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Operation Regimes: A Comparison Based on *Nannochloropsis oceanica* Biomass and Lipid Productivity

Inês Guerra [1,2], Hugo Pereira [3], Margarida Costa [1,*], Joana T. Silva [1], Tamára Santos [4], João Varela [3,4], Marília Mateus [2] and Joana Silva [1]

1 ALLMICROALGAE Natural Products S.A., R&D Department, Rua 25 de Abril s/n, 2445-413 Pataias, Portugal; ines.funico@gmail.com (I.G.); joanatlfsilva@gmail.com (J.T.S.); joana.g.silva@allmicroalgae.com (J.S.)

2 iBB—Institute for Bioengineering and Biosciences, IST, Universidade de Lisboa, Av. Rovisco Pais, nº1, 1049-001 Lisbon, Portugal; marilia.mateus@tecnico.ulisboa.pt

3 GreenCoLab—Associação Oceano Verde, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal; galvaohugo@gmail.com (H.P.); jvarela@ualg.pt (J.V.)

4 CCMAR—Centre of Marine Sciences, University of Algarve, Gambelas, 8005-139 Faro, Portugal; tamarafilipasantos@gmail.com

* Correspondence: costa.anamarg@gmail.com

Abstract: Microalgae are currently considered to be a promising feedstock for biodiesel production. However, significant research efforts are crucial to improve the current biomass and lipid productivities under real outdoor production conditions. In this context, batch, continuous and semi-continuous operation regimes were compared during the Spring/Summer seasons in 2.6 m^3 tubular photobioreactors to select the most suitable one for the production of the oleaginous microalga *Nannochloropsis oceanica*. Results obtained revealed that *N. oceanica* grown using the semi-continuous and continuous operation regimes enabled a 1.5-fold increase in biomass volumetric productivity compared to that cultivated in batch. The lipid productivity was 1.7-fold higher under semi-continuous cultivation than that under a batch operation regime. On the other hand, the semi-continuous and continuous operation regimes spent nearly the double amount of water compared to that of the batch regime. Interestingly, the biochemical profile of produced biomass using the different operation regimes was not affected regarding the contents of proteins, lipids and fatty acids. Overall, these results show that the semi-continuous operation regime is more suitable for the outdoor production of *N. oceanica*, significantly improving the biomass and lipid productivities at large-scale, which is a crucial factor for biodiesel production.

Keywords: tubular photobioreactor; pilot-scale; operation regimes; outdoor cultivation; *Nannochloropsis oceanica*

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1. Introduction

Major climate changes have been observed since 1950, and human impact, due to industrial activity, is one of the main leading causes. The emissions of greenhouse gases, including CO_2, CH_4 and NO_2, have been increasing since the pre-industrial era, and those gases remain in the atmosphere, soil and oceans [1]. This concern emphasizes the importance of fossil fuel replacements, like the ones based on biomass feedstocks for biofuel production [2].

Microalgae are ubiquitous microscopic photosynthetic organisms mainly found in aquatic environments (freshwater and saline), but also on the surface of soil and stone, from deserts to polar sea habitats [3]. These organisms are known to efficiently fix CO_2, through photosynthesis, and convert it into organic matter with an efficiency up to 10-fold faster than terrestrial plants [4]. Additionally, when compared to terrestrial plants, microalgae have the advantage of requiring less area to reach the same amount of biomass, being able

to grow in non-arable soil and having the ability to grow using non-potable water, namely in saline or wastewater [2,5].

Nannochloropsis oceanica is a fast growing microalga known to intracellularly accumulate high amounts of lipid, up to 53% of biomass dry weight [6,7], making this species suitable to be used as biodiesel feedstock [8,9]. This small unicellular marine microalga (2–4 µm in diameter) is an ochrophyte, belonging to the class Eustigmatophyceae [10] and is also of considerable interest as a source of polyunsaturated fatty acids, namely for the production of eicosapentaenoic acid for human disease-prevention [8].

Although significant progress has been made in the final microalgae biodiesel properties [11,12], the high cost of culture growth and biomass harvesting and the limited biomass productivity are still major limitations for the use of this rich biomass as a biodiesel feedstock [13]. Several laboratory studies have shown that the operation regime is crucial to significantly increase biomass and lipid productivity in *Nannochloropsis* sp., underlining the importance of testing it in settings closer to an industrial scenario [14,15].

Biomass production of microalgal feedstocks can be achieved using different operation regimes, namely batch, continuous and semi-continuous [16,17]. A batch operation regime consists of introducing all needed nutrients in the bioreactor, being this culture entirely harvested after the production period [17,18]. Under a continuous regime, the medium and all needed nutrients are continuously added to the cultivation system. At the same time, the culture is continuously removed from the system, at the same flow rate [17,18]. Semi-continuous operation regime is a combination between the batch and continuous operations. Usually, a percentage of the cultures (10–50%) is removed when cultivation reaches the mid to late exponential phase and the volume is replaced with fresh medium [18]. The major difference between these operation regimes is the achieved productivity, which is usually higher in the continuous and semi-continuous systems, as they allow maintaining the culture near the maximum growth rate [19]. On the other hand, the susceptibility to contamination is much lower and the accumulation of target substances is usually higher using the batch regime [19,20]. Therefore, identifying the adequate production regime for effective microalgae production considering all the mentioned factors is of the utmost importance. Although there is no overall better production method, the tendency in bioprocessing has been to adopt increasingly more continuous processes [21].

Production of microalgal biomass can occur using the aforementioned operation regimes, in open and closed systems. Open systems are usually more economically viable and can be divided into three major types, including natural water bodies, circular ponds, raceway ponds, and thin layer cascade systems [6,22]. Nevertheless, closed systems are known to display better growth performances since they limit the direct gas exchange, reduce the contaminants in the culture, and better control important physicochemical variables. Closed systems can be classified into three main groups: vertical column, flat panel, and tubular photobioreactors (PBRs) [23,24].

The present work aimed to cultivate *N. oceanica* in pilot-scale tubular PBRs with the outdoor light and temperature conditions, using three operation regimes: batch, continuous and semi-continuous. The main goal was to identify the most suitable operation regime that can ensure the highest biomass productivity, uses fewer resources while providing high-quality biomass with a high lipid percentage for further biodiesel application.

2. Materials and Methods

The outdoor work was performed at the facilities of Allmicroalgae (Pataias, Portugal) between 1 March and 8 July 2019.

2.1. Microalgae Strain and Culture Media

The microalga *Nannochloropsis oceanica* CCAP849/10 was obtained from the culture collection Algae and Protozoa (Oban, Scotland, UK) and is kept at Allmicroalgae culture collection. The culture medium used for growth assays was Guillard's F/2 medium at

0.31 g/L of NO_3^-, supplemented with 12 µM of iron, 30 g/L of NaCl (Salexpor, Coimbra, Portugal) and magnesium-enriched supplementation (Necton, Faro, Portugal).

2.2. Culture Scale-Up

Initially, the cultures were grown in 5 L airlift reactors, in the laboratory. The aeration of these reactors was made by compressed air pre-mixed with 1% CO_2, to maintain the pH below 8.2, sterilized by 0.2 µm filters (Sartorius, Gottingen, Germany). These reactors were maintained under constant irradiance of approximately 700 µmol of photons m^{-2} s^{-1} at room temperature (24 °C). Five of these 5 L reactors were used to inoculate an outdoor 125 L Flat Panel (FP) PBR, which served as inoculum for an 800 L FP. The aeration conditions were similar to the 5 L reactors, although the CO_2 was added using a pulse system that maintained the pH close to 8.2, and the temperature was maintained below 30 °C by an irrigation system. The 800 L FP was later used to inoculate a 2.6 m^3 tubular PBR. This PBRwas subsequently used to inoculate three 2.6 m^3 tubular PBRs used for the assay. In these systems, the agitation of the culture was performed by pumping the culture through the PBR, using centrifugal pumps. The pH was measured in real-time and kept at 8.2 by an automated system that injected CO_2 on demand. The temperature was maintained below 30 °C through an irrigation system.

2.3. Operation Regime Trial

N. oceanica was grown in three tubular PBRs (Figure 1), each being operated in batch, semi-continuous, or continuous operation regime. The horizontal tubular PBRs used in this trial had a serpentine configuration, with a working volume of 2.6 m^3 and an illuminated volume of 1.6 m^3. In order to begin the assay the PBRs were inoculated atan initial biomass concentration of 0.4 g L^{-1} for the batch regime and 1 g L^{-1} for the continuous and semi-continuous regimes.

Figure 1. Pilot-scale tubular photobioreactors (PBRs) used in the trials with 2.6 m^3 of working volume (Allmicroalgae, Pataias, Portugal).

The cultures were allowed to grow for a day to adapt to the new reactor conditions, and then each of the reactors was operated differently, depending on the correspondent operation regime. The culture grown in the batch operation regime was left to grow until the end of the trial. In the semi-continuous regime, the culture was left to grow for two days and on the second day it was diluted to 1 g L^{-1}. This dilution process was repeated every

two days until the end of the trial. In the continuous system, the culture was continuously diluted using peristaltic pumps; the flow rate was adjusted daily, to maintain the culture at around 1 g L^{-1}. Simultaneously, the culture was also continuously removed from the PBR, at the same rate. In all conditions, the Guillard's F/2 medium supplemented with iron was added manually when needed, in order to maintain the NO_3^- concentration between 0.12 and 0.31 g L^{-1}. Three replicates of each trial were performed, with a consecutive rotation of the PBR used in an operation regime.

2.4. Growth Assessment

Culture growth was followed daily through optical density (OD) until the culture operated in batch reached the stationary phase. The OD was measured at 540 nm in a UV/Vis spectrophotometer (Zuzi, Seville, Spain) while the dry weight (DW) was obtained through a calibration curve established previously (Figure S1). The DW was determined by filtering a known amount of culture through a 0.7 µm glass microfiber filter (VWR International, Radnor, PA, USA), which was later washed with an equal volume of 35 g L^{-1} ammonium formate (Biochem Chemopharma, Cosne-Cours-sur-Loire, France).

The specific growth rate was calculated for the batch operated cultures through Equation (1), where X_1 and X_2 represent, respectively, the cellular concentration in the beginning and end of the exponential phase and t_2 and t_1 are the times, in days, corresponding to those respective concentrations.

$$\mu\left(\text{day}^{-1}\right) = \frac{ln\frac{X_2}{X_1}}{t_2 - t_1} \tag{1}$$

The volumetric productivity (P) was calculated as the ratio of the sum of the produced biomass in each day considered ($m_{produced}$, g) by the total reactor volume (V_t, L) and whole growth time (t, day), as shown in Equation (2). The daily produced biomass was calculated by multiplying the biomass concentration reached in that day (X_i, g L^{-1}) by the respective removed volume (V_{ri}, L). The only exception was the last day of the whole cycle, day n, in which the entire working volume of the reactor was processed, being the produced biomass calculated by the difference of the final and initial cell concentration of the last day (X_i and X_0, g L^{-1}) multiplied by the total working volume of the last and first day

$$P\left(\text{gL}^{-1}\text{day}^{-1}\right) = \frac{\sum m_{produced}}{V_t t} \tag{2}$$

$$\begin{aligned} \sum_{i=1}^{n-1} m_{produced} &= X_i V_{ri} \\ \sum_{i=n}^{n} m_{produced} &= (X_i - X_0) V_t \end{aligned} \tag{3}$$

Areal biomass productivity (P_a) (Equation (4)) was determined by multiplying the volumetric biomass productivity by the volume of the reactor (V_t) divided by the ground area occupied by the reactor (A, m^2).

$$P_a\left(\text{gm}^{-2}\text{day}^{-1}\right) = \frac{P\ V_t}{A} \tag{4}$$

The photosynthetic efficiency (PE) was determined by the ratio between the increase of the higher heating value (HHV) and the total sun irradiation that reached the reactor (Equation (5)). The outside solar radiation and temperature were measured using a Watch-Dog 2000 weather station (Spectrum Technologies. Inc., Aurora, IL, USA). The specific HHV (HHV, kJ g^{-1}) was calculated according to a previous correlation reported by [25], present

in Equation (6), where C represents the percentage of carbon, H the percentage of hydrogen and N the percentage of nitrogen obtained by the CHN analysis of the final biomass.

$$PE\ (\%) = \frac{HHV * \left(biomass_{final} - biomass_{initial}\right)}{Total\ incident\ radiation} \times 100 \quad (5)$$

$$HHV\ \left(\text{kJ g}^{-1}\right) = -3.393 + 0.507\text{C} - 0.341\text{H} + 0.067\text{N} \quad (6)$$

The nitrogen source concentration was measured at least once a day and added when needed. Nitrates were determined according to Armstrong (1963), modified [26]. Briefly, the collected supernatant was diluted and hydrochloric acid was added at 30 mM. The absorbance of samples was measured spectrophotometrically (4251/50, Zuzi, Seville, Spain) at 220 and 275 nm. The organic matter interference was corrected by subtracting twice the absorbance read at 275 nm from the reading at 220 nm. The final absorbance was compared to a sodium nitrate calibration curve (Figure S2).

2.5. Elemental Analysis

The biomass was collected and centrifuged (Hermle Labortechnik Z300, Wehingen, Germany) at 2050× *g* for 15 min at the end of each culture trial. The resulting pellet was frozen and stored at −18 °C. Before the biochemical analysis was performed, the biomass was freeze-dried (Telstar, Lisbon, Portugal).

The lyophilized biomass was weighed into aluminum vessels, using a precision balance and inserted in a Vario el III (Vario EL, Element Analyser System, GmbH, Hanau, Germany). The CHN composition was determined according to the procedure provided by the manufacturer. The total protein was determined by multiplying the percentage of nitrogen by the factor 6.25 [10].

The total ash content was determined by gravimetric analysis. Samples were weighed before and after being burned in a muffle (J. P. Selecta, Sel horn R9-L, Barcelona, Spain) for 8 h at 550 °C.

The total lipid content was determined following the Bligh and Dyer method (1959) [27] with few modifications [28]. In brief, lipids were extracted using a mixture of distilled water, chloroform, methanol (1.8:2:2 *v*/*v*/*v*) (Fisher Chemical, NH, USA) and an IKA Ultra-Turrax disperser (IKA-Werke GmbH, Staufen, Germany), for homogenization. Afterwards, the mixture was centrifuged at 2000× *g* for 10 min for phase separation. The organic phase was transferred to a clean tube with a Pasteur pipette and later a known volume of the chloroform phase was pipetted to a pre-weighed tube and placed in a dry bath at 60 °C. Lipids were gravimetrically determined after the chloroform evaporation.

The carbohydrate content was determined by subtracting the weight of proteins, lipids and ashes from the total DW of biomass.

2.6. Fatty Acid Profile

Fatty acids were converted into the corresponding fatty acid methyl esters (FAME) according to the protocol of Lepage and Roy (1984) [29], modified by Pereira et al. (2011) [30]. FAME were analyzed in a GC-MS analyzer (Bruker SCION 456/GC, SCION TQ MS, MA, USA) equipped with a ZB-5MS column (length of 30 m, 0.25 mm of internal diameter, 0.25 µm of film thickens, Phenomenex), using helium as the carrier gas. The temperature program was 60 °C for 1 min, an increase of 30 °C per min up to 120 °C, an increase of 5 °C per min up to 250 °C, and a final increase of 20 °C per min up to 300 °C. The temperature in the injector was 300 °C. For the identification and the quantification of FAME five different concentration of Supelco 37 component FAME Mix standard (Sigma-Aldrich, Sintra, Portugal) were analyzed in order to establish 37 different calibration curves. Then the peak area of each component in each sample was compared to the correspondent calibration curve in order to have a quantitative analysis of that specific FAME.

2.7. Statistical Analyses

Statistical analyses were performed using R software (version 3.6.1) through the RStudio IDE (version 1.2.1335). Experimental results are presented with a 95% confidence level. The normal distribution of data was tested through the Shapiro–Wilk test and the data homogeneity was tested through the Bartlett test. The data was then compared using one-way ANOVA, followed by Tukey's multiple comparison tests. The non-homogeneous data was compared with the Kruskal–Wallis test followed by Dunn's test.

3. Results and Discussion

3.1. Growth Performance

N. oceanica was grown under batch, semi-continuous and continuous operation regimes in three 2.6 m^3 horizontal tubular PBRs, during three consecutive replicas, and the temperature and the incident solar radiation were registered in the three independent trials (Figure 2). The average ambient temperature throughout the whole trial was 17.5 ± 0.8 °C, with a maximum and minimum of 21.1 ± 2.8 °C and 15.6 ± 2.3 °C, respectively. The temperature of the culture inside the reactor was usually higher than the ambient temperature, but maintained below 30 °C through the thermoregulation system. The average daily solar radiation throughout the trial was 20.1 ± 3.5 MJ m^2, with a maximum registered average daily radiation of 23.8 ± 2.4 MJ m^{-2} and a minimum of 15.4 ± 1.6 MJ m^{-2}.

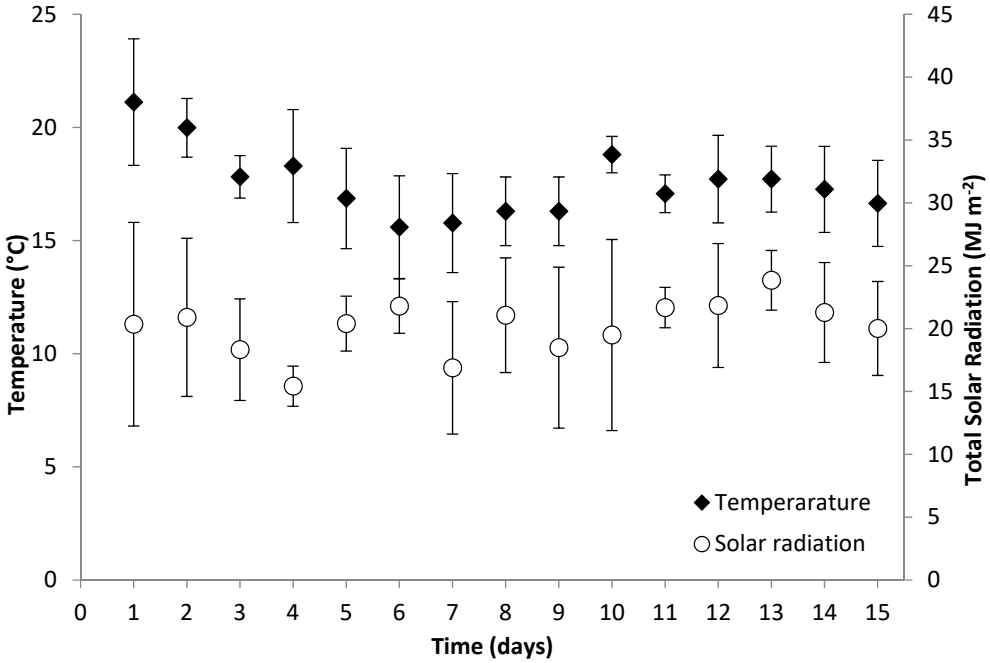

Figure 2. Average daily ambiance temperature and total solar radiation incident in the 2.6 m^3 PBRs, while culturing *Nannochloropsis oceanica*. The presented values are the average values obtained in three biologically independent replicates and the error bars represent the respective standard deviations.

The assay lasted until the batch culture ceased its growth, which happened on the 15th day with a maximum DW of 2.0 g L^{-1} (Figure 3). The batch operated culture presented no lag phase and an exponential phase of 9 days with amaximum specific growth rate of 0.129 day^{-1}, calculated from the 4th to 9th culture day.

The semi-continuous culture was renewed every second day, beginning on the 3rd day and was renovated five more times during each trial. The water volumes spent in each renovation are shown in Figure 4 and ranged between 17.8 ± 1.8% and 45.4 ± 1.0% of the total volume, having an average dilution rate of 0.122 ± 0.011 day^{-1} (Table 1). The culture DW ranged from 1.0 to 1.5 g L^{-1}. The continuous operated culture started to be diluted on the 2nd day, presenting maximum and minimum renovation volumes of 15.5 ± 0.5% and

12.2 ± 1.4% of total volume, respectively (Figure 4). The average dilution rate throughout the whole trial was 0.140 ± 0.010 day^{-1}. After the second day, until the end of the trial, it was possible to carry a steady-state, with the DW ranging from 1.1 to 1.3 g L^{-1}.

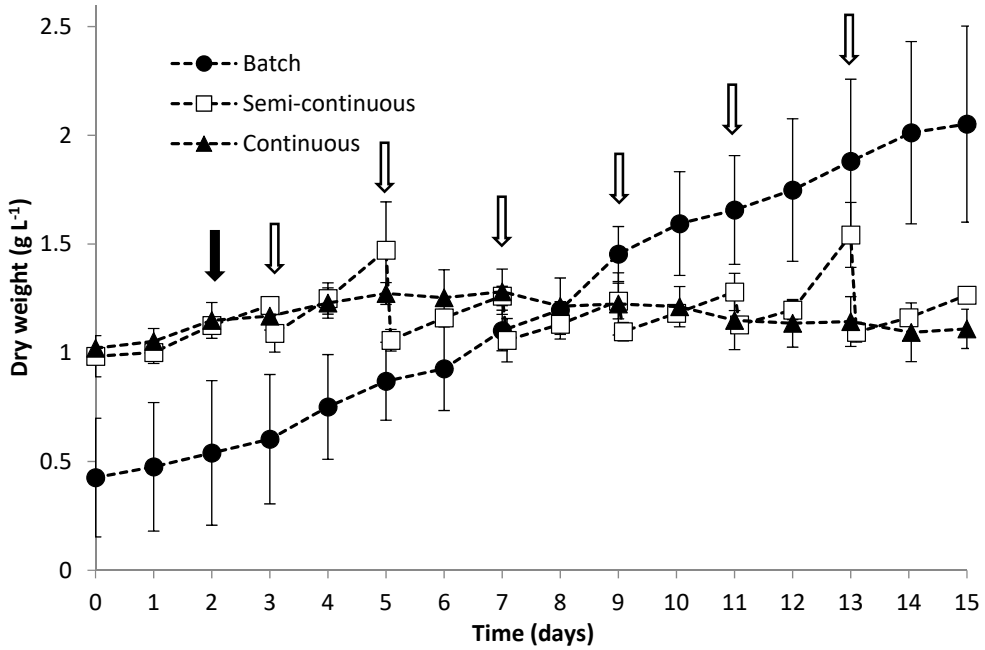

Figure 3. Growth of *Nannochloropsis oceanica* in 2.6 m^3 tubular PBRs using three different operation regimes: batch, semi-continuous and continuous. The values presented are the average of the three independent biological replicates, and the error bars are the respective standard deviations. The black arrow represents the day in which the continuous regimes started to be diluted, and the white arrows represent the moments of medium renewals under the semi-continuous regime.

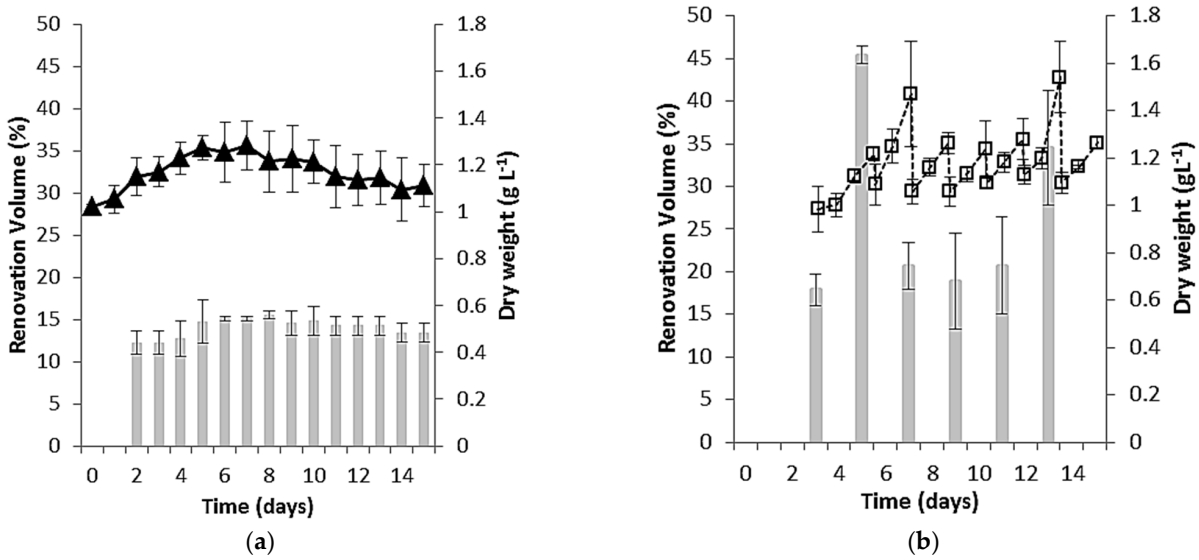

Figure 4. *Nannochloropsis oceanica* growth in 2.6 m^3 PBRs in continuous (**a**) and semi-continuous (**b**) operation regimes. The bars represent the percentage of volume renewed with fresh medium. All the values represent an average of three biologically independent replicates, with the respective standard deviation.

In terms of total water used (Table 1) the batch regime was, by far, the one that needed the least amount of water, more specifically only the initial 2.6 m^3. It was followed by the

semi-continuous and continuous operation regimes that used over 2.5 times the water used in the batch regime. Spent water per one kg of produced biomass revealed no significant differences between the semi-continuous and continuous operation systems. However, these values (1.04 ± 0.03 m^3 kg^{-1} and 1.25 ± 0.14 m^3 kg^{-1} respectively) were almost twice those verified for the batch regime (0.62 ± 0.07 m^3 kg^{-1}). Yet, these values do not consider the water spent during cleaning, which would considerably increase the water spent in the batch regime, since the reactor would have to be cleaned at the end of each cycle (±every 15 days). For the other two operation regimes, cultivation would only be discontinued for cleaning if contamination occurred.

Table 1. Specific growth rate, average dilution rate and amount of water spent, either per cycle or per amount of produced biomass, in the growth of *Nannochloropsis oceanica* in 2.6 m^3 tubular PBRs, in three different operation regimes. The values represent the average and standard deviation of three biologically independent replicates. Different letters within the same column represent significantly different values (p-value < 0.05).

Production Regime	Specific Growth Rate (Day^{-1})	Water (m^3/Cycle)	Water (m^3 kg^{-1} Produced Biomass)	Average Dilution Rate (Day^{-1})
Batch	0.129 ± 0.020	2.6 ± 0.0 ^{a}	0.62 ± 0.07 ^{a}	-
Semi-continuous	-	6.7 ± 0.4 ^{ab}	1.04 ± 0.03 ^{b}	0.122 ± 0.011 ^{a}
Continuous	-	7.4 ± 0.1 ^{b}	1.25 ± 0.14 ^{b}	0.140 ± 0.010 ^{a}

The total and maximum volumetric and areal productivities were calculated and the values obtained are presented in Table 2. The semi-continuous and continuous operation regimes reached similar productivity values, with a volumetric productivity of 0.165 ± 0.013 and 0.154 ± 0.021 g L^{-1} day^{-1}, respectively, and areal productivities of 16.3 ± 1.3 and 15.2 ± 2.0 g m^{-2} day^{-1}, respectively. On the other hand, these values were around 1.5-fold higher than the volumetric (0.108 ± 0.01 g L^{-1} day^{-1}) and areal productivity (10.7 ± 1.1 g m^{-2} day^{-1}) obtained by the batch regime. The batch and continuous operation systems presented similar values regarding the maximum volumetric and areal productivities, respectively, 0.333 ± 0.036 g L^{-1} day^{-1} and 0.266 ± 0.028 g L^{-1} day^{-1}, while the semi-continuous showed a significantly higher value of 0.427 ± 0.020 g L^{-1} day^{-1}.

Table 2. Volumetric and areal productivities of biomass during the whole test, and the maximum in a short interval, in the cultivation of *Nannochloropsis oceanica*, for three operation regimes in 2.6 m^3 PBRs. The values represent the average and standard deviation of three biologically independent replicates. Different letters within the same column represent significantly different values (p-value < 0.05).

Production Regime	Volumetric Productivity (g L^{-1} day^{-1})	Maximum Volumetric Productivity (g L^{-1} day^{-1})	Areal Productivity (g m^2 day^{-1})	Maximum Areal Productivity (g m^2 day^{-1})	Photosynthetic Efficiency (%)
Batch	0.108 ± 0.011 ^{a}	0.333 ± 0.03 ^{a}	10.7 ± 1.1 ^{a}	31.6 ± 3.4 ^{a}	0.358 ± 0.016 ^{a}
Semi-continuous	0.165 ± 0.013 ^{b}	0.427 ± 0.020 ^{b}	16.3 ± 1.3 ^{b}	40.4 ± 1.9 ^{b}	0.436 ± 0.043 ^{a}
Continuous	0.154 ± 0.021 ^{b}	0.266 ± 0.028 ^{a}	15.2 ± 2.0 ^{b}	25.2 ± 2.6 ^{a}	0.481 ± 0.073 ^{a}

The registered batch productivity value is in accordance with the 0.15 g L^{-1} day^{-1} reported by Quinn et al. (2012) for flat panel outdoor batch growth of *Nannochloropsis oculata* in the same period of the year (May to June) in the northern hemisphere (Fort Collins, CO, USA) [31].

Ledda et al. (2015) and Chini Zittelli et al. (1999) reported productivities of 0.48 g L^{-1} day^{-1} in a 340 L vertical tubular reactor operated in a semi-continuous regime, with a dilution rate of 0.33 day^{-1}, and 0.56 g L^{-1} day^{-1} for a 36.6 L horizontal tubular reactor operated in the semi-continuous regime, in the same period of the year in the northern hemisphere (Florence, Italy), respectively [32,33]. These values are higher than the 0.165 ± 0.013 g L^{-1} day^{-1} obtained in this study for semi-continuous production of *N. oceanica*. These differences are probably related to the different size and geometries of the

PBRs used in the different works, and the optimized dilution rate used by Ledda et al. (2015). In addition, Ledda et al. (2015) reported a lower maximum areal productivity of 27 g m^{-2} day^{-1}, against 40.4 ± 1.9 g m^{-2} day^{-1} in this study, suggesting that the PBR configuration used by the authors is less efficient than the one used in the present study, in what concerns the occupied area [32]. In a laboratory study, Cai et al. (2013) reported volumetric productivity of 0.068–0.092 g L^{-1} day^{-1} for the batch regime, against 0.087–0.121 g L^{-1} day^{-1} for the semi-continuous operation regime when growing *Nannochloropsis salina* in a 2 L flask reactor and a constant photosynthetic photon flux of approximately 200 µmol $m^{-2}s^{-1}$ with a harvesting frequency of three times a week [14]. However, the productivity values cannot be directly compared with those of the present study, as there is a significant gap in the volumes used. It is important to note, though, that similarly to the present study, Cai et al. (2013) also reported an increase in volumetric productivity when switching the operation regime from batch to semi-continuous [14].

The continuous operation regime presented a volumetric productivity of 0.154 ± 0.021 g L^{-1} day^{-1}, for a dilution rate of 0.14 day^{-1}. This value of global productivity is in the range of the ones reported by Camacho-Rodriguez et al. (2014) for *Nannochloropsis gaditana* in a 2.5 m^3 tubular PBR of 0.12–0.20 g L^{-1} day^{-1} with similar temperature and radiance conditions in Almeria, Spain, for a dilution rate of 0.3 day^{-1} [34]. Despite this, San Pedro et al. (2014) reached maximum volumetric productivity of 0.25 g L^{-1} day^{-1}, for *N. gaditana*, in an outdoor 340 L vertical tubular PBR with 0.1 day^{-1} dilution rate in similar weather conditions [15]. This higher volumetric productivity is probably the consequence of the different configurations and considerably smaller scale used by the author [15].

In what concerns photosynthetic efficiency, there were no significant differences between the 0.358 ± 0.016, 0.436 ± 0.043 and 0.481± 0.073% obtained using batch, semi-continuous and continuous operation regimes, respectively. During the present study, lower photosynthetic efficiency values than the ones reported in the literature were obtained: 0.358–0.481%, in contrast with the 1.2–1.8% obtained during the cultivation of *Nannochloropsis* sp. in an outdoor 0.56 m^3 horizontal tubular reactor [35]. The reason can be attributed to the higher illuminated area in the DeVree et al. (2015) study, 73% [35] against 61%.

The maximum volumetric and maximum areal productivities were higher when using a semi-continuous regime, even though this regime did not present a significantly different global volumetric productivity when comparing with the batch and continuous operation regimes.

3.2. Biochemical Profile

The biomass produced using the different operation regimes was biochemically characterized at the end of the trial (Table 3).

Table 3. Proximate composition, in percentage of total dry weight, of *Nannochloropsis oceanica* grown in 2.6 m^3 tubular PBRs, using different operation regimes. For proteins and lipids, the values represent the average and standard deviation of two biologically independent replicates and two analytical replicates. Different letters within the same column represent significantly different values. Regarding ashes and, consequently, carbohydrates, the presented values are the average of two biological replicates and the minimum and maximum values (p-value < 0.05).

Production Regime	Protein (%)	Lipids (%)	Ash (%)			Carbohydrates (%)		
			Average	Min	Max	Average	Min	Max
Batch	29.6 ± 3.6 [a]	22.0 ± 3.1 [a]	10.7	9.4	11.9	37.8	34.7	40.8
Semi-continuous	28.6 ± 2.8 [a]	24.0 ± 5.3 [a]	14.9	13.0	16.8	32.5	26.4	38.6
Continuous	28.9 ± 2.5 [a]	19.1 ± 2.5 [a]	12.9	12.0	13.8	39.1	36.4	41.8

The macronutrient composition of *N. oceanica* biomass, regarding proteins and lipids, ranged between, 28.6 ± 2.8 to 29.6 ± 3.6%, and 19.1 ± 2.5 to 24.0 ± 5.3% of biomass DW, respectively. No significant differences were observed regarding macronutrients' composition between the biomass produced among the different operation regimes. The

global lipid productivity (Table 4) was 22.9, 39.6 and 29.5 mg L^{-1} day^{-1}, for the batch, semi-continuous and continuous operation regimes, respectively; the productivity in the semi-continuous regime was significantly higher than that obtained under batch conditions.

Table 4. Global lipid productivity in the cultivation of *Nannochloropsis oceanica*, for each of the operation regimes in 2.6 m^3 PBRs. The values represent the average and standard deviation of three biologically independent replicates. Different letters within the same column represent significantly different values (p-value < 0.05).

Production Regime	Lipid Productivity (mg L^{-1} day^{-1})
Batch	22.9 ± 3.7^{a}
Semi-continuous	39.6 ± 3.1^{b}
Continuous	29.5 ± 3.9^{ab}

San Pedro et al. (2014) obtained an average protein content of 36.9% in biomass DW of *N. gaditana* grown in 340 L outdoor tubular reactors operated in continuous mode, for dilution rates varying from 0.1 to 0.35 day^{-1} [15]. The same study reported a lipid content of biomass DW within the range 17.7 and 26.7%, values similar to the ones obtained in this work [15]. In the present study, the lipid content was the same in all the operation regimes, which was also verified by Zhang et al. (2014) when growing *Nannochloropsis* sp. in continuous and batch regimes using 2 L glass bubble column PBRs at the laboratory scale [36]. Regarding the lipid productivity, the values in the present study (22.9–39.6 mg L^{-1} day^{-1}) were higher than the ones obtained by Nogueira et al. (2020) for *N. gaditana* grown in an outdoor 100 L vertical column PBR operated in the semi-continuous regime with a dilution rate of 0.5 day^{-1} in winter at Porto Santo, Portugal (7.2–17.82 mg L^{-1} day^{-1}) [37]. The difference can be attributed to the temperature and irradiance differences between seasons since the literature shows a positive correlation between temperature and lipid content in *Nannochloropsis* sp. [38,39]. San Pedro et al. (2014) reported lipid productivity values between 50–60 mg L^{-1} day^{-1} in a 340 L vertical tubular PBR operated in continuous mode using a 0.1 day^{-1} dilution rate under similar weather conditions. The higher values are due to higher biomass productivity achieved by the authors, since the attained lipid content was similar (20–30%) [15]

The lipid content values reached in the present study in a tubular PBR for *N. oceanica* (19.1–24.0%) were higher than the ones obtained by Cunha et al. (2020) (13.2–19.0%), in a raceway reactor using the same strain, in the same location and time of the year [40]. This result highlights that the tubular horizontal PBR produces biomass with higher quality for biofuel than that cultivated in open systems.

The FAME profile represents an important factor for biofuel microalgal biomass applications. The FAME profiles obtained in this study are presented in Table 5; it shows only the fatty acids above 0.50% of total FAME.

The major FAME observed in *N. oceanica* grown in an outdoor 2.6 m^3 tubular reactor were C16:1 and C16:0, together representing more than 60% of total FAME, followed by C18:1 and C20:5 and C14:0. Comparing the three operation regimes, there were only significant differences in the C16:0 and in the C18:0, which were higher in the semi-continuous than in the batch operation regime, not being significantly different from the continuous regime. In terms of saturation ratios, the present work obtained a higher percentage of saturated fatty acids (SFA), around 45% of total FAME, against around 35% of total FAME obtained by San Pedro et al. (2014) in an outdoor tubular reactor [15]. Additionally, in the present study the percentage polyunsaturated fatty acids (PUFA) was lower than that commonly reported in the literature, 10–14% of total FAME, against the 20% of total FAME, reported by San Pedro et al. (2014) [15]. Regarding the saturation degree, the only significant difference was a higher percentage of SFA in the semi-continuous operated culture (46.19 ± 1.64%) when compared to the batch (41.52 ± 1.60%). This difference did not spread to the PUFA percentage and PUFA/SFA ratio, which was not significantly different between the operation regimes, ranging between 10.45–13.73% and 0.23–0.33, respectively.

Table 5. Fatty acid methyl esters (FAME) content and profile, presented in percentage of total FAME and saturation distribution of the FAME profile in three different operation regimes of *Nannochloropsis oceanica* grown in 2.6 m^3 outdoor tubular PBR. The values represent the average and standard deviation of two biologically independent replicates and two analytical replicates. Different letters within the same row represent significantly different values (p-value < 0.05).

FAME	Batch	Semi-Continuous	Continuous
C 14:0 (%)	6.88 ± 0.44 ^{a}	5.99 ± 0.66 ^{a}	6.02 ± 0.39 ^{a}
C 16:1 (%)	32.79 ± 0.55 ^{a}	32.28 ± 0.65 ^{a}	32.28 ± 1.79 ^{a}
C 16:0 (%)	33.58 ± 1.76 ^{a}	38.24 ± 2.10 ^{b}	36.20 ± 0.94 ^{ab}
C 18:2 ω6 (%)	0.75 ± 0.09 ^{a}	0.59 ± 0.59 ^{b}	0.76 ± 0.08 ^{a}
C 18:1 (%)	11.96 ± 2.98 ^{a}	11.07 ± 0.76 ^{a}	12.40 ± 2.46 ^{a}
C 18:0 (%)	1.05 ± 0.26 ^{a}	1.95 ± 0.26 ^{b}	1.59 ± 0.19 ^{ab}
C 20:4 ω6 (%)	1.85 ± 0.48 ^{a}	1.42 ± 0.30 ^{a}	1.46 ± 0.11 ^{a}
C 20:5 ω3 (%)	11.12 ± 3.4 ^{a}	8.44 ± 2.00 ^{a}	9.16 ± 0.08 ^{a}
SFA (%)	41.52 ± 1.60 ^{a}	46.19 ± 1.64 ^{b}	43.80 ± 1.40 ^{ab}
MUFA (%)	44.76 ± 2.43 ^{a}	43.36 ± 0.92 ^{a}	44.68 ± 067 ^{a}
PUFA (%)	13.73 ± 1.00 ^{a}	10.45 ± 2.31 ^{a}	11.52 ± 0.88 ^{a}
PUFA/SFA	0.33 ± 0.11 ^{a}	0.23 ± 0.06 ^{a}	0.26 ± 0.03 ^{a}

Literature reports that a higher percentage of SFA is necessary for a good biodiesel production feedstock as the percentage of SFA is positively correlated with the cold filter plugging point (CFPP) and the cetane number, both important parameters in biodiesel quality [41,42]. Chen et al. (2012) reported a *Nannochloropsis* sp. biomass with a similar FAME profile and SFA content (35%) from which resulted a biodiesel with an HHV comparable to fossil fuels [43].

4. Conclusions

Nannochloropsis oceanica was successfully grown in pilot-scale tubular PBRs in batch, semi-continuous and continuous operation regimes, with the outdoor light and temperature conditions. Regarding biomass productivity, the semi-continuous and continuous regimes achieved higher values when compared to the batch regime. In terms of protein and lipids (19–24%) content as well as in the fatty acid profile there were no significant differences between the three operation regimes. In addition, the semi-continuous operation regime allowed obtaining higher lipid productivities, indicating that it was the most promising operation regime to grow *N. oceanica* as biodiesel feedstock.

Nevertheless, further work in biomass productivity optimization is needed, such as the optimization of dilution rates in the semi-continuous and continuous operation regimes. The ideal period of the day to harvest the biomass also needs to be explored to maximize lipid content and the SFA/PUFA ratio.

Supplementary Materials: The following are available online at https://www.mdpi.com/1996-1073/14/6/1542/s1. Figure S1: Correlation between the optical density at 540 nm and the dry weight of an autotrophic culture of Nannochloropsis oceanica. Figure S2: Correlation between the difference of the optical density at 220 nm and two times the optical density at 275 nm and nitrate concentration in mM. Figure S3: Microscopic picture (400×) of N. oceanica grown in Allmicroalgae's pilot-scale horizontal tubular photobioreactors.

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