



Nutritional value changes in response to temperature, microalgae mono and mixed cultures

Alterações no valor nutricional de microalgas em resposta a variação de temperatura, cultivo unialgal e misto

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Abstract: Aim: The response of mixed cultures and monocultures of *Pseudopediastrum boryanum* (Turpin) E. Hegewald and *Scenedesmus obliquus* (Turpin) Kützing was assessed in the laboratory at three different temperatures: 20, 30 and 40 °C. The change in biomass and biochemical composition of these cultures was evaluated. **Methods:** Microalgae were placed in a batch culture over 21-days in ASM1 medium. Cell density was directly counted every two days using a Fuchs-Rosenthal hemocytometer. Chlorophyll 'a' and total carotenoids were extracted twice. Protein, carbohydrate, total lipid and fatty acid contents were determined at the end of the experiment. **Results:** Cultures grown in 40 °C exhibited no growth. However, there was increased cell density (13.6×10^6 cell.mL⁻¹) and biomass (55 g.L⁻¹) in the *S. obliquus* monoculture at 30 °C. High protein concentrations (672.6 mg.g⁻¹) were observed in monocultures *P. boryanum* and *S. obliquus* at 20 °C treatments. There were high carbohydrate concentrations (6.17 mg.g⁻¹) in *P. boryanum* unialgal at 30 °C. There was no significant difference in total lipid content between *S. obliquus* (95.5 mg.g⁻¹), *P. boryanum* (96.3 mg.g⁻¹) and the mixed culture at 20 °C (105.3 mg.g⁻¹). FAMES varied significantly regarding the number of unsaturated components, which predominantly consisted of fatty acids with two or more unsaturated bonds. **Conclusions:** The biomass of the treatments analyzed was rich in proteins and essential fatty acids (such as linolenic acid), yet low in carbohydrate content, suggesting its potential use as a food supplement. Our results indicate that mixed culture of *P. boryanum* and *S. obliquus* was inefficient in cultivating biomass or biochemical compounds as compared to the unialgal cultivations. However, algae grown together showed better stability in their biochemical composition in response to changes in temperature, an important factor for microalgae production in open ponds and in food safety measures. These results suggest the consortia among different species of microalgae should be tested to determine better methodologies for the production of biomass and metabolites with greater stability towards environmental factors.

Keywords: mixed cultivation; *Pseudopediastrum*; *Scenedesmus*; biomass; food security.



Resumo: Objetivo: Avaliar o desenvolvimento de *Pseudopediastrum boryanum* (Turpin) E. Hegewald e *Scenedesmus obliquus* (Turpin) Kützing em cultivos uniaxiais e mistos, em três diferentes temperaturas, 20, 30 e 40 °C, em escala laboratorial, em termos de biomassa e composição bioquímica. **Métodos:** O cultivo foi do tipo batelada, em meio ASM1. A densidade celular foi determinada por contagem direta em hemocitômetro a cada dois dias. As análises dos principais pigmentos se deram em dois tempos e as análises de proteínas, carboidratos, lipídeos totais e perfil de ésteres metílicos apenas ao final do experimento. **Resultados:** Não foi registrado crescimento celular nas cepas cultivadas em 40 °C. Incremento na densidade celular ($13.6 \times 10^6 \text{ cell.mL}^{-1}$) e biomassa (55 g.L^{-1}) foram registrados na cultura unialgal de *S. obliquus* em 30 °C. Elevadas concentrações de proteínas (672.6 mg.g^{-1}) foram observadas nos cultivos uniaxiais de *P. boryanum* e de *S. obliquus* em 20 °C e de carboidratos (6.17 mg.g^{-1}) apenas no cultivo unialgal de *P. boryanum* em 30 °C. Os FAMES (fatty acid methyl esters) variaram significativamente em relação ao número de insaturações, sendo constituídos em sua maioria por ácidos graxos com duas ou mais insaturações. **Conclusões:** A biomassa dos tratamentos analisados foi rica em proteínas, ácidos graxos essenciais, como o ácido linolênico, e apresentou baixo teor de carboidratos, sugerindo seu potencial uso como suplemento alimentar. Nossos resultados indicam que a cultura mista de *P. boryanum* e *S. obliquus* não foi uma condição eficiente para a produção de biomassa ou compostos bioquímicos. No entanto, algas cultivadas em consórcio apresentaram melhor estabilidade em sua composição bioquímica em resposta a mudanças de temperatura, fator importante para a produção de microalgas em lagoas abertas e para garantir a segurança alimentar. Estes resultados sugerem que os consórcios entre diferentes espécies de microalgas devem ser testados para determinar melhores metodologias para produção de biomassa e metabólitos com maior estabilidade à fatores ambientais.

Palavras-chave: cultivo misto; *Pseudopediastrum*; *Scenedesmus*; biomassa; segurança alimentar.

1. Introduction

Microalgae present high rates of growth and biomass production (Becker, 2013; Dismukes et al., 2008), extensive chemical and metabolic diversity, and great ecological plasticity. All of these traits allow microalgae to respond quickly to varying environmental conditions (Hannon et al., 2010; Lourenço, 2006). These attributes also suggest that microalgae show promise in producing molecules that can be used in biofuel production, as well as molecules with properties that are beneficial for humans and animals (Ohse et al., 2007).

There is currently considerable effort in researching species with improved lipid production for technologies that require biodiesel; however, the yield is still not economically viable (Clarens et al., 2010; Milledge, 2011; Soratana et al., 2014). Current yields are estimated at between \$9 and \$25 USD per gallon of oil extracted from cultivation in open ponds and between \$15 and \$40 per gallon of the same oil extracted from microalgae grown in closed photobioreactors (Kirrolia et al., 2013).

Therefore, investigating endogenous or bioengineered co-products together with microalgae oil would beneficially impact the economics of algae-based biofuels. Nutrients and secondary metabolites constitute a considerable fraction of the residual biomass and present a wide variety of nutraceutical and pharmaceutical applications, as well as agricultural inputs (Dismukes et al., 2008; Hannon et al., 2010; Khan et al., 2009; Kirrolia et al., 2013).

Manipulating the algal growth environment can alter the growth characteristics and chemical composition of cultured cells. Consequently, there is considerable research that focuses on altering the physical, chemical and biological conditions of various microalgae crops to induce the production of target compounds and generate biomass, thereby improving the feasibility of the production of algae biofuels (Lourenço, 2006; Miao et al., 2004).

Currently, commercial-scale microalgae cultivation is mainly carried out with monocultures in open pond systems (Chisti, 2007), due to its low cost of implantation, maintenance and expansion (Coplin, 2012; Milledge, 2011). However, monocultures in open pond systems are subject to temperature variations, which directly affects the growth and metabolic activities of these microalgae (Lourenço, 2006; Li et al., 2011), and allows the entry of other species of microalgae and zooplankton, compromising the uniformity of the population and the quality of the final product. Therefore, using a greater number of species in the same cultivation unit may be advantageous in open pond systems, given that mixed crops have a higher stability and can better utilize available resources, producing more biomass and lipids (Coplin, 2012; Hannon et al., 2010; Smith et al., 2010).

This study evaluated the performance of mixed and monocultures of *Pseudopediastrum boryanum* (Turpin) E. Hegewald and *Scenedesmus obliquus* (Turpin) Kützing under three different temperatures (20, 30 and 40 °C). Both biomass and metabolite production (fatty acid profile and total lipid

content, carbohydrates, proteins and pigments) were analyzed to determine the effect of differing culture conditions on the biomass and aggregate products of microalgae.

2. Material and Methods

2.1. Experimental design

The species of microalgae, *P. boryanum* and *S. obliquus*, were obtained from the culture collection of microalgae at the Laboratory of Taxonomy and Ecology of Continental Algae (LATEAC), Federal University of Espírito Santo. Both species were collected and isolated from the Juara Lagoon (Serra, Espírito Santo, Brazil). These species were selected through previous tests that showed biochemical potential of their biomass, when cultivated separately.

Microalgae were cultivated in batch incubators (Electrolab, EL 202/3; Brazil) to investigate the effect of three different temperatures (20, 30 and 40 °C). For the incubations, algae were grown in 3 L Erlenmeyer flasks, containing 2.5 L of ASM1 medium (Gorham et al., 1964), with an initial pH of 7 ± 0.05 . A constant aeration of $3.5 \text{ L}\cdot\text{min}^{-1}$ of air, without direct sprinkling of CO_2 , was maintained under a 12/12 h light/dark photoperiod with maximum illuminance of $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Three treatments were carried in triplicate at each temperature. The treatments are P20/P30/P40, S20/S30/S40 and M20/M30/M40 corresponding to the monocultures of *P. boryanum*, *S. obliquus* and the mixed culture at the different temperatures, respectively. The initial inoculum was $1.5 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$ for the unialgal cultures and $7.5 \times 10^4 \text{ cells}\cdot\text{mL}^{-1}$ of each species for the mixed cultures, totaling $1.5 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$. A predetermined sequence of rotation of the Erlenmeyer flasks in the incubators was established, to ensure uniformity of the culture conditions. The experiment lasted 21 days.

2.2. Growth assessment

Cell density was determined by direct counting in a Fuchs-Rosenthal hemocytometer under an optical microscope (Olympus, CX41RF; Philippines), every 2 days of the experiment.

Dry biomass was determined every 4 days by filtering 15 mL aliquots through glass fiber filters (Macherey-Nagel, GF1 47 mm; Germany), followed by oven drying at 65 °C until constant weight (Lourenço, 2006).

Growth rate (K) and mean doubling time (G) were calculated using the values obtained from the exponential growth phase, by the following equation:

$K = (\ln(N_2) - \ln(N_1)) / (T_2 - T_1)$, where N_1 and N_2 are the numbers of cells at times T_1 and T_2 , respectively. Taking the values of K , the mean doubling time was calculated as $G = \ln(2) / K$. The maximum yield (Y_{max}) was calculated, by subtracting the highest density value obtained ($\text{cells}\cdot\text{mL}^{-1}$) by the initial inoculated value (Fogg & Thake, 1987).

2.3. Carbohydrates, proteins, total lipids and pigments

Total carbohydrate concentrations were determined following the procedure initially described by Dubois et al. (1956) and modified by Cuzzuol & Clippel (2009). The Quick Start™ Bradford Protein Assay kit (Biorad; United States) was used to quantify the total soluble protein, based on the Bradford (1976) method. Total lipids were extracted and analyzed according to Bligh & Dyer (1959). Chlorophyll 'a' and total carotenoids were extracted in 90% acetone and their concentrations were measured spectrophotometrically (Thermo Scientific, Aquamate plus; United States), following the established protocols of Lorenzen (1967) and Strickland & Parsons (1968), respectively. The concentrations of chlorophyll 'a' and carotenoids were evaluated on the 10th and 21st days of the experiment; protein, carbohydrate and total lipid analyses were evaluated only at the end of the experiment.

2.4. Analysis of fatty acids

The macerated lyophilized algae were suspended in a phosphate-buffered saline solution, followed by the addition of 42 μL C13:0 analytical standard (glyceryl tritridecanoate) solution (5 $\text{mg}\cdot\text{mL}^{-1}$ in hexane) and 4.15 mL chloroform/methanol/water (2:2:1 v/v/v). The mixture was centrifuged, and the chloroform phase was transferred to another flask and dried under N_2 gas. The total lipid content was determined gravimetrically, and the dry lipid extract was methylated to assess fatty acid content.

The methylation reaction of fatty acids to fatty acid methyl esters (FAMES) was performed by dissolving the dry lipid extract in 500 μL BF_3 (7% in methanol). The mixture was then incubated for one hour at 100 °C. Next, 1.25 mL of water were added at room temperature, and the FAMES were extracted with 500 μL hexane.

The FAMES were analyzed by gas chromatography coupled with mass spectrometry (Shimadzu, QP2010; Japan), using a 30-m fused silica capillary column (VF-Wax with 0.25 μm film; Agilent). A sample (1 μL) was injected at 220 °C in split mode. Helium was used as the carrier gas, at a flow

rate of 1 mL.min⁻¹. The procedure had the following temperature ramp: initial temperature of 60 °C with an increase of 5 °C.min⁻¹ up to 260 °C, which was maintained for 10 minutes.

The standard used to identify the peaks was Supelco 37 (47885-U). The fatty acids were identified through comparing retention times with standards and/or by comparing their mass spectra with the existing NIST spectral library. The FAMES that were not included in the standard and that presented a similarity index below 90% were not considered.

Most FAMES were quantifiable with the regression of the standard curve for the respective FAMES of the Supelco 37 standard. For those FAMES not in the Supelco standard, quantification was performed through considering the concentration of the internal standard (C13:0), because the peak area was proportional to the FAME concentration. Analysis of fatty acids were evaluated only at the end of the experiment. Based on the fatty acid profile, the ratio between omega-3 and omega-6 fatty acids (ω -3/ ω -6) was calculated.

2.5. Statistical analysis

The data obtained from the growth, dry mass, total water-soluble protein, lipid, carbohydrate, pigment and fatty acid analyses

were subjected to the Shapiro-Wilk normality analysis. The parametric data were then evaluated by (2 temperature × 3 culturing modes) factorial analysis of variance (ANOVA), for the pigment and dry mass data, the time factor was included, and Tukey's test at 5% probability level. All statistical analyses were performed using ASSISTAT (version 7.7 beta; Federal University of Campina Grande, PB, Brazil) (Silva & Azevedo, 2016).

3. Results

3.1. Growth and dry mass

There was no apparent growth for those cultures grown at 40 °C. Temperature did affect the growth parameters *K* ($F = 21.2867$, $p < 0.001$) and *G* ($F = 7.9202$, $p = 0.0064$). However, although the Y_{\max} value was significantly different between treatments ($F = 64.4840$, $p < 0.001$), it was not affected by temperature ($F = 1.5237$, $p = 0.2572$) (Table 1).

Temperature did not affect biomass ($F = 2.4984$, $p = 0.052$). Therefore, the cultivation time was primarily responsible for this improved biomass yield ($F = 15.2790$, $p < 0.001$) (Table 2). There was a significant increase in biomass, compared to days 5 and 21, in all treatments ($F = 8.9783$,

Table 1. Parameters of growth of unialgal and mixed cultures.

^a Sample	<i>K</i> (d ⁻¹)	<i>G</i> (day)	Y_{\max} (cells.mL ⁻¹)	Log phase (days)
P20	0.39 ± 0.11 bA	1.79 ± 0.05 aB	2.24 × 10 ⁶ aC	3-5
S20	0.21 ± 0.00 aB	3.25 ± 0.05 bA	10.94 × 10 ⁶ aA	1-19
M20	0.21 ± 0.01 aB	3.49 ± 0.37 bA	6.42 × 10 ⁶ bB	1-19
P30	0.72 ± 0.02 aA	0.99 ± 1.40 aB	2.26 × 10 ⁶ aC	3-9
S30	0.13 ± 0.03 bB	5.29 ± 0.78 aA	13.67 × 10 ⁶ aA	1-21
M30	0.13 ± 0.00 bB	5.73 ± 0.23 aA	9.16 × 10 ⁶ aB	1-21

^aP20, S20 and M20 correspond to *P. boryanum*, *S. obliquus* and the mixed culture at 20 °C; P30, S30 and M30 correspond to *P. boryanum*, *S. obliquus* and the mixed culture at 30 °C. Values followed by different letters differ statistically from each other ($p < 0.01$). Lowercase letters compare the respective treatment at both temperatures. Uppercase letters compare treatments within the same temperature. *K* = growth rate; *G* = doubling time; Y_{\max} = maximum yield. Mean ± SD (n = 3 cultivations).

Table 2. Biomass variation (dry weight) during cultivation in g.L⁻¹.

^a Sample	Day 5 (g.L ⁻¹)	Day 10 (g.L ⁻¹)	Day 13 (g.L ⁻¹)	Day 17 (g.L ⁻¹)	Day 21 (g.L ⁻¹)
P20	0.09 ± 0.01 bAe	0.16 ± 0.03 bAd	0.22 ± 0.01 aBc	0.36 ± 0.02 aAb	0.48 ± 0.04 aBa
S20	0.03 ± 0.01 aBd	0.15 ± 0.02 aAc	0.20 ± 0.06 aBc	0.33 ± 0.02 aAb	0.56 ± 0.06 aAa
M20	0.12 ± 0.02 aAc	0.15 ± 0.00 aAc	0.28 ± 0.06 aAb	0.27 ± 0.05 aBb	0.42 ± 0.07 aCa
P30	0.16 ± 0.03 aAd	0.21 ± 0.01 aAc	0.25 ± 0.03 aAc	0.30 ± 0.04 aAb	0.40 ± 0.05 bBa
S30	0.04 ± 0.00 aBd	0.15 ± 0.01 aBc	0.18 ± 0.01 aBc	0.27 ± 0.00 bAb	0.55 ± 0.00 aAa
M30	0.08 ± 0.01 bBd	0.15 ± 0.01 aBc	0.27 ± 0.03 aAb	0.32 ± 0.06 aAb	0.37 ± 0.01 aBa

^aP20, S20 and M20 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 20 °C; P30, S30 and M30 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 30 °C. Values followed by different letters differ statistically from each other ($p < 0.01$). Lowercase letters compare the respective treatment at both temperatures. Uppercase letters compare the treatments within the same temperature. Lowercase italics compare the treatments at the same temperature at different times. Mean ± SD (n = 3 cultivations).

$p < 0.001$), with S30 and S20 crops presenting the highest biomass at the end of the experiment.

3.2. Total carbohydrates, water-soluble proteins and lipids

The total carbohydrate, water-soluble protein and lipid contents of the different treatments are listed in Table 3. Although temperature had a significant influence on the carbohydrate concentrations ($F = 13.0323$, $p = 0.0009$), they were mainly correlated with being in a mono- or mixed culture ($F = 36.5491$, $p < 0.001$).

The highest carbohydrate concentration, 6.17 mg.g^{-1} dry weight (DW), was recorded in the monoculture of *P. boryanum* at 30°C (P30). It was higher ($F = 30.8861$, $p = 0.0006$) than the corresponding values found for the mixed culture (M30) and the unialgal *S. obliquus* culture (S30) at the same temperature, respectively.

A comparative test of means showed that as the temperature lowered from 30 to 20°C , there was significant decrease in carbohydrate content

of the monoculture of *P. boryanum* ($F = 14,1867$, $p = 0.0196$). In contrast, there was an increase in carbohydrate content in the monoculture of *S. obliquus* ($F = 11.0115$, $p = 0.0294$). The carbohydrate content of the mixed culture did not change with respect to temperature ($F = 0.0908$, $p = 7781$).

The temperature and the type of culture did not have a significant influence on the total soluble protein concentration ($F = 3.4336$, $p = 0.0662$). There was an increase in the protein content only in monocultures of *P. boryanum*, when temperature decreased to 20°C ($F = 13.4697$, $p = 0.214$). However, total lipid content is significantly affected to the temperature ($F = 94.430$, $p < 0.001$). In all treatments, the decrease in temperature to 20°C corresponded to an increase in the concentration of total lipids.

3.3. Chlorophyll 'a' and total carotenoids

The concentrations of chlorophyll 'a' and total carotenoids (Table 4) were significantly affected by culture time ($F = 123.0587$, $p < 0.001$).

Table 3. Content of total carbohydrates, water-soluble proteins and total lipids of *Pseudopediastrum boryanum*, *Scenedesmus obliquus* grown in mono- and mixed culture at 20 and 30°C .

^a Sample	Carbohydrate (mg.g ⁻¹)	% (DW)	Protein (mg.g ⁻¹)	% (DW)	Total lipid (mg.g ⁻¹)	% (DW)
P20	4.36 ± 0.23 bA	0.44	672.60 ± 5.7 aA	67.30	96.30 ± 3.1 aA	9.60
S20	3.33 ± 0.50 aA	0.33	672.60 ± 62.6 aA	67.30	95.50 ± 2.5 aA	9.50
M20	3.98 ± 0.30 aA	0.40	359.50 ± 17.1 aB	35.90	105.30 ± 4.7 aA	10.50
P30	6.17 ± 0.80 aA	0.62	466.60 ± 97.1 bA	46.70	78.50 ± 2.5 bAB	7.80
S30	2.17 ± 0.34 bC	0.22	581.80 ± 10.0 aA	58.20	82.50 ± 7.5 bA	8.30
M30	3.86 ± 0.64 aB	0.39	326.50 ± 85.3 aB	32.60	68.70 ± 6.6 bB	6.90

^aP20, S20 and M20 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 20°C ; P30, S30 and M30 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 30°C . Values followed by different letters differ statistically from each other ($p < 0.01$). Lowercase letters compare the respective treatment at both temperatures. Uppercase letters compare treatments within the same temperature. DW = dry weight. Mean \pm SD ($n = 3$ cultivations).

Table 4. Chlorophyll 'a' and total carotenoid contents of *Pseudopediastrum boryanum* and *Scenedesmus obliquus*, grown individually and in mixed culture, at 20 and 30°C , in two periods of cultivation.

^a Sample	First period (Day 10)		Second period (Day 21)	
	Chlorophyll 'a' ($\mu\text{g.mL}^{-1}$)	Carotenoids ($\mu\text{g.mL}^{-1}$)	Chlorophyll 'a' ($\mu\text{g.mL}^{-1}$)	Carotenoids ($\mu\text{g.mL}^{-1}$)
P20	1.8 ± 0.32 aAa	0.5 ± 0.04 aAb	2.3 ± 0.06 aBa	0.8 ± 0.05 bAa
S20	1.3 ± 0.28 aAa	0.5 ± 0.04 aAb	1.7 ± 0.23 aBa	0.7 ± 0.12 aAa
M20	1.8 ± 0.31 aAb	0.6 ± 0.09 aAb	3.2 ± 0.60 aAa	1.0 ± 0.20 aAa
P30	1.2 ± 0.22 bBb	0.4 ± 0.12 aABb	3.0 ± 0.30 aAa	1.0 ± 0.04 aAa
S30	1.1 ± 0.13 aBb	0.3 ± 0.06 aBb	2.3 ± 0.08 aBa	1.0 ± 0.04 aAa
M30	1.8 ± 0.27 aAb	0.6 ± 0.03 aAb	3.1 ± 0.61 aAa	0.9 ± 0.25 aAa

^aP20, S20 and M20 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 20°C ; P30, S30 and M30 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 30°C . Values followed by different letters differ statistically from each other ($p < 0.01$). Lowercase letters compare the respective treatment at both temperatures. Uppercase letters compare the treatments within the same temperature. Lowercase italics compare the treatments at the same temperature at different times. Mean \pm SD ($n = 3$ cultivations).

3.4. Profile of Fatty Acid Methyl Esters (FAMES)

Concentrations of the FAMES and their unsaturation contents for mono and mixed cultures of *P. boryanum* and *S. obliquus* at different temperatures, as well as the ω -3/ ω -6 ratio, are listed in Table 5. The composition of the FAMES varied significantly with regard to the number of unsaturated components. One exception is for the M20 treatment, in which there was a higher content of monounsaturated fatty acids (MUFAs);

all other treatments, however, were constituted predominantly by fatty acids with two or more unsaturated components.

There was an inverse relationship between the ω -3/ ω -6 ratio and the total concentration of the FAMES in the temperature treatments ($F = 21.3996$, $p = 0.0005$). The only exception was the unialgal cultivation of *P. boryanum*, where was directly proportional to the ω -3/ ω -6 ratio.

The composition of the FAMES extracted from the microalgal biomass of each treatment is shown

Table 5. Concentration (mg.g⁻¹ dry weight) of total fatty acids, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and ω -3/ ω -6 ratio of treatments.

^a Sample	^b SFAs	^b MUFAs	^b PUFAs	^c ω -3/ ω -6 ratio	^c Total
P20	21.1 ± 0.80 c	33.7 ± 0.61 b	45.2 ± 1.11 a	0.2:1.0 ± 0.0 aB	79.2 aAB
S20	16.0 ± 1.32 c	27.9 ± 3.23 b	56.2 ± 2.68 a	3.4:1.0 ± 0.1 aA	82.7 aA
M20	21.7 ± 0.33 c	41.8 ± 1.23 a	36.5 ± 0.97 b	3.4:1.0 ± 1.0 aA	73.5 aB
P30	26.2 ± 1.34 c	30.3 ± 1.50 b	43.5 ± 2.83 a	0.4:1.0 ± 0.5 aB	49.2 bB
S30	17.2 ± 1.49 b	14.0 ± 1.65 b	68.8 ± 2.97 a	1.9:1.0 ± 0.2 bA	68.4 bA
M30	28.0 ± 1.88 b	22.2 ± 0.53 c	49.8 ± 1.59 a	1.5:1.0 ± 0.4 bA	46.0 bB

^aP20, S20 and M20 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 20 °C; P30, S30 and M30 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 30 °C. Values followed by different letters differ statistically from each other ($p < 0.01$); ^bLowercase letters compare the concentrations of SFA, MUFA, and PUFA in the same treatment; ^cLowercase letters compare the respective treatment at both temperatures. Uppercase letters compare the treatments within the same temperature. Averages followed by the same letter do not differ statistically from each other ($P < 0.01$). Mean ± SD (n = 3 cultivations).

Table 6. Composition of methyl esters of fatty acids (% of total FAME) extracted from the biomass of *Pseudopediastrum boryanum* and *Scenedesmus obliquus* in their unialgal and mixed cultures at 20 and 30 °C.

Fatty acid	20 °C (%)			30 °C (%)		
	^a P20	^a S20	^a M20	^a P30	^a S30	^a M30
Tetradecanoic (C14)	1.0 ± 0.05h	1.1 ± 0.01e	1.0 ± 0.03g	1.4 ± 0.03f	1.0 ± 0.02h	1.8 ± 0.04h
Pentadecanoic (C15)	0.3 ± 0.01i	0.4 ± 0.01e	0.4 ± 0.02g	0.6 ± 0.01f	0.5 ± 0.03h	0.7 ± 0.01i
Palmitic (C16)	15.0 ± 0.48c	12.2 ± 0.39c	15.2 ± 0.27c	20.9 ± 0.16b	14.4 ± 0.97c	22.6 ± 0.69a
Palmitoleic (C16:1 Δ9)	0.2 ± 0.01i	0.6 ± 0.03e	0.4 ± 0.02g	0.6 ± 0.02f	0.7 ± 0.02h	0.8 ± 0.04i
Hexadecadienoic (C16:2 Δ7. 10)	0.6 ± 0.02h	0.5 ± 0.02e	0.4 ± 0.01g	2.7 ± 0.02e	1.5 ± 0.06g	1.9 ± 0.28h
Hexadecatrienoic (C16:3 Δ4. 7. 10)	0.6 ± 0.01h	0.3 ± 0.03e	0.3 ± 0.01g	0.9 ± 0.03f	0.4 ± 0.01h	0.4 ± 0.03i
Hexadecatrienoic (C16:3 Δ7. 10. 13)	0.7 ± 0.09h	0.8 ± 0.03e	0.5 ± 0.02g	1.2 ± 0.01f	0.7 ± 0.01h	1.2 ± 0.04i
Hexadecatetraenoic (C16:4 Δ4. 7. 10. 13)	4.5 ± 0.08e	10.6 ± 0.38c	4.7 ± 0.07e	2.6 ± 0.16e	9.8 ± 0.13d	7.4 ± 0.17e
Heptadecanoic (C17)	0.4 ± 0.02i	0.3 ± 0.00e	0.3 ± 0.04g	0.5 ± 0.01f	0.2 ± 0.01h	0.4 ± 0.02i
Heptadecenoic (C17:1 Δ11)	0.3 ± 0.01i	0.3 ± 0.02e	0.3 ± 0.04g	0.5 ± 0.01f	0.4 ± 0.03h	0.5 ± 0.03i
Stearic (C18)	1.6 ± 0.12g	1.9 ± 0.32d	4.1 ± 0.06e	2.4 ± 0.01e	1.2 ± 0.01h	2.0 ± 0.03h
Oleic (C18:1 Δ9)	30.3 ± 0.54a	21.8 ± 2.78b	35.9 ± 1.47a	22.7 ± 0.12a	8.2 ± 0.94e	13.6 ± 0.22d
Elaidic (C18:1 Δ9)	2.1 ± 0.09g	2.8 ± 0.08d	2.1 ± 0.19f	2.1 ± 0.06e	1.7 ± 0.45g	3.0 ± 0.11g
Linoleic (C18:2 Δ9. 12)	8.5 ± 0.28d	11.4 ± 0.89c	9.4 ± 0.09d	18.8 ± 0.47c	22.0 ± 1.10b	15.7 ± 0.53c
G-Linolenic (C18:3 Δ6. 9. 12)	27.4 ± 0.10b	0.4 ± 0.04e	0.2 ± 0.06g	15.8 ± 1.11d	1.4 ± 0.04g	0.7 ± 0.05i
Linolenic (C18:3 Δ9. 12. 15)	2.0 ± 0.13g	29.0 ± 1.78a	19.4 ± 0.25b	0.9 ± 0.09f	30.7 ± 0.47a	20.6 ± 0.37b
Steriodonic (C18:4 Δ6. 9. 12. 15)	1.1 ± 0.12h	3.2 ± 0.08d	1.7 ± 0.03f	0.8 ± 0.30f	2.3 ± 0.22f	2.0 ± 0.07h
Eicosenoic (C20:1 Δ11)	0.9 ± 0.04h	Nd	0.7 ± 0.23g	0.8 ± 0.05f	Nd	Nd
Docosanoic (C22:0)	2.7 ± 0.10f	0.04 ± 0.05e	0.5 ± 0.01g	0.4 ± 0.01f	Nd	0.4 ± 0.05i
Docosenoic (C22:1 Δ13)	Nd	2.4 ± 0.08d	2.5 ± 0.01f	3.5 ± 0.03e	3.0 ± 0.07f	4.4 ± 0.03f

^aP20, S20 and M20 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 20 °C; P30, S30 and M30 correspond to *P. boryanum*, *S. obliquus* and mixed culture at 30 °C. Averages followed by different letters differ statistically from each other ($p < 0.01$). Lowercase letters compare the levels of different fatty acids in the total composition of FAME in the same treatment. Mean ± SD (n = 3 cultivations). FAME = fatty acid methyl esters; Nd = not detected.

as a proportion of the total FAMES (Table 6). The most representative fatty acids in all treatments were oleic (8.2-35%), linolenic (0.9-30.7%), linoleic (8.5-22%), palmitic (12.2-22.6%) and gamma-linolenic (0.2-27.4%) acids. Other fatty acids were recorded in concentrations considered moderate to low, for example, docosenoic acid (2.4-4.4%) and stearic acid (1.2-4.1%).

Oleic fatty acid was predominant in treatments P30 (22.7%), P20 (30.3%) and M20 (35.95), which also presented high levels of palmitic (20.9%), gamma-linolenic acid (27.4%) and linolenic acids (19.4%). In S30 (30.7%) and S20 (29%), linolenic was the predominant fatty acid, followed by linoleic (22%) and oleic (21.8%). The M30 treatment was comprised mostly of palmitic (22.6%) and linolenic (20.65%) fatty acids.

4. Discussion

4.1. Growth

Although the treatments with the highest K and the shortest G were P30 and P20, the Y_{\max} values for both were statistically lower than those of S30, S20, M30 and M20. When compared to monocultures, mixed cultures showed no increase in biomass and the increase in cell density was only higher relative to the monoculture of *P. boryanum*. Additionally, there was no notable influence of temperature on the calculated parameters. These results differ from those of Phatarpekar et al. (2000), Arkronrat et al. (2016) and Arkronrat & Oniam (2014) but are similar to the results observed in one treatment in the study by Huang et al. (2011); although all of these studies have worked with mixed cultures, the species and cultivation conditions applied were different from those used in the present study.

The P30 and P20 cultures were the only ones to present a two-day growth induction phase; the stationary growth phase commenced on days 5 and 9 in P30 and P20, respectively. Cultures S20 and M20 did not present an adaptation phase and remained in the exponential phase of growth until the last day of cultivation. The S30 and M30 cultures also did not present an adaptation phase, yet on day 19 showed signs of entry into the steady-state growth phase on day 19 (Table 1).

Although the species in this study are considered cosmopolitan and adaptable to different environmental conditions, there was a dominance relationship in mixed cultures where *S. obliquus* exhibited much higher growth than *P. boryanum* and corresponded to about 80% of the total culture density. Characteristics such as the size and

complexity of the reproductive forms affect the growth rate; when competing for resources, smaller species grow faster. This faster growth rate is because these species have an advantageous surface: volume ratio that facilitates the assimilation of nutrients and available carbon into the culture medium (Phatarpekar et al., 2000; Arkronrat et al., 2016).

4.2. Total carbohydrates, water-soluble proteins and total lipids

Past studies indicate an increase of total protein and lipid concentrations as a function of increasing temperature (Juneja et al., 2013; Sayegh & Montagnes, 2011). However, this trend was not observed in *S. obliquus* and *P. boryanum* cultures, likely due to their specific growth conditions.

Koru & Cirik (2003) cultivated the Cyanophyceae *Spirulina platensis* (Gomont) Geitler microalgae, commonly used as a food supplement, and noticed a protein content of 58.3% DW in cultures performed at 30 °C, which decreased to 45.7% at 43 °C. Similarly, Rhee & Gotham (1981) working with *Scenedesmus* sp., reported this same trend in decreased protein concentration with increasing temperature. In the present study, the protein concentrations increased significantly, from 466.6 to 672.6 mg.g⁻¹ DW at 30 and 20 °C, respectively, only in the monoculture of *P. boryanum*.

The biomass produced in the mono and mixed cultures of *S. obliquus* and *P. boryanum* presented protein values considered suitable for application in aquaculture and animal feeding (Tibbetts et al., 2015a). Cultures of *Scenedesmus* sp., with protein content similar to this study (32-44% DW), presented six of the ten essential amino acids in higher concentrations than those found in plants, such as soybean and corn (Tibbetts et al., 2015a).

The protein contents found in the cultivation in this research are similar or superior to those described in the literature for plants, such as soybean (40.4% DW in grain) and bran (46.7% DW) (Silva et al., 2006) with variations from 36.82 to 39.85% DW, depending on the region of the plant in which the grain was harvested (Sales et al., 2016). The values found in the present study are higher than those found in quinoa (14.1% DW), maize (9.4% DW) and rice (6.8% DW) (Nowak et al., 2016).

Contrary to proteins and lipids, there is no effect of temperature on carbohydrate concentrations (Piorreck et al., 1984; Sayegh & Montagnes, 2011). Fast-growing cells typically have a high protein content and low carbohydrate content.

The opposite effect is expected with slow growth, when more carbon is directed towards the synthesis of carbohydrates and lipids than proteins (Henderson & Sargent, 1989; Piorreck et al., 1984; Zhu et al., 1997).

In this study, total carbohydrate levels were below average when compared to other studies on microalgae and vegetables. For instance, 10-17% carbohydrate (DW basis) was recovered from *S. obliquus* (Um & Kim, 2009), and *S. platensis*, grown at 30 °C, presented 29.7% DW basis that significantly increased to 37.6% when the temperature was raised to 43 °C (Koru & Cirik, 2003). Even greater values (581.4 mg.g⁻¹ DW) were observed in *Scenedesmus* sp., AMDD, grown in wastewater at 20 °C (Dickinson et al., 2015).

For many species, the total lipid content varies according to growth stage. Values close to the present study were recorded for *Selenastrum bibraianum* Reinsch (4.7-7.73% DW), *Scenedesmus quadricauda* Chodat (6.9-10.6% DM), *Scenedesmus falcatus* Chodat (6.4-9.6% DW), *Fragilaria* sp. (8.4-9.9% DW), *Chlorococcum* sp. (8.7-10.4% DW) and *Chlorella* sp. (9.8% DW) (Abdelkhalek et al., 2016). Total lipid contents higher than those noted for the monoculture of *S. obliquus* (8.3% DW at 30 °C), were previously reported, indicating a production of up to 27% DW (Mata et al., 2013).

According to the results presented in this study, there is potential use of this biomass as an alternative supplement for human and animal food. The microalgae of the genus *Chlorella*, *Haematococcus*, *Dunaliella*, *Spirulina*, *Nannochloropsis*, *Scenedesmus* and *Schizochytrium* are already globally commercialized for this purpose and represent more than 95% of the current market (Matos et al., 2016; Souza et al., 2018).

The lack of change of carbohydrate and protein concentrations in mixed cultures with the change in temperature suggests that these mixed cultivations are more stable than the monocultures. They are also better suited to withstand great thermal variations, which can vary up to 20 °C in a single day. This stability ensures uniformity in the biochemical composition of the biomass generated and a constant productivity of the cultivations grown outdoors (Alabi et al., 2009; Coplin, 2012; Smith et al., 2010).

The present study showed an increase in the concentration of total lipids at 20 °C, implying that when targeting the production of these

compounds, lower temperatures are recommended when cultivating *P. boryanum* and *S. obliquus* in pre-established nutrient and light conditions.

4.3. Chlorophyll 'a' and total carotenoids

Cultivation time had a marked impact on the production of photosynthetic pigments in all treatments except for P20 and S20, which did not show a significant increase in chlorophyll 'a' content at the end of the experiment. Similar results were recorded in mixed cultures of *Chlorella vulgaris* Beyerinck [Beijerinck] and *Hyaloraphidium contortum* Pascher & Korshikov. In that instance, the chlorophyll 'a' concentration increased from 0.41-0.98 to 0.66-3.17 µg.mL⁻¹ and total carotenoids from 0.19-0.39 to 0.38-1.11 µg.mL⁻¹ from the 6th to the 24th day, respectively, in each culture (Brito et al., 2013).

In this study, the values for total carotenoids were well below those found in microorganisms currently used for this purpose in the market. The microalgae *Dunaliella salina* (Dunal) Teodoresco produces up to 14% DW of β-carotene, with an estimated yield of 102.5 mg.m⁻².d⁻¹ of total carotenoids and 13.5 mg.L⁻¹.d⁻¹ β-carotene under ideal conditions (Guedes et al., 2011).

4.4. Profile of Fatty Acid Methyl Esters (FAMES)

MUFAs are an easily digestible energy source. Moreover, in contrast to SFAs, which are non-essential nutrients that increase low-density lipoprotein cholesterol in the blood, MUFAs and polyunsaturated fatty acids (PUFAs) both reduce it, with PUFAs being more potent than MUFAs. Additionally, PUFAs are important in the prevention of immunological, inflammatory and cardiovascular diseases (Huang et al., 2016; Tibbetts et al., 2015a). The high levels of MUFAs and PUFAs, low content of SFAs in the final lipid fraction of the treatments (notably those cultivations at 20 °C) and an increase in the total FAMES, imply that the biomass of *S. obliquus* and *P. boryanum* is a promising nutrient source.

The mixed cultures (M20 and M30), as well as monocultures of *S. obliquus* (S20 and S30), possessed high levels of the essential fatty acid alpha-linolenic acid, a natural precursor of the ω-3 fatty acid eicosapentaenoic (EPA). These results are explained by the density dominance of *S. obliquus* over *P. boryanum* at the end of the experiment. The alpha-linolenic acid values were also close to or superior to those found in other studies of microalgae, marketed as *Chlorella* sp.

(17.1%) (Kent et al., 2015) and *S. platensis* (1.7%) (Matos et al., 2016). The M20, S20, M30 and S30 cultures also presented lower concentrations of linoleic fatty acid as compared to *Chlorella* sp. (30.4%) and higher values than in *S. platensis* (2%).

The ω -3/ ω -6 ratios recorded in treatments S20 and M20 are optimal. Relatively lower values (2.45:1) were measured in the cultivation of *Scenedesmus* sp. and cultures of *C. vulgaris* (0.31:1), *Micractinium reisseri* R.Hoshina, M.Iwataki & N.Imamura (0.2:1), *Nannochloris bacillaris* Naumann (0.65:1) and *Tetracystis* sp. (0.67:1) (Tibbetts et al., 2015b). The ω -3/ ω -6 ratios established in this study are lower than those verified for fish oils (3-24:1 ω -3/ ω -6), which are the most common dietary source of this fatty acid (Tibbetts et al., 2015b). Nonetheless, the ratios were higher than those found in oils produced from commonly consumed oleaginous plants, such as canola (0.46:1), corn (0.02:1), olive (0.08:1) and soybean (0.13:1) oils (Martin et al., 2006). In this context, the biomass from the algae studied here can be used as a dietary supplement in ω -3-deficient diets.

Although animals can convert linoleic (ω -6) and linolenic (ω -3) fatty acids into arachidonic acid (AA, C20:4) and EPA (C20:5), respectively, their syntheses are affected by several factors (Martin et al., 2006). Among them, the ω -3/ ω -6 ratio is fundamental, because AA and EPA compete for the same lipoxygenase or cyclooxygenase enzymes. Under conditions where there is a low ω -3/ ω -6 ratio, the synthesis of AA is increased, and that of EPA is reduced, causing immune disorders, cardiovascular and inflammatory diseases (Simopoulos, 2004).

5. Conclusion

In this study, the biomass of the analyzed treatments was rich in proteins, essential fatty acids (such as linolenic acid), and had low carbohydrate content, suggesting its potential use as a food supplement. Moreover, the biomass obtained in the S20 treatment presented a high ω -3/ ω -6 ratio. However, risks should be evaluated and further studies are necessary to assess the approximate chemical composition, evaluation of protein quality and quantity of essential amino acids, fiber content, digestibility, biogenic toxic substances and non-biogenic toxic compounds. Additionally, toxicological and safety evaluations should be considered in order to classify this biomass as a safe functional food.

The results obtained from this research reveal that mixed culture of *P. boryanum* and *S. obliquus*

did not efficiently produce biomass or biochemical compounds when compared to the monocultures. However, algae grown in consortium showed greater stability in their biochemical composition in response to changes in temperatures, an important factor for microalgae cultivation in open ponds and for ensuring the food safety. The interactions between cultivated species, apart from their interactions with external factors, must be considered before the application of mixed cultures in open pond systems. Therefore, more studies should test the interaction between species in mixed cultures, with special focus on the use of species with complementary functional groups to maximize the use of the resources such as light and nutrients.

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