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Biochemical composition of green microalgae *Pseudoneochloris marina* grown under different temperature and light conditions

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ABSTRACT

Environmental factors such as temperature, light intensity, and nutrient availability, are central in microalgae metabolism, affecting their growth and composition. This study evaluated the effects of nitrogen concentration (37.1–78.8 mg L^{-1} N-NO_3), temperature (20–36 °C) and light intensity (140–364 μmol m^{-2} s^{-1}) on biomass productivity and biochemical composition of *Pseudoneochloris marina*. The highest biomass productivity (0.26 g L^{-1} d^{-1}) was achieved with 74.1 mg L^{-1} N-NO_3 at 28 °C and 252–364 μmol m^{-2} s^{-1}. Protein content decreased with the increment of factors levels and the highest content was 236 ± 18 mg g^{-1} at 20 °C and 140 μmol m^{-2} s^{-1}. The carotenoids and lipids were affected negatively by light intensity. The highest xanthophylls and carotenes contents were, respectively, 2.55 ± 0.39 mg g^{-1} and 1.25 ± 0.01 mg g^{-1} at 20 °C/28 °C and 140 μmol m^{-2} s^{-1}. The major carotenoids were all-*trans*-lutein (36.0 ± 2.9%) and all-*trans*-β-carotene (13.2 ± 1.2% of total carotenoids). The saturated, monounsaturated and polyunsaturated fatty acids represented 46.1 ± 1.5%, 10.6 ± 1.7% and 43.3 ± 1.0% of total fatty acids. The main fatty acids were C16:0, C18:2n-6, C18:3n-3 and C18:1. The lowest ω6:ω3 ratio was 1.6 ± 0.1, at the lowest temperature.

1. Introduction

Microalgae are a source of a wide range of natural products including carbohydrates, proteins, lipids and high-value products, such as carotenoids and polyunsaturated fatty acids. Microalgal biomass is able to accumulate significant amounts of these compounds and can be used in pharmaceutical products, food additives, feed supplements or in the production of biofuels (i.e. biodiesel, bioethanol, biohydrogen or methane) (Anjos et al., 2013; Benavente-Valdés et al., 2016; D'Alessandro and Antoniosi Filho, 2016).

Interest in green algae has been growing. However, it is estimated that more than 5000 species have not yet been described (Hadi et al., 2016). Due to the complexity of identifying single-celled green algae and ancient taxonomic identification conflicts, this group is often undergoing new taxonomic revisions (Darienko et al., 2010; Krienitz et al., 2015, 2004; Neustupa et al., 2009; Pegg et al., 2015; Somogyi et al., 2013). Currently, DNA barcoding techniques that identify species based on DNA sequence similarity comparing with the database is the most used method to differentiate species with similar morphology (Hadi et al., 2016).

Through the photosynthesis, CO_2 from the atmosphere is converted into biomass by autotrophic microorganisms when associated with nutrient supply and under adequate growth conditions. Microalgae are known to have a higher growth rate and a higher capacity for CO_2 biofixation when compared to terrestrial plants (Zeng et al., 2011).

The photosynthesis process is affected by nutrient availability and environmental parameters of cultivation. Nitrogen is an essential nutrient for the metabolic activities of microalgae cells. Nitrogen limitation decreases cell growth, protein synthesis, and increases reserve compounds, like carbohydrates and lipids (Pancha et al., 2014). Temperature and light intensity are known as the most critical extrinsic factors in photoautotrophic cultures, affecting microalgae metabolism, cell growth rate and biomass composition (Ho et al., 2014a; Thawechai et al., 2016). Also, the inorganic carbon acquisition depends directly on light intensity and indirectly on temperature, which modulates the enzymatic activity. The simultaneous impact of these two environmental factors are worth to be studied to better understand the daily pattern of carbon acquisition and storage (Bonnefond et al., 2016; Minhas et al., 2016).

Considering the interest of the biotechnological industry to explore alternative sources for extraction of high-value compounds, the investigation of untraditional microalgae species and different culture conditions are essential steps in the development of strategies to large-scale microalgae production. The green microalgae *Pseudoneochloris marina* (Chlorophyta) was identified by Watanabe et al. (2000) and belongs to the class of Ulvophyceae. To the best of our knowledge, few

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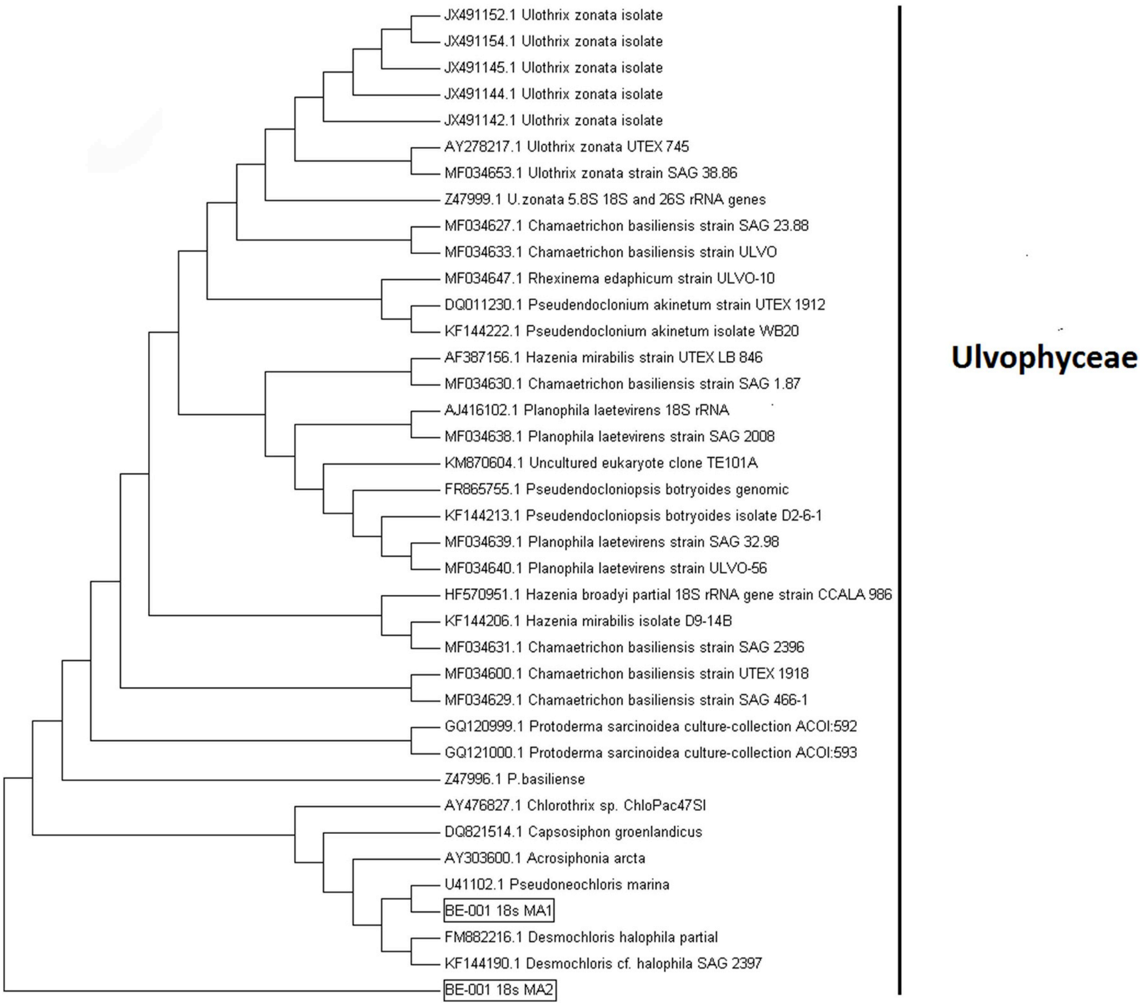

Fig. 1. Phylogenetic tree showing the relationships among sequences de 18S rDNA – BE 001 18sMA1, BE 001 18sMA2 and the most similar sequences retrieved from databases.

Table 1
Biomass concentration of *P. marina* grown under different nitrogen conditions.

N-NO_3 (mg L^{-1})	Biomass (g L^{-1})
37.1	1.33 ± 0.09^{b}
49.4	1.47 ± 0.21^{b}
61.8	1.46 ± 0.09^{b}
68.4	1.58 ± 0.04^{ab}
74.1	2.02 ± 0.21^{a}
78.2	1.76 ± 0.01^{ab}

Different letter in the same column indicates significant difference by Tukey test at 5% significance level.

studies report *P. marina,* none of them studying culture parameters on cell growth and biochemical composition of its biomass. Thereby, this study evaluated the effects of nitrogen, temperature and light intensity in *P. marina* cultures on biomass productivity and composition (carbohydrates, proteins, carotenoids, lipids). Additionally, the carotenoid and fatty acids profiles were determined for each culture condition.

2. Material and methods

2.1. Analysis of phylogenetic identification

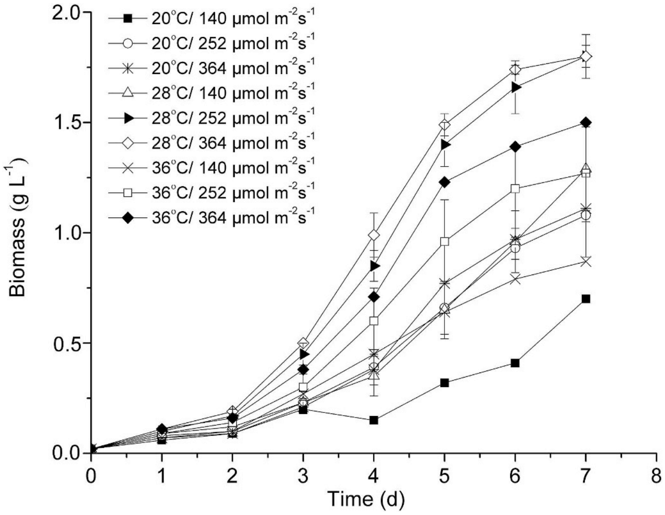

Fig. 2. Kinetics of *P. marina* growth under different conditions of temperature and light intensity.

In the present study, we investigated the eukaryotic microalgal strain BE-001 isolated originally by the Department of Marine Biology, Fluminense Federal University (Niteroi, RJ, Brazil). The cultures were maintained in a growth chamber at 22 °C under a photoperiod of 12/12 h (light/dark) in 250 mL Erlenmeyer flasks containing 100 mL of

Table 2
Regression coefficients of the coded variables for biomass productivity, protein, carbohydrate, total carotenoid and lipid content in *P. marina* biomass cultured under different temperatures (X_1, 20–36 °C) and light intensities (X_2, 140–364 μmol m^{-2} s^{-1}).

Regression coefficients	Biomass Productivity (g L^{-1} d^{-1})		Protein (mg g^{-1})		Carbohydrate (mg g^{-1})		Carotenoid (mg g^{-1})		Lipid (mg g^{-1})	
	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value
β_0	0.249	< 0.001	108.8	< 0.001	439.3	< 0.001	2.836	< 0.001	108.038	< 0.001
β_1	0.017	0.05	−22.56	0.010	–		–	–	–	–
β_2	0.370	< 0.001	−28.17	< 0.001	42.9	0.002	−0.492	< 0.001	−17.513	< 0.001
β_{11}	−0.080	< 0.001	–	–	47.6	0.010	–	–	–	–
β_{22}	−0.027	0.028	28.89	0.013	–		0.245	0.048	6.075	0.048
β_{12}	–		23.93	0.010	–		–	–	–	
Blocks	–		−11.35	0.013	–		−0.428	< 0.001	–	
R^2	0.893		0.924		0.651		0.896		0.865	
Regression *p*-value	2.8×10^{-5}		2.8×10^{-5}		0.001		3.5×10^{-6}		2.2×10^{-6}	
Lack of fit *p*-value	0.910		0.752		0.386		0.046		0.059	

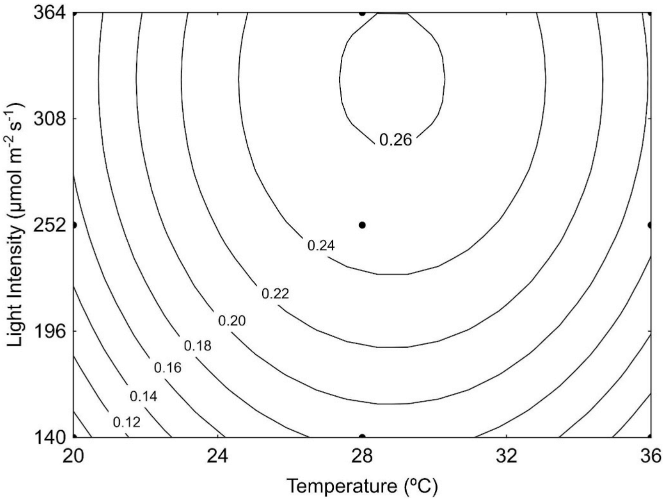

Fig. 3. Effects of temperature and light intensity on biomass productivity (g L^{-1} d^{-1}) of *P. marina* grown in batch culture.

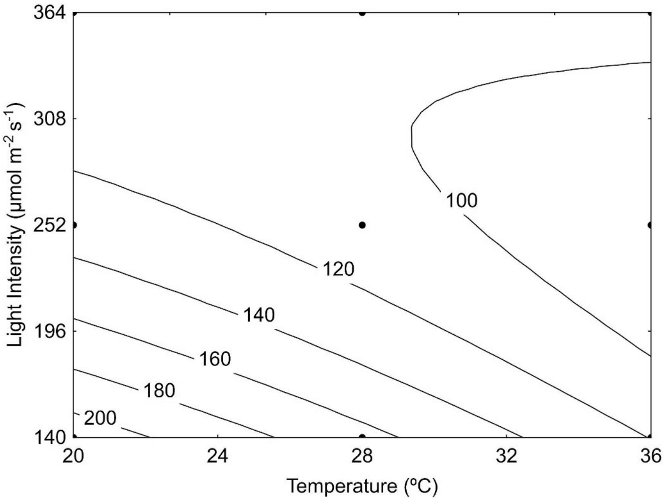

Fig. 4. Effects of temperature and light intensity on protein content (mg g^{-1}) of *P. marina* grown in batch culture.

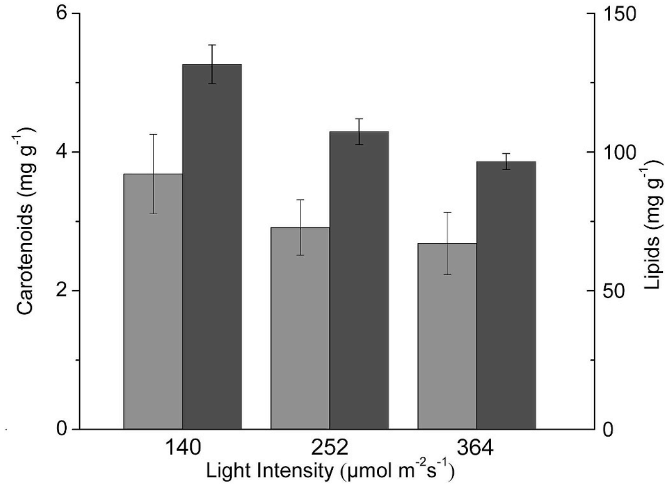

Fig. 5. Carotenoids (▨) and lipids (■) content in *P. marina* biomass cultured under different light intensities. Error bars represent the standard deviation of each culture condition.

culture medium f/2 (Guillard, 1975) with modified $NaNO_3$ concentration (300 mg L^{-1}) and an additional amount of NaCl (17 g L^{-1}) to induce saline stress.

The DNA analysis was performed by Macrogen Inc. (Seoul, Korea). Genomic DNA samples were extracted using an MG Plant Genomic DNA extraction SV kit (Doctor protein INC, Korea). The 18S rDNA region was subjected to PCR amplification using the primers MA1 (5′ GTAGTCAT ATGCTTGTCTC 3′) and MA2 (5′ CTTCTGCAGGTTCACC 3′). The PCR reaction was performed for both markers with initial denaturation at 96 °C for 5 min, 40 cycles of denaturation (96 °C for 1 min) and annealing (72 °C for 1 min), with a final extension at 72 °C for 5 min (Hadi et al., 2016). The purified PCR products were then Sanger-sequenced with the BigDye Terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were determined on both strands of PCR amplification products. The resulted 18S rDNA sequences were compared to sequences deposited in the GenBank nucleotide sequence database using the Basic Local Alignment Search Tool (BLAST) (Kent, 2002). Sequences obtained from GenBank were manually added using the Molecular Evolutionary Genetics Analysis (MEGA7) software (Kumar et al., 2016) and the alignments were performed automatically using multiple sequence alignment ClustalW (Thompson et al., 1994). The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993), as implemented by MEGA7 software.

2.2. Pre-cultures and cultivation

The pre-cultures were prepared in 500 mL Erlenmeyer flasks with 240 mL working volume using modified f/2 medium, as described above, and inoculated with 24 mL of stock culture. The flasks were

Table 3

Regression coefficients of the coded variables for xanthophylls, carotenes, saturated (ΣSFA), monounsaturated (ΣMUFA) and polyunsaturated (ΣPUFA) fatty acids contents in *P. marina* biomass cultured under different temperatures (X_1, 20–36 °C) and light intensities (X_2, 140–364 µmol m^{-2} s^{-1}).

Regression coefficients	Xanthophylls (mg g^{-1})		Carotenes (mg g^{-1})		ΣSFA (mg g^{-1})		ΣMUFA (mg g^{-1})		ΣPUFA (mg g^{-1})	
	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value	Coeff.	*p*-value
β_0	1.945	< 0.001	0.960	< 0.001	13.332	< 0.001	2.794	< 0.001	12.524	< 0.001
β_1	−0.210	0.010	0.118	< 0.001	−0.651	0.006	0.459	< 0.001	–	–
β_2	−0.301	< 0.001	−0.192	< 0.001	−0.884	< 0.001	−0.264	0.017	−1.223	< 0.001
β_{11}	–	–	–	–	−0.680	0.030	0.0358	0.021	−0.593	0.036
β_{22}	–	–	0.105	0.010	–	–	–	–	–	–
β_{12}	–	–	–	–	–	–	–	–	–	–
Blocks	−0.283	< 0.001	−0.145	< 0.001	–	–	–	–	–	–
R^2	0.838		0.939		0.759		0.756		0.800	
Regression *p*-value	5.0×10^{-5}		1.3×10^{-6}		0.001		0.001		2.9×10^{-5}	
Lack of fit *p*-value	0.044		0.012		0.380		0.383		0.354	

Table 4

Carotenoids composition of *P. marina* biomass grown under different conditions of temperature and light intensity.

Peak[a]	Temperature (ºC)	20	20	20	28	28	28	36	36	36
	Light intensity (µmol m^{-2} s^{-1})	140	252	364	140	252	364	140	252	364
Xanthophylls (mg g^{-1})										
1	all-*trans*-violaxanthin	0.29 ± 0.00	0.08 ± 0.00	0.04 ± 0.00	0.2 ± 0.16	0.18 ± 0.05	0.17 ± 0.03	0.22 ± 0.01	0.13 ± 0.02	0.26 ± 0.02
2	*cis*-violaxanthin	0.20 ± 0.01	0.12 ± 0.03	0.11 ± 0.00	0.16 ± 0.05	0.12 ± 0.01	0.09 ± 0.04	0.14 ± 0.02	0.10 ± 0.03	0.14 ± 0.01
3	all-*trans*-luteoxanthin	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.04 ± 0.00	0.06 ± 0.01	0.03 ± 0.00
4	13-cis-lutein	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.00	0.08 ± 0.02	0.09 ± 0.01	0.05 ± 0.01	0.10 ± 0.01	0.08 ± 0.03	0.14 ± 0.01
5	13′-cis-lutein	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
6	all-*trans*-lutein	1.38 ± 0.02	1.24 ± 0.20	0.99 ± 0.01	1.27 ± 0.28	1.02 ± 0.10	0.91 ± 0.14	0.97 ± 0.07	1.06 ± 0.11	1.09 ± 0.06
7	all-*trans*-zeaxanthin	0.52 ± 0.01	0.61 ± 0.14	0.49 ± 0.00	0.57 ± 0.08	0.41 ± 0.07	0.34 ± 0.11	0.17 ± 0.03	0.18 ± 0.02	0.22 ± 0.01
	Total	2.51 ± 0.02	2.13 ± 0.40	1.73 ± 0.00	2.34 ± 0.61	1.86 ± 0.20	1.61 ± 0.34	1.65 ± 0.11	1.64 ± 0.23	1.91 ± 0.09
Carotenes (mg g^{-1})										
8	15-cis-β-carotene	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.06 ± 0.02	0.04 ± 0.00	0.03 ± 0.00
9	all-*trans*-α-carotene	0.52 ± 0.00	0.21 ± 0.02	0.15 ± 0.01	0.30 ± 0.15	0.26 ± 0.04	0.24 ± 0.05	0.47 ± 0.00	0.37 ± 0.04	0.40 ± 0.03
10	13-cis-β-carotene	0.05 ± 0.00	0.08 ± 0.01	0.06 ± 0.00	0.11 ± 0.05	0.12 ± 0.02	0.10 ± 0.03	0.13 ± 0.01	0.11 ± 0.01	0.12 ± 0.00
11	all-*trans*-β-carotene	0.34 ± 0.00	0.38 ± 0.03	0.29 ± 0.02	0.47 ± 0.17	0.36 ± 0.05	0.32 ± 0.05	0.44 ± 0.01	0.41 ± 0.04	0.42 ± 0.03
12	9-cis-β-carotene	0.11 ± 0.00	0.15 ± 0.02	0.13 ± 0.01	0.18 ± 0.05	0.18 ± 0.03	0.16 ± 0.03	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
	Total	1.25 ± 0.01	0.85 ± 0.08	0.66 ± 0.03	1.10 ± 0.44	0.95 ± 0.15	0.84 ± 0.17	1.24 ± 0.03	1.09 ± 0.09	1.15 ± 0.03
	Total carotenoids	3.77 ± 0.03	2.99 ± 0.48	2.39 ± 0.03	3.44 ± 1.05	2.81 ± 0.35	2.45 ± 0.51	2.89 ± 0.08	2.73 ± 0.51	3.06 ± 0.12

[a] Numbered according to the chromatogram shown in Fig. S1.

incubated in a rotatory shaker (Oxylab, Oxy 304T) at 28 °C and 180 rpm with continuous illumination of 42 µmol m^{-2} s^{-1} for 7 days.

All cultures were performed in flat-panel airlift photobioreactors (Kochem et al., 2014) filled with 2.16 L of modified f/2 medium, according to the specific experiment, and 240 mL of pre-cultures. The cultures were daily supplemented with 1 mL L^{-1} of phosphate solution and 1 mL L^{-1} of trace-metals solution to avoid nutrient limitation (Chagas et al., 2015). The airflow was kept at 1 L min^{-1} of CO_2-enriched filtered air (0.22 µm Midisart®2000, Sartorius Stedim Biotech) controlled by rotameters. The mixture of CO_2 and compressed air was adjusted by rotameter to maintain the culture at pH 7.0. The pH was measured using pH-indicator strips (MColorpHast™, Merck, Germany). The light intensity was measured using a digital luximeter.

The experiments to determine optimum nitrogen concentration in the culture medium (f/2 plus 17 g L^{-1} NaCl) used different $NaNO_3$ concentrations (225–475 mg L^{-1} $NaNO_3$ equivalent to 37.1–78.2 mg L^{-1} N-NO_3), 28 °C, 252 µmol m^{-2} s^{-1} for 7 days (Da Fré et al., 2016; Menegol et al., 2017). Experiments to determine the effect of temperature (20 °C, 28 °C and 36 °C) and light intensity (140 µmol m^{-2} s^{-1}, 252 µmol m^{-2} s^{-1}, and 364 µmol m^{-2} s^{-1}) on microalgae growth and composition were performed in modified f/2 medium (addition of 17 g L^{-1} NaCl and 450 mg L^{-1} $NaNO_3$) for 7 days, according to a face-centered design split into two blocks.

Biomass concentration was measured during cultivation by optical density at 750 nm and correlated with dry cell weight (X). At the end of cultures, the entire content of the bioreactors was centrifuged (10,000 × *g*, 5 min, 4 °C), the supernatant was discarded, and the biomass was washed with distilled water and centrifuged again. The resulting biomass was lyophilized and stored at −18 °C.

2.3. Carotenoids analysis

The lyophilized biomasses (0.020 g) were used for carotenoids extraction using the method described by Mandelli et al. (2012) and adapted by Diprat et al. (2017). The exhaustive extraction consists of the previous hydration of the samples, rupture of cell walls by maceration using ethyl acetate followed by methanol. The samples were dissolved using methanol/methyl *tert*-butyl ether (MeOH:MTBE 1:1, v v^{-1}). Carotenoids were quantified by Waters HPLC 2695 series system (Wilmington, EUA) equipped with a diode array detector (Waters 2998 dual series) and using YMC-C30 column (5 µm particle size, 250 mm × 4.6 mm) (Waters, Wilmington, USA). The flow rate was 0.9 mL min^{-1} and column temperature 29 °C, eluted by linear gradient mobile phase of MeOH:MTBE from 95:5 to 70:30 in 30 min, followed by 50:50 in 20 min and maintaining this proportion for 15 min (Rodrigues et al., 2014). Analytical β-carotene curve (0.13–15 mg L^{-1}) was used for carotenoid quantification (Diprat et al., 2017). The carotenoid identification was performed according to Menegol et al. (2017) using

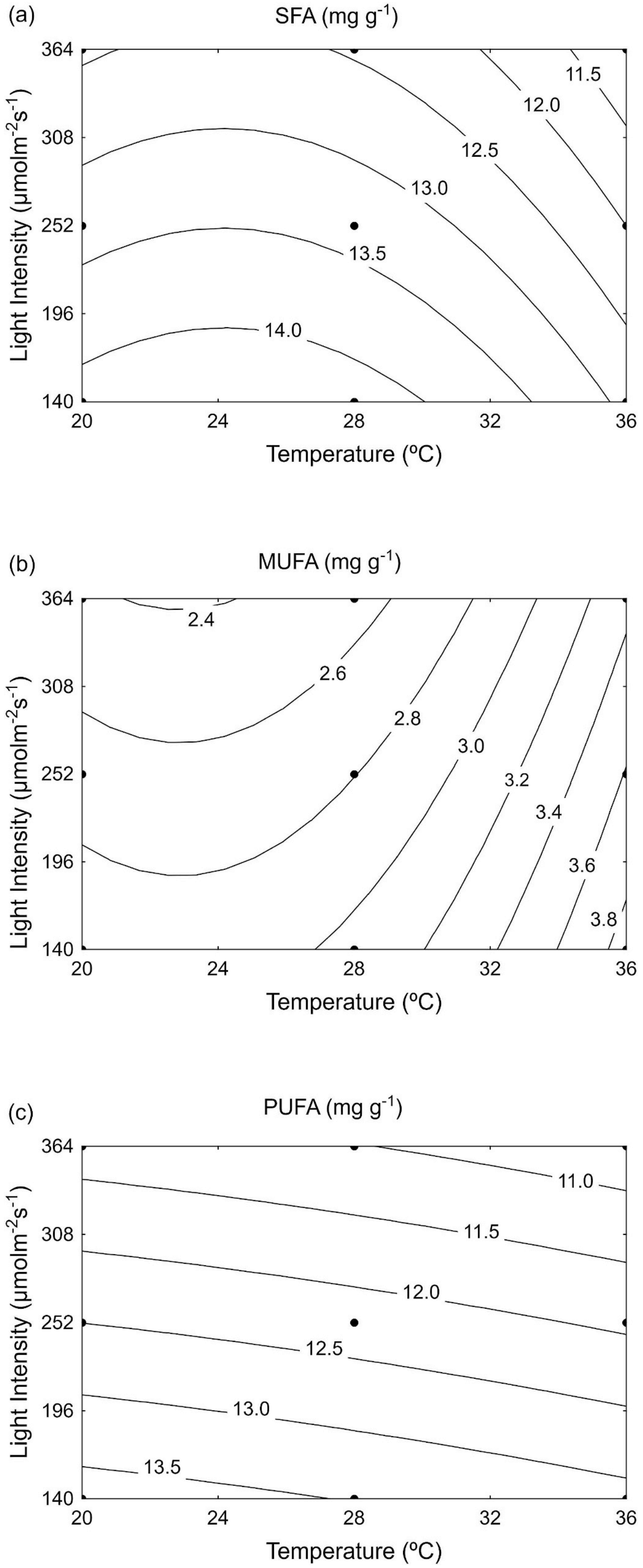

Fig. 6. Effects of temperature and light intensity on saturated fatty acid (a), monounsaturated fatty acid (b), polyunsaturated fatty acid (c) contents in *P. marina* biomass grown in batch culture.

an HPLC (Shimadzu, Kyoto, Japan) connected in series with a diode array detector (DAD) and a mass spectrometer (MS) with an ion trap analyzer and atmospheric pressure chemical ionization (APCI) source (Bruker Daltonics, Esquire model 6000, Bremen, Germany). The carotenoid chromatogram is shown in Supplementary Material (Fig. S1).

2.4. Lipids content and fatty acid composition

The total lipid content was determined according to Bligh and Dyer (1959) using 0.30 g of lyophilized biomass. For fatty-acids quantification, the lipid fraction was boiled for 30 min with a 14% BF_3-methanol solution under N_2 atmosphere according to the method described by Joseph and Ackman (1992). The samples were dissolved in hexane solvent and identified using gas chromatography (GC Model, 2010, Shimadzu, Kyoto, Japan) equipped with automated sampler and injector, flame ionization detector and a capillary column of fused silica (30 m × 0.25 mm, 0.25 μm film thickness, Rtx-Wax, Restek). One milliliter of samples were injected using split ratio of 1:50. The injector and flame ionization detector (FID) temperatures were 250 °C. The oven temperature was set at 50 °C for 1 min and increased to 200 °C at a gradient of 25 °C min^{-1}, followed by a gradient of 3 °C min^{-1} until 230 °C. The fatty acids quantification was performed according to the AOCS (1997) method. The identification of the fatty acids present in the samples was performed comparing the retention time with a standard mixture of fatty acid methyl esters (FAME-MIX 37 standard Sigma) previously analyzed by gas chromatography-mass spectrometry (GC–MS). The fatty acids chromatogram is shown in Supplementary Material (Fig. S2).

2.5. Measurement of carbohydrates and proteins content

The lyophilized biomass was hydrated overnight with 5 mL of distilled water and kept at 4 °C. The samples were centrifuged (10,000 × *g*, 5 min), the supernatant was discarded, and 1 mL of NaOH (1 N) was added to the pellet, mixed by vortex stirrer and heated at ebullition temperature for 30 min. The samples were cooled to room temperature and centrifuged (10,000 × *g*, 5 min). The protein content was measured in the supernatant according to Lowry et al. (1951). The results were correlated by calibration curve using bovine serum albumin as protein standard. The total carbohydrate analysis was measured after acid hydrolysis of the microalgae biomass. Aqueous sulfuric acid (80%, v v^{-1}) was added to the biomass and the mixture was kept overnight at 4 °C. The samples were centrifuged (10,000 × *g*, 5 min) and the supernatant was used for carbohydrate measurement by the phenol-sulfuric acid method (DuBois et al., 1956). The results were correlated to a calibration curve of glucose.

2.6. Statistical analysis

The statistical analyses were performed using Statistica 10.1 software (StatSoft, Inc.). The results were compared by Tukey test at a significance level of 5%. The experimental data of the face-centered design were analyzed by multiple regression analysis using a second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

Where Y is the predicted response value; β_0, β_j, and β_{jj} are the parameters estimated from the regression results. The responses described above were used to assess the interaction outcomes of temperature (X_1) and light intensity (X_2). The responses evaluated were biomass productivity, carotenoids, xanthophyll, lipids, fatty acid methyl esters

Table 5

Fatty acids composition of *P. marina* biomass grown under different conditions of temperature and light intensity.

Peak[a]	Temperature (ºC)	20	20	20	28	28	28	36	36	36
	Light intensity (μmol m^{-2} s^{-1})	140	252	364	140	252	364	140	252	364
Saturated fatty acids (% of total fatty acids)										
1	C14:0	4.6 ± 0.2^{a}	4.7 ± 0.4^{a}	4.6 ± 0.2^{a}	4.4 ± 0.1^{a}	3.9 ± 0.1^{ab}	4.2 ± 0.1^{a}	2.5 ± 0.1^{c}	2.5 ± 0.3^{bc}	2.6 ± 0.0^{c}
3	C16:0	40.0 ± 0.8^{a}	40.9 ± 0.1^{a}	40.4 ± 0.2^{a}	39.5 ± 0.0^{a}	41.4 ± 0.9^{a}	40.9 ± 0.1^{a}	38.6 ± 0.4^{a}	40.3 ± 0.8^{a}	40.5 ± 0.8^{a}
5	C18:0	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.6 ± 0.0^{a}	0.7 ± 0.0^{a}	0.8 ± 0.1^{a}	0.7 ± 0.2^{a}	0.7 ± 0.0^{a}
14	C22:0	0.4 ± 0.1^{ab}	0.3 ± 0.1^{ab}	0.4 ± 0.0^{a}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{ab}	0.2 ± 0.1^{ab}	0.2 ± 0.0^{ab}	0.2 ± 0.0^{ab}
15	C23:0	0.5 ± 0.0^{a}	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}	0.9 ± 0.1^{a}	0.5 ± 0.0^{a}	0.7 ± 0.2^{a}	0.5 ± 0.0^{a}
16	C24:0	0.4 ± 0.1^{ab}	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}	0.2 ± 0.0^{bc}	0.3 ± 0.1^{bc}	0.2 ± 0.0^{bc}	–	–	–
	Total	46.5 ± 0.8^{abc}	47.6 ± 0.6^{a}	47.2 ± 0.2^{ab}	45.5 ± 0.2^{abc}	47.1 ± 0.8^{a}	47.1 ± 0.1^{ab}	42.6 ± 0.2^{c}	44.4 ± 0.2^{bc}	44.6 ± 0.7^{abc}
Monounsaturated fatty acids (% of total fatty acids)										
2	C14:1	1.7 ± 0.0^{a}	0.9 ± 0.0^{bc}	0.8 ± 0.1^{bc}	1.1 ± 0.0^{b}	0.6 ± 0.1^{c}	0.5 ± 0.1^{c}	1.0 ± 0.1^{bc}	0.8 ± 0.2^{bc}	0.7 ± 0.0^{bc}
4	C16:1	0.7 ± 0.1^{a}	0.7 ± 0.1^{a}	1.2 ± 0.3^{a}	1.2 ± 0.7^{a}	0.9 ± 0.1^{a}	0.6 ± 0.0^{a}	0.7 ± 0.1^{a}	0.8 ± 0.3^{a}	0.5 ± 0.1^{a}
6	C18:1	8.2 ± 0.0^{bc}	7.3 ± 0.3^{c}	8.0 ± 0.1^{c}	7.0 ± 0.2^{c}	8.5 ± 0.3^{c}	8.5 ± 0.3^{c}	11.2 ± 0.5^{ab}	12.2 ± 0.7^{a}	11.3 ± 0.6^{a}
	Total	10.7 ± 0.4^{abcd}	8.8 ± 0.4^{d}	10.0 ± 0.0^{bcd}	9.4 ± 0.5^{cd}	10.0 ± 0.6^{bcd}	9.6 ± 0.2^{cd}	12.9 ± 0.5^{ab}	13.8 ± 0.5^{a}	12.4 ± 0.8^{abc}
Polyunsaturated fatty acids (% of total fatty acids)										
7	C18:2n-6	23.6 ± 0.0^{c}	24.9 ± 0.2^{c}	23.4 ± 0.4^{c}	28.6 ± 0.5^{b}	29.1 ± 0.3^{b}	29.6 ± 0.3^{b}	33.1 ± 0.1^{a}	32.4 ± 1.0^{a}	33.4 ± 0.0^{a}
8	C18:3n-6	1.9 ± 0.0^{a}	1.6 ± 0.1^{b}	1.4 ± 0.1^{bc}	1.1 ± 0.1^{cd}	0.6 ± 0.0^{e}	0.6 ± 0.0^{e}	1.0 ± 0.1^{d}	0.7 ± 0.0^{e}	0.6 ± 0.0^{e}
9	C18:3n-3	14.1 ± 0.2^{a}	13.1 ± 0.1^{a}	13.0 ± 0.1^{a}	12.5 ± 0.0^{a}	9.7 ± 0.5^{b}	9.7 ± 0.4^{b}	6.4 ± 0.2^{c}	5.6 ± 0.6^{c}	6.0 ± 0.2^{dc}
10	C20:3n-6	0.5 ± 0.1^{a}	0.6 ± 0.0^{a}	0.7 ± 0.0^{a}	0.5 ± 0.0^{a}	0.6 ± 0.1^{a}	0.5 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}
11	C20:3n-3	1.8 ± 0.0^{b}	2.1 ± 0.1^{ab}	2.5 ± 0.1^{ab}	2.1 ± 0.0^{ab}	2.2 ± 0.1^{ab}	2.1 ± 0.1^{ab}	2.8 ± 0.1^{a}	2.6 ± 0.1^{b}	2.6 ± 0.0^{ab}
12	C20:4n-6	0.2 ± 0.1^{abcd}	0.4 ± 0.0^{ab}	0.5 ± 0.0^{a}	0.1 ± 0.1^{bcd}	0.2 ± 0.1^{abc}	0.2 ± 0.0^{abcd}	-*	-*	-*
13	C20:5n-3	0.8 ± 0.0^{abcd}	1.0 ± 0.0^{ab}	1.3 ± 0.1^{a}	0.4 ± 0.0^{cd}	0.5 ± 0.1^{bcd}	0.7 ± 0.1^{bcd}	1.0 ± 0.6^{abc}	0.2 ± 0.0^{d}	0.2 ± 0.1^{d}
	Total	42.9 ± 0.5^{ab}	43.6 ± 0.1^{ab}	42.9 ± 0.3^{ab}	45.2 ± 0.3^{a}	43.0 ± 0.6^{b}	43.3 ± 0.4^{ab}	44.6 ± 0.7^{ab}	41.8 ± 0.3^{b}	43.0 ± 0.1^{ab}
	Total fatty acids (mg g^{-1})	30.0 ± 0.3^{ab}	28.6 ± 0.0^{ab}	25.6 ± 0.1^{ab}	31.5 ± 0.7^{a}	28.6 ± 1.4^{ab}	25.8 ± 0.3^{b}	28.2 ± 1.0^{ab}	27.9 ± 0.4^{ab}	25.1 ± 1.1^{b}
	ω6:ω3 ratio	1.6 ± 0.1^{c}	1.7 ± 0.0^{c}	1.5 ± 0.0^{c}	2.0 ± 0.1^{bc}	2.4 ± 0.1^{bc}	2.5 ± 0.0^{bc}	3.3 ± 0.1^{ab}	4.0 ± 0.5^{a}	3.9 ± 0.0^{a}

Different letter in the same column indicates significant difference by Tukey test at 5% significance level.

[a] Numbered according to the chromatogram shown in Fig. S2.

(FAMES), carbohydrates and proteins.

3. Results and discussion

3.1. Microalgae identification

The 18s rDNA of the green algae culture BE 001 was successfully amplified by the MA1 primer and showed high similarity (96%) to *Pseudoneochloris marina* U41102.1. However, the MA2 primer used in this study presented a low similarity (56%) with *Pseudoneochloris marina* U41102.1 and high similarity to *Urospora* genus (83%). Watanabe et al. (2000) reported that *Pseudoneochloris marina*, formerly described as *Neochloris* sp., is grouped in a clade with members of Ulvophyceae together with members Ulotrichales and very close to the genus *Acrosiphonia*. This report may explain the results obtained in this study, the low similarity of the sequence obtained with the MA2 primer with the genus *Urospora* and *Pseudoneochloris*. Since BE 001-MA2 sequence was not conclusive, the microalga identification was based on BE 001-MA1 that clearly binds BE 001 to *Pseudoneochloris marina* U41102.1 (Fig. 1).

3.2. Microalga growth and composition

In closed systems of microalgae cultures, CO_2 and nutrients supply, in addition to external parameters such as temperature and light intensity, are crucial for photosynthetic efficiency, affecting cell growth rate and biomass composition. *P. marina* is a green microalgae species identified recently (Watanabe et al., 2000) and its biotechnological potential has not been explored yet.

Preliminary tests were performed using different nitrogen concentration in culture medium to determine the best condition for biomass production by *P. marina* (Table 1). The highest biomass concentration (2.02 ± 0.21 g L^{-1}) was achieved using 74.1 mg L^{-1} N-NO_3, but with no significant difference to the values obtained using 68.4 mg L^{-1} or 78.2 mg L^{-1} N-NO_3. Decreasing $NaNO_3$ concentration resulted in lower cell growth, probably due to nutrient limitation. This way, the 74.1 mg L^{-1} N-NO_3 (450 mg L^{-1} Na-NO_3) concentration was chosen for further experiments to evaluate the effect of temperature and light intensity on biomass growth and composition (carbohydrates, lipids, proteins, carotenoids and FAMES).

The cell growth kinetics for the different conditions of temperature and light intensity is shown in Fig. 2. The highest biomass concentration (1.80 g L^{-1}) was achieved using light intensities of 252 μmol m^{-2} s^{-1} or 364 μmol m^{-2} s^{-1} at 28 °C. Cell growth limitation was observed in the experiments where the lower levels of light were applied: biomass of 0.70 g L^{-1} and 0.87 g L^{-1} were obtained under the conditions of 140 μmol m^{-2} s^{-1} and temperature of 20 °C and 36 °C, respectively. The temperature is known to affect nitrate uptake, changing the biochemistry of nitrogen metabolism. The high temperature increases the activity of intracellular proteolytic enzymes, resulting in the degradation of nitrogen reductase. In association with light, extreme temperature levels contribute to the inhibition of cell growth (Ho et al., 2014b; Kiran et al., 2016). The optimization of light intensity and temperature parameters are essential to reach maximal biomass productivity.

The mathematical models for biomass productivity (P_X) of *P. marina* under different temperature and light, as well as its major biomass components (proteins, lipids and carbohydrates), are shown in Table 2. Biomass productivity was affected by both temperature and light intensity. The response surface generated from the mathematical model (Fig. 3) shows that the increase of light availability favors the biomass productivity of *P. marina*, while for temperature, there is a maximum between 26 °C and 32 °C. The highest biomass productivity of *P. marina* (0.26 g L^{-1} d^{-1}) was reached at 28 °C and light intensity between 252 and 364 μmol m^{-2} s^{-1}. Ho et al. (2014b) studied the biomass production of *Desmodesmus* sp. under different temperatures (30–40 °C) and light intensities (300–1100 μmol m^{-2} s^{-1}). The authors verified that biomass productivity increased with the rising of temperature and light intensity and the optimum level of biomass productivity (0.76 g L^{-1} d^{-1}) was achieved when the cells were grown at 35 °C and 700 μmol m^{-2} s^{-1}.

The protein and carbohydrate contents were also affected by both variables. The highest protein content (236 ± 18 mg g^{-1}) was obtained at the lowest temperature (20 °C) and light intensity (140 μmol m^{-2} s^{-1}) conditions, that also correspond the lowest biomass productivity of all experiments. At low light intensity, temperature showed a pronounced positive effect on protein content, while at high light intensity, protein content was little affected by temperature (Fig. 4).

Renaud et al. (2002) related that high temperatures decrease protein content significantly in some tropical microalgal species, this fact that can be explained by the breakdown of protein conformation and interference with enzyme regulators. The high light intensity promotes better photosynthesis efficiency. Also, the nitrogen starvation at the end of the cultures (data not shown) resulted in low protein content in consequence of intracellular nitrogen consumption for cell growth (Paes et al., 2016). Therefore, under high light, nitrogen starvation and CO_2 supply, the protein in biomass microalgae can be consumed as a nitrogen source and, carbohydrate accumulation may increase during cell growth (Chen et al., 2013).

The carbohydrate content was positively affected by both light intensity and temperature (Table 2), however, as it showed a low determination coefficient ($R^2 = 0.651$), the contour lines have not been plotted. The carbohydrates content ranged between 379 ± 10 mg g^{-1} at 28 °C and 140 μmol m^{-2} s^{-1} and 538 ± 13 mg g^{-1} at 36 °C and 364 μmol m^{-2} s^{-1}. The synthesis of carbohydrates depends on temperature and light intensity, which influences cell growth rate when applied to cultures at optimal levels. Also, the accumulation of carbohydrates in microalgae biomass is due to CO_2 biofixation during the photosynthetic process (Chen et al., 2013; Minhas et al., 2016).

Carvalho et al. (2009), in *Pavlova lutheri* cultivation, demonstrated that an increase on the light intensity in the range of 30–400 μmol m^{-2} s^{-1} subtly increased the accumulation of carbohydrates. Additionally, cultures of *Chlorella vulgaris* in airlift photobioreactors showed an increase in carbohydrates content from 8.5% to 40% (d.w.) when increasing light intensity from 215 to 330 μmol m^{-2} s^{-1} (Fernandes et al., 2010).

Lipids and carotenoids contents were affected only by light intensity (Table 2, Fig. 5). For carotenoids, the increase in light intensity on cultures resulted in a slight decrease from 3.6 ± 0.6 mg g^{-1} to 2.6 ± 0.4 mg g^{-1}. This result suggests that carotenoids damage is related with the photoprotective role of carotenoids in cells, which stabilizes light-harvesting by membranes of chloroplasts, reducing the singlet oxygen production and scavenging free radicals induced by high light stress (Huang et al., 2017). The same behavior was observed for lipid content, whereby the rise in light intensity level from 140 to 364 μmol m^{-2} s^{-1} provided a reduction of 132 ± 7 mg g^{-1} to 97 ± 3 mg g^{-1}.

Light intensity in photoautotrophic microalgae systems strongly acts on cell growth, CO_2 fixation, and may change the biomass biochemical composition, such as carbohydrates and lipids (Chen et al., 2013). Zhu et al. (2016) showed that, for many microalgae species, lipid accumulation is associated with cell growth, thereby an increase in the light intensity promotes high cell growth and lipid content. On the other hand, the authors exposed that the relationship between light intensity and intracellular lipid accumulation is microalgae-specific. Additionally, some stress conditions can cause photoinhibition, which damages microalgae photosystems and thus reduces lipids accumulation. This correlation agrees with our study, which was observed that an increase in light intensity was favorable to rise the carbohydrate content but not lipid accumulation.

3.3. Carotenoid identification

The carotenoid biosynthesis differs from one species to another. Carotenoids absorb light and quench the excess of energy in photosynthetic metabolism. Some primary carotenoids, such as lutein, are used as accessory pigments, which can transfer absorbed energy to chlorophylls (Ye et al., 2008). Temperature and light intensity are both critical environmental factors that affect carotenoid accumulation in microalgae cells.

Carotenoids are divided into two groups: xanthophylls and carotenes. The increment in the temperature and light intensity affected xanthophylls negatively, while carotenes content was influenced positively by temperature and negatively by light intensity (Table 3). Xanthophylls and carotenes contents ranged between 1.61 ± 0.34 mg g^{-1} and 2.51 ± 0.02 mg g^{-1}, 0.66 ± 0.03 mg g^{-1} and 1.25 ± 0.01 mg g^{-1}, respectively (Table 4). The major carotenoids identified from *P. marina* were all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-carotene and all-*trans*-α-carotene.

Carotenoids have an essential function in membrane stabilization acting in light harvesting, thermal energy dissipation and scavenging reactive oxygen species (ROS) (García-Cañedo et al., 2016). Thereby, under high light exposure, the reduction of carotenoids can be associated with photoprotection of membrane cells of some species of microalgae, including *P. marina* evaluated in this study. In batch cultures of *Neochloris oleabundans* with CO_2 input and nitrogen supply, the authors observed that an increment on light intensity from 240 to 400 μmol m^{-2} s^{-1} slightly reduced the total carotenoids (9.4 ± 0.1 to 8.9 ± 0.1 mg L^{-1} d^{-1}) and lutein productivities (3.3 ± 0.0 to 2.6 ± 0.1 mg L^{-1} d^{-1}) (Urreta et al., 2014). The highest β-carotene content (118 mg L^{-1}) of *D. salina* cultivated under different environmental conditions was achieved in the range of 22 °C to 25 °C and light intensity of 135 μmol m^{-2} s^{-1}. The authors observed that an increment in the light intensity showed no difference in β-carotene accumulation and that lower or higher light intensity level could restrain algae growth (Wu et al., 2016).

3.4. Fatty acids methyl esters (FAMEs) identification

The fatty acid profile of *P. marina* was strongly affected by both light intensity and temperature (Table 3). The contour lines of saturated fatty acids (SFA - Fig. 6a), monounsaturated fatty acids (MUFA - Fig. 6b) and poly-unsaturated fatty acids (PUFA - Fig. 6c) showed different behavior, as high temperature affected negatively the SFA content, positively the MUFA content, and showed low influence on PUFA content in *P. marina* biomass. The temperature in FAMEs content is vital to maintain the specific membrane functions, such as of light uptake complexes, photosystems, and membrane protein. At low temperature, more PUFA are synthesized for the correct regulation of membrane fluidity (Los et al., 2013; Schüler et al., 2017; Sharma et al., 2012).

The high light intensity slightly decreased both the content of SFA, MUFA and PUFA as well as the lipid content discussed above. *P. marina* presented fatty acids profile similar to other marine microalgae species (Table 5) (Breuer et al., 2012; Jaeschke et al., 2016; Sahu et al., 2013). The FA are part of the structure of cell membranes and are produced in association with cellular growth during carbon assimilation as reserve product. The amount and profile depend on the conditions of saline stress, nitrogen depletion and factors such as temperature and light (Schüler et al., 2017). The major SFA identified in all conditions were palmitic acid (C16:0, 40.3 ± 0.8% of total FA), followed by C14:0 (2.5%–4.7% of total FA), and small amounts of C18:0 and C23:0. The MUFA content ranged between 8.8% and 13.8% of total FA. The major MUFA identified was C18:1, whose synthesis was favored at the highest temperature, followed by C14:1 and C16:1.

The essential ω6 and ω3 FA linoleic acid (C18:2n-6, LA) and α-linolenic acid (C18:3n-3, ALA) were the main PUFAS identified in all experimental conditions, followed by C20:3n-3 C18:3n-6 and C20:5n-3 (eicosapentaenoic acid, EPA). The ω3 series protect microalgal cells from oxidative damage by ROS acting as an antioxidant (Okuyama et al., 2008). The LA content increased with temperature, ranging from 23.4% to 33.4% of total FA, while the ALA content decreased with temperature, ranging from 5.6% to 14.1% of total FA. *Nannochloropsis salina* grown under different temperature and light intensity presented similar responses for FA composition compared with this study (Van Wagenen et al., 2012). Overall, the FA content is inversely proportional to cell growth, which indicates that the faster growth needs more material to assembly components and do not accumulate lipids quickly.

The ω6:ω3 ratio from human dietary is vital to maintain human health (Simopoulos, 2008). Modern lifestyle leads to the consumption of food with unhealthful high ω6:ω3 ratios (around 20:1). An ω6-rich diet shifts the physiological body state to a predisposition to develop inflammatory and thrombotic diseases and increases blood viscosity (Simopoulos, 2016). In this study, *P. marina* showed the lowest ω6:ω3 ratio, around of 1.6:1, when cultured at the lowest temperature (Table 5). This result is similar to the one reported for *H. luteoviridis* cultured under different temperature and nitrogen concentration, where the lowest ω6:ω3 ratio, around of 1.4:1, was observed at the lowest temperature (Menegol et al., 2017). Thereby, microalgae biomass can be used as an important source of PUFA to improve ω6:ω3 ratio in human alimentation, especially if cultured under low temperature.

4. Conclusion

Pseudoneochloris marina showed high potential to be used in microalgae large-scale cultures. It reached 1.80 g L^{-1} of dry biomass, with biomass productivity of 0.26 g L^{-1} d^{-1}, under photoautotrophic growth conditions. *P. marina* biomass can be an alternative source of high-value products such as carotenoids (all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-β-carotene and all-*trans*-α-carotene) and essential polyunsaturated fatty acids (linoleic acid and α-linolenic acid). Moreover, depending on light and temperature conditions, it was possible to reach high carbohydrate (53.77%) and saturated fatty acid (44.11% of total FA) content, showing that this microalga can also be explored for biofuels production.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101032.

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