different conditions were experimented. Each condition was characterized by a different nitrogen loading rate, opportunely set to test the culture both under nitrogen limitation and nitrogen excess conditions (or light excess and light limitation, respectively) (Table 1). Specifically, the nitrogen loading rate was gradually raised from a concentration that enabled complete nitrogen consumption (nitrogen limitation and excess light) until it reached a concentration that resulted in incomplete nitrogen consumption (nitrogen excess and light limitation). Besides nitrogen, all the other nutrients of the medium were kept in excess, at a constant concentration for all experimental conditions. Following the individuation of the condition leading to the DGL (transition zone from nitrogen limiting to light limiting conditions), the effect of vitamin supplementation (vitamin absence or vitamin availability using concentrations reported in paragraph 2.1) and of the type of nitrogen source (ammonium vs. nitrate) was tested.

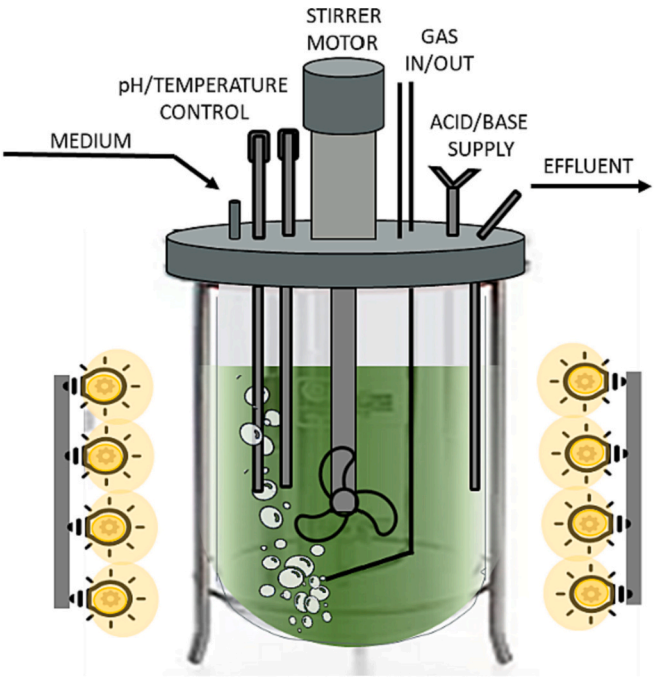

Fig. 1. Scheme of the continuous photo bioreactor used to enrich a microalgae consortium accumulating lipid.

Each condition was tested until a steady state was reached (i.e., when dioxygen and carbon dioxide in the off-gas did not vary of more than the 10 % for three consecutive HRTs). Successively, samples were collected to be analyzed in terms of lipid and carbohydrates. All experimental conditions are reported in Table 1.

2.4. Analytical methods to measure biomass, lipid and starch productivities

2.4.1. Detection of steady state

Biomass levels were measured daily by OD_{680} using a DR 3900 spectrophotometer (Hach, Netherlands) and correlating OD measures to volatile solids (VS). The off-gas was continuously monitored for CO_2 and O_2 using a Rosemount NGA off-gas analyzer (Emerson, USA). When the steady state was reached, samples were collected to measure lipid and starch content in cells.

2.4.2. Dissolved nitrogen measurement

Nitrogen concentrations were measured spectrophotometrically using Dr. Lange Ammonium cuvette tests (Hach Lange, Germany).

2.4.3. Biomass measurement

Biomass concentration was analyzed by measuring the volatile solids (VS) content of the mixed liquor according to Standard Methods (APHA, 2017). A sample of 50 mL of effluent was dried overnight in a pre-weighed aluminum foil container at 104 °C, providing the total solids

(TS) content. The ash content was then determined by calcinating the dried sample at 550 °C for 3 h. VS was calculated by subtracting ash from TS. Eq. (1) was used to estimate the total biomass productivity:

$$\text{Total Biomass Productivity}\left(\text{mg L}^{-1}\ \text{d}^{-1}\right) = \text{VS}\left(\text{mg L}^{-1}\right) \times \text{D}\left(\text{d}^{-1}\right) \quad (1)$$

where VS is the concentration of volatile solids and D the dilution rate of the chemostat.

2.4.4. Lipid extraction and analysis

Lipids were extracted using a modified protocol used for polyhydroxybutyrate (PHB) (Johnson et al., 2009), due to the similar nature of these compounds, both being hydrophobic polymers that accumulate as intracellular storage materials. In digestion tubes, 1.5 mL of a mixture of 1-propanol, hydrochloric acid (4:1 *v*/v) and 1.5 mL of 1,2-dichloroethane were added to samples of 0.2 g weighed freeze-dried biomass collected from the photobioreactor. The tubes were heated at 100 °C for 2 h. A volume of 1 mL of bi-distilled water obtained from a MilliQ water process was added to facilitate the separation of the two phases. The lower layer, containing the organic phase, was extracted from tubes. Successively, the total lipid content was measured according to Coelho et al. (2014). In short, the organic solvent was evaporated, and the remaining material (total lipid) was left in the oven at 50 °C, cooled down at room temperature in a desiccator, and weighted until constant weight.

The lipid productivity was calculated according to Eq. (2):

$$\text{Lipid Productivity}\left(\text{mg L}^{-1}\ \text{d}^{-1}\right) = \text{Lipid mass fraction}\left(\text{mg mg}^{-1}\ \text{biomass}\right) \times \text{VS}\left(\text{mg biomass L}^{-1}\right) \times \text{D}\left(\text{d}^{-1}\right) \quad (2)$$

The lipid to active biomass ratio was estimated according to Eq. (3):

$$\text{Lipid/Active biomass}\left(\text{g g}^{-1}\right) = \left(\text{Lipid mass fraction}\left(\text{g g}^{-1}\ \text{biomass}\right) \times \text{VS}\left(\text{g biomass L}^{-1}\right)\right) / \left(\text{VS}\left(\text{g total biomass L}^{-1}\right) - \text{Starch}\left(\text{g L}^{-1}\right) - \text{Lipid}\left(\text{g L}^{-1}\right)\right) \quad (3)$$

2.4.5. Starch extraction and analysis

Starch extraction was performed as follows: 5 mL of 0.6 M HCl were added to 4 mg of freeze-dried biomass. Digestion tubes were heated for 3 h at 100 °C in the digestion block. After centrifugation, the total carbohydrates content was measured in terms of glucose equivalent using the colorimetric method according to Nielsen (2017). The starch productivity was calculated according to Eq. (4):

$$\text{Starch Productivity}\left(\text{mg L}^{-1}\ \text{d}^{-1}\right) = \text{Starch mass fraction}\left(\text{mg glucose mg}^{-1}\ \text{biomass}\right) \times \text{VS}\left(\text{mg biomass L}^{-1}\right) \times \text{D}\left(\text{d}^{-1}\right) \quad (4)$$

The starch to active biomass ratio was estimated according to Eq. (5):

$$\text{Starch/Active biomass}\left(\text{g g}^{-1}\right) = \left(\text{Starch mass fraction}\left(\text{g g}^{-1}\ \text{biomass}\right) \times \text{VS}\left(\text{g biomass L}^{-1}\right)\right) / \left(\text{VS}\left(\text{g biomass L}^{-1}\right) - \text{Starch}\left(\text{g L}^{-1}\right) - \text{Lipid}\left(\text{g L}^{-1}\right)\right) \quad (5)$$

All chemical analyses were performed in triplicate. Results are reported as the mean value ± the standard deviation.

2.5. Microscope observations and molecular analyses of the biomass

A stereo zoom microscope (M205 FA, Leica Microsystems, Germany) equipped with Qwin image analysis software (V3.5.1, Leica Microsystems, Germany) was used to observe the culture. The acquisition of color microscopic color images was performed using an Axio Imager M2 (Carl Zeiss, Germany) equipped with ZEN (blue edition) software. A volume of 4 μL of BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diazasindacene) in anhydrous dimethyl sulfoxide (DMSO; 1 mg mL^{-L}) was added to 0.2 mL of algal culture for visualizing the lipid droplets in the cells. The excitation and emission wavelengths for monitoring the fluorescence of BODIPY 505/515 were 488 and 515 nm, respectively. Starch staining was observed by adding 4 μL of Lugol's solution to 0.2 mL of culture sample.

Molecular analyses of the microbial community were conducted by amplicon sequencing which provided rapid fingerprints of the enrichment culture. The genomic DNA was extracted from 250 mg wet biomass, using the DNeasy UltraClean Microbial Kit (Qiagen, The Netherlands), following the manufacturer's instructions. This included a combination of 5 min of heat (65 °C) followed by 5 min of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After extraction the DNA was checked for quality by gel electrophorese and quantified Concentrations of DNA extracts were measured with a Qubit fluorometer (ThermoFisher Scientific, USA). The DNA extracts were processed by Novogene Co. (UK) for 16S and 18S rRNA gene amplicon sequencings on an Illumina paired-end platform. 16S rRNA/18SrRNA/ITS genes of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5, 18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified using specific primer (e.g. 16S V4: 515F-806R, 18S V4: 528F-706R, 18S V9: 1380F-1510R, et al.) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

After sequencing, the raw data were quality filtered and chimeric sequences were removed. The predominant microbial populations involved in the chemostat were identified from operational taxonomic units (OTUs) affiliated to closest bacterial and eukaryal relatives on the base of ≥97 % identity, using the Mothur & Qiime software (V1.7.0). For phylogenetical determination a SSURef database from SILVA (http://www.arb-silva.de/) was used. Relative abundances were expressed versus sequencing read counts with normalisation.

3. Results

3.1. Nitrogen and light limitation regimes in continuous microalgae mixed culture

The enrichment chemostat was operated with a constant light intensity (500 μmol m^{-2} s^{-1}) and variable nitrogen loading rate (from 20 mgN $L^{-1}d^{-1}$ to 63 mgN $L^{-1}d^{-1}$). Fig. 2 reports the effect of nitrogen loading rate on biomass and residual nitrogen at steady states. The biomass and nitrogen concentrations over time are reported in Supplementary Information (Fig. A1). With increasing nitrogen loading rate, the culture shifted from a nitrogen-limited to a light-limiting regime. In between, the conditions led to a dual limitation of light and nitrogen. This was deduced by observing the biomass production and the effluent nitrogen concentration at the different nitrogen loading rates (Fig. 2). Indeed, the nitrogen concentration in the effluent was below the detection limit for nitrogen loading rates of 20, 32.5, and 42 mg N L^{-1} d^{-1}, indicating that these were nitrogen-limited conditions. At the highest nitrogen loading rate of 63 mg N L^{-1} d^{-1}, the microalgae were

Table 1
Conditions used during different experimental tests aimed at investigating the effect of the dual growth limitation strategy (nitrogen and light limitation), the nitrogen source and the vitamin addition in a phototrophic chemostat enrichment culture.

Test name[a]	N1_va	N2_va	N3_va	N4_va	N3_vn	N3_a	N3_n
Nitrogen loading rate (mgN $L^{-1}d^{-1}$)	20	32.5	42	63	42	42	42
Incident irradiance (μmol m^{-2} s^{-1})	500	500	500	500	500	500	500
Nitrogen source	NH_4Cl	NH_4Cl	NH_4Cl	NH_4Cl	$NaNO_3$	NH_4Cl	$NaNO_3$
Vitamin addition	yes	yes	yes	yes	yes	no	no

[a] Note that Ni, where $i = 1,2,3,4$, represent the NLR (influencing both nitrogen and light availability), while v, a and n indicate vitamin, ammonium and nitrates, respectively.

unable to consume all the supplied nitrogen. This indicated a different growth limitation, which was attributed to light limitation in the dense microalgae culture (1072 mgVS L^{-1}). The maximum volumetric rate of nitrogen uptake under this constant light supply was 42.9 mgN L^{-1} d^{-1}.

At a loading rate of 30–40 mgN L^{-1} d^{-1}, the biomass concentration was already maximal, whereas the residual ammonium nitrogen concentration in the broth was still below the detection limit. This indicated a combined limitation of nitrogen and light for microalgae growth in the system.

3.2. Effect of nitrogen loading rate on lipid and starch contents in the biomass

The impact of the ammonium nitrogen loading rate on the lipid and starch contents of the microalgae enrichment culture is given in Fig. 3.

The lipids to active biomass mass ratio increased from 0.54 to 0.67 g g^{-1} when the nitrogen loading rate was doubled from 20 to 42 mg N L^{-1} d^{-1}. This change also led to the system transition from single nitrogen limitation to double limitation (light and nitrogen).

When the nitrogen loading rate was increased further to 63 mg N L^{-1} d^{-1}, the nitrogen residual in the effluent increased, indicating that light becomes the sole limiting factor for growth. As a result, the lipid to active biomass ratio decreased to 0.28 g g^{-1}.

A similar trend was observed for the starch storage content. The starch to active biomass ratio increased from 0.15 to 0.52 and 0.50 g g^{-1} as a result of the increase in the ammonium nitrogen loading rate from 20 to 32.5 and 42 mg N L^{-1} d^{-1}. When the nitrogen loading rate was increased further to 63 mg N L^{-1} d^{-1}, the starch to active biomass ratio decreased to 0.13 g g^{-1}.

The highest lipid productivity of 387 mg L^{-1} d^{-1}, corresponding to 30 % of the volatile solids of the biomass, was achieved for the enrichment growing at a nitrogen loading rate of 42 mg N L^{-1} d^{-1}. Besides lipids also the starch content of the microalgae biomass selected in the chemostat increased under the dual growth limiting conditions. Lipid accumulation was higher compared to starch for all the tested nitrogen loading rates.

3.3. Effect of ammonium or nitrate as nitrogen sources and of vitamin supplementation on lipid and starch contents

The effect of applying different nitrogen sources (ammonium or nitrate) and vitamin addition on the accumulation of storage compounds in the phototrophic community is reported in Fig. 4. The effect on lipid accumulation is given in Fig. 4a and on starch accumulation in Fig. 4b. These runs were conducted at a nitrogen loading rate of 42 mg N L^{-1} d^{-1}, i.e., in the window of chemostat operation under dual limitation of nitrogen and light.

The characteristic values (namely volumetric productivities and mass fractions of lipids and starch in the microalgae biomass) of these chemostat test runs conducted with ammonium or nitrate and with or without vitamins are reported in Table 2.

The addition of ammonium as nitrogen source increased the biomass concentration and biomass volumetric productivity to some extent when compared nitrate. The mass fractions of storage products in the biomass were very similar with the two different nitrogen sources. With nitrate, the lower total biomass productivity therefore led to a lower storage polymer production. The addition of vitamins strongly increased by about 2-fold the production of lipids by the microalgae, while no apparent impact was detected on their starch production. The conjunction of ammonium as nitrogen source with the addition of vitamins led to the highest volumetric productivity of 387 mg lipids per day per litre of reactor.

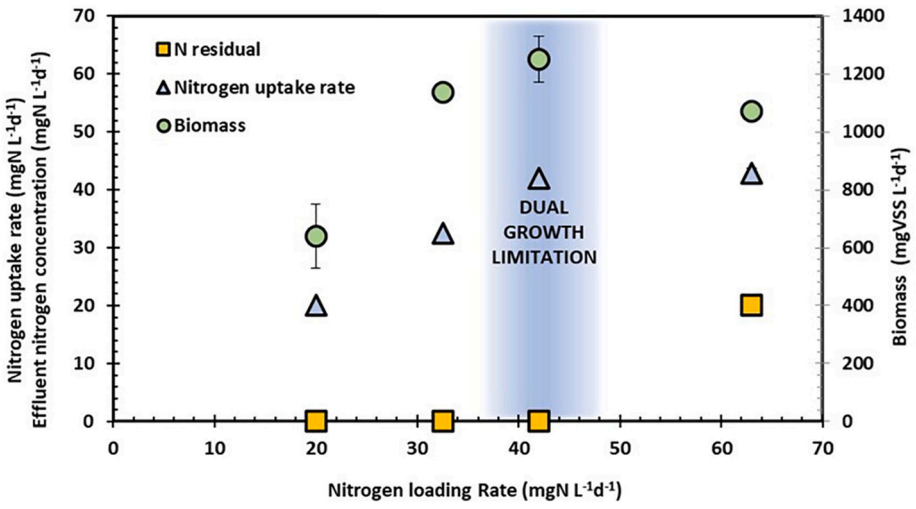

Fig. 2. The effect of the ammonium loading rate on biomass, residual nitrogen, and nitrogen uptake rate in a phototrophic chemostat enrichment culture fed with a vitamin containing autotrophic medium.

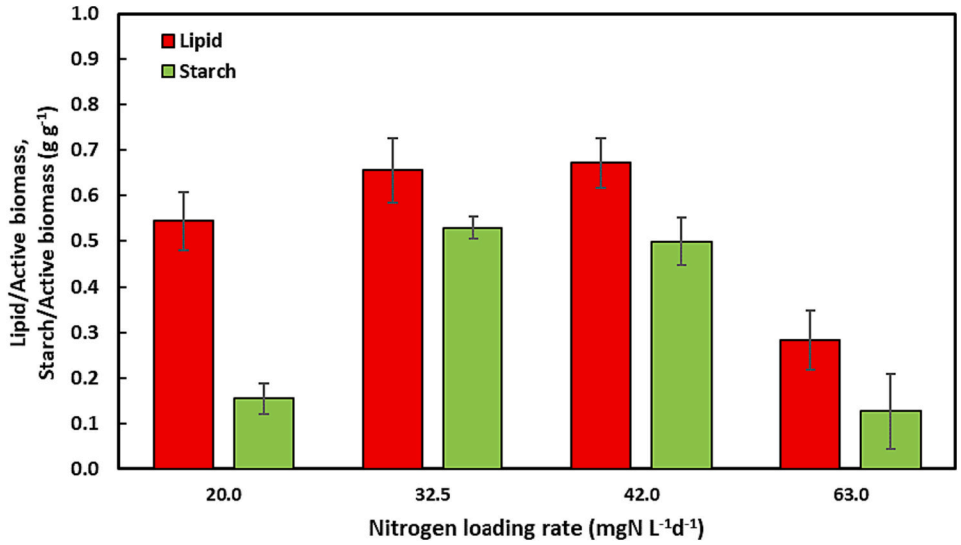

Fig. 3. Effect of the ammonium nitrogen loading rate on the lipid/active biomassand starch/active biomass ratios in the microalgae chemostat enrichment culture.

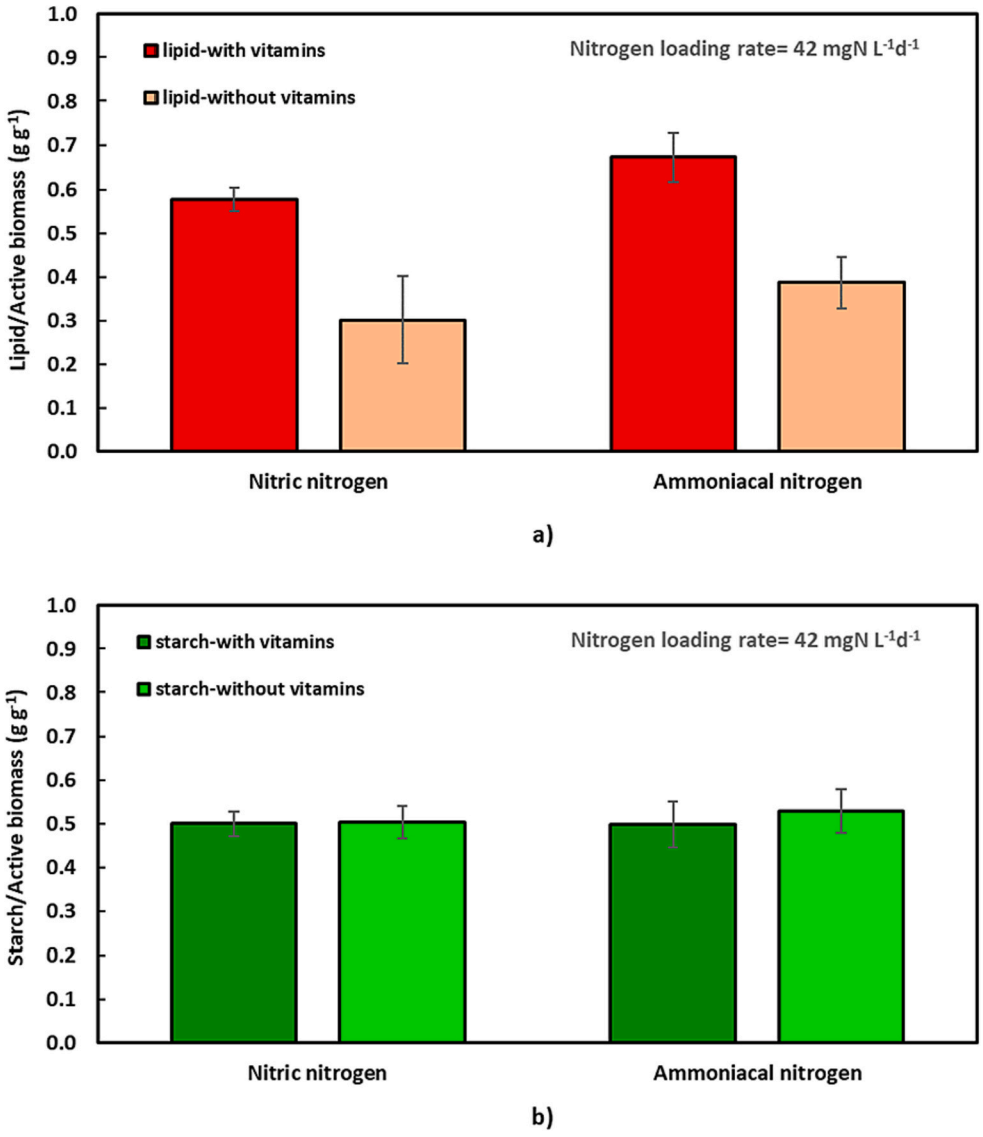

Fig. 4. The effect of nitrogen source and vitamin availability on lipid/active biomassratio (a) and on starch/active biomass ratio (b) in a phototrophic chemostat enrichment culture.

Table 2
Effect of vitamin availability and nitrogen sources on lipid and starch accumulation in a phototrophic chemostat enrichment culture. The volumetric productivities of lipids and starch were calculated by multiplying the biomass productivity by the mass fractions of lipids and starch, respectively, measured from the microalgae biomass.

Nitrogen source	Vitamins addition	Volumetric productivities			Mass fractions	
		Biomass (gVS $L^{-1}d^{-1}$)	Lipid (mg $L^{-1}d^{-1}$)	Starch (mg $L^{-1}d^{-1}$)	Lipid/VS (% m/m)	Starch/VS (% m/m)
NH_4Cl (42 mgN $L^{-1}d^{-1}$)	**Yes**	1.25 ± 0.09	387 ± 21	288 ± 15	31 ± 2 %	29 ± 1 %
$NaNO_3$ (42 mgN $L^{-1}d^{-1}$)	**Yes**	1.03 ± 0.03	286 ± 8	248 ± 7	28 ± 1 %	24 ± 1 %
NH_4Cl (42 mgN $L^{-1}d^{-1}$)	No	0.91 ± 0.01	184 ± 11	251 ± 13	20 ± 1 %	27 ± 1 %
$NaNO_3$ (42 mgN $L^{-1}d^{-1}$)	No	0.88 ± 0.03	147 ± 16	245 ± 9	17 ± 2 %	27 ± 1 %

3.4. Microscopy and molecular analyses of the microalgae mixed culture

Microscopic observations of the microalgae and their storage compounds present in the different mixed cultures obtained along the chemostat experiments are given in Fig. 5.

In the culture supplemented with vitamins, both diatoms and green microalgae were present (Fig. 5a). Accumulation of starch (brown Lugol-stained dots in the cells) seemed to occur mainly in the green microalgae (Fig. 5a). Lipid storage occurred in all community members, as visualised by BODIPY staining (Fig. 5b). Comparing green microalgae and diatoms, the latter seemed to have the highest lipid storing capacity, as highlighted by Fig. 5b.

In the absence of vitamins, the microalgae community displayed a clearly different morphology mostly composed of filamentous phototrophs (i.e., cyanobacteria), along with a minor presence of green microalgae and diatoms. (Fig. 4c and e). Cyanobacteria selected without vitamins were not able to accumulate lipids (Fig. 4d and f), but no appreciable differences due to presence of vitamins was observed in their accumulation of carbohydrates. No differences could be observed for lipid accumulation in diatoms under the presence or absence of vitamins (Fig. 5f).

Amplicon sequencing analyses were conducted by targeting both the 16S and 18S rRNA gene pools of the bacterial and eukaryal communities, respectively, in order to identify the main microbial populations selected in the phototrophic mixed cultures.

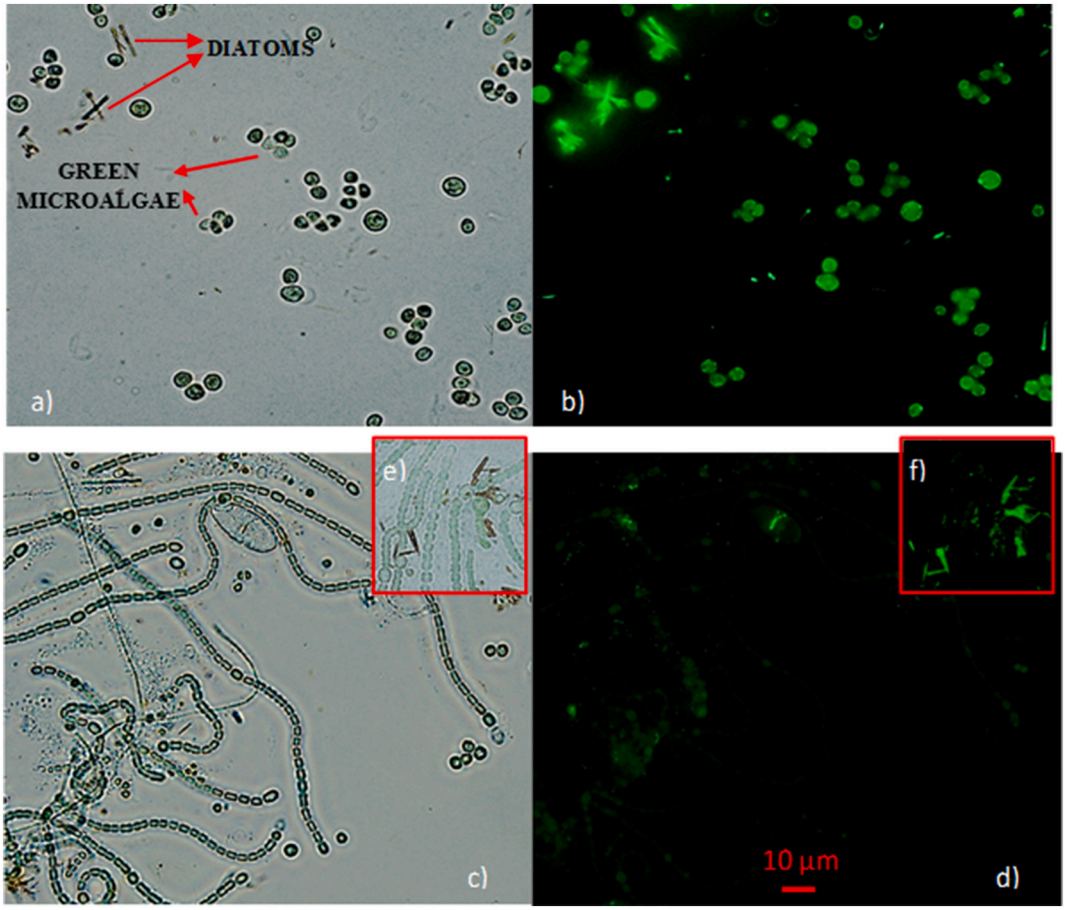

Fig. 5. Microscopic pictures of microalgae present at steady state in the enrichment cultures in the chemostat operated at a nitrogen loading rate of 42 mg N $L^{-1}d^{-1}$ (relating to dual limitation in nitrogen and light) with (a, b) and without (c, d, e, f) vitamins in the medium. The digital images were recorded with bright light and Lugol staining for starch (a, c, e) as well as with fluorescent light and BODIPY staining for lipids (b, d, f).

The vitamin-rich system fed with ammonium was dominated by a eukaryotic population of the family of *Trebouxiophyceae* (82 % in 18S dataset), a class of green microalgae forming cocci, in the division of Chlorophyta. Concerning the 16S dataset, the most abundant prokaryotes were *Rhizobiales* (16.7 %) and *Cytophagales* (14.2 %).

In the system deprived of vitamins and fed with ammonium, the *Trebouxiophyceae* relatives accounted for only 11 % of the eukaryotic community dataset. In this system, the most abundant eukaryotes were the cercozoan *Thecofilosea* (62 % in 18S dataset). The most abundant prokaryotes were bacteria belonging to the family of *Kapabacteriales* (7.7 %) while the observed filaments were related to microorganisms belonging to the class of cyanobacteria (9.7 % in total in the 16S dataset).

In both systems, other eukaryotic microalgae populations were present affiliating to the divisions of *Ochrophyta*, *Ascomycota*, *Phragnoplastophyta*, *Diatomea* and *Ciliophora*. The detailed molecular results are given in Supplementary Information.

4. Discussion

4.1. The effect of dual growth limitation of light and nitrogen

The experimental results indicated that dual growth limitation of light and nitrogen induced the best condition to select for a photolithoautotrophic green microalgae community with high lipid storage activity. Under these conditions, the highest total biomass production (measured as volatile solids, VS) was observed. This increase in biomass was not attributed to a higher proportion of active biomass, but rather to the highest storage of carbon compounds.

As long as nitrogen is limiting, it sets the maximal amount of active biomass that can be formed. Light is in surplus, and the overproduction of photosynthesis products is directed to a carbon storage pool. Under strong nitrogen limiting conditions the large surplus of photosynthesis products is directed for most part to lipid storage. This can be related to the fact that lipids form a stronger electron sink then sugars. On the other hand, when light became limiting for photosynthesis while nitrogen availability remained limited, the lipid production per unit of biomass remained constant. In this case, the excess photosynthesis products were observed to accumulate as polysugars. Future metabolic flux studies could unravel the exact cellular regulation processes behind these observations.

The maximum lipid productivity obtained in the present microalgae mixed culture (387 $mgL^{-1}d^{-1}$) was compared to those achieved in previous continuous pure cultures of microalgae reported in literature (Table 3). The literature is dominated by pure culture studies, whereas knowledge on enrichment cultures for phototrophic lipid production is limited. One of the most used strategies to enhance the lipid accumulation was nitrogen limitation, using a continuous low nitrogen loading rate (Kandilian et al., 2019; Klok et al., 2013; Wen et al., 2014). Chu et al. (2019) adopted a two-step feeding strategy. The first feeding step was conducted under non-limiting nitrogen conditions while the second feeding step was under complete nitrogen starvation (0 $mgL^{-1}d^{-1}$). Remmers et al. (2017) adopted an alternating light/dark strategy (500 $\mu mol\ m^{-2}\ s^{-1}$, 16 h light and 8 h dark) to induce the substrate storage condition. In the same study, vitamin addition (B_1, B_7, B_{12}) was performed to enhance the productivity. The latter strategy was also adopted by Tang et al. (2012).

The comparison between the results achieved in this study with literature reveals that the lipid content (%DW) was lower compared to values reported for pure cultures. On the other hand, the overall lipid productivity was higher than for previous studies, due to the higher biomass accumulation. These results can be attributed to the fact that the system accumulated both lipids and carbohydrates. Future studies should focus on the ecological significance of lipid or polyglucose accumulation in order to define better selection criteria for lipid accumulating organism only. Considering the total mass fraction of storage compounds, the maximum value of 54 % m/m obtained here was among the highest reached, to date. These results underline that the dual growth limitation continuously operated bioreactor is a very promising methodology to enhance the storage compounds productivity by a stable enrichment culture.

As suggested by Cavaillé et al. (2016), to apply the dual limitation strategy, the dilution rate has to be carefully selected. Indeed, the wideness of the dual limitation zone strongly depends on the dilution rate, being broader at lower dilution rates and narrower at higher dilution rates; the zone diminishes when the dilution rate approaches the maximal growth rate of the community. In the present study, the dilution rate and observed maximal growth rate (estimated by considering the growth kinetics of the community as a whole, rather than individual strains) were 0.5 d^{-1} and 0.67 d^{-1}, respectively.

4.2. Ammoniacal nitrogen as preferred nitrogen source

Most microalgae culturing is performed with nitrate as nitrogen source. This is in general done for practical reasons such as lower toxicity of nitrate versus ammonium, certainly at elevated pH (free ammonia). From the comparison of results obtained under cultivation with different nitrogen sources (Fig. 4 and Table 2), it emerged that the ammoniacal nitrogen was the most effective source in terms of both total biomass and storage compounds productivity. While the starch/active biomass ratios of the biomass were very similar with the two different nitrogen sources, the lipid/active biomass ratio increased under ammoniacal nitrogen availability. Such results agree with a previous study on lipid accumulation, which was conducted in batch conditions and using pure cultures. The authors have compared the use of ammonium and nitrate, and found that ammoniacal nitrogen enhanced lipid productivity, due to the overall biomass increase (Zhu et al., 2019). In the present study, conducted under nitrogen limiting conditions and carbon excess, the presence of ammoniacal nitrogen led to the biomass increase, mainly due to the accumulation of intracellular compounds. When ammoniacal nitrogen was replaced with nitric nitrogen, microalgae prioritized the reduction of nitrate to ammonium over carbon fixation. This was due to the fact that energy and reducing equivalents (electrons) are diverted towards nitrate reduction, which is energetically less favourable than using ammonium directly. As a result, microalgae allocated their energy resources to nitrate reduction rather than carbon fixation.

In detail, microalgae use different assimilation mechanisms, based on the available nitrogen form (Salbitani and Carfagna, 2021). Before being assimilated into macromolecules, nitrate enters into the algal cells across cell membranes, where it is reduced to ammonium (Kronzucker et al., 1999). Due to nitrogen reducing enzymes, nitrate is assimilated by sequentially reducing nitrate into nitrite, and then nitrite into ammonium (L'Helguen et al., 2008). Therefore, nitrate is energetically more difficult to be assimilated compared to ammonium, because of its oxidized form (Wheeler and Kokkinakis, 1990). Conversely, using ammonium, microalgae avoid energy consumption due to nitrate and nitrite reduction, as well as nitrate reductase and nitrite reductase enzymes production (Salbitani and Carfagna, 2021). Ammonium is already in the reduced state of amino acids, favouring the production of proteins at a lower energy and electron cost for the microbial cells.

It is worth noticing (Table 3) that the majority of studies performed in continuous mode have been conducted using nitrate as nitrogen source. However, based on our results, the use of ammonium is advisable. The possibility of using ammonium will have to be evaluated based on the other operating conditions as well. For instance, pH, temperature, and salinity regulate the equilibrium between ammonium (NH_4^+; ionized form) and free ammonia (NH_3; undissociated form), as the two chemical species are readily interchangeable. In an alkaline environment, free ammonia represents the main form. It can diffuse rapidly via microalgae membranes exerting a toxic direct impact on the photosynthetic apparatus (Li et al., 2019). Consequently, ammonium should be used under

Table 3
Overview of continuous microalge cultivation for lipid production reported in the literature.

Species	Reactor	Carbon	Nitrogen	Dilution rate	Lipid productivity	Other conditions	Lipid production strategy	Ref
D. tertiolecta	Type = Cylinder; V = 6 L; d = NR.	4 % *v*/v CO_2-enriched air; Gas flow rate = 30 mL min^{-1}; Other sources = no.	Source = $NaNO_3$; [N] = 1.64 mg L^{-1}.	0.17–0.74 d^{-1} (0.42 optimal value).	9.8 $mgL^{-1}d^{-1}$ 10.8 % DW	*T* = 25 °C; pH = initial 7.8, not controlled; Mechanical agitation = 600 rpm; Incident irradiance= 50 μmol m^{-2} s^{-1}.	Optimization of dilution rate; Vitamin B_{12} addition.	(Tang et al., 2012)
Chlorella pyrenoidosa XQ-20044	Type = Cylinder; V = 2 L; d = 10 cm.	1 % *v*/v CO_2-enriched air; Gas flow rate = 2 L min^{-1}; Other sources = Na_2CO_3 (0.18 mM).	Source = $NaNO_3$; [N] = 3.29, 6.59, 9.88, 13.2, 16.4, 31.3 and 49.4 mg L^{-1} (9.88 optimal value)	0.24–2.4 d^{-1} (0.48 optimal value).	144 $mgL^{-1}d^{-1}$ 34.69 %DW	*T* = 30 °C; pH = 7–8; Mechanical agitation = NP; Incident irradiance = 600 μmol m^{-2} s^{-1}.	Optimization of nitrogen concentration and dilution rate.	(Wen et al., 2014)
Choricystis minor B. Fott	Type = Cylinder; V = 3.5 L; d = 17.8 cm.	1.58 % *v*/v CO_2-enriched air; Gas flow rate = 4.7 L min^{-1}; Other sources = Na_2CO_3 (0.009 mM).	Source = $NaNO_3$; [N] = 252 mg L^{-1}.	0.12–4.8 d^{-1} (0.33 optimal value).	82 $mgL^{-1}d^{-1}$ 29.7 % DW	*T* = 10–30 °C, optimal value = 25 °C; pH = 6; Mechanical agitation = 200 rpm; Incident irradiance = 500 μmol m^{-2} s^{-1}.	Optimization of dilution rate and temperature; Investigation on the lipid extraction method.	(Sobczuk and Chisti, 2010))
Parachlorella kessleri UTEX2229	Type = Flat panel; V = 1 L; d = 3 cm (thickness).	Air; Gas flow rate = 100 mL min^{-1}; 100 % CO_2 was injected to control the pH. Other sources = $NaHCO_3$ (0.005 mM).	Source = $NaNO_3$; [N] = 214, 69.1, 51, 31.2, 14 mg L^{-1} (51 optimal value).	0.24 d^{-1}.	85.8 $mgL^{-1}d^{-1}$ 20 % DW	*T* = 22 °C; pH = 8; Mechanical agitation = NP; Incident irradiance = 250 μmol m^{-2} s^{-1}.	Optimization of the nitrogen concentration	(Kandilian et al., 2019)
Neochloris oleoabundans	Type = Flat panel; V = 1.7 L; d = 2 cm (thickness).	2 % *v*/v CO_2-enriched N_2; Gas flow rate = 1.2 L min^{-1}; Other sources = $NaHCO_3$ (10 mM).	Source = $NaNO_3$; [N] = 19.7, 5.76, 3.46, and 2.14 mg $L^{-1}d^{-1}$ for Low Light and 33.9, 11.5, 9.05, 5.76 (optimal) and 3.46 g $L^{-1}d^{-1}$ for High Light.	0.18–1.15 d^{-1}.	46 $mgL^{-1}d^{-1}$ 12.4 % DW	T = 30 °C; pH = 7.5; Mechanical agitation = NP; Incident irradiance = Low Light - 200 μmol m^{-2} s^{-1}, High Light - 500 μmol m^{-2} s^{-1} (optimal value).	Optimization of the nitrogen concentration and the incident irradiance; Constant energy intake by turbidostat operation.	(Klok et al., 2013)
Acutodesmus obliquus starchless mutant	Type = Flat panel; V = 1.7 L; d = 2 cm (thickness).	1 % *v*/v CO_2-enriched N_2; Gas flow rate = 1 L min^{-1}; Other sources = $NaHCO_3$ (10 mM).	Nitrates Optimal: 7 ± 1 mgN $mol^{-1}_{photons}$	Not reported	157 $mgL^{-1}d^{-1}$ 33 % DW	*T* = 27.5 °C; pH = 7; Mechanical agitation = NP; Incident irradiance = 500 μmol m^{-2} s^{-1},16 h light and 8 h dark.	Nitrogen limitation under light/dark cycles; Vitamin addition (B_1, B_7, B_{12}).	(Remmers et al., 2017)
Chlorella sp. A2	Type = NR; V = 4 L; d = NR.	Air; Gas flow rate = NR; Other sources = Na_2CO_3 (0.009 mM).	Source = CH_4N_2O; [N] = First mode: 2.1, 4.2, 9.4, 4.2× Int (OD_{680}/2.5 + 1) mg L^{-1} (optimal). It means that 4.2 mgN L^{-1} were added daily if OD < 2.5 and additional 4.2 for every increase in OD_{680} 2.5 Second mode: 8.4 mgN L^{-1} daily (optimal), every 2,3 or 4 days.	Not reported	29.9 $mgL^{-1}d^{-1}$ 22.7 %DW	T = outdoor temperature; pH = NR; Mechanical agitation = NP; Incident irradiance = Natural light at a light intensity >6000 lx.	Minimal nitrogen supply.	(Dang et al., 2016)

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Table 3 (*continued*)

Species	Reactor	Carbon	Nitrogen	Dilution rate	Lipid productivity	Other conditions	Lipid production strategy	Ref
*Chlorella*PY-ZU	Type = Cylinder; V = NR; d = NR.	15 % *v*/v CO_2-enriched N_2; Gas flow rate = 0.1 vvm; Other sources = Na_2CO_3 (0.009 mM).	Source = $NaNO_3$; [N] = First stage - 247 mg L^{-1}, second stage - 0 gL^{-1}.	Not reported	191.3 $mgL^{-1}d^{-1}$ 42.10 % DW	T = 27 °C pH = NR; Mechanical agitation = NP; Incident irradiance= Light intensity of approximately 8500 lx, 16 h light and 8 h dark	Two steps process: First step-nitrogen sufficient, second step-nitrogen starvation and phosphorous addition.	(Chu et al., 2019)
Ettlia sp. YC001	Type = Cylinder; V = 0.8 L; d = 9.5 cm.	10 % v/v CO_2-enriched air; Gas flow rate = 150 mL min^{-1}; Other sources = no.	Source = $NaNO_3$; [N] = 252 mg L^{-1}.	0.2–0.8 d^{-1} (0.79 optimal value).	291.4 $mgL^{-1}d^{-1}$ 23.1 % DW	*T* = 25–28 °C; pH = 6.5–10.5 (optimal value 6.5); Mechanical agitation = NP; Incident irradiance= 260–1500 μmol m^{-2} s^{-1} (1500 optimal value).	Optimization of pH, light intensity and dilution rate.	(Seo et al., 2017)
Chlorella vulgaris OW-01	Type = Cylinder; V: 3 L; d: 17 cm.	Air, 3 % *v*/v CO_2-enriched air (optimal); Gas flow rate = 600 mL min^{-1}; Other sources = Na_2CO_3 (0.009 mM).	Source = $NaNO_3$; [N] = 247 mg L^{-1}.	0.08–1.5 d^{-1} (0.75 optimal value).	270 $mgL^{-1}d^{-1}$ 32 % DW	T: 25 °C; pH = not controlled; Mechanical agitation = 120 rpm; Incident irradiance= 500 μmol m^{-2} s^{-1}.	Two steps process: First step-compensation of limiting growth factors to reach the highest biomass productivity, second step-optimization of the dilution rate.	(Cho et al., 2016)
Ettilia sp. YC001	Type = Cylinder; V: 5 L; d: 25 cm.	10 % v/v CO_2-enriched air (optimal); Gas flow rate = 1 L min^{-1} at 5 s intervals; Other sources = Na_2CO_3 (0.009 mM).	Source = $NaNO_3$; [N] = 247 mg L^{-1}.	0.2 d^{-1}.	49.7 $mgL^{-1}d^{-1}$ 51 % DW	T = 25 °C; pH = 6.5, 8.5 (optimal value), 10.5; Mechanical agitation = NP; Incident irradiance= 500 μmol m^{-2} s^{-1}.	pH control via CO_2 rather than chemical pH control.	(Seo et al., 2017)
Scenedesmus obliquus	Type = Flat panel; V: 0.25 L; d:1.2 cm.	CO_2-enriched air; Other sources = Na_2CO_3 (0.009 mM).	Source = $NaNO_3$; [N] = 247 mg L^{-1}.	0.31 d^{-1}.	318 $mgL^{-1}d^{-1}$ 39 % DW	*T* = 23 °C; pH = 8; Mechanical agitation = NP; Incident irradiance = 100 μmol m^{-2} s^{-1}.	Investigation on biomass recycle (optimal condition without recycle).	(Sforza et al., 2014)
Open mixed culture dominated by *Nitzschia sp*	Type = Cylinder; V: 1.5 L; d:11 cm	5 % v/v CO_2-enriched air; Gas flow rate = 50 mL min^{-1} with gas recycle Other sources = no	Source = $NaNO_3$; [N] = 122 mg L^{-1}.	0.58 d^{-1}	10 % VS	*T* = 28 °C; pH = 7.5; Mechanical agitation = 200 rpm; Incident irradiance = 650 μmol m^{-2} s^{-1} (16 h–8 h light-dark periods)	Light-dark cycles; Silicate addition	(Mooij et al., 2016)
Open mixed culture dominated by diatoms	–	–	Nualgi (US patent application No.: 70275856)	–	37 $mgL^{-1}d^{-1}$ 30%DW	*T* = 26 °C; pH = 7.5; Mechanical agitation = NP; Incident irradiance = 100 μmol m^{-2} s^{-1} (12 h–12 h light-dark periods)	Light-dark cycles; Silicate addition	(Marella et al., 2018)
Open mixed culture dominated by *Trebouxiophyceae*	Type = Cylinder; V: 2 L; d:12 cm	6 % v/v CO_2-enriched air; Other	Source = NH_4Cl; [N] = 42 mg L^{-1}.	0.5 d^{-1}.	387 $mgL^{-1}d^{-1}$ 31 % VS	T = 30 °C; pH = 7.5; Mechanical agitation = 150 rpm;	Vitamin addition; Dual growth limitation (nitrogen and light) strategy.	This study

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Table 3 (*continued*)

Species	Reactor	Carbon	Nitrogen	Dilution rate	Lipid productivity	Other conditions	Lipid production strategy	Ref
		sources = no.				Incident irradiance = 500 μmol m^{-2} s^{-1}.		

N.R. = Not reported.
N.P. = Not performed.

more controlled and circumneutral pH condition or a proper CO_2 dissolution to assimilation rate, which avoids alkaline conditions.

Ammoniacal nitrogen represents the most common N-containing pollutant generally present in domestic wastewater, originating from the ammonification of urea released in urine. The removal of nitrogen is one of the principal secondary biological wastewater treatment process, which is usually more expansive than primary treatment (Abdel-Raouf et al., 2012). The ammoniacal nitrogen concentration in wastewater is usually in the range of 27–100 mg NH_4^+ L^{-1} (Salbitani and Carfagna, 2021). Such values are similar to those used in the present study. In particular, the best condition was conducted using the ammoniacal nitrogen concentration of 42 mg N-$NH_4^+L^{-1}$, corresponding to 54 mgNH_4^+ L^{-1}. Even though ammoniacal nitrogen is a suitable nitrogen source, the use of ammoniacal nitrogen may cause changes in the pH of the culture medium. In the present study, the pH was controlled to keep a stable value of 7.5. For practical applications the right N-source will have to be defined, from pH regulation ammonium nitrate is the often preferred nitrogen fertiliser.

4.3. The effect of vitamin addition on lipid accumulation

Vitamins supplementation strongly enhanced the lipid accumulation (Figs. 2 and 3 and Table 2). From the comparison of the conditions conducted with and without vitamins, it emerged that vitamins increased the lipid productivity from 17 % to 28 % when nitrate was used as N-source and from 20 % to 31 % with ammonium. Interestingly, carbohydrate storage (starch) was not affected by the addition of vitamins. The enhancement of lipid storing capacity under vitamin B availability has been observed in previous works as well (Remmers et al., 2017; Tang et al., 2012). A majority of works using vitamin supplementation as strategy to enhance lipid accumulation has been conducted using pure cultures. Little is known about the possibility of creating a selective environment under vitamin availability using open mixed culture systems. Nonetheless, in a previous study on batch mixed cultures, green microalgae belonging to *Chlorella* species grew in a vitamin-rich medium with no sign of growth in the control treatment deprived of vitamins (Fazeli Danesh et al., 2018). These findings align with the present study, confirming that the supply of vitamins promotes the growth of microalgae populations with a lipid production ability.

From microscopy pictures (Fig. 5a and b), under vitamin availability, both diatoms and green microalgae produced lipid. The presence of diatoms was likely due to the silicate availability in the medium. Silicate is an essential nutrient for the development of the silica shell of diatoms (Tramontano et al., 2020). From microscopy images the lipid accumulation of diatoms seemed higher compared to green microalgae. This result matches with Mooij et al. (2016), who have shown that diatoms are enriched when silicates are added to the medium and that lipid production is higher in diatoms-dominated cultures than in cultures dominated by green microalgae. In the present study, the observation of lipid storing green microalgae is consistent with literature results: *Trebouxiophyceae*, which dominated the vitamin-rich system, have been identified in many pure culture studies as possible lipid-producing microalgae (Arrojo et al., 2022; Maltsev et al., 2019).

With the vitamin-free medium, the microbial community was dominated by filamentous heterocyst cyanobacteria, and much less lipid was stored in the biomass. Microscopy images indicated that the cyanobacteria were not storing lipid. The detected mass fraction of lipid, which was measured under this condition, was therefore likely due to the residual *Trebouxiophyceae* and other microalgae which were present in the community. This observation is in agreement with previous studies indicating the limited capacity of cyanobacteria to store lipid (Lynch et al., 2015).

Previous research has indicated that certain bacterial species, such as cyanobacteria and heterotrophic bacteria (e.g. *Rhodococcus* and *Alcanivorax*), are known lipid producers (although with a low content compared to microalgae) (Alvarez et al., 2019; Manilla-Pérez et al., 2011). However, these bacterial genera were absent in the studied enrichment culture. According to Amplicon Prokaryotes analyses, the majority of bacteria in the vitamin-deprived system belonged to *Kapabacteriales* and cyanobacteria (Supplementary Information). *Kapabacteriales* are not traditionally associated with lipid production, and microscopic observations (Fig. 5) confirmed that lipid production was not associated to cyanobacteria.

In the vitamin-rich system, the predominant bacterial strains in the culture were identified as belonging to the *Cytophagales* and *Rhizobiales* genera. *Rhizobiales* are commonly found in microalgae-rich environments, benefiting from soluble organic compounds produced by microalgae. Additionally, *Cytophagales* are recognized for their ability to degrade complex organic matter and can be found in various environments, including microalgae-rich ecosystems (Sánchez Zurano et al., 2020). However, there is no evidence supporting lipid-producing capabilities of these microorganisms.

Regarding the factors influencing the selection of the two different observed communities: Eukaryotic microalgae cannot synthesize B vitamin de novo (Fallahi et al., 2021). From a study by Tandon et al. (2017) more than half of 312 microalgae species required exogenous vitamin B12 and 23 % required vitamin B1. Moreover, there is currently no research indicating vitamin production within the *Trebouxiophyceae* class. Conversely, cyanobacteria are known to synthesize various vitamins, including B-group vitamins such as thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pantothenic acid (vitamin B5), pyridoxine (vitamin B6), biotin (vitamin B7), and cobalamin (vitamin B12) (Nandagopal et al., 2021). While cyanobacteria can synthesize vitamins, their vitamin requirement is lower compared to green microalgae. Green microalgae, on the other hand, have a higher demand for vitamins compared to cyanobacteria (Helliwell et al., 2016). As a result, the findings observed in this study regarding the selection of a lipid-storing community can be explained as follows: In the presence of vitamins, eukaryotic microalgae, which have a high requirement for vitamins but limited vitamin production capacity, dominate the culture. This is because the vitamins are supplied to them. The selected microalgae community is capable to accumulate lipid. In the absence of vitamins, cyanobacteria dominate the culture since they have the ability to produce vitamins while having a lower vitamin requirement compared to green microalgae. Cyanobacteria are not capable to accumulate lipid.

It is worth mentioning that, in the present study, the identification of the prevalent microorganisms among green microalgae and cyanobacteria relied on qualitative observations through microscopy. In the future, fluorescence in situ hybridization (FISH) techniques targeting 18S rRNA and 16S rRNA could be utilized to identify and localize specific cell types within populations on microscopy images. These FISH-

based measurements would provide precise quantitative information about the abundance of eukaryotes vs. prokaryotes in such complex samples.

The high lipid content observed in the presence of vitamins may also be attributed to vitamin as the cofactors for enzymes involved in the lipid biosynthesis (Fazeli Danesh et al., 2018). Vitamin B1 is involved in the decarboxylation process of pyruvate to acetyl CoA, which is a lipid precursor (Higgins et al., 2018). Vitamin B5 is essential for the synthesis of CoA and ACP enzymes, which are involved in the biosynthesis of lipids (Rébeillé et al., 2007). Vitamin B8 acts as a cofactor of enzymes which are responsible for the hydrolysis of ATP (Tandon et al., 2017). Vitamin B12 is converted to coenzyme forms of B12 (e.g. methylmalonyl-CoA mutase), which contributes to lipid synthesis (Palacios et al., 2014).

From a practical point of view, vitamin supplementation to industrial-scale microalgae system for biofuels and/or value-added bioproducts production would be economically challenging. Indeed, vitamins B are very expensive micronutrients. Therefore, a co-culture of microalgae and vitamin B-producing bacteria has been suggested in several studies to overcome this issue (Yao et al., 2019). Examples of symbiotic relationships include *Rhizobium* sp. enhancing the growth of *Botryococcus braunii* and *Mesorhizobium loti* providing vitamin B12 to *Lobomonas rostrata*. These associations involve the exchange of nutrients and inhibit the growth of other bacteria which can lead to the collapse of microalgae, demonstrating mutual symbiosis and the competitive exclusion principle. Such "synthetic ecology" strategies are therefore advantageous, particularly in non-sterile environments (Magdouli et al., 2016). How to efficiently enrich for such communities can be the aim of future research.

The presence of vitamins in wastewater might be an interesting feature, as well. Selimoğlu et al. (2015) measured the vitamin B1, B2, B3, B6, B11 and B12 content in domestic wastewater from different treatment plants. Very different values were detected. For instance, vitamin B12 content was in the range of 0.05 and 5.16 mg L^{-1}. The other B vitamin values ranged between 0.06 and 11 mg L^{-1}. Despite the very high variability of vitamin concentration in wastewater, the possibility of using wastewater as vitamin source is worth of consideration. The vitamin concentration used in the present work was of the same order of magnitude compared to the limited values reported for wastewater. The possibility of using wastewater as vitamin supplementation source is particularly interesting considering our observations on the beneficial effect of ammoniacal nitrogen. Indeed, domestic wastewater can be used as source of both ammonium and vitamins in biofuels microalgae production systems, with the additional advantage to perform the concomitant tertiary treatment of wastewater.

5. Conclusions

A continuous bioreactor operated under combined nitrogen and light limitation showed a strong potential to enrich for a phototrophic microalgae community with high lipid storage capacity. Supplementation of vitamins and using ammonium as nitrogen source further enhanced the lipid storage by the eukaryotic microalgae affiliating with *Trebouxiophyceae*. The mass fraction and volumetric productivity (31 % VS and 387 mg$L^{-1}d^{-1}$) of lipids accumulated in the mixed culture biomass are among the highest vales reported hitherto in literature. These findings represent one important step forward in lipid production by mixed-culture microalgae systems. This is one of the first studies highlighting the benefits of mixed-culture photo-biotechnologies for the production of lipids not only for biofuels but also for food ingredients in the circular economy. The use of enrichment cultures allows for a relative cheap cultivation in open ponds or other scalable reactors in a continuous operation.

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CRediT authorship contribution statement

Grazia Policastro: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sirous Ebrahimi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **David G. Weissbrodt:** Writing – review & editing, Supervision, Methodology. **Massimiliano Fabbricino:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Mark C.M. van Loosdrecht:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Abdel-Raouf, N., Al-Homaidan, A.A., Ibraheem, I.B.M., 2012. Microalgae and wastewater treatment. Saudi J. Biol. Sci. 19, 257–275. https://doi.org/10.1016/j.sjbs.2012.04.005.

Alvarez, H.M., Herrero, O.M., Silva, R.A., Hernández, M.A., Lanfranconi, M.P., Villalba, M.S., 2019. Insights into the metabolism of oleaginous Rhodococcus spp. Appl. Environ. Microbiol. 85 https://doi.org/10.1128/AEM.00498-19.

APHA, 2017. 6200 volatile organic compounds. In: Standard Methods For the Examination of Water and Wastewater, Standard Methods for the Examination of Water and Wastewater. American Public Health Association. https://doi.org/10.2105/SMWW.2882.118.

Arrojo, M.Á., Regaldo, L., Calvo Orquín, J., Figueroa, F.L., Abdala Díaz, R.T., 2022. Potential of the microalgae *Chlorella fusca* (Trebouxiophyceae, Chlorophyta) for biomass production and urban wastewater phycoremediation. AMB Express 12. https://doi.org/10.1186/s13568-022-01384-z.

Behera, B., Unpaprom, Y., Ramaraj, R., Maniam, G.P., Govindan, N., Paramasivan, B., 2021. Integrated biomolecular and bioprocess engineering strategies for enhancing the lipid yield from microalgae. Renew. Sust. Energ. Rev. 148, 111270 https://doi.org/10.1016/j.rser.2021.111270.

Bumbac, M., Nicolescu, C.M., Olteanu, R.L., Gherghinoiu, S.C., Bumbac, C., Tiron, O., Manea, E.E., Radulescu, C., Gorghiu, L.M., Stanescu, S.G., Serban, B.C., Buiu, O., 2023. Preparation and characterization of microalgae styrene-butadiene composites using *Chlorella vulgaris* and *Arthrospira platensis* biomass. Polymers (Basel) 15.

Cavaillé, L., Albuquerque, M., Grousseau, E., Lepeuple, A.S., Uribelarrea, J.L., Hernandez-Raquet, G., Paul, E., 2016. Understanding of polyhydroxybutyrate production under carbon and phosphorus-limited growth conditions in non-axenic continuous culture. Bioresour. Technol. 201, 65–73. https://doi.org/10.1016/j.biortech.2015.11.003.

Cho, D.H., Ramanan, R., Heo, J., Shin, D.S., Oh, H.M., Kim, H.S., 2016. Influence of limiting factors on biomass and lipid productivities of axenic *Chlorella vulgaris* in photobioreactor under chemostat cultivation. Bioresour. Technol. 211, 367–373. https://doi.org/10.1016/j.biortech.2016.03.109.

Chu, F., Cheng, J., Zhang, X., Ye, Q., Zhou, J., 2019. Enhancing lipid production in microalgae Chlorella PY-ZU1 with phosphorus excess and nitrogen starvation under 15% CO2 in a continuous two-step cultivation process. Chem. Eng. J. 375, 121912 https://doi.org/10.1016/j.cej.2019.121912.

Coelho, R.S., Vidotti, A.D.S., Reis, É.M., Franco, T.T., 2014. High cell density cultures of microalgae under fed-batch and continuous growth. Chem. Eng. Trans. 38, 313–318. https://doi.org/10.3303/CET1438053.

Dang, H., Perreault, F., Stewart, J., Wang, Q., Zhu, J., Chen, W., Chen, H., Zhang, X., He, C., Rong, J., 2016. Improved productivity of neutral lipids in *Chlorella* sp. A2 by minimal nitrogen supply. Front. Microbiol. 1, 557. https://doi.org/10.3389/fmicb.2016.00557. | www.frontiersin.org.

Durner, R., Witholt, B., Egli, T., 2000. Accumulation of poly[(R)-3-hydroxyalkanoates] in Pseudomonas oleovorans during growth with octanoate in continuous culture at different dilution rates. Appl. Environ. Microbiol. 66, 3408–3414. https://doi.org/10.1128/AEM.66.8.3408-3414.2000.

Ebadian, M., van Dyk, S., McMillan, J.D., Saddler, J., 2020. Biofuels policies that have encouraged their production and use: an international perspective. Energy Policy 147, 111906. https://doi.org/10.1016/j.enpol.2020.111906.

Egli, T., Quayle, J.R., 1986. Influence of the carbon: nitrogen ratio of the growth medium on the cellular composition and the ability of the methylotrophic yeast Hansenula polymorpha to utilize mixed carbon sources. J. Gen. Microbiol. 132, 1779–1788. https://doi.org/10.1099/00221287-132-7-1779.

Fallahi, A., Rezvani, F., Asgharnejad, H., Khorshidi, E., Hajinajaf, N., Higgins, B., 2021. Interactions of microalgae-bacteria consortia for nutrient removal from wastewater: a review. Chemosphere 272, 129878. https://doi.org/10.1016/j.chemosphere.2021.129878.

Fazeli Danesh, A., Mooij, P., Ebrahimi, S., Kleerebezem, R., van Loosdrecht, M., 2018. Effective role of medium supplementation in microalgal lipid accumulation. Biotechnol. Bioeng. 115, 1152–1160. https://doi.org/10.1002/bit.26548.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea Cleve. Can. J. Microbiol. 8, 229–239.

Helliwell, K.E., Lawrence, A.D., Holzer, A., Kudahl, U.J., Sasso, S., Kräutler, B., Scanlan, D.J., Warren, M.J., Smith, A.G., 2016. Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B12. Curr. Biol. 26, 999–1008. https://doi.org/10.1016/j.cub.2016.02.041.

Higgins, B.T., Wang, Q., Du, S., Hennebelle, M., Taha, A.Y., Fiehn, O., VanderGheynst, J. S., 2018. Impact of thiamine metabolites and spent medium from Chlorella sorokiniana on metabolism in the green algae Auxenochlorella protothecoides. Algal Res. 33, 197–208. https://doi.org/10.1016/j.algal.2018.05.019.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621–639. https://doi.org/10.1111/j.1365-313X.2008.03492.x.

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G., Van Loosdrecht, M.C.M., 2009. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromolecules 10, 670–676. https://doi.org/10.1021/bm8013796.

Kandilian, R., Taleb, A., Heredia, V., Cogne, G., Pruvost, J., 2019. Effect of light absorption rate and nitrate concentration on TAG accumulation and productivity of Parachlorella kessleri cultures grown in chemostat mode. Algal Res. 39, 101442 https://doi.org/10.1016/j.algal.2019.101442.

Kleerebezem, R., van Loosdrecht, M.C., 2007. Mixed culture biotechnology for bioenergy production. Curr. Opin. Biotechnol. 18, 207–212. https://doi.org/10.1016/j.copbio.2007.05.001.

Klok, A.J., Martens, D.E., Wijffels, R.H., Lamers, P.P., 2013. Simultaneous growth and neutral lipid accumulation in microalgae. Bioresour. Technol. 134, 233–243. https://doi.org/10.1016/j.biortech.2013.02.006.

Kronzucker, H.J., Glass, A.D.M., Siddiqi, M.Y., 1999. Inhibition of nitrate uptake by ammonium in barley. Analysis of component fluxes. Plant Physiol. 120, 283–291. https://doi.org/10.1104/pp.120.1.283.

L'Helguen, S., Maguer, J.F., Caradec, J., 2008. Inhibition kinetics of nitrate uptake by ammonium in size-fractionated oceanic phytoplankton communities: implications for new production and f-ratio estimates. J. Plankton Res. 30, 1179–1188. https://doi.org/10.1093/plankt/fbn072.

Li, T., Gargouri, M., Feng, J., Park, J.J., Gao, D., Miao, C., Dong, T., Gang, D.R., Chen, S., 2015. Regulation of starch and lipid accumulation in a microalga Chlorella sorokiniana. Bioresour. Technol. 180, 250–257. https://doi.org/10.1016/j.biortech.2015.01.005.

Li, X., Li, W., Zhai, J., Wei, H., Wang, Q., 2019. Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. Bioresour. Technol. 273, 368–376. https://doi.org/10.1016/j.biortech.2018.11.042.

Lynch, F., Santana-Sánchez, A., Jämsä, M., Sivonen, K., Aro, E.M., Allahverdiyeva, Y., 2015. Screening native isolates of cyanobacteria and a green alga for integrated wastewater treatment, biomass accumulation and neutral lipid production. Algal Res. 11, 411–420. https://doi.org/10.1016/j.algal.2015.05.015.

Magdouli, S., Brar, S.K., Blais, J.F., 2016. Co-culture for lipid production: advances and challenges. Biomass Bioenergy 92, 20–30. https://doi.org/10.1016/j.biombioe.2016.06.003.

Maltsev, Y., Maltseva, I., Maltseva, S., Kociolek, J.P., Kulikovskiy, M., 2019. Fatty acid content and profile of the novel strain of *Coccomyxa elongata* (Trebouxiophyceae, Chlorophyta) cultivated at reduced nitrogen and phosphorus concentrations. J. Phycol. 55, 1154–1165. https://doi.org/10.1111/jpy.12903.

Manilla-Pérez, E., Lange, A.B., Luftmann, H., Robenek, H., Steinbüchel, A., 2011. Neutral lipid production in *Alcanivorax borkumensis* SK2 and other marine hydrocarbonoclastic bacteria. Eur. J. Lipid Sci. Technol. 113, 8–17. https://doi.org/10.1002/ejlt.201000374.

Marella, T.K., Parine, N.R., Tiwari, A., 2018. Potential of diatom consortium developed by nutrient enrichment for biodiesel production and simultaneous nutrient removal from waste water. Saudi J. Biol. Sci. 25, 704–709. https://doi.org/10.1016/j.sjbs.2017.05.011.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renew. Sust. Energ. Rev. 14, 217–232. https://doi.org/10.1016/j.rser.2009.07.020.

Mooij, P.R., 2016. On the Use of Selective Environments in Microalgal Cultivation.

Mooij, P.R., Stouten, G.R., Tamis, J., Van Loosdrecht, M.C.M., Kleerebezem, R., 2013. Survival of the fattest. Energy Environ. Sci. 6, 3404–3406. https://doi.org/10.1039/c3ee42912a.

Mooij, P.R., de Jongh, L.D., van Loosdrecht, M.C.M., Kleerebezem, R., 2016. Influence of silicate on enrichment of highly productive microalgae from a mixed culture. J. Appl. Phycol. 28, 1453–1457. https://doi.org/10.1007/s10811-015-0678-2.

Nandagopal, P., Steven, A.N., Chan, L.W., Rahmat, Z., Jamaluddin, H., Noh, N.I.M., 2021. Bioactive metabolites produced by cyanobacteria for growth adaptation and their pharmacological properties. Biology (Basel) 10, 1–24. https://doi.org/10.3390/biology10101061.

Mandl, Nicole, Marion, Pinterits, Mandl, N., Pinterits, M., 2020. Annual European Union Greenhouse Gas Inventory 1990–2018 and Inventory Report 2020, p. 977.

Nielsen, S.S., 2017. Total carbohydrate by phenol-sulfuric acid total carbohydrate method S.Suzanne Nielsen. Food Anal. Lab. Man. 137–141. https://doi.org/10.1007/978-3-319-44127-6.

Palacios, O.A., Bashan, Y., de-Bashan, L.E., 2014. Proven and potential involvement of vitamins in interactions of plants with plant growth-promoting bacteria-an overview. Biol. Fertil. Soils 50, 415–432. https://doi.org/10.1007/s00374-013-0894-3.

Poblete-Castro, I., Escapa, I.F., Jäger, C., Puchalka, J., Chi Lam, C.M., Schomburg, D., Prieto, M.A., Martins dos Santos, V.A.P., 2012. The metabolic response of *P. putida* KT2442 producing high levels of polyhydroxyalkanoate under single- and multiple-nutrient-limited growth: highlights from a multi-level omics approach. Microb. Cell Factories 11, 1–21. https://doi.org/10.1186/1475-2859-11-34.

Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F., 2013. Biodiesel from microalgae: a critical evaluation from laboratory to large scale production. Appl. Energy 103, 444–467. https://doi.org/10.1016/j.apenergy.2012.10.004.

Rébeillé, F., Ravanel, S., Marquet, A., Mendel, R.R., Smith, A.G., Warren, M.J., 2007. Roles of vitamins B5, B8, B9, B12 and molybdenum cofactor at cellular and organismal levels. Nat. Prod. Rep. 24, 949–962. https://doi.org/10.1039/b703104c.

Remmers, I.M., Hidalgo-Ulloa, A., Brandt, B.P., Evers, W.A.C., Wijffels, R.H., Lamers, P. P., 2017. Continuous versus batch production of lipids in the microalgae Acutodesmus obliquus. Bioresour. Technol. 244, 1384–1392. https://doi.org/10.1016/j.biortech.2017.04.093.

Salbitani, G., Carfagna, S., 2021. Ammonium utilization in microalgae: a sustainable method for wastewater treatment. Sustainablity 13, 1–17. https://doi.org/10.3390/su13020956.

Sánchez Zurano, A., Garrido Cárdenas, J.A., Gómez Serrano, C., Morales Amaral, M., Acién-Fernández, F.G., Fernández Sevilla, J.M., Molina Grima, E., 2020. Year-long assessment of a pilot-scale thin-layer reactor for microalgae wastewater treatment. Variation in the microalgae-bacteria consortium and the impact of environmental conditions. Algal Res. 50, 101983 https://doi.org/10.1016/j.algal.2020.101983.

Selimoğlu, F., Öbek, E., Karataş, F., Arslan, E.I., Tatar, Ş.Y., 2015. Determination of amounts of some vitamin B groups in domestic wastewater treatment plants. Turkish J. Sci. Technol. 10, 1–5.

Seo, S.H., Ha, J.S., Yoo, C., Srivastava, A., Ahn, C.Y., Cho, D.H., La, H.J., Han, M.S., Oh, H.M., 2017. Light intensity as major factor to maximize biomass and lipid productivity of Ettlia sp. in CO2-controlled photoautotrophic chemostat. Bioresour. Technol. 244, 621–628. https://doi.org/10.1016/j.biortech.2017.08.020.

Sforza, E., Enzo, M., Bertucco, A., 2014. Design of microalgal biomass production in a continuous photobioreactor: An integrated experimental and modeling approach. Chem. Eng. Res. Des. 92, 1153–1162. https://doi.org/10.1016/j.cherd.2013.08.017.

Sharma, K.K., Schuhmann, H., Schenk, P.M., 2012. High lipid induction in microalgae for biodiesel production. Energies 5, 1532–1553. https://doi.org/10.3390/en5051532.

Sibi, G., Shetty, V., Mokashi, K., 2016. Enhanced lipid productivity approaches in microalgae as an alternate for fossil fuels – a review. J. Energy Inst. 89, 330–334. https://doi.org/10.1016/j.joei.2015.03.008.

Sobczuk, T.M., Chisti, Y., 2010. Potential fuel oils from the microalga Choricystis minor. J. Chem. Technol. Biotechnol. 85, 100–108. https://doi.org/10.1002/jctb.2272.

Tandon, P., Jin, Q., Huang, L., 2017. A promising approach to enhance microalgae productivity by exogenous supply of vitamins. Microb. Cell Factories 16, 1–13. https://doi.org/10.1186/s12934-017-0834-2.

Tang, H., Chen, M., Simon Ng, K.Y., Salley, S.O., 2012. Continuous microalgae cultivation in a photobioreactor. Biotechnol. Bioeng. 109, 2468–2474. https://doi.org/10.1002/bit.24516.

Tramontano, C., Chianese, G., Terracciano, M., de Stefano, L., Rea, I., 2020. Nanostructured biosilica of diatoms: from water world to biomedical applications. Appl. Sci. 10 https://doi.org/10.3390/app10196811.

Wen, X., Geng, Y., Li, Y., 2014. Enhanced lipid production in *Chlorella pyrenoidosa* by continuous culture. Bioresour. Technol. 161, 297–303. https://doi.org/10.1016/j.biortech.2014.03.077.

Wheeler, P.A., Kokkinakis, S.A., 1990. Ammonium recycling limits nitrate use in the oceanic subarctic Pacific. Limnol. Oceanogr. 35, 1267–1278. https://doi.org/10.4319/lo.1990.35.6.1267.

Yao, S., Lyu, S., An, Y., Lu, J., Gjermansen, C., Schramm, A., 2019. Microalgae–bacteria symbiosis in microalgal growth and biofuel production: a review. J. Appl. Microbiol. 126, 359–368. https://doi.org/10.1111/jam.14095.

Zhou, J., Wang, M., Saraiva, J.A., Martins, A.P., Pinto, C.A., Prieto, M.A., Simal-Gandara, J., Cao, H., Xiao, J., Barba, F.J., 2022. Extraction of lipids from microalgae using classical and innovative approaches. Food Chem. 384, 132236 https://doi.org/10.1016/j.foodchem.2022.132236.

Zhu, L., Li, S., Hu, T., Nugroho, Y.K., Yin, Z., Hu, D., Chu, R., Mo, F., Liu, C., Hiltunen, E., 2019. Effects of nitrogen source heterogeneity on nutrient removal and biodiesel production of mono- and mix-cultured microalgae. Energy Convers. Manag. 201, 112144 https://doi.org/10.1016/j.enconman.2019.112144.