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Fucoxanthin production from *Tisochrysis lutea* and *Phaeodactylum tricornutum* at industrial scale

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ABSTRACT

Fucoxanthin is a xanthophyll carotenoid with high market value. Currently, seaweeds are the primary source for fucoxanthin industrial production. However, marine microalgae reach 5 to 10 times higher concentrations (2.24 to 26.6 mg g^{-1} DW) and are considered a promising feedstock. In this work, two marine microalgae were produced at industrial scale to evaluate biomass and fucoxanthin production: *Phaeodactylum tricornutum* for autumn/winter and *Tisochrysis lutea* for spring/summer. Both strains were grown in 15 m^3 tubular flow-through photobioreactors; for 170 consecutive days of semi-continuous cultivation regime. The average volumetric biomass productivities of *P. tricornutum* and *T. lutea* were 0.11 and 0.09 g DW L^{-1} day^{-1}. *P. tricornutum* reached higher maximum biomass concentration (2.87 g DW L^{-1}) than *T. lutea* (1.47 g DW L^{-1}). *P. tricornutum* fucoxanthin content ranged between 0.2 and 0.7% DW, while *T. lutea* between 0.2 and 0.6% DW. The fucoxanthin content was correlated with the irradiation (MJ m^{-2}) and biomass concentration in the photobioreactor (g L^{-1}). This is the first work in literature reporting a long-term industrial production of *T. lutea*. Overall, we showed possible scenarios for fucoxanthin production from microalgae, increasing the window to supply the industry with steady production throughout the year.

1. Introduction

Fucoxanthin is a xanthophyll carotenoid known for its benefits in human health. Due to its chemical structure, fucoxanthin has important bioactive properties as anti-cancer, anti-obesity, and anti-inflammatory. As an antenna pigment, fucoxanthin is in the light-harvesting complexes, and it is a strong antioxidant [1–6]. Due to these characteristics, this carotenoid has been in high demand over the last years, increasing its commercial value [4].

Fucoxanthin can be obtained from three main sources. Chemical synthesis has been reported, although the process is not efficient for industrial-scale production [3,7]. Brown macroalgae are currently the primary feedstock for fucoxanthin industrial production. However, its content in this feedstock is low, between 0.01 and 2.08 mg g^{-1} dry weight (DW). Also, brown algae are considered a traditional food in many Asian countries, and their use for fucoxanthin extraction is in direct competition for this resource [1,2]. Marine microalgae, such as diatoms and haptophytes, are considered a suitable and cost-effective option due to their higher intracellular contents, between 2.24 and 59.2 (standard deviation of 22.8) mg·g^{-1} DW, and the possibility for higher biomass production without competing for food resources [1–4,7].

Industrial production of fucoxanthin from microalgae can take place in photobioreactors, i.e. in semi-controlled environments, enabling continuous production [4]. Solar radiation is the most sustainable and cost-efficient light source at industrial scale, but it leaves microalgae exposed to daily and seasonal variations [8]. Fucoxanthin content is strictly correlated with light conditions (i.e., light/cell). When exposed to low light conditions, microalgae increase their fucoxanthin content to increase their capacity to harvest light; whereas under high light

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conditions, the opposite occurs [4,5].

Experimental data from industrial-scale systems are rare, making it difficult to design operational strategies to maximize biomass and product formation. This work intends to close this gap by evaluating the effect of outdoor light availability on biomass and fucoxanthin productivities in an industrial set-up. For that, two distinct microalgae were used: the diatom *Phaeodactylum tricornutum* and the haptophyte *Tisochrysis lutea*. The reason for using them is their high fucoxanthin productivities and their response to temperature. *P. tricornutum* was selected for its capacity to grow at temperatures between 10 and 20 °C, which is adequate for autumn/winter temperatures in the south of Portugal [7]. On the other hand, *T. lutea* was selected for its capacity to grow at temperatures between 25 and 35 °C, suitable for spring/summer [1].

2. Materials and methods

2.1. Microalgae strains and inoculum production

Biomass production was performed at Necton S.A. facilities (Belamandil, Portugal), between 11th of November 2017 and 11th of September 2018, including the whole scale-up steps (data not shown) and the industrial trials. *Phaeodactylum tricornutum* (N017) and *Tisochrysis lutea* (N023) strains were obtained from Necton's culture collection. The inoculum of the two strains was prepared in 5 L round-bottom flasks using sterilized natural seawater supplemented with NutriBloom® plus (Phytobloom by Necton, Belamandil, Portugal) at an average nitrate concentration of 4 mM. All flasks were kept under natural sunlight, at a controlled temperature of 23 ± 2 °C and constant aeration (0.2 μm filtered) with air and CO_2 (1%, v v^{-1}).

2.2. Process pipeline

2.2.1. Scale up to outdoor photobioreactors

A schematic representation of the process pipeline is presented in Fig. 1. The scale-up process from laboratory to outdoor production started with the inoculation of 100 L flat panel (FP) PBR, using two 5-L round-bottom flasks. Thereafter, the 100 L FP was used to inoculate an 800 L FP, which was later divided into two or four 800 L FPs, for *P. tricornutum* or *T. lutea*, respectively. These FPs were later used to inoculate a 15 m^3 industrial-scale tubular PBR. All FPs were grown using NutriBloom® plus (nitrate concentration of 4 mM), using constant aeration (0.2 μm filtered), and the pH was kept between 8.0 and 8.2 for both strains, through the injection of CO_2 using a pulse system developed in-house. Each scale-up step lasted between 7 to 15 days depending on the on-site environmental conditions.

2.2.2. Industrial biomass production, harvesting, and freeze-drying

The large-scale production of microalgal biomass was performed in 15 m^3 tubular PBRs. Each PBRs is composed of two main areas: a photosynthetic zone of polymethyl methacrylate (PMMA) tubes (56 mm of internal diameter), and a degassing tank. This corresponds to a total ground area of 340 m^2, and a volume of 44 L per m^2 of ground area. The pH was set to 8.0 and 8.2, for *P. tricornutum* and *T. lutea*, respectively; controlled by CO_2 injection. The cooling of the PBRs was ensured by a water sprinkling system. The cooling set-points were 26 and 33 °C, for *P. tricornutum* and *T. lutea*, respectively.

After biomass production, both cultures were transferred to a harvesting tank and centrifuged at 11000*g* (Westfalia KB25-06-76, Oelde, Germany). After centrifugation, the paste was stored at −20 °C and later freeze-dried overnight (Frozen in Time Ltd., model F50, North Yorkshire, United Kingdom). The ambient temperature and radiation were registered in Necton's production facility using a WatchDog WD2700 metrological station. Both measurements were registered every 15 s.

2.3. Determination of growth parameters and nitrates

The growth of both strains was assessed by determining the optical density and dry weight of cultures at different time points. Optical density was measured at 540 and 740 nm, in a UVmin-240 spectrophotometer (Shimadzu, Japan). Dry weight (DW) was determined by filtering the culture through a 0.7-μm microfibre filter (VWR, Pennsylvania, USA) in a vacuum filtration system, and washed with ammonium formate (31.5 g L^{-1}). The filters were dried at 60 °C for 72 h, or until a

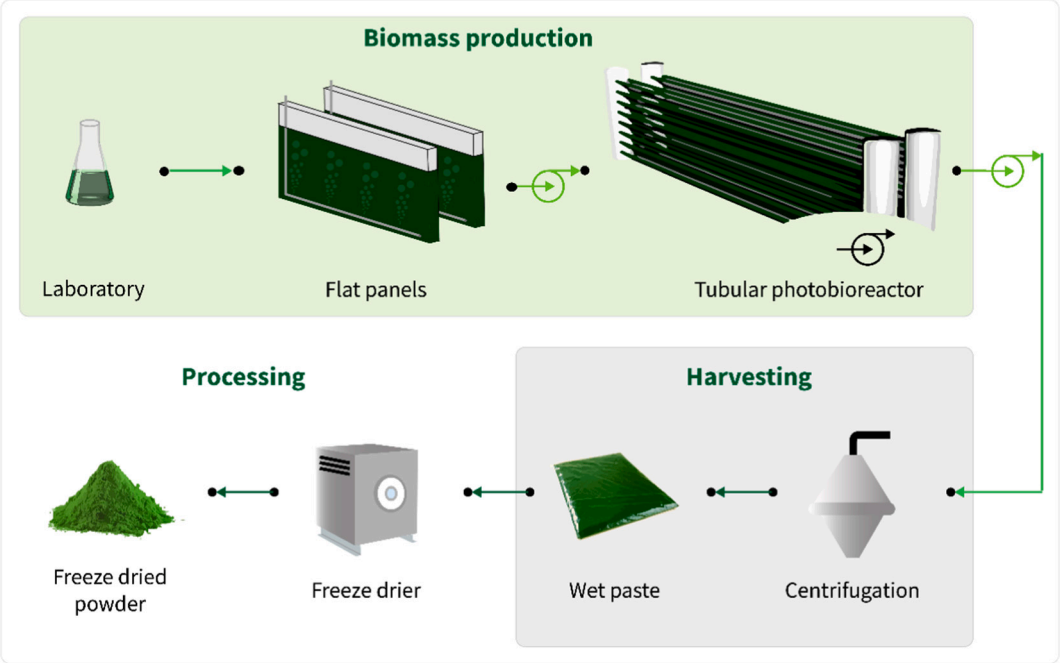

Fig. 1. Process pipeline used at Necton for the industrial production of microalgal biomass. The process started with the scale-up of cultures using production systems of different volumes, followed by biomass production in tubular photobioreactors. Produced biomass was harvested via centrifugation, and the wet microalgal paste was directly frozen and later freeze-dried.

constant weight was achieved, and the DW was measured by gravimetry.

Volumetric biomass productivity (V_p) was calculated according to Eq. (1), where X_1 and X_2 is the accumulated biomass harvested between two sampling points, and t_1 and t_2 is the time of the sampling:

$$V_p\ (g\ L^{-1}\ d^{-1}) = \frac{X_2 - X_1}{t_2 - t_1} \quad (1)$$

The areal productivity (A_p) was calculated according to Eq. (2), where V_p is the volumetric biomass productivity, PBR_V is the volume (15,000 L), and PBR_A is the implementation ground area (340 m^{-2}):

$$A_p\ (g\ m^{-2}\ d^{-1}) = V_p \times PBR_V \div PBR_A \quad (2)$$

Nitrates were determined following the methodology described by Armstrong [9] with modifications. Briefly, microalgal samples were centrifuged for 15 min at 4500 *g*, the supernatant was collected and HCl was added. A blank solution of 0.1 mol L^{-1} of HCl was used, and samples were measured at an optical density of 220 and 275 nm, in a UVmin-240 spectrophotometer (Shimadzu, Japan).

2.4. Fucoxanthin analysis

Fucoxanthin quantification was performed with freeze-dried biomass, using the procedure described by Gao et al. [10]. The biomass, around 5 mg, was extracted twice with 100% Ethanol in beat beating tubes. The supernatants were collected and dried in a N_2 stream. The residue was recovered with 100% Methanol, filtered through a syringe filter (Whatman® SPARTAN® RC 30, pore size 0.2 μm) into a HPLC amber vial, and analyzed in an UPLC Shimadzu Nexera X2, equipped with a quaternary pump and DAD, and Kinetex C18 column (5 μm, 150 × 4.6 mm). The injection volume was 10 μL. The solvents used for the elution were (A) 0.5 M ammonium acetate in methanol:milliQ water (85:15), (B) acetonitrile:milliQ water (90:10), and (C) 100% ethylacetate. Each run takes 53 min with the following elution program (time in minutes: solvent concentration in %): 5 min: A(60)B(40)C(0), 10 min: A(0)B(100)C(0), 40 min: A(0)B(30)C(70), 45 min: A(0)B(30)C(70), 46 min: A(0)B(0)C(100), 47 min: A(0)B(100)C(0), 48 min: A(60)B(40)C(0), 53 min: A(60)B(40)C(0). A calibration curve (R^2 0.998) was performed using analytical grade Fucoxanthin (from Sigma Aldrich), ranging from 0 to 10 μg mL^{-1}. The results were expressed as % DW.

2.5. Statistical analysis

The growth data for each PBR was expressed as means ± standard deviation. Student's *t*-test at a 0.05 probability level was performed in R computing software.

3. Results and discussion

3.1. Industrial production of Phaeodactylum tricornutum

Industrial scale cultivation of *P. tricornutum* has been recently reported in literature [8,11–13]. This microalga can produce high concentrations of omega-3 fatty acids, such as eicosapentaenoic acid (EPA), and pigments, such as fucoxanthin [5,12,14]. Also, *P. tricornutum* is a robust microalga suitable for large-scale production systems and with the capacity for targeted genome manipulation [15], increasing widely further applications of this microalga.

In the current work, industrial production of *P. tricornutum* took place between January 3rd and April 4th of 2018, representing a total production period of 89 days (Fig. 2). The mean ambient temperature was 12.7 ± 2.2 °C during the whole production period (Fig. 2). Daily radiation increased from 12.7 ± 3.8 MJ m^{-2} d^{-1} (first half of the trial) to

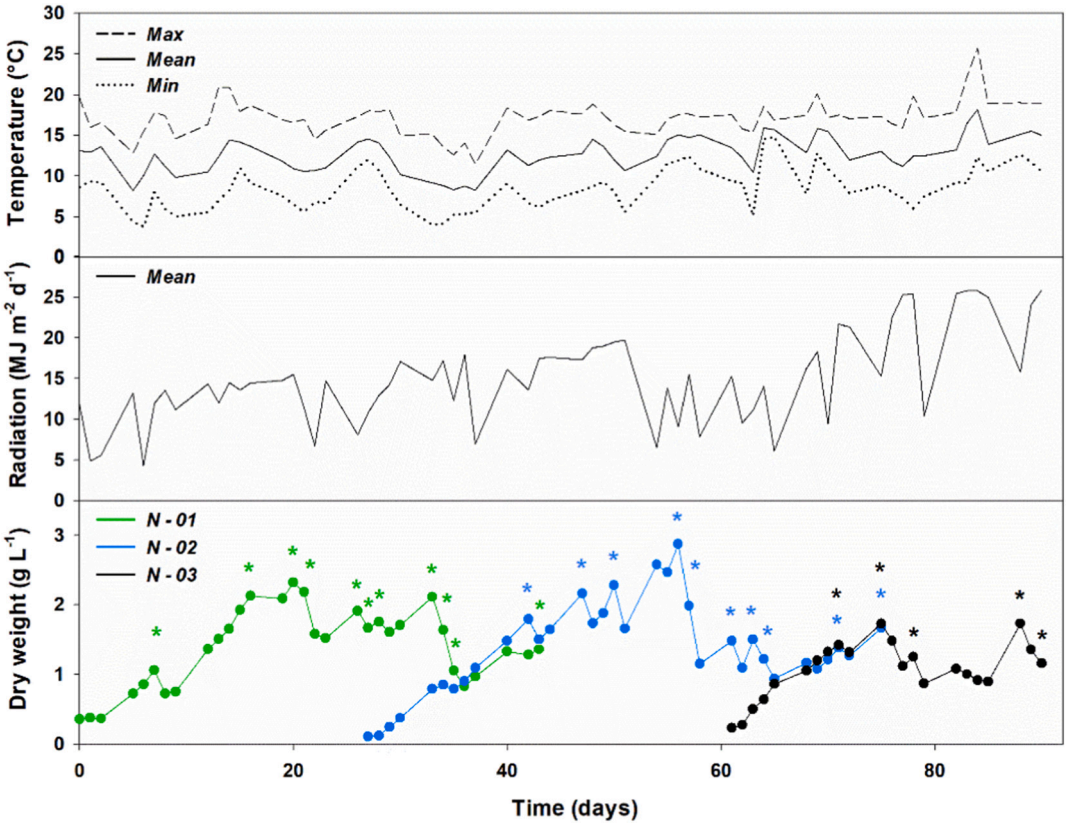

Fig. 2. Mean, minimum and maximum ambient temperature (°C) and radiation (MJ m^{-2} d^{-1}) registered during *Phaeodactylum tricornutum* growth experiments. Three tubular photobioreactors (N-01, N-02, and N-03) were performed between January 3rd and April 4th (2018) at Necton S.A. production facility. The asterisks represent the days of harvesting and medium renewal.

16.9 ± 6.6 MJ m^{-2} d^{-1} (second half), reaching a maximum of 25.9 MJ m^{-2} d^{-1} in early April (Fig. 2). The outdoor scale-up of *P. tricornutum* cultures in flat panels lasted 24 ± 3 days, and the production PBRs were inoculated with low biomass concentrations: from 0.11 to 0.36 g L^{-1}. During the production period, three independent PBRs, N-01, N-02 and N-03, were operated for 40, 48 and 30 days, respectively. All PBRs were operated using semi-continuous cultivation, where the biomass concentration was maintained between 1.5 and 2.5 g L^{-1}, by renewing with fresh culture media (marked by asterisks in Fig. 2).

The average biomass concentration in N-01 and N-02 ranged between 1.5 and 2.2 g L^{-1}, while the maximum biomass concentration reached was 2.3 and 2.9 g L^{-1}, respectively. However, in N-03, the microalgae growth was almost halved, ranging between 1 and 1.5 g L^{-1}; and a maximum of 1.7 g L^{-1}. This decrease in *P. tricornutum* growth, observed in the end of N-02 experiments and throughout the whole production period of N-03, is related with the approaching of the spring season. Spring season in the South of Portugal is characterized by high light intensity and high temperatures that compromised the performance of *P. tricornutum*, since temperatures above 24 °C significantly reduce its growth performance [16,17].

P. tricornutum biomass concentrations observed in this work were lower than the 25 g L^{-1} obtained by Sevilla et al. (2004) [8]. Although in this reference *P. tricornutum* was also grown in outdoor conditions, the authors used a mixotrophic approach by adding glycerol, and the cultivation was performed in a 60 L split-cylinder airlift PBR. On the other hand, the biomass concentrations achieved in this study were considerably higher than the 0.96 g L^{-1} previously reported by Branco-Vieira et al. [13], where *P. tricornutum* was grown autotrophically outdoors in an 800 L bubble column PBR. Likewise, higher biomass concentrations were obtained in our work when compared with the experiments performed by Quelhas et al. [11], using 10 and 35 m^3 tubular flow-through PBRs, which ranged between 0.8 and 1.2 g L^{-1}.

3.2. Industrial production of *Tisochrysis lutea*

T. lutea is one of the most used strains due to its interesting biochemical profile. This microalga has a high content of omega-3 fatty acids, such as docosahexaenoic acid (DHA), ascorbic acid, and fucoxanthin [18,19]; it can also adapt to a wide range of temperatures and light intensities, ideal for industrial production [18,20].

T. lutea was grown between June 28th and September 11th, 2018 (Fig. 3). The combined operation time of the three PBRs was operated for 76 days. The ambient temperature was optimum for the production of this strain, with average and maximum temperatures of 23.9 ± 2.6 and 29.1 ± 3.5 °C. However, cultures were exposed to a particularly hot period between the 34th and 42nd days of cultivation, where the average temperature reached 27.8 ± 2.7 °C (maximum temperature 34.7 ± 3.5 °C).

Regarding the daily radiation, it was higher in the first half of the production period, 29.9 ± 2.1 MJ m^{-2} day^{-1}, decreasing steadily to 22.4 MJ m^{-2} day^{-1} in the end of the trial. The outdoor scale up period of cultures in flat panels was similar to the one of *P. tricornutum*, 22 ± 6 days. N-01, N-02, and N-03 were inoculated with a biomass concentration of approximately 0.3 g L^{-1} DW and grown for different time periods, 51, 39 and 34 days, respectively. All PBRs displayed a high growth rate, with a high renovation frequency, normally with an average biomass concentration between 0.8 and 1.3 g L^{-1} DW. The cultures reached maximum biomass concentrations of 1.39, 1.25, and 1.47 g L^{-1} for N-01, N-02, and N-03, respectively. Although the third PBR registered the highest biomass concentration in the whole production period (1.47 g L^{-1} DW), a decrease of biomass concentration was observed at the end of August and early September, which is related with the decrease of radiation observed on site (Fig. 3).

Although several reports describe the production of *T. lutea* at laboratory scale, information on growth performance in outdoor industrial scale is inexistent. The biomass concentration of *T. lutea* obtained in our

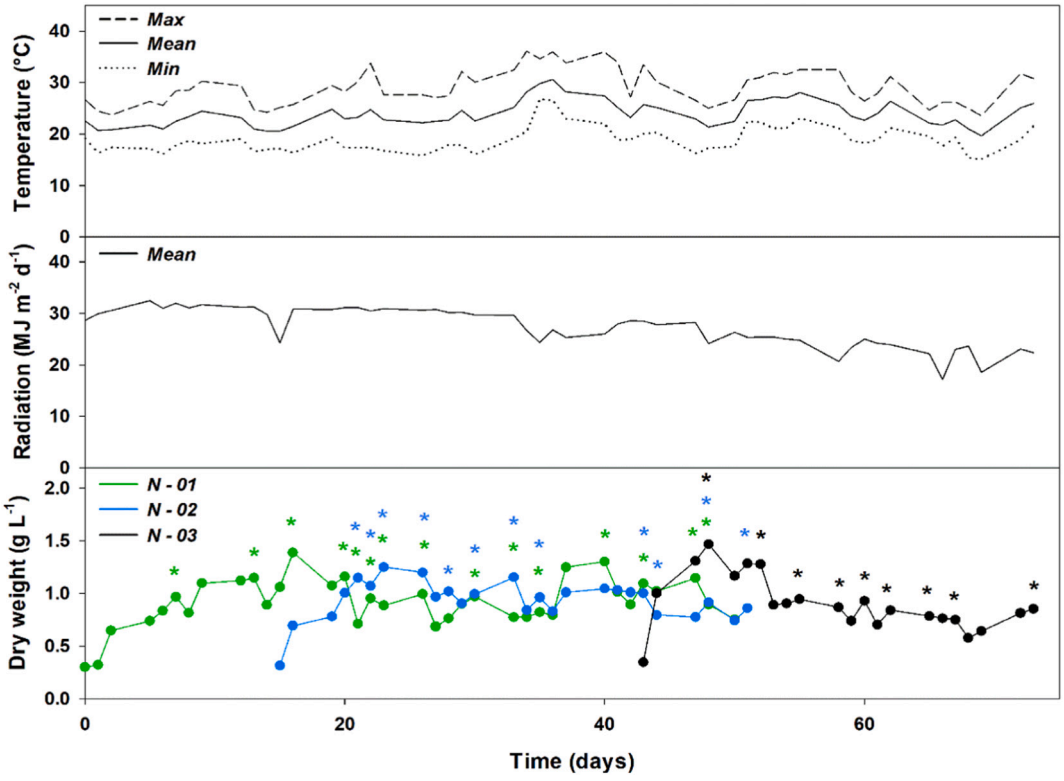

Fig. 3. Mean, minimum and maximum ambient temperature (°C) and radiation (MJ m^{-2} d^{-1}) registered during *Tisochrysis lutea* growth experiments. Three tubular photobioreactors (N-01, N-02, and N-03) were performed between June 28th and September 11th (2018) at Necton S.A. production facility. The asterisks represent the days of harvesting and culture medium renewal.

experiments is similar or higher than those obtained in different laboratory production systems [20–22]. To the authors knowledge, the results obtained in this work can only be compared with the data reported by Ippoliti et al. (2016), where *T. lutea* was grown in a 3 m^3 tubular PBR, and biomass concentrations of approximately 1 g L^{-1} were observed using a dilution rate of 0.15 day^{-1} [18].

3.3. Growth performance assessment and strain comparison

The results on production time and volume, biomass harvested, and volumetric and areal productivities of *P. tricornutum* and *T. lutea* achieved in the experiments described in this work can be found in Table 1.

During 89 days of production, a total volume of approximately 125 m^3 of *P. tricornutum* was harvested, which corresponded to approximately 200 Kg of dried biomass. On the other hand, for *T. lutea*, a total volume of 165 m^3 of concentrated culture was harvested, which represented about 161 Kg of dried biomass in 79 days of production. These results show that similar volumetric and areal biomass productivities were obtained for *P. tricornutum* (0.11 ± 0.04 g L^{-1} day^{-1} and 4.78 ± 1.86 g m^{-2} day^{-1}, respectively) and *T. lutea* (0.09 ± 0.01 g L^{-1} day^{-1} and 3.78 ± 0.25 g m^{-2} day^{-1}, respectively) in the three PBRs operated semi-continuously ($p > 0.05$).

The volumetric productivity of *P. tricornutum* obtained in this work is higher than the values reported for the same strain, grown in 10 and 35 m^3 tubular photobioreactors (0.07 g L^{-1} day^{-1}), whereas the areal productivity (19–23 g m^{-2} day^{-1}) is lower [11]. These differences in the volumetric and areal productivities are related to the lower volume per ground area occupied by the tubular PBRs used in this work, i.e. 44 L m^{-2} compared to 323 and 263 L m^{-2}, used by Quelhas et al. [11]. This comparison indicates that the PBRs used in this work might display a low stocking density. The areal productivity of *P. tricornutum* was also lower than the values previously reported for a 51 L tubular PBR (outdoor) and a 250 L flat panel PBR (indoor) that registered 13.1 and 21.0 g m^{-2} day^{-1}, respectively [8,11]. Similarly, the average areal productivity obtained for *T. lutea* was lower than the 20 g m^{-2} day^{-1} obtained outdoors by Ippoliti et al. in a 3 m^3 tubular PBR [18]. Altogether, these results highlight that the PBRs used in this work display a low culture volume per ground area. We, therefore, suggest a higher stocking density of tubes to increase culture volumes of culture. This would maintain the same ground area of implementation, while improving areal productivities.

3.4. Fucoxanthin productivity vs. light intensity

Fucoxanthin, a primary pigment, is correlated with the light intensity perceived by microalgal cells [1,3,6,7,15]. During the winter season of 2017 in Olhão, light intensity varied between 7 and 20 MJ m^{-2} (with exception of a few sunnier days, where it reached 25 MJ m^{-2}); and in summer between 21 and 31 MJ m^{-2} (Figs. 2 and 3). The day length varies greatly between the two seasons, being winter characterized for shorter days when compared with summer. During these experiments, the day length between January and March varied between 9.5 and 12.5 h, and between July and September varied between 15 and 12 h (https://dateandtime.info/).

The results obtained for *P. tricornutum* (Fig. 4A) show a trend between biomass concentration and fucoxanthin content, whereas the increase of biomass resulted in an increase of fucoxanthin. This tendency can be explained by the amount of light available per cell (Fig. 4B). The light per cell was constant in the first two months of the experiment, with an average of 206 MJ kg^{-1} (8.6 MJ kg^{-1} d^{-1}), increasing to 352 MJ kg^{-1} in March. Due to this high light intensity, the biomass production is stimulated, increasing the fucoxanthin content in the culture broth (mg L^{-1}), but not per cell (mg g^{-1}). The same trend was observed in *T. lutea* production (Fig. 4C and D). Low dry weight, together with high light intensities resulted in an average specific light supply of 600 MJ kg^{-1} (throughout the cultivation period), leading to an increase of biomass and therefore fucoxanthin.

A maximum fucoxanthin content of 0.7% DW and 0.54% DW was reached in *P. tricornutum* and *T. lutea*, respectively. These results are in agreement with what is reported in literature, between 0.2 and 1.6% DW for *P. tricornutum* [15], and 0.6 and 1.9% DW for *T. lutea* [1]. However, higher contents were obtained in laboratory-controlled experiments, with constant light intensity every day, contrary to what takes place outdoors, as in the present work. Additionally, the samples of this work were subjected to the standard Necton' industrial process, including storage in harvesting tanks, centrifuging and freeze-drying steps, that might have contributed to a lower fucoxanthin content. This should also be further investigated.

This work studies the production of fucoxanthin from microalgae throughout different seasons. For that purpose, the authors suggest using different microalgae species, with different optimal growth temperatures, in the different seasons. As expected, an increase in fucoxanthin is observed under low light per cell cultivation conditions [3,4,23]. When producing microalgae in outdoor facilities, the amount of light per cell cannot be directly controlled. But it is possible to control the biomass concentration inside the PBRs, by increasing the self-shading effect and indirectly controlling the light perceived per cell. For this reason, the authors advise cultivating in a turbidostat mode in outdoor industrial facilities. In a turbidostat, the biomass concentration is kept constant by automatic adjustment of the dilution rate. Additionally, identifying the optimal time for biomass harvesting can be crucial to maximize the intracellular fucoxanthin content, since diatoms are known to present diurnal changes in the pigment composition [24]. This work shows the first steps to an "all-year fucoxanthin production by microalgae" concept, proving that both microalgae can be cultivated for long periods of time without culture crashes. Nevertheless, it is important to continue optimizing this process. To do so, the importance of online monitoring biomass and fucoxanthin concentrations needs to be considered and developed. In line with this, incorporating climatic conditions is fundamental for the prediction of biochemical parameters

Table 1
Production time (days), volume (m^3), biomass harvested (Kg), volumetric (g L^{-1} day^{-1}) and areal (g m^{-2} day^{-1}) productivities of *Phaeodactylum tricornutum* and *Tisochrysis lutea*. Three independent tubular photobioreactors were performed for each microalga at Necton S.A. production facility. Mean and standard deviation (Stdev) of each parameter were calculated (n = 3).

PBR	Production time (days)	Volume harvested (m^3)	Biomass harvested (Kg)	Volumetric productivity (g L^{-1} day^{-1})	Areal productivity (g m^{-2} day^{-1})
Phaeodactylum tricornutum					
N-01	40	54	89	0.15	6.51
N-02	48	48	82	0.11	5.03
N-03	30	23	29	0.06	2.81
Mean ± Stdev	39 ± 9	42 ± 16	66 ± 33	0.11 ± 0.04	4.78 ± 1.86
Tisochrysis lutea					
N-01	51	71	71	0.09	4.07
N-02	39	49	48	0.08	3.64
N-03	34	45	42	0.08	3.64
Mean ± Stdev	41 ± 9	55 ± 14	54 ± 15	0.09 ± 0.01	3.78 ± 0.25

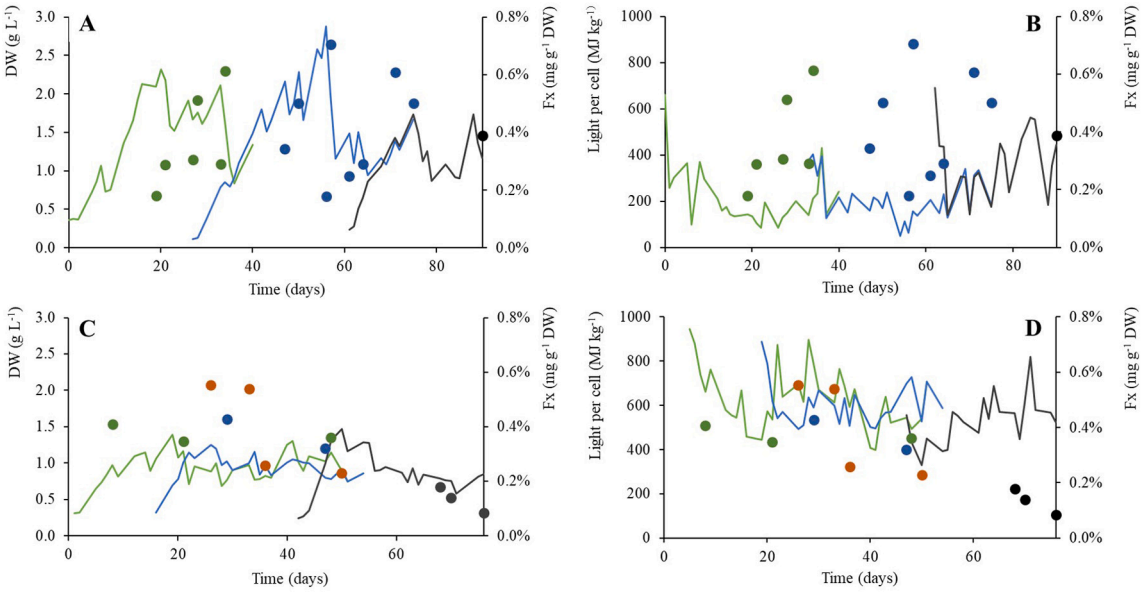

Fig. 4. Dry weight (DW; g L^{-1}), fucoxanthin (Fx; % DW) and light per cell (MJ kg^{-1}) in *Phaeodactylum tricornutum* (A and B) and *Tisochrysis lutea* (C and D) growth experiments, performed between January – March and June – September, respectively. Three tubular photobioreactors were perfumed per microalgae (N-01 in green, N-02 in blue, and N-03 in black). Data points of fucoxanthin content are represented in the same colors of the respective photobioreactor, with exception of orange points that represent the combined biomass from two photobioreactors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and process control of microalgae cultivation.

4. Conclusions

First steps were taken to produce all year round microalgal fucoxanthin at industrial scale, by combining *P. tricornutum* (autumn/winter seasons) and *T. lutea* (spring/summer seasons). Both microalgae were successfully produced semi-continuously in industrial scale tubular photobioreactors. Overall, *P. tricornutum* displayed higher areal biomass productivity than *T. lutea.* The average fucoxanthin content was 0.4% DW for *P. tricornutum*, and 0.3% DW for *T. lutea*. These results cement the idea of using different microalgae during different seasons to produce a specific high-value compound.

CRediT authorship contribution statement

Hugo Pereira[†]**:** Investigation, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing.

Marta Sá[†]**:** Investigation, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing.

Inês Maia: Investigation, Methodology, Writing - review & editing.

Alexandre Rodrigues: Methodology, Writing - review & editing.

Iago Teles: Project administration, Methodology, Formal analysis, Writing - review & editing.

René H Wijffels: Project administration, Writing - review & editing.

João Navalho: Project administration, Methodology, Writing - review & editing.

Maria J Barbosa: Project administration, Formal analysis, Funding acquisition, Methodology, Writing - review & editing.

[†] These authors contributed equally to this work.

Declaration of competing interest

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

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