Effect of nitrogen limitation on growth, biochemical composition, and cell ultrastructure of the microalga *Picocystis salinarum*

Ronald Tarazona Delgado [1,2] · Mayara dos Santos Guarieiro [1] · Paulo Wagnner Antunes [3] · Sérvio Túlio Cassini [3] · Haydee Montoya Terreros [2] · Valéria de Oliveira Fernandes [1]

Abstract

In recent years biodiesel production has attracted worldwide attention due to the awareness of fossil fuel depletion and microalgae biomass is considered a promising raw material for its formulation. The present study evaluated the effects of different levels of nitrogen limitation (37.5, 18.75, 9.375 mg L^{-1} $NaNO_3$) on the growth, cell ultrastructure, and biochemical composition of a halophilic native strain of the green alga *Picocystis salinarum* as a potential raw material source for biodiesel. During a culture period of 20 days, growth measurements and photosynthetic pigments were estimated. Cell density, dry weight, and chlorophylls *a*, *b* content decreased with time as nitrogen limitation increase; however, carotenoid content increased. In addition, nitrogen limitation caused an progressive increase in the lipid and carbohydrate yield and a decrease in protein. The high N limitation (9.375 mg L^{-1}) had a significant effect on the accumulation of total lipid content (33.87% dry weight). Carbohydrate content (30.98% dry weight) and protein content (1.89% dry weight) decrease. The lipid content showed a differential FAME profile with high saturated fatty acid values (996.08 $\mu g\ g^{-1}$ dry weight) mainly palmitic acid, compare with the unsaturated ones that showed low values under high N limitation. The gradual increase of lipid content was also corroborated by transmission electron microscopy images with a single large lipid droplet cell formation. Therefore, evaluation of the algal culture conditions such as N limitation, as a strategy to maximize lipid content and improve the fatty acid profile in unexplored strain of *P. salinarum*, showed a potential biomass yield as a suitable candidate for biodiesel production.

Introduction

The common use of petroleum-based fuels is widely recognized as unsustainable, it has been considered a global concern due to the exhaustion of its stocks, and the huge emission of greenhouse gases into the atmosphere contribute to climate change (Tan et al. 2018). Among substitute alternatives to petroleum products (fossil fuels), biodiesel has become a potential renewable fuel and its use leads to a reduction of harmful CO_2 emissions and the elimination of sulfur oxide emissions (Francisco et al. 2010).

Biodiesel can be derived from food crops such as edible oilseeds (sunflower, palm, soy, coconut), considered first-generation raw materials. However, mass production of alternative fuel source as terrestrial oil crops may cause shortage for food supply as well as deforestation (Chen et al. 2018; Rawat et al. 2013; Tandon and Jin 2017). Recently, non-food crops (non-edible seeds: pine nut, karanja, jojoba, mahua), edible oil residues, and animal fats have gained importance as second-generation raw materials. Nevertheless, they do not have enough lipid content to replace biofuel current needs (Rawat et al. 2013; Tandon and Jin 2017).

In this context, microalgae have emerged as a potential biodiesel source to cover global demand for fuel due to their high growth rate, photosynthetic efficiency, and high lipid content biosynthesis (Chisti 2007; Chen et al. 2018). Consequently, microalgae biomass is

✉ Ronald Tarazona Delgado
   ronald.mtd@hotmail.com

1   Department of Biological Sciences, Federal University of Espírito Santo, Vitória, ES 29075-015, Brazil

2   Natural History Museum, National University of San Marcos, LIM 14-0434 Lima, Peru

3   Department of Environmental Engineering, Federal University of Espírito Santo, Vitória, ES 29075-910, Brazil

considered as the third-generation raw material for biodiesel. Their use does not interfere with food production and competition for arable land is reduced, and the water volume requirement is much lower for their biomass production compare to cultivable plants in the agronomic activity. Besides, certain microalgae biomass contains other biomolecules including carbohydrates, proteins, and pigments that can be used for different secondary value-added products such as food, pharmaceutical, or cosmetic additives (Demirbas and Demirbas 2011; Tandon and Jin 2017).

Microalgae can be grown in media based on freshwater or seawater. To avoid competition for freshwater and significantly contribute to the biodiesel economy from microalgal biomass, the selection of a cultivable strain in seawater is mandatory (San Pedro et al. 2013). Although many oleaginous microalgae have already been studied, there are a large number of unexplored species, mainly from extremophile aquatic ecosystems (acidophilic, alkaliphilic, halophilic, or thermophilic). As a result of thriving in such diverse and extreme environments, they produce a variety of unique and complex lipids and fatty acids that are not generally present in freshwater and marine microalgae and have an even greater potential as a source of lipid raw material for biodiesel production and due to their robustness, their adaptability to open raceways ponds can be successfully developed (Malavasi et al. 2020). Furthermore, it is necessary to manipulate the biochemical composition of the strain to increase their lipid content with synthesis of specific fatty acids for biodiesel formulation by adjusting the nutrient composition, salinity, or pH of the media, and varying culture conditions such as light, temperature, or photoperiod (Juneja et al. 2013).

Nutrient depletion is an approach to target metabolic pathways in lipid synthesis as the main reserve substance in microalgae. Although it has been reported that phosphorus and iron channel the metabolic flux to lipid biosynthesis under normal conditions, nitrogen is considered the most effective nutritional limiting factor for triggering high oil contents (Courchesne et al. 2009). Such is the case of the culture under nitrogen limitation of the halophilic microalga *Picocystis salinarum*, which has a significant increase of lipid contents stored in numerous intracellular lipid droplets, suggesting their potential as future biofuel strain (Wang et al. 2014).

Therefore, the present research aimed to evaluate the effect of nitrogen limitation as a strategy to increase the lipid and fatty acid productivity of the biomass of a local strain of *P. salinarum* as a potential raw material for biodiesel. In addition, analysis of changes in biochemical composition, growth, and cell ultrastructure of this understudied species of microalga is reported.

Materials and Methods

Strain and culture conditions

The chlorophyte *Picocystis salinarum* USM 303650 was isolated from the salinas of Chilca, located in the central arid coastal region of Peru (12° 32′ 40″ S, 76° 43′ 23″ W). The salinas are formed of several aquatic environments such as small lagoons, ponds, and pools. The water budget is governed by seawater seepage and evaporation. The strain has been deposited in the Herbarium of the Natural History Museum, National University of San Marcos, and reported with some culture characteristics in our previous research (Tarazona-Delgado et al. 2017).

The microalga was cultured in f/2 medium (Guillard 1975), prepared with filtered seawater, pH was adjusted to 8, and the medium was autoclaved (121 °C, 20 min). The cultures were maintained under constant aeration and controlled conditions of temperature (29 ± 1 °C), light intensity (47.25 μmol photons m^{-2} s^{-1}) using 40 W daylight fluorescent lamps with a 12:12 h light:dark photoperiod. Batch cultures were grown in 3 L Erlenmeyer flasks (2.7 L culture medium and 0.3 L of axenic inoculum with $9.5 \pm 0.5 \times 10^5$ cells mL^{-1}) for 20 days. Lyophilization was set used to obtain microalgal biomass.

Experimental design

Cultivation experiments were conducted to evaluate and compare the algal growth, cell ultrastructure, and biochemical composition of the *P. salinarum* strain under different nitrogen limitation conditions. The original sodium nitrate concentration (75 mg L^{-1}) in f/2 medium is reported as 1 N (normal conditions). This was modified to serial dilutions: 1/2, 1/4, and 1/8 times, receiving the labels 0.5 N, 0.25 N, and 0.125 N, respectively.

Growth assessment

Cell density (10^6 cells mL^{-1}) was determined by direct counting with a Neubauer hemocytometer every 2 days during the culture period (20 days). Dry weight (g L^{-1}) was calculated by gravimetry every 4 days, culture aliquots (20 mL) were filtered in a glass fiber microfilter (Macherey Nagel, GF-1 47 mm, Germany), followed by oven drying at 60 °C until constant weight.

Quantification of photosynthetic pigments

Chlorophyll *a* and *b* and total carotenoids were extracted in 90% acetone and measured every 4 days using the equations of Jeffrey and Humphrey (1975) and Strickland and Parsons (1972), respectively.

Determination of protein, carbohydrate, and total lipid

Total protein content was determined according to Lowry et al. (1951). For alkaline hydrolysis, 4 mL of 1 N NaOH was added to 5 mg of microalgal biomass and the mixture was incubated at 100 °C for 1 h and centrifuged at 3000 rpm for 30 min. Then, 5 mL of solution of 2% Na_2CO_3 in 0.1 N NaOH, 0.5% CuSO4, and 1% $KNaC_4H_4O_6$ (100:1:1, v/v/v) was added to 0.5 mL aliquot of the alkaline extract. The mixture was kept for 10 min at room temperature. Then, 0.5 mL of Folin-Ciocalteau reagent in distilled water (1: 1, v/v) was added and the mixture was incubated for 30 min. The blue complex was analyzed in a spectrophotometer set at 750 nm against a calibration curve of albumin solution of known concentration as the standard.

Total carbohydrate content of the microalgal biomass was determined using the phenol-sulfuric acid method of Kochert (1978). Briefly, 5 mg of biomass was used for alkaline hydrolysis. Afterward, 1 mL of 1 N NaOH and 0.05 mL of 4% phenol were added to 0.5 mL aliquot of alkaline extract. The mixture was kept in an oven at 25 °C for 30 min. Then, 2.5 mL of sulfuric acid was added to the mixture and was kept for 5 min at room temperature. The yellow-brown complex was spectrophotometrically at 485 nm against a calibration curve (anhydrous glucose solution).

Total lipid was extracted according to Bligh and Dyer (1959). 7.5 mL chloroform and methanol (1: 2, v/v) was added to 0.5 g biomass. The mixture was vortexed for 2 min followed by addition of 2.5 mL chloroform and 2.5 mL distilled water, and vortexed again. Subsequently, the mixture was centrifuged at 3500 rpm at 4 °C for 8 min. The organic phase with the extracted lipids was separated and placed in an oven at 30 °C for organic solvent evaporation. Finally, the total lipids were determined gravimetrically.

The total protein, carbohydrate, and lipid concentrations are given as mg g^{-1} and biomass dry weight (% DW).

Transmission electron microscopy (TEM)

Cell ultrastructure was studied using the TEM according to the method of Souza et al. (2017) with minor modifications. Samples (0.05 mg microalgal biomass) were washed in phosphate-buffered saline and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4 °C for 12 h. Fixed samples were washed three times with cacodylate buffer for 10 min. Post-fixation in 1% OsO_4 in 0.1 M cacodylate buffer and 1.25% $K_4Fe(CN)_6$ (1: 1, v/v) was followed by dehydration in an acetone series (30, 50, 70, 90, and 100%). Later, the samples were infiltrated in Epon resin and polymerized in an oven at 60 °C for 48 h. The resulting blocks were cut at 60 nm in an ultramicrotome and stained with 5% uranyl acetate and 1% lead citrate for 30 min each. The ultrathin sections were mounted on mesh n° 400 grids coated with formvar, and examined in the transmission electron microscope (JEOL, JEM-1400, Brazil), operated at 120 kV with a LaB6 filament.

Analysis of fatty acid methyl esters (FAMEs)

Fatty acids were determined using high-performance liquid chromatography (HPLC) of methyl esters from the microalgal biomass. The transesterification of biomass was performed according to Menezes et al. (2013) with few modifications. Two gram of biomass was suspended in 3 mL of 0.5 M NaOH in methanol, followed by heating at 70 °C for 10 min. Then, samples were cooled in an ice-water bath and 9 mL of an esterifying solution of NH_4Cl:methanol:H_2SO_4 (1:30:1.5, g/v/v) was added. Samples were again heated at 70 °C, cooled in an ice-water bath, and 5 mL heptane and 2 mL distilled water were added to the mixture and vortexed. The heptane phase containing FAMEs were transferred into a tube and dried under a stream of nitrogen.

The FAMEs were dissolved in 10 μL acetonitrile and analyzed in a chromatography system (Shimadzu, CBM-20A, Japan), equipped with a DGU-20AS solvent degasser, LC-20AT gradient quaternary pump, SIL-20AHT automatic sample injector, SPD-M20A diode array detector, and a 100 mm × 2.1 mm × 2.6 μm Kinetex C18 HPLC column (USA).

A binary mobile phase consisting of (A) trifluoroacetic acid solution and distilled water (0.1:99.9, v/v) and (B) acetonitrile were filtered using a vacuum filtration system through 0.45 μm membrane filters and degassed in an ultrasound bath (Limpsonic, Brazil). The HPLC system was programmed to operate under controlled conditions of column temperature (37 °C), detection wavelength (210 nm), and flow rate (0.25 mL min^{-1}). The following gradient elution was employed: 0–1 min: 100% A; 1–12 min: 90–70% A, 20–40% B; 12–32 min: 100–90% A, 62–40% B; 32–32.5 min (column equilibration): 100% A. The fatty acids were analyzed by comparing their retention time of the corresponding peaks with a known standard mixture of FAMEs added to each sample as the standard. LCSolutions 2.1 software was used for data acquisition and analysis.

Statistical analysis

The tests were performed using triplicates for each treatment. Means and standard deviation (SD) were calculated for all treatments, and significant differences were determined by analysis of variance according to Tukey's highly significant differences test ($p < 0.05$). Comparison among the treatments was performed by one-way ANOVA test ($p < 0.05$). Principal component analysis (PCA) was used to determine the relationship between all tests analyzed. ANOVA and Tukey's test were performed using SPSS 20.0 software, and the PCA using XLSTAT 2020 software.

## Results and discussion

Growth measurements

The growth of microalgae depends on an adequate supply of nutrients —mainly nitrogen, phosphorus, and micronutrients. Nitrogen (N) is a major component in many biological macromolecules like chlorophylls, proteins, and DNA. Under N depletion, microalgae grow in a medium lacking of N source, while under N limitation, there is a constant but insufficient N availability. Therefore, the N nutrient stress on cellular physiology negatively affects microalgal growth such as cell density and dry biomass (Ördög et al. 2012; Benavente-Valdés et al. 2016).

The *P. salinarum* growth response was proportional to the N concentration in the medium during the culture period (20 days). The control culture (1 N) reached the highest values of cell density from the 18th day until the end of culture period on the 20th day (13.1×10^6 cells mL^{-1}), and a gradual decrease in the population growth with the increase of nitrogen limitations (0.5, 0.25, and 0.125 N) (Fig. 1). These findings corroborate the results obtained by others who have reported that microalgae cell density is directly proportional to the concentration of N in the culture medium (Illman et al. 2000; Chen et al. 2011; Zhu et al. 2014).

Regarding the biomass yield as dry weight (Fig. 2), the highest values were obtained in 1 N (0.96 g L^{-1}) and 0.5 N (0.88 g L^{-1}) on the 20th day. They were followed by a slight dry weight decrease in response to N limited availability under 0.25 (0.86 g L^{-1}) and 0.125 N (0.74 g L^{-1}) treatments. In certain microalgae, the dry mass yield under nutrient-limited conditions may not differ much from that under normal conditions, since they can accumulate some specific biomolecule. Similar studies in some green microalgae showed the dry mass slightly decreased under N limitation or depletion (Ördög
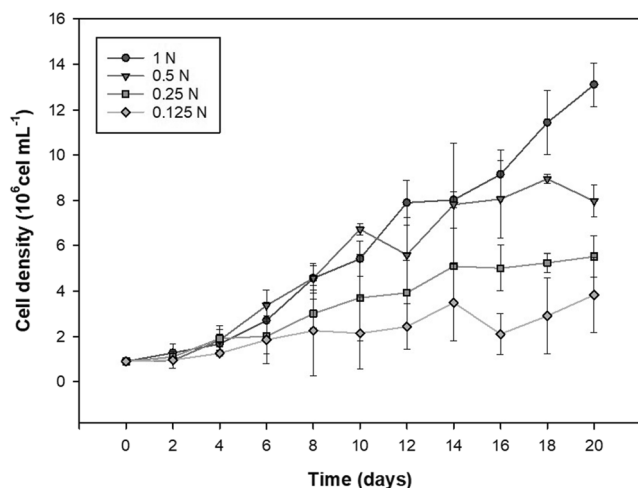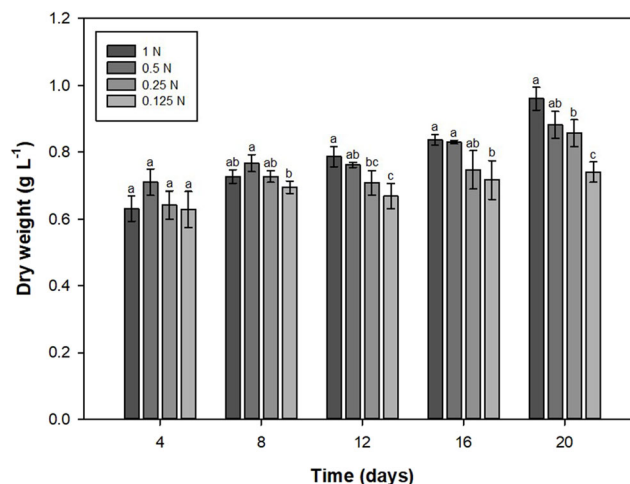
Fig. 2 Effect of nitrogen limitation on the dry weight of *P. salinarum*. Data are mean ± SD ($n = 3$). Values with the different letters represent significant difference ($p < 0.05$) between treatments

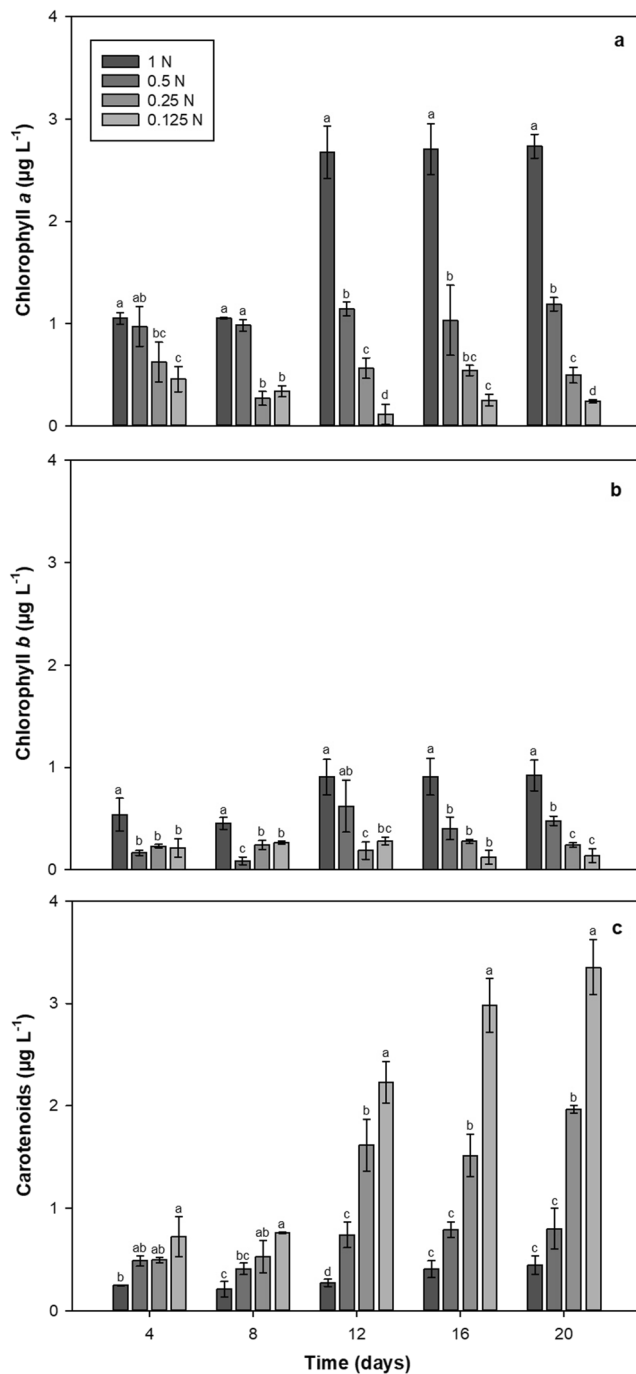et al. 2012; Anand and Arumugam 2015; Sathasivam et al. 2018).

Photosynthetic pigments

N limitation decreased the chlorophyll content of *P. salinarum* (Fig. 3 a, b). Chlorophyll *a* content was highest in the control from the 12th day and reached 2.73 μg mL^{-1} on the 20th day. However, a sharp drop of 91.2% in chlorophyll *a* content was evidenced under the 0.125 N treatment by the end of the culture period. High chlorophyll *b* content was obtained from the 16th day to the 20th day (0.92 μg mL^{-1}) in the control culture. A high decrease of chlorophyll *b* (84%) was observed under nitrogen limitation (0.125 N treatment). However, *P. salinarum* N limitation was related positively with the carotenoid content. The highest carotenoid content started on the 12th day with a gradual increase up to 3.35 μg mL^{-1} on the 20th day in 0.125 N treatment, which is seven times higher than algal growth in the control culture (Fig. 3 c).

The photosynthetic pigments of the 0.5 and 0.25 N treatments showed intermediate values between 1 and 0.125 N treatments throughout the culture period. Thus, the chlorophyll content was related positively to the nitrogen levels tested, but carotenoid accumulation was related negatively to the nitrogen levels. These results can be seen in the culture flask coloration with greenish pigmentation at the beginning, later with time the cultures with normal nitrogen supply had an intense green color; however, those with the lowest nitrogen supply changed to yellowish.

Similar results have been recorded in *Dunaliella salina*, with the chlorophyll content decreasing from 27.90 to 10.20 μg mL^{-1} when the nitrogen concentration was reduced in half. Conversely, the carotenoid content increased from 99.43 to 177.10 μg mL^{-1} (Sathasivam et al. 2018). Chlorophylls decrease and carotenoids increase in cultures

Fig. 1 Effect of nitrogen limitation on the cell density of *P. salinarum*. Data are mean ± SD ($n = 3$). Values with the different letters represent significant difference ($p < 0.05$) between treatments

Fig. 3 Effect of nitrogen limitation on the photosynthetic pigment content of *P. salinarum*. Data are mean ± SD (*n* = 3). Values with the different letters represent significant difference (*p* < 0.05) between treatments

under N stress conditions also have been reported for the green freshwater microalgae *Chlamydomonas reinhardtii* (Cakmak et al. 2012) and *Dunaliella tertiolecta* (Young and Beardall 2003).

Therefore, there was a progressive loss of certain plastid functions, with impact in photosynthetic pigments such as the decrease in chlorophyll synthesis and an increase in carotenoids with the limiting nitrogen nutrient. This occurrence is

related to the reorganization of the photosynthetic apparatus to maximize the efficiency of absorption of specific spectra of light under situations of nutritional stress (Young and Beardall 2003). Chlorophyll is a nitrogen-rich compound utilized as an intracellular nitrogen pool to support cell growth. Then, chlorophyll concentration would also account the high values of cell density and biomass production in the control culture (75 mg L^{-1}) and 0.5 N treatment (37.5 mg L^{-1}).

Biochemical composition

Differential responses in total protein, carbohydrate, and lipid contents of *P. salinarum* under N limitation culture treatments are shown in Table 1. Nitrogen is an essential element for amino acid synthesis; its deficiency dramatically reduces protein biosynthesis, triggers inhibition of the citric acid cycle, and results in a drastic cell division decrease due to protein reduction in the photosystem reaction center and photosynthetic electron transport (Deng et al. 2011; Msanne et al. 2012).

The total protein content of *P. salinarum* under 1 and 0.5 N was 13.73 ± 0.25 mg g^{-1}, and under 0.25 and 0.125 N treatments showed a remarkable decrease of 7.81% DW and 1.8 % DW, respectively (Table 1). Cobos et al. (2017) reported a similar decrease in protein content under N depletion for freshwater green microalgae: *Acutodesmus obliquus* from 12.8 to 9.7% DW, *Ankistrodesmus* sp. from 14.5 to 10.5% DW, and *Chlorella lewinii* from 31.2 to 14.2% DW.

Under N depletion or N limitation, alternative metabolic pathways for fixed inorganic carbon such as the synthesis of carbohydrates or lipids in microalgae are activated (Deng et al. 2011; Msanne et al. 2012; Pancha et al. 2014). The carbohydrate production is mainly related to the cell wall structural components and nutritional reserves (Markou et al. 2012). Our work demonstrated that carbohydrate content was the main biochemical fraction for cultures with high nitrogen concentrations: 1 N and 0.5 N with 43.35 ± 0.21% DW. However, low carbohydrate contents were obtained in *P. salinarum* grew under 0.25 and 0.125 N treatments with 34.48% and 30.98% DW, respectively. Therefore, under N extreme stress conditions of 9.375 mg L^{-1} NaNO$_3$ (0.125 N), carbohydrate content decreased and became the main second biomolecule followed by the lipid content (Table 1).

Similar results of the effect of N limitation on carbohydrate accumulation were reported for *Chlorella vulgaris* with 41% DW (Dragone et al. 2011) and *Tetraselmis subcordiformis* with 35% DW (Yao et al. 2012). However, this increase can be substantial in some species, *C. reinhardtii* registers a carbohydrate accumulation of 80% DW (Siaut et al. 2011).

The lipids have a main role in cell membrane structural composition. Nevertheless, under nutritional limitation, due to their hydrophobic nature, lipids are derived as a storage product. They present very low states and are efficiently

Table 1 Effect of nitrogen limitation on biochemical composition (total protein, carbohydrate, and lipid content) of *P. salinarum*

Treatment	Total protein		Total carbohydrate		Total lipid	
	(mg g^{-1})	(% DW)	(mg g^{-1})	(% DW)	(mg g^{-1})	(% DW)
1 N	134.75 ± 9.92[a]	13.48	431.42 ± 11.34[a]	43.14	212.33 ± 5.57[c]	21.23
0.5 N	139.75 ± 5.42[a]	13.98	435.58 ± 9.38[a]	43.56	218.67 ± 4.04[c]	21.87
0.25 N	78.08 ± 5.05[b]	7.81	344.75 ± 7.5[b]	34.48	255.33 ± 4.16[b]	25.53
0.125 N	18.92 ± 4.73[c]	1.89	309.75 ± 11.46[c]	30.98	338.67 ± 7.21[a]	33.87

Data are expressed as mean ± SD (*n* = 3). Values with the different letters represent significant difference (*p* < 0.05) between treatments. *DW* dry weight

packaged in the cell and can be metabolized under adverse conditions for survival and subsequent cell proliferation (Courchesne et al. 2009). The increase in total lipid content could be explained by a boost in transcript levels of genes encoding enzymes of the lipid biosynthesis pathways, specifically in the last step in the Kennedy pathway of triacylglycerol biosynthesis (Deng et al. 2011; Weiss et al. 1960).

In the present study, the lipid content of *P. salinarum* under 1 N and 0.5 N treatment was 21.55 ± 0.32% DW and the N limitation under 0.25 N and 0.125 N treatments caused an increase in the lipid content of 25.53 and 33.87% DW, respectively. In this last treatment, the high lipid content represented the main biomolecule. The lipid content increases in variable ways in other algae species. For example, in *Nannochloropsis oceanica*, it almost doubles from 7.9 to 15.31% DW, and in *C. vulgaris*, from 5.9 to 16.41% DW (Converti et al. 2009). Other species of *Chlorella*, *C. emersonii* and *C. minutissima*, showed a high increase of lipids in the order of 63% and 56% DW, respectively (Illman et al. 2000). However, in *C. lewinii*, there was an increase from 9.5 to 13.2% DW, in *Acutodesmus obliquus*, from 15.2 to 18.8% DW, and in *Ankistrodesmus* sp., from 23.7 to 39.5% DW (Cobos et al. 2017).

Cell ultrastructure

The results previously described suggest that, depending on the N concentration supplied and the type of species, microalgae synthesize certain biomolecules to acclimate to the nutritional deficit and continue with their development. In this context, the biomolecules produced are mainly carbohydrates and lipids. They are storage in reserve subcellular structures and accumulated at expense of reduced growth rate (Siaut et al. 2011; Msanne et al. 2012).

In addition, electron micrographs of *P. salinarum* vegetative cells under different N concentrations showed cell structural changes (Fig. 4). Under normal conditions, longitudinal sections of cells showed oval shape with a typical chloroplast occupying most of the cell volume (Fig. 4 a). This observation was similar to other published TEM images of *P. salinarum* (Lopes Dos Santos et al. 2017; Glabonjat et al. 2020). Under

nitrogen depletion treatments, *P. salinarum* accumulated organic material reserve as starch grains and lipid droplets (LD) (oil body or oleosome) in variable numbers and sizes for each treatment (Fig. 4 b, c, d), which are not observed in previous TEM reports for this species.

It has been proposed that the lipids are synthesized and packaged initially in the plastid and then transported to the cytoplasm, where they form the LDs (Eltgroth et al. 2005). These structures are the main storage structure for neutral lipids in eukaryotic cells and support evidence they are involve in other cellular processes such as lipid homeostasis and communication signaling between other organelles. The LD synthesis in response to specific cellular needs and their number per cell change according to the nutritional status (Goold et al. 2015). Also, under N limitation or N depletion conditions, both the number and size of the LDs can increase and the chloroplast became imperceptible, because the LDs act as a sink for membrane-derived fatty acids, including plastid membrane lipids that are degraded (Siaut et al. 2011; Roopnarain et al. 2014; Goold et al. 2015).

The biochemical composition of *P. salinarum* was in agreement with our TEM results. In 0.5 N treatment, several dispersed starch grains were observed compared to LDs, and the chloroplast was hardly visible (Fig. 4 b). However, under 0.25 N treatment, the development of large LDs was obvious and starch grains decreased (Fig. 4 c). Furthermore, in the 0.125 N treatment, a dominant single LD occupied most of the cell volume as well as several small ones around it (Fig. 4 d).

Under normal growth conditions, *C. reinhardtii* has a single cup-shaped plastid that occupies more than two-thirds of the total cell volume, and in some strains, neither starch grains nor lipid droplets were detected. The appearance and accumulation of these reserve structures, as well as the reduction of plastid organelle, were common in cells under N depletion (Siaut et al. 2011). Zhu et al. (2014) observed in *Chlorella zofingiensis* an increase in starch grains both in size and number after the first days under N stress, with a few LDs. Through the coming days, the cells exhibited more LDs instead of starch granules. Then, small LD fusion formed larger

Fig. 4 Cell ultrastructure of *P. salinarum* under nitrogen limitation. Treatments: 1 N (a), 0.5 N (b), 0.25 N (c), 0.125 N (d). Symbols: chloroplast (c), lipid droplet (L), small lipid droplets (arrows), starch grain (*)
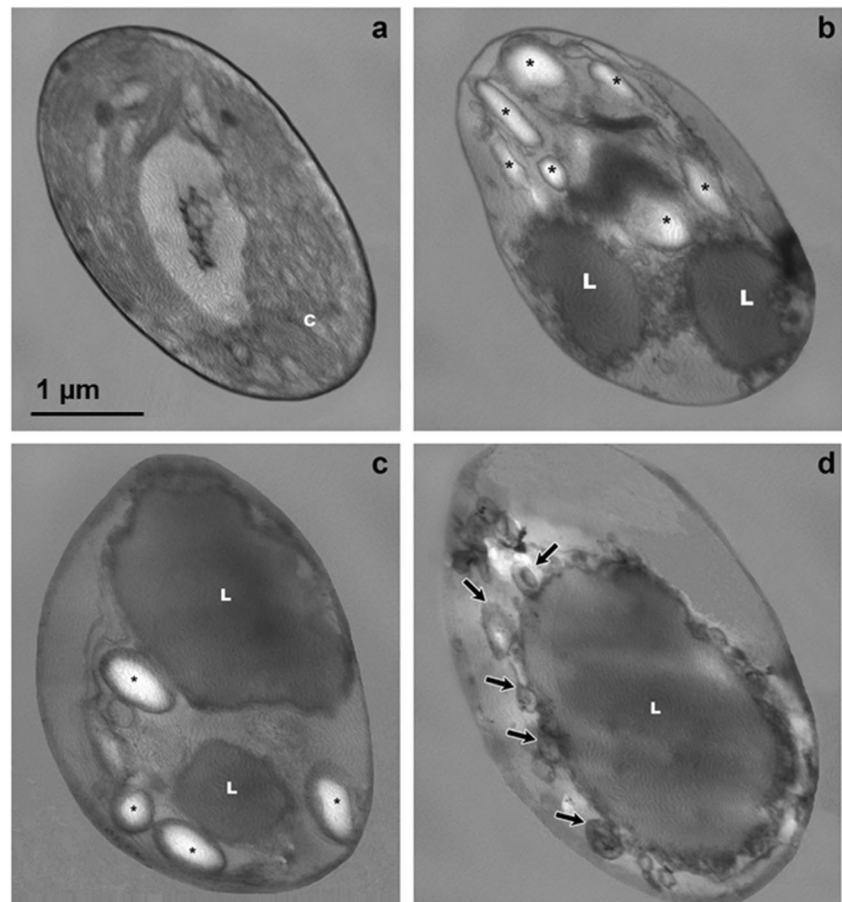
ones. Other studies have reported that the starch granules can fuse and be converted into LDs, suggesting that the carbon flux of starch must provide some of the precursors for lipid synthesis (Ito et al. 2013; Mizuno et al. 2013).

These findings are in agreement with our results and suggest that the presence of a single large LD in *P. salinarum* cells under the high N limitation tested (0.125 N treatment) was a storage lipid.

Profile of fatty acid methyl esters

The FAME analysis with the profile of saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) of *P. salinarum* under N limitation is presented in Table 2. Among SFAs, the lauric acid (C12:0) content proportionally reduced as the N decreased and was not detected in the 0.125 N cultures. In contrast, palmitic acid (C16:0) and myristic acid (C14:0) contents increased. Under the highest N limitation (0.125 N), C16:0 was the most abundant fatty acid with 923.95 µg g^{-1} DW. Regarding to the UFAs, oleic acid (C18:1) content also decreased in response to the lowest N supply in cultures. However, linoleic acid (C18: 2) and linolenic acid (C18: 3) contents remained constant or were not detected in all

treatments under limitation of N, representing the lowest values of fatty acids.

Several authors have also reported nitrogen limitation as good strategy for stimulating the C16:0 production between the SFAs, as reported for the green microalgae *C. vulgaris*,

Table 2 Composition of fatty acid methyl esters (µg g^{-1} DW) of *P. salinarum* under nitrogen limitation

Fatty acid	Treatment			
	1 N	0.5 N	0.25 N	0.125 N
C12:0	274.01[a]	157.83[b]	53.53[c]	-
C14:0	59.15[b]	28.99[c]	79.65[a]	72.13[a]
C16:0	59.24[d]	300.49[c]	721.96[b]	923.95[a]
C18:1	111.32[b]	96.38[b]	254.24[a]	0.61[c]
C18:2	16.99[a]	-	18.07[a]	19.87[a]
C18:3	0.92[a]	-	1.64[a]	-
SFA	392.40[c]	487.31[b]	855.15[a]	996.08[a]
UFA	129.23[b]	96.38[b]	273.95[a]	20.48[c]

Data are expressed as mean ($n = 3$). Values with the different letters represent significant difference ($p < 0.05$) between treatments. *SFA*, saturated fatty acid; *UFA*, unsaturated fatty acid; (-), not detected

Fig. 5 Principal component analysis of the growth measurements and biochemical composition of *P. salinarum* under nitrogen limitation

Biplot (F1 and F2: 89.45%)
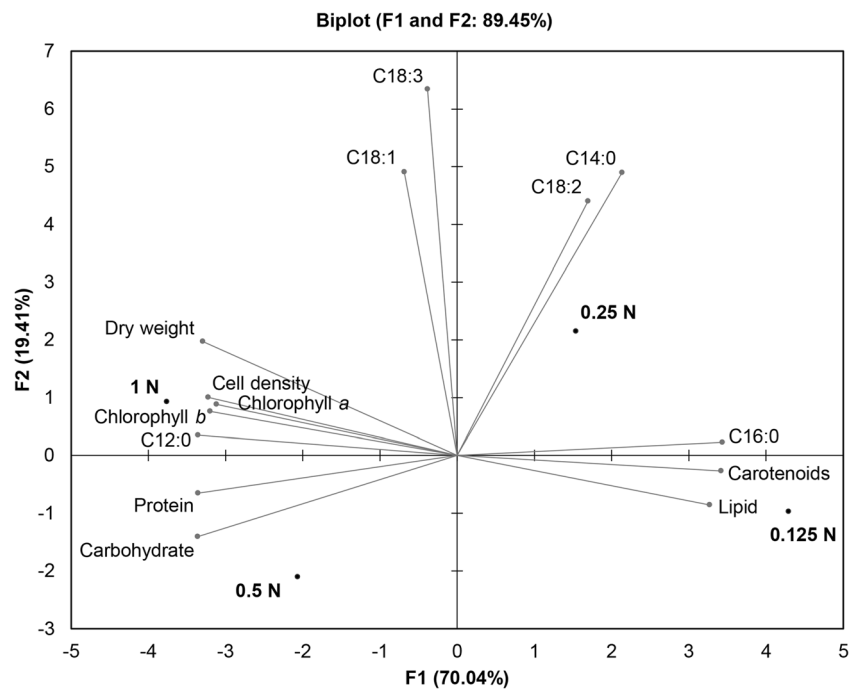
Nannochloropsis oculata, C. reinhardtii, D. salina, and D. tertiolecta (Converti et al. 2009; Chen et al. 2011; Msanne et al. 2012; Lv et al. 2016). Furthermore, Anand and Arumugam (2015) reported that the main fatty acid detected (C18:1) in *S. quadricauda* was drastically reduced under N limitation. On the other hand, this fatty acid was not detected in *D. salina* (Lv et al. 2016). In our study, the minimum values of UFAs mainly C18:2 and C18:3 due to N limitation were similar those recorded for *Coccomyxa* sp. in similar stress condition (Msanne et al. 2012). Therefore, it is confirmed that microalgae cells tend to decrease the degree of fatty acid unsaturation in response to the N deficit.

The FAME profile plays an important role in biodiesel quality. It determines its viscosity, lubricity, total unsaturation (iodine value), density, oxidative stability, cetane index (ignition quality indicator), cold flow property, and calorific value (Knothe 2005, 2013; Francisco et al. 2010). The FAMEs of the microalgae are different from those of higher plants, the latter being especially rich in polyunsaturated fatty acids (PUFAs) such as C18:2 and C18:3. These have four or more double bonds, being more susceptible to oxidation during storage which reduce its acceptability for use in biodiesel use. Besides, as the PUFA concentration increases, the biodiesel nitrate and nitrite emission rate increases (Chisti 2007; Francisco et al. 2010; Chen et al. 2018).

On the other hand, when the SFAs are high, they result in a lower cetane index and increase the biodiesel stability since the SFAs are more resistant to auto-oxidation (Knothe 2005). International and regional guidelines for biofuel like the requirements of the European Norms EN 14213 and EN 14214 require that the C18:3 amount must have a limit lower than 12% of the total FAMEs for motor vehicles use (Knothe 2006). Thus, biomass highly rich in oils with high levels of saturated fatty acids is sought and meets local criteria for use and biofuel production.

The PCA provides an overview of the N limitation effects on *P. salinarum* (Fig. 5). Its biochemical composition under control conditions (1 N) had the expected results in green microalgae, and high growth (cell density and dry mass) was related to high contents of chlorophyll *a* and *b*, proteins, and carbohydrates. The same patterns were also followed with the 0.5 N treatment. Furthermore, the degree of fatty acid saturation was recognized under the 0.25 and 0.125 N treatments, mainly with the last treatment related to the lipid content, carotenoids, and C16:0. The *P. salinarum* biomass under 0.125 N treatment showed a high total lipid yield with and adequate fatty acid composition with high SFA content (C:16) and a low C18:3 (PUFA) meeting certain European Norms requirements. Therefore, it showed to be a suitable potential raw material source for the biodiesel production.

Conclusions

The findings of this study suggest that *P. salinarum* biomass is a potential source of lipid raw material suitable for the production of biodiesel, which could contribute to sustainable development as a viable alternative to petroleum. However, it will be necessary to develop cultivation systems for biomass

production on a large scale for biofuel production and additional bioactive compounds (carbohydrates and carotenoids) useful in other applications.

Declarations

Competing interests The authors declare no competing interests.

References

Anand J, Arumugam M (2015) Enhanced lipid accumulation and biomass yield of *Scenedesmus quadricauda* under nitrogen starved condition. Bioresour Technol 188:190–194

Benavente-Valdés JR, Aguilar C, Contreras-Esquivel JC, Méndez-Zavala A, Montañez J (2016) Strategies to enhance the production of photosynthetic pigments and lipids in chlorophycae species. Biotechnol Rep 10:117–125

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Cakmak T, Angun P, Demiray YE, Ozkan AD, Elibol Z, Tekinay T (2012) Differential effects of nitrogen and sulfur deprivation on growth and biodiesel feedstock production of *Chlamydomonas reinhardtii*. Biotechnol Bioeng 109:1947–1957

Chen M, Tang H, Ma H, Holland TC, Ng KYS, Salley SO (2011) Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresour Technol 102:1649–1655

Chen J, Li J, Dong W, Zhang X, Tyagi RD, Drogui P, Surampalli RY (2018) The potential of microalgae in biodiesel production. Renew Sust Energ Rev 90:336–346

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306

Cobos M, Paredes JD, Maddox JD, Vargas-Arana G, Flores L, Aguilar CP, Marapara JL, Castro JC (2017) Isolation and characterization of native microalgae from the Peruvian Amazon with potential for biodiesel production. Energies 10:1–16

Converti A, Casazza AA, Ortiz EY, Perego P, Del Borghi M (2009) Effect of temperature and nitrogen concentration on the growth

and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process Process Intensif 48: 1146–1151

Courchesne NMD, Parisien A, Wang B, Lan CQ (2009) Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. J Biotechnol 141:31–41

Demirbas A, Demirbas MF (2011) Importance of algae oil as a source of biodiesel. Energy Convers Manag 52:163–170

Deng X, Fei X, Li Y (2011) The effects of nutritional restriction on neutral lipid accumulation in *Chlamydomonas* and *Chlorella*. Afr J Microbiol Res 5:260–270

Dragone G, Fernandes BD, Abreu AP, Vicente AA, Teixeira JA (2011) Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl Energy 88:3331–3335

Eltgroth ML, Watwood RL, Wolfe GV (2005) Production and cellular localization of neutral long-chain lipids in the haptophyte algae *Isochrysis galbana* and *Emiliania huxleyi*. J Phycol 41:1000–1009

Francisco ÉC, Neves DB, Jacob-Lopes E, Franco TT (2010) Microalgae as feedstock for biodiesel production: carbon dioxide sequestration, lipid production and biofuel quality. J Chem Technol Biotechnol 85: 395–403

Glabonjat RA, Blum JS, Miller LG, Webb SM, Stolz JF, Francesconi KA, Oremland RS (2020) Arsenolipids in cultured *Picocystis* strain ML and their occurrence in biota and sediment from Mono Lake, California. Life 10:1–21

Goold H, Beisson F, Peltier G, Li-Beisson Y (2015) Microalgal lipid droplets: composition, diversity, biogenesis and functions. Plant Cell Rep 34:545–555

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, NY, pp 29–60

Illman AM, Scragg AH, Shales SW (2000) Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzym Microb Technol 27:631–635

Ito T, Tanaka M, Shinkawa H, Nakada T, Ano Y, Kurano N, Soga T, Tomita M (2013) Metabolic and morphological changes of an oil accumulating trebouxiophycean alga in nitrogen-deficient conditions. Metabolomics 9:178–187

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem Physiol Pflanz 167:191–194

Juneja A, Ceballos RM, Murthy GS (2013) Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review. Energies 6:4607–4638

Knothe G (2005) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86:1059–1070

Knothe G (2006) Analyzing biodiesel: standards and other methods. J Am Oil Chem Soc 83:823–833

Knothe G (2013) Production and properties of biodiesel from algal oils. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Dordrecht, pp 207–221

Kochert G (1978) Carbohydrate determination by the phenol sulfuric acid method. In: Hellebust J, Craigie J (eds) Handbook of phycological methods. Physiological and biochemical methods. Cambridge University Press, Cambridge, pp 95–97

Lopes Dos Santos A, Pollina T, Gourvil P, Corre E, Marie D, Garrido JL, Rodríguez F, Noël MH, Vaulot D, Eikrem W (2017) Chloropicophyceae, a new class of picophytoplanktonic prasinophytes. Sci Rep 7:1–20

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lv H, Cui X, Wang S, Jia S (2016) Metabolic profiling of *Dunaliella salina* shifting cultivation conditions to nitrogen deprivation. Metab Open Acess 6:2153

Malavasi V, Soru S, Cao G (2020) Extremophile microalgae: the potential for biotechnological application. J Phycol 56:559–573

Markou G, Angelidaki I, Georgakakis D (2012) Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Appl Microbiol Biotechnol 96:631–645

Menezes RS, Leles MIG, Soares AT, Brandão PI, Franco M, Filho NRA, Sant'anna CL, Vieira AAH (2013) Avaliação da potencialidade de microalgas dulcícolas como fonte de matéria-prima graxa para a produção de biodiesel. Quim Nova 36:10–15

Mizuno Y, Sato A, Watanabe K, Hirata A, Takeshita T, Ota S, Sato N, Zachleder V, Tsuzuki M, Kawano S (2013) Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. Bioresour Technol 129:150–155

Msanne J, Xu D, Konda AR, Casas-Mollano JA, Awada T, Cahoon EB, Cerutti H (2012) Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa* sp. C-169. Phytochemistry 75:50–59

Ördög V, Stirk WA, Bálint P, van Staden J, Lovász C (2012) Changes in lipid, protein and pigment concentrations in nitrogen-stressed *Chlorella minutissima* cultures. J Appl Phycol 24:907–914

Pancha I, Chokshi K, George B, Ghosh T, Paliwal C, Maurya R, Mishra S (2014) Nitrogen stress triggered biochemical and morphological changes in the microalgae *Scenedesmus* sp. CCNM 1077. Bioresour Technol 156:146–154

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

Roopnarain A, Gray VM, Sym S (2014) Influence of nitrogen stress on *Isochrysis galbana* strain U4, a candidate for biodiesel production. Phycol Res 62:237–249

San Pedro A, González-López CV, Acién FG, Molina-Grima E (2013) Marine microalgae selection and culture conditions optimization for biodiesel production. Bioresour Technol 134:353–361

Sathasivam R, Pongpadung P, Praiboon J, Chirapart A, Trakulnaleamsai S, Roytrakul S, Juntawong N (2018) Optimizing NaCl and KNO₃ concentrations for high β-carotene production in photobioreactor by *Dunaliella salina* KU11 isolated from saline soil sample. Chiang Mai J Sci 45:106–115

Siaut M, Cuiné S, Cagnon C, Fessler B, Nguyen M, Carrier P, Beyly A, Beisson F, Triantaphylidès C, Li-Beisson Y, Peltier G (2011) Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnol 11:7

Souza LS, Simioni C, Bouzon ZL, Schneider RCS, Gressler P, Miotto MC, Rossi MJ, Rörig LR (2017) Morphological and ultrastructural characterization of the acidophilic and lipid-producer strain *Chlamydomonas acidophila* LAFIC-004 (Chlorophyta) under different culture conditions. Protoplasma 254:1385–1398

Strickland JDH, Parsons TR (1972) A practical handbook of seawater analyses. Fisheries Research Board of Canada, Ottawa

Tan XB, Lam MK, Uemura Y, Lim JW, Wong CY, Lee KT (2018) Cultivation of microalgae for biodiesel production: a review on upstream and downstream processing. Chin J Chem Eng 26:17–30

Tandon P, Jin Q (2017) Microalgae culture enhancement through key microbial approaches. Renew Sust Energ Rev 80:1089–1099

Tarazona-Delgado R, Terreros HM, Astocondor MM, Huatuco MM (2017) *Picocystis salinarum* (Prasinophyceae, Chlorophyta) en las Salinas de Chilca, Lima, primer registro para el Perú. Arnaldoa 24: 557–566

Wang S, Lambert W, Giang S, Goericke R, Palenik B (2014) Microalgal assemblages in a poikilohaline pond. J Phycol 50:303–309

Weiss SB, Kennedy EP, Kiyasu JY (1960) The enzymatic synthesis of triglycerides. J Biol Chem 235:40–44

Yao C, Ai J, Cao X, Xue S, Zhang W (2012) Enhancing starch production of a marine green microalga *Tetraselmis subcordiformis* through nutrient limitation. Bioresour Technol 118:438–444

Young EB, Beardall J (2003) Photosynthetic function in *Dunaliella tertiolecta* (Chlorophyta) during a nitrogen starvation and recovery cycle. J Phycol 39:897–905

Zhu S, Huang W, Xu J, Wang Z, Xu J, Yuan Z (2014) Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalga *Chlorella zofingiensis*. Bioresour Technol 152:292–298