A novel two-phase bioprocess for the production of *Spirulina (Arthrospira) maxima* LJGR1 at pilot plant scale during different seasons and for phycocyanin induction under controlled conditions

D. A. García-López[a], *E. J. Olguín*[a*], *R. E. González-Portela*[a], *G. Sánchez-Galván*[a], *R. De Philippis*[b], *R. W. Lovitt*[c], *C. A. Llewellyn*[d], *C. Fuentes-Grünewald*[d], and R. Parra Saldívar[e].

[a]*Environmental Biotechnology Group, Institute of Ecology (INECOL). Carretera Antigua a Coatepec 351, Xalapa, Veracruz, 91073, Mexico*.

[b]*Department of Agriculture, Environment, Food and Forestry (DAGRI), University of Florence, Piazzale delle Cascine 18, Florence, 50144, Italy.*

[c]*Membranology Ltd., Swansea Enterprise Park, Rainbow Business Centre, Llansamlet, Swansea, Wales, SA79PF, United Kingdom.*

[d]*Department of Biosciences, Swansea University, Singleton Park, Swansea, Wales, SA28PP, United Kingdom.*

[e]*Tecnológico de Monterrey, School of Engineering and Sciences, Campus Monterrey, Ave. Eugenio Garza Sada 2501, Monterrey, N.L., 64849, Mexico.*

corresponding author:* eugenia.olguin@inecol.mx

Abstract

A two-phase outdoor cultivation bioprocess for *Arthrospira maxima* LJGR1 combined with phycocyanin induction in concentrated cultures under controlled conditions, was evaluated using a modified low-cost Zarrouk medium. Growth was monitored during 4 cycles in 2018 and 4 cycles in 2019. Biomass was harvested and concentrated using membrane technology at the end of each cycle for further phycocyanin induction using blue LED light (controlled conditions, 24h). The highest biomass productivity was observed during spring and summer cycles; during mid-fall and mid-end fall, a decrease was observed. Under favorable growth conditions, phycocyanin induction was successful. However, during cycles with unfavorable growth condition, phycocyanin induction was not observed. Reagent grade phycocyanin was recovered and purified using microfiltration and ultrafiltration technologies. It was concluded that this novel two phase process has a potential to be implemented at larger scale within a biorefinery approach including the production of sub products from residual biomass and exopolysaccharides.

Keywords

Cyanobacteria; pigments induction; membrane harvesting; membrane ultrafiltration; urea.

1. Introduction

The cyanobacterium of the genus Arthrospira, commonly referred to as *Spirulina,* has attracted special interest due to its nutritional value and pharmacological activities (Lee *et al*., 2017). Species of the Arthrospira genus are cultured at large scale for commercial exploitation in several regions, major companies that are involved in its commercialization have presence in USA (California and Hawaii), in some European Countries including United Kingdom, Germany, France, Poland, Netherlands and Asian countries such as Japan, China, India, Taiwan and Thailand (Soni *et al*., 2017), primarily to produce food supplements for humans or feed for animals. Arthrospira species are valued due to their rich composition of minerals and antioxidants, and high protein content and essential amino acids. Additionally, Arthrospira biomass can be used for the extraction of high-value products, such as pigments, including chlorophyll a, beta-carotene and phycocyanin (Lima *et al*., 2018). Pigment production using cyanobacteria offers advantages over its production from other sources like higher plants, as cyanobacteria have faster growth rates and high photosynthesis efficiencies and also require less space (Khazi *et al*., 2018). Many species of cyanobacteria also thrive in extreme medium conditions e.g. high alkaline medium in which *Spirulina* grows and thus avoid culture contamination., The potential for using cyanobacteria for pigment production is remarkable since the demand for natural colorants is growing as consumers and regulatory food administrations increasingly question the use of artificial

colorants (Newsome *et al*., 2014; Vaz *et al*., 2016). Additionally, pigments produced from microalgae have been approved by several food administrations and nowadays are demanded to replace synthetic pigments in food, cosmetic and nutritional markets (Rahman *et al*., 2017).

Phycocyanin (PC) is a blue-colored accessory photosynthetic pigment that is part of the light harvesting complex in cyanobacteria and some eukaryotic algae (Kissoudi *et al*., 2018). The PC produced in cyanobacterium Arthrospira is gaining commercial interest due to its nutritional and health care value (Xie *et al*., 2015). Several industrial applications of PC have been recognized, which include its use as antioxidant, food coloring, cosmetics, pharmaceutical and nutraceutical (Eriksen, 2008, Sekar & Chandramohan, 2008). Generally, PC is extracted from outdoors open-pond cultures of the cyanobacterium *Arthrospira platensis,* however these cultures are not highly productive. Despite remarkable results reported at lab scale (Markou, 2014, Chen *et al*., 2016, Prates *et al*., 2018), different results have been observed under outdoor settings where the fluctuating conditions of both light and temperature, limiting the growth of Arthrospira and PC accumulation. It has been reported that in open ponds or raceways, biomass density can hardly reach beyond 0.8 gL^{-1} and PC content only reaches around 7% of cell of dry weight (DW) (del Rio-Chanona *et al*., 2015); besides, these amounts usually vary in relation to seasonal conditions and location. Therefore, major challenges associated with PC production in Arthrospira include increasing biomass productivity, reducing cultivation costs and increasing PC intracellular accumulation (Manirafasha *et al*., 2016, 2018).

A potential solution to accomplish these goals is the integration of different strategies aimed at reducing cultivation cost and increasing PC content in Arthrospira biomass. Reducing cultivation costs is normally achieved by replacing nutrients from basic culture media for others with lower cost; *i.e*. replacing sodium nitrate, the nitrogen source, by urea (Matsudo *et al*., 2009, Sassano *et al*., 2014; Affan *et al*., 2015) or by sea water enriched with anaerobic effluents or digestates (Olguín *et al*., 1997, 2001, 2003). On the other hand, the induction of pigment content using narrow wavelengths is an attractive route since photosynthetic pigments have specific responses to different light conditions (Schulze *et al*., 2014, 2016; Baer *et al*., 2016, Coward *et al*., 2016). However, the use of artificial light instead of sunlight for long periods represents an increasing cost, compromising the total production cost (Blanken *et al*., 2013).

The effect of light wavelength and intensity has been reported in *Arthrospira* sp. with different results for both growth and pigment production. Chen *et al.*, (2010) studied the effect of different light wavelengths at various intensities, their results demonstrated the best growth of *A. platensis* with red LED light (3000 $\mu molm^{-2}s^{-1}$) whereas blue LED light (3000 $\mu molm^{-2}s^{-1}$) showed the best yield of pigments chlorophyll and PC. Markou in 2014 reported the highest growth of *A. platensis* using pink and red LED light, while the maximum PC content was obtained with blue LED light. Rizzo *et al.*, (2015) studied the effect of white and green fluorescent lamps on *A. platensis* growth and pigment production, while white light (150 $\mu molm^{-2}s^{-1}$) provided the best growth, no differences were observed in pigment production due to the effect of light wavelength. More

recently, Prates *et al.*, (2018) reported the maximum growth of *Spirulina* (*Arthrospira*) sp. LEB 18 under red LED light, but green LED light promoted the highest phycocyanin content.

In order to develop a commercial process, one of the major challenges is to choose a proper reactor. In fact, massive cultivation of microalgae has been developed in open pond raceway (OPR) reactors worldwide, mainly due to their low capital expenditure (CAPEX) and costs of operation (OPEX) which allow scaling up the production to 10,000 - 100,000 L. However, the use of these reactors has been limited to microalgae genera such as *Nannochloropsis* sp., *Chlorella* sp., *Tetraselmis* sp., *Spirulina* (*Arthrospira* sp.), *Dunaliella salina*, *Scenedesmus* sp., *Haematococcus pluvialis*, *Anabaena* sp., *Phaeodactylum tricornotum*, *Micractinium* sp., *Actinastrum* sp. (Kumar *et al*., 2015). According to Grobbelaar (2007), some factors that limit the productivity of microalgae in OPR are: 1) depth of the culture or the cross-section of light, 2) water flow, 3) content and supply of nutrients, 4) cultivation mode (batch, semi-continuous, continuous), 5) biomass concentration or areal density, and 6) photo acclimation. It is important to note that even under the best operating conditions, microalgal cultures growing in OPR are limited due to environmental conditions (mainly daily and seasonal fluctuations of light and temperature), thus, they generally operate with microalgal concentrations between 0.5 - 1 gL^{-1} and they achieve an average productivity of 10 $g_{DW}m^{-2}d^{-1}$ (Morais *et al*., 2009, Kumar *et al*., 2015). Nonetheless, despite these disadvantages, OPR are currently the most widely used type of reactor for microalga large scale cultivation due to their low cost and easy operation compared to closed

photobioreactors (Gupta *et al*., 2015). Therefore, it is desirable to find strategies to achieve higher yields of microalgae per growth area in this type of reactors. In this work, a set of experiments were performed with the aim to develop and evaluate a novel two-phase process for the mass cultivation of *Spirulina (Arthrospira) maxima* LJGR1 at pilot plant level, utilizing a low-cost medium and open pond raceways (OPR) reactors during different seasons. In the second phase, after harvesting, biomass was concentrated using membrane technology and exposed to blue LED lamps, under controlled conditions for a short period, to increase its PC content.

2. Materials and Methods

2.1. Location

Cultivation of *A. maxima* LJGR1 was carried out in an outdoor OPR pilot-scale biorefinery facility located at the City of Xalapa, Veracruz, Mexico (18°36′40.51″N; 95°39′21.93″W), which is inside a greenhouse (400 m^2) for rain protection. The area of Xalapa is considered as “subtropical cloud forest”, characterized by a non-predictable pattern of irradiance due to alternate periods of sunshine and shade by clouds. Climatic conditions of this area are considered as temperate. Light intensity was measured using a quantum meter (model MQ-500, Apogee Instruments®) three times per day (10:00, 14:00, 17:00 h) in six different points located on the surface of each raceway to obtain a representative light profile on the surface of the reactors. Similarly, temperature data were recorded daily as average temperature per day,

maximum and minimum temperatures per day and internal average temperature of the culture medium.

Two open pond raceways (10 m^2, 2000 L) constructed with cement and covered by a PVC membrane were used. The construction of raceways was in accordance to the design proposed by Acien *et al*., (2013) with continuous agitation provided by paddle wheels operating at 30 rpm.

2.2. Microorganism and culture conditions

The strain of *Spirulina (Arthrospira) maxima* LJGR1 was obtained from the Algae Continental Laboratory collection at the Facultad de Ciencias, UNAM, Mexico.

The inoculum cultures were maintained under controlled conditions (100 $\mu molm^{-2}s^{-1}$, 16h light / 8h dark cycle, 32 ºC) at laboratory scale in 4 L bubbled columns, using Zarrouk medium. During the preparation of inoculum for cultivation outdoors, cultures were acclimated (during a period of 8 days) to non-controlled conditions at the pilot plant. This was made using 200 L flat-plate photobioreactors (made of transparent plastic bags) to scale-up the culture volume and then using it as inoculum for the raceway reactors.

A modified version of Zarrouk's medium (1966) was used as culture medium in the raceway reactors. This contained (gL^{-1}): 8.4 g $NaHCO_3$ (reduced 50% from original medium), 0.5 g K_2HPO_4, 0.5 g K_2SO_4 (reduced 50% from original medium), 1.0 g NaCl, 0.04 g $CaCl_2$, 0.08 g EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$; trace metals were added by addition of 1.0 mL L^{-1} of solution A (gL^{-1}): 2.860 g H_3BO_3, 1.810 $MnCl_2 \cdot 4H_2O$, 0.222 $ZnSO_4 \cdot 7H_2O$, 0.079 $CuSO_4 \cdot 5H_2O$, 0.252 Na_2MoO_2; and 1.0 mL L^{-1} of solution B (mgL^{-1}): 22.96

NH_4NO_3, 96.00 $K_2Cr_2(SO_4)_4 \cdot 2H_2O$, 47.85 $NiSO_4 \cdot 7H_2O$, 17.94 $Na_2SO_4 \cdot 2H_2O$, 43.98 $Co(NO_3)_2 \cdot 6H_2O$. Additionally, the original nitrogen source, sodium nitrate ($NaNO_3$), was replaced by urea; this was added daily in fed-batch mode at 0.03 $gL^{-1}\,d^{-1}$ using a stock solution. All salts used were of analytical-grade. Tap-water was used in the preparation of this modified culture medium; for chemical sterilization of the tap water before Arthrospira inoculation, chlorine was used and then neutralized with sodium thiosulfate.

During the experimental stage, 2000 L raceways (10 m^2) were used. The working volume was 1800 L and the depth of the culture was maintained at 18 cm by replacing with tap-water (with sodium thiosulfate) the evaporated water daily. The cultures of *A. maxima* LJGR1 were maintained during a total of 8 cycles (8 days each one): 4 cycles corresponded to year 2018: end of summer (Sept 13-20), early fall (Oct 11-18), mid fall (Oct 24-31), and mid-end fall (Nov 14-21); the rest 4 cycles corresponded to year 2019: mid-end spring (June 5-11), end spring (June 14-22), early summer (June 24-July 2) and mid-early summer (July 4-12). At the end of each cycle, 200 L of culture medium, from each raceway, were harvested and concentrated to 15 L using a membrane filtration platform (membrane microfiltration 0.2 μm) designed and built by Membranology® (Swansea, UK). The concentrated culture was used for the induction stage, whereas the culture remaining in the raceway reactors was used to start a new cycle, with the addition of 200 L of fresh medium.

2.3. Biochemical analysis of the biomass

The protein content was determined according to Lowry *et al*., (1951). Total carbohydrates were determined with the method reported by Dubois *et al.*,

(1956). The lipid content was determined according to the method of Folch *et al*., (1957). Polyphosphates (Poly-P) were determined by the methodology described by Ray & Mukherjee (2015). Ashes were obtained by gravimetric technique burning biomass at 550 ºC during 4 hours in a furnace (AOAC, 1980). Additionally, the presence of exopolysaccharides (EPS) were detected in permeate water using Fourier Transformed Infra-red (FTIR) analysis after harvesting. Previous to EPS chemical precipitation the samples were centrifuged for 10 min at 4500 rpm, the supernatant was used in order to recover the EPS. The EPS were extracted from the sample using ethanol in the ratio 1:1 ethanol:supernatant in a 50 mL tube. Then, the tube was shaken using a vortex (5 min) and stored at 4 ºC for 12 h. After storage, the sample was centrifuged 10 min at 4500 rpm. The remaining ethanol:supernatant was discarded and the sample, with the precipitated wet EPS pellet, was dried for 4 h at 100 ºC. FTIR attenuated total reflectance (ATR) spectrum was collected on Cary 630 FTIR Spectrometer (Agilent®), approximately 3-5 mg of finely powdered dried sample was scan over the wavenumber range of 4000-450 cm^{-1} at a resolution of 4 cm^{-1}. Scans were recorded using the spectroscopic software MicroLab Quant® FTIR Software. Spectra were background corrected for ambient air.

2.4. Kinetic parameters of growth

The samples used to determine algal biomass were first washed with HCl (0.01 M) followed by deionized water (twice), in order to remove salts. The dry weight of microalgal biomass was obtained by gravimetric methods until obtaining a constant weight at 100 ºC. The areal density growth (Chisti, 2016) was

calculated as the total density (X_{max}) produced in the total volume (V_L) between the operation area (A); $Areal\ density(gDWm^{-2}) = (X_{max} * V_L)/A$; Similarly, average productivity (P_x) was calculated as the ratio between the difference in areal density from the beginning to the end of culture to the cultivation time(T_c); *Productivity ($g_{DW}m^{-2}\ d^{-1}$)=(Final (areal density-Initial areal density))／T_c .* The maximum specific growth rate (μ_{max}) was calculated according to Wood *et al*., (2015).

2.5. Phycocyanin induction, extraction and analysis

At the end of each cycle, a volume of 200 L from each raceway was concentrated to a final volume of 30 L using membrane microfiltration rig, as it was mentioned before. For the PC induction, the concentrated 30 L culture was distributed in 2 mini raceways of 15 L capacity each (1 m long, 20 cm wide, and 18 cm high, with a surface area of 0.1914 m^2, and the water column was 8 cm), agitation was provided by paddles at a constant speed of 30 rpm. The mini raceways were placed in a growth chamber (maintained at 30 °C) adapted with blue LED lamps (450 nm), with an effective luminous intensity of 475 $\mu molm^{-2}s^{-1}$. The induction stage lasted for 24 hours, and then the biomass was harvested by microfiltration (0.2 µm).

For PC extraction and quantification, samples of 0.5 ±0.1 g fresh biomass were collected by filtration (Whatman® GF/C membrane, 0.45 microns) and then were washed with HCl (0.01 M) followed by deionized water (twice), to remove salts. The extraction procedure consisted in the addition of solution containing 0.01 M sodium phosphate and 0.15 M NaCl, pH 7.0, to wet biomass (1:10 w/v), followed by four consecutive freezing (-20 ºC) and thawing (4 ºC) cycles of the

biomass to disrupt the cell walls and finally to isolate the PC by centrifugation (14,500 rpm, 10 minutes); at the end of this procedure, the PC crude extract was recovered for further analysis. The calculation of PC content in the crude extract was according to the equation (Eq. 1) described by Bennett & Bogorad (1973).

(Eq. 1) PC (mg/mL) = $[OD_{615}-0.474(OD_{652})] / 5.35$

Using the previous formula and data from the harvested biomass during the extraction procedures (biomass dry weight and volume), the final PC content, expressed as mgPC g^{-1}_{DW} cell was calculated.

Additionally, the absorption spectrum of the PC crude extract was recorded using a spectrophotometer (HACH® Mod. DR/5000), reading from 400 to 700 nm.

2.6. Phycocyanin concentration and purification

The remaining concentrated cell suspension harvested from the mini raceways was disrupted using four consecutive freezing (-20 ºC) and thawing (4 ºC) cycles as mentioned previously.

The PC crude extract was separated from the cell debris using membrane technology. During this procedure, the PC crude extract was concentrated and purified through microfiltration (MF) using 0.2 µm pore size membranes, followed by ultrafiltration (UF) using membranes 10,000 Molecular Weight Cut Off (MWCO) Daltons or 10 kDa. The harvesting rig consists of circulating liquid through 1-inch stainless steel tubular loop fitted with a small 0.9 m^2 of hollow fiber membranes module (GE® Life Sciences) and powered by a small centrifugal pump to provide 1 to 1.5 bar operating pressure regulated by a

diaphragm value in the loop. Through continuous liquid circulation, retentate is concentrated in the rig container and the permeate is collected after passing through the membrane module.

During the MF (regarded as a first stage of purification), the cell debris was separated as retentate in the rig container, while, the PC pigment was separated as permeate. The UF, regarded as a second stage of purification, was performed in order to concentrate and purify the PC found in the retentate. Thus, the continuous circulation of the PC crude extract through the UF membrane, allowed the concentration and purification of PC.

The PC concentration was calculated using the equation (Eq. 1) as described earlier, and the purity was estimated by the ratio between two wavelengths (620 nm as maximum absorbance for PC divided by 280 nm, the absorbance in the UV region) as described in Eq. 2, according to Boussiba & Richmond (1979).

(Eq.2) Purity = OD_{620}/OD_{280}

2.7. Statistical analysis

Multiple linear regression (MLR) was used to analyze the effect of the factors time and cycle on the growth of *A. maxima* LJGR1. One-way ANOVA was performed to compare biochemical composition among data obtained from different cycles, and Tukey multiple comparison test was conducted to determine differences between means with interval confidence 95%. Finally, *t-test* was used to analyze differences in phycocyanin content before and after induction during each cycle. All statistical analyses were performed using Statistica® software, version 7, for Windows®.

3. Results and Discussion

3.1. Climatic conditions during outdoor cultures in raceways

Light intensity and temperature temporal changes are presented in Figures 1 and 2, respectively. It is important to notice that raceways were located inside a greenhouse.

On average, light intensities were 459.0 ±61.9, 524.0 ±73.4, 264.0 ±48.6 and 210.0 ±42.5 $\mu molm^{-2}s^{-1}$ during the 4 first cycles carried out in 2018, respectively and 382.66 ±130.62, 614.53 ±104.56, 528.00 ±118.88 and 732.46 ±112.54 during the second group of cycles performed in 2019, respectively. In the case of temperature inside the culture medium, the average temperature decreased during the first 4 cycles as follows: 22.0 ±2.0, 21.5 ±1.7, 19.0 ±0.8 and 16.1 ±3.2 ºC, respectively but during the second group of cycles, the temperature was similar among the reactors: 22.20 ±1.74, 21.96 ±1.11, 21.24 ±1.86 and 21.55 ±1.35 ºC, respectively. Notably, the maximum temperature inside the greenhouse during some of the first cycles in 2018, was around 35 °C while during the cycles of spring and summer in 2019, maximum temperatures exceeded 40 ºC. Nonetheless, as described above, the average temperature inside the culture medium during the majority of cycles was around 21 - 22 °C, with exception of mid fall and mid-end fall due to climatic conditions, indicating that the raceways had a good agitation and maintained a temperature well below the maximum ambient temperature. This is one of the advantages of this type of reactor compared to closed photobioreactors that require to be cooled down during their operation under high temperatures.

As shown in Figures 1 and 2, the average light intensity showed a higher variability compared to the temperature in the culture during each cycle and throughout the experimental period. This was the result of climatic conditions inherent to the area, showing a clear decrease in both parameters especially during the first four cycles from the end of the summer to early winter. The conditions during the first four cycles and especially during mid fall and mid-end fall were extremely different compared to those observed during the spring and early and mid-early summer in the following year (2019). Such variable conditions affected both, the areal density and the productivity of *A. maxima* LJGR1 cultures as explained in the next section.

3.2. Kinetic parameters of growth

The growth of *A. maxima* LJGR1 in every cycle, reported as areal density ($g_{DW}m^{-2}$), are presented in Fig. 3 and growth parameters are presented in Table 1. It can be observed that the highest areal density was achieved during the end of spring (179.50 ±4.85 $g_{DW}m^{-2}d^{-1}$), early summer (168.00 ±7.00 $g_{DW}m^{-2}d^{-1}$) and mid-early summer (165.20 ±16.34 $g_{DW}m^{-2}d^{-1}$) of 2019 being statistically similar (p<0.05) among the three seasons tested.

The different biomass densities observed (Table 1), could be explained by the environmental conditions (Figs. 1 and 2), which affected *A. maxima* LJGR1 areal density growth performance under outdoor conditions. During the mid-end fall, longer periods of low light intensity (below 210.0 ±42.5 $\mu molm^{-2}s^{-1}$) and temperatures below 15 ºC were observed. Similarly, de Jesus *et al*., (2018) reported that temperatures below 17 ºC reduced the metabolic activity of *Arthrospira (Spirulina)* sp. LEB-18 and therefore reduced its growth. For large-

scale cultivation, in addition to prolonged periods of natural light, Arthrospira strains require high temperatures for optimal growth (Jimenez *et al*., 2003). To analyze the effect of environmental conditions during the growth (as areal density in $g_{DW}m^2$) of *A. maxima* LJGR1 in sequential cycles, analysis of simple linear regression was ran, testing if light intensity and/or temperature could be explanatory variables. However, such statistical analysis was unsuccessful to prove consistent results due to the high dispersion of data (due to changing climatic conditions). Therefore, the effect of cycles (assuming each one with its respective conditions) as factors determining growth was analyzed. Additionally, since each cycle is composed from different days, time was included as second factor, in the model. Using a MLR with cycles and time (days) as factors, the 3D model (Fig. 4) achieved a prediction power of 82% (adjusted R^2=0.82, p<0.0001) and confirmed that the highest areal density was achieved during the end of spring, early summer and mid-early summer.

Concerning the productivities achieved during the different cycles (Table 1), the best results were observed during end spring (18.97 ±0.63 $g_{DW}m^{-2}d^{-1}$) which is 144% higher than the lowest productivity (mid-end fall) followed by mid-early summer (16.24 ±1.73 $g_{DW}m^{-2}d^{-1}$) and the rest of cycles. The lowest productivities corresponded to those cycles carried out under the less unfavorable of climatic conditions, mid fall and mid-end fall (9.93 ±0.88 and 7.76 ±0.80, $g_{DW}m^{-2}d^{-1}$ respectively). Previous studies using open pond raceways for cultivation of Arthrospira showed similar results to those obtained in our study under unfavorable climatic conditions. Olguín *et al*., (1997) cultivated *Arthrospira (Spirulina)* sp. strain (SF) using untreated sea water supplemented

with anaerobic effluents from pig waste, under temperate conditions at Xalapa, Mexico; these authors obtained a maximum productivity of 10.9 $g_{DW}m^{-2}d^{-1}$. In another report, Jimenez *et al*., (2003) studied the annual productivity of *Arthrospira (Spirulina) platensis* strain Laporte M132-1 and the influence of different physicochemical and biological variables, using open pond raceways on the Mediterranean coast in Malaga Spain; they obtained an average productivity of 8.2 $g_{DW}m^{-2}d^{-1}$ with a maximum of 13.95 $g_{DW}m^{-2}d^{-1}$ during summer season (July), whereas productivity decreased drastically during winter season (December-January) to 2 - 2.62 $g_{DW}m^{-2}d^{-1}$. They concluded that not only seasonal variation impacted on Spirulina growth but that physicochemical variables such as pH and dissolved oxygen concentration also exerted a strict control of the productivity in large scale ponds as a consequence of a high photosynthetic activity during this seasonal variation. A control over these variables is therefore required to maintain annual productivity when moving to outdoors conditions. Olguín *et al*., (2003) reported the annual productivity of *Arthrospira (Spirulina)* sp. strain (SF) under tropical conditions (average temperature 28 - 30 ºC, light intensity around 1000 $\mu molm^{-2}s^{-1}$) at La Mancha beach in Veracruz, Mexico, using sea water supplemented with anaerobic effluents from pig waste, maintaining a constant pH of 9.5 in semi-continuous cultures. They reported an average annual productivity of 11.8 $g_{DW}m^{-2}d^{-1}$, (average annual productivity in this work was 13.78 $g_{DW}m^{-2}d^{-1}$) whereas the highest productivity 14.4 - 15.1 $g_{DW}m^{-2}d^{-1}$ was observed during the summer period. Such productivity values during the summer period are quite comparable to the productivities observed in the present work during spring and

summer cycles. In another work reported by Kim *et al*., (2007), the outdoor cultivation of *Arthrospira (Spirulina) platensis* NIES 46 was carried out using open pond raceways and underground-water-based medium inside a green house facility located in Gongju-Si, South Korea. The experimental stage was carried out during summer with an average culture temperature of 30 ºC and a mean sunlight intensity of 807 $\mu molm^{-2}s^{-1}$, ranging from 319 to 1,500 $\mu molm^{-2}s^{-1}$ due to the rainy season. The mean productivity was 10.5 $g_{DW}m^{-2}d^{-1}$ varying from 4.2-12.3 $g_{DW}m^{-2}d^{-1}$ due to the weather conditions of the rainy season. Finally, Morais *et al*., (2009) reported an average productivity of *Arthrospira (Spirulina)* strain LEB-18 of 21.59 $g_{DW}m^{-2}d^{-1}$ under tropical conditions, in Rio Grande south Brasil, using Mangueira lagoon water supplemented with 20% (v/v) Zarrouk medium. Such supplementation of mineral salts and bicarbonate might provide an explanation for achieving the highest productivity among various reports. The productivities of *A. maxima* LJGR1 achieved in the present work are quite comparable to most of those previously reported and discussed above. To further improve productivity,as recommended in Olguín *et al*., 2003 , the depth of the water column in the raceways could be adjusted to a lower level during fall and winter compared to during summer, ().

The maximum specific growth rate (μ_{max}) followed a similar pattern as the other parameters, the highest value was observed during the end of spring being statistically superior with those observed during other periods (Table 1).

Regarding the biomass composition in Arthrospira species, it is well known that protein is the main constituent and under appropriate growing conditions, protein content can be around 50 to 70 % of its dry mass (Ciferri, 1983). In our

study, the protein content of *A. maxima* LJGR1 biomass ranged from 51 to 64 % during the cycles observed, being highest (>60%) during the four periods in 2019, corresponding to spring and summer. In contrast, the biomass at the end summer and fall showed a lower protein content (Table 2). For carbohydrates and lipids, the pattern was the opposite, with highest contents during end of summer and fall. This is in agreement with previous reports showing that under stress periods, such as exposure to lower intensities and/or temperatures not optimal for growth, that carbohydrates and lipids contents increase as storage molecules (Magro *et al*., 2017). Our observed percentages of carbohydrates and lipids are very similar as those reported previously in Arthrospira strains (Satyanarayana *et al*., 2011).

3.3. Phycocyanin induction, extraction and analysis

The phycocyanin (PC) content before and after induction is presented in Fig. 5. The use of blue LED light increased the PC content during most of the cycles with exception of during mid fall and mid-end fall when growth conditions were less favourable. This is consistent with PC, which serves as a light harvesting compound, requiring favorable growth conditions such as constant high light intensities and temperatures above 30ºC (de Jesus *et al*, 2018).

It has been reported that PC is affected as a result of non-favorable growth conditions (de Jesus *et al*., 2018; Hidasi & Belay, 2018), since the production of PC is totally or partially associated with cell growth (Deamici *et al*., 2016).

This hypothesis is reinforced by the profile of ammonium nitrogen ($N\text{-}NH_4^+$) content in the culture medium observed during different cycles (Fig. 6). Since

urea, in the culture medium, is hydrolyzed and transformed into $N\text{-}NH_4^+$, the increased concentration of this compound could indicate an inefficient use of this alternative nitrogen source by *Arthrospira maxima* LJGR1 during the unfavorable growth conditions observed in mid fall and mid-end fall. It should be noted that during favorable conditions, the $N\text{-}NH_4^+$ concentration was fluctuating due to the daily addition of urea following a fed-batch mode at 0.03 $gL^{-1}d^{-1}$. According to Danesi *et al*., (2002), temperature influences cultivation in different ways: higher temperatures favor growth of the microorganisms and increases the rate of $N\text{-}NH_4^+$ loss; under such conditions growth can be promoted and urea is used efficiently since $N\text{-}NH_4^+$ accumulation is avoided. Similarly, the highest growth of *A. maxima* LJGR1 was observed when $N\text{-}NH_4^+$ concentration was lower than 4 mgL^{-1}, during spring and summer cycles in which the temperature inside the cultures was above 21 ºC. Conversely, during the fall, the lowest growth and maximum ammonium accumulation was observed. Additionally, maximum environmental temperatures decreased from 33 to 24ºC during mid fall, and from 31 to15 ºC during mid-end fall cycles. These results are similar to those reported by Olguín *et al*., (1997) during the cultivation of *Spirulina* sp. under temperate conditions during autumn (with the lowest temperature and intensity, 22 ºC and 245 $\mu molm^{-2}s^{-1}$, respectively) with daily addition of anaerobic effluents. Under these conditions, the lowest productivity (3.6 $g_{DW}m^{-2}d^{-1}$) and protein content (34%) were observed, together with $N\text{-}NH_4^+$ accumulation in the culture medium as a result of a lower productivity. In this work, the solar radiation and temperature, during cycle 3 and 4, probably not only affected the growth of *A. maxima* LJGR1 but also the phycocyanin content.

The growth of *Arthrospira* and its PC content are influenced not only by the environmental conditions (light and temperature) but also by the components in the medium, mainly the nitrogen source. Different studies have evaluated these factors separately at laboratory scale (Chaiklahan *et al*., 2007, Khazi *et al*., 2018, Kumar *et al*., 2018). However, the evaluation of the influence of various parameters in outdoor cultures is very complex since it is not possible to consider individual effects without considering the rest of them. Example of this complexity is the study reported by Hidasi & Belay (2018) where it was demonstrated that the composition of photosynthetic pigments under outdoor conditions not only varies seasonally but also diurnally.

Light intensity and quality of light, are some of the main factors evaluated in the production of PC, (Pagel *et al*., 2019). Various studies related to the influence of light intensity have found that low light intensities (lower than 200 $\mu molm^{-2}s^{-1}$) increase the PC content (Olguin *et al*., 2001, Chaiklahan *et al*., 2007, Ogbonda *et al*. 2007, Rizzo *et al*., 2015). During the attenuation of light, the accessory pigments are necessary to guarantee the maximum possible absorption (Olivera *et al*., 2014). However, most of such studies are the result from experiments performed at laboratory indoors conditions that lack the variability observed at outdoors. It has been reported by Goksan & Kilic (2009), that under outdoor cultivation of *A. platensis* during summer in Turkey, the highest concentration of PC was observed during maximum conditions of intensity and temperature (1416 $\mu molm^{-2}s^{-1}$ and 33.8 °C) compared to a lower PC concentration during the minimum conditions of such parameters (571 $\mu molm^{-2}s^{-1}$ and 23.6 °C). Similarly, de Jesus *et al*., (2018) evaluated the growth of the

Spirulina sp. LEB-1 under outdoor condition in two sites in Brazil. These authors found that the highest PC content (31% of cell DW) was observed in the region with higher light intensity and temperature (75.85 - 1800 $\mu molm^{-2}s^{-1}$ and 27 - 37 °C) in contrast to a lower PC content (7% of cell DW) observed in the region of lower light intensity and temperature (22.29 - 558.75 $\mu molm^{-2}s^{-1}$ and 19 - 25 °C).

Furthermore, results on the use of light quality in the literature have shown contrasting results. Although it is known that PC has its maximum absorption peak in the red light range at 620 nm (Li *et al*., 2019), the results in the literature differ in the conclusions in terms of the quality of light required to increase PC content. Some authors report that red light (Walter *et al*., 2011; Yim *et al*., 2016; Kilimtzidi *et al*., 2019) and others that blue light (Akimoto *et al*., 2012; Markou 2014; Lee *et al*., 2016; Park & Dinh 2019) give higher increases. It has recently been suggested (Hsieh-Lo *et al*., 2019) that the effect of blue light to induce PC is due to the short wavelength of the blue light, which is not easily absorbed, demanding the production of PC as accessory pigment. Furthermore, a combination of 70% red and 30% of blue light has been reported with good results (Lima *et al*., 2018).

Additionally, the effect of light quality cannot be considered isolated and its effect may vary depending on the variation and composition of nutrients in the medium, as recently evidenced by Wicaksono *et al*., (2019). It is important to notice that most of these studies correspond to laboratory-scale experiments with the exception of the one reported by Kilimtzidi *et al*., (2019) in which red filters were used to cover the surface of raceways exposed to outdoors

conditions. Such lack of experiments for the evaluation of the effect of light quality under outdoor conditions, represent a limitation and it is difficult to compare the results obtained under indoor conditions. Furthermore, such controversial results open the question if the kind of light quality should be a variable to induce PC at larger scales.

Finally, the source of nitrogen, its concentration and application mode (batch, fed-batch, continuous), is the other main factor involved in the PC content, since PC is a protein pigment and nitrogen plays a key role during its production (Boussiba & Richmond, 1980). The most studied nitrogen source in the culture of Arthospira is nitrate, since it is the nitrogen component of the Zarrouk medium. However, the use of alternative nitrogen sources may be even more effective for the production of PC such as urea (Ajayan *et al*., 2012). Nonetheless, it is difficult to isolate the effect of urea on PC production, laboratory scale studies have shown that efficient urea consumption varies with light intensity. Xie *et al*., (2015) reported the highest PC content (16.1%) in lab experiments under a light intensity of 300 $\mu molm^{-2}s^{-1}$ and a urea fed batch feeding of 5 mM.

In this study, the feeding of the nitrogen source, urea, was not a limiting growth factor as it occurred at the end of cultures subjected to a batch feeding mode. Furthermore, since urea was hydrolyzed releasing $N\text{-}NH_4^+$ constantly, the cells were able to use this N source efficiently when the light intensity and temperature were favorable. Such behavior was observed during the majority of periods with exception of mid fall and mid-end fall. However, when these environmental parameters were not favorable such as in the cycles performed

during mid fall and mid-end fall, $N\text{-}NH_4^+$ was accumulated in the culture medium. It has already been reported that ammonium nitrogen inhibits the growth of most microalgae when it is found in concentrations higher than 25 - 35 mgL^{-1} due to excess of N (Converti *et al*., 2006).This could explain the lowest growth and the low induction of PC that were observed during such periods where the highest ammonium increase were observed (Fig. 6). These results suggest that for a successful induction of PC using blue light, the inoculum must be a culture under non-limited N conditions and the utilization of the nitrogen source should be at a high uptake rate, which in turn depends on favorable environmental conditions for optimum growth.

In summary, in this study, the feeding of urea under a fed batch mode outdoors may be interacting with the daily fluctuating environmental conditions and the conclusion is that multiple factors determined the PC contents during the different tested periods. In other words, cultures of Arthrospira under outdoor conditions respond in a complex manner where all the factors affect the metabolism involved for the production of PC. Thus, the results under outdoor conditions are not necessarily consistent with data reported from lab indoors experiments under controlled conditions.

Results from different previous reports using Arthrospira strains for PC production are shown in Table 3. These values are usually around 7 to 17% PC (% dry weight), similar to those achieved in the present work. It is important to note that all works reported previously were operated at lab scale volume (0.25 to 10 L) using artificial light source. Therefore, the present work provides

interesting information at pilot plant scale with attractive results regarding the PC content in the biomass of this cyanobacteria.

3. 4. Phycocyanin concentration and purification

For commercial purposes PC requires purification. To achieve this PC pigment was separated from the cell debris through microfiltration (MF), to a concentration of PC in the crude extract of 0.14 ±0.02 $mgmL^{-1}$ and a purity of 2.65 ±0.18. During the second filtration step using ultrafiltration (UF, 10 kDa), the PC concentration increased to 0.47 ±0.02 mg mL^{-1} and the purity to 3.72 ±0.14. These results represent an increase of 3.37-fold with respect to PC content and 1.43-fold with respect to the purity obtained after MF, respectively (Fig. 7). The use of MF followed by UF to concentrate and purify PC was efficient for obtaining PC of analytical grade, *i.e.* Abs_{680}/Abs_{280} between 0.7-3.9 (Fernández-Rojas *et al*., 2014). Filtration using membranes is an attractive technique for PC recovery and purification since it can be applied to large-scale, it is simple to operate and considerable cheaper and energy efficiently compared to chromatographic columns or PC concentration using centrifuges; additionally, it is normally carried out at ambient temperature reducing thermal damage (Chaiklahan *et al*., 2011). Nonetheless, filtration needs to be applied efficiently using proper Molecular Weight Cut Off (MWCO) in order to recover PC with high purity grade. Thus, only few articles have been published exploiting this technique and other methods such as chromatography, aqueous two-phase system (ATPS), precipitation and reverse osmosis.

Rito-Palomares *et al*., (2001) reported a method, for PC recovery and purification from an *A. maxima* strain, composed of two ATPS extraction steps,

followed by UF with polymeric membrane 30 kDa, and finally a precipitation step with ammonium sulphate. At the end of this process they obtained PC of reagent grade with a purity of 3.8 ±0.10. A similar value was reported in the present study using a membrane of 10 kDa in the harvesting rig. Similarly, Patil *et al*., (2008) reported the extraction and purification of PC from *A. platensis*, through multiple ATPS stages integrated with a final UF step (30 kDa). The use of UF facilitated the separation of polyethylene glycol used during ATPS, which in turn increased the concentration of PC with purity from 3.23 to 4.02. Finally, Chaiklahan *et al*., (2011) described the purification of PC from *A. platensis* by a filtration process, using a first step filtration to separate cell debris (0.5 µm), then a MF filtration step was included (0.8/0.2 µm) and finally a UF (50 kDa) was applied; with this process they obtained phycocyanin with purity around 1.0.

The process employed during the present study allowed a PC recovery of reactive grade with the use of an efficient membrane technology and suitable for large-scale process. Using this downstream process approach also allows the extraction of other interesting metabolites such as carbohydrates or lipids in the remaining biomass debris after PC extraction. This double advantage is remarkable since 50 - 90% of pigment production costs are related to the purification step (Patil *et al.,* 2006).

3.5. Biorefinery potential, valorization of sub-products

The residual biomass *of A. maxima* LJGR1 after phycocyanin extraction was sun-dried, and subjected to a biochemical analysis showing a protein content of 31.45 ±1.99 of DW cell, carbohydrates 13.20 ±1.44 of DW cell, lipids 4.39 ±1.44

of DW cell and ash content of 29.86 ±1.66 of DW cell. The high ash content restricts the use of residual biomass as a food additive in aquaculture or animal feed. However, the residual biomass has potential for biogas generation through bio-digestion (Vonshak & Richmond 1988, Wuang *et al*., 2016). The use of this residual biomass in biofuel production could be attractive, since increases the value from waste biomass.

Additionally, exopolysaccharides, which have potential commercial value, were detected in the permeate water and were extracted with a chemical precipitation. It has been reported that EPS has anti-inflammatory, hypoglycemic, and anticoagulant/antithrombotic activities with potential uses in medicine (Raposo *et al*., 2013). Additionally, EPS can be used in the metal wastewater bioremediation, as chelating agents for the removal of positively charged heavy metal ions (De Philippis *et al*., 2011).

Finally, in the context of a biorefinery where multiple products are produced, increasing the economic feasibility of the integrated system (Cherubini, 2010; Olguín, 2012), a schematic approach for the recovery of PC, residual biomass and EPS is suggested. One of the advantages of this process is the use of microfiltration for harvesting, which allows the pigment recovery, microalgal biomass and permeate water, reducing labor time and energy costs. It is important to notice that although several techniques have been optimized to purify PC from Arthrospira *(Spirulina)*, only few works have used membrane separation processes. Membrane separation has several characteristics that ensures minimum denaturation, deactivation, and/or degradation of highly labile biological products (Figueira *et al*., 2018). A potential biorefinery based on *A.*

maxima LJGR1 could, therefore, be developed through innovative and cost-efficient processes using biomass to produce different bio-based products.

4. Conclusions

It has been demonstrated that a two-phase process for PC production is feasible using membrane technology. This approach includes a first outdoor production stage using low cost-medium with daily addition of urea promoting a high productivity of *A. maxima* biomass, followed by an induction stage of PC using blue LED light under controlled conditions. Additionally, residual biomass after pigment extraction could be recovered and permeate water contained EPS as well. The potential exploitation and effective recovery of these sub-products could improve the overall economic viability of the cultivation of *A. maxima* through a biorefinery approach at industrial microalgae culture scale.

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Figure Captions

Figure Captions

Fig. 1. Light intensity during cycles in 2018: end summer (Sept 13-20), early fall (Oct 11-18), mid fall (Oct 24-31), mid-end fall (Nov 14-21), and cycles in 2019: mid-end spring (June 5-11), end spring (June 14-22), early summer (June 24-July 2) and mid-early summer (July 4-12).

Fig. 2. Temperature during cycles in 2018: end summer (Sept 13-20), early fall (Oct 11-18), mid fall (Oct 24-31), mid-end fall (Nov 14-21), and cycles in 2019: mid-end spring (June 5-11), end spring (June 14-22), early summer (June 24-July 2) and mid-early summer (July 4-12).

Fig. 3. Areal density ($g_{DW}m^{-2}$) of *A. maxima* LJGR1 in two raceway reactors, during cycles at 2018: end summer (Sept 13-20), early fall (Oct 11-18), mid fall (Oct 24-31), mid-end fall (Nov 14-21), and cycles at 2019: mid-end spring (June 5-11), end spring (June 14-22), early summer (June 24-July 2) and mid-early summer (July 4-12).

Fig 4. 3D model representing the effect of cycle and time on the growth (as areal density in $g_{DW}m^{2}$) of *A.maxima* LJGR1.

Fig. 5. Phycocyanin content (mg g_{DW}^{-1}) in *Arthrospira maxima* LJGR1 during different growth cycles. Different letters indicate significant differences inside cycles, between PC content induced and non-induced, t-student test (p <0.05), n=6.

Fig. 6. Ammonium nitrogen content (mg L^{-1}) in the culture medium of *A. maxima* LJGR1 during cycles in 2018: end summer (Sept 13-20), early fall (Oct 11-18), mid fall (Oct 24-31), mid-end fall (Nov 14-21), and cycles in 2019: mid-end

spring (June 5-11), end spring (June 14-22), early summer (June 24-July 2) and mid-early summer (July 4-12).

Fig. 7. (a) Phycocyanin concentration obtained after microfiltration (MF) and ultrafiltration (UF), and **(b)** phycocyanin purity obtained after microfiltration (MF) and ultrafiltration (UF).

Tables

Table 1. Maximum areal density (X_{max}), average productivity (P_x), maximum specific growth rate (μ_{max}) of *Arthrospira maxima* LJGR1 during different cycles carried out in 2018 (marked with *) and during 2019 (marked with **).

Period	Kinetic parameters		
	X_{max} ($g_{DW}m^{-2}$)	P_x ($g_{DW}m^{-2}$ d^{-1})	μ_{max} (d^{-1})
End summer*	140.63 ±1.05^{c}	13.76 ±0.19^{c}	0.282 ±0.083^{c}
Early fall*	145.46 ±6.24^{b,c}	14.17 ±0.63^{c}	0.307 ±0.030^{c}
Mid fall*	123.57 ±4.41^{c}	9.93 ±0.88^{d}	0.235 ±0.075^{c}
Mid-end fall*	95.70 ±3.38^{d}	7.76 ±0.80^{d}	0.200 ±0.023^{c}
Mid-end spring**	132.90 ±19.09^{c}	13.63 ±2.42^{c}	0.242 ±0.051^{c}
End spring**	179.50 ±4.85^{a}	18.97 ±0.63^{a,b}	0.452 ±0.048^{a,b}
Early summer**	168.00 ±7.00^{a}	15.81 ±1.02^{c}	0.327 ±0.047^{b,c}
Mid-early summer**	165.20 ±16.34^{a}	16.24 ±1.73^{b,c}	0.321 ±0.051^{b,c}

Different letters indicate significant differences, Tukey test ($p < 0.05$), n=6

Table 2. Biochemical composition of *Arthrospira maxima* LJGR1 in cultures exposed outdoors during different cycles, carried out in 2018 (marked with *) and during 2019 (marked with **).

Cycle	Composition (%)				
	Proteins	**Carbohydrates**	**Lipids**	**Poly-P**	**Ashes**
End summer*	53.05 ±2.63^{b}	12.74 ±2.26^{d}	8.85 ±0.17^{a}	7.19 ±0.05^{a}	9.29 ±0.49^{c}
Early fall*	55.90 ±2.33^{b}	14.53 ±0.46^{c,d}	9.66 ±0.42^{a}	6.99 ±0.13^{a}	10.09 ±0.98^{c}
Mid fall*	51.64 ±2.84^{b}	16.31 ±1.56^{b,c}	9.66 ±0.70^{a}	7.05 ±0.0.08^{a}	10.06 ±0.56^{c}
Mid-end fall*	52.63 ±1.32^{b}	17.78 ±2.63^{a,b}	6.51 ±0.77^{b}	4.14 ±0.25^{c}	11.49 ±1.22^{a,b}
Mid-end spring**	61.47 ±1.44^{a}	14.52 ±2.02^{c,d}	4.10 ±0.46^{c}	4.60 ±0.53^{c}	9.21 ±0.53^{c}
End spring**	64.45 ±3.40^{a}	10.86 ±0.92^{e}	3.91 ±0.63^{c}	4.73 ±0.26^{c}	10.39 ±0.71^{b,c}
Early summer**	64.73 ±2.76^{a}	10.30 ±0.70^{e}	5.36 ±1.02^{b}	4.31 ±0.18^{c}	10.65 ±0.32^{b,c}
Mid-early summer**	64.79 ±2.27^{a}	9.58 ±0.30^{e}	6.21 ±0.75^{b}	5.88 ±0.82^{d}	9.24 ±0.11^{c}

Different letters indicate significant differences, Tukey test ($p < 0.05$), n=6

Table 3. Phycocyanin content (% dry weight) obtained from different Arthrospira strains reported previously, and in this work.

Strain	Working volume (L)	Light (source)	PC content (% DW)	Reference
A. platensis	0.25	White fluorescent lamp 4.8-6 Klx	14.1±2.2	Ajayan *et al*., (2012)
A. platensis SAG 21.99	0.4	Blue LED 9±0.5 Klx	17.6±2.4	Markou, (2014)
A. platensis	1	White fluorescent lamp 150 µmol	7-10	Rizzo *et al*., (2015)
A. platensis	1	White tungsten filament lamps 300µmol	16.1	Xie *et al*.,(2015)
A. platensis	1	White fluorescent lamp 700 µmol	16.8	Chen *et al*., (2016)
A. platensis	0.8	White LED 400 µmol	14.9	Ho *et al*., (2018)
A. sp. LEB18	10	Green LED 500 µmol	12.6	Prates *et al*., (2018)
A. maxima LJGR1 (UNAM)	2000	Sun light 459µmol (growth phase), Blue LED 475±10 µmol (induction phase)	9.5 (growth), 13.5 (induction)	This work*

* end-summer 2018.

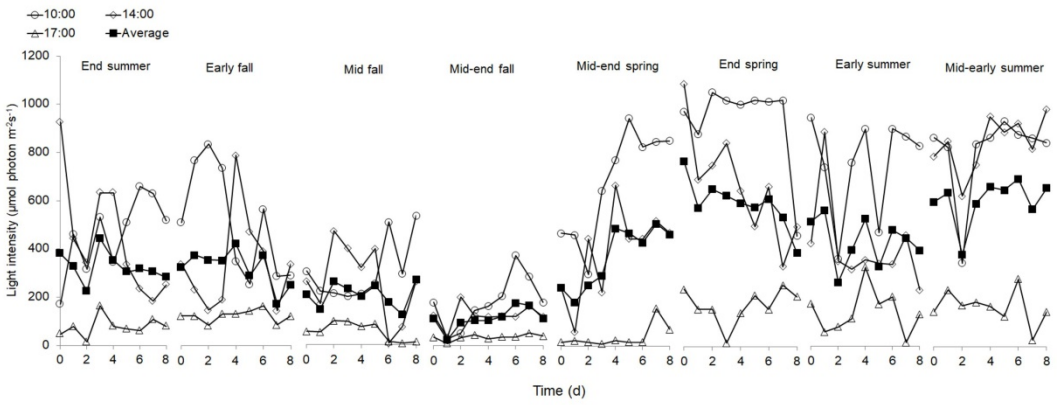
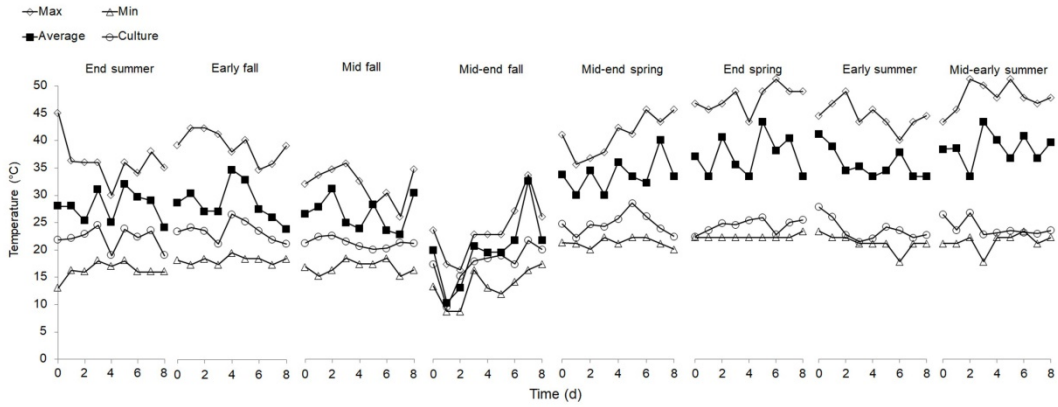


Figure 1.



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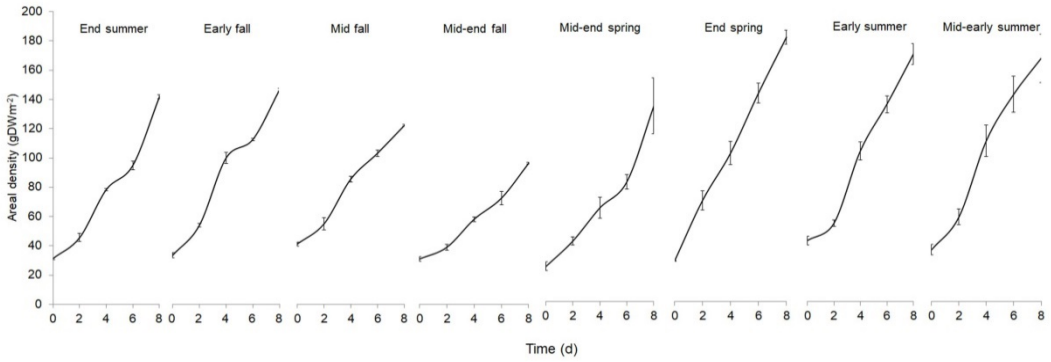

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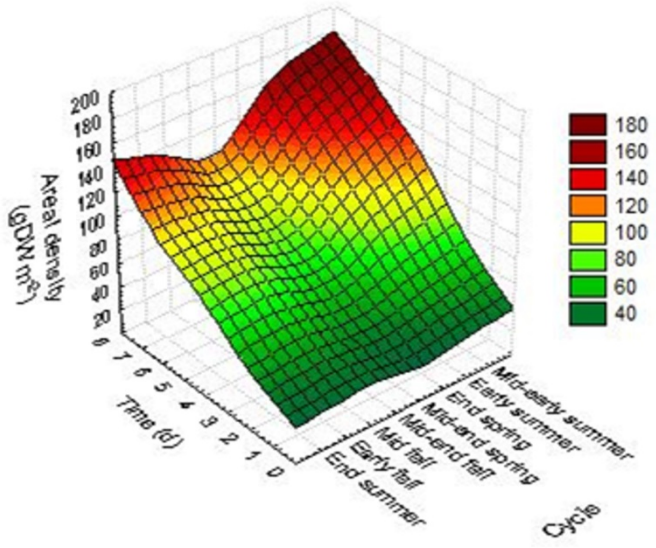

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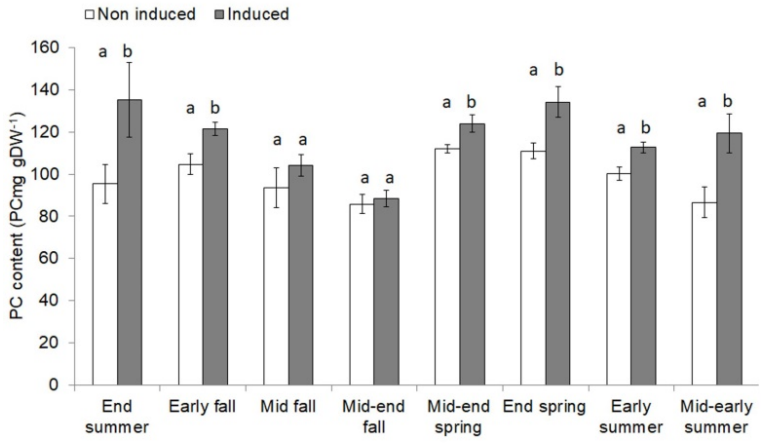
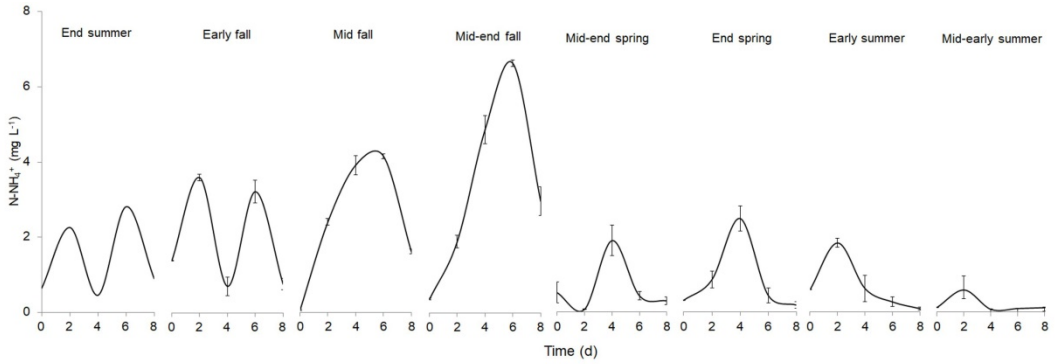


Figure 5.



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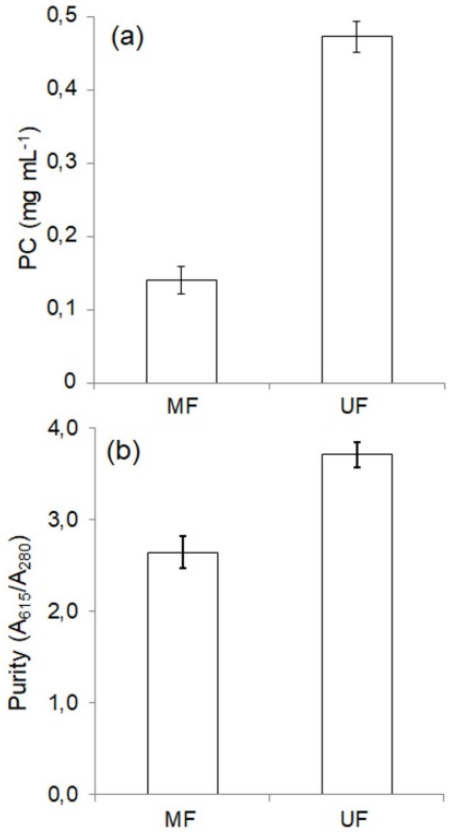
(a)
PC (mg mL-1)
0,5
0,4
0,3
0,2
0,1
0
MF
UF
(b)
Purity (A615/A280)
4,0
3,0
2,0
1,0
0,0
MF
UF

Figure 7.