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Research Article

Determinants of astaxanthin industrial-scale production under stress caused by light photoperiod management of *Haematococcus pluvialis* cultivation

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ABSTRACT. *Haematococcus pluvialis* (Chlorophyta) is a microalga with the greatest capacity to generate pure natural astaxanthin with powerful antioxidant properties, through special cysts, in response to disruptions caused by stress conditions. This research tested the design of a prototype for applying artificial light photoperiods controlled by light-emitting diodes on an industrial scale for generating forced stress in *H. pluvialis* cells during two seasons of the year (winter and spring 2019) in the Coquimbo region in Chile. Three different culture structures were used for the four stages of the alga production cycle. Two containers, A and B, were used for the first and second culture stages, while a raceway (large pool) was used for the third and fourth culture stages. Experiments with four different photoperiods (PP) that represent hours of light:darkness were conducted in two trials (spring 16:8 and winter 18:6) and two for control (spring 11:13 and winter 10:14). In the experiment, an exponential increase of β-carotenoid was achieved, used in human and animal food for its health properties and as a natural colorant in the salmonids industry. Biomass and astaxanthin production under forced stress were measured with physical and chemical variables such as light intensity, temperature, pH, and dissolved oxygen. Results show that the spring culture showed a considerable increase of cysts and, therefore, of astaxanthin reservoirs, reaching a pigment production density of 276 g m^{-3}, with the consequent increase in density of up to 22% more than the control PP.

Keywords: *Haematococcus pluvialis*; astaxanthin; stress; LED; light photoperiods; industrial-scale culture

INTRODUCTION

Haematococcus pluvialis (Chlorophyta) is a freshwater green flagellate microalga considered one of the most important natural astaxanthin sources, representing 90% of total carotenoids (Margalith 1999, Galarza et al. 2019). Astaxanthin ($C_{40}H_{52}O_4$, 3.3′-dihydroxy-β,β-carotene-4.4′-dione) is a secondary ketocarotenoid derived from β-carotene belonging to the xanthophylls family (Molino et al. 2018, Galarza et al. 2019). Carotenoids are natural bioactive compounds that protect microalga cells by physically stabilizing their membrane (Liaaen-Jensen & Frode-Lutnaes 2005, Novoveská et al. 2019), and β-carotene is a carotenoid antioxidant that reacts with peroxyl lipid radicals to form a carotenoid radical (Kobayashi & Sakamoto 1999, Domínguez et al. 2019).

Astaxanthin is a natural pigment with three stereoisomers: 3R, 3'R, and 3'S (Hewlings 2019) and has powerful antioxidant properties, being the second most important carotenoid on the planet. It is used by many manufactures linked to the nutraceutical, cosmetic, and food industries due to its known antioxidant, anti-inflammatory, and anti-tumor properties (Gajardo-Solari et al. 2011, Turan 2018, Do et al. 2019, Koyandea et al. 2019). There is a great demand for astaxanthin in the aquaculture industry, specifically in salmon farming, making intensive use of this natural pigment (Christiansen & Torrissen 1996). Furthermore, some studies have evaluated the effect of the inclusion

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of astaxanthin and β-carotene in diets to enhance growth, reproduction, and pigmentation of ornamental invertebrates, showing results that indicate that both carotenoid sources are promoters of increased survival, quality of pigmentation, and growth of the tested individuals (Díaz-Jiménez et al. 2019).

Astaxanthin esters are accumulated in lipochromes called aplanospores located in the lipid vesicles of its perinuclear cytoplasm in the *H. pluvialis*, producing up to 4% of the dry mass of this coveted dye (Machado Jr. et al. 2016, Butler et al. 2018, Novoveská et al. 2019, Ramos-Tramontin et al. 2019). Different varieties of *H. pluvialis* have proportions of and astaxanthin in different percentages of their dry weight basis (Table 1). This pigment seems to be produced in response to the disruption of homeostasis caused by external stress conditions (Borowitzka et al. 1991, Zlotnik et al. 1993, Domínguez et al. 2019). Therefore, the greater the stress, the greater the production of vesicles or cysts in the alga's defensive response (Galarza et al. 2019). The astaxanthin production by *H. pluvialis* helps protect the cells from oxidative damage caused mainly by high energy radiation and a lack of nitrogen (Wang et al. 2013a). The microalgae *H. pluvialis* changes its metabolism to adapt to light-induced oxidative stress. These metabolic changes include increased levels of glutathione (GSH) and the production of astaxanthin. Then, adding exogenous GSH to the culture medium inhibited astaxanthin biosynthesis, and *H. pluvialis* cell density decreased, indicating that an adequate redox state is essential for astaxanthin accumulation and cell survival (Hu et al. 2020).

The astaxanthin production is directly related to light exposure, pH and temperature, through stress shocks. In this sense, two recent studies were selected. One, from Azizi et al. (2020), has been applied to the growth of *H. pluvialis* and its astaxanthin production in 5 L bioreactors, using different light intensities, light application cycles, and nutritionally optimized culture media. They observed that the application of light was the most important factor for the production of astaxanthin, determining that the exposure to artificial light with an intensity of 700 µmol m^{-2} s^{-1} increases the production of astaxanthin up to 50% more (30 mg L^{-1}) than a culture exposed to natural light, using the optimal culture medium (14 mg L^{-1}).

Another study evaluated the effect of light quality, using narrow-band light-emitting diode (LED) light sources, irradiation, and temperature on the production of red and green blood cells of *H. pluvialis*. They established that monochromatic red light at 20°C was optimal for producing red blood cells, while the maximum cumulative rate of astaxanthin was achieved with a variety of red and blue light at 30°C, reaching astaxanthin productivity of 25 mg L^{-1} d^{-1}. Irradiation affected astaxanthin content, but not dry weight gain. The authors estimated that *H. pluvialis* is highly dependent on the composition of light and that the use of LEDs is an excellent tool to increase the production of metabolites, such as astaxanthin (Pereira & Otero 2020).

Various types of culture systems for growing microalgae have been proposed for decades, where open pond systems (raceway) have dominated (Xu et al. 2009). Nowadays, the progress of bioengineering and biotechnology has made the mass production of microalgae more efficient. Among the improved processes are the photobioreactors, either tube or plate, which have emerged as an alternative to scale the production of microalgae from laboratory to pilot scale. Recently, they have started to be used in astaxanthin production (Pérez-López et al. 2014, Deniz 2020, Onorato & Rösch 2020). However, the luminosity applied to the cultures varies as the tube's diameter or the plate's thickness increases. For this reason, it is necessary to carry out lighting and control tests on an industrial scale to optimize growing systems, making them more efficient and controllable (Xu et al. 2009).

After reviewing the different studies on the incidence of light and temperature on *H. pluvialis* for the production of astaxanthin at the laboratory level, the following question arises: what would be the incidence of the photoperiod using LED lights in *H. pluvialis* that allow establishing a sustainable and scalable production of astaxanthin over time, while maintaining profitable operating costs?

Although there is scientific evidence that the application of light and stress results in increased production of astaxanthin cells and cysts, there are no documented studies of intensive application of light on an industrial scale carried out throughout the year with different photoperiods (PP). Therefore, based on this gap, the objective of this research is to develop a prototype of LED lights that allows increasing cell density during the vegetative cultivation phase of *H. pluvialis*, controlling two different photoperiods (one in the winter season and another in the spring) to visualize tangible results on an industrial scale during one year.

MATERIALS AND METHODS

The photoperiod (PP) experience required manufacturing a prototype of lighting curtains to provide artificial light to the *Haematococcus pluvialis* crops, controlling the lighting time through timers associated with each curtain. In this way, natural lighting in the culture systems was extended through these curtains to adjust the test PP of 18:6 and 16:8, to the natural winter

Table 1. *Haematococcus pluvialis* varieties as sources of astaxanthin percentage (%) on the dry weight basis (Ranga-Rao et al. 2014).

Haematococcus strain	Astaxanthin content (%) on the dry weight basis	Reference
Haematococcus pluvialis	3.8	Ranga-Rao et al. (2009, 2010)
Haematococcus pluvialis (K-0084)	3.8	Aflalo et al. (2007)
Haematococcus pluvialis (local isolation)	3.6	Torzillo et al. (2003)
Haematococcus pluvialis (AQSE002)	3.4	Ranga-Rao et al. (2014)
Haematococcus pluvialis (K-0084)	2.7	Wang et al. (2013a)

in Chile (southern hemisphere, July 2019; 10 light:14 darkness) and spring in Chile (southern hemisphere, October 2019; 11 light:13 darkness). A total of 32 curtains were installed, each 1.26 m long × 1.0 m high. Each curtain contained 120 full RGB color LED bulbs, with a homogeneous position distribution of approximately 10 cm of equidistance between each LED. Each bulb consumed 0.25 W and emitted 37.5 lumens. Thus, values for each 1.26 m^2 curtain to be applied to A and B containers consumed 30 W h^{-1} and, by transforming lumens emission to lux, a total of 3.57 Klux per curtain is generated (https://www.calculadoraconversor.com/lumenes-a-lux).

The LED light prototypes were divided into two formats, a 1.26 × 1 m curtain for containers A, representing the growth phase of the inoculum, and a total of six curtains for each of the mass growth units in containers B, because these two phases have different specific requirements of light conditions and culture media, running in the same physical location (cultures in 600 L bags). Each culture was separated into modules so that the curtains' light intensities did not interfere with each other. In addition, a control culture was included (associated with the natural photoperiod of the season) to be contrasted with the cultures submitted to the experimental photoperiods.

Cultivation of *Haematococcus pluvialis*

We worked with the strain *H. pluvialis* isolated from strains in the Coquimbo region, Chile, where the experiment was carried out. The region of Coquimbo is located approximately between 29°20' and 32°15'S. The climatic conditions of the experimental crops during winter were at an average and maximum temperature of 10.5 and 18.5°C, and with an average and maximum solar radiation of 155.6 and 660 W m^{-2}, respectively. For the spring conditions, the climatic conditions were 14.2 and 21.1°C and an average and maximum solar radiation of 243.8 and 111.7 W m^{-2}, respectively. The data were extracted from the Centre for the Study of Arid Zones meteorological station (www.ceazamet.cl).

From the *H. pluvialis* strains obtained, cultures were made in Bold's Basal Medium (BBM), prepared as described by Stein (1980); this culture medium is highly enriched in minerals and allows the development of green microalga. Initial cultures were made in Erlenmeyer flasks with a capacity of 150 mL, with 100 mL of culture. The obtained cells were inoculated into a 500 mL Erlenmeyer flask at a concentration of 3×10^3 cell mL^{-1}. The cells were then transferred to 20 L carboys at an initial concentration of 15,000 cell mL^{-1}, where they were allowed to propagate for 10 to 15 days. When a density of 300,000 cell mL^{-1} were obtained, cells were transferred to the A containers, thus initiating the industrial growth stage. All cultures made in this stage were incubated at 20°C, without agitation.

Astaxanthin production process

A series of stages are needed to produce astaxanthin through the cultivation of *H. pluvialis*, which coincides with the growth cycle of this green microalga.

Stage I: this initial phase was carried out in the laboratory, where the *H. pluvialis* cells were propagated in 500 mL Erlenmeyer flasks and then in carboys to 20 L (Fig. 1).

Stage II: the A containers were inoculated with a cell density of 35,000 cell mL^{-1}. They were incubated for approximately 15 to 25 days, and cells were collected when they reached a density of 250,000 cell mL^{-1}. Such containers built-in wood and a plastic bag to grow the alga had a working volume of 600 L and supplemented seawater was used to generate stress to increase cell propagation (Fig. 1).

Stage III: once the desired cell density was reached, we inoculated six plastic bags, the B containers, which had a total volume of 3600 L (near 600 L each). The cultures were incubated for 10 to 25 days until a cell density of at least 120,000 cell mL^{-1} was obtained (Fig. 1).

Stage IV: subsequently, all bags B (n = 12) were transferred to a 90,000 L raceway pool. Cultures were incubated for 10 to 25 days until a cell density of

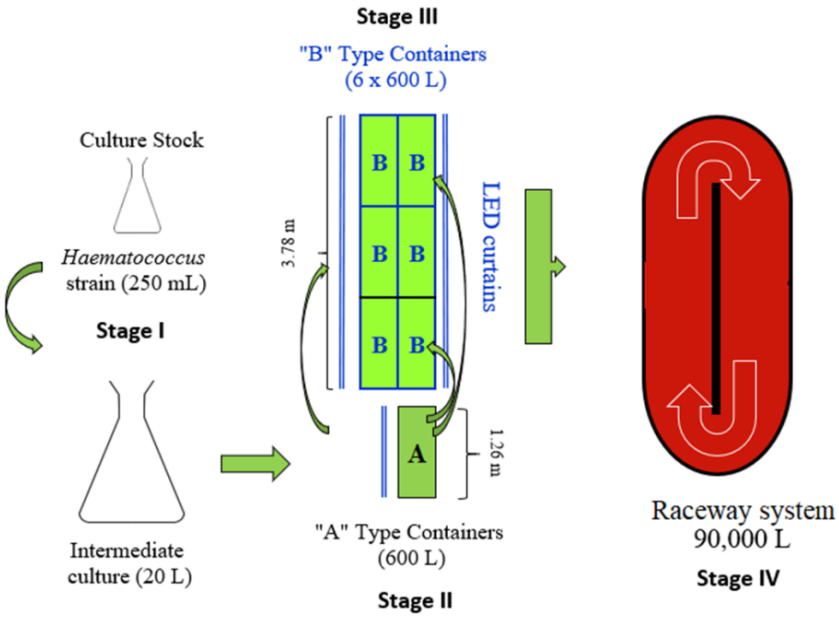

Figure 1. Schematic diagram from the beginning with *Haematococcus* strain inoculation and intermediate culture (stage I), then to mass culture in containers A (stage II), containers B (stage III), and final culture stage IV in raceway systems.

100,000 cell mL^{-1} was reached, and orange-red cell coloration was observed (Fig. 1).

Stage V: finally, the cells were collected and subjected to a crushing treatment and heat spray to break the cell wall, thus recovering the astaxanthin found inside. At this stage, an impure extract is obtained, in which the percentage of astaxanthin present as carotenoid is determined. The extract is then purified through a physical and chemical treatment and weighed, obtaining the final pure astaxanthin (kg).

The maximum concentration achieved by any treatments was established to calculate cellular products and make effective comparisons in the same period. In the A (winter) containers, the 18^{th} was established as the maximum concentration achieved for the 18:6 treatment. Afterward, this treatment did not register notoriously higher densities.

Determination of the effect of illumination on *Haematococcus pluvialis* cell growth and astaxanthin production

Two tests were carried out in 2019 to study the impact of light-emitting diode (LED) lighting on the cellular growth of *H. pluvialis* and the production of astaxanthin, one in winter (July) and the other in spring (October), each lasting three months with the respective variables in light and temperature and compared to the natural PP control culture. *H. pluvialis* has a life cycle that includes vegetative growth and astaxanthin production using cysts, for which an inoculation scheme was designed that included two phases.

Determination of physical and chemical variables

Light intensity

The incident light measurement in the bags' culture containers (A and B) was conducted between 12:00 and 12:30 h every day, recorded with a PCE-172 Luxmeter (PCE Instruent™, Spain). The measurements were made in different positions in the culture vessels to take homogeneous measurements. The results were obtained in Kilolux and converted to µmol m^{-2} s^{-1} using the Plantekno® online converter (https://www.plantekno.com), considering the sunlight spectrum which irradiated the bags during the natural phase of each season.

Monitoring of culture parameters

Samples were taken periodically, always at noon, to monitor the culture. The incident light was measured, and a sample of each bag was taken from the bottom of each containers. The volume of the sample was standardized to 100 mL. Once the samples were taken, they were evaluated with a digital oxygen meter, which uses an H01PST sensor (Handy Polaris Oxigard™, Denmark), thus determining the dissolved oxygen expressed in mg L^{-1}. In addition, the temperature (°C) and pH of each sample were also measured using a HI98127 multiparameter instrument (Hanna Instru-

ment™, USA). Finally, the cell density of the cultures was determined by counting *H. pluvialis* cells using a binocular microscope (Olympus™, Japan). Total carotenoids were determined according to the protocol described by Davis et al. (1953).

Statistical analysis

The industrial culture format of *H. pluvialis* was used, which comprises an inoculum grown in 600 L containers (bag), so the samples from the A containers were only taken in duplicate, which will present only a descriptive analysis. Each containers A subsequently generates six units of containers B. For type B containers, a total of 12 units in separate containers were used. A two-way repeated measures ANOVA between the 18:6; 16:8 PP treatments and control group for the 13 winter days, and a second two-way repeated measures ANOVA analysis between the 16:8 (1); 16:8 (2) PP treatments and control group for the six spring days in B containers, were used. Normality assumptions were checked through a Shapiro-Wilks test and sphericity assumption through Mauchly's test to examine intragroup variability. Multiple comparisons were evaluated by *t*-test, adjusting the *P*-value by Bonferroni to avoid type I error. Statistical analyses were performed with the statistical package rxtatix v0.5 of the R v3.6.3 environment. A significance level of $P < 0.05$ was used.

RESULTS

Winter cell production test I. Cell production in A containers

The test I in A containers had a total duration of 23 days. The cell densities generated in both treatments did not present significant differences ($P > 0.05$), mainly because the samples are duplicates. The 18:6 PP treatment reaches a value of 252,188 cell mL^{-1} on day 18; however, on day 19 of culture, increasing cell density was observed in the treatment with the 16:8 PP, reaching 18:6 PP with an average value of 253,750 cell mL^{-1} (Fig. 2). Both experimental PP (LED) increased close to 30% cell density (>50,000 cell mL^{-1}). It was determined that the cell density obtained on day 19 for the 18:6 and 16:8 PP is the optimal one to inoculate the next stage (containers B; Fig. 1).

The cultures that used the 18:6 PP had a higher cell density on day 18 of the culture, where the cell concentration remained close to 250,000 cell mL^{-1}. It should be noted that the control culture did not reach 200,000 cell mL^{-1} on day 20 (Fig. 2).

Evaluation of the physical and chemical variables associated with the production

As complementary information to the production data, the physical and chemical variables of light intensity, temperature, pH, and dissolved oxygen were measured in the vegetative cultures of *H. pluvialis*. Mean light intensity on A containers was highly variable (Table 2), ranging from 7.5 to 46.6 Klux (138.7 to 851 µmol m^{-2} s^{-1}). This variability coincides with the presence of cloudy or clear days.

During the first 15 days, the highest light intensity levels were recorded (7.5-46.6 Klux; Table 2). On day 16, it was necessary to install a cover over the containers to regulate light intensity, thus keeping it between 8 and 15 Klux (148 and 277.5 µmol m^{-2} s^{-1}). At this stage, it is very important to avoid stress factors as much as possible to enhance cell propagation and prevent caking. Additionally, this action prevented the progressive increase of mean temperature in the culture containers, which ranged from 12 to 23°C (Table 2). As for pH, mean values varied between 7 and 8 (Table 2).

Dissolved oxygen in each culture ranged from 6.48 to 11 mg L^{-1} (Table 2). An interesting finding was that, during the last days of culture, the treatment with more hours of light (18:6) presented an average increase of this parameter compared to the other PP treatments.

Winter cell production test II. Cell production in B containers

Tests were conducted in the B containers using PP as treatments (16 h light:8 h darkness; 18 h light:6 h darkness; and the control culture with 10 h light:14 h darkness, which lasted 13 days.

A two-way ANOVA for repeated measures was performed to evaluate the effect of PP and time on the increase in cell density (cell mL^{-1}). The results of the two-way ANOVA showed a significant effect ($F[26,286] = 8.56$, $P < 0.0001$; Table 3) on the variable time and on the interaction of PP × time. The variable PP alone had no significant effect on the increase of cell density in B containers. The interaction between PP and time on cell density was highly significant ($P = 2.59\times10^{-23}$; Table 3). Thus, the effect of the PP variable was analyzed in each period (day). *P*-values were adjusted using the Bonferroni multiple testing correction method. The effect of PP was significant at time T02, where the control PP group presented a higher average density (13,750 cell mL^{-1}) respecting both PP with LED lights. Subsequently, at times T12 and T13, the 16:8 PP presented the greatest differences ($P < 0.05$).

It is necessary to emphasize that the cultures that used the 16:8 PP fulfilled the cell density requirements

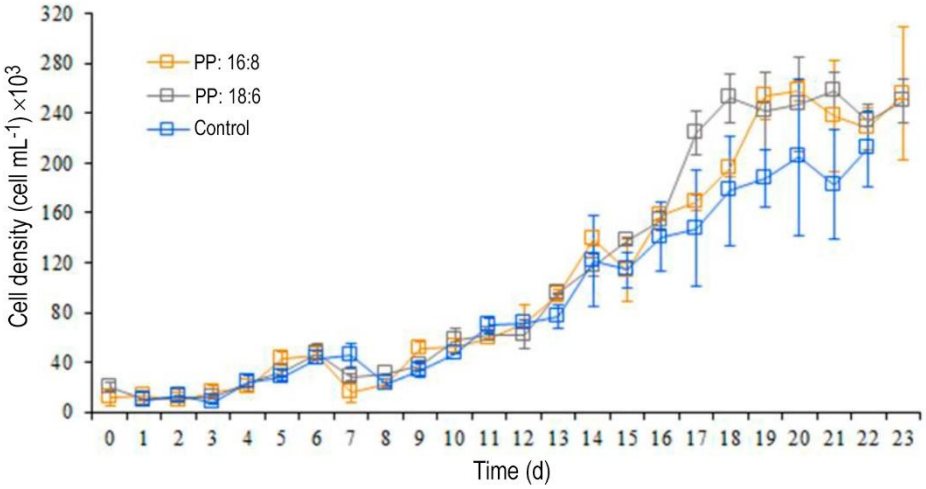

Figure 2. Growth in cell density of *Haematococcus pluvialis* in type A containers with experimental photoperiods (PP) (light:darkness), PP (16:8), PP (18:6) and control group equivalent to photoperiod 10:14 for the winter season. Data are shown as mean ± standard error (n = 2). Type A containers are made only in duplicate.

Table 2. Mean records of physical and chemical variables for *Haematococcus pluvialis* growing bags (A and B containers) during winter and spring conditions for each photoperiod (PP) treatment. Values represent the mean of the total culture period. Min: minimum value recorded, Max: maximum value recorded, Ave: average value during culture days.

Season	Containers type	PP	Culture days	Light intensity (Klux)			Temperature (°C)			pH			Dissolved oxygen (mg L^{-1})		
				Min	Max	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max	Ave
Winter	Containers A	18:06	22	7.52	46.60	26.00	11.85	22.91	18,51	7.00	7.75	7.48	7.05	11.10	9.02
		16:08		7.70	42.85	25.36	12.20	22.15	18.38	7.17	7.75	7.41	7.16	9.84	8.57
		Control		7.90	42.65	26.32	12.30	22.95	18.46	7.25	7.90	7.51	6.48	9.48	8.54
	Containers B	18:06	13	4.12	14.09	11.78	17.64	22.30	19.14	7.23	7.43	7.33	8.16	8.61	8.44
		16:08		4.33	17.57	14.91	17.52	22.31	19.09	7.30	7.47	7.34	7.90	8.56	8.36
		Control		4.75	18.20	14.97	17.57	22.71	19.51	7.28	7.61	7.39	8.18	8.72	8.43
Spring	Containers A	16:8 (1)	21	7.43	44.65	27.59	19.30	22.75	21.33	7.25	8.00	7.53	7.19	8.47	7.94
		16:8 (2)		7.06	44.45	27.84	19.35	22.60	21.35	7.24	7.85	7.49	7.23	8.52	7.97
		Control		7.72	46.90	29.18	19.50	22.60	21.36	7.25	7.70	7.47	7.33	8.60	8.08
	Containers B	16:8 (1)	6	15.86	24.76	18.24	21.62	23.32	22.59	7.36	7.53	7.44	7.74	8.52	8.17
		16:8 (2)		22.63	25.62	24.11	11.03	23.50	22.90	7.32	7.46	7.38	7.70	8.62	8.25
		Control		27.24	31.27	29.72	22.18	23.98	23.10	7.33	7.48	7.39	7.78	8.57	8.25

for harvest on day 11. The maximum density obtained in the control treatment (10:14) was 95,181 cell mL^{-1} on day 10 of cultivation and remained similar until harvest (day 13). Interestingly, there was a difference of approximately 60,000 cell mL^{-1} on day 13 of culture between both tests (Fig. 3).

Evaluation of the physical and chemical variables associated with the production

The physical and chemical variables of light intensity, temperature, pH, and dissolved oxygen in each B containers of the vegetative culture of *H. pluvialis* were evaluated. During this second phase of cultivation, the cover was maintained on the containers roof to keep the mean incidence of light intensity between 10 and 18 Klux (135 to 243 µmol m^{-2} s^{-1}).

Similarly, the temperature in B containers was more stable during the development of the culture, with an average temperature between 18.3 and 19.5ºC (Table 2). Except for day six of cultivation, when the mean temperature increased three degrees above average (>22ºC), coinciding with one of the days with the greatest light and heat intensity.

Table 3. Two-way ANOVA for repeated measures for the photoperiod treatments (16:8 and 18:6 *vs*. control (10:14)) and time (days) for the growth experiments (cellular density) of the microalgae *Haematococcus pluvialis* in type-B containers (stage III) during wintertime. DFn: numerator degrees of freedom, DFd: denominator degrees of freedom, F: F-value, and *P*: associated probability. Statistically significant results ($P < 0.05$) are indicated in bold.

Effect	DFn	DFd	F	*P*
Photoperiod	2	22	1.169	3.29×10^{-1}
Time	13	143	101.291	**2.90×10^{-65}**
Photoperiod × time	26	286	8.559	**2.59×10^{-23}**

The variables of light intensity and temperature had similar behavior. However, the mean light intensity (Klux) in the 18:6 group was permanently lower than the control and the 16:8 group. As for the pH values, means values showed variations between 7.23 and 7.61. On the other hand, dissolved oxygen values varied between 7.9 and 8.7 mg L^{-1} (Table 2).

Finally, in the analyses carried out in winter, it is observed that the light intensity parameter varies between the crops (the other parameters were constant), observing that the higher biomass formation coincides with moments of lower light intensity. Both PP used in this improvement stage showed higher biomass than the control in lower light intensity conditions.

Spring cell production test I. Production in A containers

According to the results of the winter experience: a) the notorious difference between the experimental PP *vs*. the control, and b) the little difference between 18:6 and 16:8 PP as in the previous test, this stage started with the inoculation of A containers, applying only the 16:8 PP (16 h light:8 h darkness) and control (natural light 11:13). PP was applied to a group of containers from the beginning of cultivation (16:8 (1)) and to another group from day 10 (16:8 (2)), conducting biological tests of the studied PP. All cultures had a total duration of 21 days.

The cell densities generated in both experimental tests did not present differences mainly because the samples were in duplicate only and that one sample was detected in high data variation. In this case (spring), the control group (daylight (11:13)) has a higher average value, but not significant with experimental LEDs groups. After day 16, the densities of the control and treatments 16:8 (2) increased and, to a lesser extent, the PP 16:8 (1) (135,630, 134,062, and 120,053 cell mL^{-1} respectively). On day 21 of culture, an increase in cell density was observed in the treatment with the 16:8 (2) PP (173,750 cell mL^{-1}) compared to the control (11:13) PP, which only reached a density of 141,875 cell mL^{-1} (Fig. 4).

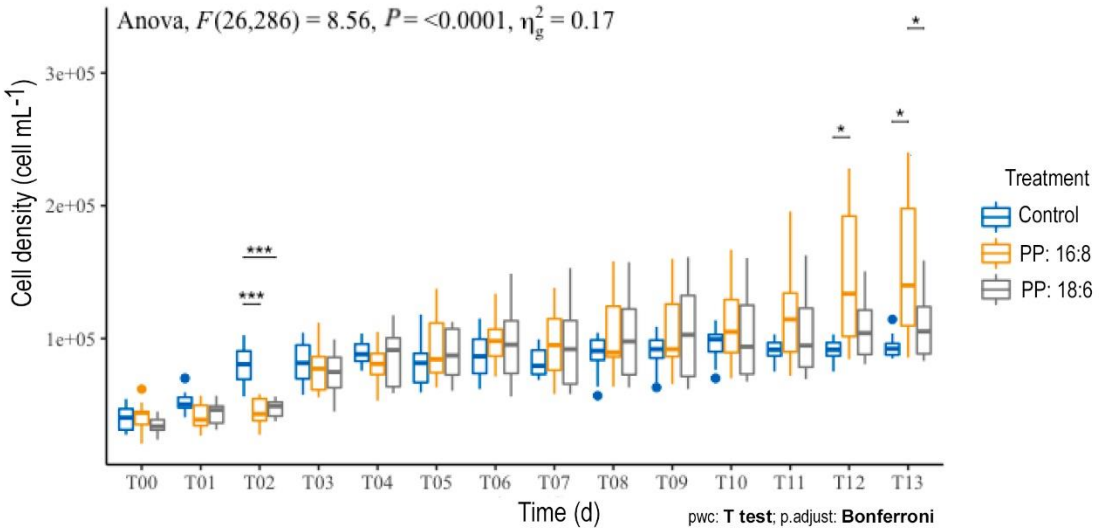

Figure 3. Growth in cell density (cell mL^{-1}) of *Haematococcus pluvialis* in type B containers in relation to experimental photoperiods (PP) (light:darkness): PP: 16:8, PP: 18:6 and control group equivalent to PP: 10:14 for winter season. Data are shown in box plots (n = 12). For growth in B containers, we used six replicates per A containers. Treatment (PP): time interaction effect (Two-way ANOVA of repeated measures, $P = < 0.0001$), effect size ($\eta_g^2 = 0.17$), multiple comparison (pwc: t test; p.ajusted: Bonferroni: *$P < 0.01$, ***$P < 0.0001$.

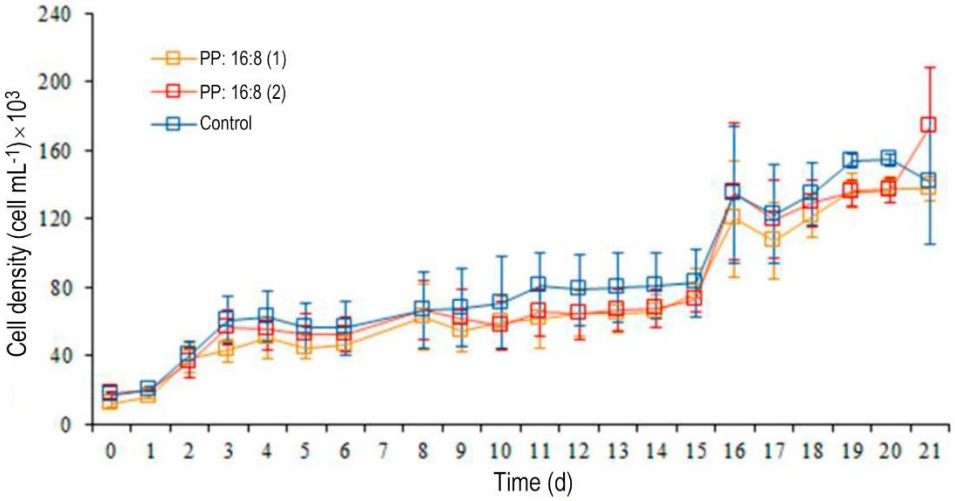

Figure 4. Growth in cell density (cell mL^{-1}) of *Haematococcus pluvialis* in type A containers with experimental photoperiods (PP) (light:darkness): (■) PP: 16:8 (1), (□) PP: 16:8 (2) and (□) control group equivalent to PP: 11:13 for the spring season. PP 16:8 (1) and (2) are explained in materials and methods. Data are shown as mean ± standard error (n = 2). Type A containers are made only in duplicate.

Evaluation of the physical and chemical variables associated with the production

Each culture's incident light intensity varied from 7.1 to 46.9 Klux (131.3-867.6 µmol m^{-2} s^{-1}), especially during the first 10 days of cultivation. This variability coincides with the presence of cloudy or clear days. The average light intensity was between 27.6 and 29.2 Klux (Table 2).

The temperature behavior in the culture medium of the containers varied between 19.3 and 22.7ºC. The light intensity and temperature variables fluctuated strongly during the whole test; however, their behavior was similar in all the cultures.

The mean pH values fluctuated between 7.2 and 8.3, but no differences were found between the treatments. The highest pH values coincided with the lowest temperature values on day 16 of cultivation (Table 2).

The mean values of dissolved oxygen in the culture medium of the containers presented variations between 7.94 and 8.08 mg L^{-1}. The lowest values occurred on the first five days of culture (7.2-7.3 mg L^{-1}), then increased slightly to between 7.6 and 8.6 mg L^{-1} (Table 2).

Spring cell production test II. Production in B containers

In this second experiment, cell densities generated in *H. pluvialis* cultures in B containers showed significant differences. As in the case of cell density increase in winter *H. pluvialis*, a two-way ANOVA for repeated measures was performed to evaluate the effect of the experimental PP 16:8 (1) and 16:8 (2) in spring (11:13) and of the variable time on the increase of cell density (cell mL^{-1}). The results of the two-way ANOVA showed a significant effect (F [2.27,24.95] = 8.36, $P < 0.0001$; Table 4) on both the variables PP ($P = 2.00\times10^{-3}$) and time ($P = 6.67\times10^{-8}$), both with a significant effect on the increase of cell density in B containers. In addition, the interaction of both variables was also significant ($P = 1.00\times10^{-3}$; Table 4). The effect of the PP variable was analyzed for each period (day). *P*-values were adjusted using the Bonferroni multiple testing correction method. The effect of the PP was significant from day 3 (T03) to the end of the experiment (T06). The control treatment reached only an average value of 59,010 cell mL^{-1}, thus establishing a difference of more than 25,000 cell mL^{-1} (Fig. 5). Likewise, a significant difference in cell density was observed between the 16:8 PP treatments (2) and the control treatment, with the former reaching a mean

Table 4. Two-way ANOVA for repeated measures for the photoperiod treatments (16:8 and 18:6 *vs*. control (10:14)) and time (days) for the growth experiments (cellular density) of the microalgae *Haematococcus pluvialis* in type-B containers (stage III) during springtime. DFn: numerator degrees of freedom, DFd: denominator degrees of freedom, F: F-value, and *P*: associated probability. Statistically significant results ($P < 0.05$) are indicated in bold.

Effect	DFn	DFd	F	*P*
Photoperiod	2	22	7.989	**2.00×10^{-3}**
Time	1.75	19.22	48.166	**6.67×10^{-8}**
Photoperiod × time	2.27	24.95	8.359	**1.00×10^{-3}**

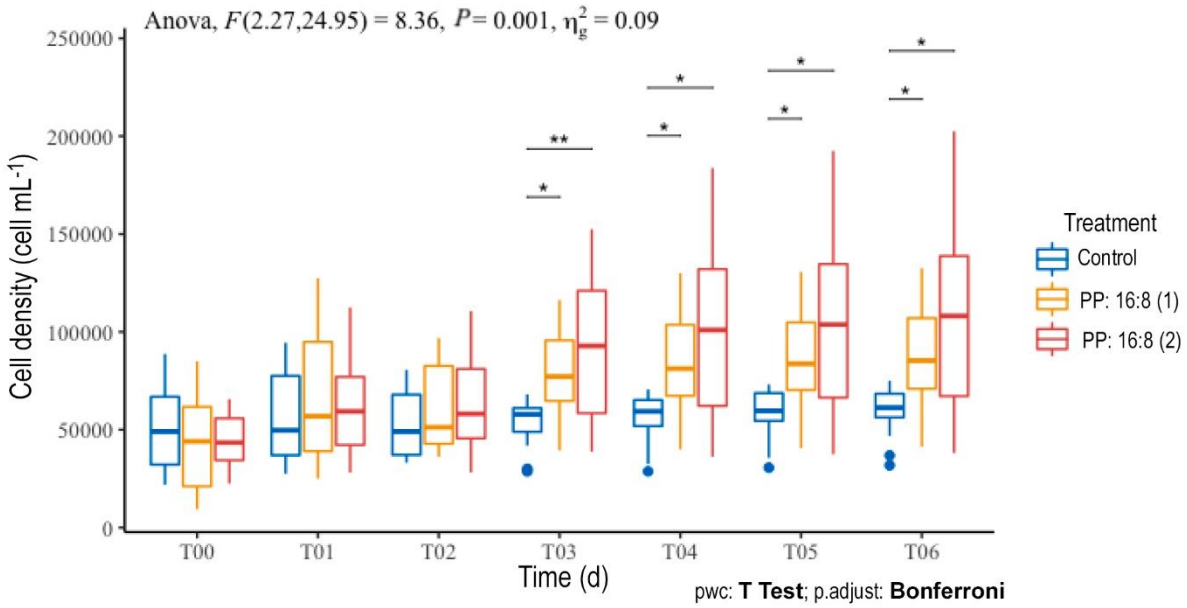

Figure 5. Growth in cell density (cell mL^{-1}) of *Haematococcus pluvialis* in type B containers in relation to experimental photoperiods (PP) (light:darkness): (■) PP:16:8 (1), (□) PP:16:8 (2) and (□) control group equivalent to PP 11:13 for spring season. PP 16:8 (1) and (2) are explained in materials and methods. Data are shown in box plots (n = 12). For growth in B containers, we used six replicates per A containers. Treatment (PP): time interaction effect (two-way ANOVA of repeated measures, $P = < 0.0001$), effect size ($\eta_g^2 = 0.09$), multiple comparison (pwc: t test; p.ajusted: Bonferroni: *$P < 0.01$, **$P < 0.001$).

density of 106,823 cell mL^{-1}, doubling the cell density of the control culture (Fig. 5). This experience in spring in containers B had a duration of only six days since the control group (smaller growth) showed signs of contamination by *Coelastrella* sp. This event determined that the 12 bags of each treatment were transferred to their respective raceway units to obtain astaxanthin to establish the respective comparisons.

Evaluation of the physical and chemical variables associated with the production

In this second phase of cultivation (B containers), the cover was maintained over each containers, making the incidence of light intensity more stable (Table 2). The highest incidence of natural light was recorded in the control treatment which a mean value of 31.2 Klux (577.2 µmol m^{-2} s^{-1}) and a lower mean value of 15.9 Klux (294.2 µmol m^{-2} s^{-1}) in the 16:8 (1) treatment. Significant differences were found between the three treatments ($P < 0.05$).

Regarding temperature, mean thermal variations were recorded between 21.6 and 23.9ºC (Table 2). The pH values in the culture medium of the containers presented variations between 7.32 and 7.53. As for dissolved oxygen, it varied between 7.70 and 8.62 mg L^{-1}, with the lowest values recorded on the first two days (7.70-7.85 mg L^{-1}), increasing slightly on the following days (8.0-8.85 mg L^{-1}) (Table 2).

In the spring period, the result was different. The use of PP has a major impact on cell productivity and the production of pure astaxanthin and is in line with Hong et al. (2019). The final results of pure astaxanthin production showed that both photoperiods performed better than the control group. The highest of these was 16:8 (1) with 22.2%.

Production of astaxanthin in raceway pool

At this stage, the final production of astaxanthin extracted from the cysts produced by the raceway system was evaluated. The production of pure astaxanthin was higher when the 16:8 PP was used, both in winter and in spring (Table 5). In the winter experiment, a pure astaxanthin production of 21.8 kg was observed when the 18:6 PP was applied, while 20.8 kg was produced when the control treatment was applied. In the spring experiment, the production of pure astaxanthin was 24.8 kg when the 16:8 (1) PP was applied, while in the control culture, the production was 20.3 kg (Table 5).

Regardless of the treatment used, total carotenoids present in the impure cell mass were close to 5% in winter and spring. At the end of the cyst production

Table 5. Cell production obtained at the end of cultures in A and B containers. MCP: maximum cell productivity (cell mL^{-1} h^{-1}). *Photoperiod (PP) application from the start of cultivation. **PP application 10 days after the start of cultivation. (β) Cell productivity was considered only in containers B. This value indicates cells obtained at the end of each culture and considers culture volume and time in which that amount of cells was obtained. (Ω) The day when a maximum number of cells was reached.

Season	Winter				Spring			
	Containers A		Containers B		Containers A		Containers B	
Treatments	MCP β	Final process day$^{\Omega}$	MCP β	Final process day$^{\Omega}$	MCP β	Final process day$^{\Omega}$	MCP β	Final process day$^{\Omega}$
16:8 (1) PP**	503	21	568	11	322	20	607	6
16:8 (2) PP*	-	-	-	-	345	21	749	6
18:6 PP	525	20	346	13	-	-	-	-
Control	385	24	302	13	296	20	410	6

process in winter, it was observed that the control and 18:6 PP cultures presented percentages of 5.65 and 5.61, respectively, higher than those obtained with 16:8 photoperiods. On the other hand, at the end of the spring process, it was observed that all the cultures presented similar percentages (Table 5).

It is important to underline that the increase in cells during the winter trial does not strongly impact the production of pure astaxanthin when scaling up the data to a monthly production. Applying the 18:6 PP (light:darkness) generates a positive difference of 3.5 kg (20.7%) in a monthly projection of astaxanthin production. In contrast, a PP with less light, such as 16:8, increased the control (Table 5) of an extra 1.1 kg (6.5%).

When calculating the production of pure astaxanthin obtained for 30 days, it was observed that the treatments with the 16:8 (1) produced 28.6 kg in spring and 18.0 kg in winter. On the other hand, the control produced 23.4 kg in spring (11:13) and 16.9 kg in winter (10:14), showing that greater exposure to light generates a greater astaxanthin production (Table 5).

DISCUSSION

Although there is abundant evidence in the literature on the production cycle of astaxanthin in various strains of *Haematococcus pluvialis*, further research focused on improving production characteristics applicable on an industrial scale is still needed (Fujii et al. 2006, Gómez et al. 2016, Shah et al. 2016, Zhang et al. 2020). In this way, several studies have been carried out on astaxanthin production using *H. pluvialis* and other microalgae, but mainly at the laboratory level, these data being difficult to extrapolate to an industrial scale. However, it is important to point out that there are some studies of commercial scaling of cultures with microalga identifying some key factors, mainly light, nutrients and temperature, but also the need for qualified and experienced personnel to produce enough inoculums to supply large ponds and the same time to keep them free from all exogenous contamination (Borowitzka & Vonshark 2017). In addition, other authors highlight the importance of the concentration of vegetative cells of *H. pluvialis* used as the initiator inoculum of the astaxanthin formation process (Wang et al. 2013a,b, Do et al. 2019).

For his reason, we apply photoperiods with controlled light intensity during the vegetative cell cycle of *H. pluvialis*, seeking in this way to increase its cell size, and therefore increase astaxanthin production during the cyst formation stage.

Among the most important facts to highlight from the research, it can be said that a strong increase in the density of pure astaxanthin production from *H. pluvialis* was observed. When the 16:8 PP (1) was applied, good results were achieved during the vegetative cycle of *H. pluvialis* (containers B). This PP was able to increase the density of pure astaxanthin production, especially in the spring months. Interestingly, this procedure increased astaxanthin production, especially in the spring months, since with the 16:8 (1) PP, it was possible to pass from 226 to 276 g m^{-3}. This increase is very substantial for this industry from an economic point of view. In other studies, a maximum production density of 9.77 g m^{-3} was achieved using *H. pluvialis* mutations (Dawidziuk et al. 2017), giving a very significant difference between 216.2-266.2 g m^{-3} in favor of our research with the 16:8 (1) PP than the 9.77 g m^{-3} obtained in the other study.

It is necessary to highlight that although the highest astaxanthin production was obtained using 16: 8 (1), the highest cell productivity (green algae) was obtained using 16:8 (2) PP. Both PP agreed that they were applied during the vegetative cycle of *H. pluvialis* and in spring.

To achieve high production of natural astaxanthin sustainably, it is necessary to fundamentally increase

cell density and astaxanthin accumulation within the cells (Ranga-Rao et al. 2014, Zhang et al. 2020). As observed, the organism that produces the best yields of natural astaxanthin is *H. pluvialis* under stress conditions (Zhang et al. 2020). In the vegetative state, these microalgae are green, but when subjected to stress, they start producing astaxanthin inside the cell, changing its color to orange-red (Solovchenko 2015, Shah et al. 2016, Pereira & Otero 2020). This stress can be produced by multiple factors, one of them is the intensity of the light applied to the culture (Azizi et al. 2020), and this is why astaxanthin production has better yields in the spring and summer months compared to autumn and winter (Hong et al. 2019).

It is known that light stress favors astaxanthin production in the curation stage because it would trigger the acceleration of carotenoid production reactions inside the cell, also contributing to increasing its size (Hu et al. 2008). An increase in incident light time and associated temperature in spring improves the production performance of several types of microalga (Oostlander et al. 2020), which coincides with a study by Aflalo et al. (2007). They concluded that the application of controlled doses of light, lower than sunlight, during the green phase, would promote the formation of larger cells that are more resistant to partial stress and, therefore, better able to respond efficiently to astaxanthin production at a later stage under conditions of total stress. However, our results are different. The 16:8 (2) PP treatment was subjected to light stress longer than 16:8 (1) because the light in the latter was applied from its inoculation. Furthermore, this treatment was exposed to a higher light intensity than 16:8 (1) PP when the cultures were made in containers B and during the spring.

On the other hand, it has been observed that there are certain ranges of tolerance to light stress in green alga and that they would also be favorable for their development and survival; these ranges go from 85 to 215 µmol m^{-2} s^{-1} (Rautenberger et al. 2009). The works carried out by Oostlander et al. (2020) show that in *Rhodomonas* sp. maintaining a 16:8 PP cycle, an increase in temperature (21 to 25°C) and light intensity (150 to 600 µmol^{-2} s^{-1}) the increase in biomass and amount of fatty acids (EPA and DHA).

The answer to the question originally posed: what would be the incidence of the PP using LED lights in *H. pluvialis* that allow establishing a sustainable and scalable astaxanthin production over time while maintaining profitable operating costs? It can be inferred that astaxanthin production from stress caused by the artificial light exposure to *H. pluvialis* has an inherent threshold like this microalga, whose production, as expected, is not exponentially infinite from the point of view of its performance in astaxanthin. Based on this natural limitation, it is important to note that once microalga reaches their threshold for astaxanthin production, it stabilizes, regardless of how much *H. pluvialis* exposure to light has increased since then. The above is a key point since any increment in the length of exposure to light leads to an increase in electricity consumption, ceasing to be environmentally and economically sustainable from the moment this threshold is reached since operating costs would far exceed the expected profitability.

As could be seen, our study involved the design of a system to control the light intensity using LED curtains to intervene in the sequential PP cycles of the vegetative *H. pluvialis* cell culture. Thus measures the results on an industrial scale, where we evaluate the intensity of light, temperature, pH, and dissolved oxygen, determining that both the intensity of the light and the temperature are the key factors for the production of vegetative cells (vesicles) of *H. pluvialis*, which are ultimately responsible for the generation of astaxanthin. We also observe that the highest biomass concentrations were reached when the light intensity was low in the cultures. On the other hand, we observe that temperatures do not strongly impact the biomass concentration obtained in each culture, neither in winter nor in spring. However, the temperature does have a great influence on the amount of astaxanthin produced. Our results were evaluated on an industrial scale, showing that the highest astaxanthin production occurred at warmer temperatures, such as those in spring (22-23°C).

If the two seasons tested (winter and spring) are considered, it is possible to make an optimal projection of the production, which amounts to an excellent 280 kg yr^{-1}, with the 16:8 (1) PP and a light intensity of 16 Klux (216 µmol m^{-2} s^{-1}), applied throughout the vegetative growth process of *H. pluvialis*. This way, astaxanthin production increase was observed on an industrial scale in a much higher percentage than that obtained by previous investigations, even exceeding the 242 kg yr^{-1} that was achieved with our control. This projection of an additional 38 kg yr^{-1} represents a 15% increase in final production, which is very encouraging in economic and financial terms for the natural astaxanthin industry, whose market price is around USD 7000 per kg (Shah et al. 2016).

This knowledge generated through the prototype design could increase the production yields of natural astaxanthin of high purity and quality, with significant economic returns for the producers of this valuable pigment. However, it is also important to mention that it is required to improve the power in the curtains of LED lights to increase the intensity in Klux for the need

that is required for growth in the vegetative stage and carotenoids production. According to our results with 16:8 (1) PP, it should be around 216 µmol m^{-2} s^{-1}; and in the cyst production stage should be more light intensity; because it requires high light and thermal stress.

Finally, new challenges arise for future research to help increase astaxanthin production using *H. pluvialis*. In this sense, the proposal would be to experiment with a curtain similar in size to the one used in the present investigation, but with a greater intensity of lumens, as well as different lengths of the white light spectrum, ranging from blue (400-460 nm) to red (620-700 nm) in order to analyze the impact of these spectra in a large-scale study. The purpose would be to test a similar design of curtains, but containing LED bulbs with greater light intensity each, respecting the same 16:8 (1) PP already used successfully in the research and where each bulb consumed 0.25 W for 37.5 lumens emission. Each 1.26 m^2 curtain involved 89 bulbs with a total consumption of 30 W to emit 3.57 Klux. The proposal for the new design would be to use curtains of similar diameter, but with a generation of among 5-10 Klux, which would represent a density of between 90-185 µmol m^{-2} s^{-1}. Additionally, it would be necessary to experiment with different sources of renewable energy, which allows an industrial project to be operational in different areas lacking traditional electricity and at the same time achieve greater environmental and financial sustainability. It would allow evaluating a different impact on astaxanthin production about the final product's cost/benefit/quality and sustainability.

REFERENCES

Aflalo, C., Meshulam, Y., Zarka, A. & Boussiba, S. 2007. On the relative efficiency of two- *vs.* one-stage production of astaxanthin by the green alga *Haematococcus pluvialis*. Biotechnology and Bioengineering, 98: 300-305. doi: 10.1002/bit.21391

Azizi, M., Moteshafi, H. & Hashemi, M. 2020. Distinctive nutrient designs using a statistical approach coupled with light feeding strategy improve *Haematococcus pluvialis* growth performance and astaxanthin accumulation. Bioresource Technology, 300: 1-45. doi: 10.1016/j.biortech.2019.122594

Borowitzka, M.A. & Vonshark, A. 2017. Scaling up microalgal cultures to commercial scale. European Journal of Phycology, 52: 407-418. doi: 10.1080/09670262.2017.1365177

Borowitzka, M.A., Huisman, J.M. & Osborn, A. 1991. Culture of the astaxanthin-producing green alga *Haematococcus pluvialis* 1. Effects of nutrients on growth and cell type. Journal of Applied Phycology, 3: 295-304. doi: 10.1007/BF00026091

Butler, T., McDougall, G., Campbell, R., Stanley, M. & Day, J. 2018. Media screening for obtaining *Haematococcus pluvialis* red motile macrozooids rich in astaxanthin and fatty acids. Biology, 7: 2. doi: 10.3390/biology7010002

Christiansen, R. & Torrissen, O.J. 1996. Growth and survival of Atlantic salmon, *Salmo salar* L. fed different dietary levels of astaxanthin. Aquaculture Nutrition, 2: 55-62. doi: 10.1111/j.1365-2095.1996.tb00008.x

Davis, E.E., Dedrick, J., French, C.S., Milner, H.W., Myers, J., Smith, J.H. & Spoehr, H.A. 1953. Laboratory experiments on *Chlorella* culture at the Carnegie Institution of Washington Department of Plant Biology. In: Burlew, J.S. (Ed.). Algal culture from laboratory to pilot plant. Carnegie Institution of Washington, Washington, DC.

Dawidziuk, A., Popiel, D., Luboinska, M., Grzebyk, M., Wisniewski, M. & Koczyk, G. 2017. Assessing contamination of microalgal astaxanthin producer *Haematococcus* cultures with high-resolution melting curve analysis, Journal of Applied Genetics, 58: 277-285. doi: 10.1007/s13353-016-0378-x

Deniz, I. 2020. Scaling-up of *Haematococcus pluvialis* production in stirred tank photobioreactor. Bioresource Technology, 310: 123434. doi: 10.1016/j.biortech.2020.123434

Díaz-Jiménez, L., Hernández-Vergara, M., Pérez-Rostro, C. & Ortega-Clemente, L. 2019. The effect of astaxanthin and β-carotene inclusion in diets for growth, reproduction, and pigmentation of the peppermint shrimp *Lysmata wurdemanni*. Carotenoids for peppermint shrimp. Latin American Journal of Aquatic Research, 47: 559-567. doi: 10.3856/vol47-issue3-fulltext-17

Do, T-T., Ong, B-N., Nguyen-Tran, M-L., Nguyen, D., Melkonian, M. & Tran, H-D. 2019. Biomass and astaxanthin productivities of *Haematococcus pluvialis* in an angled twin-layer porous substrate photobioreactor: effect of inoculum density and storage time. Biology, 8: 68. doi: 10.3390/biology 8030068

Domínguez, A., Pereira, S. & Otero, A. 2019. Does *Haematococcus pluvialis* need to sleep? Algal Research, 44: 101722. doi: 10.1016/j.algal.2019.101722

Fujii, K., Imazato, E., Nakashima, H., Ooi, O. & Saeki, A. 2006. Isolation of the non- fastidious microalga with astaxanthin-accumulating property and its potential for application to aquaculture. Aquaculture, 261: 285-293. doi: 10.1016/j.aquaculture.2006.07.014

Gajardo-Solari, S., Benites-Vílchez, J., López-Vivar, J., Burgos-Hermosilla, N., Caro-Galán, C. & Rojas-

Arredondo, M. 2011. Astaxanthin: natural antioxidants with various applications in cosmetics. Biofarbo, 19: 6-12.

Galarza, J., Arredondo-Vega, B., Villón, J. & Henríquez, V. 2019. Deesterification of astaxanthin and intermediate esters from *Haematococcus pluvialis* subjected to stress. Journal of Applied Biotechnology Reports, 23: e00351. doi: 10.1016/j.btre.2019. e00351

Gómez, P., Haro, P., Lagos, P., Palacios, Y., Torres, J., Sáez, K., et al. 2016. Intraspecific variability among Chilean strains of the astaxanthin-producing microalga *Haematococcus pluvialis* (Chlorophyta): an opportunity for its genetic improvement by simple selection. Journal of Applied Phycology, 28: 2115-2122. doi: 10.1007/s10811-015-0777-0

Hewlings, S.J. 2019. Human health benefits of astaxanthin derived from *Haematococcus pluvialis*: a review. EC Nutrition, 14: 902-916.

Hong, M., Chang, W., Patel, A., Oh, M., Lee, J. & Sim, S. 2019. Microalgal-based carbon sequestration by converting LNG-fired waste CO2 into red gold astaxanthin: the potential applicability. Energies, 12: 1718. doi: 10.3390/en12091718

Hu, Z., Li, Y., Sommerfeld, M. & Hu, Q. 2008. Enhanced protection against oxidative stress in an astaxanthin-overproduction *Haematococcus* mutant (Chlorophyceae). European Journal of Phycology, 43: 365-376. doi: 10.1080/09670260802227736

Hu, C., Cui, D., Sun, X., Shi, J. & Xu, N. 2020. Primary metabolism is associated with the astaxanthin biosynthesis in the green alga *Haematococcus pluvialis* under light stress. Algal Research, 46: 101768. doi: 10.1016/j.algal.2019.101768

Kobayashi, M. & Sakamoto, Y. 1999. Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*. Biotechnology Letters, 21: 265-269. doi: 10.1023/A:1005445927433

Koyandea, A., Wayne-Chewa, K., Rambabub, K., Taoc, Y., Chud, D-T. & Show, P-L. 2019. Microalga: a potential alternative to health supplementation for humans. Food Science and Human Wellness, 8: 16-24. doi: 10.1016/j.fshw.2019.03.001

Liaaen-Jensen, S. & Frode-Lutnaes, B. 2005. Charged carotenoid species. Studies in Natural Products Chemistry, 30: 515-557.

Machado Jr., F. Trevisol, T., Boschetto, D., Burkert, J., Ferreira, S., Oliveira, V. & Burkert, C. 2016. Technological process for cell disruption, extraction, and encapsulation of astaxanthin from *Haematococcus pluvialis*. Journal of Biotechnology, 218: 108-114. doi: 10.1016/j.jbiotec.2015.12.004

Margalith, P.Z. 1999. Production of ketocarotenoids by microalga. Applied Microbiology and Biotechnology, 51: 431-438. doi: 10.1007/s002530051413

Molino, A., Rimauro, J., Casella, P., Cerbone, A., Larocca, V., Chianese, S., et al. 2018. Extraction of astaxanthin from microalga *Haematococcus pluvialis* in red phase by using generally recognized as safe solvents and accelerated extraction. Journal of Biotechnology, 283: 51-61. doi: 10.1016/j.jbiotec. 2018.07.010

Novoveská, L., Ross, M., Stanley, M., Pradelles, R., Wasiolek, V. & Sassi, J.-F. 2019. Microalgal carotenoids: a review of production, current markets, regulations, and future direction. Marine Drugs, 17: 640. doi: 10.3390/md17110640

Onorato, C. & Rösch, C. 2020. Comparative life cycle assessment of astaxanthin production with *Haematococcus pluvialis* in different photobioreactor technologies. Algal Research, 50: 102005. doi: 10.1016/j.algal.2020.102005

Oostlander, P., Van Houcke, J., Wijffels, R. & Barbosa, M.J. 2020. Growth and fatty acid content of *Rhodomonas* sp. under day:night cycles of light and temperature. Algal Research, 51: 102034. doi: 10.1016/j.algal.2020.102034.

Pereira, S. & Otero, A. 2020. *Haematococcus pluvialis* bioprocess optimization: effect of light quality, temperature and irradiance on growth, pigment content and photosynthetic response. Algal Research, 51: 102027. doi: 10.1016/j.algal.2020.102027

Pérez-López, P., González-García, S., Jeffryes, C., Agathos, S., McHugh, E., Walsh, D., et al. 2014. Life cycle assessment of the production of the red antioxidant carotenoid astaxanthin by microalga: from lab to pilot scale. Journal of Cleaner Production, 64: 332-334. doi: 10.1016/j.jclepro.2013.07.011

Ramos-Tramontin, L., Rueksomtawin-Kildegaard, K., Sudarsan, S. & Borodina, I. 2019. Enhancement of astaxanthin biosynthesis in oleaginous yeast *Yarrowia lipolytica* via microalgal pathway. Microorganisms, 7: 472-479. doi: 10.3390/microorganisms7100472

Ranga-Rao, A., Sarada, R., Baskaran, V. & Ravishankar, G.A. 2009. Identification of carotenoids from green alga *Haematococcus pluvialis* by HPLC and LC-MS (APCI) and their antioxidant properties. Journal of Microbiology and Biotechnology, 19: 1333-1341.

Ranga-Rao, A., Siew-Moi, P., Sarada, R. & Ravishankar-Gokare A. 2014. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications. A review. Marine Drugs, 12: 128-152. doi: 10.3390/md12010128

Ranga-Rao, A., Raghunath-Reddy, R.L., Baskaran, V., Sarada, R. & Ravishankar, G.A. 2010. Characterization of microalgal carotenoids by mass spectrometry and their bioavailability and antioxidant

properties elucidated in rat model. Journal of Agricultural and Food Chemistry, 58: 8553-8559. doi: 10.121/jf101187k

Rautenberger, R., Mansilla, A., Gómez, I., Wiencke, C. & Bischof, K. 2009. Photosynthetic responses to UV-radiation of intertidal macroalga from the Strait of Magellan (Chile). Revista Chilena de Historia Natural, 82: 43-61. doi: 10.4067/S0716-078X2009000100003

Shah, M., Liang, Y., Cheng, J. & Daroch, M. 2016. Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high-value commercial products. Frontiers in Plant Science, 7: 531. doi: 10.3389/fpls.2016.00531.

Solovchenko, A.E. 2015. Recent breakthroughs in the biology of astaxanthin accumulation by microalgal cells. Photosynthesis Research, 125: 437-449. doi: 10.1007/s11120-015-0156-3

Stein, J. 1980. Handbook of phycological methods. Culture methods and growth measurements. Cambridge University Press, Cambridge.

Torzillo, G., Goksan, T., Faraloni, C., Kopecky, J. & Masojidek, J. 2003. Interplay between photochemical activities and pigment composition in an outdoor culture of *Haematococcus pluvialis* during the shift from the green to red stage. Journal of Applied Phycology, 15: 127-136. doi: 10.1023/A:1023854904163

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Turan, T. 2018. Isolation and cultivation of a newly-discovered astaxanthin-rich green microalga - *Haematococcus* sp. flotow strain from Homeros Valley (Bornova Creek, Izmir, Turkey). Turkish Journal of Fisheries and Aquatic Sciences, 19: 785-791. doi: 10.4194/1303-2712-v19_9_07

Wang, J., Sommerfeld, M., Lu, C. & Hu, Q. 2013a. Combined effect of initial biomass density and nitrogen concentration on growth and astaxanthin production of *Haematococcus pluvialis* (Chlorophyta) in outdoor cultivation. Algae, 28: 193-202. doi: 10.4490/Alga.2013.28.2.193

Wang, J., Han, D., Sommerfeld, M.R., Lu, C. & Hu, Q. 2013b. Effect of initial biomass density on growth and astaxanthin production of *Haematococcus pluvialis* in an outdoor photobioreactor. Journal of Applied Phycology, 25: 253-260. doi: 10.1007/s10811-012-9859-4

Xu, L., Weathers, P.J., Xiong, X.R. & Liu, C.Z. 2009. Microalgal bioreactor: challenges and opportunities. Engineering in Life Sciences, 9: 178-189. doi: 10.1002/elsc.200800110

Zhang, C., Chen, X. & Too, H.P. 2020. Microbial astaxanthin biosynthesis: recent achievements, challenges, and commercialization outlook. Applied Microbiology and Biotechnology, 104: 5725-5737. doi: 10.1007/s00253-020-10648-2

Zlotnik, I., Sukenik, A. & Dubinsky, Z. 1993. Physiological and photosynthetic changes during the formation of red aplanospores in the chlorophyte *Haematococcus pluvialis*. Journal of Applied Phycology, 29: 463-469. doi: 10.1111/j.1529-8817.1993.tb00147.x