TESIS DE DOCTORADO

# MICROALGAE CULTURE OPTIMIZATION AND INDUSTRIAL UPSCALING: INFLUENCE OF LIGHT COMPOSITION ON GROWTH, PIGMENT CONTENT AND PHOTOSYNTHETIC RESPONSE OF FOUR MICROALGAL SPECIES WITH COMMERCIAL INTEREST

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**ESCUELA DE DOCTORADO EN CIENCIAS.**
**PROGRAMA DE DOCTORADO EN AVANCES EN BIOLOGÍA MICROBIANA Y PARASITARIA**

SANTIAGO DE COMPOSTELA
2019

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**Microalgae culture optimization and industrial upscaling: influence of light composition on growth, pigment content and photosynthetic response of four microalgal species with commercial interest**

D./Dña. Simón Pereira Bouzas

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**Microalgal culture optimization and industrial upscaling: influence of light composition on growth, pigment content and photosynthetic response of four microalgal species with commercial interest**

D./Dña. Ana María Otero Casal

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"Life did not take over the globe by combat,
but by networking"

Lynn Margulis and Dorion Sagan

Microcosmos, 1986

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I would like to express my gratitude to Professor Ana Otero for her guidance throughout these last years, as a mentor and director of the present work. Despite one of the things that I have realized developing this project is the amount of things I am ignorant of regarding this topic, you have greatly contributed to my understanding of the things I do know and therefore to the things I have achieved. The works where you have been involved have always been a reference. Special mention to your contributions in microalgae research that, without a shadow of a doubt, have helped greatly to the industrial development of this field. I am grateful that you have trusted me to add my thoughts to the interpretation of part of yours and your colleagues' work with *H. pluvialis*, and that we could finally see justice done. Additionally, every discussion with you contributes either to better interpretations, or to plant the seed of ideas that feed my mind and keep it busy for a while, rising new questions. All that has an immense value for me. I am glad we have teamed up.

I would like to refer to all the colleagues I have worked with during these last years in the Netherlands, and specially those within the *H. pluvialis* team at Astaco Technologies, with whom I have shared so much time. Without their effort this work could not have ever be completed. In part, this work belongs to them as well. I have learnt something from each one of them and I hope I have given something back.

Taco Neeb, I am glad that you have placed your trust in me to take part in the shaping of Astaco Technologies as a company, and I largely appreciate the freedom that is given in this company to develop your own ideas and projects, growing together. For this, in all honesty: thank you.

I would like to extent these words to all those with whom I have shared some time at the IEO-Vigo and at the research group directed by Prof. Ana Otero in the USC. Special thanks to Blanca Álvarez, who showed me a photobioreactor for the first time, as well as to the very smart Hugo Milhazes, who did a good work accompanying me during my first steps with microalgae. I am certain he is doing a perfect job keeping time "on time".

To my family and my friends, most of which probably don't know I've ever enrolled and completed a PhD. Regardless of this fact, I am sure they will all be up for a celebration as soon as they hear the good news.

To my parents, for obvious reasons. Both of you are references for me, and for others in your respective fields. You have taught me to live simply and to value and enjoy basic pleasures, which has shaped me enormously. I have been influenced by the way you both think and act, and I feel proud every time someone tells me that this and that reminds them to any of you. To a great extent, I am myself because of you. Thank you.

I extent these words to my sister, who has helped me complete this work. She is sharp and smart, a good combination to achieve whatever someone aims for. I am sure this will be the case.

To Elena, for her uniqueness and the time spent together, full of joy above all else.

Finally, I would like to remember those who, surely, would have loved to hear how a microalgae looks like and what are the reasons why we grow them, what is a photobioreactor,

what do I do at work, how is life in the Netherlands, how photosynthesis works, and how light, as life, is full of colours.

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**ABBREVIATIONS**

ADP: Adenosin diphosphate
ANOVA: Analysis of variance
APC: Allophycocyanin
ARA: Araquidonic acid
ATP: Adenosin triphosphate
Ax: Antheraxanthin
CAPEX: Capital expenditure
CH: Carbohydrate
Chl: Chlorophyll
CIP: Clean in place
$CO_2$: Carbon dioxide
Cyt bf: Cytochrome $b_6f$ complex
DCW: Dry cell weight
DHA: Docosahexaenoic acid
DMF: N, N-Dimethylformamide
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
$Ek$, $Ik$: Photosynthesis saturation point
EPA: Eicosapentaenoic acid
$E_{PAR}$: Incident irradiance, PAR region
EPS: Exopolysaccharides
ETR: Electron transport rate
$Fo$: Minimal fluorescence
FA: Fatty acid
$Fm$: Maximal fluorescence
FNR: Ferredoxin nicotinamide adeninde dinucleotide phosphate reductase
$Fv$: Variable fluorescence
$Fv/Fm$: Variable to maximum fluorescence ratio
GGPP: Geranylgeranyl pyrophosphate
$H^+$: proton
$H_2O$: Water
$H_2SO_4$: Sulfuric acid
HPLC: High-performance liquid chromatography
hv: Photon
$Ic$: Photosynthesis compensation point
$Ik$, $Ek$: Photosynthesis saturation point
LC(s): Light curve(s)
LED: Light emitting diode
LHC: Light harvesting complex
LYC-B: Lycopene β-cyclase
$NADP^+$: Nicotinamide adenine dinucleotide phosphate
$NADPH_2$: Reduced Nicotinamide adenine dinucleotide phosphate
Nm: nannometer
NaOH: Sodium hydroxide
NPQ: Non-photochemical quenching
$O_2$: Molecular oxygen

OHM: Optimum *Haematococcus* medium
OPEX: Operational expenditure
PAM: Pulse amplitud modulation (fluorometry)
PAR: Photosynthetic active radiation
PBR(s): Photobioreactor(s)
PBS: Phosphate buffer saline
PC: Phycocyanin
PC: Plastocyanin
PCB: Phycocyanobilin
PE: Phycoerythrin
PEB: Phycoerythrobilin
PEC: Phycoerythrocyanin
Pheo: Pheophytin
P-I curve: Photosynthesis-irradiance or photosynthesis-response curve
$P_{max}$: Maximum photosynthesis
PQ: Plastoquinone
$PQH_2$: Reduced plastoquinone
PSI, P700: Photosystem one
PSII, P680: Photosystem two
PSY: Phytoene synthase
PUB: Phycourobilin
PUFA: Polyunsaturated fatty acid
PXB: Phycobiliviolin
$Q_A$: Primary quinone electron acceptor
$Q_B$: Secondary quinone electron acceptor
$qN$: Non-photochemical quenching
$qP$: Photochemical quenching
QY: Quantum Yield
$rETR$: Relative electron transport rate
$R_f$: Ratio of front
ROS: Reactive oxygen species
Rpm: Revolutions per minute
RuBisCO: Ribulose-1,5-biphosphare carboxylase/oxygenase
SCP: Single cell protein
SD: Standard deviation
$S/V$: Illuminated surface to volume ratio
TLC: Thin-layer chromatography
USD: United state dollars
UV-VIS: Ultraviolet-visible (spectroscopy)
Vx: Violaxanthin
Zx: Zeaxanthin

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# INTRODUCTION

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# 1. Microalgae. General aspects

Microalgae do not constitute a taxonomic group by their own. Instead, microalgae is a term that is used to define a group of unicellular microorganisms highly diversified, which exist in practically every aquatic environment and conduct, in general, oxygenic photosynthesis (Richmond and Qiang, 2013). Microalgae show a huge variability in terms of size, shape, habitat, cellular structure, mode of reproduction, or biochemical composition (Barsanti and Gualtieri, 2006; Richmond and Qiang, 2013). A wide array of diverse carbohydrates, lipids, proteins, and a variety of photosynthetic pigments can be found along the entire microalgae spectrum (Spolaore *et al.*, 2006; Borowitzka, 2013). Microalgae are primary producers, and it is generally accepted that they contribute to around half of the photosynthetic productivity on earth that is mainly occurring in the oceans, meaning that they are the primary source of atmospheric oxygen, which is generated as a by-product of photosynthesis (Geider and Osborne, 1992). In the field of biotechnology, the term microalgae includes not only eukaryotic species that have the typical plant-cell eukaryotic organelles, but also include cyanobacteria, a group of prokaryotes that carry out oxygenic photosynthesis.

As for some evolutionary aspects of this cluster, cyanobacteria are the oldest group as it was evidenced by the fossil remanants of stromatoliths dated around 3500 million years ago. The origin of photosynthetic eukaryotes is dated around 2000 million years ago (Richmond and Qiang, 2013). However, there seems to be less general agreement on the comparison with the datation of the cyanobacterial origin, since eukaryotic algae are not a single evolutionary linage. This date has been proposed with respect to the genomic traces of the single primary endosymbiotic event that generated plastids once a non-photosynthetic eukaryote engulfed a cyanobacteria. The event produced a successful symbiosis and, over time, the cyanobacteria became a chloroplast. After that, other secondary and tertiary endosymbiotic events occurred consisting on the phagocytosis and following retention of a primary or secondary endosymbiont by a second heterotrophic eukaryote (Moreira and Philippe, 2001; Lewis and McCourt, 2004). As a consequence, linage classification of eukaryotic microalgae is based on the endosymbiotic origin of its plastids. In this respect, the groups Glaucophyta, Chlorophyta, and Rhodophyta contain double membrane-bound plastids and originated from the first endosymbiotic event. Among these groups, the Glaucophyta are considered the most ancient before the linage diverged to Chlorophyta and Rhodophyta, and represent an intermediate group in transition between the endosymbiont and the plastid, preserving some prokaryote characteristics such as the presence of peptidoglycan in the plastid membranes. The groups Cryptophyta, Haptophyta and Heterokonts seem to be derived from secondary endosymbiosis events, while Dinoflagellates represent the major group originated by tertiary endosymbiosis (Parker *et al.*, 2008).

Chlorophyta and Rhodophyta represent the main groups with regards to the species that have been used in this work. Regarding the Chlorophyta, or green algae, the species of this group are characterized by the presence of chlorophylls *a* and *b* as the main photosynthetic pigments, while red algae, Rhodophyta, contain chlorophyll *a* and phycobiliproteins. In both groups, under standard growth conditions, the main storage compound is starch or starch-like products (Barsanti and Gualtieri, 2006; Richmond and Qiang, 2013). The diversity of lipids is very high among the different species and, in brief, lipids are found either in cellular membranes or accumulated in lipid bodies or oil droplets in the cytosol (Borowitzka, 2013).

With respect to proteins, the quality is, as well, exceptional. Microalgae are recognized as a very rich source of proteins and in some cyanobacteria this fraction can represent a value as high as 60 % of the dry weight due to the petidoglycan cell walls, but also high protein contents can be found in green or red algae (Richmond and Qiang, 2013).

Metabolic and physiological flexibility of microalgae is remarkable and what is described under standard conditions may change radically when a cell is submitted to conditions of restricted cellular division such as nitrogen starvation and therefore stationary-phase (Pessarakli, 2011; Richmond and Qiang, 2013; Borowitzka *et al.*, 2016). In this case, protein content may decrease in favour of carbon-based compounds. Lipids instead of carbohydrates may become the main storage compounds, and lipid profile may change with respect to logarithmic-phase conditions in some algae (Otero and Fábregas, 1997). Some species may exude compounds to the medium like extracellular polysaccharides (Ramus, 1972). Moreover, pigment composition can change drastically and in most species pigments like chlorophylls or phycobilins, that may serve as intracellular nitrogen storage (Figueroa *et al.*, 1995), will be degraded in favour of pigments with a more protective role such as carotenoids or xanthophylls (Latowski *et al.*, 2011; Solovchenko, 2013; Stange, 2016).

The extensive microalgal diversity, in terms of species variety and physiological flexibility reflected in a diverse and dynamic biochemical composition, together with their unicellular nature, determine the potential of these organisms for the production of biomolecules with industrial interest. Microalgae combine the characteristic properties of higher plants, such as an interesting biochemical profile being a source of photosynthetic pigments and other molecules with pharmacological potential, with the biotechnological advantages of microorganisms, such as the higher productivity and a short life cycle. These properties make microalgae susceptible to be produced massively. In this sense, microalgae play a crucial role in aquaculture industry, as they are irreplaceable food for aquatic farmed-animals like molluscs and shrimps, early-stage development of fish, and for the improvement of the nutritional value of supporting cultures such as rotifers or *Artemia* sp. (Domínguez *et al.*, 2005; Coutinho *et al.*, 2006; Ferreira *et al.*, 2008, 2011; Seixas *et al.*, 2010). Species like *Chaetoceros* spp., *Skeletonema costatum*, *Phaeodactylum tricornutum*, *Isochrysis galbana*, *Tetraselmis suecica*, *Nannochloropsis* spp., *Nitzschia* spp., or *Rhodomonas* sp., are commonly grown *in situ* in aquaculture facilities (Borowitzka and Borowitzka, 1990; Coutinho *et al.*, 2006; Ferreira *et al.*, 2009; Seixas *et al.*, 2009). Additionally, species such as *Tetraselmis chuii*, *Chlorella* sp., and *Arthrospira* spp., had been brought into the human nutrition and animal feed market due to their nutritional value, rich in proteins, fatty acids, dietary fibers, carotenoids, vitamins, minerals, and other bioactive compounds (reviewed by Borowitzka, 1999, 2013; Richmond and Qiang, 2013). Two green algae, *Dunaliella salina* and *Haematococcus pluvialis*, have been industrialized due to their ability to accumulate the secondary carotenoids β-carotene and astaxanthin, respectively, which are used in the nutraceutical and cosmetic market and as food and feed additives (Borowitzka and Borowitzka, 1990; Olaizola, 2000, 2003; Guerin *et al.*, 2003; Borowitzka, 2013). Other species represent good candidates for the production of specific compounds such as lutein, that could be produced from the Chlorophyta *Muriellopsis* sp., or from *Scenedesmus almeriensis* (reviewed by Richmond and Qiang, 2013). Other pigments of the phycobiliprotein family, phycoerythrin or phycocyanin, with application as natural colourants in food, cosmetics, and within the pharmaceutical and analytical industry, can be extracted from biomass of Rhodophyta, Cryptophyta, or Cyanobacteria (Dumay and Morançais, 2016;

Morançais *et al.*, 2018). The algae *Botryococcus braunii* produces a high amount of hydrocarbons, of which hydrocarbon-oils are the most readily available source for converting bio-oils into fuels (reviewed by Richmond and Qiang, 2013). Also, other microalgae species where storage and structural compounds such as lipids can range from 10 to 60 % of the cell content, depending on cellular state, combined with a high productivity, have made microalgae a promising feedstock for the sustainable production of biofuels (Borowitzka, 2013; Richmond and Qiang, 2013; Zittelli *et al.*, 2013). As for red microalgae, Rhodophyta, produce a significant amount of polysaccharides with potential pharmacological applications (Arad and Levy-Ontman, 2010). For now, sulfated polysaccharides of red microalgae have already been introduced for applications in the cosmetic and food industry (Borowitzka, 2013; Richmond and Qiang, 2013). *Chlamydomonas reinhardtii* is the main species being used for the research with regards to hydrogen production by microalgae (Hemschemeier *et al.*, 2008; Vijayaraghavan *et al.*, 2009; Batyrova and Hallenbeck, 2017). Furthermore, the capacity to consume different nitrogen and phosphorous sources, as well as the ability of absorption and adsorption of heavy metals makes microalgae a good alternative for its use in wastewater treatment and bioremediation programmes (Larsdotter, 2006; Bux, 2013).

## 2. Carotenoid biosynthesis in *H. pluvialis* and *Dunaliella* spp.

*Dunaliella* spp., and *H. pluvialis* have been industrialized due to their ability to accumulate carotenoids under specific culture conditions. Non-limited culture conditions, which are normally referred to as favourable environmental conditions, produce vegetative growth of *H. pluvialis* and *D. salina* (Ben-Amotz and Avron, 1983; Fábregas *et al.*, 2000, 2001). During vegetative growth, cell division is active and the cells of both species are characterized by their motility due to the presence of two flagella in one end of the cell, and their green colour due to the abundance of chlorophyll (Borowitzka, Beardall and Raven, 2016). Primary carotenoids, that play a major role in oxygenic photosynthesis, are present as structural and functional components of the photosynthetic apparatus, in the thylakoid membranes, and are essential for cell viability at this cellular state (Stange, 2016). When the algae are submitted to conditions that increase oxidative stress, cellular state changes and secondary carotenoid synthesis genes are upregulated (Jin *et al.*, 2003; Richmond and Qiang, 2013). The extent to which each of the conditions affects the expression level of carotenogenic-related genes is variable but some of the conditions that induce the most stress are nutrient starvation, high light, continuous illumination, or high salinities (Ben-Amotz and Avron, 1983; Harker *et al.*, 1996; Boussiba *et al.*, 1999a; Aflalo *et al.*, 2007; Ben-Amotz *et al.*, 2009; Borowitzka *et al.*, 2016; Domínguez *et al.*, 2019). During these cellular states, cell division is diminished or completely restricted and cells change colour to red and orange due to the accumulation of secondary carotenoids, accompanied by morphological changes such as the loss of flagella and the development of a thick cell wall in *H. pluvialis*, or the development of an extensive glycocalix layer outside the membrane in cells of *D. salina* (Richmond and Qiang, 2013; Baudelet *et al.*, 2017). These carotenoids are not essential for photosynthesis and are not located in the thylakoid membranes of the chloroplast, but either in lipid globules in the interthylakoid space of the chloroplast's stroma, in the case of β-carotene, or in lipid vesicles in the cytoplasm, in the case of astaxanthin (Ben-Amotz *et al.*, 2009; Richmond and Qiang, 2013; Stange, 2016). Therefore, in both species a relation exists between lipid metabolism and carotenogenesis. In fact, astaxanthin sysnthesis was inhibited in *H. pluvialis* cells incubated with an inhibitor of fatty acid synthesis, suggesting that active fatty acid synthesis is an

essential requirement for this process (Schoefs *et al.*, 2001), and in *D. salina* a coordinated triacylglycerol synthesis for the formation of lipid droplets is a requisite for β-carotene accumulation (Rabbani *et al.*, 1998).

Part of the biosynthetic pathway for carotenogenesis is common in *H. pluvialis* and *D. salina* (Ben-Amotz *et al.*, 2009; Richmond and Qiang, 2013; Stange, 2016). Phytoene, a 40-carbon molecule, is the precursor for all other carotenoids. Phytoene is synthesized from the condensation of two 20-carbon molecules of geranylgeranyl pyrophosphate (GGPP), and this reaction is catalyzed by the enzyme phytoene synthase (PSY). A number of enzymes similar to PSY catalyze the successive desaturation reactions needed to convert phytoene into lycopene. Then lycopene can be cyclized on both ends of the molecule to form β-carotene, which is catalyzed by lycopene β-cyclase (LYC-B). This chain of reactions occurs in the plastid, and in carotenogenic *Dunaliella* spp. β-carotene is then accumulated in lipid vesicles in the intherthylakoid spaces of the chloroplast stroma. Furthermore, in *Dunaliella,* an isomerization reaction must occur somewhere in the pathway since carotenoids may exist in *trans*- and *cis*- configurations (Ben-Amotz *et al.*, 2009). The mechanism that regulates spatial configuration of carotenoids has not been fully elucidated yet, but it has been hypothesized that it could be photoregulated (Ben-Amotz *et al.*, 2009). Species that do not show massive carotenoid accumulation, like *D. tertiolecta*, may present other carotenoid-based photoprotective strategies, such as the activation of a xanthophyll cycle (Latowski *et al.*, 2011; Segovia *et al.*, 2015). In these, β-carotene is hydroxilized to zeaxanthin, which in turn is epoxidated once to form antheraxanthin, and twice to form violaxanthin (Stange, 2016). These epoxidation/de-epoxidation reactions are reversible (Latowski *et al.*, 2011), and zeaxanthin accumulates under high irradiance, correlating with energy dissipation processes (Walker, 1987; Richmond and Qiang, 2013).

In *H. pluvialis*, astaxanthin accumulation occurs in plastid globules in the cytoplasm, mainly esterified to fatty acids. β-carotene is transported to the endoplasmatic reticulum and converted to free astaxanthin through a series of reactions that add two keto groups forming echinenone, followed by a double hydroxylation (Richmond and Qiang, 2013; Stange, 2016). Finally, free astaxanthin is esterified to fatty acids and accumulated in cytoplasmatic lipid vesicles. Esterification may be mediated by a group of enzymes of the family of the diacylglycerol acyltransferases (Schittenhelm *et al.*, 1994).

With regards to the physiological role of the accumulation of these carotenoids, although it seems demonstrated that there is a correlation between oxidative stress and secondary carotenoid synthesis, researchers have not yet achieved an agreement on how the protective mechanism exactly works. Astaxanthin was suggested to act as a physical barrier blocking the penetration of light, protecting the photosynthetic apparatus against excess of photons (Hagen *et al.*, 1994). Moreover, light-induced migration of the astaxanthin containing globules towards the cell periphery was documented under high irradiance, while the pigment tend to accumulate in the center of the aplanospores when irradiance was less severe (Peled *et al.*, 2012; Ota *et al.*, 2018). Other authors have proposed that the main protective mechanism was not the accumulation of the pigment itself but the steps of its synthesis, which would consume excess electrons, protecting the cells against oxidative stress (Fan *et al.*, 1998). In the same line, it has been suggested that astaxanthin biosynthesis pathway may consume a significant amount of subcellular molecular oxygen, reducing ROS production and therefore protecting against oxidative stress (Li *et al.*, 2008). Also, the antioxidant properties of astaxanthin may

prevent DNA damage and the degree of esterification has been correlated with the pigment antioxidant properties (Kobayashi *et al.*, 1997; Kobayashi and Sakamoto, 1999), despite it is not clear how would it act over the nucleus or the photosynthetic apparatus due to their location outside the chloroplast.

Similar roles have been described for β-carotene that, due to its absorbtion spectrum, would act as a screen against excess irradiance, especially against blue photons. However, due to the intraplastidic location of β-carotene, there seems to be a higher consensus on its antioxidant role, quenching singlet oxygen and other free radicals protecting the cells against oxidative stress (Murthy *et al.*, 2005; Ben-Amotz *et al.*, 2009).

With respect to the factors that influence carotenogenesis, there seems to be a general consensus about the major effects that nutrient limitation and high levels of irradiance have on the accumulation of astaxanthin in *H. pluvialis* and the accumulation of β-carotene in *D. salina*. Nevertheless, nutrient limitation was only established as a key parameter for carotenogenesis in *H. pluvialis* after the works of Fábregas et al. (Fábregas *et al.*, 1998, 2001, 2003). Therefore, the combination of these two factors, irradiance and nutrient limitation, was proven to be the optimum (Fábregas *et al.*, 2001; Aflalo *et al.*, 2007; Scibilia *et al.*, 2015), on top of which other secondary factors could be added in order to accelerate and enhance the accumulation. High temperatures (Tjahjono *et al.*, 1994), the use of an optimized light composition (Kobayashi *et al.*, 1992) or continuous illumination (Domínguez *et al.*, 2019) seem to have a clear effect, while other factors, such as the limitation of other culture medium elements or the addition of NaCl, affect the accumulation rate to a lesser extent or are not yet fully understood (Kobayashi *et al.*, 1993; Fábregas *et al.*, 1998; Sarada *et al.*, 2002). In the same way, high levels of irradiance (Loeblich, 1982; Ben-Amotz and Avron, 1983) and nutrient limitation (Ben-Amotz and Avron, 1983; Ben-Amotz *et al.*, 2009) induce the accumulation of β-carotene in *D. salina*. On top of these, higher temperatures also affect accumulation rate (Pisal and Lele, 2005) and despite no effect was attributed to light wavelength composition at first (Ben-Amotz and Avron, 1989), recent studies suggest that light composition may affect β-carotene accumulation in this algae (Fu *et al.*, 2013, 2014; Xu and Harvey, 2019a, 2019b). Apart of irradiance and nutrient limitation, high salinity has been suggested as primary in *D. salina* relating directly to carotenogenesis, which would not only induce the synthesis of β-carotene but also increase intracellular glycerol production, that functions as an osmoregulator (Ben-Amotz and Avron, 1990; Ben-Amotz *et al.*, 2009). Highest astaxanthin pigment contents are reported between 4 to 7 % of dry weight (Aflalo *et al.*, 2007; Scibilia *et al.*, 2015), while carotenogenic *Dunaliella* spp. can accumulate β-carotene up to 10 to 14 % of dry weight (Ben-Amotz *et al.*, 1982, 2009).

## 3. Microalgae cultivation

Microalgae cultivation in small-scale is relatively easy. It is possible to isolate a certain species and grow monocultures in small photobioreactors (PBRs) under aseptic conditions using standard microbiological techniques (Andersen, 2005). Harvesting and extracting a small fraction containing metabolites with potential industrial interest can be done using common procedures. However, these processes start to gain complexity as soon as the cultivation unit increases in size, since a number of elemental things that did not constitute any problem at lab-scale suddenly become challenging, preventing successful

industrialization (Burlew, 1953; Olaizola, 2000). This is one of the main reasons why, despite the diversity of microalgae species and biotechnological applications is very high, only a few species have been industrialized (Borowitzka *et al.*, 2016).

Extensive cultivations of *Haematococcus pluvialis*, *Dunaliella* spp., or *Spirulina* spp., have been upscaled successfully in outdoor conditions in locations that allow the maintenance of optimal environments for these organisms. The species mentioned above, some of which are cultivated for the extraction of a certain metabolites like the carotenoids astaxanthin and β-carotene from *Haematococcus* and *Dunaliella* respectively, or *Spirulina*, which is mainly commercialized for human consumption due to its rich nutritional value, represent the first successful business models of a growing industry that has allowed the establishment of large companies such as Cyanotech (Hawaii), Parry Nutraceuticals (India), BGG (China), Fuji-AstaReal (Japan, Sweden, US), NBT (Part of Nikken Sohonsha Corporation, Japan) or Algaetechnologies (Israel). The successful scale up is directly related with the natural environmental conditions that represent the optimum for these species, most of which are extremophiles, having the ability of living in non-neutral environments. *Spirulina* and *Dunaliella* are obvious cases. Massive blooms of *Spirulina* and *Dunaliella* were occurring in nature in very alkaline lakes or in marine environments with high salinity before its commercial use (Hudnell, 2008; Ben-Amotz *et al.*, 2009). Despite the naturally occurrence of massive blooms denotes that, somehow, certain conditions boost-up certain species growth, it is not a guarantee for an easy industrialization, as it occurs with the cyanobacteria *Aphanizomenon flos-aquae*, which is harvested every year from the blooms in the Klamath lake (Oregon, US) but it is not cultivatable yet at an industrial level outside of that environment. *Haematococcus pluvialis* might have represented the first successful story of a non-extremophyle that required the solution of some major problems for its production, since *H. pluvialis* blooms were not occurring in nature and the conditions under which this algae accumulated astaxanthin had to be elucidated first (Droop, 1954; Fábregas *et al.*, 2003; Aflalo *et al.*, 2007). Nevertheless, it was found that the optimal conditions under which *H. pluvialis* accumulated astaxanthin massively implied nitrogen starvation and high irradiance. This findings were an advantage for the development of its commercial cultivation since just as *Spirulina* or *Dunaliella*, *Haematococcus* demanded some extreme conditions for the production of astaxanthin, allowing an easier control against other factors that would prevent success, such as culture contamination by other fast-growing phototrophic organisms, which constitutes one of the main challenges in industrial-scale cultivation facilities. In summary, taking into account current applications and production costs, only species with a clear competitive advantage constitute target organisms with a chance of scale up success in open system cultivations in outdoor conditions, which represent the most cost-effective way of production, due to the lower investment costs of open with respect to enclosed systems. Accordingly, outdoor systems do not need much sophistication. The most common systems for this type of productions are raceways, which are extensively described in the literature (Borowitzka, 1999; Posten and Chen, 2016). Outdoor open systems do not offer much control over the culture conditions and are mainly optimized to have a large illuminated surface that is achieved by occupying a large area and working with culture depths no larger than 10-15 cm. Turbulence is normally provided by the action of a paddle wheel, however, mixing is considered to be poor in these systems producing stratification of the culture. Then, a strain adapted to the conditions that prevail in the production location must be inoculated.

During the last decades the popularity of closed production systems known as photobioreactors (PBRs) (Borowitzka, 1999) has augmented. PBRs allow to further optimize culture conditions in order to achieve massive cultures, maximize productivities, and potentially diversify the range of species that could be cultivated, since the producer could modify the environment in order to adapt it to the optimum conditions of a specific species or strain, reduce impurities and control contamination to a larger extent. In this respect, it is now common to find production facilities that have found advantageous the use of closed PBRs to produce *Spirulina* (Ecoduna, Austria) or *H. pluvialis* (Algaetechnologies Ltd., Israel; BGG, China). Furthermore, the development of this technology has allowed the recent incorporation of new microalgal-derived products from species such as *Tetraselmis chuii* or *Phaeodactylum tricornutum* that can now be steadily cultivated.

PBRs can achieve a higher stability and productivity than open systems, since the unit configuration is designed to maximize light distribution, irradiance, and allow a more sensible pH, dissolved oxygen, turbulence, and temperature control. PBRs allow different operational modes that have been proven to affect largely the biochemical composition of the biomass (Sukenik *et al.*, 1993; Fábregas *et al.*, 1995; Otero and Fábregas, 1997; Otero *et al.*, 1997; Ferreira *et al.*, 2011). Furthermore, closed PBRs can be equipped with artificial light sources allowing the production of massive cultures in regions of the planet where sunlight is not available or far from optimal. Contrary to open systems, and besides a higher cost, enclosed systems require a major degree of sophistication and the benefits and functionalities described above are only attainable when the operation and the PBR design are optimized accordingly.

At laboratory-scale, sterile conditions are easily achieved (Andersen, 2005). PBRs, culture medium, and lab equipment used to take samples by a skilled microbiologist may be easily sterilized by autoclaving. Once the cultivation unit scales to a size that cannot be easily fit inside an autoclave the challenge starts. Full sterilization of cultivation hardware becomes almost impossible since the efficacy of the techniques available are reduced significantly with respect to lab-scale PBRs. Operators should aim, instead, a high level of sanitation, normally achieved through cleaning protocols with biologically hazardous chemical compounds such as hydrogen peroxide, hypochlorous acid, or bleach (Andersen, 2005; Richmond and Qiang, 2013). Steam sterilization, normally used in fermentation productions, would be optimal since results would be similar to those obtained with an autoclave for microalgal contaminants, and steam can reach difficult spots in the cultivation hardware compared to other techniques. However, the combination of the different materials that are used to assemble large PBRs such as stainless steel, glass, plastic, or rubber, may limit significantly the use of any sterilization treatment implying extreme temperatures or pressures and make it challenging when it comes to eliminate small gaps and spaces during the assembly of the units, that constitute "dead spots" where biofilm may accumulate complicating long-term sanitation. The engineering complexity, in terms of the amount of different materials, has to do solely with the photosynthetic nature of the organisms for which these units are intended for, since light must be available and therefore reach the culture through a translucent material. For this reason, sterilization is less of a problem in the industrial cultivation of heterotrophs, where industrial stainless-steel fermenters allow full sterilization (Richmond and Qiang, 2013). Technical solutions and protocols to sample large cultures aseptically may be easily designed and implemented. Nevertheless, air and water inputs represent a major contamination source. Sanitation of these in a larger scale requires the use of UV, ozone, or filters with a minimum pore size to ensure the highest degree of filtration (Andersen, 2005). The efficiency of these

filters decreases with use, meaning that a strict maintenance and quality control is needed to guarantee reliability. In summary, no matter the type of PBR, the design of an operation-friendly unit must be focused on preventing the so-called "dead spots", as well as integrate a well-designed air and water pre-treatment unit together with a clean-in-place solution (CIP).

PBRs are a relatively new cultivation technology with a growing number of users, but the design of the units is far from standardized. There are many designs described in literature, some of which have succeeded while others represented just a one-time trial (Andersen, 2005; Richmond and Qiang, 2013). Among those that have been better established, column, tubular, and flat-plate PBRs should be outlined since they are becoming the standards. As a very simplified description, all these systems are enclosed, optimized for a high light availability, allow a controlled environment, and may be automated to a certain extent. The main differences among them has to do with the associated investment costs and application. Generally speaking, column PBRs are cheaper and are common in locations like hatcheries and aquaculture facilities that produce live feed for supporting cultures and other aquaculture-specific uses, while tubular and flat-plate PBRs normally imply a higher investment cost and are reserved for higher-end products such as the production of specific biomolecules. In the present work we have used flat-plate PBRs for the scale up of a *H. pluvialis* culture process. PBRs design and operation are described in Materials and Methods, section 2.4.

No matter the application or the PBR type and functionalities, the degree of sophistication in closed PBRs correlates with the degree of culture conditions optimization that can be achieved for the species that aims to be produced. The optimization must be in accordance with the feasibility of controlling almost every growth parameter. This means that, in any case, an extensive optimization of the culture conditions and the production process needs to be done at lab-scale prior to the scale up to industrial-size PBRs. Otherwise, maximized productivity will not be achieved and, as a consequence, investment costs of the units can hardly be justified. In microalgae, growth is affected by the parameters that affect photosynthesis. Factors such as pH, carbon source, dissolved oxygen concentration, or nitrogen source and concentration largely affect growth (Borowitzka *et al.*, 2016). However, and despite the huge interplay among them, within all factors light and temperature should be outlined since the adjustment of these is crucial and both have the largest impact on productivity. Moreover, the optimization of these two is more species-dependant than the optimization of parameters like pH, carbon or nitrogen source. In this sense, temperature is, in some areas of the world, the parameter limiting productivity even above light availability. Photosynthesis, as any other metabolic process, is largely affected by temperature and optimum and non-viable may be, in some cases, only a few degrees apart. Moreover, temperature is one of the parameters that has a larger effect in the synthesis of metabolites that must be produced in conditions that disrupt cellular homeostasis to a certain extent (Tjahjono *et al.*, 1994; Pisal and Lele, 2005; Bonnefond *et al.*, 2016). Besides, temperature is one of the parameters that interacts less with PBR geometry. As a consequence, the results tend to be directly scalable between cultivation units of different types and sizes. On the other hand, light is the most important factor in photosynthesis and its optimization is certainly more complex than any other culture parameter. Light optimization is a broad concept. It includes light quantity (irradiance and lenght of light: dark cycles, when applicable), light distribution (homogeneity), and light quality (wavelength composition). Effective light reaching the cells depends strongly on both the culture and the cultivation unit, and it is an essential substrate that creates a large gradient distribution in the cultivation medium. Due to

its nature, light can be reflected, refracted, absorbed, or scattered, and every parameter will interact differently depending on the material of the PBRs or the action spectra derived from the pigment composition of the species that is being cultured, among other factors. Moreover, light might be the parameter that raise more controversy in between biologists and engineers, since each of these groups of professionals measures and understands light in a different manner, difficulting mutual understanding. Consequently, for the highest chance of business success, procedures need to be developed in order to facilitate an operational framework between these two disciplines. During the present work, this framework for mutual understanding has been developed based on the measurement of the photosynthetic parameters described in the Introduction section 4.2.

## 4. Photosynthetic efficiency and mass cultures of microalgae

Understanding photosynthesis is essential in microalgae biotechnology. In simple terms, photosynthesis is a unique process in which inorganic compounds and light energy are converted into organic matter (Walker, 1987; Geider and Osborne, 1992; Larkum *et al.*, 2003). For photoautotrophs like algae, cyanobacteria or land plants, oxigenic photosynthesis is the main way of nutrition. Photosynthesis can be divided in two parts: light and dark reactions (Walker, 1987; Richmond and Qiang, 2013). The first ones include the split of water molecules and the release of oxygen ($O_2$) as a by-product, and the production of biochemical reductant and energy ($NADPH_2$ and ATP). In the latter, $NADPH_2$ and ATP are subsequently used for inorganic carbon fixation, normally $CO_2$, and its posterior reduction to carbohydrates, which are accumulated as storage compounds such as starch, or as structural components of the cell walls (Barsanti and Gualtieri, 2006). Despite it looks like there is a clear separation between the two parts the truth is that, in practice, both reaction groups are active at the same time. In cyanobacteria, which have a prokaryotic internal organisation, photosynthetic membranes are located in the chromoplast, a region in the periphery of the cell, and the major presence of hydrophylic phycobilisomes, characteristic photosynthetic units of this microalgal group, prevents membranes to be stacked. On the other hand, in eukaryotic microalgae the photosynthetic apparatus is organised in plastids, chloroplasts, where two regions are differentiated: thylakoids (stacked lipidic membranes where photosynthetic units are located, except in Rhodophyta where due to the presence of phycobilisomes, as in cyanobacteria, thylakoids are not stacked), and stroma (aqueous intraplastidial space). The interthylakoid space between lipidic membranes is denominated thylakoid lumen (Barsanti and Gualtieri, 2006).

The energy needed to run photosynthesis is dispensed by light. Nevertheless, only wavelengths that correspond to visible light, approximately between 400 and 700 nm is considered as photosynthetic active radiation (PAR light) (Richmond and Qiang, 2013). Energy decreases with wavelength size so, in PAR light, blue photons, those closer to 400 nm, are more energetic than photons closer to 700 nm, red. In plant physiology, the amount of light that is used to illuminate a culture is expressed in relation with the surface that is being illuminated: irradiance, which is expressed as the amount of photons reaching a surface area per unit time, normally μmol photon $m^{-2}$ $s^{-1}$.

Photosynthetic pigments harvest light. These pigments are present in every photosynthetic organism. However, the composition and the relative abundance of each pigment varies

depending on the species and the cellular state. There are three major groups of pigments: chlorophylls, carotenoids and xanthophylls, and phycobilins (Roy *et al.*, 2011; Richmond and Qiang, 2013). Chlorophylls are considered the main photosynthetic pigments. Chlorophyll molecules consist of a tetrapyrrole ring containing a centered magnesium atom, and a long-chain terpenoid alcohol. Chlorophylls are bound to apoproteins. There are different type of chlorophylls: *a*, *b*, *c*, and *d*, and the main difference among them are the side-groups on the tetrapyrrole ring. Chlorophylls absorb light in the blue and red region of the spectrum, around 400-450 and 620-660 nm. Among chlorophylls, Chl *a* is common in all oxygenic phototrophs (Barsanti and Gualtieri, 2006). Chl *a* is located in the reaction centre of the photosystems and in the light-harvesting antennae complexes, where it can be found together with other chlorophyll molecules (Chl *b* or *c*) and other antennae pigments like carotenoids.

Carotenoids are a highly diverse group of pigments that absorb light in the blue-green region of the spectrum with absorption maxima in the range of 400-500 nm, and present a yellow, red or orange colour. Structurally, carotenoids are long hydrocarbon chains conjugated with double-bonds. When these chains are oxygenated are referred to as xanthophylls. Carotenoids can either act as light-harvesting pigments channelling excitation energy to Chl *a*, have a structural function within the light-harvesting and reaction centre complexes, or act as protective molecules against excessive irradiance, preventing the formation of chlorophyll triplets or scavenging reactive oxygen species (ROS) that may accumulate in the cell, damaging the photosynthetic apparatus in conditions of disrupted homeostasis (Latowski *et al.*, 2011; Jahns and Holzwarth, 2012; Solovchenko, 2013). Due to the importance that the mechanisms of photoacclimation and photoprotection have within this work, Introduction section 4.1 will be entirely dedicated to it.

Located in the thylakoid membranes, Cyanobacteria and Rhodophyta present large extrinsic protein light-harvesting antennae complexes called phycobilisomes, consisting in phycobiliproteins bound to apoproteins (Glazer, 1984; Bernard *et al.*, 1996). Phycobiliproteins are divided into four classes depending on their light absorption proteins: phycoerythrins (PE), phycocyanins (PC), allophycocyanins (APC), and phycoerythrocyanins (PEC). Phycoerythrin is the main phycobiliprotein found in Rhodophyta (Larkum *et al.*, 2003). Each phycobiliprotein consists of a linker polypeptide, called γ subunit, that assists in the orientation of the antennae components, and an heterodimer comprised of an α and β subunit that, in turn, are constituted by a maximum of three chromophores denominated phycobilins. Four types of phycobilins have been described: phycourobilin (PUB), phycoerythrobilin (PEB), phycocyanobilin (PCB), and phycobiliviolin (PXB) (Morançais *et al.*, 2018).

Photosynthetic pigments absorb photons, and this is the basis of the photosynthetic process. However, a larger mechanism is required in order to convert those photons into energy and, ultimately, into biomass. In this respect, photosynthetic membranes contain five major complexes: light-harvesting complex (LHC), photosystem II (PSII, P680), photosystem I (PSI, P700), cytochrome $b_6$/f, and ATP synthase (Figure 0.1) (Larkum *et al.*, 2003). Light energy is received and trapped in PSI and PSII, which work in equilibrium and are interconnected by an electron transport chain, in standard conditions (Figure 0.2). In brief, when the photosystems are illuminated, a water molecule is split, oxygen is released, and two electrons are extracted and transferred through the photosynthetic apparatus to produce $NADPH_2$. In parallel, this reaction involves the transport of protons ($H^+$) from the stroma into

the lumen forming a pH gradient between thylakoid's lumen and the plastid's stroma. This imbalance state needs to be solved by the release of protons from the lumen to the stroma via ATP synthase, photophosphorylation, producing ATP.

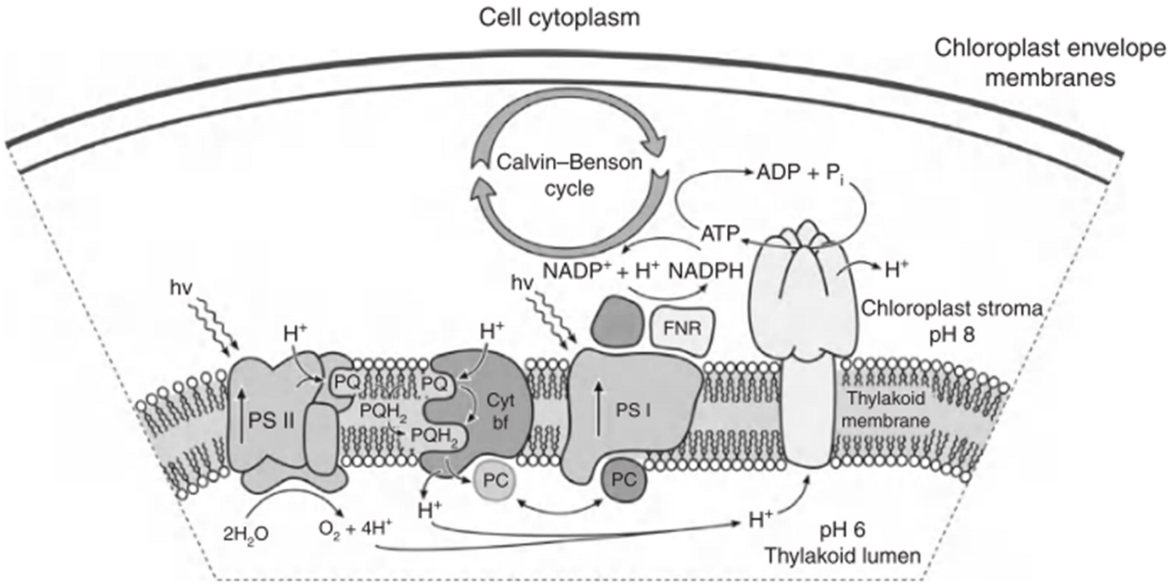



**Figure 0.1. Schematic representation of the four major structures within the thylakoid: photosystem I and II, cytochrome $b_6$/f complex, and ATP synthase. Electrons removed from molecules of $H_2O$ result in the evolution of $O_2$ as a byproduct that is transported outside the thylakoid. Protons are translocated from an external space (stroma) into the intrathylakoid space during the light-induced electron transport. The flow of protons through the ATP synthase to the stroma leads to the generation of ATP from ADP and Pi in the stroma, where the Calvin-Benson cycle reactions are carried out. NADPH2 is also formed on the stromal side of the thylakoid. From J. Masojídek, G. Torzillo and M. Koblízek (2013), with permission of the publisher.**

Light energy is received by the antennae pigments located in the LHCs and it is funnelled from a higher to a lower energetic level to the reaction centres of the photosystems (Walker, 1987; Richmond and Qiang, 2013). During this process, some energy can be dissipated in the form of fluorescence or heat, and only part of the energy may actually be used in photochemistry. The oxidation of the primary electron donor of P680 (PSII) leads to a charge separation. On the acceptor side of the PSII, pheophytin reduces the primary acceptor, plastoquinone $Q_A$. Then, two electrons are transferred from $Q_A$ to the secondary acceptor $Q_B$. The reduced plastoquinone is oxidised by the cytochrome $b_6$/f complex, then plastocyanin acts as the electron donor for P700 (PSI). A similar cascade reaction occurs in the acceptor side of PSI but instead of plastoquinone the electron acceptor is ferredoxin, which ultimately reduces $NADP^+$ into $NADPH_2$. This reaction scheme, where electrons are firstly donated by water and end up reducing $NADP^+$ it is the so called "Z scheme" or acyclic photophosphorylation. Under certain conditions, like a cellular state demanding a large amount of ATP, a higher quota of cyclic photophosphorylation can happen, and electrons are maintained in a closed loop around PSI resulting in a higher production of ATP (Figure 0.2).

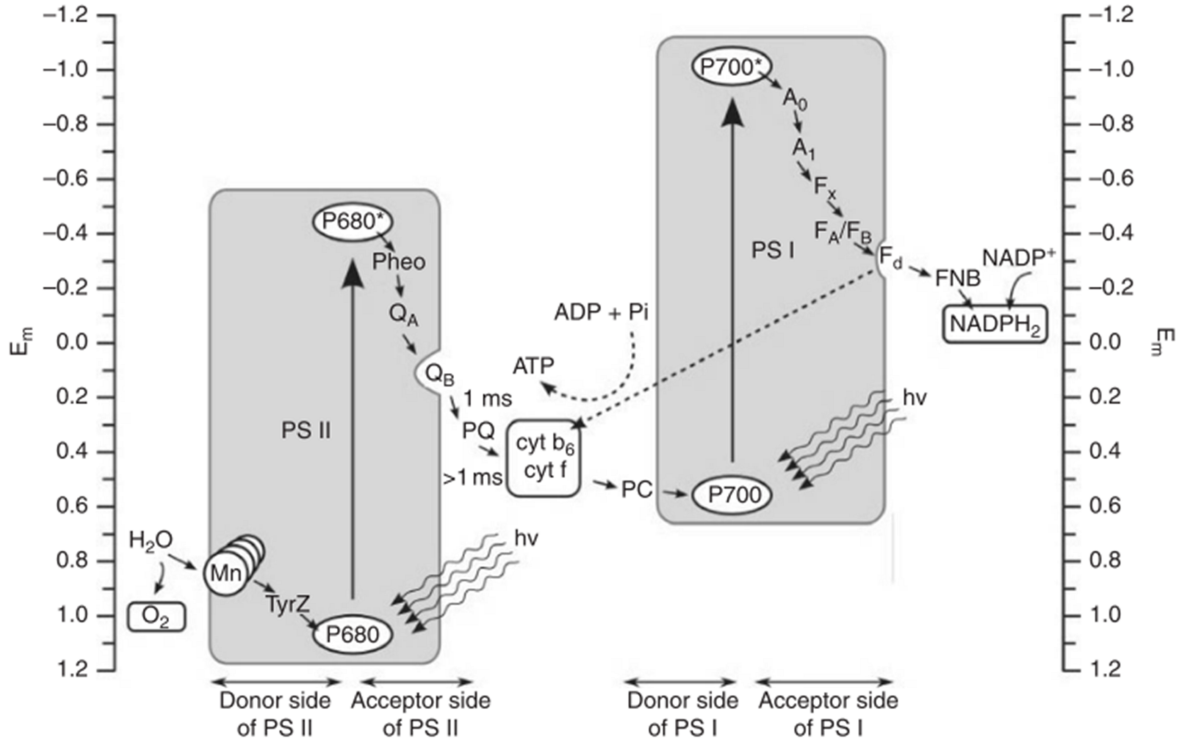



**Figure 0.2. Photosynthetic electron flow from water to reductant power, Z scheme, and cyclic photophosphorylation around P700 (PSI). From J. Masojídek, G. Torzillo and M. Koblízek (2013), with permission of the publisher.**

Physiologically, once $NADPH_2$ and ATP are available, these products can be used to assimilate $CO_2$ into storage compounds, carbohydrates, via the Calvin-Benson cycle. The stoichiometry of this reaction shows that to fix one molecule of $CO_2$, 2 molecules of $NADPH_2$ and 3 ATP are needed. Within the cycle, $CO_2$ is added to ribulose bisphosphate (5C) to form two molecules of phosphoglycerate (2 x 3C), and this reaction is catalysed by the enzyme ribulose bisphosphate carboxylase/ oxygenase (RuBisCO), which can function as a carboxylase or oxygenase depending on the relative concentrations of $O_2$ and $CO_2$. Paradoxically, RuBisCO has a higher affinity for $O_2$ than for $CO_2$ (Geider and Osborne, 1992; Richmond and Qiang, 2013), therefore oxygen concentration must be kept under control in mass microalgal cultures in order to avoid photorespiration, maximizing efficiency and productivity, and reducing metabolic damage.

### 4.1. Photoacclimation and photoprotective mechanisms against photoinhibition

Microalgae exhibit different mechanisms to respond to changes in their environment. In microalgal biotechnology, the knowledge about the specific response of each organism to these changes and understanding how the parameters trigger these responses may serve as clues to optimize cultivation conditions and to increase the productivity of certain metabolites like pigments, fatty acids, or certain enzymes, that are synthesized during the cellular response to these conditions.

Microalgae can respond to quick changes in light by modulating their light harvesting capacity or by modulating the amount of PSII reaction centres (Walker, 1987; Richmond and Qiang, 2013). In general, under light limiting conditions pigmentation increase in either number of photosynthetic units or size of LHCs, and the opposite happens when light is saturating. During photosynthetic activity under high irradiance for an extended period of time, a pH gradient is built up between the chloroplast stroma and the thylakoid lumen due to the accumulation of $H^+$ in the latter at a higher rate than the maximum photophosporilation rate, which is in charge of keeping the balance under standard conditions. It is believed that the generation of this differential pH between both sides of the electron transport chain triggers energy dissipation mechanisms, for instance, in the form of heat reducing the amount of energy delivered to PSII, that accounts for non-photochemical processes. PSII can be, in extreme occasions, completely inactivated. Under overexcitation conditions an accumulation of $O_2$, over-reduction of $NADP^+$ and accumulation of $NADPH_2$ occurs together with an increase of ROS via Mehler's reaction. Additionally, an increased excited chlorophyll state can lead to Chl *a* triplet formation that can react with oxygen forming additional ROS (Borowitzka *et al.*, 2016). In some algae a reversible conversion of epoxidized to de-epoxidized xanthophylls occurs under high irradiance, creating effective ROS quenchers and triggering dissipation energy processes reflected as non-photochemical fluorescence quenching (Walker, 1987; Richmond and Qiang, 2013). At list six different types of xanthophyll cycles have been described. Among them, the most common in both Chlorophyta and Rhodophyta is the de-epoxidation of violaxanthin (Vx) to zeaxanthin (Zx) (Latowski *et al.*, 2011), where it has been evidenced that the amount of Zx correlated with the amount of non-photochemical fluorescence quenching (NPQ) and therefore with energy dissipation as heat in LHC-II, which is essential to prevent photoinhibition (Walker, 1987; Torzillo *et al.*, 2003; Masojídek *et al.*, 2004; Jahns and Holzwarth, 2012). Other microalgae respond by accumulating certain metabolites like secondary carotenoids (Solovchenko, 2013; Solovchenko and Neverov, 2017). This may be the case for certain carotenogenic *Dunaliella* spp. or *H. pluvialis* that accumulate β-carotene and astaxanthin, respectively. Nevertheless, these carotenoids do not transfer excitation energy to PSII and neither seem to activate any strong non-photochemical quenching processes like the ones observed when a xanthophyll cycle is active (Richmond and Qiang, 2013). Although the authors that have studied this topic have not yet reached an agreement on how exactly the protective mechanism involving the accumulation of astaxanthin works (Hagen *et al.*, 1994; Kobayashi *et al.*, 1997; Li *et al.*, 2008), the main hypothesis is that these pigments serve mainly as a physical screen to protect the photosynthetic apparatus from excessive irradiance.

Regardless of the photoprotective mechanism, the ultimate goal is to adjust the excitation energy reaching PSII by any means, preventing the photosynthetic apparatus overload originated by an overexcitation of PSII that can lead to photoinhibition. Therefore, the susceptibility of cells to photoinhibition increases under non-optimal culture conditions leading to lower saturation irradiances. Photoinhibition mechanism has been described more precisely (Walker, 1987; Murata *et al.*, 2007). Apparently, the light energy that is absorbed by antennae pigments accelerates photoinhibition by the suppression of PSII repair mechanisms. PSII is a dimer composed of two main proteins: D1 and D2 (Walker, 1987; Richmond and Qiang, 2013). D1 needs to be continuously repaired during photosynthetic activity, regardless irradiance. The repair process demands protein synthesis. At the same time, reactive oxygen species (ROS) are continuously created and constantly being scavenged by enzymatic (superoxide dismutase, catalase, or peroxidase, among others) and non-enzymatic

(xanthophyll cycle, tocopherols, ascorbic acid, among others) antioxidants, in an equilibrium state (Latowski *et al.*, 2011). When the rate of repair is lower than the rate of damage the equilibrium is lost and results in oxidative stress. Photon-saturated photosynthetic apparatus affects the oxygen-evolving complex leading to an increase of intracellular ROS. ROS accumulation inhibits *de novo* protein synthesis and, as a result, PSII repair is inactivated. Non-optimal environmental factors may also affect PSII protein synthesis and therefore compromise D1 repair. As a result, photoinhibition symptoms can be observed in conditions of disrupted homeostasis not necessarily generated by an excessive irradiance but a nutrient deficiency or very high temperatures, and all these factors interact to generate a photoinhibitory condition.

### 4.2. Measuring photosynthesis

Theoretical knowledge of photosynthesis is essential for the correct interpretation of investigations and to make the right decisions when it comes to work with photosynthetic organisms such as microalgae. However, theoretical concepts and its interpretation remain in a conceptual framework when these can not be measured. Industrial microalgal biotechnology is a field where different professional disciplines must work together. A framework of understanding is indispensable when biologists and engineers need to collaborate close together to develop industrial-sized cultivation technology that fulfils the biological demands of photosynthetic microorganisms growth. The relationship could be described as if biologists are more used to work with concepts and relative values, while engineering demands pragmatism and exact values. As a consequence, biological concepts like those related with photosynthesis (light-limited, light-saturated, photoinhibition, or high light, among others) must be translated into measurable data, in order to facilitate understanding between both worlds.

Photosynthesis relates to any parameter directly correlated with growth (Walker, 1987; Larkum *et al.*, 2003; Richmond and Qiang, 2013). It is common to measure photosynthetic activity via oxygen evolution, since oxygen is a by-product of photosynthesis, or with carbon fixation rate, since the rate of carbon assimilation must be in line with growth and therefore with photosynthetic activity. Other methods such as respiration or growth rate derived from cellular division or dry weight could also be used to estimate photosynthetic activity. However, none of these parameters correlate to photosynthetic activity as accurately as chlorophyll fluorescence, which has become the most powerful technique in photosynthesis research (Walker, 1987; Demmig-Adams *et al.*, 1996; Torzillo *et al.*, 1996; Maxwell and Johnson, 2000; Figueroa *et al.*, 2013; Kalaji *et al.*, 2014; Malapascua *et al.*, 2014). While the ease of use of other methods is restricted to lab and medium-scale, chlorophyll fluorescence techniques can be easily used at any scale in a timely manner and require minimum interaction with the culture, which is really advantageous in industrialized facilities where the minimization of this interaction will greatly contribute to prevent contamination.

Chlorophyll fluorescence techniques measure PSII activity, mainly. The data collected by this method reveals photosynthetic performance of an organism (Walker, 1987). When PSII is illuminated, energy is ultimately transferred to the Chl *a* in the reaction centre. However, when the excitation energy is saturating, part of it can be dissipated as heat or re-emitted as fluorescence. The equilibrium between these three parameters (energy reaching

Chl *a* and energy dissipated either as heat or fluorescence) will provide useful information about the state of the photosynthetic apparatus. Evidently, when the acceptor side of PSII is empty, the ability to accept electrons is high. Generally speaking, this happens when microalgae have been maintained in the dark for sufficient time to de-saturate their photosynthetic apparatus. In this situation, reaction centres of photosystems are in "open state", ready to accept electrons and use them for photochemistry. The fluorescence that can be measured in this state is very low and it is denominated $Fo$. If PSII is exposed for a short period of time to a pulse of light that saturates the photosystems, reaction centres will react moving the electrons to $Q_A$, reducing it. At this point, photosystems are in a "closed state" because photochemistry is momentarily blocked, since there is no available capacity at the acceptor side of the reaction centres to accept more electrons. Excessive energy must be dissipated as heat or fluorescence. The highest fluorescence measured is denominated $Fm$ (maximal fluorescence). Therefore, fluorescence yield is in line with the ratio between "open" and "closed" reaction centres, which represents a saturation level. The difference between $Fm$, which accounts for "closed" reaction centres and therefore reduced $Q_A$, and $Fo$ that accounts for "open state" (oxidised $Q_A$), is denoted variable fluorescence ($Fv$). The ratio $Fv/Fm$ is used to calculate PSII quantum yield, a measure of photosynthetic efficiency. Therefore, the more "closed" reaction centres before the photosystem is saturated, the lower PSII quantum yield, thus the lower photosynthetic efficiency because there is less available capacity to use electrons. Good interpretation of changes in variable to maximum chlorophyll fluorescence, supported with additional data, allows researchers to know if a culture can accept more irradiance without compromising efficiency, as well as know when irradiance is too much and efficiency has decreased, or if a culture is under the risk of or being photoinhibited. In addition, changes in nutrient availability or an accumulation of dissolved oxygen leading to photorespiration in a PBR may be quickly detected and prevented with these techniques (Walker, 1987; Richmond and Qiang, 2013). If the same measurement is conducted in cultures that are exposed to light, $Fm$ will yield lower values since reaction centres are "closed" to a certain extent. Moreover, energy dissipation and energy derived to photochemistry, non-photochemical (NPQ) and photochemical fluorescence quenching ($qP$), respectively, as well as electron transport rate ($ETR$) can also be derived from chlorophyll fluorescence data. Data from $ETR$ could be used to plot photosynthesis-irradiance curves (P-I curves), which can then be used to determine absolute values of important parameters for culture conditions and light optimization. These paramenters will be introduced in section 4.3.

### 4.3. Photosynthetic parameters as estimates of microalgal physionlogical status

Despite photosynthetic responses are diverse among microalgae, all share the same foundation. For a good understanding of microalgal growth, it is important to have a clear schematic representation of the key parameters used to characterize photosynthesis. As it has been described previously, measuring photosynthesis is essential in order to translate theoretical concepts into data and put them in practice. Photosynthesis can be measured with respect to any process that relates directly with its activity. Oxygen evolution, carbon assimilation rate, respiration rate, growth rate, or chlorophyll fluorescence relate to photosynthetic rate. Photosynthesis can be represented graphically with the data collected by any of these methods (y-axis) with respect to irradiance (x-axis). The curve that will be generated is called light-response or photosynthesis-irradiance curve (P-I curve) (Figure 0.3) (Walker, 1987; Larkum *et al.*, 2003; Barsanti and Gualtieri, 2006; Reynolds, 2006; Buchner

and Ewingen, 2009; Richmond and Qiang, 2013), from which some key points can be extrapolated: a first part with a positive slope ($\alpha$) that increases linearly with irradiance until it reaches a plateau where photosynthetic rate is maximum: $P_{\max}$. At very low irradiances, respiration might be higher than photosynthesis and therefore net photosynthetic activity might be negative. The point where photosynthesis and respiration are in equilibrium, no net gains or losses, is denominated compensation point ($Ec$ or $Ic$) (Fig. 3). At high irradiances, photosynthetic rate may drop below $P_{\max}$. This phenomenon reflects photoinhibition symptoms, which can lead to irreversible cellular damage. In theory, the highest photosynthetic efficiency would be the point in the intersection between the maximum slope of the curve with $P_{\max}$, denominated saturation point ($Ek$ or $Ik$). $E_k$ delimits light limited (below $Ek$) from light saturated (over $Ek$) conditions and represents the moment when the photosynthetic rate per additional photon starts dropping. In practice, in the light limited region of the curve, electron transport capacity exceeds photon absorption rate meaning that most of the photons are used for photochemical reactions, yielding high photochemical fluorescence quenching ($qP$). Whereas when absorbed photons exceed electron transport chain capacity, the photosynthetic apparatus will be saturated and the excess energy will need to be dissipated and therefore non-photochemical fluorescence quenching increases (NPQ, $qN$) that, in turn, makes photosynthetic efficiency drop (PSII QY, $Fv/Fm$).

P-I curves serve as good tools to characterize the photosynthetic response of a species and to optimize light in every aspect. However, P-I curves are not immutable, meaning that the curve and the parameters derived from the curve change depending on environmental and physiological factors. Factors like the photoacclimation of cells to high or low light can affect the curve's parameters. From a biotechnological perspective, which looks to maximize photosynthetic activity, the closer a culture is to its optimum the higher $P_{\max}$ that translates to growth rate. Therefore, factors like temperature or dissolved oxygen largely affect this curve. In practice, it would mean that two identical cultures exposed to the same irradiance but maintained under optimal and non-optimal conditions would not be able to use the same amount of photons equally. The closer the conditions are to optimum, the higher growth using identical irradiance (Walker, 1987; Malapascua *et al.*, 2014), since optimized conditions imply a higher saturation point and a higher electron transport chain capacity. At the same time, maximum slope and height of the curve may as well change depending on the physiological state of the cells (Walker, 1987; Richmond and Qiang, 2013). Like for prokaryotes, growth phases in microalgae are clearly defined in lag, exponential, and stationary. Photosynthetic rate will be maximal during exponential phase, when primary metabolism is highly operative and cells are actively dividing being able to accept and use every photon effectively. In industrial cultivation of microalgal cells that point is defined by a certain cell density. At the same time, a certain cell density is accompanied by a certain culture turbidity, and therefore an optical density of that liquid, all of which is affected by PBR design and will interact with light absorption that is regulated by Beer-Lambert's law. Beer-Lambert law explains how light is absorbed not linearly but exponentially in a dense microalgal culture, meaning that most of the photons are absorbed in the first layers of culture, which creates a huge gradient of light availability (Geider and Osborne, 1992; Barsanti and Gualtieri, 2006; Borowitzka *et al.*, 2016).

The application of the information provided by the P-I curves will only be successfully transferred to improve mass microalgae cultivation if the PBR design allows it, meaning that the knowledge is really valuable when the PBR has been designed to permit a good light

availability, reflected in the illuminated surface of the cultivation unit. Regarding to this, a common factor used to characterize light distribution is illuminated surface to volume ratio ($S/V$). High $S/V$ ratios pre-determine cultures for fast growth, since light is distributed better over the culture (Figueroa *et al.*, 2013; Malapascua *et al.*, 2014).

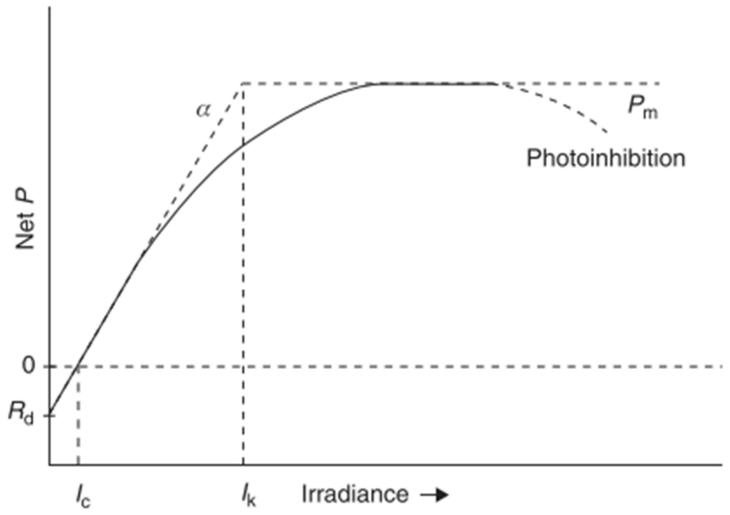



**Figure 0.3. Schematic diagram of a P-I curve, showing the typical photosynthetic paramenters. The light-saturated rate is denoted $P_{max}$. At low irradiance, photosynthesis rate is approximately a linear function of irradiance, and the ratio between photosynthesis and irradiance is often denoted by the symbol $\alpha$. The saturation irradiance, $Ik$, is given as intercept between $\alpha$ and $P_{max}$. At irradiance above the optimum, photosynthesis rates usually shows a decline from the light-saturate value. Dark respiration is denoted by $Rd$. The compensation irradiance $Ic$, where no photosynthetic net gains or losses are observed. From G. Torzillo and A. Vonshank (2013), with permission of the publisher.**

## 5. The influence of light quality in microalgae cultivation

Biochemical composition of a microalgal cell, including pigment composition, changes depending on the environment. Photoacclimation and photoprotection responses to conditions of high, low, or excesive irradiance, where cells modify its pigment content to adapt to the amount of photons received, are common phenomena in nature. However, not only irradiance but also its wavelength composition matters, since not all the photons yield the same photosynthetic efficiency, which is reflected in each species photosynthetic action spectra (Geider and Osborne, 1992; Frank *et al.*, 1999), which measures the ability of a species to photosynthesize in light of different spectral qualities. This capacity is given, mainly, by the pigment composition of the photosynthetic apparatus. As an example, green and brown algae exhibit lower absorption in the green region of the light spectrum, while red algae show a marked peak between 500 and 600 nm translated in a more efficient use of the available green photons and therefore a lower light requirement compared to the photosynthetic activity in the red and blue regions of the spectrum (Emerson and Lewis, 1942, 1943; Haxo and Blinks, 1950; Fork and Amesz, 1969; Lüning and Dring, 1985; Neori *et al.*, 1986; Leukart and Luning, 1994; Larkum *et al.*, 2003).

Although primary regulation of pigmentation is under genetic control and determined taxonomically, the composition and abundance of pigments responds also to a secondary

phenotypic regulation that depends on environmental conditions, such as light quality or nutrient deficiency (Sosik and Mitchell, 1991). In some cyanobacteria, the culture can completely change its colour depending on the quality of the irradiance received, adjusting its action spectrum (Flores and Herrero, 2014). During this process, called chromatic acclimation, LHCs or phycobilisomes are modified with respect to the spectral quality received in order to maximize photosynthetic efficiency. Tandeau De Marsac (1977) proved that around 70 % of the total cyanobacteria collection of the Pasteur Institute (France) had the capacity to perform chromatic acclimation, being some of these effects very marked in terms of the change of the physical appearance of the culture. The works of Kehoe et al. (Kehoe and Grossman, 1994; Kehoe and Gutu, 2006; Flores and Herrero, 2014) have extensively reviewed the different types of chromatic acclimation.

In eukaryotes, different light spectrum trigger different responses. However, these are complex and species-specific since, in some cases, it is unclear if the differences are triggered by the light wavelength changing parameters like growth rate, or directly by the light wavelength itself affecting other light-mediated metabolic processes (Richmond and Qiang, 2013). Results reported in the literature do not allow a generalization. Changes in the biochemical composition of different species such as *Thalassiosira rotula* or *Dunaliella tertiolecta* have been reported with respect to different light spectrum (Rivkin, 1989). Different growth rates and biochemical composition were reported for *Cyclotella caspia*, *Tetraselmis gracilis*, *Phaeodactylum tricornutum* or *Thalassiosira gravida* with respect to light quality (Holdsworth, 1985; Aidar *et al.*, 1994). Changes in cell number, biomass concentration, and biochemical composition with respect to light composition have also been observed in *Chatoceros* spp. cultured in semicontinuous mode (Saavedra and Voltolina, 1994). Related to this, differences in photosynthetic efficiency have also been reported for diatoms like *Skeletonema costatum* and other benthic diatoms (Mercado *et al.*, 2004; Miao *et al.*, 2012). Furthermore, in *Dunaliella bardawil* grown under different wavelengths in batch mode (Sánchez-Saavedra *et al.*, 1996), protein content and carbon fixation and allocation, reflected in the amount of either carbohydrates or lipids and its composition, was also influenced by light quality. Changes in the fatty acid profile of different *Nannochloropsis* spp. with respect to incident light quality have also been reported (Das *et al.*, 2011; Teo *et al.*, 2014; Ra *et al.*, 2016; Schulze *et al.*, 2016). Moreover, lipid fraction changed with respect to light quality in cultures of *Chlorella vulgaris* (Pérez-Pazos and Fernandez-Izquierdo, 2011; Atta *et al.*, 2013), and nutrient consumption rate has been reported as affected by light composition in cultures of *Scenedesmus* spp. (Kim *et al.*, 2013).

It is obvious that these responses may have a direct applicability in microalgal biotechnology. In this respect, progress has been made to enhance the production of light-related metabolites from microalgae. Ben-Amotz et al. (Ben-Amotz and Avron, 1989; Ben-Amotz *et al.*, 1989) were among the first authors studying the effect of different wavelengths in *Dunaliella,* describing how β-carotene rich *Dunaliella* spp. was protected against blue light, mainly. Recently, Fu et al. (2013) tried to enhance carotenoid production in this species with different light wavelengths, remarking the importance of the use of blue light for such purpose. However, recent studies have shown different outcome (Xu and Harvey, 2019a, 2019b), reflecting the heterogeneity of the results obtained regarding this topic.

In *H. pluvialis,* the first studies with regards of light composition were performed by Kobayshi's group (Kobayashi *et al.*, 1992), who observed that carotenoid synthesis was higher

in cultures cultivated under blue light, in mixotrophic conditions. Later other authors reported a similar behaviour (Park and Lee, 2001; Katsuda *et al.*, 2004; Kim *et al.*, 2009; Xi *et al.*, 2016). However, most of these studies are performed with non-aerated cultures, non-optimized culture medium, or under low irradiances, which yielded low-biomass cultures. Besides, the importance of nitrogen starvation conditions for massive astaxanthin accumulation is sometimes dismissed and this results in very low astaxanthin accumulation rates. Park and Lee (2001) reported maximum astaxanthin concentrations of 6 mg $L^{-1}$ after 16 days of batch growth under 160 μmol $m^{-2}$ $s^{-1}$, while Xi et al. (2016) reached only 15 mg $L^{-1}$ after 10 days.

With respect to phycobiliproteins, Takano et al. (1995) reported that under red light *Synechococcus* synthesized twice the phycocyanin than cultures maintained under blue or green light. In *Spirulina platensis*, a similar response was observed together with an enhancement of the pigment purity (Walter *et al.*, 2011). In *P. cruentum*, changes in the photosynthetic pigments profile were first described by Brody and Emerson (1959) and, more recently, studies have shown the potential of optimizing light composition in this species (You and Barnett, 2004; Baer *et al.*, 2016; Coward *et al.*, 2016) with varied results that range from the use of monochromatic red and blue lights for the production of exopolysaccharides (You and Barnett, 2004) to the addition of green light to a broader light spectrum to enhance phycoerythrin content per cell (Baer *et al.*, 2016; Coward *et al.*, 2016).

It seems evident that it is possible to grow microalgae under a non-complete PAR spectrum or even with monochromatic lights and that the spectral quality of the light source can affect culture characteristics largely. Nevertheless, enough knowledge has not yet been generated in order to characterize repetitive patterns and formulate generalizations, as it is evidenced from recent revisions of the topic (Schulze *et al.*, 2014; Glemser *et al.*, 2016). It is plausible that the patent highly specific responses may be magnified by the heterogeneity of the materials and methods used across the different studies found in the literature, as well as the non-standardised approaches, which tend to be very technical and therefore the biological perspective is left behind, or vice versa. The development of technologies such as light emitting diodes (LEDs), light sources that emit narrow bandwidth monochromatic light and cover most of the visible light spectrum, may allow more accurate studies. LEDs bypass most of the limitations of older spectrographic (Monk and Ehret, 1956) and monochromator technologies (Geider and Osborne, 1992). LEDs can be used *in vivo* in continuous cultures allowing the study of long-term effects, are small and accessible devices, produce less heat than other light sources, require less amperage currents to produce the same amount of photons, allow a wide range of irradiances, are cheap, stable, and have a long shelf life (Walker, 1987). With LEDs researchers may be able to study the effect of very specific wavelengths or precise delimited regions of visible light effectively and trigger, understand, and characterize light-dependant responses meticulously. Moreover, the application of these light sources in a larger scale would be advantageous compared to other artificial light sources if the enhancement of certain metabolites with industrial interest, such as anti-oxidant enzymes or photosynthetic pigments, by narrowing down the light spectrum, is significant. Besides, the higher energy to photons conversion efficacy of LED's compared to fluorescent or halogen lamps, would anyway allow a more efficient energy usage reducing operational costs of indoor cultivations.

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# OBJECTIVES

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The main objective of this work was the study of the effect of wavelength composition on growth, pigment content, and photosynthetic response in different microalgae.

Specific obejtives include:

1. Culture of *H. pluvialis* at laboratory-scale. Effect of temperature and light composition on growth and carotenogenesis, and characterisation of photosynthetic response.

2. Optimization of culture conditions and industrial proof of concept of an indoor two-stage cultivation of *H. pluvialis* for the production of astaxanthin using light emitting diodes and flat-plate photobioreactors.

3. Effect of light composition on growth, pigment content and pigment composition in cultures of carotenogenic *D. salina* and non-carotenogenic *D. tertiolecta* in conditions of nitrogen starvation, and photosynthetic response characterisation.

4. Effect of light composition on growth, pigment content and exopolysaccharide production, and photosynthetic response characterisation, in cultures of *P. cruentum* under nitrogen limitation.

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# MATERIALS AND METHODS

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# 1. Organisms description

## 1.1. *Haematococcous pluvialis*

*H. pluvialis* is a green alga (Chlorophyta) reported to be found, naturally, in freshwater shallow water bodies such as small ponds in between rocks, where it is more visible when it forms red layers attached to the pond walls (Droop, 1953). The alga goes through three clear stages during its life cycle. During vegetative growth, while cells are dividing actively, *H. pluvialis* cells have an oval to pear-shaped morphology. In this stage, cells are motile due to the presence of two flagella and display a green colour. The green cells present a clear periplasm, an extracellular gelatinous matrix, that is connected to the protoplast by thin cytoplasmic strands. Green cells contain a large chloroplast that contains a variable number of clear pyrenoids that are surrounded by starch capsules. The size of green vegetative cells varies depending on the strain, and have been reported in the range of 15 to 30 μm (Wayama *et al.*, 2013; Allewaert *et al.*, 2015; Borowitzka, 2018a). Optimum temperature is strain-specific but for the vegetative stage it is usually reported between 15 and 20 ºC (Tjahjono *et al.*, 1994; Allewaert *et al.*, 2015). In a later stage of its life cycle, when entering stationary-phase and therefore conditions of dimished to restricted cellular division, *H. pluvialis* cells lose the flagella and the oval shape and acquire a non-motile dark-green rounded appearance denominated palmella stage. If conditions remain unchanged, palmella stage will continue developing towards bigger rounded cells, the aplanospores. Aplanospores develop a thick cell wall, and the periplasm that was clear in the previous stages of the life cycle is not evident anymore since the cells grow and occupy that space completely (Baudelet *et al.*, 2017). Cells in an aplanospore stage change colour completely with respect to the green stages of the life cycle. This change of colour is the result of the accumulation of the carotenoid astaxanthin, which accumulates mainly esterified to C18 and C16 fatty acids in lipid droplets (Holtin *et al.*, 2009) that, eventually, occupy the entire cytoplasm. The increase in weight of the cells results not only from the accumulation of astaxanthin, which can be found in the range of 1-8 % of the dry weight, but the cells accumulate a large amount of storage compounds during this process. Difference in cell weight between vegetative motile stage and an aplanospore filled with astaxanthin may be ten-fold. Conditions that restrict cellular division to a certain extent trigger the accumulation of the carotenoid, being the combination of nitrogen starvation and high light the conditions that accelerate the most this physiological response (Aflalo *et al.*, 2007). However, some authors have also described the formation of small amounts of astaxanthin during active cellular division when *H. pluvialis* was cultured in continuous light and a nitrogen-replete medium (Domínguez *et al.*, 2019). Therefore, it is plausible that the oxidative damage provoked by different conditions can trigger the synthesis of this pigment when the disruption of homeostasis reaches a certain treshold, and the extent and synthesis rate depends on the combination of each condition with the cellular state. When environmental conditions are favourable for cellular division, aplanospores can germinate to release vegetative cells (Fábregas *et al.*, 2003; Borowitzka, 2018a). Mitosis and cytokinesis inside aplanospores produce flagellated daughter cells that are released to the medium by the rupture of the parental cell wall. In general, despite sexual reproduction has been reported in this species (Triki *et al.*, 1997), it is unusual, and *H. pluvialis* undergoes asexual division by mitosis (Wayama *et al.*, 2013; Borowitzka, 2018a).

With respect to carbon metabolism, *H. pluvialis* can grow, principally, photoautotrophically and mixotrophically in different organic carbons (Kobayashi *et al.*,

1992; Orosa *et al.*, 2001), but also heterotrophically to a lower extent (Kobayashi *et al.*, 1992). However, only illuminated cultures achieve massive pigment accumulation (Borowitzka *et al.*, 1991; Boussiba, 2000; Fábregas *et al.*, 2003; Aflalo *et al.*, 2007).

Astaxanthin is one of the most valuable microalgae-derived products. It is used in aquaculture industry, mainly added to salmon dry feed formulations or to colour flesh of other animals that are unable to synthesize it *de novo* (Lorenz and Cysewski, 2000). Astaxanthin is also used in cosmetic industry, and as a nutraceutical for human consumption. Current commercial processes generally follow a two-stage production process (Fábregas *et al.*, 2001), where green and red cells are grown separately under optimum conditions for each stage in enclosed or in a combination of closed and open systems. Once the red biomass is harvested, the cells are disrupted, dried, and stabilised to prevent oxidation until further processing.

The strain used in this work (*H. pluvialis* SAG 34-1a) was obtained from the Culture Collection of Algae at the University of Göttingen (Germany)

### 1.2. *Dunaliella* spp.

The genus *Dunaliella* (Chlorophyta) includes 28 recognized species separated in 2 subgenera: *Pascheria* and *Dunaliella*. The latter is subdivided in 4 sections: *Tertiolectae*, *Duneliella*, *Viridis*, and *Peirceinae* (Ben-Amotz *et al.*, 2009). The taxonomy of this group is complex, and rises controversy. At first, morphological and physiological features were used to characterize the different species of this group. However, molecular data has evidenced the inadequacy of that approach, and therefore taxonomy is being rearranged based on new molecular studies. In this respect, the subgenus *Pascheria* (5 species) includes only freshwater species and may not even belong to the genus at all. With regards to the subgenus *Dunaliella*, it includes a huge diversity of species that tolerate a wide range of saline environments. Species can be found from brackish to hypersaline environments, with a global distribution. Cells are uninucleate with a single chloroplast and lack cell wall. Instead, cells are covered by a glycocalyx. An eyespot can be found in some species. Morphology is very diverse, ranging from almost spherical cells to ellipsoid, ovoid, or fusiform shapes (Oren, 2005; Borowitzka, 2018a). Both vegetative cell division by longitudinal division of the flagellated cell and sexual reproduction by fusion of two motile cells to form a zygote has been reported for species of the genus (Oren, 2005; Borowitzka and Siva, 2007). Moreover, as in *H. pluvialis,* under certain conditions that disrupt cellular homeostasis, some species of *Dunaliella* may form a palmella and aplanospore stages, diminishing or restricting cellular division, loosing their flagella and showing a more rounded shape. These forms return to a vegetative motile stage when environmental conditions are back to "normal" (Borowitzka and Huisman, 1993). The first *Dunaliella* species studied were the ones found in hypersaline environments, such as *D. salina*, *D. parva*, or *D. viridis*. These species have the ability to accumulate intracellular glycerol to survive the huge osmotic pressure of living in such environments (Borowitzka and Brown, 1974). This characteristic, together with the absence of a rigid polysaccharid cell wall (Baudelet *et al.*, 2017), allows a quick adjustment of the intracellular osmotic pressures by tuning water and glycerol content, and therefore survive rapid environmental changes (Herrero *et al.*, 1994). Later, it was found that some of these hypersaline species of *Dunaliella* could accumulate β-carotene massively, which lead to the development of large-scale facilities exploiting the algae for this purpose (Ben-Amotz and Avron, 1981, 1983, 1990; Ben-Amotz *et al.*, 1989; Borowitzka, 2013).

### 1.2.1. *Dunaliella tertiolecta*

*D. tertiolecta* was first described by Butcher in 1959, and belongs to the section *Tertiolectae*. It can be found, mainly, in brackish and marine habitats of America and Europe. *D. tertiolecta* grows optimally at salinities between 2-4 % and lower or higher salt concentrations compromise its growth. Optimum temperature of this species has been reported at 20 ºC (Ben-Amotz *et al.*, 2009). *D. tertiolecta* has been used as the feed for supporting cultures in aquaculture facilities, the rearing of fishes and molluscs. Its use has also been proposed as Single Cell Protein (SCP), mineral source in fish diets, or as a source of vitamins (Fábregas *et al.*, 1986, 1989, 1994; Abalde *et al.*, 1991; Ben-Amotz *et al.*, 2009). Due to the ease of cultivation, this microalga has been extensively used for photosynthesis-related studies. In these, the microalga is characterized by fast and clear responses to changes in irradiance. Moreover, *D. tertiolecta* has also been used to study the responses of microalgae to nitrogen starvation conditions, and in toxicity studies (Fisher *et al.*, 1984; Sukenik *et al.*, 1987; Abalde *et al.*, 1995; Geider *et al.*, 1998; Young and Beardall, 2003; Oren, 2005; Ben-Amotz *et al.*, 2009).

The strain used in the present work (*D. tertiolecta* Butcher CCAP 19/6B) has been obtained from the Culture Collection of Algae and Protozoa (UK).

### 1.2.2. *Dunaliella salina*

*D. salina* was first described by Teodoresco in 1905, despite in 1983 Dunal had already described a red-coloured unicellular biflagellate in saturated salty environments naming it *Haematococcus salinus* (Oren, 2005). The species belongs to the section *Dunaliella*, and it is clasified among the hypersaline *Dunaliella* spp. In fact, *D. salina* has been proposed as the most salt tolerant eukaryote, able to survive in salinities as high as 35 % (Borowitzka *et al.*, 2016). Therefore, the species inhabits in hypersaline environments throughout the world. According to Teodoresco's first description of the species, under certain conditions the cells of *D. salina* synthesized massive amounts of carotenoids colouring the cells orange-red (Oren, 2005). However, some isolates originally deposited as *D. salina* in the culture collections do not comply with those physiological characteristics and must be misidentified (Ben-Amotz *et al.*, 2009). This disorganization resulted in an evident confusion, reflected in the literature, which needs revision in order to base the organization of the genus also on molecular data instead on only morphological and physiological aspects. Some authors, in order to step away of this confusion, named new species such as *D. bardawil* Ben-Amotz at Avron (Ben-Amotz *et al.*, 1982), which fits the original description of the species made by Teodoresco and, as a consequence, is considered by many as a *D. salina* (Borowitzka, 2010).

The species *D. salina* must be able to massively accumulate the carotenoid β-carotene under certain environmental conditions including high temperatures, high salinity, high irradiance, or low nutrient availability. Irradiance and salinity are the most crucial factors (Ben-Amotz *et al.*, 1989, 1991). The cells accumulate up to 14 % of dry weight as β-carotene, which is higher than other species of the genus such as *D. parva*, or the non-carotenogenic *D. viridis* and *D. tertiolecta* (Borowitzka, 2010). Within the cell, β-carotene is accumulated as granules in lipid droplets in the interthylakoid space of the chloroplast and occur as a mixture of *cis*- and *trans*- isomers (Oren, 2005; Borowitzka, 2010). β-carotene is a valuable chemical, highly demanded as colouring agent in food. Furthermore, it serves as a precursor of retinol,

and is added in cosmetic formulations (Oren, 2005). Due to its interest, large-scale cultures of *D. salina* are being exploited as a source of this compound. Currently, *D. salina* is being produced extensively in unmixed hypersaline open ponds in Australia, or intensively in more sophisticated systems, raceways, in Israel (Borowitzka and Borowitzka, 1990; Oren, 2005; Ben-Amotz *et al.*, 2009; Richmond and Qiang, 2013; Borowitzka *et al.*, 2016; Borowitzka and Vonshak, 2017).

The strain used in the present work (*D. salina* Teodoresco CCAP 19/18) has been obtained from the Culture Collection of Algae and Protozoa (UK).

### 1.3. *Porphyridium cruentum*

*P. cruentum* is an unicellular and non-motile Rhodophyta species described originally by Carl Nägeli in 1849 (Nägeli, 1849; Barsanti and Gualtieri, 2006). The algae from the genus have a wide habitat distribution since they occur naturally on rocks and soil of humid areas, but also in brackish and marine environments (Jones *et al.*, 1963). Morphologically, cells are small and generally rounded, about 5 to 10 μm diameter, depending on the cellular state (Ramus, 1972). The cells are composed of a single chloroplast with a central pyrenoid, and a cytoplasmatic region with all the typical eukaryotic organelles. The cells have a cellular membrane and lack a rigid cell wall (Gantt and Conti, 1965). Instead, cells are surrounded by a gelatinous cover that varies in thickness depending on the cellular status (Arad and Levy-Ontman, 2010). Starch grains appear randomly distributed throughout the cytoplasm, and unlike other alga, starch is absent in the chloroplast nor surrounding the pyrenoid (Jones *et al.*, 1963; Gantt and Conti, 1965). The chloroplast lamellae of *P. cruentum* are in conformity with that of red algae and cyanobacteria and consists of single layered membranes where the photosynthetic apparatus is located (Gantt and Conti, 1965). Rhodophyta, and therefore *P. cruentum*, possess chlorophyll *a* and lack chlorophyll *b*. However, the main photosynthetic pigment in *P. cruentum* is phycoerythrin, a phycobiliprotein that confers the red colour to this microalgae (Barsanti and Gualtieri, 2006). In terms of cell division, sexual reproduction has not been described in *P. cruentum* and cells divide asexually by mitosis (Gantt and Conti, 1965). Optimum growth temperature has been found for *Porphyridium* sp. at 25 ºC, despite no cellular damage was observed until the culture was exposed to temperatures as high as 38 ºC, reflected in a decrease in $O_2$ evolution (Vonshak *et al.*, 1985). As for carbon metabolism, the microalgae grows photoautotrophically, but can also activate myxotrophic metabolism when certain sugars are available (Fábregas *et al.*, 1999).

The biochemical composition of this microalgae varies a lot depending on the cellular status and practically every fraction includes a product with potential industrial application. For this reason, from a biotechnological perspective, culture conditions may need to vary accordingly in order to maximize the production of the target metabolite. Lipid density in the lipid bodies of nitrogen-starved cells decreased with respect to exponentially growing cells as a consequence of a higher content of neutral with respect to polar lipids (Köst *et al.*, 1983; Otero *et al.*, 1997). With respect to fatty acid profile, when the cells are actively dividing, they are rich in eicosapentaenoic acid (EPA, 20:5 n-3). However, the abundance shifts towards araquidonic acid (ARA, 20:4 n-6) when growth rate is reduced (Ahern *et al.*, 1983; Cohen *et al.*, 1988; Cohen, 1990; Nuutila *et al.*, 1997), and specially under nitrogen starvation. Furthermore, carbohydrate content is high and its composition is interesting with respect to other microalgae. Starch accumulates intracellularly as the main storage compound and its amount is directly correlated with culture age. Additionally, the gelatinous cell wall is

composed of cell bound polysaccharides, of which part of them dissolve in the culture medium increasing its viscosity. These polysaccharides have been studied for their properties and potential applications in cosmetics and pharmaceutical industry (Arad and Levy-Ontman, 2010). In this respect, *P. cruentum* polysaccharides produce a higher viscosity with lower concentrations than other industrial phycocolloids such as carrageenan (Arad *et al*., 1985), and have a superior stability (Arad *et al.*, 2006). Moreover, in recent years, potential applications of *P. cruentum* and other *Porphyridium* species in the pharmaceutical industry were described (Falaise *et al.*, 2016). For instance, polysaccharides from *P. cruentum* inhibited viral replication in rats. Also, *in vivo* administration in mice increased macrophage population. EPS extracts of *P. cruentum* have also shown other antiviral, antibacterial, and anti-inflammatory properties (Fábregas *et al.*, 1999; Matsui *et al.*, 2003; Raposo *et al*., 2014). It has been hypothesized that the sulfate groups would be the responsible for the bioactivity since blocking sulfate groups significantly decreased antiviral activity (Geresh *et al*., 2000). These polysaccharides also promote anti-oxidant activity suggesting a protecting mechanism against ROS formation in the cells (Tannin-Spitz *et al.*, 2005), and it has also been shown that polysaccharides from *P. cruentum* induced cytokine activity (Abdala-Díaz *et al.*, 2010) and could have potential for the treatment of certain cancers (Gardeva *et al.*, 2009). The phycobiliprotein phycoerythrin has a wide range of applications including its use as food or cosmetic colourant, or as a fluorescence compound that could be used as a biomarker in cytometry and inmunology (Glazer and Stryer, 1984), or as a pharmacological compound in the therapy against certain cancers (Hu *et al.*, 2008). Phycoerythrin content is closely related to both protein synthesis and photosynthetic apparatus response to environmental conditions.

Despite *P. cruentum* is not as largely exploited as *H. pluvialis* or *D. salina* spp., large-scale cultivation systems for its production and downstream processing have been developed (Richmond and Qiang, 2013), where most optimistic estimates have reported potential productivities of 50 tons $ha^{-1}$ $year^{-1}$ of dry biomass (Vonshak *et al.*, 1985).

The strain used in the present work (*P. cruentum* UTEX 161) has been obtained from the Culture Collection of Algae at the University of Texas (US).

# 2. Cultivation systems

### 2.1. Strains and strain preservation

*Haematococcus pluvialis* (strain SAG 34-1a) was obtained from the Culture Collection of Algae at the University of Göttingen (Germany). *Dunaliella tertiolecta* Butcher (strain CCAP 19/6B) and *Dunaliella salina* Teodoresco (strain CCAP 19/18) were obtained from the Culture Collection of Algae and Protozoa (UK), and *Porphyridium cruentum* (UTEX 161) was obtained from the Culture Collection of Algae at the University of Texas (US). Upon arrival, strains were transferred to both solid and liquid medium for preservation and further use (Figure. 0.4). Culture mediums were demineralized water enriched with OHM (Fábregas *et al.*, 2000) for *H. pluvialis,* and filtered seawater enriched with Algal Medium modified from Fábregas et al. (1984) for *D. tertiolecta*, *D. salina*, and *P. cruentum*. Nitrate concentration was adjusted in every culture media to 4 mM $L^{-1}$, and the rest of the elements were increased accordingly. Salinity was adjusted to 35 ppt for *D. tertiolecta* and *P.*

*cruentum*, and to 75 ppt for *D. salina*, as recommended by the culture collection. For solid medium, 1.5 % (w/ v) agar was added to the liquid medium. All the culture media were sterilized by autoclaving at 121 ºC for 21 minutes before usage. Liquid cultures were maintained in a volume of 20 mL in 100 mL erlenmeyers and agitated manually once a day. Both liquid and solid stock cultures were incubated and preserved under dim warm light at room temperature (20 ± 2 ºC) and submitted to a photoperiod of 12 h light 12 h dark.

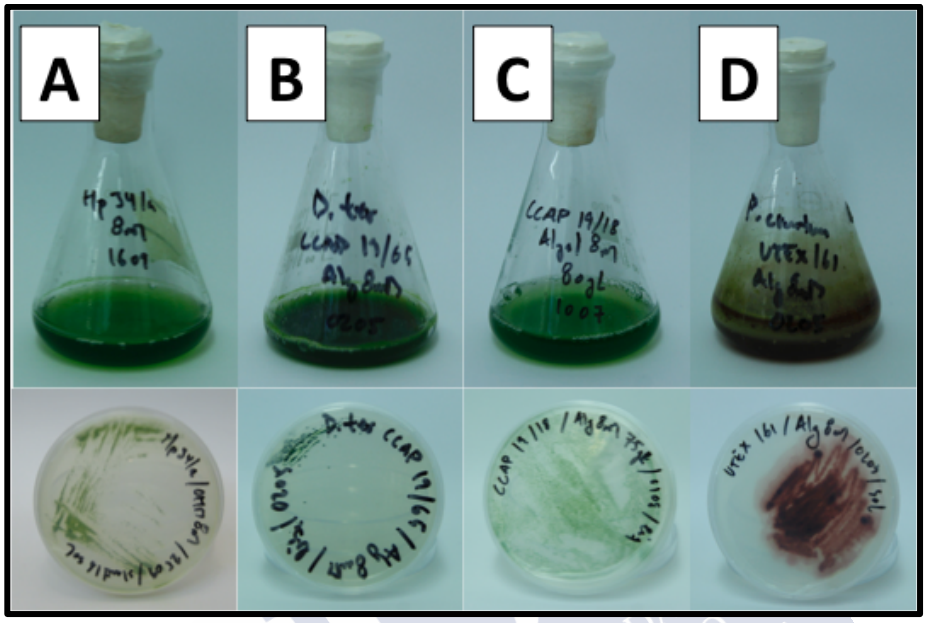



**Figure 0.4. Liquid and solid stock cultures of *H. pluvialis* SAG 34-1a (A), *D. tertiolecta* Butcher CCAP 19/6B (B), *D. salina* CCAP 19/18 (C), and *P. cruentum* UTEX 161 (D).**

### 2.2. Laboratory-scale pre-cultivation setup

Pre-cultures of every strain were carried out in 1 L cylindrical glass photobioreactors (100 mm diameter) (Figure 0.5A) illuminated from one side with a maximum irradiance of 100 µmol photon $m^{-2}$ $s^{-1}$ provided by fluorescent cool white lamps. Irradiance was measured with a Li-Cor LI-250A light meter equipped with a Li-Cor QUATUM probe. Cultures were grown in the same culture medium as the one used to preserve them (Materials and methods, section 2.1). These cultures were submitted to a photoperiod of 12 h: 12 h light/ dark with constant aeration. The pH of the cultures was kept between 7.0 and 7.5 for *H. pluvialis* and between 7.2 and 8.0 for the other species, with pulses of $CO_2$ during the light period. Temperature was kept at 20 ± 2 ºC by controlling room temperature. Regular dilutions to these cultures with the same culture medium and salinity were carried out to ensure that cultures were kept actively growing in exponential phase.

### 2.3. Laboratory-scale experimental setup

Every laboratory-scale experiment was performed in the same experimental setup with except of the optimization of light quality in *H. pluvialis* vegetative stage.

For every microalgae species, exponentially growing pre-cultures (Materials and methods, section 2.2) were used as inoculation material. Microalgae were cultivated in total volumes of 100 mL, in glass mini-photobioreactors with a diameter of 50 mm (Figure 0.5B).

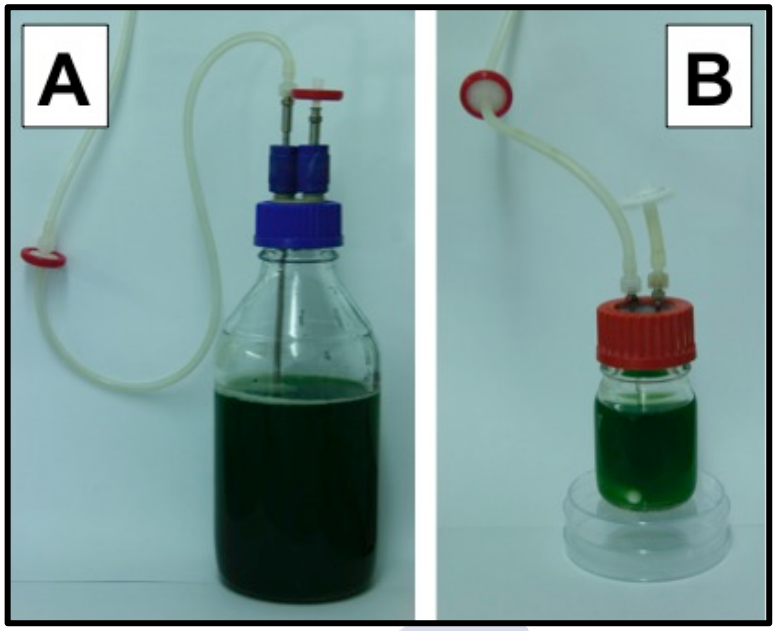



**Figure 0.5. 1 L cylindrical glass photobioreactor (100 mm diameter) (A), and 100 mL cylindrical glass photobioreactor (50 mm diameter) (B).**

The mini-photobioreactors consisted in 100 mL Duran® glass bottles with a modified cap for the input of air and $CO_2$. The experimental setup consisted in three independent groups with a capacity for three replicates per group. Each group of three mini-photobioreactors (Figure 0.6) had independent photoperiod, irradiance, and light wavelength control, which could be adjusted via proprietary software (Astaco Technologies B.V.). Moreover, every group of mini-photobioreactors had an independent temperature control since the units were standing in a glass water bath with a stainless steel coil that recirculated water at the respective temperature through a laboratory-scale heat exchange unit. Despite cultures were aerated continuously with an air flow of 0.12 L $min^{-1}$, the mini-photobioreactors were also equipped with a stirring magnet. For this reason, the water baths were standing on top of stirring plates that allowed mechanical mixing of the cultures at 60 rpm. pH was maintained between 7.0 and 7.5 in every species by the enrichment of the air stream with regular pulses of $CO_2$. For every red-stage *H. pluvialis* laboratory-scale experiment, as well as for every *D. tertiolecta*, *D. salina*, or *P. cruentum* experiment, cultures were continuously illuminated with light produced by light-emitting diodes (LEDs, assembled by Astaco Technologies B.V.) with different combinations of monochromatic narrowband light wavelengths, which varied depending on the experiment. Available wavelengths were: 420, 450, 470, and 660 nm. Irradiance could be adjusted between 0 and 350 µmol photon $m^{-2}$ $s^{-1}$ without compromising light distribution between treatments. Most of the experiments compared batch cultures maintained for seven days at 300 µmol photon $m^{-2}$ $s^{-1}$ under monochromatic blue (450 nm), monochromatic red (660 nm) and a mix of red (660 nm) and blue (450 nm) with a red: blue ratio of 1: 1. However, in red-stage *H. pluvialis*, experiments were also done comparing mixes of red and different blue wavelengths (420, 450, and 470 nm), or comparing different irradiance levels (50 to 300 µmol photon $m^{-2}$ $s^{-1}$) with a fixed light composition. Mini-photobioreactors could easily be handled for sampling in order to maintain sterility using aseptic microbiological technique.

The optimization of temperature in vegetative-stage *H. pluvialis* was carried out in the same experimental setup but cultures were maintained under a photoperiod of 12 h light 12 h dark, and $CO_2$ pulses were only provided during the light period.

For the optimization of light quality in *H. pluvialis* vegetative-stage, 1 L photobioreactors consisting on 1000 mL Duran® glass bottles with a modified cap for the input of air and $CO_2$ (Fig. 0.5A) were used, in a temperature controlled chamber (20 ± 2 ºC).

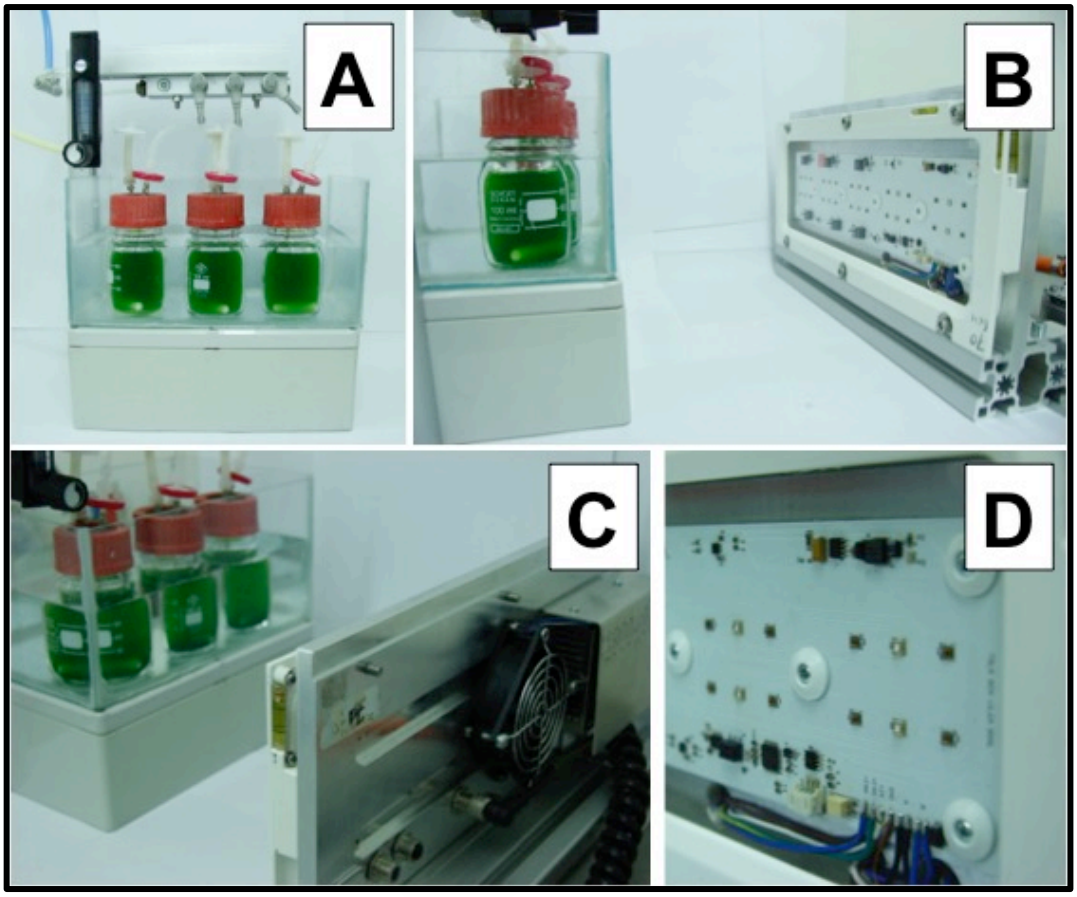



**Figure 0.6. Laboratory-scale experimental setup (one group with a capacity for three replicates), consisting on 100 mL cylindrical glass photobioreactors (50 mm diameter) standing in a water bath for temperature control. Photobioreactors are aerated and mechanically mixed with stirring magnets. Front view of the cultivation unit (A), side view of the experimental setup including the light source (B), back view of the experimental setup (C), and detail of the LED light sources (D).**

### 2.4. Medium-scale and pilot-scale experimental setup

#### 2.4.1. Internally illuminated fermentor-type photobioreactor

A fermentor-type photobioreactor was the first system used to validate laboratory-scale results at a larger scale (Figure 0.7A). The PBR consisted in a fermentor-type column of stainless steel equipped with a submersed lamp and a conical bottom with an approximate slope of 15º. The unit had a working volume of 100 L and an illuminated surface of 1.3 $m^2$, accounting for a S/ V of 13 ($m^2$/ $m^3$). The PBR was equipped with a water jacket that allowed an accurate temperature control by recirculating water that was passed through a heat exchanger that could cool down or warm up the liquid with respect to the process needs.

The unit had an input point for the aeration at the vertex of the conical bottom, and an air outflow via an airlock installed in the lid. Attached to the bottom, the unit was equipped with a stainless steel impeler. Sensors for culture monitoring and automation of the operation were introduced via sensor ports in-built in the stainless steel lid, in the center of which a glass cover for a submersed lamp was installed. When light was on, the air was enriched with $CO_2$ on demand when the pH was higher than the set-point.

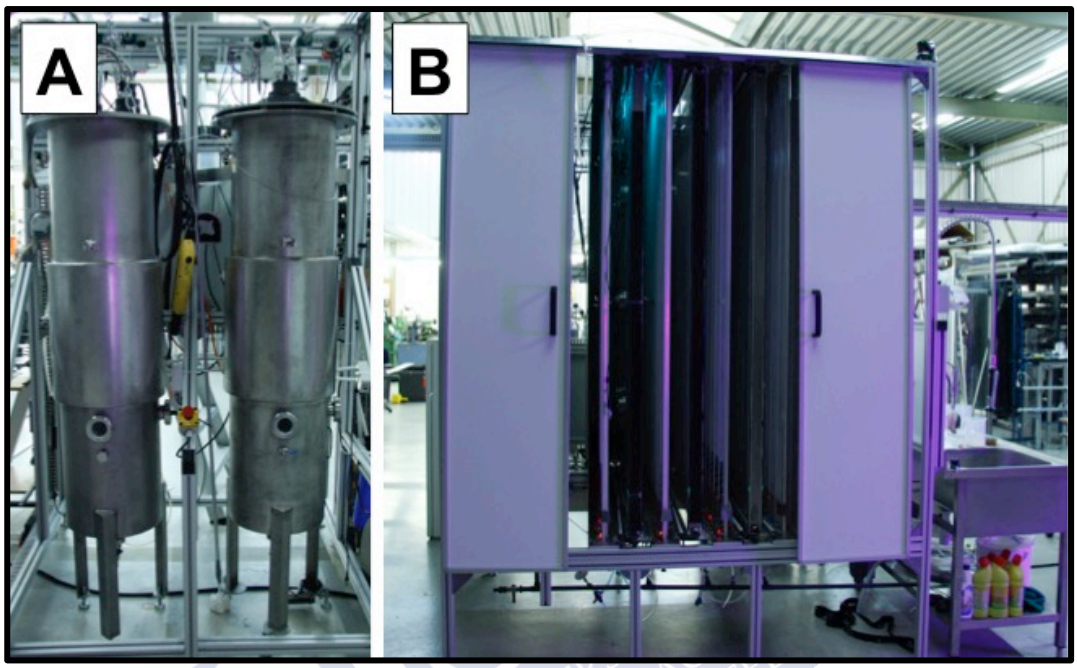



**Figure 0.7. Two 100 L internally illuminated fermentor-type photobioreactors (A), and three 150 L flat-plate photobioreactors (B).**

### 2.4.2. Flat-plate photobioreactors

Prototypes with a working volume of 20 L and industrial pilot-scale cultivation units of 150 L were built based on the classic flat-plate design, and consisted in two glass plates attached to a stainless steel frame. The pilot-scale units (150 L. Figure 0.7B and figure 0.8) were a faithful reproduction of the prototype units (20 L). The units were completely enclosed with a bottom piece that integrated an aeration system that produced turbulence in the cultures and a homogeneous mixing, and a lid that covered the top part of the reactor that included input points for sensors, sampling port, and other cultivation hardware. Both bottom and top parts were made in stainless steel. Glass plates composition was low in iron oxide, which was specifically chosen because it allowed more transparency, translated in 15 % more transmittance of light compared to standard glass. The separation between glass plates was 50 mm but, since the photobioreactors were illuminated from both sides, the effective optical path was 25 mm. A water jacket was integrated in the stainless steel frame of the reactors that allowed temperature control by recirculating water that was connected to a heat exchanger, which could cool-down or warm-up the recirculated liquid depending on the needs of the process. A number of sensors were integrated in the system in order to facilitate automation and remote monitoring.

At the prototype level, the reactors were equipped to be able to test both green and red *H. pluvialis* stages regardless of the unit, while there were differences in the pilot-scale 150 L units between photobioreactors meant for green growth and photobioreactors meant for induction. In this respect, both green and red stage photobioreactors included volume, pH, temperature and optical density sensors. pH and temperature were controlled accurately on demand either by adding $CO_2$ to the air stream when the pH was higher than the set-point, or opening a valve that allowed water recirculation when temperature was above the set-point. Additionally, cultivation units dedicated to the continuous production of green vegetative cells were equipped with a dissolved oxygen sensor, a nutrient dosing unit, a water input point, and a peristaltic pump meant to outflow and transfer green culture into red-stage PBRs. Both green and red stage reactors included an anti-foam dosing system that prevented the formation and accumulation of foam in the PBR headspace, and an aseptic sampling port. Photobioreactors were illuminated from both sides with LED lamp racks (assembled by Astaco Technologies B.V.) that were situated 70 mm away from the glass plates. The lamp racks included 660 and 450 nm LED light sources with an optimized illumination angle that directed the major part of the photons to the reactor walls, preventing any major loss of light out of the illuminated surface. Irradiance homogeneity across the illuminated surface was ± 15 %. Moreover, lamp racks were hollow, so that coolant could be recirculated, which allowed to run the light sources at the optimum temperature in terms of power conversion into photons. Each cultivation unit occupied an approximate area of 0.85 $m^2$.

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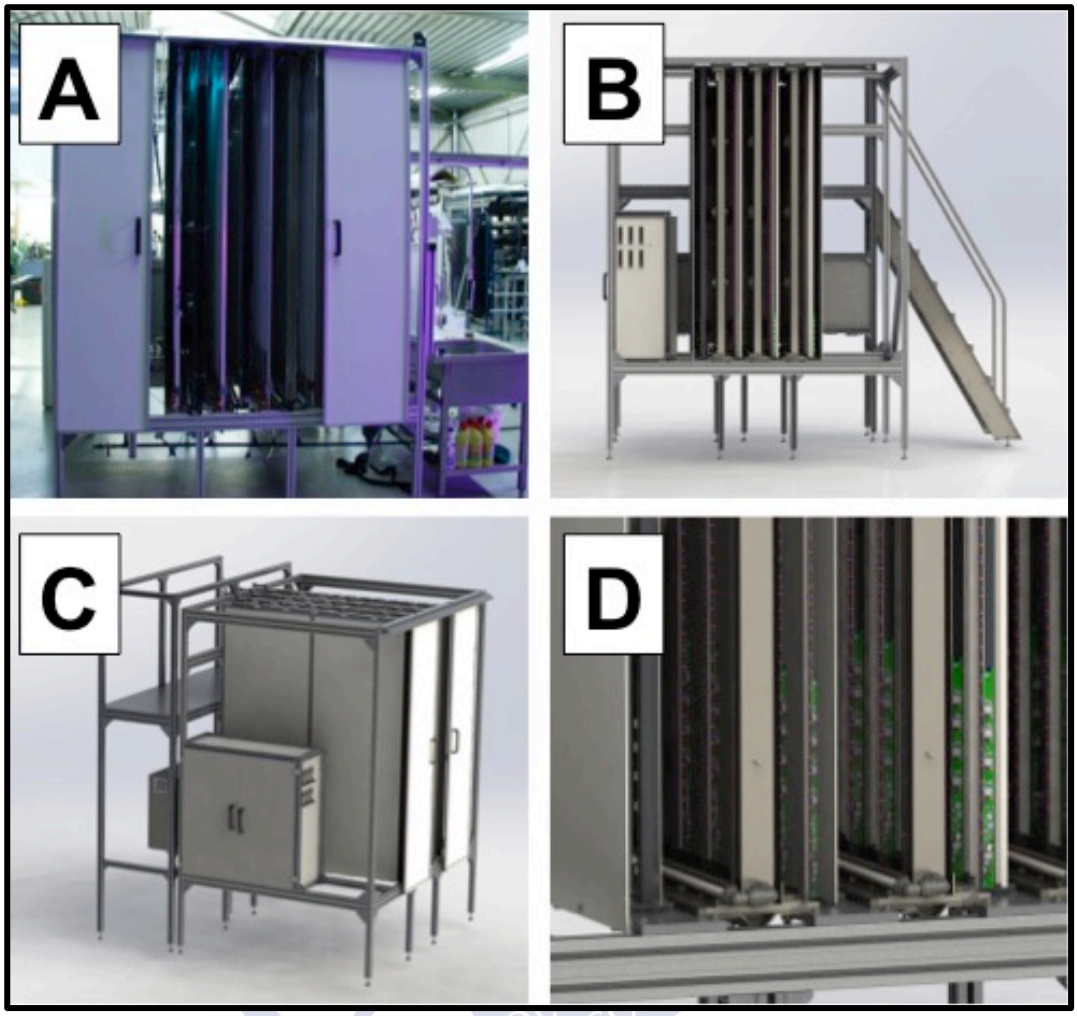



**Figure 0.8. 150 L pilot-scale flat-plate photobioreactors. Constructed side view (A), rendered side view (B), rendered general view (C), and rendered close-up of the stacked flat-plates and light sources (D).**

### 2.4.3. Side equipment and photobioreactor operation

In addition to the cultivation units, a number of side equipment was installed. Air was supplied by a compressor and was first de-moisturized and then filtered through a 1 μm followed by 0.01 μm nominal filter pore size, prior to be introduced in the cultures. 100 % $CO_2$ was filtered and diluted into the air stream on demand when the pH sensor read a signal above the set-point (7.5). Water was first filtered through a 20 μm filter followed by a 5 μm filter in order to remove any big particles, it was then passed through a UV filter, followed by an ultrafiltration step through a membrane filter with a pore size of 0.01 μm before entering the cultivation unit.

In terms of operation, inoculum for the 150 L green-stage flat-plate PBR was cultured in the 20 L flat-plate prototypes and these, in turn, were inoculated with 5 L cultures grown in a light bench at laboratory-scale. Therefore, a dilution factor of 1: 7.5 was used to inoculate the 150 L units. Inoculation was done manually. Once the green culture reached late exponential phase, which normally occurred after two days of growth, continuous cultivation mode started by transferring 6.7 % of the green volume every 2 hours into a 150 L red-stage PBR to induce secondary metabolism. Continuous cultivation was fully automated. Once

induction period ended, harvesting was done pumping the red volume through a centrifuge that concentrated and dewatered the biomass to an algal paste. Red-stage *H. pluvialis* cultures were harvested by centrifugation, which dewatered the biomass producing a red microalgal paste that contained around 20-23 % of solid content. Red paste was then spread in aluminium trays on a 1 cm thick layer and stored frozen at - 28 º C for further analysis and downstreaming. Water was not reused in this pilot-concept, and neither air or $CO_2$ were recirculated.

Before green-stage cultivation started and between each induction period, PBRs were sanitized. In every photobioreactor described in this section, including the pilot-scale units constructed as a proof of concept, sanitation was done manually. Any visible biofilm build-up was brushed out manually from the PBR walls. Any carbonate build-up was removed with the aid of commercial anti-scaling products. Once solids were not detected visually, the cultivation vessel was rinsed with filtered water. Then, the vessel was filled with pre-treated water and hydrogen peroxide was added to a concentration of 1.5 % (v/ v). This solution was allowed to act for 1.5 hours before the unit was emptied, and rinsed with filtered water until no trace of peroxide could be detected by a colorimetric method. A similar protocol was followed on a daily basis to sanitize water lines. Sampling port, together with any tubing used for liquid transfer or dosing of the nutrients was autoclaved for 21 minutes at 121 º C and +1 atm. 10 L nutrient stock solutions were prepared separately and sterilized before use by autoclaving. Ethanol 70 % was used to maintain hygienic conditions and was sprayed over any surface or tube before and after any of these required some kind of intervention during operation, as well as pre and post sampling through the sampling port with pre-autoclaved syringes.

## 3. Analyses

### 3.1. Cellular count and growth rate

Cell concentration was evaluated by microscope counting using a Bürker-Türk hemocytometer. Serial dilutions with sterilized demineralized water for *H. pluvialis*, and with sterilized seawater adjusted to the respective salinity for *D. tertiolecta*, *D. salina*, and *P. cruentum*, were made so that the number of counted cells per field (1 $mm^2$) was between 30 and 300. In motile species (*H. pluvialis*, *D. tertiolecta*, and *D. salina*) a drop of Lugol solution (Sigma-Aldrich #62650) was added to fix the cells prior to cellular counting.

Growth rates, as doublings per day ($k$), were calculated as the rate of increase in cells per unit time following the equation $k = log_2(Nt/No) \ / \ \Delta t$ , where $No$ is the population size at the beginning of the time interval and $Nt$ is the population size at the end of the time interval. "$t$" is expressed in days and $\Delta t$ is the length of the time interval ($t_t - to$) (Andersen, 2005).

### 3.2. Dry weight

For biomass concentration, 3 mL samples were filtered through GF/A glass microfiber filters with a particle retention of 1.6 µm (VWR®, European Cat. No. 516-0861), which were previously dried at 80 ºC for 24 h and then weighted on a Mettler AE166 high precision scale.

In the species that were grown in seawater (*D. tertiolecta*, *D. salina*, and *P. cruentum*) the filtered sample was washed with 9 mL of a solution of 0.5 M of ammonium formate (CAS Number 540-69-2) in order to wash out the salts, and then dried again at 80 ºC during 24 h before the final weighting (Zhu and Lee, 1997). Biomass concentration was calculated by weight difference with the filter.

### 3.3. Nitrogen quantification

Presence or absence of nitrogen was monitored, for every species, with MQuant® Nitrate Test strips (Merck® ref. 110020) by a colorimetric method. The lower detection limit of this colorimetric semi-quantitative method is 10 mg $L^{-1}$ $NO_3^-$ (0.16 mM $L^{-1}$). Additionally, for pilot-scale *H. pluvialis* experiments, which required a more accurate quantification of nitrate, commercial nitrate quantification kits were used (Hach Lange® LCK 340 and 339) with a range of detection of 1 to 155 mg $L^{-1}$ of $NO_3^-$ (DIN 38405 D9-2).

### 3.4. Dissolved oxygen

Dissolved oxygen was measured with a Hach® HQ40d multi-meter introducing a pre-calibrated dissolved oxygen probe in the cultures right after a $CO_2$ pulse occurred.

### 3.5. Pigment analyses

#### 3.5.1. Total chlorophyll and carotenoids

For the quantification of total chlorophyll and carotenoids, 1 mL samples were centrifuged at 13000 rpm, supernatant was discarded, and pellets were stored at -20 ºC until analysis. Cells were broken with DMSO (CAS Number 67-68-5) and the extract was adjusted to a ratio of 1: 5 DMSO: acetone. Extractions were done under dimmed light and pellets were extracted until complete discolouration. Extracts absorption was measured by UV-VIS spectrophotometry in 10 mm optical path glass cuvettes. After corroborating that the addition of DMSO did not produce significant changes in the absorption spectra of the extract with respect to the use of 80% acetone in water, total chlorophyll and carotenoids were quantified as described by Lichtenthaler (1987).

#### 3.5.2. Pigment profile characterization in *D. tertiolecta* and *D. salina*

##### 3.5.2.1. Thin-layer chromatography

By the end of the experiments, on day 7, samples were centrifuged, dewatered, and biomass was freeze-dried (Snijders Scientific, model LY-3-TT) in the dark. Then, a pellet of 2 mg was extracted with a mixture of hexane and ethanol (3: 2, v: v) until complete discolouration. Solids were removed by centrifugation followed by extract filtration through a 0.22 μm filter. Extract composition was analysed by silica gel thin-layer chromatography (TLC, Sigma-Aldrich Pcode: 1311P06), loading 40 μL of extract and using a mixture of hexane and acetone (3: 2, v: v) as mobile phase. When a clear separation was achieved, TLCs were removed from the chromatographic chamber and allowed to dry in the dark. When TLCs were dried, each major pigment band was scraped out of the surface of the

TLC with the aid of a scalpel and the solids containing the pigments were resuspended in 1 mL pure acetone and centrifuged at 13000 rpm to remove solids. Then, supernatant was transferred to 10 mm optical path glass cuvettes for spectrophotometric determination (modified from Kobayashi *et al.,* 1991).

3.5.2.2. Hihg-performance liquid chromatography

Extracts were obtained from 15 ± 2 mg of freeze-dried biomass that was dissolved in N, N-Dimethylformamide (DMF, CAS Number 68-12-2). Extracts were incubated in the dark for 12 hours at 4 ºC. Then, solids were removed by centrifugation of the extracts for 10 minutes at 4000 rpm followed by extract filtration through a 0.22 µm filter. The whole process was conducted under low temperature and dimmed light. Extract composition was analysed by high-performance liquid chromatography (HPLC) as described by Lubián & Montero (1998) using a C18 5-µm column (Symmetry C18 of 5-µm 4.16 x 150 mm) and two solutions containing distilled water + 0.05 M Tetrabutylammonium and ammonium acetate 1 M + methanol (1), and acetone + methanol (2) as mobile phase. Elution time was 40 minutes with a flow of 1 mL $min^{-1}$. Chlorophyll and carotenoids including lutein, zeaxanthin, β-carotene, anteraxanthin, neoxanthin, and violaxanthin, were detected by a Photodiode Array Detector at 450 nm and identified and quantified by comparison with commercial standards (DHI LAB Products).

### 3.5.3. Phycoerythrin quantification in *P. cruentum*

For phycoerythrin determination in *P. cruentum*, the method proposed by Bennett and Bogorad (1973) was used. 1 mL samples were centrifuged at 13000 rpm and pellets were stored at -20 ºC until analysis. Pellets were resuspended in phosphate buffer saline (PBS, 0.01M, pH 7.0) and submitted to several freezing/ thawing (-20/ 4 ºC) cycles followed by ultrasonication (BRANSON® digital sonifier 450) for 30 seconds, in ice. Samples were then centrifuged at 13000 rpm in order to recover the supernatant and repeat the extraction of the pellet until a clear supernatant was obtained. Aqueous extracts were transferred to 10 mm optical path plastic cuvettes for spectrophotometric determination.

### 3.6. Exopolysaccharide concentration

Soluble carbohydrate concentration analysis was used to estimate exopolysaccharide synthesis in *P. cruentum*. Soluble carbohydrates were determinated spectrophotemetrically by the phenol-sulfuric acid method using glucose as a standard (DuBois *et al.*, 1956). On day 7, at the end of the experiment, cultures were centrifuged at 5000 rpm for 10 minutes. Pellets were discarded. 1 mL of each supernatant was transferred to a 10 mL glass tube and 4 mL of NaOH 1 N were added to each tube. Then, 1 mL alicuotes were separated and 25 µL of phenol 80 % followed by 2.5 mL of $H_2SO_4$ were added to each sample. When the samples were cooled to room temperature, sample absorbance was determinated spectrophotometrically at 485 nm in 10 mm optical path glass cuvettes. Measurements were compared against a calibration curve made with glucose (D-(+)-Glucose, CAS Number: 50-99-7) in the range of 0-1 g $L^{-1}$ that followed a linear regression.

### 3.7. Chlorophyll fluorescence

For in vivo chlorophyll fluorescence analysis, the PAM fluorometer Aquapen AP-C

100 (Photon Systems Instruments) was used. Measurements were done with a blue excitation light (455 nm). Saturating pulses for fluorescence measurements were set at 3000 μmol photon $m^{-2}$ $s^{-1}$.

For dark-adapted measurements, cultures were maintained without any source of illumination for 30 minutes before the measurement was performed, while samples were maintained under continuous illumination and measured directly as a measure of light-adapted state. Effective PSII quantum yield, $Y_{II}$, was calculated from measurements with light-adapted samples as $Fv'/Fm'$, where $Fv'$ is the variable fluorescence and $Fm'$ is the maximum fluorescence yield. $Fv' = Fm' - Fo'$, where $Fo'$ is the steady-state fluorescence at respective irradiance level. Same measurement in dark-adapted cultures was used to calculate the maximum PSII quantum yield. In this case, $Fo$ would be the fluorescence level when maximum PSII reaction centres are fully oxidized (open), and $Fm$ when they are fully reduced (closed) (Malapascua *et al.*, 2014).

With the data provided by this set of measurements, photochemical ($qP$) and non-photochemical ($qN$) fluorescence quenching can be determined as $qP = (Fm' - Fo)/(Fm' - Fo')$ and $qN = (Fm - Fm')/(Fm - Fo)$ (Sánchez-Saavedra *et al.*, 1996; Richmond and Qiang, 2013; Malapascua *et al.*, 2014; Zhao *et al.*, 2017).

For the rapid fluorescence induction kinetics, an OJIP test was performed with samples that were dark-adapted shortly in order to not loose the photoacclimation state. To allow comparison of transients measured on different samples, a double nomalisation was performed calculating the relative variable fluorescence at time $t$: $Vt = (Ft - Fo)/(Fm - Fo)$ (Malapascua *et al.*, 2014).

For the characterization of the point of maximum photosynthesis ($P_{max}$), photosynthesis saturation point ($Ek$), light limited and light saturating irradiances, photosynthesis-irradiance curves (P-I curves) were performed following a protocol integrated in the PAM fluorometer denominated "Light Curves (LCs)". During a LC, the sample was submitted to different levels of actinic light (10, 20, 50, 100, 300, 500, and 1000 μmol photon $m^{-2}$ $s^{-1}$) for 1 minute per level. During each level, the samples are submitted to different pulses in order to monitor the adaptation level to each intensity. After each level, samples are submitted to a saturating pulse that measures PSII effective quatum yield for each irradiance. From these data, relative electron transport rate ($rETR$) was calculated as $Y_{II}\ x\ E_{PAR}$, where $E_{PAR}$ is the incident irradiance and $Y_{II}$ is the effective PSII quantum yield at the respective irradiance (Malapascua *et al.*, 2014), and photosynthesis-irradiance curves are plotted. After a curve fitting, maximum slope ($\alpha$) was calculated. Then, $Ek$ was calculated as the intersection between $Pmax$ and $\alpha$, $Ek = P_{max}/\alpha$ (Zittelli *et al.*, 2013; Malapascua *et al.*, 2014).

# 4. Light measurements

### 4.1. Irradiance

Every irradiance specified in this work was measured with a Li-Cor LI-250A light meter, pre-calibrated in a darkroom. Submersed measurements were done with a Li-Cor UNDERWATER probe (Serial number UWQ 8595), while non-submersed measurements were done with a Li-Cor QUANTUM probe (Serial number Q 41169)

### 4.2. Light spectrum

Every light source spectrum was measured with a Jaz spectrometer (Ocean Optics®). Data was analyzed with the software OceanView®.

## 5. Statistical analyses

Unless otherwise specified, data were calculated as means of three replicates ± standard deviation (SD). Statistical treatment of data was performed with the software GraphPad Prism 6. Comparisons between treatments were done for days 3 and 7, which stand for the end of logarithmic phase and the end of the experiment with cultures on stationary phase, respectively, by an analysis of variance (one-way ANOVA) with a Tukey correction for multiple comparisons, at a significance level of 0.05.

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# RESULTS AND DISCUSSION

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# 1. OPTIMIZATION OF A TWO-STAGE PROCESS FOR THE PRODUCTION OF ASTAXANTHIN FROM *H. pluvialis* AT LABORATORY-SCALE: LIGHT QUALITY, IRRADIANCE, TEMPERATURE, AND PHOTOSYNTHETIC RESPONSE

## Rationale

Despite the main factors that induce massive carotenogenesis in *H. pluvialis* are already widely described (Droop 1954; Fábregas *et al*., 1998, 2003; Boussiba *et al*., 1999), the success on the industrial cultivation of this species for the production of astaxanthin requires the optimization of every parameter involved in the process to the best extent possible (Olaizola, 2000). In this respect, there is room for improvement when it comes to new technology that is quickly being integrated in microalgae mass production such as LED artificial lightning, which allows the design of very specific light sources adapted for the needs of the process (Ramanna *et al.*, 2017).

The current metodology for the production of *H. pluvialis* involves a two-stage process where green vegetative cells and red aplanospores are produced separately (Olaizola, 2000; Fábregas *et al.*, 2001). The vegetative stage is produced under favourable culture conditions, adjusting culture parameters so that light becomes the limiting growth factor, while the other factors are kept in the optimal range, meaning nutrient saturation, optimum temperature, optimum irradiance, optimum aeration, pH, etcetera. Then, the green culture is normally transferred to a different cultivation unit where the culture conditions change dramatically. Here the main concern is to provide the cells a high irradiance, and ensure carbon bioavailability and a nitrogen-deplete medium. One of the key points to adjust this process successfully is the optimization of the remaining nitrogen concentration in the green culture at the beginning of the induction period, which should be minimized so that astaxanthin accumulation is accelerated, since nitrogen starvation in the presence of high irradiance is the main combination of factors that trigger massive carotenogenesis in *H. pluvialis* (Fábregas *et al.*, 2003). There might be slight variations and strategies to this process depending on the degree of process-control and the technology that is available but, in general, this process-scheme is considered the most efficient method (Aflalo *et al.*, 2007) and every successful industrial cultivation has been built up around this approach.

Apart of nitrogen depletion and illumination, which are considered "primary factors" for massive astaxanthin synthesis, other parameters may have an influence on the rate and the amount of pigment that is accumulated once carotenoid synthesis is triggered. In the

literature, these are referred to as "secondary factors". Addition of organic carbon (mixotrophy), deprivation or addition of a certain element of the culture medium such as magnesium or iron, or temperature modification, are among the most frequently cultivation factors reviewed in the literature (Kobayashi *et al*., 1991, 1993; Tjahjono *et al.,* 1994; Fábregas *et al.,* 1998). High irradiance is considered as a must-have when it comes to the induction of carotenogenesis (Fábregas *et al.,* 1998). In this respect, absolute irradiance is the most studied factor. However, the use of technologies that allow a tailor-made light emission spectrum, such as LEDs, add a new dimension to this parameter, allowing not only the optimization of the absolute amount of photons but also its wavelength composition. Moreover, temperature is known as a determining factor in almost every biological process, and from our point of view, the most promising secondary factor to achieve an enhancement of astaxanthin synthesis.

In this work, the influence of light quality and temperature have been investigated for both green and red stages in a two-stage culture process of *H. pluvialis* at laboratory-scale, in order to define the optimum conditions with the purpose to scale up the process. From the industrial perspective, the output of this investigation would be succesful if an improved two-stage process could be established by optimizing light quality and temperature in both green and red stages, focusing on the enhancement of growth rate in the first one, and astaxanthin productivity in the latter. Moreover, to achieve a better understanding of the process, the effect of absolute irradiance on biomass concentration and carotenoid synthesis has been studied in the red stage. Additionally, the photosynthetic response to different light qualities has been described in *H. pluvialis* cells under inductive conditions in order to characterize the effects that this parameter has on the photosynthetic apparatus.

# Results

## 1. Effect of light quality and temperature on *H. pluvialis* vegetative growth

The effect of blue (450 nm), red (660 nm), and a mix of red and blue light with different red: blue ratios of 1: 4, 1: 1, and 4: 1, with an incident irradiance of 100 μmol photon $m^{-2}$ $s^{-1}$ was studied on vegetative phase *H. pluvialis* cultures maintained in 1 L glass cylindrical photobioreactors (Figure 0.5A; 10 mm diameter) at 20 ºC. Growth, measured as cell density was, in late exponential phase, the highest in the cultures grown under monochromatic red light, and lowest in the cultures grown under monochromatic blue light. Within mixed red and blue wavelengths, no differences in growth rate were observed (Figure 1.1A), presenting intermediate values between those achieved with pure red and pure blue light sources. Significant differences were only observable from day 3 of cultivation. Biomass concentration at late exponential phase followed the same pattern as cell density (Figure 1.1B).

In a second experiment, the effect of different temperatures set at 20, 25, and 30 ºC was studied in cultures grown under pure red light with an irradiance of 100 μmol photon $m^{-2}$ $s^{-1}$. Maximum growth rates were achieved with a cultivation temperature of 20 ºC, although no significant differences were found with respect to 25 ºC ($p > 0.05$). With 30 ºC, maximum growth rate dropped by 20 % with respect to 20 and 25 ºC (Figure 1.2A). Additionally, a

temperature of 30 ºC caused cellular aggregates and loss of cell motility, which was reflected in the quick precipitation of the culture once mixing was stopped (Figure 1.2B). Nitrogen was available in the cultures during the entire cultivation period. However, nitrogen consumption, measured by a colorimetric method, was the highest at 20 ºC, followed by 25 and 30 ºC (data not shown).

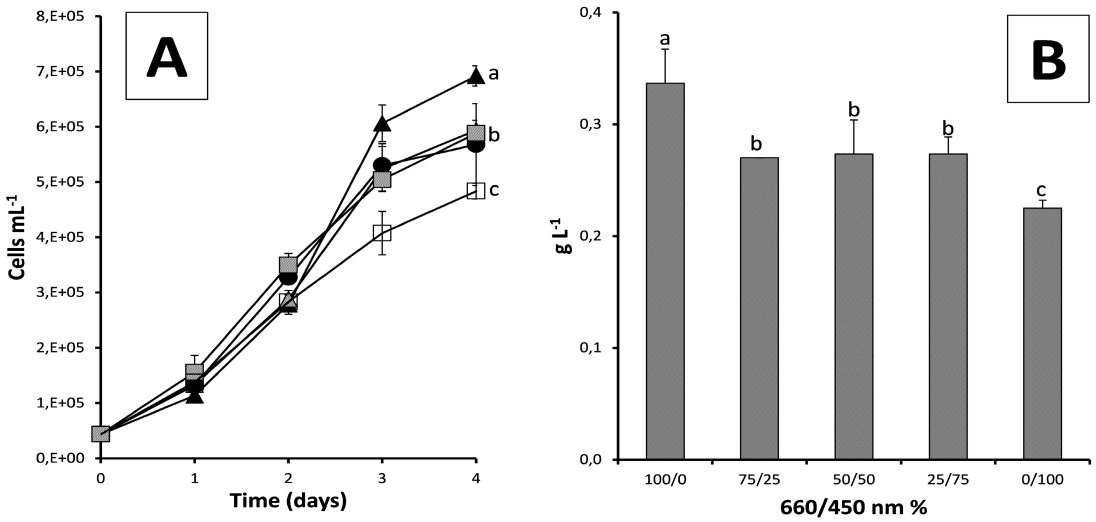



**Figure 1.1 Cell density (cells mL$^{-1}$) (A) and biomass concentration (g L$^{-1}$) on late exponential phase (B) for *H. pluvialis* vegetative phase cultures grown under different light qualities at 100 µmol photon m$^{-2}$ s$^{-1}$: Blue (open squares), red (closed triangles), and different ratios of mix of red and blue (grey squares 1: 4, closed circles 1: 1, closed squares 4: 1). Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments (one-way ANOVA, with Tukey correction, p < 0.05).**

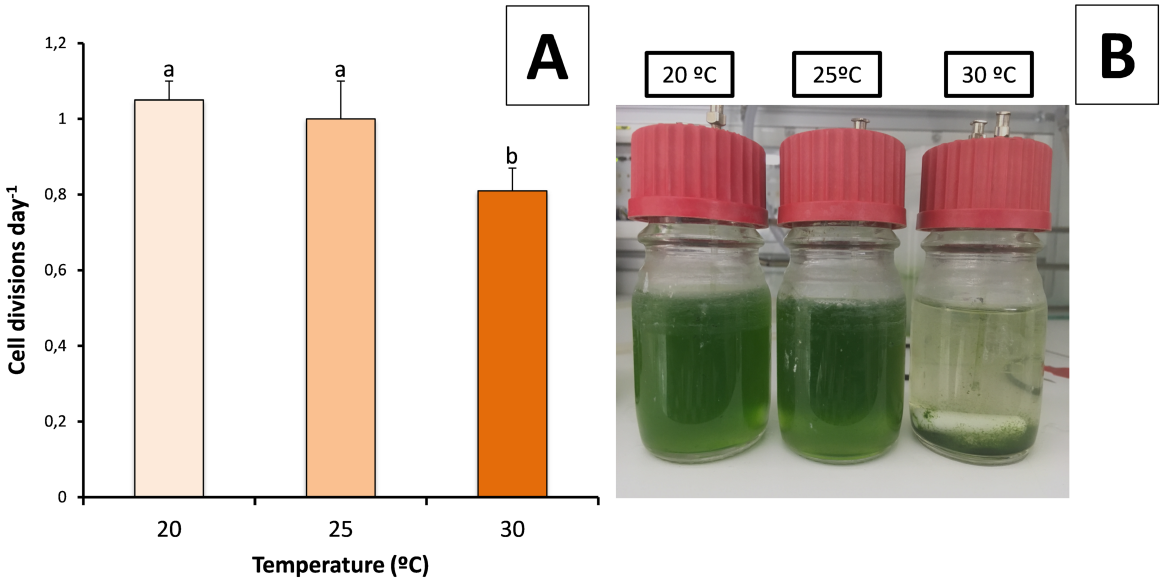



**Figure 1.2. Maximum growth rate (cell divisions day$^{-1}$) (A) of *H. pluvialis* vegetative phase cultures grown for 5 days under 100 µmol photon m$^{-2}$ s$^{-1}$ red light with different temperatures: 20 °C (ligh orange bars), 25 °C (pale orange bars), and 30 °C (dark orange bars). Cellular aggregates and cell motility loss were observed when cultures were maintaned at 30 °C, reflected on the precipitation of the culture once mixing was stopped (B). Vertical bars represent S.D. (*n = 3*). Different superscript letters indicate significant differences among treatments (p < 0.05).**

## 2. Effect of light quality on *H. pluvialis* red stage: biomass concentration and carotenoid accumulation

After having observed differences in vegetative cells growth rate with respect to light quality, the response of biomass concentration and astaxanthin accumulation rate was studied in the process of formation of *H. pluvialis* aplanospores grown under blue (450 nm), red (660 nm), and a mix of red and blue light with a 1: 1 ratio, at an incident irraciance of 300 μmol photon $m^{-2}$ $s^{-1}$ and a temperature of 20 ºC. Exponentially growing pre-cultures, as described in section 2.2 of materials and methods, were used to inoculate the 100 mL photobioreactors in the experimental setup (Figure 0.6). Biomass concentration after 7 days of transference to inductive conditions was slightly higher in cultures maintained with red-light, while cultures maintained with blue-light achieved the lowest biomass concentration. Although differences in biomass concentration were not significant by the end of the experiment ($p > 0.05$), they were evident up to day 5 after the transference to inductive conditions (Figure 1.3A). Opposite to biomass concetration, carotenoid content was maximal in red-blue light, which was about 75 % higher than the content achieved in red-light on day 7 (Figure 1.3B). No astaxanthin accumulation was observed until day 3, which co-occurs with nitrogen depletion in the culture medium of every treatment. The synergistic effect of light quality on growth and pigment accumulation resulted in the highest astaxanthin productivity in the cultures maintained with a mixture of red and blue light (Figure 1.3C). Productivity was calculated by dividing astaxanthin concentration by the days of cultivation after the transference to inductive conditions. Productivity of the cultures maintained in red-blue light was around 30 % and 70 % higher than in blue-light and red-light treatments, respectively. Chlorophyll content dropped drastically with nitrogen depletion by day 3, and then continued decreasing until the end of the experiment, where blue-light cultures maintained the highest content, 9.3 mg $g^{-1}$ with respect to the cultures where red light was present, that finished with 6.4 mg $g^{-1}$ (Figure 1.3D). In consequence, a mix of red and blue wavelengths with a 1: 1 red to blue ratio was chosen as the optimum light quality for future experiments carried out to study the influence of other parameters in carotenogenesis, such as temperature.

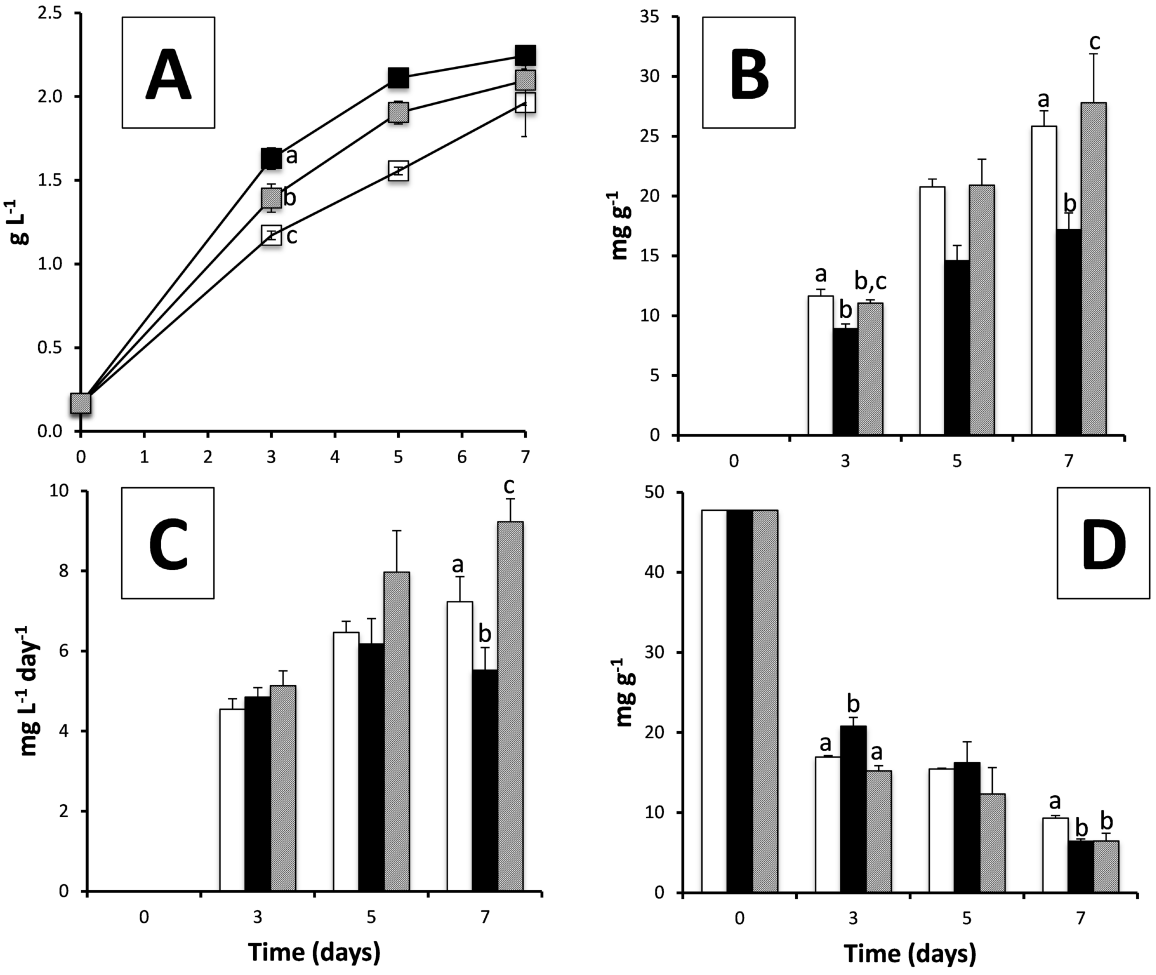



**Figure 1.3. Biomass concentration (g $L^{-1}$) (A), astaxanthin content (mg $g^{-1}$) (B), astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) (C), and chlorophyll content (mg $g^{-1}$) (D) of *H. pluvialis* aplanospores maintained under different light qualities at an irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open symbols/ bars), red (closed symbols/ bars), and mix of red and blue (grey symbols/ bars). Temperature was 20 °C. Nitrogen was completely depleted on all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

## 3. Effect of temperature on *H. pluvialis* red stage: biomass concentration and carotenoid accumulation

A mix of red and blue light with a 1: 1 ratio at an incident irradiance of 300 μmol photon $m^{-2}$ $s^{-1}$ resulted in the highest astaxanthin productivity at 20 ºC, which was around 10 mg $L^{-1}$ $day^{-1}$ (Figure 1.3C). In the next experiment, the effect of different temperatures that ranged between 20 and 40 ºC on biomass concentration and astaxanthin accumulation was studied under those light conditions.

Opposite to the green stage, biomass concentration during the red stage was similar in cultures maintained at 20 and 25 ºC, but was maximal in cultures grown at 30 ºC (Figure 1.4A). 35 ºC inhibited cellular growth. Carotenogenesis was not observed under this condition either, and complete cellular lysis was observed a few days after the transference to this temperature. At 40 ºC cultivation was not viable since cellular lysis occurred within a few hours after inoculation (data not shown). Temperature produced clear differences in astaxanthin accumulation among treatments, which were only observable from day 3, when no nitrogen could be detected in the culture medium of cultures maintained at 20, 25, and 30 ºC (data not shown). Within this temperature range, a direct correlation between cultivation temperature and astaxanthin accumulation could be observed. A temperature of 30 ºC produced the highest increase in pigment content, reaching around 55 mg $g^{-1}$, being approximately double than with the treatment of 20 ºC (Figure 1.4B). Since biomass concetration was also maximal at 30 ºC, greatest differences were observed in pigment productivity, where the use of this cultivation temperature enhanced astaxanthin productivity two-fold and three-fold compared to 25 and 20 ºC, respectively, from day 5 of the experiment (Figure 1.4C). Chlorophyll content decreased from 50 mg $g^{-1}$ on day 0 to 5 mg $g^{-1}$ by the end of the experiment. Although the difference was not large, there seemed to be an inverse correlation between temperature, and therefore astaxanthin synthesis, with chlorophyll content (Figure 1.4 D). Therefore, 30 ºC was selected as the optimum temperature for massive carotenoid synthesis.

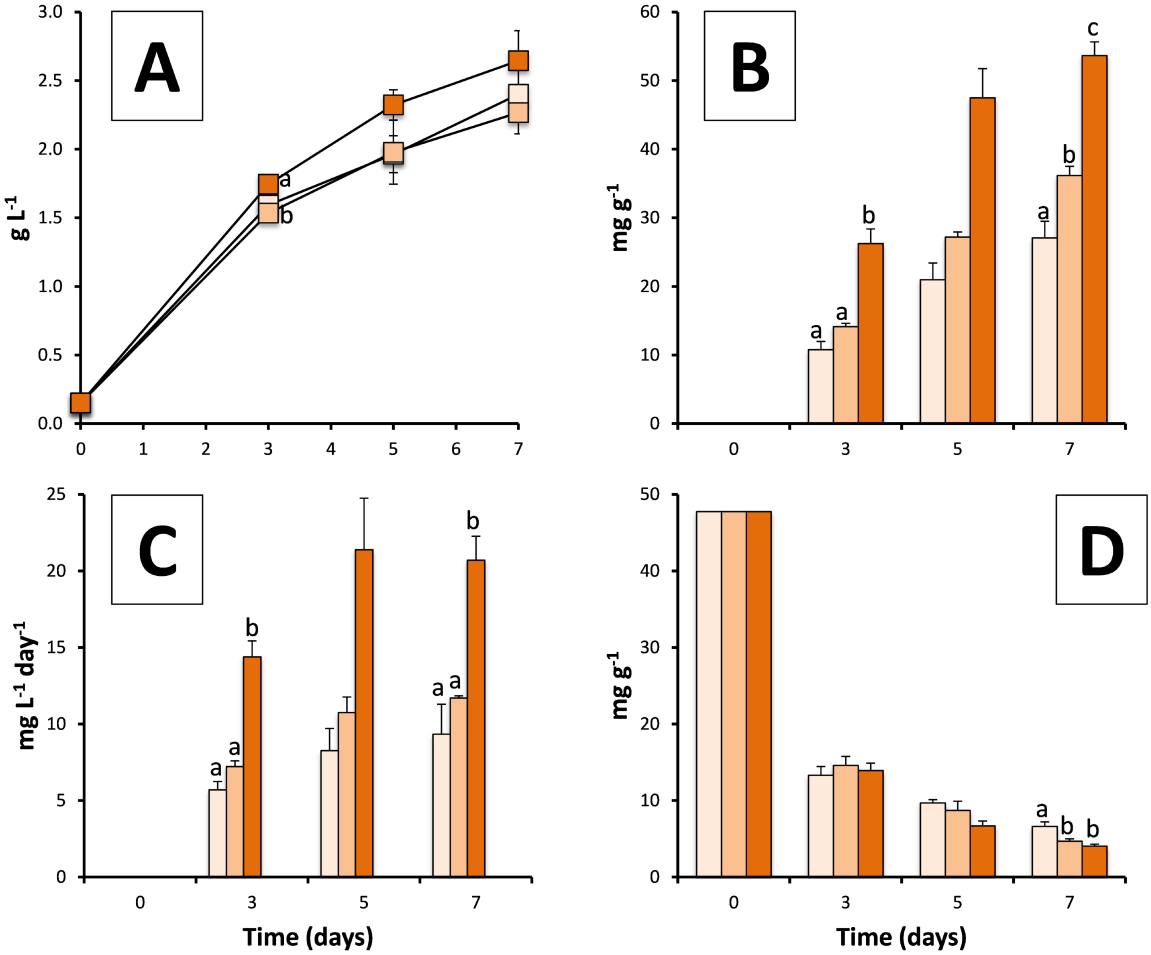



**Figure 1.4. Biomass concentration (g $L^{-1}$) (A), astaxanthin content (mg $g^{-1}$) (B), astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) (C), and chlorophyll content (mg $g^{-1}$) (D) of *H. pluvialis* aplanospores grown with an incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$ of a mix of red and blue light with a 1: 1 ratio, under different temperatures: 20 °C (light orange), 25 °C (pale orange), 30 °C (dark orange). 35 and 40 °C are not represented. Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

## 4. Effect of light quality on *H. pluvialis* red stage under optimum temperature: biomass concentration, pigment content, and photosynthetic response

Previous experiments allowed to select the optimum temperature for massive astaxanthin accumulation was at 30 ºC, using a mix of red and blue light, which was previously described as the most productive light quality at 20 ºC compared to monochromatic red and blue lights (Figure 1.3 and 1.4). In order to validate that the combined effect of optimum temperature and optimum light quality would maintain its efficiency over other combinations of optimum temperature and different light qualities, an experiment that compared cultures maintained at 30 ºC with blue (450 nm), red (660 nm), and the mix of red and blue with an incident irradiance of 300 μmol photon $m^{-2}$ $s^{-1}$ was set. Additionally, the photosynthetic response of *H. pluvialis* to these conditions was monitored to characterize the mechanisms that opperate during carotenogenesis in this species. As expected, the results have shown that at a temperature of 30 ºC, the different wavelenghts produced similar relative differences among treatments as the ones that were observed previously with 20 ºC (Figure 1.5), where astaxanthin productivity was maximal with red-blue and minimum with red-light. This result was considered as a confirmation that a mix of red and blue light was optimal to obtain maximal astaxanthin content and productivities (Figure 1.5B and 1.5C). Moreover, at the higher temperature the effect that light quality had on every monitored value was larger than with 20 ºC. Biomass concentration was, by the end of the cultivation period, around 0.5 to 1 g $L^{-1}$ higher at 30 ºC compared to 20 ºC (Figure 1.3A and 1.5A). Astaxanthin content increased from maximum values of around 30 mg $g^{-1}$ in the lower temperature to maximum values of around 50 mg $g^{-1}$ at 30 ºC (Figure 1.3B and 1.5B). As a consequence, astaxanthin productivities doubled at 30 ºC with respect to 20 ºC (Figure 1.3C and 1.5C). Moreover, the absolute chlorophyll content for every treatment was lower with the higher temperature and differences between treatments were larger (Fig. 1.3D and 1.5D). Blue-light cultures maintained the highest chlorophyll content, while a reduction to values around 1 mg $g^{-1}$ was measured in red-light cultures (Fig. 1.5D).

Additionally, the photosynthetic response was studied to characterize the response of *H. pluvialis* to different light wavelengths under optimized inductive conditions. In this respect, total chlorophyll concentration decreased in the treatments that contained red light from day 3. Interestingly, an increase of chlorophyll concentration was measured from day 3 to 5 in blue-light cultures, a period in which nitrogen was already absent from the culture medium (Figure 1.6A). After day 5, a decrease in chlorophyll concentration was also observed in blue-light cultures. Overall, chlorophyll concentration was highest in blue-light, followed by red-blue and red-light cultures, which showed the lowest chlorophyll concentration among the treatments (Figure 1.6A). After 7 days under induction conditions all the cultures lost completely the green colour that characterizes vegetative cells and developed a strong red color due to the activation of secondary metabolism that triggered high carotenoid accumulation. However, the difference in chlorophyll content made an impact in the physical appearance of the cultures since lighter and orange-coloured tones were observed in red-light cultures compared to blue-light and red-blue light cultures, where the colour was darker and with brownish tones (Figure 1.6B).

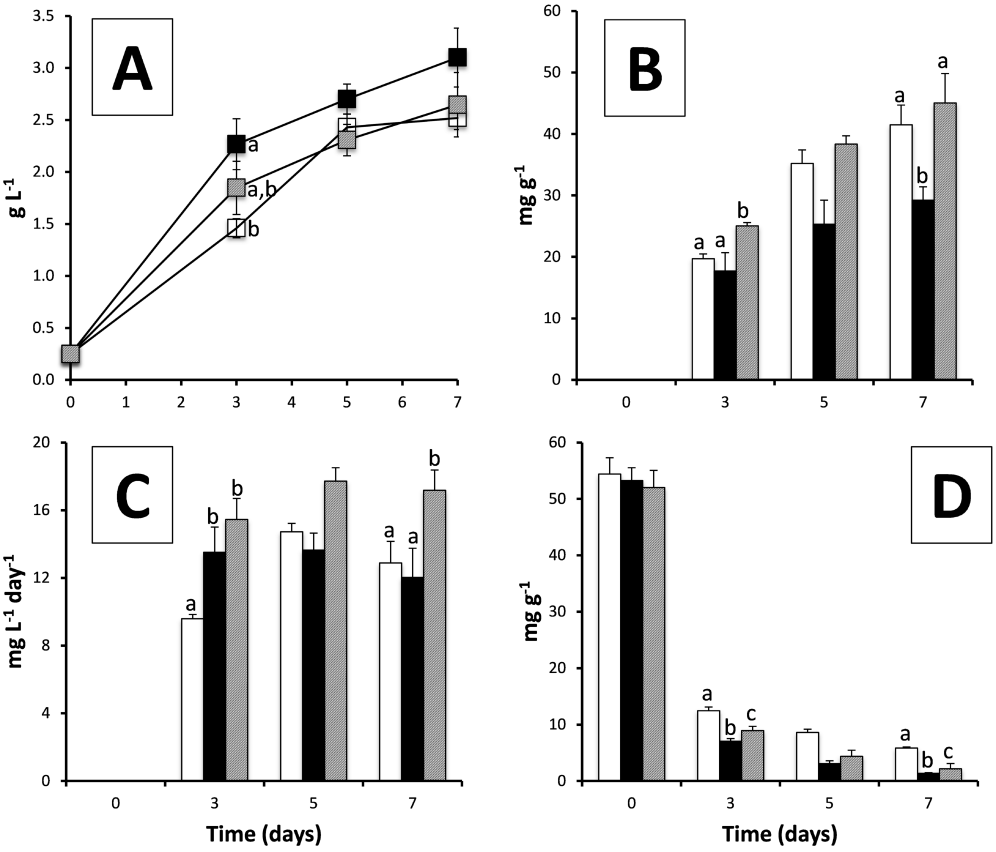



**Figure 1.5. Biomass concentration (g $L^{-1}$) (A), astaxanthin content (mg $g^{-1}$) (B), astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) (C), and chlorophyll content (mg $g^{-1}$) (D) of *H. pluvialis* aplanospores grown under different light qualities of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open symbols), red (closed symbols), and mix of red and blue (grey symbols). Temperature was 30 °C. Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

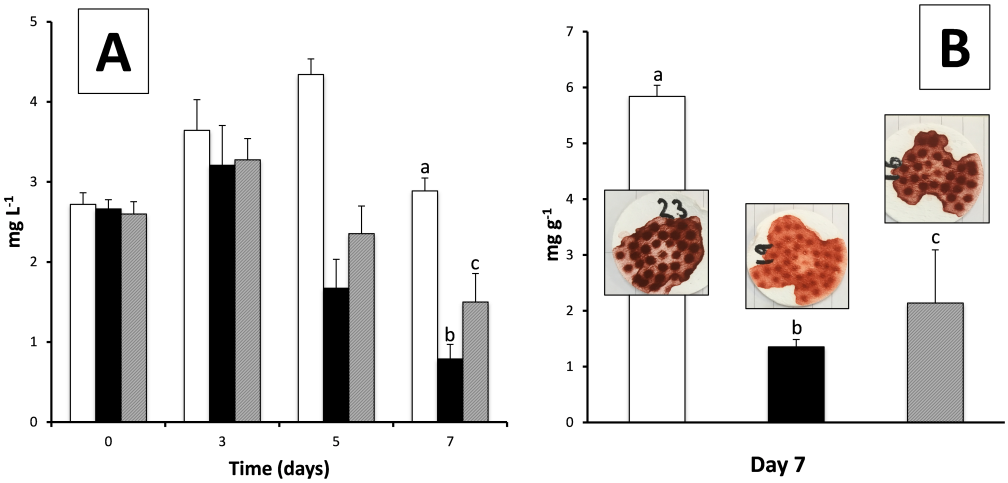



**Figure 1.6. Chlorophyll concentration (mg $L^{-1}$) (A) and final chlorophyll content (mg $g^{-1}$) and physical appearance on day 7 (B) of *H. pluvialis* aplanospores grown under different light qualities at an incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was maintained at 30 °C. Nitrogen was absent in all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

With respect to photosynthetic efficiency, measured as effective PSII quantum yield, blue-light treatment maintained the highest values throughout the experiment that were stable around 0.6. On the contrary, red-blue and red-light treatments showed a decreasing trend throughout the experiment, although in the cultures maintained under red-light the reduction rate was higher than in the mixed light treatment, where quatum yield stabilized around 0.5 from day 5 of the experiment (Figure 1.7).

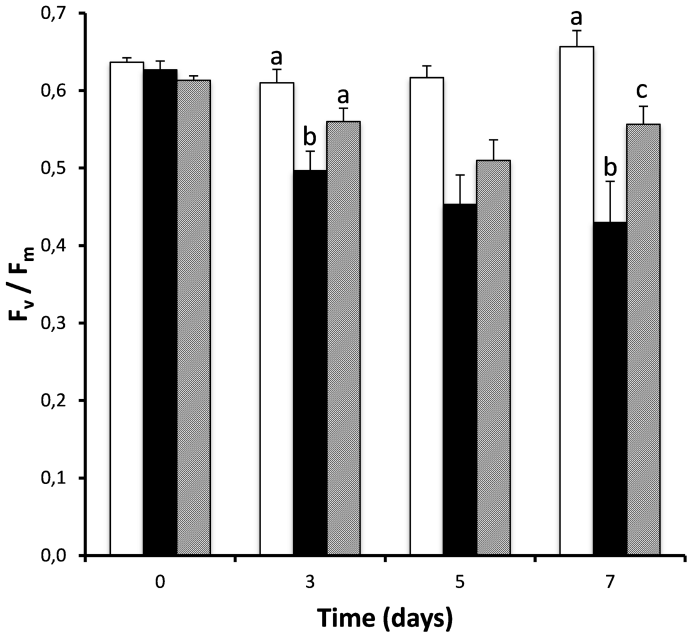



**Figure 1.7. Photosynthetic efficiency, measured as effective quantum yield, of *H. pluvialis* aplanospores grown under different light qualities at an incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open symbols), red (closed symbols), and mix of red and blue (grey symbols). Temperature was maintained at 30 °C. Nitrogen was absent in all treatments by day 3. Vertical bars represent S.D. (*n* = *3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

Photochemical ($qP$) and non-photochemical ($qN$) fluorescence quenching showed opposite results (Figure 1.8). $qP$ showed a continuous decreasing trend with time for cultures maintained in blue-light, while cultures maintained in red-light and red-blue suffered a sudden drop on day 3 with respect to the start of the experiment, and remained stable thereafter (Figure 1.8A). Opposite to $qP$, $qN$ showed an increase with time for every treatment. However, while $qN$ in blue-light cultures raised gradually, a sharp increase was measured at day 3 in the treatments where red light was present, which then decreased slightly towards the end of the experiment (Figure 1.8B).

Dissolved oxygen concentration decreased in every treatment from values close to 20 mg $L^{-1}$ on day 0, when cells were still on vegetative growth mode, to values around 10 mg $L^{-1}$ when cells were fully induced by the end of the experiment (Figure 1.9). The sharpest drop was measured between day 0 and 3, when no nitrogen could be detected in the culture medium of any treatment. Then, the cultures showed a more moderate and constant decreasing trend until the end of the experiment, where differences were not significant. No correlation is found between chlorophyll concentration and dissolved oxygen concentration (Figures 1.6 and 1.9).

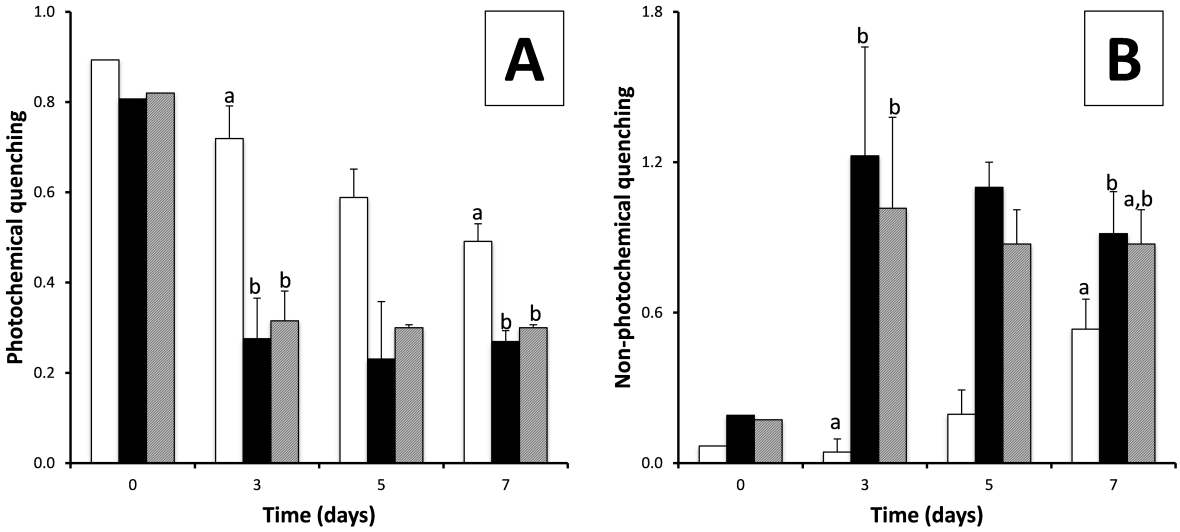



**Figure 1.8.** Photochemical $(qP)$ (A) and non-photochemical $(qN)$ (B) fluorescence quenching of *H. pluvialis* aplanospores grown under different light qualities at a irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was maintained at 30 °C. Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).

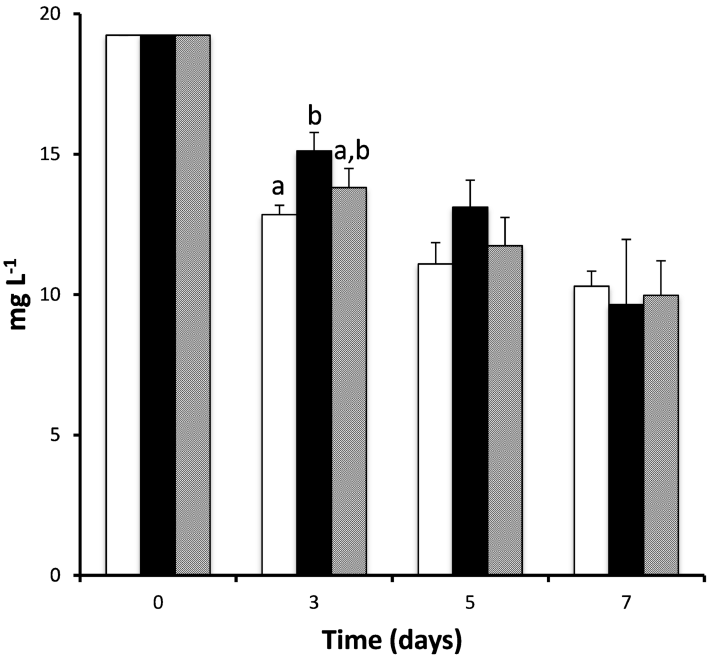



**Figure 1.9.** Dissolved oxygen concentration (mg $L^{-1}$) of *H. pluvialis* aplanospores grown under different light qualities at an incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was maintained at 30 °C. Nitrogen was absent in all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).

## 5. Effect of blue light wavelengths on *H. pluvialis* aplanospores: biomass concentration, pigment content, and photosynthetic response

Preceding experiments evidenced that the ideal conditions to maximize astaxanthin productivity in *H. pluvialis* were the use of a mix of red and blue wavelengths together with a temperature of 30 ºC. It was hypothesized that this response resulted from the combined effect that both colours, isolated, had on the physiology of the algae. Red wavelengths would enhance carbon fixation resulting in higher biomass concentrations than blue wavelengths, which in turn would enhance carotenogenesis resulting in biomass with higher astaxanthin content. Narrowband LED light sources allow the study of the effect that isolated wavelengths have on microalgae physiology. Therefore, it was thought that the study of combinations of red light (660 nm) with different blue wavelegths (420, 450, and 470 nm) might have potential for improving astaxanthin accumulation since it could produce different responses.

The results showed a direct correlation between wavelength energy and biomass concentration. The use of the most energetic 420 nm blue LED, in combination with red light, resulted in the highest biomass concentration, followed by 450 and 470 nm (Figure 1.10A). Regarding astaxanthin content, 450 nm seems to have a slightly higher effect compared with the other two blue light sources, which showed similar results, despite after day 5 differences were not statistically significant (Figure 1.10B). Productivity was the highest in either 420 or 450 nm (data not shown).

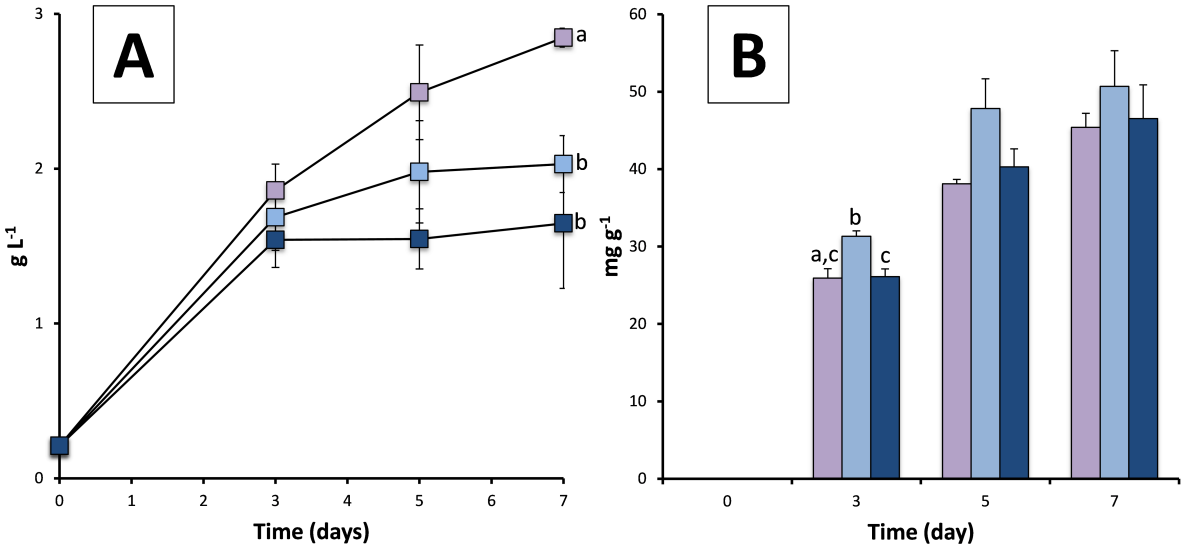



**Figure 1.10. Biomass concentration (g $L^{-1}$) (A) and astaxanthin content (mg $g^{-1}$) (B) of *H. pluvialis* aplanospores grown under a mix of red (660 nm) and different blue wavelengths with a 1: 1 ratio and an incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: 420 nm (purple symbols), 450 nm (light blue symbols), and 470 nm (dark blue symbols). Temperature was maintained at 30 ºC. Nitrogen was absent for all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

Photosynthetic efficiency did not show significant differences among treatments, resulting in cultures that maintained a high PSII quantum yield, with values around 0.6, throughout the experiment (data not shown). As already recorded in foregoing experiments (Figure 1.8A), qP

showed a sharp drop for every wavelength combination on day 3 (Figure 1.11A). After day 3 differences were observed between treatments, which were significant by the end of the experiment ($p < 0.05$). It appeared to be an inverse correlation between wavelength energy and $qP$ after day 3. On the other hand, $qN$ showed an increasing trend for every treatment until day 5 that stabilized thereafter. Contrary to previous experiments carried out with a mix of red and blue light (Figure 1.8B), a sharp increase in $qN$ from day 3 of cultivation was not observed. Instead, a gradual increase with a direct correlation to wavelength's energy was measured (Figure 1.11B). This effect was more similar to that observed earlier in monochromatic blue (Figure 1.8B).

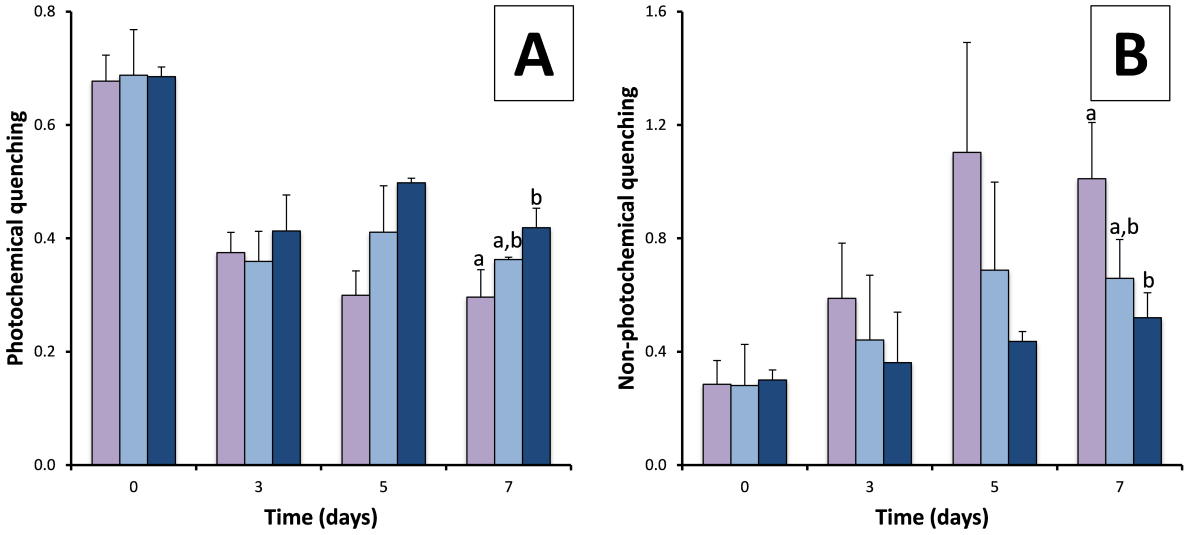



**Figure 1.11. Photochemical ($qP$) (A) and non-photochemical ($qN$) (B) fluorescence quenching of *H. pluvialis* aplanospores grown under a mix of red (660 nm) and different blue wavelengths with a 1: 1 ratio and an incedent irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: 420 nm (purple symbols), 450 nm (light blue symbols), and 470 nm (dark blue symbols). Temperature was maintained at 30 °C. Nitrogen was absent in all treatments by day 3. Vertical bars represent S.D. ($n = 3$). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

## 6. Effect of absolute irradiance of optimized wavelength composition on *H. pluvialis* red stage: biomass concentration, photosynthetic efficiency and carotenogenesis

In the previous experiments, optimum light quality and temperature for maximum carotenogenesis were established in *H. pluvialis* cultures under inductive conditions. Cultures grown with a mix of red and blue light maintaned at a temperature of 30 ºC resulted in pigment productivities up to 20-25 mg $L^{-1}$ $day^{-1}$ (Figures 1.4D and 1.5D). It was assumed that high irradiance was a must have when it comes to fast astaxanthin accumulation in *H. pluvialis* red stage, therefore all the previous experiments were done with an incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$. However, a study of the effect that irradiance had on biomass concentration and carotenogenesis was missing. Since the ultimate purpose of these experiments was the industrial scale up of an optimized two-stage culture process, a superior understanding on how this variable affected cultivation was required. Green cultures were submitted, in parallel, to a light quality of red (660 nm) and blue (450 nm) wavelengths with a 1: 1 ratio at different incident irradiances that ranged from 50 to 300 µmol photon $m^{-2}$ $s^{-1}$. Temperature was maintained at 30 ºC.

Biomass concentration increased in every treatment and showed a direct correlation with irradiance up to 200 µmol photon $m^{-2}$ $s^{-1}$, where maximum biomass concetration was reached (Figure 1.12A). Higher irradiances produced slightly lower biomass concentrations from day 5 of the cultivation period. This pattern was not observed when astaxanthin content was studied, which appeared to continue increasing with irradiance (Figure 1.12B), as well as for chlorophyll content where the content decreased with irradiance (Figure 1.12C). The combination of both effects resulted in the saturation of pigment productivity from an incident irradiance of 200 µmol photon $m^{-2}$ $s^{-1}$ on day 7 with values around 20 mg $L^{-1}$ $day^{-1}$. Complete nitrogen depletion was achieved in all treatments by day 3 except with 50 µmol photon $m^{-2}$ $s^{-1}$, where carotenoid content did not increased with respect to day 0 and the cells remained green throughout the experiment.

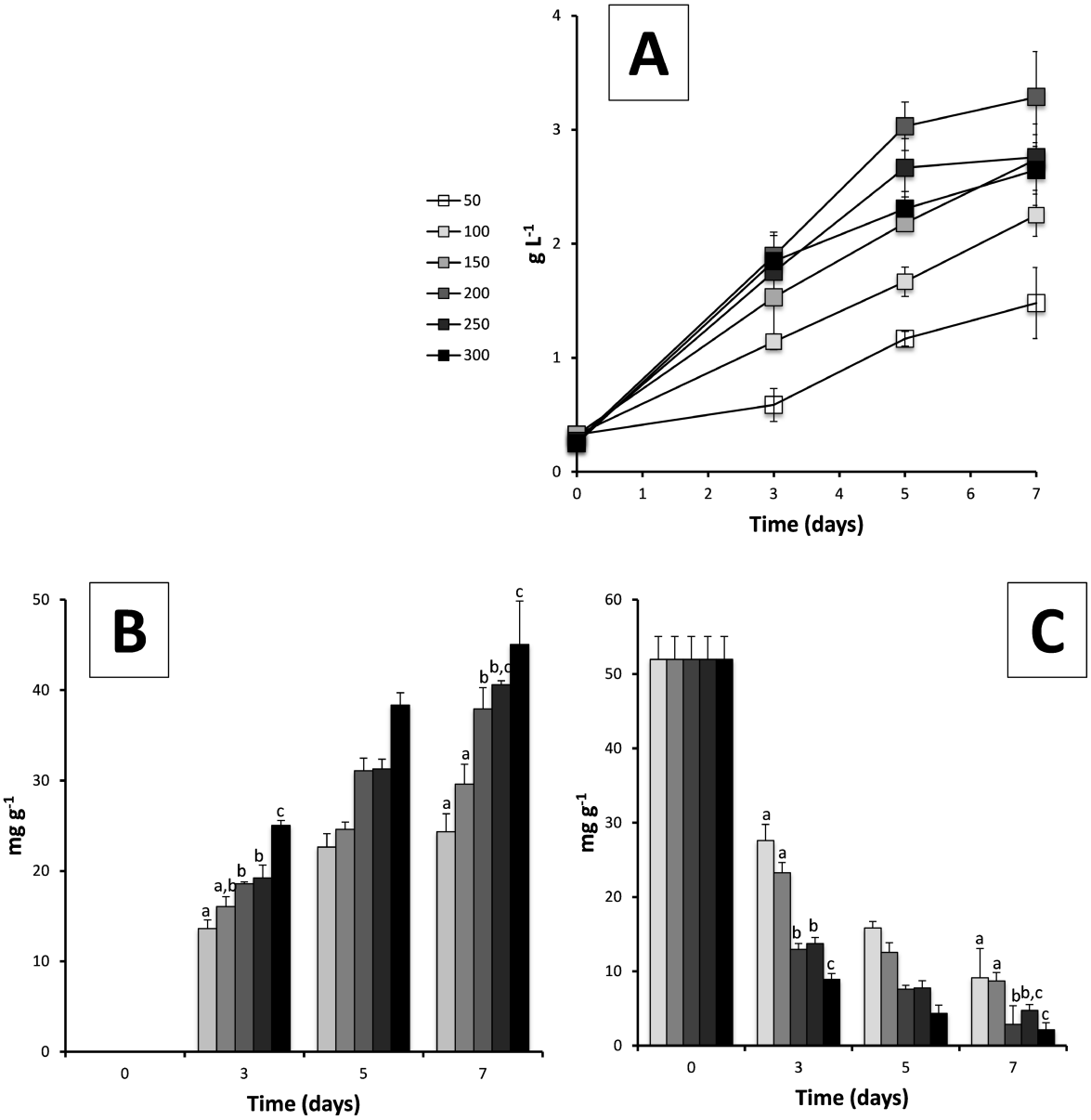



**Figure 1.12. Biomass concentration (g $L^{-1}$) (A) astaxanthin content (mg $g^{-1}$) (B) and chlorophyll content (mg $g^{-1}$) (C) of *H. pluvialis* aplanospores grown under a mix of red and blue light with a 1: 1 ratio and an incident irradiance ranging from 50 to 300 µmol photon $m^{-2}$ $s^{-1}$. Temperature was 30 ºC. Nitrogen was absent for all treatments by day 3 except for 50 µmol photon $m^{-2}$ $s^{-1}$, where nitrogen could be detected in the culture medium until the end of the experiment. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

As for photosynthetic efficiency, PSII quantum yield showed an inverse correlation to incident irradiance (Figure 1.13). The drop of $Fv\ /\ Fm$ was barely noticeable with the lowest irradiance tested, 50 µmol photon $m^{-2}$ $s^{-1}$, while it was more pronounced the higher the irradiance and the longer the time exposed to inductive conditions.

Despite dissolve oxygen showed a decreasing trend throughout the experiment, differences between treatments were observed (Figure 1.14). Dissolved oxygen concentration showed a stable value around 12 mg $L^{-1}$ in cultures maintained at 50 µmol photon $m^{-2}$ $s^{-1}$, which remained green and nitrogen-saturated during the entire cultivation period. On the contrary, cultures maintained at higher irradiances showed a clear reduction of dissolved oxygen concentrations from day 0 to the end of the induction period. Moreover, it is interesting to analyze the values measured on day 0, when cells were still actively growing on vegetative mode in every treatment, since nitrogen was still available. In this scenario, dissolved oxygen production increased with irradiance until a plateau was reached with 200 µmol photon $m^{-2}$ $s^{-1}$ indicating photosynthesis saturation at that light intensity (Figure 1.14), which correlates with the increase observed in biomass concentration between days 0 and 3 (Figure 1.13A).

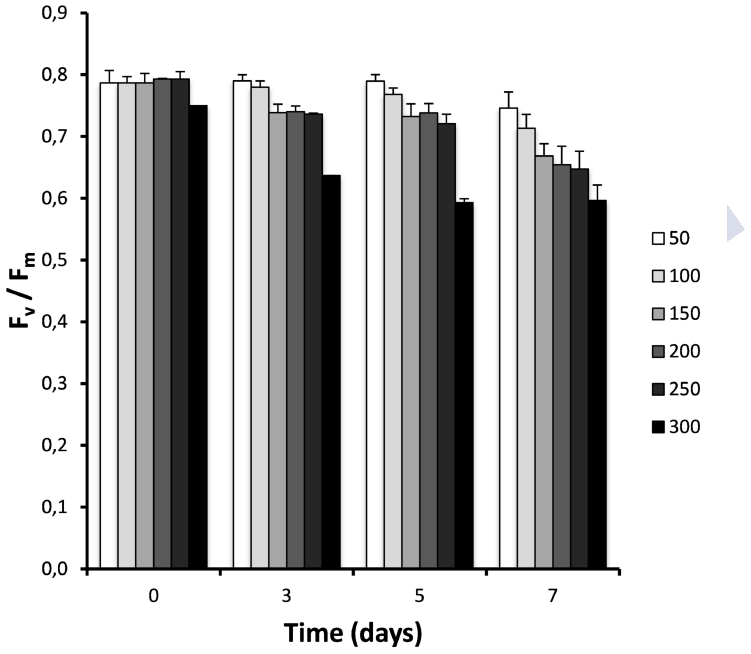



**Figure 1.13. Photosynthetic efficiency, measured as quantum yield, of *H. pluvialis* aplanospores grown under a mix of red and blue light with a 1: 1 ratio and an incident irradiance ranging from 50 to 300 µmol photon $m^{-2}$ $s^{-1}$. Temperature was 30 ºC. Nitrogen was absent for all treatments by day 3 except for 50 µmol photon $m^{-2}$ $s^{-1}$, where nitrogen could be detected in the culture medium until the end of the experiment. Vertical bars represent S.D. (*n = 3*).**

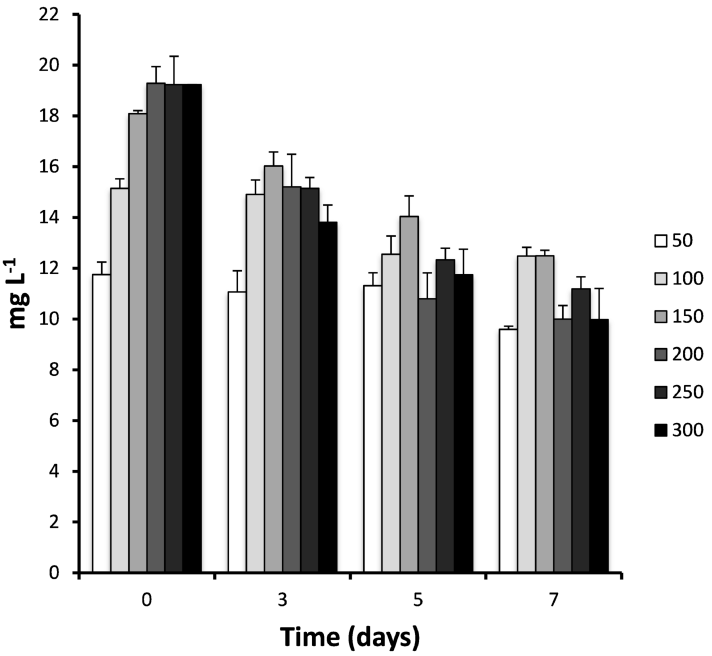



**Figure 1.14. Dissolved oxygen concentration (mg $L^{-1}$) of *H. pluvialis* aplanospores grown under a mix of red and blue light with a 1: 1 ratio and an incident irradiance ranging from 50 to 300 µmol photon $m^{-2}$ $s^{-1}$. Temperature was maintained at 30 °C. Nitrogen was absent for all treatments in day 3 except for 50 µmol photon $m^{-2}$ $s^{-1}$, where nitrogen could be detected in the culture medium until the end of the experiment. Vertical bars represent S.D. (*n* = 3).**

## Discussion

The optimization of microbial mass cultivation is a complex field (Olaizola, 2003; Borowitzka and Vonshak, 2017). The interaction between crucial cultivation factors such as temperature, irradiance or aeration rate, among others, makes it challenging when it comes to designing an experimental setup that is able to minimize this interplay. Moreover, if the aim of such investigation is the industrial upscale of that process, outcome must be interpreted carefully, since part of the results will be specific to the experimental setup where the research has been carried out. Nevertheless, lab-scale experimentation provide usefull information about the response of a certain microbe to a production parameter, even though it may not be directly scalable to a larger cultivation unit. This circumstances are specially evident with photosynthetic organisms due to the inevitable use of light, which can be considered as an "essential nutrient", which adds an extra layer of cultivation complexity compared to heterotrophs, due to the creation of steep gradients within a dense microalgal culture and its physical properties, that interact with every element of the experimental setup: from reactor design, including optical path and homogenity of light distribution, to reflection and refraction in the photobioreactor walls. Harker et al. (1996) described the difficulty of reproducing laboratory-scale results at a larger scale with *H. pluvialis*. They could achieve carotenoid contents higher than 5.5 % of dry weight at small-scale, but only half of that value was obtained when they tried to reproduce those results in 30 L photobioreactors, due to the impossibility to maintain the same irradiance and distribution as in the laboratory-scale cultivation units.

Some parameters are directly scalable from the lab bench. These include parameters which application to industrial cultivation is more simple and are more independent from photobioreactor's size or shape than irradiance and light distribution. This implies that it is easier to mimic small-scale conditions at a larger scale. Light quality or temperature comply with these characteristics, and their scalability depends mainly on the engineering capability of building that specific light source or providing a solution to maintain the desired temperature constant. The technologies requires to do this are common in other fields such as horticulture or fermentation, and therefore already developed and proven. These technologies are less subjected to the innovation and lack of standardization common in photobioreactor design where, despite some designs like raceways, columns, tubular or flat-plate photobioreactors have proven their success and the industry slowly embraces them as the standard cultivation units, every company still tries to develop their one-of-a-kind photobioreactor design (Richmond and Qiang, 2013).

The elements related to the scale up of microalgal cultures will be discussed thoroughly in other chapters of this work, but it is important to start taking them into consideration when it comes to the interpretation of the results obtained during laboratory-scale experimentation.

Our results confirmed that, under the conditions that were tested during this work, no astaxanthin accumulation occured until nitrogen was depleted of the culture medium. The same was observed and described by Fábregas et al. (2001). Therefore, during the experiments that were described in this chapter, nitrogen depletion acted as the stressor that triggered the activation of secondary metabolism and, as a response, massive carotenoid accumulation occurs (Borowitzka, 2018b). Only under this circumstance the cultures were in strict carotenogenic conditions. Other authors have observed minor astaxanthin synthesis in nitrogen-repleted cultures when these were exposed to continuous illumination (Domínguez *et al.*, 2019). Despite the pre-cultures, maintained under circadian light regime, were submitted to continuous illumination in the experimental setup, carotenoid accumulation was only obvious once nitrogen was absent of the culture medium. Once the cultures reached this stage the results showed that light quality, irradiance, and temperature are key factors that must be taken into account when it comes to the optimization of a two-stage *H. pluvialis* cultivation process.

Light quality had an effect on both vegetative cells growth (Figure 1.1) and aplanospores weight and pigment content (Figure 1.3). Green vegetative cells showed the fastest cell division rate when illuminated with red-light, while the growth rate was minimum in the cultures maintained under blue-light (Figure 1.1A). This is in line with recent results that found that, in *H. pluvialis*, red light produced higher cell density and biomass concentration than white light (Zhu *et al.*, 2018) or blue light (Xi *et al.*, 2016). This is consistent with the action spectra and photosynthetic apparatus of green algae, that contain chlorophyll as the major photosynthetic pigment and therefore should be able to absorb and use warm light efficiently. An up-regulation of cell-division related genes under red light has also been described in *H. pluvialis* (Lee *et al.*, 2018)**.** Interestingly, differences in cell density were not significant between cultures that were grown under a combination of red and blue light in different proportions (Figure 1.1A). Biomass concentration followed the same pattern as cell density, proving that in green-stage *H. pluvialis* cultures, both parameters are affected equally by light wavelength (Figure 1.1B). Aplanospores show the same pattern of respose to light wavelength than green cells regarding biomass concentration at 20 ºC (Figure 1.3A. Despite

differences by day 7 are minimal, at this temperature maximum dry weights were achieved in red-light followed by red-blue and blue-light. At a higher temperature of 30 ºC no differences could be measured between blue-light and red-blue light cultures (Figure 1.5A). Dissolved oxygen measurements support this result showing higher concentrations in red-light throughout the experiment, which could be explained by a higher photosynthetic rate and a higher carbon fixation (Figure 1.9). As a result, more oxygen is produced as a by-product of photosynthesis, which is then released and dissolved in the culture medium.

Carotenoid content was heavily affected by light quality. It was enhanced in cultures grown under a light source that contained blue light, being maximal under red-blue light on day 7 regardless of the temperature, since content was at least 50 % higher than the content measured in red-light cultures at both 20 and 30 ºC (Figure 1.3B and 1.5B). Similar results were obtained in phototrophic and mixotrophic cultures of *H. pluvialis* Flotow where pigment accumulation was maximal under blue light and a combination of red and blue light (Kobayashi *et al.* 1992; Xi *et al.* 2016). However, these studies only obtained poor astaxanthin concentrations not higher than 20 mg $L^{-1}$. It is essential to emphasize that the use of light quality alone will not enhance carotenoid accumulation dramatically if it is not used on top of more critical inductive conditions such as the absence of nitrogen, carbon availability and high irradiance. This topic has been reviewed (Aflalo *et al.*, 2007) comparing the most popular "stress conditions" used in the literature showing clear differences between the different methods, being the combination of high irradiance and nitrate deprivation the optimum condition that yielded higher carotenoid content and productivity, together with the highest astaxanthin fraction among other carotenoids that was around 95 %. Other works showed molecular data on carotenogenesis-related genes that were up-regulated by either red or blue light, but blue light producing a higher astaxanthin concentration than red (Steinbrenner and Linden, 2003). More recently, a clear up-regulation of astaxanthin-synthesis-related genes and ROS-related genes was recorded under blue light cultivation compared to a control of white light and cultures grown in red light (Lee *et al.*, 2018).

Due to the synergistic effect of red-blue wavelengths, that resulted in a balanced increase in biomass concentration and carotenoid content, carotenoid productivity under these conditions was the highest (Figure 1.3C and 1.5C). Interesting differences among cultures that were illuminated with a combination of red light (660 nm) and 420, 450, or 470 nm blue light were observed (Figure 1.10 and 1.11). Apparently, the choice of the blue wavelength clearly affected biomass concentration showing a direct correlation between wavelength's energy and culture's dry weight (Figure 1.10A). Katsuda et al. (2004) observed similar differences between two light sources that peaked at 420 and 470 nm (Katsuda *et al.*, 2004), although the astaxanthin concentrations reported in their study are low, with maximal values of 40 mg $L^{-1}$ of astaxanthin compared to the 120 to 150 mg $L^{-1}$ that were achieved during the present investigation. In this work, carotenoid content was maximal with the combination of red light with 450 nm blue light (Figure 1.10B). Despite not comparing specifically the same wavelengths and reporting very low pigment concentrations of 1 mg $L^{-1}$, Park and Lee (2001) found the highest astaxanthin concentration in *H. pluvialis* cultures illuminated with fluorescent light supplemented with 470 nm compared to fluorescent light alone or supplemented with 680 nm red light.

No differences could be observed in PSII effective quatum yield nor dissolved oxygen concentration between treatments. However, there seems to be a direct correlation between

energy dissipation, reflected in qN, with wavelength's energy (Figure 1.11B), which may be explained by the *in vivo* astaxanthin's absorption at those wavelengths, which is around two-fold for 470 nm with respect to 420 nm (Lu *et al.*, 2017).

Chlorophyll content and concentration during induction phase were clearly affected by light quality (Figures 1.3D, 1.5D, and 1.6). Chlorophyll content dropped with the onset of stationary phase and therefore with nitrogen starvation, and continued decreasing with time in both 20 and 30 ºC for every wavelength (Figures 1.3D and 1.5D). In this sense, chlorophyll content was inversely correlated with astaxanthin content. Moreover, at 30 ºC, differences were larger between treatments by the end of the experiment, and blue light maintained the highest chlorophyll content, around five times higher than that observed in cultures maintained under red light (Figure 1.5D). Chlorophyll concentration increased equally in every treatment between day 0 and day 3. Thereafter, coinciding with nitrogen depletion, differences were significant among treatments. Surprisingly, chlorophyll concentration in the blue-light treatment increased until day 5, after complete depletion of nitrogen, and only afterwards a drop could be measured (Figure 1.6A). Decrease of either chlorophyll content or concentration are described by some authors for *H. pluvialis* in conditions that stimulate astaxanthin synthesis (Kobayashi *et al.* 1992; Harker *et al.* 1996; Grünewald *et al.* 1997;), despite other authors did not observe this phenomenon (Zlotnik *et al.* 1993; Fábregas *et al.* 2001). Anyway, blue-light treatment maintained the highest chlorophyll content and concentration followed by red-blue and red-light and the difference influenced the physical appearance of the cultures. Despite every treatment developed an intense red colour, cultures maintained in blue-light showed a dark red with brown tones, derived from the higher chlorophyll content, while red-light cultures showed a brighter and lighter red with orange-coloured tones due to the lower chlorophyll content in the background (Figure 1.6B).

As in other well-known carotenogenic species like *D. salina*, which will be discussed extensively in chapter 3 of the present work, the photoprotection provided by secondary carotenoid accumulation was more effective against blue light than against red light. This was reflected in a higher and more constant PSII effective quantum yield of 0.65 measured in blue-light cultures compared to red-light cultures, which showed a decreasing PSII quatum yield that reached 0.4 by the end of the experiment (Figure 1.7). The reason must be related to the interaction between biomass absorption and light source emission spectra. Carotenoids in *H. pluvialis* aplanospores absorb light in the blue region of the spectrum. Astaxanthin, accumulated in cytoplasmatic lipid bodies, shields light of those wavelengths and keeps chlorophylls in the thylakoid membranes protected from absorbing too much energy, which is translated in a lower chlorophyll degradation than the one occurring under 660 nm red light (Figure 1.5D), a wavelength out of astaxanthin's action spectra and where most of the light absorption is assumed by chlorophylls. Additionally, a clear energy dissipation mechanism reflected by an increase of non-photochemical quenching and a decrease of photochemical quenching was measured in the treatments where red light was present in comparison with blue-light cultures (Figure 1.8) that may indicate that, in the event that astaxanthin action spectra is useless for shielding excess light, a different mechanism must be active to dissipate energy and avoid severe photodamage and cell death. Some authors have hypothesized about the precise protective mechanism of carotenogenesis in *H. pluvialis* that has been suggested to derive from the steps of the synthesis pathway of the pigment itself, consuming excess electrons (Fan *et al.*, 1998) or subcellular molecular oxygen (Li *et al.*, 2008), reducing ROS. Moreover, in accordance with other authors (Fábregas *et al.*, 2003), astaxanthin accumulation

occured only after the complete consumption of nitrogen in the culture medium (Figure 1.5B). Therefore the photoprotective role associated to astaxanthin would only be active under inductive conditions. During vegetative growth other mechanisms for photoprotection and energy dissipation may be active.

As for the effect of absolute irradiance during the induction phase, all the cultures were able to deplete nitrogen in the culture medium and activate secondary metabolism except for the cultures that were maintained at 50 μmol photon $m^{-2}$ $s^{-1}$, where carotenoid accumulation was not observed since the cells remained in vegetative stage. The cells under this condition kept a carotenoid content stable on 1 % during the 7 days of the experiment (Figure 1.12B). Biomass concentration reached a maximum in *H. pluvialis* aplanospores around 200 μmol photon $m^{-2}$ $s^{-1}$ (Figure 1.12A). Higher irradiances produced slightly lower biomass concentrations. Interestingly, maximum dissolved oxygen concentrations were measured on a lower incident irradiance of 150 μmol photon $m^{-2}$ $s^{-1}$ and oxygen production dropped in higher irradiances (Figure 1.14). On the other hand, in the cultures that achieve nitrogen depletion and trigger carotenoid accumulation, carotenoid content correlated positively with incident irradiance and was maximal with the highest irradiance tested, despite the clear detrimental effect of such high irradiance values on cell photosynthesis (Figures 1.12B and 1.13), which was in line with chlorophyll content that decreased with time under inductive conditions and was inversely correlated with carotenoid content (Figure 1.12C). This is in accordance with various results obtained with *H. pluvialis* that showed that the higher irradiance, the higher the astaxanthin accumulation rate (Fan *et al.*, 1994; Park and Lee, 2001; Steinbrenner and Linden, 2003). Still, astaxanthin content of these studies is, in the best case, not higher than 2.5 %, even though the maximum incident irradiance tested, 250 μmol photon $m^{-2}$ $s^{-1}$, was similar to the one used in the present study (Steinbrenner and Linden, 2003). During the present investigation, maximum carotenoid content reached a maximum of 5 % of dry weight in the cultures maintained at 300 μmol photon $m^{-2}$ $s^{-1}$ by the end of the experiment. However, it is important to remark that this result is specific for the initial concentration of nitrogen in the medium, cell density and irradiance, since the time required to reach nitrogen depletion could be shorter and, consequently, carotenogenesis would be triggered earlier in time in a different cultivation setup. Moreover, a higher cell density at the beginning of the induction period would decrease light penetration in the culture and light availability per cell. The interaction between initial nitrogen concentration, cell density, and surplus nitrogen concentration in the culture medium has been described in detail by Fábregas et al. (2001).

PSII quantum yield showed an inverse correlation with incident irradiance during the induction period (Figure 1.13) and dropped in every treatment with time. The drop was specially evident in the treatments where carotenoid accumulation was active, therefore the treatments with enough light to be in pure inductive conditions. tThese observations point out the importance of the development of a production process with a high degree of process control when it comes to the flexibility to generate different products: maximum astaxanthin productivity is not necessarely achieved with the highest incident irradiance and it would be essential to find that equilibrium point between saturated growth conditions and astaxanthin content. On the other hand, maximizing astaxanthin content of the biomass could be interesting due to the potential higher market price and lower downstream processing costs. This could be achieved either by reducing initial nutrient concentration, that would result in lower green-stage vegetative cell density, increasing incident irradiance, or tunning light quality.

Anyhow, according to the present results, the parameter that had the largest influence in carotenoid accumulation was temperature. Temperature during the induction stage influenced biomass concentration only slightly (Figure 1.4A), but a huge effect was measured on carotenoid synthesis (Figure 1.4B), which peaked at 30 ºC resulting in a three-fold increase in carotenoid productivity with respect to 20 ºC (Figure 1.4C), partly due to the most probable accumulation of a higher endogenous ROS content. Interestingly, chlorophyll content also showed an inverse correlation with temperature and therefore with astaxanthin content but the differences among treatments were smaller than for carotenoid content (Figure 1.4D). Tjahjono et al. (1994) reported similar results in terms of carotenoid synthesis per cell for cultures maintained at 30 ºC and 33 ºC compared to 20 ºC, although in their case secondary metabolism was induced by these higher temperatures, which inhibited cell division and, subsequently, activated carotenogenesis. In the present investigation carotenogenesis was not observed either in the cultures maintained at 35 ºC or 40 ºC. Instead, growth was inhibited and complete cellular lysis occurred after a few days or a few hours after the transference to these conditions, respectively. A temperature of 30 ºC produced problems in vegetative green-phase cultivation such as cellular aggregation, cell motility loss and a decrease in nutrient consumption (Figure 1.2B). These issues were absent with 20 ºC and 25 ºC (Figure 1.2); moreover, nutrient consumption was fastest with 20 ºC. A similar behaviour was described in *H. pluvialis* green cultures, where an increase in temperature beyond 30 ºC resulted in a decrease in growth rate, oxygen evolution and finally in non-viable cultures (Fan *et al.*, 1994). In general, these observations are in line with normal temperatures for various strains of *H. pluvialis* which showed optimum values between 18 and 22 ºC, and maximum viable temperatures between 30 and 35 ºC (Allewaert *et al.*, 2015). In summary, it seems evident that the best strategy for the optimization of a two-stage process is the use of two different temperatures optimized individually for each of the two stages of the production process.

In comparison with other works related with the optimization of *H. pluvialis* two-stage process, this investigation could be in line with the works of Fábregas group (Fábregas *et al.* 2000, 2001, 2003) due to the similarities in experimental setup. In every study, including this one, a phototrophic two-stage cultivation was established using the same culture medium (OHM), which was proven to be crucial for the maintenance of high-density continuous cultures (Fábregas *et al.*, 2000), and equal initial nitrate concentration (4 mM N $L^{-1}$). Theoretically, the use of equal nitrogen concentration would produce similar biomass concentrations at the begining of the stationary phase, after the complete depletion of nitrogen from the culture medium, even though the *H. pluvialis* strains used in their and our investigation were different. Also, cultivation units and irradiance for the induction of aplanospores were similar, being cylindrical mini-photobioreactors with an optical path of 30 mm illuminated with 240 µmol photon $m^{-2}$ $s^{-1}$ for Fábregas et al. (2001), compared to a diameter of 50 mm and 300 µmol photon $m^{-2}$ $s^{-1}$ for the present study. The results they reported achieve maximum astaxanthin concentrations of 50 mg $L^{-1}$ and maximum productivities of 10 mg $L^{-1}$ $day^{-1}$ (Fábregas *et al.*, 2001), which are comparable with the results reported in the present work for blue-light cultivation at 20 ºC (Fig 1.3B and 1.3C). Despite they worked with 12 h: 12 h circadian light/ dark cycles while the present study uses continuous light, the absolute hours of light were equal due to the difference in cultivation time, which is twice in the experiments of Fábregas compared to this work, both accountting for 168 illuminated hours of induction period. Nevertheless and despite these similarities, at a temperature of 30 ºC, the present study reports maximum pigment concentrations three times

higher (125-150 mg $L^{-1}$), with productivities of 20 to 25 mg $L^{-1}$ $day^{-1}$ (Figures 1.4 and 1.5), proving that a combination of factors can be applied successfully during the second stage of the process to enhance carotenogenesis, as proposed by Fábregas et al. (2001).

After an extensive literature review, it was found that the results of this investigation are at the high-end of reported carotenoid content and productivity. This may be because despite it seems that there is enough evidence about the use of a combination of high irradiance and nitrogen depletion as the optimum inductive conditions that result on the highest astaxanthin content and productivity, already observed by Droop in 1954 (Droop, 1954), most authors still do not use these as the elementary conditions on top of which additional stressors can be tested. Therefore, many of those studies are done under suboptimal conditions. Consequently, very low values are reported in comparison with the ones that follow the "high light, no nitrate" strategy. In this respect Xi et al. (2016), Kobayashi et al. (1992), or Katsuda et al. (2004), all of which have investigated the effect of light quality in carotenogenesis, report astaxanthin concentrations not higher than 20 mg $L^{-1}$ due to the use of a nitrogen-replete medium or very low irradiances. This certainly hinders the full potential that these secondary cultivation factors would have when applied correctly. The same happens with the studies that investigate the effect of the addition of organic carbon to the culture medium in order to provide an extra carbon source with the aim of decoupling carbon fixation from light bioavailability, taking advantage of the heterotrophic growth capacity of *H. pluvialis*. The reported carotenoid content is in most cases not higher than 2 % of dry weight (Kobayashi *et al.,* 1991, 1992; Orosa *et al.,* 2000; Wen *et al.,* 2015). Additionally, values not higher than 3 % astaxanthin content are, in the best case, reported for the studies made in non laboratory-scale cultivation units, either prototypes or industrial productions run with artificial light or sunlight (Harker *et al.,* 1996; Olaizola 2000; López *et al.,* 2006; Wang *et al.,* 2013). The heterogenity of the so called "inductive factors" together with the use of suboptimal conditions in the different reports available in the literature makes the comparison of data almost impossible.

Among the studies that use the nitrogen limitation together with high irradiance as inductive conditions, the present investigation also reports higher values for astaxanthin content and productivity. These high values should be derived from the optimization of the light quality and temperature that are used in combination with nitrogen depletion and high incident irradiance. Authors such as Fábregas et al., aforementioned, or Boussiba et al., who reported 4 % astaxanthin content with a maximum pigment concentration around 100 mg $L^{-1}$ (Boussiba *et al.*, 1999b) still keep some of these factors suboptimal. Only Kang et al. (2005) with 7.7 % astaxanthin content, Scibilia et al. (2015) with maximum astaxanthin concentrations of 305 mg $L^{-1}$, and Domínguez et al. (2019) with a concentration of around 250 mg $L^{-1}$ reported better results (Kang *et al.*, 2005; Scibilia *et al.*, 2015; Domínguez *et al.*, 2019). These studies used N-deprivation and high irradiance to achieve this values, and did not use a higher temperature with respect to the green stage cultivation. However, Kang's impressive astaxanthin content was achieved only after 18 days of cultivation under inductive conditions, which would account for a productivity of around 7 mg $L^{-1}$ $day^{-1}$ in comparison with around 20-25 mg $L^{-1}$ $day^{-1}$ obtained in our system (Figure 1.4C). On the other hand, astaxanthin concentrations reported by Scibilia et al. (2015) were achieved after 10 days in cultures maintained at 400 µmol photon $m^{-2}$ $s^{-1}$ in 50 mL reactors, while Domínguez et al. achieved their concentration after 7 days. Only in these two cases the productivity would actually be higher than the maximum 25 mg $L^{-1}$ $day^{-1}$ achieved in the present work. As for other

cultivation methodologies different from any variation of the two-stage process, efforts have been made to develop an efficient one-stage continuous cultivation process for astaxanthin-rich *H. pluvialis* cells (Del Río *et al.*, 2005). This approach worked around the idea of keeping the culture parameters precisely on the intersection between nitrogen suficiency and nitrogen deficiency, which produced an equilibrium between young aplanospores and germinating cysts. This strategy could be interesting for some industries such as fish feed due to the higher bioavailability of the pigment as a result of the more porous cell wall of germinating cysts compared to fully induced aplanospores (Domínguez *et al.*, 2005). However, reported pigment productivities in the one-stage system were not higher than 15 mg $L^{-1}$ $day^{-1}$ and the cultivation method would be more challenging in terms of scalability than a two-stage process (Del Río *et al.,* 2010).

As a conclusion, the results achieved during this investigation represent a promising starting point for the industrial scale up of such process, which will be discussed in the next chapters of this work.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

# 2. UPSCALING OF A TWO-STAGE PRODUCTION PROCESS OF *H. pluvialis* CULTIVATED WITH ARTIFICIAL LIGHT: CONSIDERATIONS, CONSTRAINTS AND OPTIMIZATION

## Rationale

The results presented in Chaper one represented a starting point for the upscale of a two-stage cultivation process for the production of *H. pluvialis*. Light quality and temperature were optimized for both stages at laboratory-scale in order to favour cell division of green cells and astaxanthin synthesis of aplanospores, separately. The investigation resulted in the choice of monochromatic red light and 20 º C as the optimum conditions for vegetative growth, while a mix of red and blue light with a 1: 1 ratio and a temperature of 30 ºC produced the highest astaxanthin productivity, which was around 20 to 25 mg $L^{-1}$ $day^{-1}$ (Figure 1.4C), among the highest values reported in literature (Scibilia *et al.*, 2015; Domínguez *et al.*, 2019). The exhaustion of nitrogen from the culture medium together with high irradiance were confirmed as the main factors triggering massive carotenoid accumulation in this species, since no astaxanthin synthesis was observed while nitrogen was present in the culture medium, even when temperatures up to 35 ºC produced the cessation of cell division prior to cellular lysis (Figure 1.4). On top of nitrogen starvation and absolute irradiance, massive carotenogenesis could be enhanced by the application of other secondary factors such as light quality or temperature.

The effect of incident irradiance on biomass concentration and astaxanthin content of the biomass was also described in Chapter one. Using an initial nitrate concentration of 4 mM $L^{-1}$ and cylindrical mini-photobioreactors with a diameter of 50 mm, complete nitrogen depletion was achieved in three days with intensities above 50 µmol photon $m^{-2}$ $s^{-1}$. Saturated growth in terms of biomass concentration was reached with irradiances of 150 to 200 µmol photon $m^{-2}$ $s^{-1}$, and astaxanthin content showed a direct correlation with irradiance, reaching about 5 % of dry weight by the end of the experiments with 300 µmol photon $m^{-2}$ $s^{-1}$, the highest irradiance tested (Figure 1.12). Additionally, the effect of light quality on the photosynthetic response of aplanospores was characterized (Figures 1.6 to 1.9).

All the former investigations were done in an industrial context, therefore it was essential that the lab-scale optimization developed in chapter one was further extended to scale up the process. Chapter two describes the scalability of those results and the upscaling process, and puts together the most informative data collected during a process that lasted 16 months. The outcome of an industrial-scale pilot plant would be considered successful if the results equal or improve what was achieved in laboratory-scale. In this chapter, ideas will be shared from the engineering perspective of photobioreactor design. Additionally, considerations and

constraints on the upscale process of microalgae cultivation will be discussed, including some brief economic considerations. One of the main topics will be the development and proposal of a simple method to figure out optimum growth irradiance of a microalgae culture grown with artificial light, related to the definition of photosynthesis key parameters such as $Ek$ and $P_{max}$. Data from prototypes and industrial photobioreactors will be provided to support the discussion. For a better understanding of the process described next, it may be important to underline that, during this work, validation of lab-scale optimization and photobioreactor development toward a highly optimized unit have been done in parallel.

In brief, the results presented in Chapter two summarize the experiments performed to upscale the optimized conditions selected in Chapter one at lab-scale for the production of both green and red stages. For the green stage, irradiance and light distribution were optimized in 20 L flat-plate photobioreactors with a light path of 25 and 50 mm. In these systems, a good light distribution was proven to be a much more important factor than incident irradiance. Additionally, the optimization of continuous cultivation of green-stage *H. pluvialis* was performed. For the red stage, the scalability of the effect that light quality and temperature had on astaxanthin accumulation rate was tested first in 100 L industrial fermenter-type column photobioreactors that had a low illuminated surface to volume ratio of 13 ($m^2$/ $m^3$) (Figure 0.7A). It was evidenced that both parameters scaled with success. However, astaxanthin productivities were only as high as 14 mg $L^{-1}$ $day^{-1}$, a significant drop with respect to the 20-25 mg $L^{-1}$ $day^{-1}$ that were achieved at lab-scale. Light distribution was identified as the main factor hindering the achievement of higher concentrations. As a consequence, the same type of flat-plate photobioreactors that were used for the green stage were the chosen cultivation system for the induction stage, since illuminated surface to volume ratio is high in this particular design, meaning a better light distribution. Then, the optimization of irradiance for the highest pigment productivity in aplanospores was performed. In this way, equal results to those obtained at lab scale, in the range of 20 to 25 astaxanthin mg $L^{-1}$ $day^{-1}$, were achieved. Finally, as an industrial proof of concept, a pilot-scale unit composed of 150 L flat-plate units based on the 20 L flat-plate prototypes was built (Figure 0.7B and 0.8). Optimization of green and red stages was integrated, and results improved with respect to prototype-scale, reaching pigment productivities that were twice those achieved at lab and prototype-scale, which outcompeted any result reported in the literature.

# Results

## 1. Green stage: optimization of irradiance, light distribution, and continuous cultivation

For the initial optimization of incident irradiance for the production of green-stage cells, 20 L flat-plate prototypes were selected. These allowed the cultivation with a surface to volume ratio of either 20 or 40 ($m^2$/ $m^3$), depending on the illumination of only one or both sides of the photobioreactor, which accounts for a light path of either 50 or 25 mm, respectively. This kind of photobioreactor design, which basically consists in two glass plates attached to a stainless steel frame was, from our perspective, the design that allowed the best light distribution, simplicity of light-modeling, and the easiness of experimentation, achieving a higher homogeneity compared to any other kind of photobioreactor composed of curved

illuminated surfaces such as tubular or columns. Moreover, the choice of this type of unit allowed the accurate definition of the irradiance that was being used.

Experiments done at laboratory-scale for green vegetative cells production, reported in Chapter one (Figures 1.1 and 1.2), were done under a fixed irradiance of 100 µmol photon $m^{-2}$ $s^{-1}$ in a system composed of cylindrical glass mini-photobioreactors with a diameter of 50 mm. This incident irradiance was defined as "low", compared with 300 µmol photon $m^{-2}$ $s^{-1}$ that were used in the experiments with aplanospores, defined as "high". However, no optimization of irradiance was done prior to those experiments. The effect of different irradiances was only tested in the red stage, where the effect on biomass concentration and astaxanthin content of the aplanospores was assessed (Figure 1.12). It was therefore necessary to perform some experiments to assess the optimal irradiance to be applied to the flat-plate photobioreactors for green stage cultivation.

Theoretically, the most efficient irradiance for photosynthetic activity under any condition would be the intersection between the maximum slope of a photosynthesis-irradiance curve with maximal photosynthesis ($P_{max}$), a point known as Photosynthesis saturation point or $Ek$ (Richmond and Qiang, 2004; Barsanti and Gualtieri, 2006; Malapascua *et al.*, 2014). The objective of the first experiment was to establish the irradiance that allowed the achievement of the maximum $Ek$ of the cultivated organism in the selected, high $S/V$ photobioreactor. $Ek$ can be calculated from any photosynthesis-irradiance curve (P-I curve) that in our case was based on relative electron transport rate ($rETR$) calculated from chlorophyll fluorescence values using a PAM-fluorometer that measured PSII photosynthetic efficiency under a number of irradiances that ranged between 0 and 1000 µmol photon $m^{-2}$ $s^{-1}$, on a daily basis. For the construction of the P-I curve, a batch culture of *H. pluvialis* was maintained in a 20 L flat-plate photobioreactor with a $S/V$ ($m^2/m^3$) of 20, illuminated with 100 µmol photon $m^{-2}$ $s^{-1}$, and a circadian light/ dark cycle of 12 h: 12h. The culture was grown with an initial nitrate concentration of 8 mM $L^{-1}$ to ensure that light would the limiting factor. Cell density and relative electron transport rate ($rETR$) for the calculation of $Ek$ were monitored daily one hour after the beginning of the light period. Highest cell density was achieved after seven days when the culture was entering early stationary phase and was 1.6 million cells $mL^{-1}$ (Figure 2.1A), which was higher than the cell density recorded at the same growth phase in lab-scale cultures maintained at the same nitrate concentration (8 mM $L^{-1}$) and illuminated with the same irradiance, which was around 0.6 million cells $mL^{-1}$ (Figure 1.1A). Maximum growth rate was measured at the early exponential phase, being 1 division per day and coincided with maximum $Ek$ that was 500 µmol photon $m^{-2}$ $s^{-1}$ (Figure 2.1B). With increasing cell density, growth rate together with $Ek$ dropped to values close to 400 µmol photon $m^{-2}$ $s^{-1}$. Therefore, 500 µmol photon $m^{-2}$ $s^{-1}$ was chosen as the optimum growth irradiance for *H. pluvialis* green stage cultivation in our system (Figure 2.1).

According to the initial hypothesis, an effective cultivation system would be the one that could distribute this irradiance over as much volume as possible. As a proof of concept, an experiment was designed to compare photosynthetic activity of a culture grown in a photobioreactor with a $S/V$ of 40 illuminated with 500 µmol photon $m^{-2}$ $s^{-1}$ (effective light path of 25 mm), with a culture maintained in a photobioreactor with a $S/V$ of 20 and 1000 µmol photon $m^{-2}$ $s^{-1}$ (effective light path of 50 mm), so that in both cultivation units the photon input was equal, but distributed differently. In theory, the culture maintained in the photobioreactor with a higher $S/V$ and $Ek$ irradiance (500 µmol photon $m^{-2}$ $s^{-1}$) would grow

faster and reach a stationary phase cell density higher than the culture maintained in the lower $S/V$ and light saturated (1000 µmol photon $m^{-2}$ $s^{-1}$), which would reach light limitation earlier, denoting the importance of light distribution above absolute irradiance. The results confirmed the hypothesis (Figure 2.2). Cell density was higher in the culture maintained with a higher $S/V$ that reached 3 million cells $mL^{-1}$ compared to the lower $S/V$ that reached 1.7 million cells $mL^{-1}$ by the end of the exponential phase (Figure 2.2A). At around mid-exponential phase, (days 2 to 3) high $S/V$ cultures showed a $qP$ of 0.65 and $qN$ of 0.13, while in the low $S/V$ cultures a $qP$ of 0.19 and a $qN$ of 1.58 were calculated (Figure 2.2B). Additionally, maximal slope, $P_{max}$, and $Ek$ were higher in the culture maintained with a $S/V$ of 40 and optimum growth irradiance (Figure 2.2C). These results evidenced that a better light distribution ($S/V$ of 40) had a more crucial effect on growth with respect to a high irradiance, which had to do, mainly, with the efficiency at which photosynthetic apparatus is capable to work at the available irradiance ($Ek$).

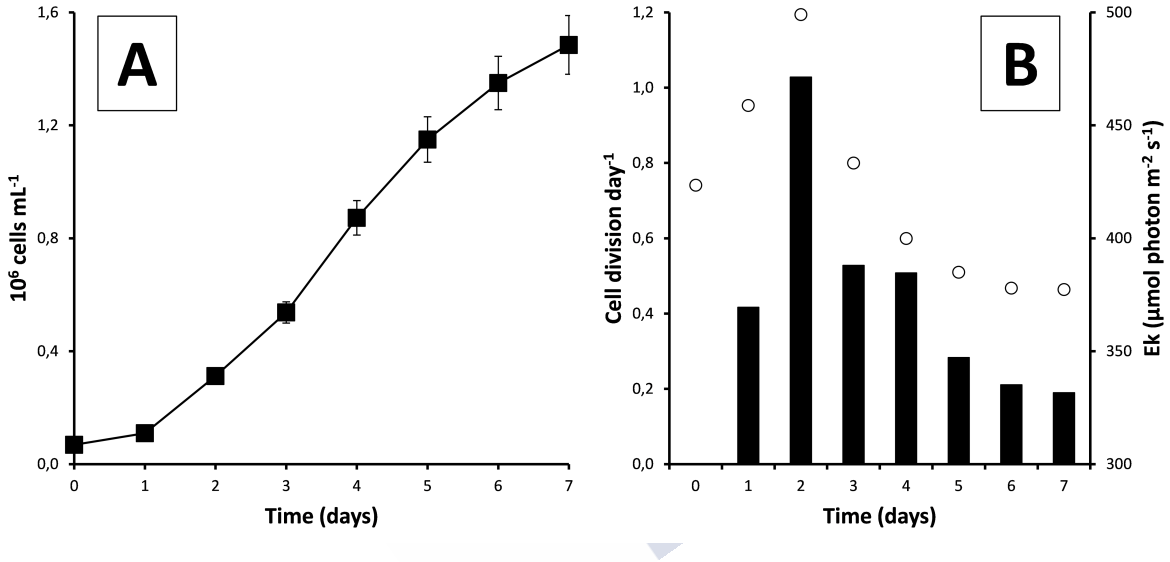



**Figure 2.1. Cell density ($10^6$ cells $mL^{-1}$) (A), cell division rate (cell division $day^{-1}$) and daily $Ek$ (µmol photon $m^{-2}$ $s^{-1}$) (B) of *H. pluvialis* vegetative green-stage culture maintained in a flat-plate photobioreactor with a $S/V$ ($m^2/m^3$) of 20, at a growth irradiance of 100 µmol photon $m^{-2}$ $s^{-1}$ with a mix of red and blue light with a 10: 1 ratio at 8 mM N $L^{-1}$. Temperature was maintained at 20 °C. In B, bars indicate cell division rate and open circles indicate calculated daily $Ek$. Vertical bars represent S.D. ($n$ = 2).**

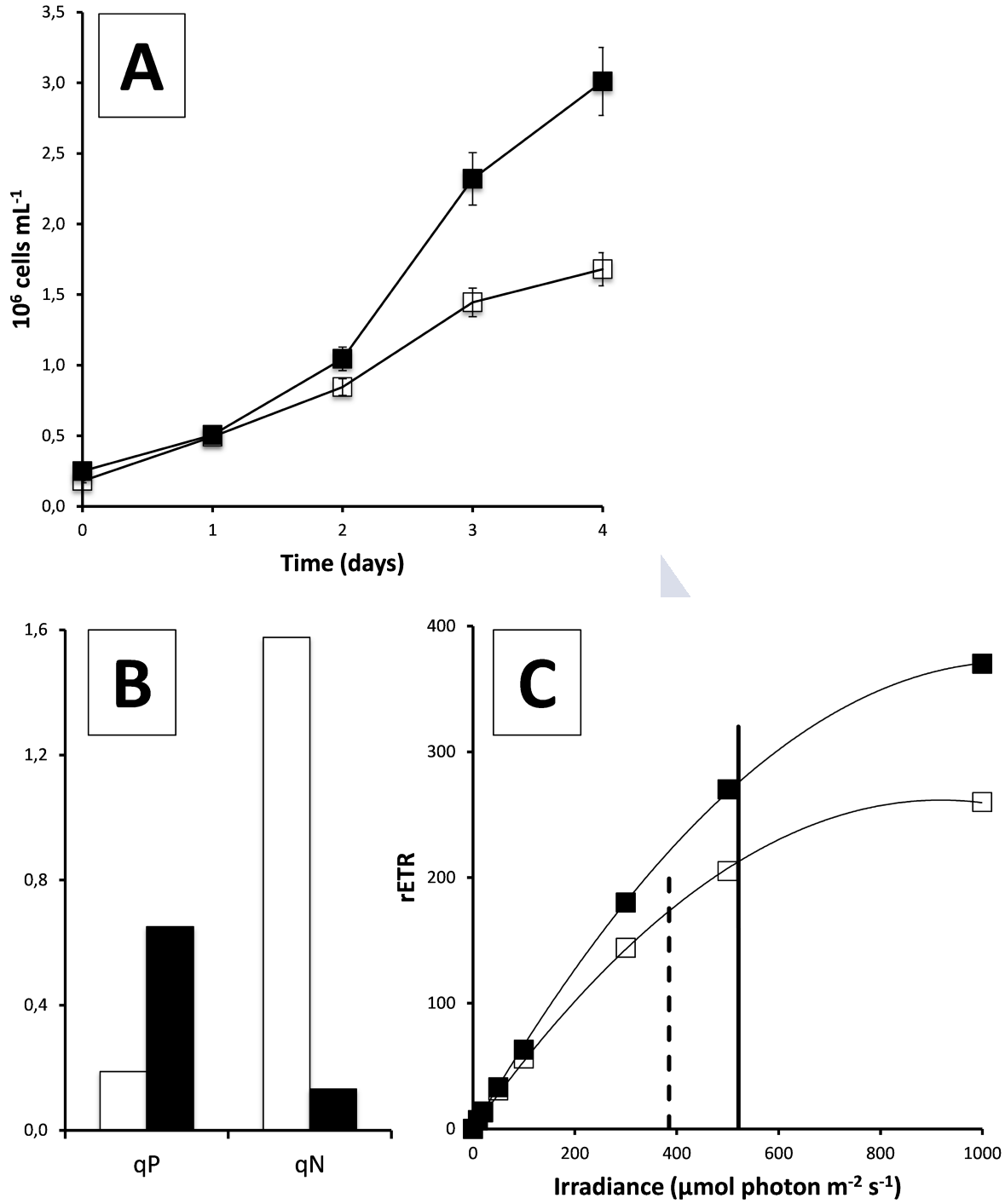



**Figure 2.2. Cell density ($10^6$ cells $mL^{-1}$) (A), photochemical ($qP$) and non-photochemical ($qN$) quenching (B), and PR curve based on $rETR$ (C), of green-stage cultures of *H. pluvialis* grown in flat-plate reactors with a $S/V$ ($m^2/m^3$) of 20 (open symbols) and 40 (closed symbols). In C, dashed line represents $Ek$ for S/ V 20, and continuous line represents $Ek$ for $S/V$ 40 at logarithmic phase. Vertical bars represent S.D. ($n$ = 2).**

After this preliminary optimization phase and since the final objective of this investigation was the establishment of a two-stage cultivation process for *H. pluvialis* similar to that established by Fábregas et al. (2001), the production of green stage *H. pluvialis* should be optimized in continuous mode, hence the optimum dilution rate of the culture required investigation. The final goal was to achieve a dilution rate with a high productivity where a minimum concentration of remaining nitrogen would be present in the medium, so that once the outflow was transferred to inductive conditions, nitrogen depletion could occur fast, triggering massive carotenogenesis in the shortest time possible. To investigate this, a semicontinuous cultivation system was set under the conditions that were defined previously: a 20 L flat-plate photobioreactor with a $S/V$ of 40 illuminated with an irradiance of 500 µmol photon $m^{-2}$ $s^{-1}$ (effective light path of 25 mm) and submitted to a circadian light/ dark cycle of 12 h: 12 h. pH was maintained at 7.5 with pulses of $CO_2$ that were dosed on demand when the pH of the culture was higher than the set-point. Temperature was kept on 20 ºC, and a nitrate concentration of 8 mM $L^{-1}$ was used to maintain the continuous cultures, that were submitted to renewal rates of 30, 40, 45, and 50 % until a steady state was reached. As expected, cell density at the steady state showed an inverse correlation with renewal rate (Figure 2.3A), being maximal with the lowest renewal rate tested, 30 %, achieving around 2.8 ± 0.2 million cells $mL^{-1}$ and lowest with 50 %, around 1.8 ± 0.05 million cells $mL^{-1}$. Cell productivity per day was maximal with a renewal rate of 40 %, achieving 1.1 ± 0.03 x $10^9$ cells $L^{-1}$ $day^{-1}$. Similar but slightly lower results were achieved with 45 % renewal rate, with a productivity of 0.99 ± 0.03 x $10^9$ cells $L^{-1}$ $day^{-1}$ (Figure 2.3A). Steady-state biomass concentration also decreased with renewal rate but achieved maximum productivities of 0.315 ± 0.03 g $L^{-1}$ $day^{-1}$ with 45 % renewal rate (Figure 2.3C). Additionally, nitrate could only be detected in the outflow of the cultures from a renewal rate of 45 % with a concentration of 10 mg $L^{-1}$ (0.16 mM $L^{-1}$), and a renewal rate of 50%, where the remaining nitrate concentration increased to 25 mg $L^{-1}$ (Figure 2.3B). Therefore, 40 to 45 % daily renewal rate represented the intersection between nutrient limited and saturated conditions in this cultivation system and at a nitrate concentration of 8 mM $L^{-1}$. Cultures maintained with lower renewal rates were nitrogen limited with this nitrogen concentration. As a result, 45 % renewal rate was selected as the most preferable renewal rate for the production process of green-stage cells.

Nevertheless, from an industrial point of view, there was still significant room for improvement to enhance the efficiency of the entire production process. While previous experiments were focused on photobioreactor design optimization to allow a better light distribution, and optimal light intensities were investigated for vegetative growth (Figure 2.1 and 2.2), the fact that the green stage cultivation was run with a circadian light/ dark cycle might not be optimal, since green cells growth was, in principle, limited to 12 hours of light period. Artificial-light cultivation of microalgae allows the control of the duration of the photoperiod, if any, so that the producer is not limited by day length as it happens in nature. For this reason, this parameter represented an obvious target in order to try to maximize productivity in the green stage, as well as to increase red stage output because, hypothetically, if light hours were doubled, production of green cells per day may be doubled as well, increasing the input for the second-stage cultivation units. Consequently a number of experiments were designed to shed some light on this matter.

Three different cultivation modes were tested in parallel in three 20 L prototypes: a green culture of *H. pluvialis* run in semicontinuous mode with a circadian light/ dark cycle of 12 h: 12 h (SC-LD) submitted to a renewal rate of 40 % right before the light period started. This

stood for one renewal every 24 h. A second green culture of *H. pluvialis* was run in semicontinuous mode but continuous illumination (SC-CL). The SC-CL unit was submitted to renewal rates of 35 % every 12 hours, since previous experiments demonstrated that the application of renewal rates of 40% every 12 hours under these conditions resulted in the wash out of the culture (data not shown). A third green culture of *H. pluvialis* was maintained in continuous light and run in semicontinuous mode where 6.7 % of the volume was renewed every 2 hours (C-CL), accounting for a daily dilution of 0.8 $day^{-1}$. This mode was the most similar to a standard chemostat operation mode where there is a continuous inflow of fresh medium at the same rate as volume is outflowing. This mode is easily operated if there is enough automation level and process control. Cultures grown in chemostat mode are normally used in cultivation of heterotrophs and produce a very stable product, since cellular density, biomass concentration, biochemical composition, turbidity, or nutrient concentration are kept constant during the entire cultivation period, which is considered advantageous from a production perspective.

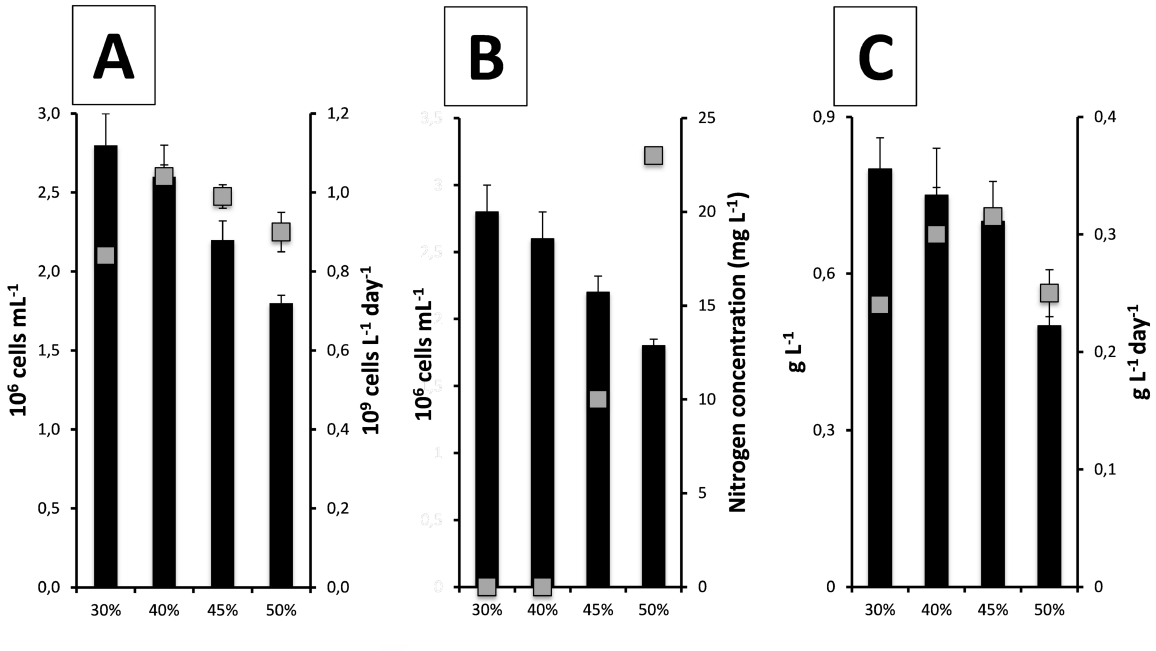



**Figure 2.3. Steady-state cell density ($10^6$ cells $mL^{-1}$) (bars) and cell productivity ($10^9$ cells $L^{-1}$ $day^{-1}$) (squares) (A); Steady-state cell density ($10^6$ cells $mL^{-1}$) (bars) and nitrogen concentration (mg $L^{-1}$) (squares) (B); Steady-state biomass concentration (g $L^{-1}$) (bars) and biomass productivity (g $L^{-1}$ $day^{-1}$) (squares) (C), in semicontinuous cultures of *H. pluvialis*. Cultures were maintained with daily renewal rates between 30 and 50% of the volume of the cultures at a N concentration of 8 mM, in flat-plate photobioreactors with a *S/V* of 40 and a growth irradiance of 500 µmol photon $m^{-2}$ $s^{-1}$ and submitted to a circadian light/ dark cycle of 12 h: 12 h. Temperature was kept constant at 20 ºC.**

Due to technical limitations, and despite previous experiments indicated an optimal renewal rate of 45%, 40 % was the largest renewal rate that could be maintained while running the three cultures in parallel. Since it was seen in previous experiments that SC-LD cultures maintained with a renewal rate of 40 % were nitrate limited with 8 mM $L^{-1}$, initial nitrate concentration of the cultures was increased to 10 mM $L^{-1}$ in all conditions to avoid limitation. The highest steady-state cell density was achieved in the SC-LD that was 2.6 ± 0.3 x $10^6$ cells $mL^{-1}$ (Table 2.1), showing a good reproducibility of the results in independent experiments (Figure 2.3A) and confirming 40 % as the accurate intersection between nutrient limited and

saturated conditions. Lower steady-state cell densities were observed in the treatments maintained under continuous light independently of the operation mode. SC-CL stabilized around 1.4 ± 0.1 x $10^6$ cells $mL^{-1}$, and C-CL stabilized in 1.3 ± 0.1x $10^6$ cells $mL^{-1}$. Despite being submitted to very different renewal rates, only small differences were found in cell productivity where maximal values were measured in C-CL and SC-LD that achieved 1.05 ± 0.1 x $10^9$ and 1.04 ± 0.1 x $10^9$ cells $L^{-1}$ $day^{-1}$, respectively. SC-CL achieved similar but slightly lower productivities of 0.98 ± 0.1 x $10^9$ cells $L^{-1}$ $day^{-1}$. Opposite to that, steady-state biomass concentration was higher in the treatments maintained under continuous illumination than in the treatment maintained in circadian light/ dark cycles (Table 2.1). Biomass concentration at steady-state was maximal in C-CL at around 1.1 ± 0.08 g $L^{-1}$, while it achieved 0.75 ± 0.06 g $L^{-1}$ in the SC-LD. In terms of biomass productivity, the differences were larger between continuous light and circadian light/ dark cycles cultivation since this value is strongly related with the percentage of culture that is harvested daily. C-CL achieved 0.9 g $L^{-1}$ $day^{-1}$, while SC-LD achieved only 0.3 g $L^{-1}$ $day^{-1}$. Nitrate was present in the outflow of every treatment, but lower concentrations were measured in C-CL in comparison with SC-LD and SC-CL. The remaining nitrate in C-CL was about 10 mg $L^{-1}$ (0.16 mM $L^{-1}$), while remaining nitrate in SC-LD and SC-CL was around 23 mg $L^{-1}$ (0.37 mM $L^{-1}$) (Table 2.1). As a result of these observations, C-CL was chosen as the preferred option for the integration of a continuous cultivation of vegetative green cells of *H. pluvialis* in a two-stage cultivation process, since this operation mode generated equal cell productivities, but almost three-fold higher biomass productivities and lower remaining nitrogen in the culture medium. This operation mode was also the most beneficial choice regarding the estimated investment costs required for an industrial-scale production process compared to the use of L/ D cycles, since three times higher dry weight productivity was obtained by only doubling the number of light hours.

**Table 2.1. Daily renewal rate, steady-state cell density and productivtiy, steady-state biomass concentration and productivity, and steady-state nitrogen concentration, in semicontinuos cultures of green-stage *H. pluvialis* grown in flat-plate photobioreactors with a $S/V$ ($m^2$/ $m^3$) of 40, maintained under a growth irradiance of 500 µmol photon $m^{-2}$ $s^{-1}$ with a mix of red and blue light with 10: 1 ratio, and a temperature of 20 °C. Different superscript letters indicate significant differences among treatments ($p < 0.05$)**

| | SC-LD | SC-CL | C-CL |
|---|---|---|---|
| Daily renewal rate (%) | 40% | 70% | 80% |
| Steady-state cell density ($10^6$ cells $mL^{-1}$) | 2,6 ± 0,3 (a) | 1,4 ± 0,15 (b) | 1,3 ± 0,15 (b) |
| Steady-state cell productivity ($10^9$ cells $L^{-1}$ $day^{-1}$) | 1,04 ± 0,1 | 0,98 ± 0,12 | 1,05 ± 0,1 |
| Steady-state biomass concentration (g $L^{-1}$) | 0,75 ± 0,06 (a) | 1 ± 0,1 (b) | 1,1 ± 0,08 (b) |
| Steady-state biomass productivity (g $L^{-1}$ $day^{-1}$) | 0,3 ± 0,1 (a) | 0,7 ± 0,12 (b) | 0,88 ± 0,04 (c) |
| Steady-state nitrate concentration (mM $L^{-1}$) | 0.37 (a) | 0.37 (a) | 0.16 (b) |

# 2. Red stage: scalability of light quality and temperature, and optimization of irradiance for the production of astaxanthin-rich *H. pluvialis*

## 2.1. Fermetor-type column reactors

In a first experiment, the results of the tests performed at laboratory-scale for the selection of optimal light quality and temperature for obtaining maximal *H. pluvialis* red stage biomass concentration and carotenoid accumulation were validated in 100 L fermentor-type column reactors with a surface to volume ratio ($S/V$, $m^2/m^3$) of 13 (Figure 0.7A). In this first experiment green-stage cultures were grown in the same photobioreactor, in batch mode, with an initial nitrate concentration of 4 mM $L^{-1}$, a temperature of 20 ºC, a circadian light: dark cycle of 12 h: 12 h, and a light quality of a mix of red and blue with a ratio of 10: 1. This light quality was used instead of monochromatic red, which resulted in the highest growth during lab-scale experiments (Figure 1.1A) due to a technical limitation of the light source in this system that could not function only with a single wavelength. Induction period was considered to start when nitrate concentration dropped below 10 mg $L^{-1}$ (0.16 mM $L^{-1}$), and had a duration of seven days. Green biomass concentration at the beginning of the induction period was 0.25 g $L^{-1}$, around the same value measured in lab-scale experiments by the end of exponential growth phase (Figure 1.1B). During the induction period two conditions were compared: standard and optimized conditions. In standard conditions, the only difference with respect to the green stage was the change from a circadian light: dark cycle to continuous illumination. On the other hand, under optimized conditions, circadian light: dark cycle was changed to continuous illumination, light quality was set as a mix of red and blue with a ratio of 1: 1, and temperature was increased to 30 ºC. Every other cultivation parameter was equal between both cultures.

Biomass concentration at the end of the induction period was higher under optimized conditions, reaching values around 2 g $L^{-1}$ after seven days under inductive conditions (Table 2.2), in comparison with 1.1 g $L^{-1}$ measured in the standard conditions. Pigment content results were similar to lab-scale (Figure 1.4B) since astaxanthin content was higher in the optimized culture, which reached values around 5 % (w/w) compared to 3 % under standard conditions. Therefore, astaxanthin productivity with optimized conditions was three-fold higher than with standard conditions, resembling lab-scale results (Figure 1.4D), but only reached a moderate productivity of 14 mg $L^{-1}$ $day^{-1}$ (Table 2.2) compared with the 25 mg $L^{-1}$ $day^{-1}$ that were achieved at laboratory scale (Figure 1.4D). This comparison confirmed the scalability of the positive effect that an optimized light quality and high temperature had on biomass concentration and astaxanthin productivity. However, results were far from those achieved at lab-scale, most likely due to the poor light distribution in this fermentor-type internally illuminated cultivation unit ($S/V$ of 13), which limited the amount of biomass compared to lab-scale results, where maximum biomass concentrations of 3 g $L^{-1}$ were recorded at the end of the induction period (Figure 1.12A).

### 2.2. Flat-plate photobioreactors

Further experimentation to optimize irradiance in the induction stage was carried out in the 20 L flat-plate prototype photobioreactors that allowed the cultivation at a $S/V$ of 40. *H. pluvialis* green vegetative cultures were maintained in flat-plate photobioreactors in semicontinuous mode with a renewal rate of 45 % and under the conditions aforementioned in section 1 of this chapter (Figure 2.3). When the cultures reached a steady-state, conditions were modified to inductive conditions: circadian light/ dark cycle was changed to continuous illumination, temperature was increased to 30 ºC, light quality was shifted to a red: blue ratio of 1: 1, and a series of irradiances were tested: 225, 550, 775, and 1100 μmol photon $m^{-2}$ $s^{-1}$. Cultures were maintained under these conditions for seven days before harvesting. Biomass concentration, astaxanthin content and productivity were quantified at the end of the experiment (Figure 2.4).

Unsurprisingly, biomass concentration was higher than in lab-scale experiments, reaching 4 g $L^{-1}$ against 2.5-3 g $L^{-1}$, due to the combination of two factors: (1) higher initial biomass concentration at the induction period derived partially from a higher initial nitrate concentration in the up-scaling experiments, (8 compared to 4 mM $L^{-1}$), and (2) the higher irradiance and better light distribution applied during the induction period in the flat-plate photobioreactors. Despite some differences in maximum biomass concentration could be observed at laboratory scale by the end of the experiments between different irradiances (Figure 1.12A), reaching a maximum of 3 g $L^{-1}$, biomass concentration by the end of the cultivation period in the flat-plate photobioreactors did not show significant differences among treatments in the prototype-scale, being around 4 g $L^{-1}$ for irradiances in the range 225-1100 μmol photon $m^{-2}$ $s^{-1}$ (Figure 2.4). As in lab-scale results (Figure 1.12B), a direct correlation was observed between irradiance and astaxanthin content in the biomass, which reached maximum values of 5.2 % of dry weight with the highest irradiance tested (Figure. 2.4). Astaxanthin content increased significantly from the lowest irradiance up to 775 μmol photon $m^{-2}$ $s^{-1}$, but only a slight increase was observed from 775 to 1100 μmol photon $m^{-2}$ $s^{-1}$. Astaxanthin productivity increased accordingly, reaching 25-30 mg $L^{-1}$ $day^{-1}$ with 775 and 1100 μmol photon $m^{-2}$ $s^{-1}$. As a result of these observations, 775 μmol photon $m^{-2}$ $s^{-1}$ was chosen as the most efficient irradiance to be used during the induction period.

**Table 2.2. Biomass concentration (g $L^{-1}$), astaxanthin content (mg $g^{-1}$), and astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) of *H. pluvialis* aplanospores grown in 100 L column reactors with a S/ V ($m^2$/ $m^3$) of 13, after seven days of cultivation. Standard treatment was maintained under continuous illumination of a mix of red and blue light with 10: 1 ratio, and a temperature of 20 °C. Optimized treatment was maintained under continuous illumination of a mix of red and blue light with a 1: 1 ratio, and a temperature of 30 °C. (*n* = 2).**

| | Standard | Optimized |
|---|---|---|
| Biomass concentration (g $L^{-1}$) | 1,13 ± 0,1 | 2 ± 0,2 |
| Astaxanthin content (mg $g^{-1}$) | 30 ± 2,3 | 51 ± 1,0 |
| Astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) | 4,4 ± 0,2 | 14,1 ± 0,3 |

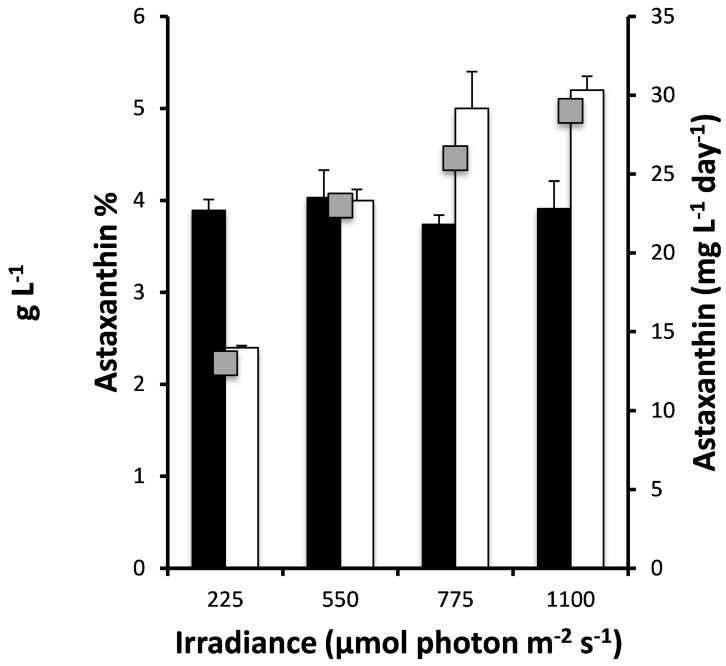



**Figure 2.4. Biomass concentration (g $L^{-1}$) (closed bars), astaxanthin content as a percentage of dry weight (open bars), and astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) (squares), of *H. pluvialis* aplanospores harvested after 7 days under inductive conditions with 225, 550, 775, and 1100 µmol photon $m^{-2}$ $s^{-1}$ in flat-plate photobioreactors with a $S/V$ of 40.**

## 3. Two-stage *H. pluvialis* production bioprocess: proof of concept at industrial pilot-scale

The results achieved in 20 L prototypes gave enough confidence to scale-up the process to industrial units with a working volume of 150 L. The design of these units was as similar as possible to the flat-plate prototypes used for light distribution, irradiance, and continuous cultivation optimization. A more detailed description of the design and operation of these units can be found in section 2.4 of Materials and Methods (Figure 0.7B and 0.8). As a proof of concept, three photobioreactors were constructed. One of them would function in continuous mode, being continuously illuminated and submitted to a daily dilution of 0.8 $day^{-1}$ (renewal rate of 6.7% every 2 hours), maintained at 20 ºC and an irradiance of 500 µmol photon $m^{-2}$ $s^{-1}$ with a red: blue ratio of 10: 1 and a nitrate concentration of 10 mM $L^{-1}$, for the continuous production of green-stage *H. pluvialis*. The steady-state outflow of that cultivation unit would be used to fill the remaining two photobioreactors, which would be used as red stage units. In these, temperature would be increased to 30 ºC, irradiance to 775 µmol photon $m^{-2}$ $s^{-1}$ and light quality changed to a red: blue ratio of 1: 1. Seven days after the maintenance under inductive conditions, the entire volume was harvested and biomass concentration, astaxanthin content and productivity was quantified. pH was maintained at 7.5 in both stages by the addition of $CO_2$ to ensure carbon bioavailability. Three independent induction cycles were analysed.

Steady-state cellular productivity, as well as biomass productivity and remaining nitrate concentration of the green stage cultivation were equal to the values obtained during optimization in the 20-L prototype unit (Figure 2.3A) at around 1.05 x $10^9$ cells $L^{-1}$ $day^{-1}$, 0.9 g $L^{-1}$ $day^{-1}$, and 10 mg $L^{-1}$ (0.16 mM), respectively. After three consecutive induction cycles, average biomass concentration was, at the moment of harvesting, 6 g $L^{-1}$ ± 0.2 while astaxanthin content increased up to 6 %. As a consequence, astaxanthin productivity calculated as astaxanthin concentration divided by the number of days under inductive conditions increased to 51 mg $L^{-1}$ $day^{-1}$. When the productivity was calculated on the basis of the astaxanthin produced at the end of the induction period per litre of semicontinuous culture per day, the value increased to 288 mg $L^{-1}$ $day^{-1}$ (Table 2.3).

**Table 2.3. Overview of the key production paramenters of red-stage *H. pluvialis* production at laboratory (100 mL) and pilot-scale (150 L). (*) Astaxanthin productivity calculated as pigment concentration per day under inductive conditions. (**) Astaxanthin productivity calculated on the basis of the astaxanthin produced at the end of the induction period per litre of semicontinuous culture per day. The duration of the induction period was seven days (*n = 3*).**

| | Lab-scale | Pilot-scale |
|---|---|---|
| Final biomass concentration (g $L^{-1}$) | 2,65 ± 0,22 | 6,0 ± 0,3 |
| Astaxanthin content (mg $g^{-1}$) | 5,5 ± 0,04 | 6,0 ± 0,2 |
| Astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) | 20,7 ± 1,6 | 50 ± 5 |
| Astaxanthin productivity (mg $L^{-1}$ $day^{-1}$) | - | 288 |

## 4. Economical considerations

Despite the focus of this work is not the economical assessment of *H. pluvialis* market, economics are a crucial part for the successful industrialization of any technological process. Economic feasibility must be ensured to reach a commercial level and biological optimization has a large effect on this. It is the intention of this section to give a general overview of some economical considerations related with the astaxanthin market and industry, and discuss how those relate with some key features of the cultivation process that has been developed during the upscaling, which may shed some light over some of the decisions that have been made during that time.

Opposite to the biological aspects of this kind of processes that may be kept constant once they are optimized, giving no reasons to expect changes in productivity once the production process is established, the economical aspects of these processes are more complicated due to the dynamic nature of the markets, which may and has suffered huge variations in the product's value over time. As a brief insight to this matter, by early 2000's the price of pure *H. pluvialis* derived natural astaxanthin was about 19000 USD $Kg^{-1}$, and has dropped since then to reach its minimum price by 2015 at 2250 USD $Kg^{-1}$. The reasons for the market crash seemed to be related with the large amounts that were introduced in the market, mainly by southeast Asian growing production capacity. After 2015, astaxanthin's market value seemed to have partially recovered and has stabilized around 6800 USD $Kg^{-1}$ as of December 2018, due to an increase in the pigment demand together with quality and delivery issues (A. Vitale, personal communication, June 2019). However, the market price of a certain product depends largely on the field of application, transaction volume, and marketing strategy (Guedes *et al.*, 2011).

Aquaculture and animal feed are the major markets (in volume) for astaxanthin, mainly synthetic (Lorenz and Cysewski, 2000), followed closely by nutraceuticals, which tends to demand the natural form. However, it is expected that astaxanthin will benefit from the huge estimated growth of the nutraceutical market (BCC Publishing, 2018) and, as a result, in the coming years the nutraceutical market may overtake over aquaculture in terms of major market (in US $) for astaxanthin. Despite smaller in terms of volume, cosmetic industry is also demanding natural astaxanthin and would be third in this list. However, quality criteria and product stability are known for its strictness in cosmetic industry and the introduction of new products in this market is not simple (Koller *et al.*, 2014; Novoveská *et al.*, 2019).

Astaxanthin global market includes natural microalgal astaxanthin produced by *H. pluvialis*, bacterial astaxanthin produced by *Paracoccus*, and synthetic astaxanthin produced by companies such as DSM (the Netherlands. AstaSana™; https://www.dsm.com/markets/human-nutrition/en/products/carotenoids.html) or BASF (Germany. Lucantin®; https://nutrition.basf.com/en/Animal-nutrition/Carotenoids.html) (Novoveská *et al.*, 2019). The synthetic version can be rapidly produced using inexpensive chemicals and due to the non-requirement of harvesting and downstreamprocessing costs. Therefore, while the the price per kg of the natural version may reach about 7000 USD, the price of the synthetic version may be half of that cost (Li *et al.*, 2011; Novoveská *et al.*, 2019). The total volume of products containing astaxanthin by 2020 (oleoresin, softgels, beadlets, tablets, etc) is expected to be around 670 metric tons; 65 % of those should be produced with microalgal astaxanthin. As for the global production capacity, reports from

2014 estimate a global *H. pluvialis* biomass production capacity of around 700 metric tons, and about 18 to 20 metric tons of pure astaxanthin (Industry Experts, 2018). Therefore, in very simplistic terms, it could be said that average astaxanthin content in worldwide *H. pluvialis* production is about 2.8 %. The Americas would be the region with a larger capacity for the production of natural astaxanthin from *Haematococcus*, with USA and Chile as the main producers, followed closely by the Asia-Pacific region, which is expected to take over the Americas as the main producer soon. In both regions, producers with capacities over 100 metric tons $year^{-1}$ can be found (BGG in China and Cyanotech Corporation in US). Israel (AlgaTechnologies Ltd.) and Europe (Algalif; Iceland. AstaReal AB; Sweden. Simris; Sweden. Algamo; Czech Republic. Algicel; Portugal, Azores), with multiple smaller producers, follow far behind the aforementioned regions in terms of production capacity (Araujo, 2019) despite Europe has been reported to be the largest market for carotenoids followed by USA (Novoveská *et al.*, 2019).

In summary, from a global perspective, it seems that astaxanthin market will continue growing and that there is room for product in the different markets (Li *et al.*, 2011; Acién, Fernández and Molina-Grima, 2013; Koller *et al.*, 2014; Novoveská *et al.*, 2019). However, when we dig deeply into the topic, it is interesting to analyse how astaxanthin production has evolved. In the early days, the industrial achievement of *H. pluvialis* biomass with a pigment content of 1.5-2 % was considered a success (Lorenz and Cysewski, 2000), since only a small part of the knowledge was available compared with what the industry knows these days, and cultivation systems and techniques were less sophisticated. As of today, only very large extensive outdoor productions still produce on that range (Cyanotech; US Hawaii. https://www.cyanotech.com/), while most of the mid-sized facilities produce biomass with a content between 3-5 % of dry weight (AlgaTechnologies Ltd; Israel. https://www.algatech.com/. AstaReal; Sweden, US, Japan. https://astareal.com/en/), and only a very few smaller producers, mainly European, produce biomass with an astaxanthin content from 6 to 8 % (Algalif; Iceland. https://algalif.com/). Gained knowledge, experience, innovative strategies and an improved production technology allowed this improvement. Therefore, a closer look into the global *H. pluvialis* production shows that a huge range of products are available, which varies between 1.5 to 8 % in astaxanthin content. This is highly relevant, since the astaxanthin content of the biomass together with the production capacity are the two main parameters affecting biomass application, which are directly related with downstream processing costs.

As a brief description of the downstream processing for aquaculture and nutraceutical market, after dewatering, cellular lysis, drying, and stabilising against oxidation, which are common steps for both applications, the main difference is the grinding step that is needed after the drying in order to provide a fine powder that can be introduced in a dry meal formula for aquaculture (salmon feed) (Lorenz and Cysewski, 2000), in comparison with the extraction of the oil fraction of the biomass together with the astaxanthin, which is normally done with high pressure (> 1000 bar) supercritical fluid extraction (M. Tippelt, R&D Engineer at NATECO$_2$, personal communication, March 2019), being $CO_2$ the main solvent (Nobre *et al.*, 2006; Kim and Chojnacka, 2015). While the grinding step required for aquaculture is a cheap process, the outsourcing of the industrial extraction of the oily fraction of the biomass has a minimum cost of about 45 € $Kg^{-1}$ in Europe, based on the highest efficiency of the extraction, which is reported to be higher than 90 % and is mainly dependant on the format in which the biomass is dried, being flakes the preferred format (M. Tippelt, R&D Engineer at NATECO$_2$, personal

communication, March 2019). Additionally, outsourced services costs depend on availability, which may be compromised by other raising industries with a need for supercritical fluid extraction as well (see services at https://www.nateco2.de/en/) (Hierro and Santa-María, 1992; Baysal *et al.*, 2000; Díaz-Reinoso *et al.*, 2006; Abbasi *et al.*, 2008; Reverchon *et al.*, 2009; Sahena *et al.*, 2009; Campardelli *et al.*, 2015; Ramirez *et al.*, 2018; Ahmad *et al.*, 2019). Considering this information, it is obvious that biomass with a higher astaxanthin content has an advantage for its use in the nutraceutical sector since the operational expenditure (OPEX) costs required for the production of a 10 % oleoresin (680 USD $Kg^{-1}$), a common format in the market composed of *H. pluvialis* oil extract standardized to 10 % astaxanthin with a vegetable oil, is inversely correlated with pigment content, due to the amount of biomass that needs to be processed. Only 1.25 Kg of 8 % biomass are processed to make 1 Kg of oleoresin at 10 % astaxanthin, while 5 Kg are required of a biomass with a pigment content of 2 %. In the same way, biomass with a lower astaxanthin content is most likely to get a wider margin when introduced into the aquaculture market, where the price of 3-5 % *H. pluvialis* biomass can be in the range of 150 USD $Kg^{-1}$ but the OPEX may be lower than the production of a higher-end astaxanthin content biomass, which are mainly derived from downstream processing costs (Molina Grima *et al.*, 2003; Guedes *et al.*, 2011). Additionally, the production capacity also influences the end application of the biomass since scale will highly influence OPEX costs. When production capacity is low in terms of kilos per year but pigment content is high, this may still not be enough for the application of the biomass in the nutraceutical market, which starts to be more favourable with mid to large sized capacities, where the investment in an in-house extraction plant may start to be profitable (Li *et al.*, 2011; Acién *et al.*, 2013). Nevertheless, innovative business structures based on the popular agricultural cooperatives may be good models to adapt for microalgae industry, where multiple small-sized producers may cooperate and centralize downstreaming in one location only (Thomassen *et al.*, 2016), bypassing downstream costs, investing low capital expenditure (CAPEX), and ensuring product availability as a cluster.

In summary, based on the market picture that has been described above, astaxanthin demand is expected to grow in the coming years. Despite the market crash experienced in 2014-15, prices have recovered partially. This has encouraged new players to start producing and, as a consequence, competition has also risen. *H. pluvialis* can be found in different formats from low percentages up to 8 % astaxanthin content. Competitive OPEX together with affordable initial CAPEX are key economical features for successful commercialization. Additionally, product quality and production stability are a must in order to be able to deliver and fulfil the demands of the market. When it comes to indoor cultivation of *H. pluvialis*, these circumstances can only be fulfilled when cultivation conditions and bioprocess design have been optimized to the highest extent and improvements justify a higher investment. In this respect, the pilot line based on the 150 L flat-plate photobioreactors that has been described in this chapter was conceived as a proof of concept for the achievement of accurate production estimates and further industrialization of a process based on those photobioreactor units.

As a practical example, when the costs together with the production estimates achieved during this investigation would be upscaled to a small commercial facility able to produce about 2000 Kg *H. pluvialis* biomass $year^{-1}$, with 6 % astaxanthin content (around 120 Kg pure astaxanthin $year^{-1}$), total CAPEX would be about 90 € $L^{-1}$ of production volume, including cultivation units and support equipment for pre-cultivation and harvesting. Main OPEX costs stand for building rent (20 %), labour (20 %), and downstream processing costs including

extraction (30 %). The rest of the OPEX costs include $CO_2$, water, nutrients, insurance, and illumination power consumption, among others, and stand for the remaining 30 %. This model is estimated from a scenario where the area for the installation needs to be rented, labour costs are average for a region such as the Netherlands, and extraction of the biomass needs to be outsourced. Probably one of the features where the biological optimization had the largest effect is in the low light energy consumption that is needed. In this concept, light energy OPEX stands, depending on the price per kWh, between 6 to 15 % of total OPEX $Kg^{-1}$ *H. pluvialis* biomass, which is in the range of 100 - 140 € $Kg^{-1}$, or around 1750 € $Kg^{-1}$ of pure astaxanthin. The optimization of irradiance, light distribution, and light quality has allowed a dramatic reduction in this parameter. Moreover, when the production facility is installed in a location where there is a $CO_2$ source of enough quality, or an excess of cooling water, or an excess of electricity, which are common features in industrialized North-European areas, a circular economy approach can be adopted, reducing OPEX costs largely. In terms of CAPEX, the optimization of continuous cultivation for green stage *H. pluvialis* allowed the reduction of CAPEX by 15 % due to the feasibility of using continuous illumination to produce green-stage cells that reduced the requirement of green photobioreactors by half, even though cell productivity was less efficient than with a circadian light regime. Additionally, economies of scale also affect CAPEX $L^{-1}$ of culture, with a potential reduction of at least 20 % that is estimated in a larger scale. Overall, it is evident that a balance between CAPEX and OPEX needs to be found, and creative formulas need to be adopted in order to maximize margins and ensure business success. Normally, large reduction of CAPEX costs decrease system sophistication in terms of efficiency or automation, and increase other OPEX costs such as power consumption, or labour cost, which could still make a viable business case depending on the facility's location. On the other hand, when reducing OPEX costs, CAPEX needs to be kept in mind, maintaining attractiveness for investment.

## Discussion

The data presented in this chapter from prototype and industrial pilot-scale units summarise the upscale process of a two-stage production of *H. pluvialis* with artificial-light. The data described scalability considerations of each cultivation parameter, optimize growth irradiance in a green culture of *H. pluvialis* and, in summary, showed the importance of photobioreactor design and culture conditions optimization when it comes to reproduce laboratory-scale results in larger cultivation units.

Despite the proof of concept units that were used during this work did not have an automated clean in place (CIP), a high degree of sanitation could be achieved with manual cleaning followed by chemical treatment as described in section 2.4 of Materials and Methods. All the measures applied for the maintenance of hygienic conditions were reflected in the high control of contamination during the entire testing period, during which barely no contaminants were detected. It is worth to mention that the choice of glass as the translucent material of the reactor benefited the cleaning operation. Glass is, on the contrary to other translucent materials normally used in microalgae cultivation such as plastic or PVC, less porous, smoother, and more resistant to the physical damage eventually caused by cleaning hardware or chemical agents, which may scratch softer materials creating regions where biofilm cannot be easily removed, or degrading them, decreasing light transmittance with use.

Despite glass is more expensive than alternative materials, our experience indicates that the investment may be worth in the long term.

With respect to the difficulty to maintain culture conditions, artificial light photobioreactors represent an obvious advantage with respect to cultivation units run with sunlight, especially for the green stage. By using artificial light, an operator can adjust the irradiance for the needs of the process at any moment, whereas in sun-driven systems the efficiency and productivity of the process depends on the location conditions, mainly. Regardless of the nature of the light source, the most important condition when it comes to photobioreactor design is that a good light availability must be ensured (Richmond and Qiang, 2013). In the 150 L flat-plates, light availability was ensured by illuminating both sides of the reactor, accounting for a $S/V$ of 40. Additionally, the design of the light sources was optimized even by carefully choosing the illumination angle of the LEDs to prevent photon loss out of the photobioreactors illuminated area as much as possible. Also, the glass used had a low iron oxide content, which allowed 15 % more transparency than standard glass.

Control of culture conditions refers, as well, to the accuracy of controlling culture temperature, which is more problematic the larger the volume to be cooled-down or warmed-up. Especially challenging in a process such as a two-stage *H. pluvialis* bioprocess where, in the case of the present work, optimum temperatures between both stages differed in 10 ºC. However, in the prototype units temperatures in both stages could be kept with an accuracy of ± 1 º C..

Photobioreactor design will have a huge impact in gas exchange, which is crucial in terms of carbon biovailability, pH control, or avoiding oxygen accumulation in the culture medium (Borowitzka *et al.*, 2016). During this work, no problems were faced in terms of maintaining pH under control. Furthermore, since a dissolved oxygen sensor was present in the green stage reactors, aeration flow could be adjusted accordingly in order to increase or decrease gas exchange to maintain dissolved oxygen concentration in values that were not causing a decrease of photosynthetic efficiency due to photorespiration. Oxygen sensor was skipped from the red stage reactors because laboratory-scale experiments already shown that dissolved oxygen decreased significantly during induction (Figure 1.9) and, most likely, would not represent any problem.

A high degree of automation will allow different options regarding operation and it may be key when the productivity of a certain metabolite increases significantly when the species is grown in continuous cultivation with respect to batch, or vice versa. In this sense, continuous cultivation of green-stage *H. pluvialis* worked smoothly during the entire testing period, and red-stage batch cultures operation was quite simple and did not suppose a challenge in terms of automation.

Results showed that successful *H. pluvialis* industrialization is not granted before a meticulous optimization process is performed under laboratory-scale culture conditions. Then, a correct interpretation of the results achieved at laboratory-scale must be done before a rigorous optimization at larger scale conditions, in order to analyse on which factors the optimization must be focused to reproduce lab-scale results, followed by the construction of a pilot-scale proof of concept. In this respect, lab-scale improved astaxanthin productivity from 10 to 25 mg $L^{-1}$ $day^{-1}$ by tuning light quality and temperature, mainly (Figure 1.5). When

those culture conditions were transferred to an industrial fermenter-type reactor prototype of 100 L, best results on astaxanthin productivity were reduced to 14 mg $L^{-1}$ $day^{-1}$ (Table 2.1). After identifying the poor light distribution, reflected in a low $S/V$ of 13 in the column reactor, as the main factor hindering astaxanthin productivity, new 20 L flat-plate prototypes with a higher $S/V$ were designed and productivity increased to 28 mg $L^{-1}$ $day^{-1}$ (Figure 2.4). This photobioreactor design was scaled up to pilot-scale units of 150 L. In parallel, green stage continuous cultivation was optimized. The integration of both resulted in an astaxanthin productivity of around 50 mg $L^{-1}$ $day^{-1}$, calculated as astaxanthin production per days under inductive conditions, or 288 mg $L^{-1}$ $day^{-1}$ when the productivity was calculated on the basis of the astaxanthin produced at the end of the induction period per litre of semicontinuous culture per day (Table 2.3).

There are only few published data of industrial scale commercial *H. pluvialis* photobioreactors (Harker *et al.*, 1996; Olaizola, 2000), so comparison in the same context is difficult, but the comparison of the obtained results with those available fully confirm the success of the present optimization and upscaling process. Productivity doubled with respect to small-scale experiments and the results were better than any published data with *H. pluvialis*. Harker et al. (1996) reported promising astaxanthin contents of 5.5 % in the biomass at lab-scale conditions, but they could only reach 2.7 % when the cultivation was upscaled to 30 L units (Harker *et al.*, 1996). They explained that the problem was that they could not keep the same culture conditions in the 30 L unit than during the small-scale experiments, mainly regarding irradiance. When compared with the few reports available on large-scale *H. pluvialis* cultivation, astaxanthin content was double with respect to the Aquasearch Growth Modules (AGM) described by Olaizola, where only maximal 3 % pigment content was achieved (Olaizola, 2000). Olaizola reported maximal biomass productivity of 19 g $m^{-2}$ $day^{-1}$, which would be low compared to the 150 L photobioreactors areal productivity for both green and red stage that achieved 158 and 151 g $m^{-2}$ $day^{-1}$, respectively.

Two fundamental concepts with respect to light availability and its usage by the photosynthetic microorganisms; optimization of irradiance based on photosynthesis saturation point ($Ek$) and surface to volume ratio ($S/V$), were highly relevant for the success of the upscaling process because they facilitated the integration of biology and engineering, and deserve a detailed discussion. First, results showed that $Ek$ changed during the growth of *H. pluvialis* and was maximal during exponential phase, which coincided with the highest cell division rate measured (Figure 2.1). Calculated $E_k$ was 500 µmol photon $m^{-2}$ $s^{-1}$. The establishment of this parameter was revealed as crucial in the optimization process. Then, the results clearly showed the greater importance of good light distribution above high irradiance (Figure 2.2). The system with a $S/V$ of 40 and 500 µmol photon $m^{-2}$ $s^{-1}$ almost doubled in terms of growth the system with $S/V$ of 20 and 1000 µmol photon $m^{-2}$ $s^{-1}$ (Figure 2.2A), which also showed a low $qP$: $qN$ with respect to $S/V$ 40 where $qP$ was high and $qN$ low (Figure 2.2B). This result was not surprising, since the highest photosynthetic efficiency of the first one should be reflected in a higher photochemical fluorescence quenching ($qP$) and low non-photochemical fluorescence quenching ($qN$) during exponential growth phase, while the opposite should be observed in the latter because the cells above $Ek$ must activate some kind of energy dissipation system to deal with the excess of absorbed photons that cannot be used efficiently. The effect of a high $S/V$, or a low optical path, has been widely described and numerous comparisons can be found in the literature (reviewed in Richmond and Qiang,

2004). Although it could be argued that the results from the culture maintained under 1000 µmol photon $m^{-2}$ $s^{-1}$ could be improved under a higher aeration rate resulting in shorter light/ dark cycles in the photobioreactor (Evers, 1991; Molina Grima *et al.*, 1993; Acién Fernández *et al.*, 1998; Brindley *et al.*, 2011), it still looks evident that a good light distribution should be prioritized with respect to a high irradiance (Figure 2.2), at least when the cultivation unit offers full control over the irradiance. Increasing turbulence may affect the exposure time of a cell to the photic zone of a reactor allowing a better dynamic of saturation and de-saturation cycles of the photosynthetic apparatus, preventing the decrease of photosynthetic efficiency and allowing the maintenance of photosynthetic efficiency at irradiances higher than $Ek$. However, despite this is a reasonable idea for sunlight driven systems, highly endorsed within the literature (Richmond and Qiang, 2013; Borowitzka *et al.*, 2016), it may be considered non-economically feasible in indoor systems due to the energy waste in excess illumination, which will not translate linearly into more photons nor a higher photosynthetic efficiency, and barely no increase in illuminated volume. According to Beer-Lambert's law, that explains how light is not absorbed linearly but exponentially, in practice, most light absorption occurs within the first centimetres of a dense microalgae culture regardless of the irradiance (Borowitzka *et al.*, 2016). Therefore, light penetration of either 500 or 1000 µmol photon is practically the same in a dense culture. Furthermore, increasing turbulence may be also limited by the sensitivity of each organism to shearing forces (Richmond and Qiang, 2013). In the case of *H. pluvialis*, in some strains a high turbulence can result in damage of the fragile green vegetative cells. Moreover, the topic may be more complex than it appears, since there is an enormous interplay between aeration rate and other parameters crucial for photosynthesis such as dissolved oxygen concentration (Olaizola, 2003), which will be lower the higher the turbulence in a photobioreactor, increasing photosynthetic efficiency. Despite the boundaries concerning maximal turbulence in green cells of *H. pluvialis* were not investigated during this work, this is an important issue that may deserve further investigation. For green-stage production the aeration flow was regulated in accordance with dissolved oxygen concentration to prevent the accumulation of more than 15 mg $L^{-1}$ in the culture. With that turbulence no damage in the green cells could be detected by microscopic examination and values for photosynthetic efficiency were as expected. In summary, an equilibrium between all these factors needs to be determined for every case.

The result of the experiment comparing light distribution is in line with the observations that have been done in outdoor cultures of *Spirulina* grown in sun-driven systems in regions with strong sunlight, in raceways. In these cultures, photosynthetic efficiency, measured as $Fv/Fm$ showed high values during the first hours of daylight, while a decrease in photosynthetic efficiency was recorded during the middle hours of the day, when sunlight was stronger. Efficiency then recovered during the last hours of the day, when light intensity decreased again (Torzillo *et al.*, 1996). Despite these *Spirulina* cultures would maintain the same $S/V$ regardless the irradiance received throughout the day, this observation can be assimilated to what was observed in *H. pluvialis* cultures maintained under 1000 µmol photon $m^{-2}$ $s^{-1}$ and $S/V$ 20 compared to cultures maintained at 500 µmol photon $m^{-2}$ $s^{-1}$ and a $S/V$ of 40 where, due to the saturation of the photosynthetic apparatus, reflected in the differences between $qP$ and $qN$ within the treatments (Figure 2.2B), $P_{max}$ was lower during exponential phase with the higher irradiance (Figure 2.2C), meaning that, in theory, the same amount of photons would yield less growth under such conditions.

Considering that optimum growth irradiance in terms of photosynthetic efficiency can be calculated, why would an operator want to run a culture with a suboptimal irradiance? Unless the use of a suboptimal irradiance in terms of photosynthetic efficiency enhances some kind of secondary metabolite, such as astaxanthin in *H. pluvialis* aplanospores, the answer is intricate. Evidently, sun-driven systems do not need to bother much about this topic since they must follow a more opportunistic approach than indoor systems. Due to the use of a free light source with an availability difficult to predict, their main concern may probably be preventing photoinhibition in low density cultures by screening strong sunlight and improve production in a sunny day or when cultures are light limited (Richmond and Qiang, 2013). However, in artificial light cultivation, where the production of every photon counts as an operational cost and irradiance can be regulated and kept constant, if the operator can keep the culture under the most efficient growth irradiance, electricity costs associated with illumination per kilo of biomass will drop dramatically. This is essential to achieve high density cultures, which at the same time increases areal productivity, since an efficient growth irradiance distributed over a high $S/V$ will automatically allow to work at a higher nutrient concentrations that will produce more biomass (Richmond and Qiang, 2013). The results of the present work reflect this idea. The cultures in the first prototypes were grown with a nitrate concentration of 4 mM $L^{-1}$ that was not limiting for green-stage (Table 2.1). When the $S/V$ was improved to 40, nitrate concentration had to be increased to 8 mM $L^{-1}$ to prevent limitation. Even with 8 mM N $L^{-1}$, semicontinuous cultures were nitrogen limited up to 40 % renewal rate (Figure 2.3B) denoting a potential higher capacity to consume nitrogen. The final unit was run with 10 mM N $L^{-1}$, due to the need of maintaining a low remaining nitrogen concentration in the outflow of green cultures, and a steady state biomass concentration around 1 g $L^{-1}$ (Table 2.1).

Results obtained during the optimization of the continuous cultivation of green-stage *H. pluvialis* were highly relevant. A comparison between three cultivation modes was carried out. Interestingly, steady-state cell density was about twice in SC-LD compared to C-CL: 2.6 against 1.3 million cells $mL^{-1}$ (Table 2.1). However, cell productivity was very similar in the three systems, around 1.0 x $10^9$ cells $L^{-1}$ $day^{-1}$. This result is in line with recent works that have compared different light regimes in green-stage *H. pluvialis*, circardian 12h light 12 h dark regime against continuous illumination in batch cultures, observing a similar relative difference (Domínguez *et al.*, 2019). This is an interesting fact if the results are interpreted with respect to most efficient growth strategy for *H. pluvialis* green cells. In this respect, SC-LD outcompeted C-CL when cell productivity is considered since steady state cell density was double in SC-LD compared with C-CL and no difference in cell productivity was observed, meaning that the production of the same number of cells in SC-LD was done with half the illumination hours and therefore half of light energy consumption.

On the other hand, bigger differences were found in terms of biomass concentration (Table 2.2). Continuous light treatments stabilized on a higher biomass concentration, around 1 g $L^{-1}$, than the condition maintained under a circadian light/ dark cycle that only reached 0.7 g $L^{-1}$. This may be due to the moment when the sample was taken, since 0.7 g $L^{-1}$ were measured in the SC-LD by the end of the dark period, right before the renewal rate occurred. During a dark cycle, the microalgae culture "burns" carbon (Fábregas *et al.*, 2002). As a result, biomass concentration measured by the end of a light period may be higher than the biomass concentration measured by the end of the subsequent dark period. Still, steady state nitrogen concentration was the lowest with C-CL with respect to SC-LD, which is in accordance with the observations made by Domínguez et al. (2019), where higher nitrogen consumption was

measured under continuous illumination, which was not translated into a higher number of cells. Moreover, Domínguez et al. (2019) detected small amounts of astaxanthin in actively dividing green cells maintained under continuous illumination prior to nitrogen depletion, proving that light regime must affect astaxanthin synthesis independently of light intensity. Despite the accumulation of astaxanthin in nitrogen-replete conditions was very low and therefore with a scarce biotechnological application, it may shed some light on other possible mechanisms for astaxanthin synthesis. Despite during this work astaxanthin was not measured in green-stage experiments, under continuous light cells certainly can look more yellowish or with orange tones compared to circadian light regime cultures by microscopical examination, especially during the first days after inoculation, before continuous mode starts.

In summary, it seems evidenced that under continuous illumination there is a decoupling among nitrogen consumption and cell division, together with a surplus of carbon fixation produced by a continuous photosynthetic activity due to the use of continuous illumination that translates into higher biomass concentration or weight per cell.

Among the treatments that were maintained under continuous illumination, SC-CL was submitted to one renewal rate every 12 hours but could not stand a total daily dilution rate of 80 %, since cell density started dropping until the culture washed out. This phenomenon was not observed when the renewal rates were decreased to 35 %. On the contrary, C-CL could be maintained with 80 % daily dilution rate without problems. This could be explained by the fact that, in practice, density fluctuations in C-CL are lower than in SC-CL, since with every renewal SC-CL lowers cell density, biomass concentration, and turbidity by 40 %, generating high fluctuations in cell density that can affect photosynthetic efficiency. On the contrary, C-CL only decreased those by around 7 % every 2 hours. SC-CL may need some extra time to re-adapt to the lower density conditions, while C-CL barely changes so growth is kept at a constant rate. In the end, C-CL cultivation mode was adopted for the production of green-stage *H. pluvialis*, even when cell productivity was less efficient, since there was a need to reduce initial investment costs and this strategy could reduce by half the required number of photobioreactors producing green-stage culture in an industrial situation compared to the use of a SC-LD strategy.

Finally, regarding the induction stage, both biomass concentration and astaxanthin content were enhanced in the 150 L units by the end of the batch period with respect to the 20 L prototypes. Astaxanthin productivity calculated on the basis of the astaxanthin produced at the end of the induction period per litre of semicontinuous culture per day increased from 90 to 288 mg $L^{-1}$ $day^{-1}$. In this respect, this study has achieved astaxanthin productivities more than 20-fold higher than those obtained by Fábregas et al. (1998, 2000, 2001, 2003) that were used as the main reference for the design of this industrial process, and proves the scalability of a process similar to the one they originally proposed, only with the addition of an extra step of culture conditions optimization and adapted to the available industrial technologies. A number of reasons could explain the improvement between prototypes and final units: on one hand, it could be caused by the use of a higher initial nutrient concentration of 10 mM N $L^{-1}$ instead of 8 mM N $L^{-1}$ during the green stage production. On the other hand, despite steady-state biomass concentration was higher in C-CL with respect to SC-LD, cell density steady-state was around half so irradiance per cell was higher. Furthermore, the higher carbon fixation of green cells produced in C-CL mode with respect to SC-LD seems to be reflected in a higher concentration of storage compounds per cell. Therefore, a different biomass composition

could have some influence in massive carotenogenesis. Future investigations for the elucidation of this phenomenon could be highly interesting.

The optimization of biological processes at a large scale is as challenging as fundamental, and normally demands a different methodology than the more puristic approach that must prevail at laboratory-scale. A scientific approach based on well interpreted data must always be present, in many cases together with a good intuition, but the methodology requires adaptation to any unavoidable technical limitation as the ones faced in some of the procedures described in this chapter. This, together with the fact that technical engineering development might be done in parallel to industrial-scale tests, certainly puts the microbiologist, more used to laboratory-scale development, out of their comfort zone. However, the opportunity to participate in the research and development of a process from the lab bench to commercial scale is highly rewarding, since it does not only give the researcher a bigger picture of the considerations and constraints of any commercial-scale bioprocess, but implies working in a synergic manner with other fields, as essential as biology, to make industrialization successful, such as engineering or economics. In the end, when done correctly, optimization of industrial-scale processes serves to find new uncertainties with a need for an answer, and to point out weak spots in the process that could potentially improve results, forcing researchers to go back to the lab table to continue making progress.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

# 3. EFFECT OF LIGHT QUALITY ON GROWTH, PIGMENT CONTENT AND PHOTOSYNTHETIC RESPONSE OF CAROTENOGENIC AND NON-CAROTENOGENIC SPECIES OF THE GENUS *Dunaliella* IN CONDITIONS OF NITROGEN DEFICIENCY AND CONTINUOUS ILLUMINATION

## Rationale

Chapters one and two evidenced the importance of light spectrum optimization in the cultivation of *H. pluvialis* with artificial light. In brief, laboratory-scale results have shown that, during vegetative growth, *H. pluvialis* achieved higher cellular densities maintained under monochromatic red light, while lowest cellular densities were achieved with monochromatic blue light. Interestingly, the use of different ratios of red and blue light did not produce significant differences in cellular density (Figure 1.1). On the other hand, under inductive conditions, the use of blue monochromatic light enhanced carotenoid synthesis, while red monochromatic light enhanced biomass concentration. However, the use of a combination of red and blue wavelengths enhanced carotenoid productivity, by stimulating a more balanced growth and carotenoid accumulation (Figure 1.3). Chlorophyll content decreased during stationary phase but, in general, chlorophyll content remained higher in blue light (Figure 1.6B). Moreover, optimum temperature for carotenogenesis was found at 30 ºC, which produced astaxanthin contents up to around 5 % of dry weight (Figure 1.4), which was twice as much as the content achieved with 20 ºC. Irradiance was directly correlated with carotenoid content and inversely correlated with chlorophyll content (Figure 1.12). Apart, carotenoids protected *H. pluvialis* cells against blue light. This phenomenon was reflected in the maintenance of a higher PSII quantum yield in the cultures illuminated with the respective wavelength (Figure 1.7). Non-photochemical fluorescence quenching increased when red light was used in comparison with the blue light (Figure 1.8).

Laboratory-scale results were proven to scale up with success in different types of photobioreactors (Chapter two, section 2). After the integration of lab-scale optimization in flat-plate photobioreactors optimized for an effective light distribution, carotenoid content and productivity were higher than lab-scale experiments (Chapter two, section 3), achieving a content of 6 % of dry weight and productivities up to around 50 mg $L^{-1}$ $day^{-1}$ (Table 2.3). The achievement of these results evidenced the importance of the optimization, among others, of light composition for the cultivation of this species with artificial light. Its importance for industrial-scale feasibility was reflected in the reduction of OPEX costs derived from lamp

power with respect to the production cost per kilo of biomass, which were predicted in the range of 6 to 15 % with the proposed strategy (Chapter two, section 4).

The manifested importance of the choice of light composition in the cultivation of *H. pluvialis* suggests that similar responses may be triggered in other species with industrial interest. Research in this direction can contribute not only to understand the physiology behind those responses, but may also have direct applicability in industry. In Chapter three, the effect that different light wavelengths have on growth, pigment content, and photosynthetic response of two *Dunaliella* species was characterized. *D. salina* Teodoresco, CCAP 19/18, was chosen due to its known capacity to massively accumulate β-carotene (Kleinegris *et al.*, 2010; Pirwitz *et al.*, 2015) in conditions similar to those governing massive astaxanthin accumulation in *H. pluvialis*. *D. tertiolecta* Butcher, CCAP 19/6B, which lacks the carotenogenic ability of its relative, was used to characterize, in parallel, a different physiological response to the same conditions of nitrogen deprivation and continuous illumination. Results suggest that a similar strategy to that shown in Chapter two for *H. pluvialis* could be used in artificial-light industrial cultivation of *D. salina*, since the response was in many ways similar to that of *H. pluvialis*, with marked differences among the different light wavenlengths. On the other hand, the response of *D. tertiolecta* to ensure culture's viability appeared to be as effective as the one of *D. salina* despite the lack of β-carotene accumulation, and was characterized by a clear increase of energy dissipation mechanisms, which were invariable among wavelengths.

Several authors have studied the effect of light intensity and spectrum quality on carotenoid accumulation of species of the genus *Dunaliella,* although variations in experimental setups and methodology hinder the achievement of general conclusions. A direct and linear relationship between irradiance and carotenoid accumulation together with a drop in chlorophyll content have been described in cultures of different carotenogenic *Dunaliella* species grown under cool-white lamps (Loeblich, 1982; Ben-Amotz *et al.*, 1989). Experiments carried out with *D. bardawil* (Ben-Amotz and Avron, 1989) pointed to irradiance as the parameter regulating the accumulation of β-carotene, with light quality having no impact on carotenoid accumulation, despite the blue region of the light spectra was not investigated. On the contrary, experiments carried out with the same strain of *D. bardawil* demonstrated that light quality had an influence, controlling growth and photosynthetic metabolism in this species (Sánchez-Saavedra *et al.*, 1996). Cultures grown under white light supplemented with far-red wavelengths ($\lambda > 700$ nm) showed an increased carotenoid content compared to those without far-red supplementation. Cell density was higher with white light but cell volume was lower compared to cultures supplemented with far-red light (Sánchez-Saavedra *et al.*, 1996). An increase in β-carotene accumulation was, as well, recorded in studies that supplemented visible light with ultraviolet-A radiation (340 - 400 nm) in cultures of *D. bardawil* and a carotenogenic *D. salina* (Jahnke, 1999), pointing to a screening effect of β-carotene against high energy wavelengths. This protective effect was manifested by a smaller drop in $Fv/Fm$ between *D. bardawil* with a high β-carotene content compared to a low β-carotene *D. bardawil* and a non-carotenogenic *D. salina,* both of which showed a significant decrease in photosynthetic efficiency (White and Jahnke, 2002). This effect was already observed by Ben-Amotz et al (1989) in a β-carotene rich *D. bardawil* exposed to high irradiances of blue, white, and red light, where only the treatment under blue light did not show any signs of photoinhibition (Ben-Amotz *et al.*, 1989). More recent works showed that monochromatic narrowband red light caused a decrease in growth rate at irradiances higher than 170 $\mu E\ m^{-2}\ s^{-1}$, but the addition of blue light allowed growth at higher intensities and up-

regulated carotenoid synthesis in *D. salina*, hypothesizing the existence of a photo-regulation system involving blue light (Fu *et al.*, 2013). However, this is controversial, since other works have shown that red light produced the highest carotenoid accumulation (Xu and Harvey, 2019a, 2019b).

Regarding non-carotenogenic species of *Dunaliella, D. tertiolecta* is characterized by a rapid decline in photosynthetic capacity, quantum yield, and a decrease in chlorophyll to carotenoid ratio as a response to nitrogen-limited conditions (Sosik and Mitchell, 1991; Geider *et al.*, 1998; Young and Beardall, 2003). However, studies are scarce on the effect of light quality on growth. Among the existing studies, no difference in cell division rate of *D. tertiolecta* was observed when different wavelengths were applied (Rivkin, 1989; Tang *et al.*, 2011), but differences on metabolism and carbon uptake and allocation were recorded (Rivkin, 1989), although the cultures reached stationary phase due to a light limitation and were not submitted to any type of high-light stress.

# Results

## 1. Effect of light quality on *D. tertiolecta* and *D. salina*: growth, pigment content, and photosynthetic response

The effect of blue (450 nm), red (660 nm), and a mix of red and blue light with a red: blue ratio of 1: 1, with a continuous incident irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$, was studied on cultures of *D. tertiolecta* and *D. salina* maintained at 20 ºC in 100 mL cylindrical glass mini-photobioreactors (50 mm diameter). Cultures were inoculated at a biomass concentration of around 0.15 g $L^{-1}$ and a chlorophyll concentration of 5 mg $L^{-1}$, equivalent to a cell concentration of 2.4 and 1.4 million cells $mL^{-1}$, respectively. pH was maintained between 7.2 and 7.5.

Growth, measured as cell density, was not affected by light quality in *D. tertiolecta* while in *D. salina* slightly higher cell densities were achieved with blue-light and red-blue light ($p < 0.05$) (Figures 3.1A and 3.1B). After the transference of the pre-culures (1 L PBR, 100 mm diameter) to the experimental setup (100 mL PBR, 50 mm diameter), stationary phase was achieved due to nitrogen depletion after 3 days in *D. tertiolecta*. In *D. salina* cell number continued to increase until day 5 in all conditions despite nitrate was exhausted and could not be detected in the culture media on day 3 in any of the conditions. On the contrary, when growth was estimated as dry weight, a clear impact of the irradiance wavelength composition was observed in both species (Figures 3.1C and 3.1D).

As expected, highest biomass concentration was achieved with red-light in both species, followed by red-blue and blue-light. In *D. tertiolecta* dry weight continued to increase until day 5, despite cell division ceased on day 3. The onset of dry-weight stationary phase was achieved on day 3 for *D. salina* maintained in red-light, despite the cell density continued to increase in those cultures. For blue-light and red-blue light treatments dry weight continued to increase until day 5, and the differences between these two treatments remained stable until the end of the experiment ($p < 0.01$). Dry weight of the blue-light treated cultures remained lower during the whole experiment ($p < 0.01$) but no significant differences could be found

between red-light and red-blue light treatments by the end of the experiment for *D. salina* ($p > 0.05$).

In *D. tertiolecta*, dissolved oxygen concentration clearly decreased from values of 14-16 mg $L^{-1}$ during logarithmic phase (days 0 to 3) to values close to 7.4 mg $L^{-1}$, the oxygen saturation value for 35 ppt salinity seawater at 20 ºC, with the onset of stationary phase on day 5, with no differences among treatments ($p > 0.05$) (Figure 3.2A). Surprisingly, while growth, measured as both, cell density and dry weight, was maintained in *D. salina* until day 5, oxygen concentration decreased drastically in all treatments from the very high concentrations observed on day 0 (20 mg $L^{-1}$), to a value of 10 mg $L^{-1}$ on day 3, which is above saturation point for dissolved oxygen in 75 ppt seawater at 20 ºC (5.84 mg $L^{-1}$), remaining stable thereafter, with no significant differences observed among treatments during stationary phase ($p > 0.05$, Figure 3.2B).

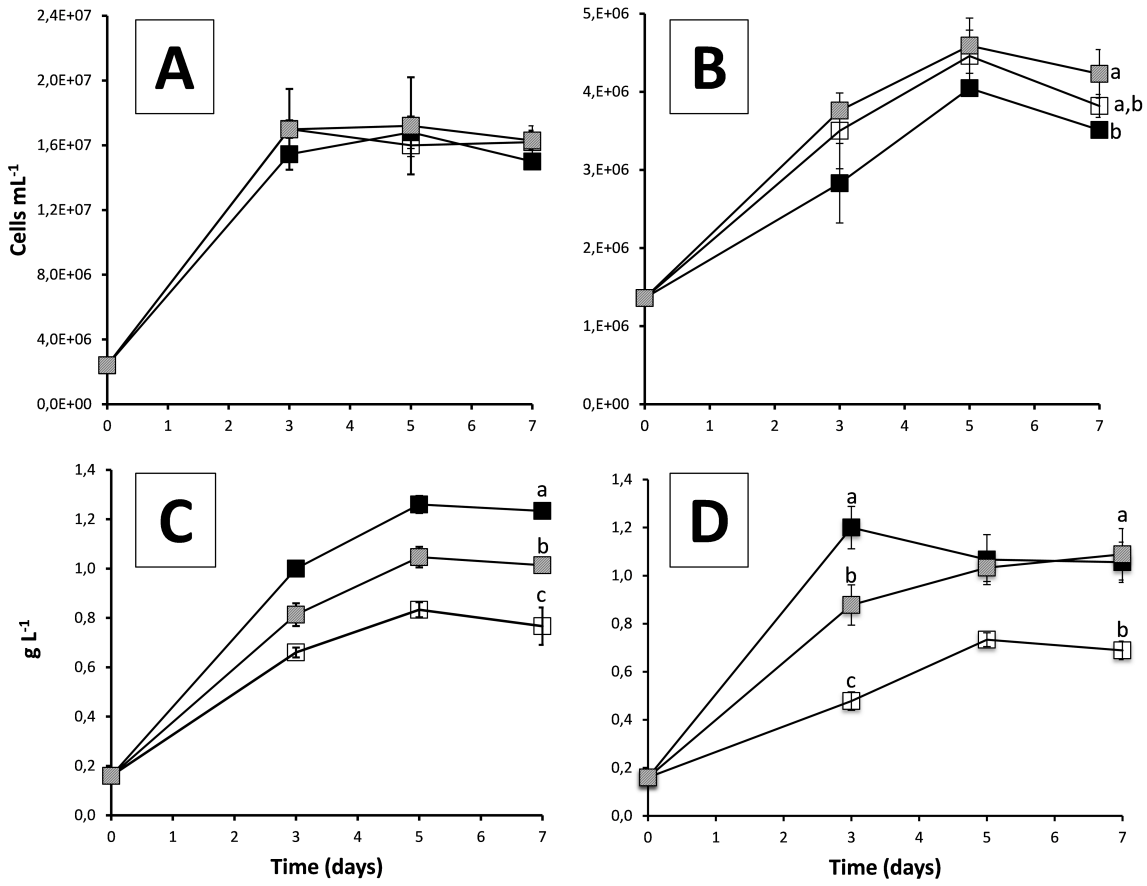



**Figure 3.1. Cell density (cells $mL^{-1}$) for *D. tertiolecta* (A) and *D. salina* (B), and biomass concetration (g $L^{-1}$) for *D. tertiolecta* (C) and *D. salina* (D) cultures grown under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open squares), red (closed squares), and mix of red and blue (grey squares). Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.01$).**

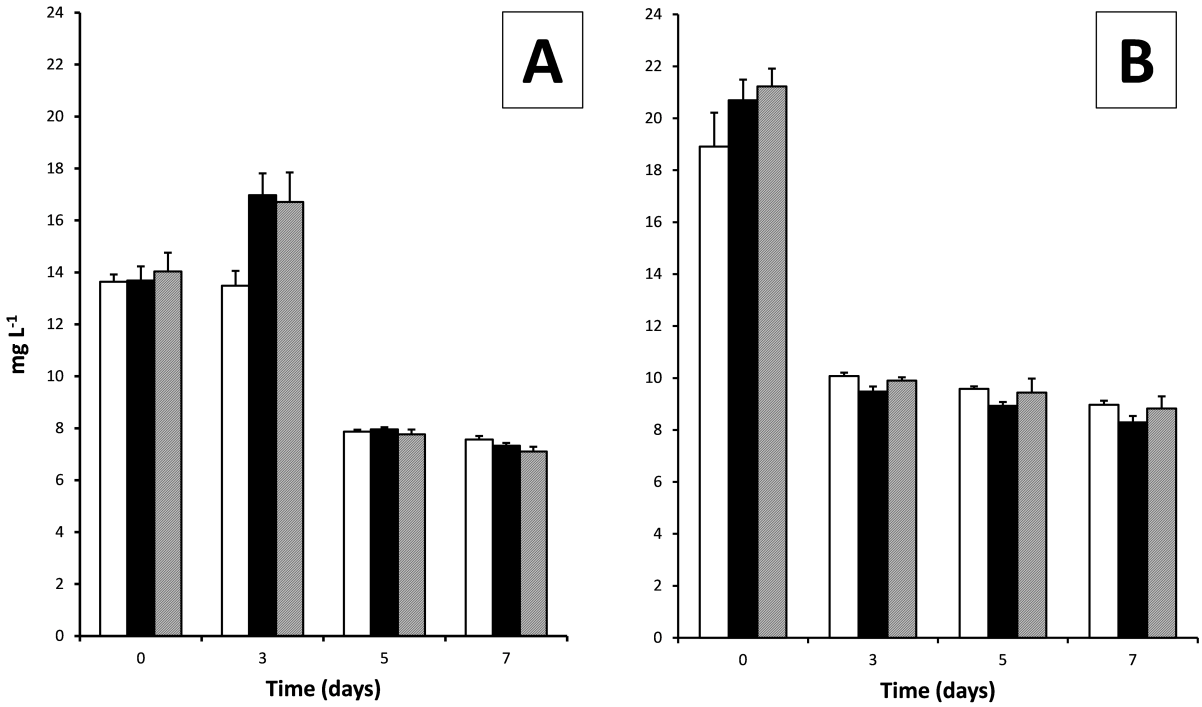



**Figure 3.2. Dissolved oxygen (mg $L^{-1}$) in the batch cultures of *D. tertiolecta* (A) and *D. salina* (B) under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n* = *3*).**

Total chlorophyll content in the biomass, expressed as mg $g^{-1}$ of dry weight, decreased for the non-carotenogenic *D. tertiolecta* from day 0 to day 3, and remained stable thereafter. Statistically significant differences were observed among treatments, since in blue-light cultures chlorophyll content remained higher than in the treatments where red light was present ($p < 0.01$) (Figure 3.3A). Total carotenoid content in this species decreased with the onset of stationary phase for red-light and red-blue treatments, while no decrease in carotenoid content was observed in blue-light cultures, which was constant throughout the experiment around 10 mg $g^{-1}$ of dry weight (Figure 3.3C).

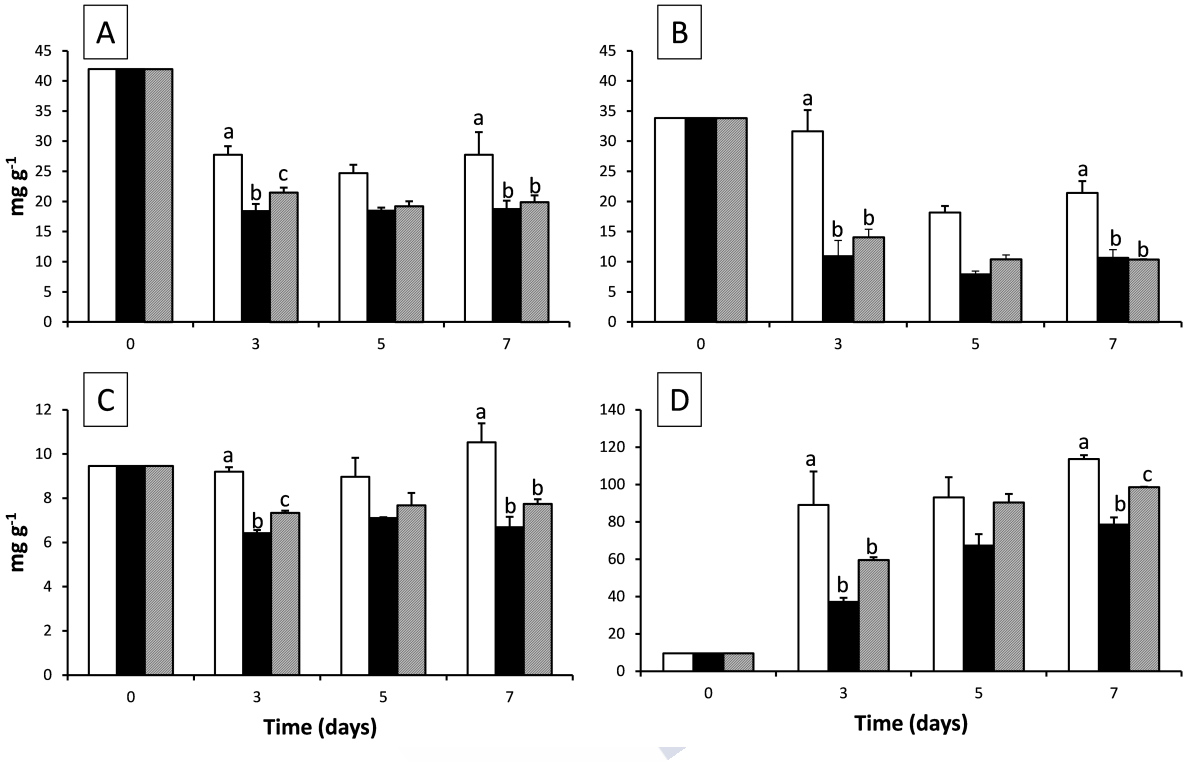



**Figure 3.3. Total chlorophyll and total carotenoid biomass content (mg $g^{-1}$) in batch cultures of *D. tertiolecta* and *D. salina* under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. A and C represent total chlorophyll and total carotenoid content for *D. tertiolecta*; B and D represent total chlorophyll and total carotenoid content for *D. salina*. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

In *D. tertiolecta* the ratio total chlorophylls to total carotenoids decreased by 30 % on day 3, when stationary phase was achieved, with respect to the value observed prior to the transfer to high light conditions on day 0, and stabilized around a value of 2.5 that remained constant during the stationary phase, showing no differences between treatments, with cultures maintaining a dark green colour (Figure 3.4A).

In *D. salina,* a reduction of total chlorophyll biomass content throughout the experiment was observed once the stationary phase had been achieved. The decrease was more significant for red-light and red-blue light treatments compared with blue-light. In the treatments that contained red light a marked decrease was observed between days 0 and 3 and then chlorophyll content remained stable around 10 mg $g^{-1}$ until the end of the experiment. On the other hand, a decrease could barely be observed in blue-light cultures from day 0 to 3, where

content stayed at about 30-35 mg $g^{-1}$. A drop to values around 20 mg $g^{-1}$ was observed on day 5 in this treatment with no further decrease until the end of the experiment (Figure 3.3B). Total carotenoid content of *D. salina* increased during the experiment up to values in the range of 80 to 120 mg $g^{-1}$ by day 7, which was reflected in the development of a yellow to orange colour in all treatments (Figure 3.5). Blue-light cultures showed the highest accumulation rate, having accumulated around 80 mg $g^{-1}$ by day 3 in comparison with red-light and red-blue cultures that only reached 40 and 60 mg $g^{-1}$ by the same day, respectively. At the end of the experiment, total carotenoid content was the highest in blue-light cultures reaching about 120 mg $g^{-1}$, followed by red-blue with values around 100 mg $g^{-1}$ and red-light with 80 mg $g^{-1}$ ($p < 0.01$) (Figure 3.3D). As a consequence of carotenoid accumulation in *D. salina*, the ratio total chlorophyll to total carotenoid dropped drastically from an initial value of 3.5 to values around 0.3 on day 3. On day 7 the ratios were 0.19 ± 0.02, 0.14 ± 0.01, and 0.11 ± 0.00 for blue-light, red-light, and red-blue light treatments respectively, being significantly higher in blue-light cultures with respect to cultures where red light was present ($p < 0.01$), mainly derived from the higher chlorophyll content (Figure 3.4B). As a consequence, the cultures grown under blue-light looked more greenish, and cultures grown in red-blue and red-light looked dark and pale orange respectively (Figure 3.5).

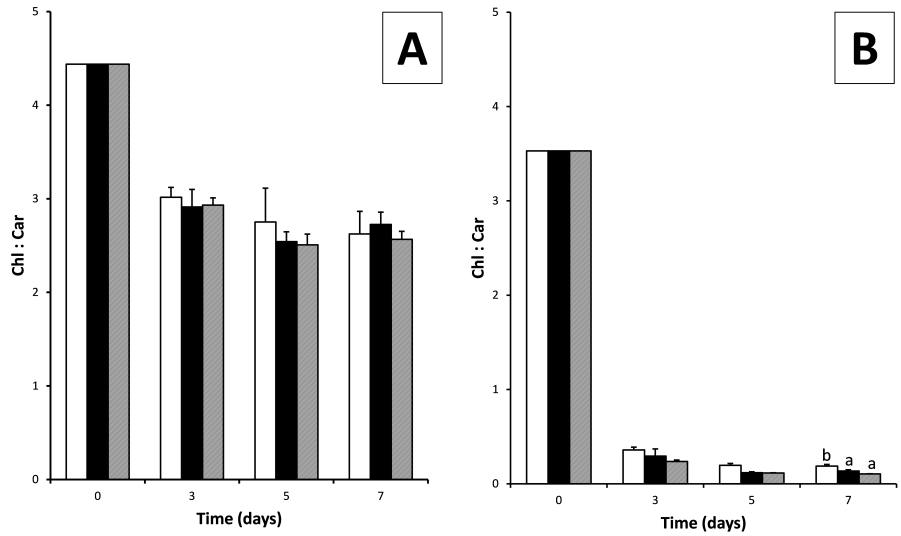



**Figure 3.4. Chlorophyll to carotenoid ratio in batch cultures of *D. tertiolecta* (A) and *D. salina* (B) under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n = 3*).**

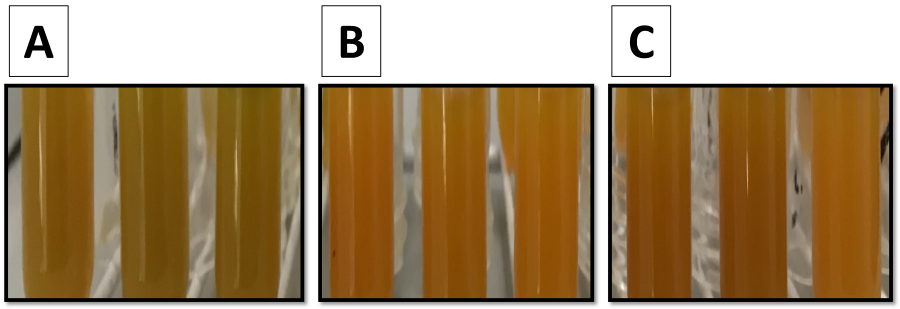



**Figure 3.5. Physical appearance of stationary phase cultures of *D. salina* grown under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (A), red (B), and mix of red and blue (C).**

When pigment concentration was computed, total chlorophyll and total carotenoids concentration for the non-carotenogenic *D. tertiolecta* increased up to day 5, showing the same trend observed in biomass concentration, with no statistically significant differences between treatments ($p > 0.05$) (Figures 3.6A and 3.6C). The total carotenoid concentration in this species remained low, with maximal concentrations around 8 mg $L^{-1}$, irrespective of the treatment (Figure 3.6C). In *D. salina* all cultures showed an almost equal initial increase in total chlorophyll and total carotenoid concentration but showed differences in pigment concentrations between treatments with the onset of stationary phase. Blue-light treated cultures maintained a higher total chlorophyll concentration than the other treatments, remaining constant around 15 mg $L^{-1}$ up to day 7 (Figure 3.6B). The highest total carotenoid concentration was achieved in red-blue treated cultures, reaching 113 ± 2 mg $L^{-1}$ on day 7 (Figure 3.6D), a value 40 % higher than the concentration achieved in red-light treated and blue-light treated cultures ($p < 0.01$).

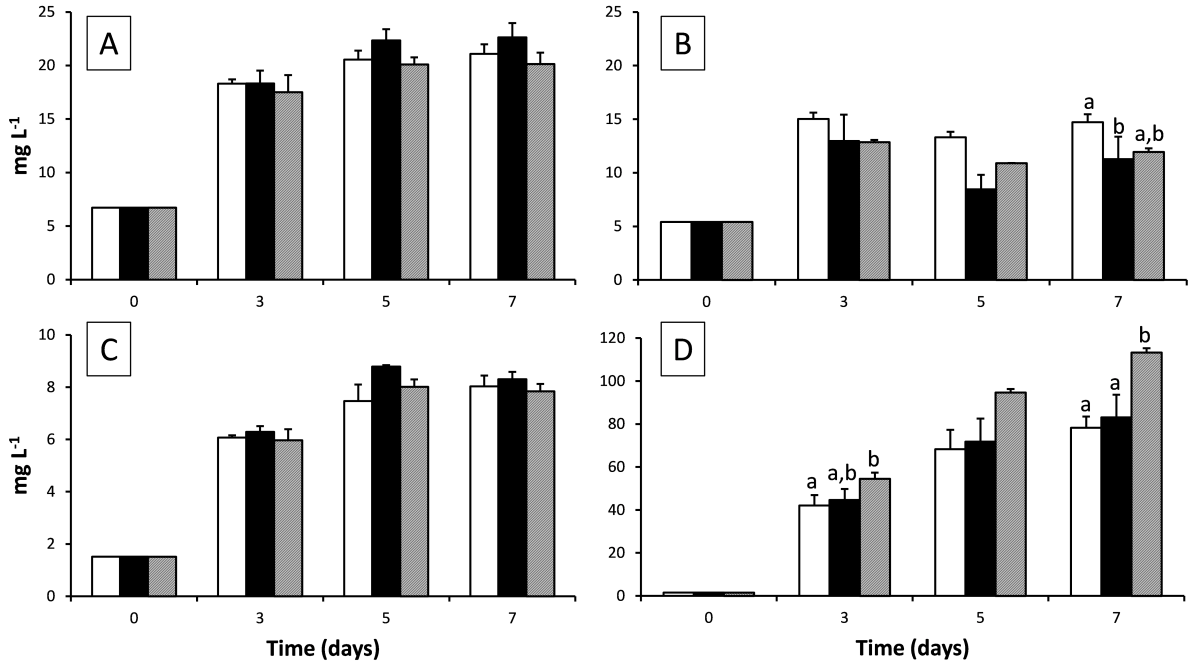



**Figure 3.6. Chlorophyll and carotenoid concentrations in batch cultures of *D. tertiolecta* and *D. salina* under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. A and C represent chlorophyll and carotenoid concentration for *D. tertiolecta*; B and D represent chlorophyll and carotenoid concentration for *D. salina*. Vertical bars represent S.D. (*n* = *3*).**

In terms of carotenoid composition, β-carotene was the dominant carotenoid in *D. salina*, which was confirmed by TLC. Carotenoid profile was more balanced in *D. tertiolecta,* where a carotene was also present with lutein as the major carotenoid (Figure 3.7).

The HPLC analysis on day 7 showed similar relative proportions of minor carotenoids, including neoxanthin, violaxanthin, anteraxanthin, and lutein in both species (Figure 3.8). Some additional peaks were present in the analysis of both species that remained unidentified using the available standards.

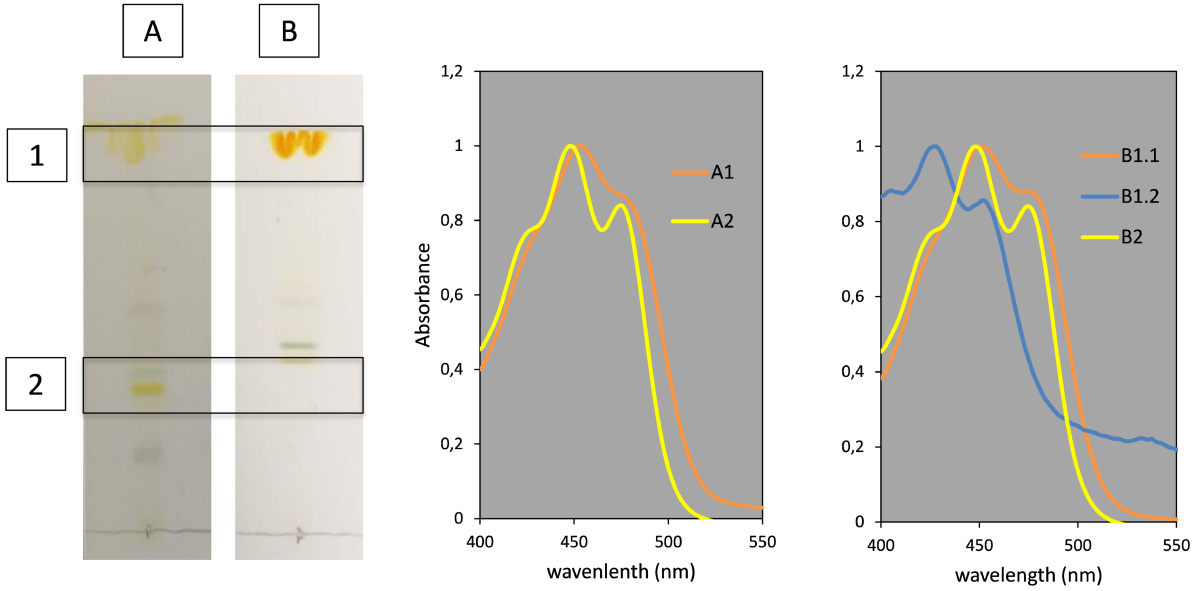



**Figure 3.7. TLC analysis and absorption spectra after elution with pure acetone of the detected carotenoids for *D. tertiolecta* (A) and *D. salina* (B) by the end of the experiments (day 7). Region 1 shows pigments with a high $R_f$ value, closer to the solvent front, lower polarity. In region 1, $R_f$ of 1.1 is higher than that of 1.2. Region 2 shows pigments with a lower $R_f$ value and a higher polarity.**

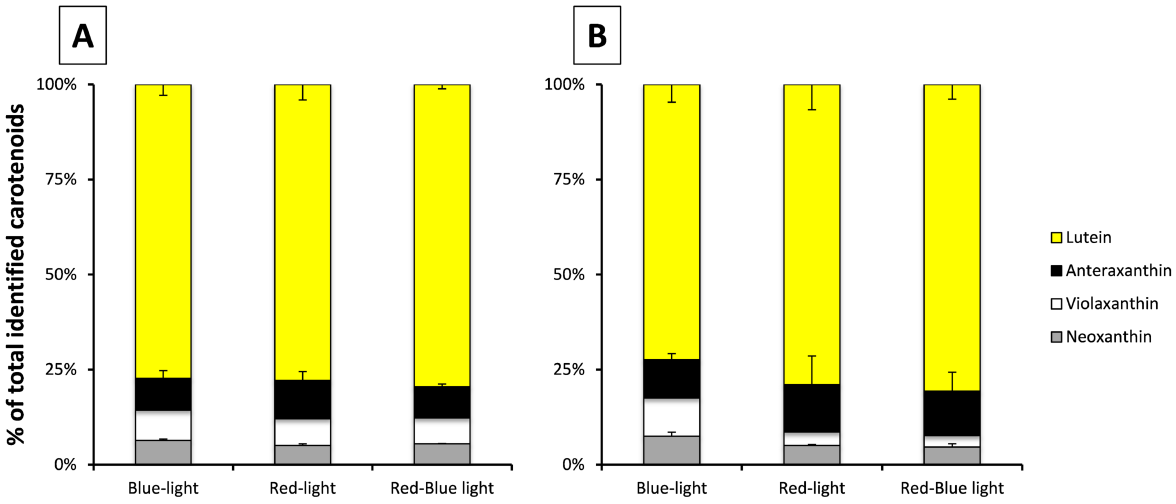



**Figure 3.8. Carotenoid composition in percentage of total identified carotenoids in day 7 of batch cultures of *D. tertiolecta* (A) and *D. salina* (B) maintained under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$. Vertical bars represent S.D. (*n* = 2).**

Effective photosystem II quantum yield ($Y_{II}$) decreased for all cultures when transferred from the pre-cultures to the experimental setup, due to the increase in effective irradiance derived from the higher light intensity and lower optical path of culture units. $Y_{II}$ in the pre-cultures was 0.76 and 0.73 for *D. tertiolecta* and *D. salina* respectively (data not shown), and dropped to around 0.55 and 0.40 for *D. tertiolecta* and *D. salina,* respectively, during the first hours after the transfer to high light conditions (Figure 3.9). Resembling dissolved oxygen trend (Figure 3.2), in *D. tertiolecta* the PSII quantum yield remained stable on day 3 but dropped thereafter to values around 0.25 for all treatments, with no significant change being recorded thereafter (Figure 3.9A). A similar drop in effective quantum yield was recorded for *D. salina* cultures when red light was present, while $Y_{II}$ remained stable in the cultures maintained in blue-light with values around 0.40 until the end of the experiment (Figure 3.9B). Relative electron transport rate showed the same pattern (data not shown).

Photochemical fluorescence quenching ($qP$) dropped to 0 in *D. tertiolecta* on day 5 while non-photochemical fluorescence quenching ($qN$) increased throughout the experiment for this species in all treatments, achieving maximum values of 1.5 (Figures 3.10A and 3.10C). For *D. salina*, $qP$ values suffered an initial drop on day 3 and remained stable thereafter for blue-light and red-blue cultures, with values around 0.6-0.8. The values of $qP$ in red-light treated cultures of D. *salina* seemed to decrease continuously (Figure 3.10B). In this species $qN$ was low irrespective of the treatment ($qN < 0.6$) in comparison with *D. tertiolecta*, remaining stable until the end of the experiment (Figure 3.10D).

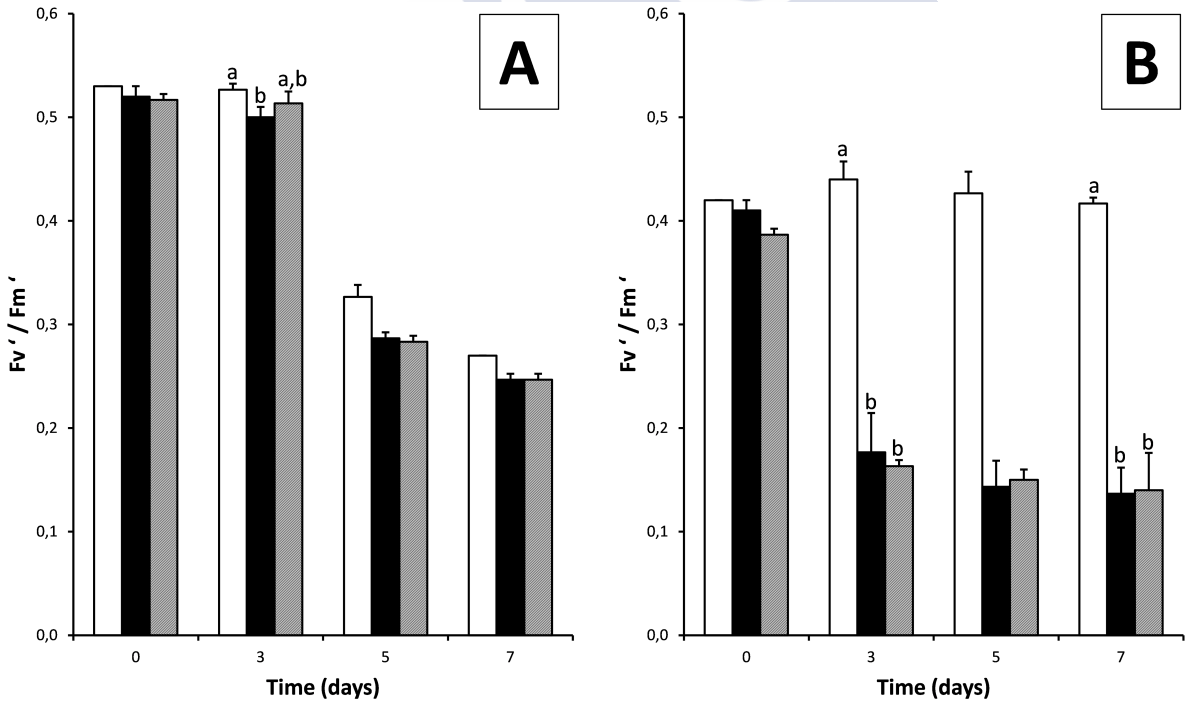



**Figure 3.9. PSII effective quatum yield in batch cultures of *D. tertiolecta* (A) and *D. salina* (B) under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n* = *3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

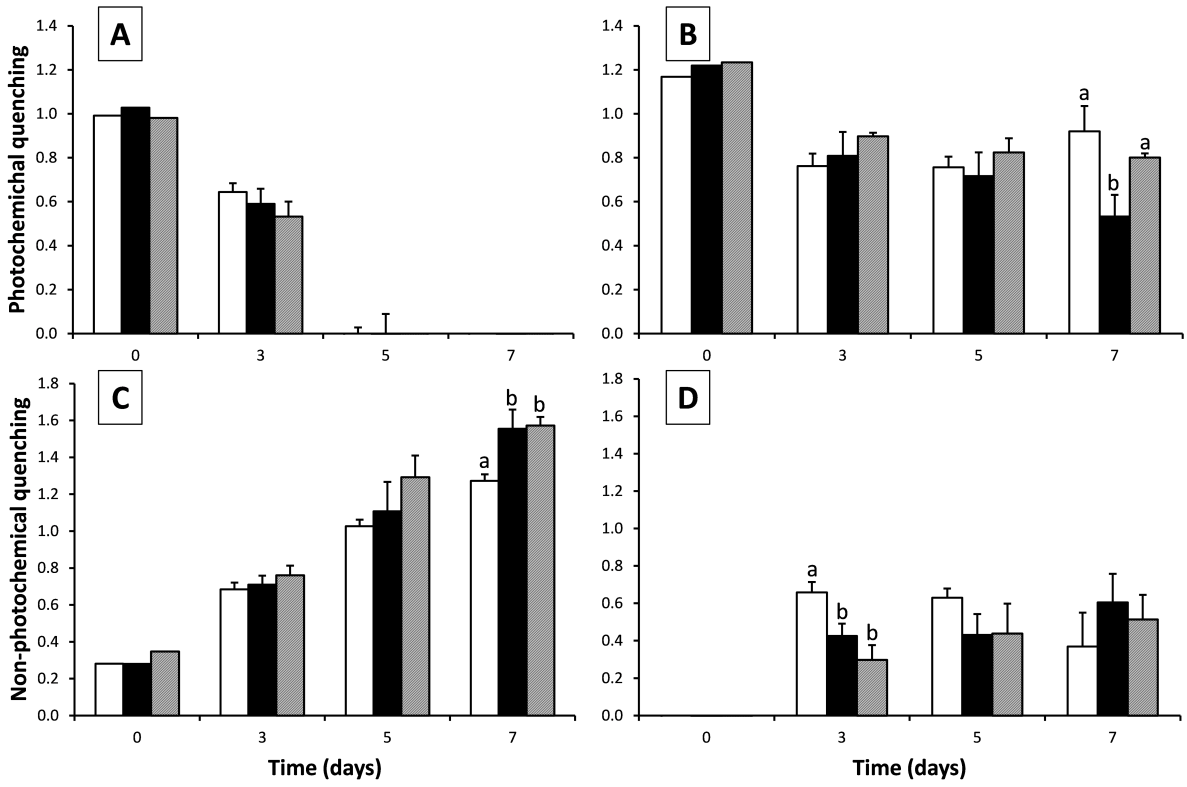



**Figure 3.10. Photochemical ($qP$) and non-photochemical ($qN$) chlorophyll fluorescence quenching in batch cultures of *D. tertiolecta* and *D. salina* under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. A and C represent photochemical and non-photochemical quenching for *D. tertiolecta*; B and D represent photochemical and non-photochemical quenching for *D. salina* Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

Differences were also visible when rapid fluorescence induction kinetics (OJIP curves) were compared at the end of the experiment. No differences in fluorescence rapid induction response were found between treatments for *D. tertiolecta*, while blue-light treated cultures of *D. salina* showed significantly lower J and I inflections in comparison with red-light and red-blue light treated cultures (Figures 3.11A and 3.11B).

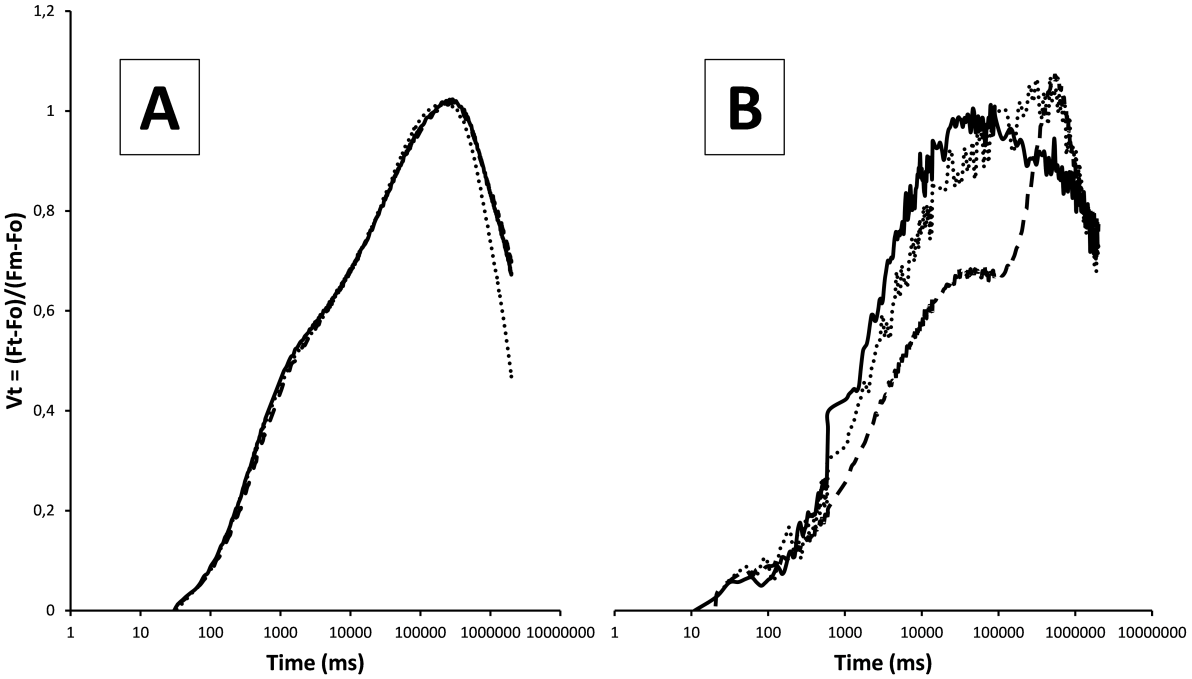



**Figure 3.11. OJIP curves of a representative stationary phase culture of *D. tertiolecta* (A) and *D. salina* (B) under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (discontinuous line), red (dotted line), and mix of red and blue (continuous line). X axis is reresented in log-scale.**

## Discussion

The results have shown that for both, the non-carotenogenic *D. tertiolecta* and the carotenogenic *D. salina*, maximum biomass concentrations were achieved under red light (Figures 3.1C and 3.1D). In this respect, the response was similar to that observed during the optimization of *H. pluvialis*, described in Chapter one, where red light enhanced biomass concentration compared to blue wavelengths (Figures 1.3A and 15A). Moreover, these results are in agreement with other studies made with marine green algae. Kumar et al. (2017) found that treatments with red light as main light source maximized biomass concentration, lipid content, and $CO_2$ sequestration rate in *D. tertiolecta.* Abiusi et al (2014) reported the same behaviour for biomass concentration of *Tetraselmis suecica* grown in red and blue monochromatic light. On the other hand, for both *Dunaliella* species little or no differences in cell density were found between treatments (Figures 3.1A and 3.1B). Same results were obtained for *D. tertiolecta* cultures grown in red compared to bright white LEDs (Tang *et al.*, 2011), and for *D. salina* grown at different light colour temperatures (Pavón-Suriano *et al.*, 2018; Xu and Harvey, 2019a). This indicates that different wavelengths may influence differently the amount of carbon fixation and allocation regardless of the cellular division, as already stated by Rivkin (1989) who reported that in *D. tertiolecta,* high intensity blue light

enhanced cell carbon and protein content per cell in contrast with the same intensity of red light, which increased polysaccharide and lipid content of the cell.

Pigment content of *D. tertiolecta* was influenced by different incident light quality (Figures 3.3A and 3.3C). Content of both chlorophylls and carotenoids was the highest in cultures maintained with blue-light, while it was about 20 % lower in cultures illuminated with red-light and red-blue light. However, no differences could be observed when total chlorophyll and total carotenoid concentration were computed, that increased with the same pattern as the increase in biomass (Figures 3.6A and 3.6C). Only total chlorophyll to total carotenoid ratio decreased once nitrogen was depleted (Figure 3.4A), but remained equal for the three wavelengths tested during stationary phase. The drop in total chlorophyll to total carotenoid ratio was accompanied by a drop in oxygen production (Figure 3.2A), $Y_{II}$ and $rETR$ (Figure 3.9A), and $qP$ (Figure 3.10A). Similar results have been described in nitrogen starved cultures of *D. tertiolecta* (Young and Beardall, 2003). Those studies showed a drop in $P_{max}$, measured in our case by $rETR$, that has been attributed to a decrease in carbon fixation capacity of RuBisCO and not to a decrease in chlorophyll content, since N stored in RuBisCO could be mobilized to support other cell functions (Sukenik *et al.*, 1987). Indeed, no chlorophyll degradation was observed between days 3 and 7, under nitrogen starvation, in our *D. tertiolecta* cultures (Figure 3.3A), indicating that the observed decrease of $rETR$, dissolved oxygen (Figure 3.2A), and $Y_{II}$ (Figure 3.9A) may be the result of other processes, which could certainly be in line with the one mentioned above.

Despite showing these typical signs of photoinhibition (Krause and Weis, 1991), *D. tertiolecta* did not present any obvious damage such as chlorosis or loss of motility, demonstrating a resourceful and flexible mechanism to deal with such adverse conditions, not dependent on massive carotenoid accumulation but on a different energy dissipation mechanism reflected in a clear increase of non-photochemical quenching (Figure 3.10C). Despite the HPLC analyses should be considered as non-conclusive, since not all the peaks could be identified, and evidenced by the TLC analysis and following spectrophotometric analyses of the eluted bands (Figure 3.7) that showed at least a high $R_f$ yellowish pigment close to the solvent front with absorption maxima at around 450 and 475 nm which must be a carotene (Lichtenthaler, 1987), it looked like in our *D. tertiolecta* cultures the energy dissipation process depended in the action of xanthophyll cycle specific pigments. Under conditions of excess light, xanthophyll cycle works de-epoxidizing violaxanthin via anteraxanthin to zeaxanthin, rising its content (Lubián and Montero, 1998). Interestingly, no zeaxanthin could be identified. Instead, a large amount of lutein was reported in the analysis (Figure 3.8) that was confirmed by elution from the TLC as the main carotenoid (Figure 3.7), which is in line with HPLC chromatograms reported in the literature for this species (Ben-Amotz *et al.*, 2009). However, we believe that, due to the similar chromatographic mobility of zeaxanthin and lutein ($R_f$ = 0.4) (Lichtenthaler, 1987), which are isomer molecules, the peak identified as lutein could very well account for both pigments. However, we could not confirm this since the ratio lutein to zeaxanthin must be very high and the absorbance of lutein from the eluted stripe must cover zeaxanthin's spectra. A direct correlation between these pigments and non-photochemical quenching processes has been described (Bilger and Björkman, 1991; Yamamoto and Bassi, 1996; Jahns and Holzwarth, 2012), which would explain the strong increase of $qN$ (Figure 3.10C) observed in all treatments during the experiment. On the contrary, photochemical quenching ($qP$) dropped to zero after day 3 independently of the light quality (Figure 3.10A). This equal response of the *D. tertiolecta*

cultures regardless of the light quality was also reflected in the rapid fluorescence induction kinetics, where no difference in redox state of PSII could be observed (Figure 3.11A). More authors have described similar photoprotective and repair mechanisms in *D. tertiolecta* submitted to ultraviolet radiation stress including cells that maintained a reduced but operative $ETR$, decreased PSII quantum yield and increase ROS scavenging mediated by xanthophyll cycle, all at the expense of growth (Segovia *et al.*, 2015).

The apparent shut down in photosynthetic activity observed in stationary phase *D. tertiolecta* was only clear for *D. salina* cultures grown in red-light and red-blue light, that showed a similar behaviour to *D. tertiolecta* for biomass concentration (Figure 3.1D), dissolved oxygen (Figure 3.2B), $Y_{II}$ (Figure 3.9B) and $rETR$. Nevertheless, dissolved oxygen values remained over the saturation values of 75 ppt salinity seawater at 20 ºC, indicating the existence of photosynthetic activity to a certain extent. On the contrary, *D. salina* cultures grown in blue-light did not show a drop in $Y_{II}$ (Figure 3.9B) nor $rETR$, which remained high until the end of the experiment. Related to this, chlorophyll content in *D. salina* cultures grown in blue-light remained twice as high with respect to the treatments where red light was present, where a larger decrease in chlorophyll content and PSII quantum yield with the onset of stationary phase were observed (Figure 3.3B and 3.9B). This response is very similar to that observed in *H. pluvialis*, where blue-light cultures also maintained the highest chlorophyll content during stationary phase (Figures 1.5D and 1.6). The phenomena, as hypothesized for *H. pluvialis*, could be related to the interaction between different incident light emission and biomass action spectrums. As in *H. pluvialis* (Chapter one, section 4) the carotenoids accumulated by *D. salina*, absorb light in the range of 400 to 500 nm, and therefore act as a perfect umbrella for the photosynthetic apparatus in the cultures maintained in the 450 nm narrowband blue-light, reducing the photodamage that may be caused by high incident irradiance over the photosynthetic apparatus.

*D. salina* cultures grown in red-light and red-blue light ($Y_{II}$ 0.15, $rETR$ 20) showed a clear increase in total carotenoid content, as well as a significant drop in chlorophyll content (Figures 3.3B and 3.3D). This could be explained by the fact that even though carotenoid synthesis was accelerated, the protection against an incident high light intensity of 660 nm would be ineffective and the chlorophyll would still be damaged in the uncovered low energy absorption region, confirming a main light-screen function of accumulated carotenoids against blue wavelengths, preventing cellular damage (Cohen, 1999), which is in line with the results observed by Ben-Amotz et al. (1989). Interestingly, despite the clear drop in $Y_{II}$, and contrary to what was observed in *H. pluvialis*, where treatments containing red light responded increasing non-photochemical fluorescence quenching ($qN$) and reducing photochemical fluorescence quenching ($qP$) (Figure 1.8), in *D. salina* these parameters remain undifferentiated among treatments (Figures 3.10B and 3.10D) revealing a probable different action mode to that of astaxanthin, which could be related with the intraplastidic location of β-carotene. Moreover, this result seems to indicate that carotenoid accumulation is produced as a result of damage of the photosynthetic apparatus, independently of its effectiveness to protect the photosystems against high incident irradiance. In this respect, some authors suggested that carotenoid synthesis was up-regulated by photosystem stress and accumulation of ROS in the cell, generating oxidative stress (Boussiba *et al.*, 1999; Murthy *et al.*, 2005; Ye *et al.*, 2008; Tammam *et al.*, 2011; Galasso *et al.*, 2017). It could be expected that, since β-carotene protects the photosystems against blue photons, the red-light treatment would generate more stress and could have triggered maximum carotenoid accumulation, but

actually was the blue-light treatment the one that presented the fastest response (9% carotenoid content on day 3) and highest carotenoid content with respect to the other treatments by the end of the experiment (120 mg $g^{-1}$), followed by red-blue and red-light (Figure 3.3D).

As it was expected, TLC evidenced that β-carotene was the major carotenoid accumulated in *D. salina,* which was confirmed by the elution of the thick orange band right next to the solvent front that showed maximum absorbance peaks at 452 and 477 nm (Figure 3.7), in line with absorption maxima of β-carotene in pure acetone (Lichtenthaler, 1987). Moreover, another carotene with a lower $R_f$ value than β-carotene could be detected with TLC and its elution showed that absorption maxima of this pigment was shifted around 25 nm toward shorter wavelengths with respect to β-carotene or lutein (Figure 3.7). This pigment remained unidentified by HPLC analyses but considering its spectrophotometric characteristics we hypothesize that it could be α-carotene or β-zeacarotene (Gross, 1991). The relative proportion among the minor carotenoids that were identified by HPLC in *D. salina* was similar to that in *D. tertiolecta* (Figure 3.8B). As a consequence of this pigment composition, the photosynthetic response in this species must be driven by massive β-carotene accumulation. Accumulation under blue-light resulted in a higher photoprotection and therefore a faster acclimation to the disruption of homeostasis caused by nutrient limitation and continuous high irradiance, denoted by the maintenance of a higher chlorophyll content (Figure 1.3B) and PSII photosynthetic efficiency (Figure 3.9B). This could suggest that the signal for *D. salina* to massively synthesize carotenoids may be accelerated and maximized by the presence of blue light. Enhanced carotenoid accumulation under blue-light cultivation has been already described for other well-known carotenogenic species like *H. pluvialis* (Kobayashi *et al.*, 1992).

Reminding *H. pluvialis* response (Figure 1.5), carotenoid concentration was the highest in *D. salina* cultures maintained with red-blue light (Figure 3.6D). Hence carotenoid production would be maximized when blue light is used in combination with red light due to the synergistic effects of the two wavelengths combined: blue-light enhancing carotenoid content per cell (Figure 3.3D) while red-light maximized biomass concentration (Figure 3.1D). Interestingly, we did not observe differences in PSII redox state (Figure 3.11B), nor photosynthetic efficiency measured by PSII effective quantum yield between red-light and red-blue treatments, which remained low despite the difference in absolute red-light content of the light source (300 and 150 photon $m^{-2}$ $s^{-1}$, respectively) (Figure 3.9B). Within the parameters that were recorded, the main difference between these treatments seemed to be related with carbon fixation and allocation. Red light alone generated the highest biomass concentration during logarithmic growth but reached a plateau on day 3 (Figure 3.1D), after which massive carotenoid synthesis started (Figure 3.3D). On the contrary, when both wavelengths were present, biomass concentration showed an increasing trend after nitrogen depletion (Figure 3.1D) together with a higher carotenoid synthesis (Figure 3.3D), which may be related with the highest oxygen uptake rate that has been reported recently for red-light grown cultures with respect to blue-light grown cultures in *D. salina* (Xu and Harvey, 2019a). These results are in agreement with recent works (Fu *et al.*, 2013) that achieved maximum carotenoid concentration when blue was added to red light, despite the ratio red to blue and irradiance were different to the ones used in the present study. They hypothesize the presence of a blue-light dependent signal transduction pathway for triggering major carotenoids synthesis that is independent from red light.

The results included in this chapter showed how the choice of the correct light quality makes an important difference ($p < 0.01$) in artificial-light microalgae cultivation, since it does not only affect carotenoid synthesis but also other important parameters such as biomass concentration (Figures 3.1C and 3.1D). In *D. salina*, similar to *H. pluvialis*, taken into account that carotenoid-rich-biomass market price may change depending on the percentage of pigment contained in the biomass, mainly due to a decrease of downstream processing costs (McWilliams, 2018), the design of the light source reveals itself as a useful tool for the optimization of the microalgal production processes.

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# 4. EFFECT OF LIGHT QUALITY ON GROWTH, PIGMENT CONTENT, AND PHOTOSYNTHETIC RESPONSE OF THE RHODOPHYTA *P. cruentum* IN CONDITIONS OF NITROGEN DEFICIENCY AND CONTINUOUS ILLUMINATION

## Rationale

The investigations described on Chapters one and three studied, among other cultivation factors, the effect of light quality on cell density, biomass concentration, photosynthetic response, and pigment content of three species of green microalgae under conditions of continuous illumination and nitrogen limitation. *H. pluvialis* and *D. salina* are popular carotenogenic species that have been cultured at industrial-scale in order to produce the carotenoids astaxanthin and β-carotene, respectively. Carotenogenesis is triggered in these species by phenomena that increase intracellular ROS, being the combination of nitrogen limitation and high irradiance the parameters that produce the major carotenoid synthesis, which were known beforehand. Therefore, the investigations were expecting to add another layer of culture conditions optimization by studying the interaction between massive carotenoid production and light composition. The effect that light quality had on the photosynthetic response and carotenogenesis in both species was similar and, in general, blue light enhanced carotenoid content (Figures 1.5B and 3.3D) while red light increased biomass concentration (Figures 1.5A and 3.1D), but was the combination of both wavelengths the treatment that yielded the highest carotenoid productivity (Figures 1.5C and 3.6D). Moreover, massively accumulated carotenoids protected cells against blue light, mainly, which was reflected in the maintenance of a higher chlorophyll content (Figures 1.5D and 3.3B) and PSII photosynthetic efficiency in those treatments compared to treatments were red light was present (Figures 1.7 and 3.9B). Those results evidenced that light composition is an additional factor to take into account with exceptional importance in artificial-light microalgae cultivation and industrialization, as described and discussed in Chapter two for *H. pluvialis*. On the other hand, the non-carotenogenic *D. tertiolecta* was used to enrich the study in terms of fundamental physiology, in order to characterize, in parallel, the expected different response of a known carotenogenic (*D. salina*) and non-carotenogenic (*D. tertiolecta*) species subjected to the same conditions of nitrogen depletion and high irradiance of different wavelengths. *D. tertiolecta* responded in a very different way than *D. salina* (Chapter 3). The response triggered a strong energy dissipation mechanism in the non-carotenogenic species reflected in an increase of non-photochemical quenching together with an extreme reduction of photochemical quenching (Figures 3.10A and C), which must be driven by xanthophyll-cycle specific carotenoids. Opposite to the carotenogenic species, PSII effective quantum

yield dropped equally in every treatment (Figure 3.9A). Nevertheless, no signs of chlorosis or motility loss could be observed during microscopical examination so, despite being completely different to that triggered in *D. salina*, this response allowed *D. tertiolecta* to withstand those environmental conditions, proving to be as effective as the response of its carotenogenic relative.

In order to explore the effect of these factors on more diverse microalgal species, in this fourth chapter the effects of light quality on growth, photosynthetic response, pigment content and polysaccharide production of the Rhodophyta *P. cruentum* were studied under the same conditions of nitrogen depletion, continuous illumination, and different light composition that were applied to the other microorganisms in this work.

*P. cruentum* is scarcely studied compared to other common microalgal species. To put this statement into perspective, when a simple search in the WOS database (Web Of Science: https://www.recursoscientificos.fecyt.es/) was carried out, during the period between 1970 to 2018, 238 publications contain *P. cruentum* in their title, of which the major part was published in the 1980's (27 %) and 90's (24 %) and most of it (51 %) was generated in USA, France, Spain, and Israel, while the same search with *D. tertiolecta* showed 370 results, *D. salina* 731, *H. pluvialis* showed 529, and *C. vulgaris*, a well studied green algae that is also used in plant physiology studies, 2130. However, *Porphyridium* spp. has a huge industrial potential and a few companies are making efforts to culture it under outdoor conditions on a commercial-scale (Arad and Levy-Ontman, 2010) where the most optimistic estimates reported a potential productivity of 50 tons $ha^{-1}$ $year^{-1}$ of dry biomass (Vonshak *et al*., 1985).

Phycoerythrin is the main pigment in *P. cruentum*, and its content is closely related to both protein synthesis and photosynthetic apparatus response to environmental conditions. In terms of protein metabolism, it is widely evidenced that protein synthesis and demand is maximal under conditions that favour cell division, when protein synthesis is high. This can be clearly observed in semicontinuous cultures of a wide range of microalgae species, where protein content increases with renewal rate and therefore with metabolic activity, growth rate and nitrogen availability, which is understandable since protein synthesis requires nitrogen as a main building block (Fábregas *et al.*, 1995, 2001; Seixas *et al.*, 2009; Ferreira *et al.*, 2011). Specifically in *P. cruentum*, a study has shown how protein content decreased from 40 to 10 % with the onset of stationary phase in batch cultures (Arad *et al.*, 1985). With respect to the response of the photosynthetic apparatus, phycoerythrin is found in the phycobilisomes, which are arranged in the thylakoid membranes of the chloroplast. Therefore, it is evident that its abundance will be dependent on factors affecting plastid metabolism. In this respect, it is well known that microalgae cells increase storage compounds content when a stationary phase is reached, this is specially evident when growth cessation is caused by a nutrient deficiency such as nitrogen limitation. Under these conditions, when nitrogen is not available and carbon fixation is still active, carbon is allocated in compounds such as starch, or allocated into neutral lipids accumulated in lipid bodies. This increase in storage compounds requires some volume in the cells, which grow in size, and is normally accompanied by a decrease in plastids volume. Also, as it has been shown with other species included in this work, chlorophyll content decreases since its synthesis depends on nitrogen bioavailability as well and photosynthetic activity has to be re-dimensioned once nitrogen is limiting. *P. cruentum* cells growing exponentially are characterized by a very recognizable large plastid volume ordered in clear thylakoid membranes containing phycobilisomes. As soon as those cells were

submitted to a severe nitrogen limitation, plastid volume was reduced more than three-fold while starch volume increased six-fold and lipid volume increased by twelve. Moreover, thylakoids were less ordered and aggregated. These changes were accompanied by an extreme reduction of both chlorophyll and phycoerythrin content per cell, which has been hypothesized to work as nitrogen storage (Zhao *et al.*, 2016). As soon as nitrogen was replenished in the culture medium, cell composition went back to normal (Köst *et al*., 1983; Zhao *et al.*, 2016). However, when nitrogen is available, phycoerythrin concentration does not show a direct correlation to it, but concentration is maximal when nitrate is available and its transformation into intracellular nitrogen is close to maximum. This normally coincides with the intersection between nitrogen limited and saturated conditions, where cell density is also high and effective irradiance per cell is lower with respect to conditions of higher growth rate where irradiance per cell is higher. This point was found in semicontinuous cultures of *P. cruentum* maintained with a renewal rate of 10 % and an initial nitrate concentration of 16 mM $L^{-1}$. With lower renewal rates, where irradiance per cell is also low but cultures were submitted to a more severe nitrogen limitation, phycoerythrin concentration dropped dramatically (Fábregas *et al.*, 1998). Also, *P. cruentum* cells grown under high irradiance may look yellowish with respect to a lower irradiance level, where the red colour was maintained, and was closely related to the amount of protein and presumably with nitrogen and light availability (Arad *et al*., 1985). Yellow cultures have, as well, been observed in conditions of severe nitrogen starvation but the red colour was restored once nitrogen was added again to the culture medium (Zhao *et al.*, 2016). Furthermore, it has been proven that not only light intensity but also quality can affect the relative concentration of pigments in *P. cruentum* cultures. Cultures were illuminated either with blue or green light, as these wavelengths represent the PAR regions that are mainly absorbed by chlorophyll and phycoerythrin, respectively, therefore matching the action spectra of *P. cruentum*. When light intensity was low, the wavelength related to the pigments absorption spectra enhanced the synthesis of that pigment, following Engelmann's theory of chromatic adaptation. However, when light intensity was high, the opposite occurred and blue light enhanced phycoerythrin synthesis while green light enhanced chlorophyll synthesis. High-intensity blue light increased phycoerythrin to chlorophyll ratio by three-fold compared with low intensity blue light (Brody and Emerson, 1959). More recently, the importance of green light for *P. cruentum* growth has been shown, with differences between monochromatic red and blue lights compared with a combination of green, blue, and red light being found, that also produced the highest phycoerythrin content per cell (Baer *et al.*, 2016; Coward *et al.*, 2016). Additionally, monochromatic red and blue cultures showed very different pigment content. Chlorophyll, zeaxanthin, and β-carotene content were reduced significantly in red light compared to blue light (Coward *et al.*, 2016).

Polysaccharide production by *P. cruentum* is also strongly related to its growth rate. Two main polysaccharide fractions are differentiated, cell-bound polysaccharides and exopolysaccharides (EPS) that are dissolved in the culture medium. Both of these polysaccharide fractions were found to be qualitatively identical (Ramus, 1972). The dynamics of polysaccharide production by *P. cruentum* are very interesting. Apparently, polysaccharides are produced intracellularly and are secreted to the cell surface via membrane-mediated Golgi vesicles exocytosis. It has been postulated that its synthesis may be light dependant (Ramus, 1972; Arad and Levy-Ontman, 2010). During logarithmic growth phase, the rate of EPS dissolution is higher than the rate of production. The medium increases in viscosity but the cellular walls look thin, since there is no time for the polysaccharides that

are synthesized to accumulate around the cell, forming a gelatinous cover. On the contrary, when cells reach a stationary phase, a thick capsule is formed around the cells due to the higher production rate with respect to dissolution rate (Ramus, 1972). Furthermore, a high irradiance also enhanced total polysaccharides compared to a low irradiance, including attached and dissolved fraction where an increase up to 36 % per cell was reported, which accounted for a concentration of about 2.5 g $L^{-1}$, despite it was not clear if this effect was caused directly by the higher irradiance or the faster depletion of nitrogen due to the higher irradiance (Arad *et al*., 1985). In fact, when total EPS production was investigated in semicontinuous cultures of *P. cruentum*, the results clearly showed an inverse correlation between nitrogen availability and EPS production, which decreased with renewal rate, that is accompained by a higher effective irradiance per cell (Fábregas *et al.*, 1998). This was in concordance with the studies made in batch cultures where total EPS production was the highest in stationary phase. However, the contrary occurred with the sulfated EPS content, since the percentage of sulfatation increased with renewal rate, therefore being correlated to a higher nitrogen availability, irradiance per cell, and primary metabolic activity (Fábregas *et al.*, 1998). Recently, some studies have investigated the effect of light quality over EPS production. Results indicate that monochromatic lights may be used for stimulating the production of EPS by *P. cruentum*. Information is scarce, and the few studies available seem to agree on the fact that EPS production is higher with blue light compared to red light (You and Barnett, 2004; Sun *et al*., 2008). However, investigations have also shown the effects of combining red and blue wavelengths with other additional colours of the PAR spectrum. In this respect, the results rise some controversy. It seems that the addition of white light to blue light did not improve EPS production with respect to monochromatic red and blue light (You and Barnett, 2004). Opposite to that, other studies have shown that the addition of green light may improve the EPS production with respect to red or blue lights alone, despite the soluble carbohydrate fraction of the total EPS was higher with red, blue, or green monochromatic lights alone (Coward *et al.*, 2016). Interestingly, You and Barnett (2004) observed normal cell division in their cultures maintained under monochromatic red or blue, while Coward et al. (2016) did not report any cell division for cultures maintained under the same light colours.

The present chapter is focused on the study of the photosynthetic response under continuous illumination of different isolated light wavelengths and nitrogen limitation, complementing the results of Zhao et al. (2017), who studied the photosynthetic response under nitrogen limitation and a broad light spectrum. In brief, the results that are presented below reflect the effect that red, blue, and a mix of red and blue light have on the response of *P. cruentum* under nitrogen limitation and continuous illumination. The use of blue light seems less harmful than the use of monochromatic red light or a mix of red and blue light, which was reflected on a lower electron transport chain saturation level throughout the experiment that increased with the increase of red light in the light composition. An obvious increase in dry weight that can be associated with an increase in storage compounds, was observed in every treatment. However the use of blue light alone showed the highest increase in biomass concentration throughout the experiment. Blue light also exhibited the highest chlorophyll and phycoerythrin content and concentration during stationary-phase. The concentration of the latter remained four times higher than the concentration measured in red light. Interestingly, PSII quantum yield and electron transport rate of blue light illuminated cultures even increased during nitrogen starvation, which may be explained by a better adaptation of *P. cruentum* to this light quality. Furthermore, EPS concentration by the end of the experiment, measured as dissolved carbohydrates, was highest in blue-light cultures, which have also

maintained a deep red colour in comparison to the treatments where red light was present, where the characteristic red colour was lost and cultures looked yellow and orange once stationary-phase was achieved.

# Results

## 1. Effect of light quality on *P. cruentum*: biomass concentration, pigment content, EPS production, and photosynthetic response

The effect of blue (450 nm), red (660 nm), and a mix of red and blue light with a red: blue ratio of 1: 1, with a continuous incident irradiance of 300 μmol photon $m^{-2}$ $s^{-1}$, was studied on cultures of *P. cruentum* maintained at 20 ºC in 100 mL cylindrical glass mini-photobioreactors (50 mm diameter). Cultures were inoculated with logarithmic-phase growing cultures at an initial cellular density of 9.13 million cells $mL^{-1}$, which accounted for an initial biomass concentration of about 0.4 g $L^{-1}$. Cultures were grown with an initial nitrate concentration of 4 mM $L^{-1}$. Nitrogen could not be detected in the culture medium of any treatment on day 3 of cultivation. Viscosity in the cultures clearly increased with culture time, and between the start of the experiment and day 3 the cells developed a visible gelatinous matrix that covered and aggregated cells microscopically. For this reason the monitoring of growth by cell counting with a Bürker-Türk hemocytometer became a problematic methodology yielding non-consistent cell density values (data not shown). Therefore, growth was monitored mainly as biomass concentration, and dry weight of the samples was determined by washing the sample with 3 volumes of 0.5 M ammonium formate (Zhu and Lee, 1997).

Biomass concentration increased in every treatment between days 0 and 3 (Figure 4.1A). On day 3, the highest biomass concentration was measured in cultures maintained under red-light, followed by red-blue, and blue-light. Interestingly, in red-light maintained cultures biomass concentration stabilised on day 3, and showed no further change from that day to the end of the experiment. On the other hand, biomass concentration did not stabilised until day 5 for red-blue cultures, while it kept increasing during the entire cultivation period with blue-light, which achieved the highest biomass concentration by day 7, at about 4 g $L^{-1}$.

Dissolved oxygen values decreased for every treatment with culture time (Figure 4.1B). However the decrease was more pronounced in red-light cultures that started with an oxygen concentration of 22 mg $L^{-1}$ when the cultures were transferred to the experimental setup, therefore still in logarithmic growth phase, with respect to the end of the experiment where only 12 mg $L^{-1}$ were recorded. Interestingly, contrary to the increase in dry weight, dissolved oxygen values were the lowest in blue-light cultures except for day 7 where the lowest value was measured in red-light. The highest values were recorded in red-blue light cultures. The decrease in dissolved oxygen concentration for blue-light and red-blue was not as marked as for red-light, since the difference between the start and the end of the experiment was only 3.5 and 5.5 mg $L^{-1}$, respectively.

As expected, pigment content decreased for every treatment after the depletion of nitrogen from the culture medium on day 3, and kept on decreasing the longer the time the cultures

were exposed to those conditions (Figures 4.2A-C). With respect to chlorophyll and carotenoids, differences were not significant between treatments by the end of the experiment. However blue-light treated cultures reached day 3 with a higher chlorophyll and carotenoid content than cultures where red light was part of their light composition. Nevertheless, the marked decrease in pigment content was not clearly reflected when chlorophyll and carotenoid concentration were computed (Figures 4.2D-F). In this respect, chlorophyll concentration decreased as well with the onset of nitrogen limitation but remained stable from day 3 until the end of the experiment in treatments where blue light was present, being higher in blue-light with respect to red-blue. Chlorophyll concentration in the red-light treatment decreased throughout the experiment (Figure 4.2D). A similar behaviour was observed with carotenoid concentration (Figure 4.2E). In this case carotenoid concentration of both blue-light and red-blue remained stable during the experiment, but carotenoid concentration decreased in red-light cultures with time. Chlorophyll to carotenoid ratio decreased from values about 2.5 at the beginning of the experiment to values around 1.8 from day 3 to the end of the experiment, showing no significant differences among treatments (data not shown).

Differences among light wavelengths were more obvious for phycoerythrin, the main pigment in *P. cruentum* that, as expected, also decreased in both content and concentration with the onset of nitrogen limitation. However, blue-light treatment maintained a higher content and concentration during nitrogen starvation (Figures 4.2C and 4.2F). Phycoerythrin concentration was close to 75 mg $L^{-1}$ for every treatment at day 0, and stabilised on day 5 around 45 mg $L^{-1}$ for blue-light, around 30 mg $L^{-1}$ for red-blue, and around 15 mg $L^{-1}$ in red-light. In terms of content, phycoerythrin accounted for about 20 % of dry weight at the beginning of the experiment and was reduced to about 1 % or less under severe nitrogen starvation.

Content was, by the end of the cultivation period, double in blue-light with respect to red-light and around one third higher than red-blue (Figure 4.2C). The relative concentration of pigments in each culture produced very noticeable differences in the colour of the biomass (Figure 4.3B). Blue-light grown cultures preserved the red colour characteristic of this species, due to the maintenance of higher phycoerythrin content. On the other hand, red-blue cultures displayed an orange colour, due to the decrease of phycoerythrin content in comparison to blue light cultures but the maintenance of a relatively high chlorophyll and carotenoid concentration with respect to red-light cultures, which looked yellowish, since this treatment generated the strongest and most clear degradation for every pigment fraction during nitrogen starvation and high irradiance.

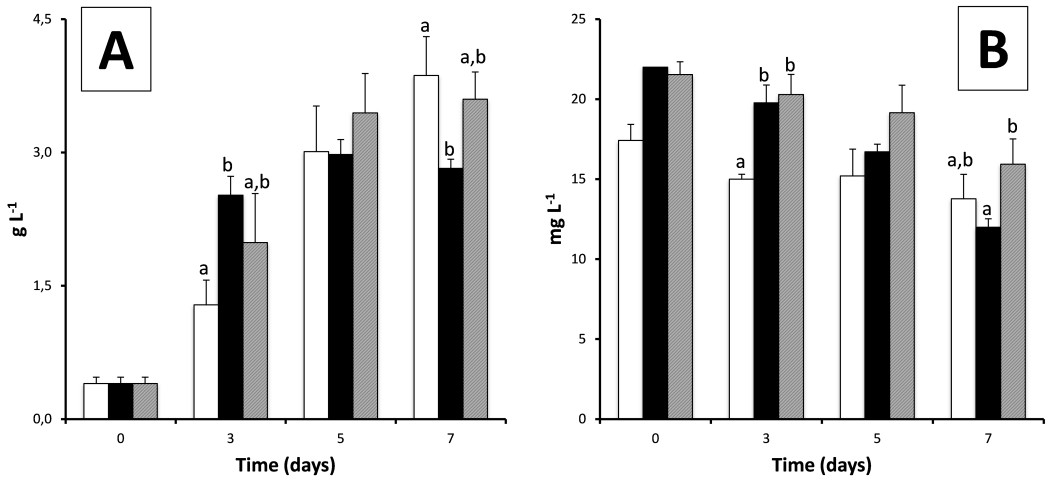



**Figure 4.1. Biomass concentration (g $L^{-1}$) (A) and dissolved oxygen concentration (mg $L^{-1}$) (B) in *P. cruentum* cultures maintained under different light qualities at a continuous irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was 20 ºC. Nitrogen was completely depleted on all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

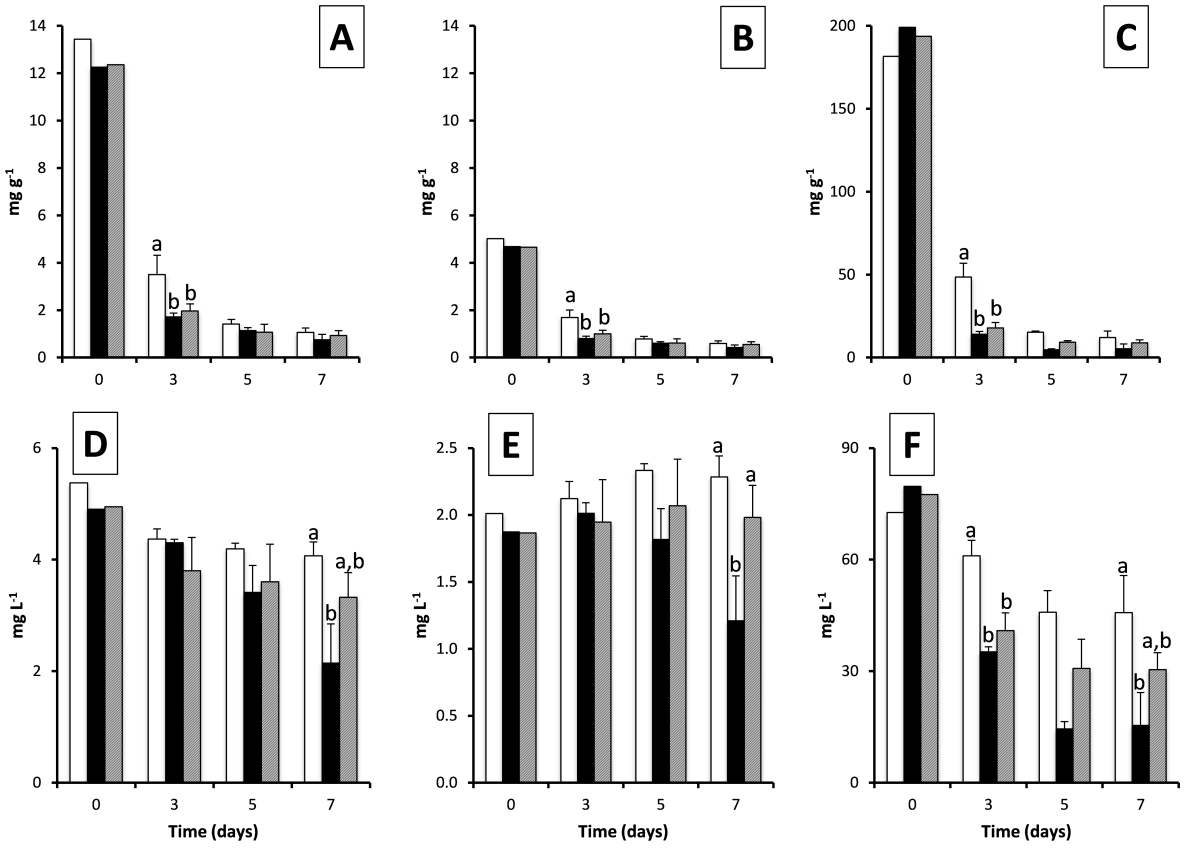



**Figure 4.2. Content (mg $g^{-1}$) and concentration (mg $L^{-1}$) of chlorophyll (A, D), carotenoids (B, E), and phycoerithrin (C, F) in cultures of *P. cruentum* maintained under different light qualities at a continuous irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was 20 ºC. Nitrogen was completely depleted on all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

With respect to the photosynthetic response of *P. cruentum*, effective PSII quantum yield was the highest in blue-light maintained treatments, followed by red-blue and red-light cultures. Interestingly, quantum yield increased for blue-light from 0.45 at the beginning of the cultivation period to 0.55 on day 3, and remained stable thereafter. Quantum yield of red-blue maintained cultures remained unaltered during the cultivation period around 0.35. On the other hand, quantum yield of the red-light maintained cultures decreased throughout the experiment from values around 0.35 on day 0 to 0.25 by the end of the experiment (Figure 4.4).

Interestingly, $qP$ remained high in every treatment throughout the experiment, with values unaltered around a value of 1 for treatments in which blue light was present. Red-light treatment even showed an increase of $qP$ during stationary phase and was significantly different to the other two treatments by the end of the experiment (Figure 4.5A). In terms of $qN$, it raised in the treatments where red light was present between days 0 and 3, and then showed a slight decreasing trend until the end of the experiment. Blue-light cultures showed a different pattern in terms of $qN$, since a continuous increasing trend with culture time was measured (Figure 4.5B).

Moreover, the study of chlorophyll fluorescence transients, so called OJIP curves, showed a consistent pattern throughout the experiment among treatments. When computed, after normalisation, red-light cultures showed a higher degree of electron transport chain saturation level, following by red-blue and blue-light treatments, which had the lower level of saturation that was reflected in the values of J and I inflections, which were higher for red-light followed by red-blue and blue-light (Figure 4.3A)

Finally, as the culture medium clearly increased in viscosity during the cultivation period, the production of exopolysaccharides (EPS) was estimated in the supernatant of cultures by the end of the experiment by measuring soluble carbohydrates in the culture media after removing the biomass by centrifugation. Carbohydrates were quantified by the phenol-sulfuric method (DuBois *et al.*, 1956) using glucose as standard. On day 7, the supernatants of cultures maintained under blue-light contained about 580 mg $L^{-1}$ of soluble carbohydrates, which was around 20 % higher than the concentration measured in red-light and red-blue light cultures (Figure 4.6).

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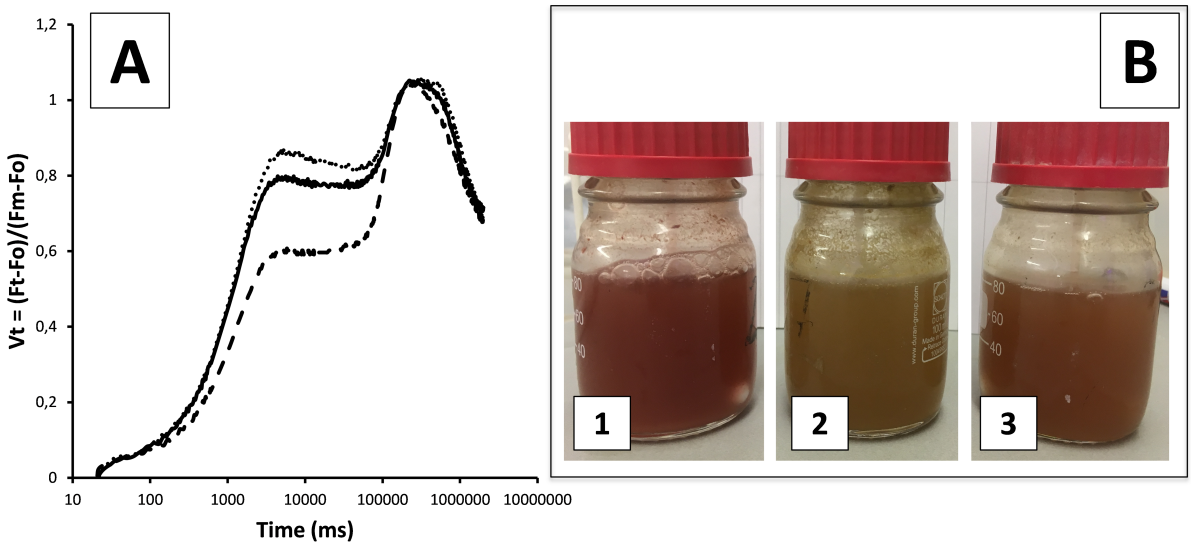



**Figure 4.3. Chlorophyll fluorescence transient (A) and physical appearance (B) on day 7 in cultures of *P. cruentum* maintained under different light qualities at a continuous irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (discontinuous line, B1), red (dotted line, B2), and mix of red and blue (continuous line, B3).**

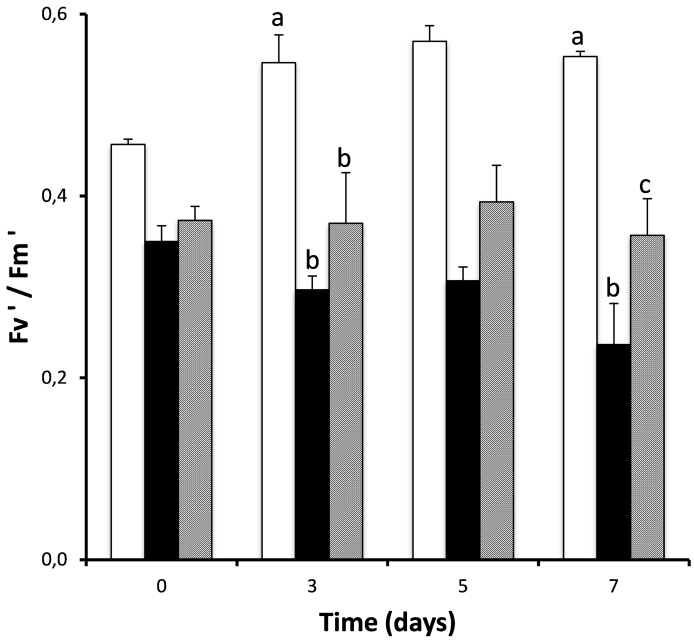



**Figure 4.4. PSII effective quantum yield in cultures of *P. cruentum* maintained under different light qualities at a continuous irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was 20 °C. Nitrogen was completely depleted on all treatments by day 3. Vertical bars represent S.D. (*n = 3*). Different superscript letters within the same day indicate significant differences among treatments ($p < 0.05$).**

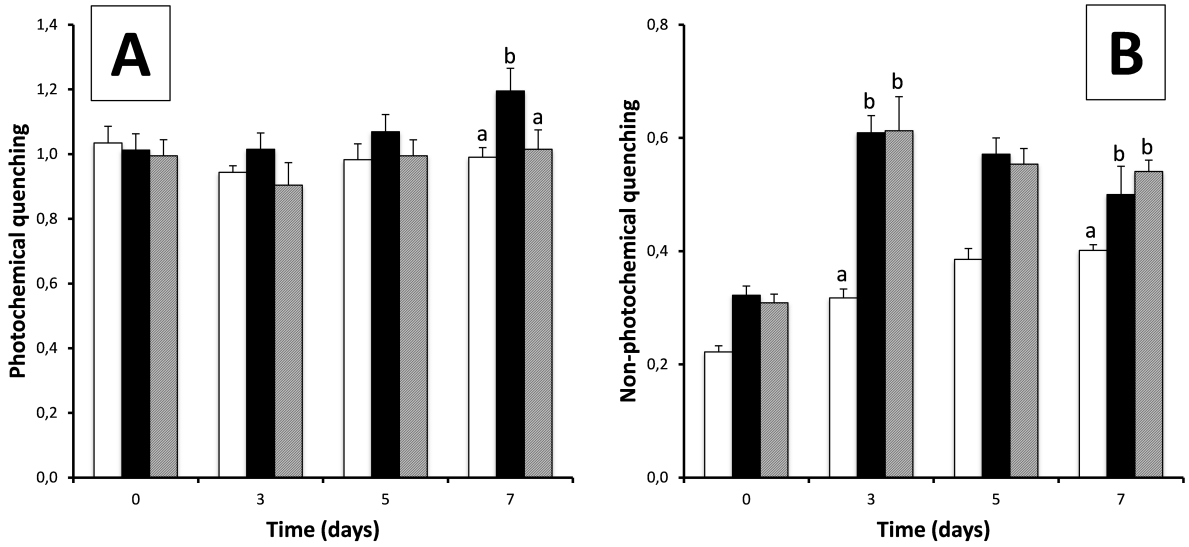



**Figure 4.5. Photochemical (qP) (A) and non-photochemical (qN) (B) fluorescence quenching in cultures of *P. cruentum* maintained under different light qualities at a continuous irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was 20 °C. Nitrogen was completely depleted on all treatments by day 3. Vertical bars represent S.D. (*n* = *3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05)**

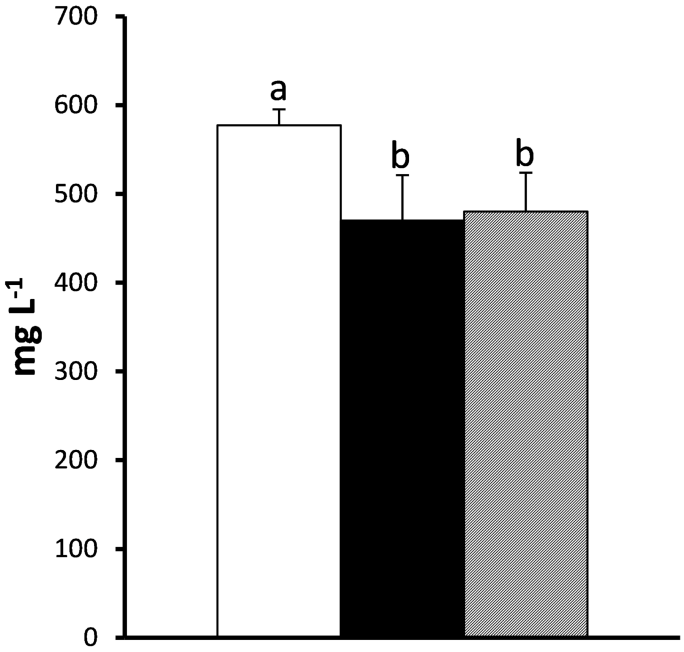



**Figure 4.6. Carbohydrates concentration in EPS fraction of *P. cruentum* cultures by the end of the experiment, maintained under different light qualities at a continuous irradiance of 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (open bars), red (closed bars), and mix of red and blue (grey bars). Temperature was 20 °C. Vertical bars represent S.D. (*n* = *3*). Different superscript letters within the same day indicate significant differences among treatments (p < 0.05).**

## Discussion

Few studies have investigated the effect that different light wavelengths have on both growth and photosynthetic apparatus response of *P. cruentum*. The results of the present study add data on the effect that blue and red light have on the growth of *P. cruentum.* The species that have been studied in previous chapters of this work were all green algae, belonging to the Chlorophyta. Among those, some recurrent patterns were described, which can be directly associated with light quality. Such patterns were, for instance, that red-light favours an increase in biomass concentration with respect to blue-light or mixed light (Figures 1.5A, 3.1C and 3.1D). Also, the higher the red to blue ratio in the light source the lower pigment content while the lower the ratio the higher pigment content in the biomass (Figures 1.5 and 3.3). Moreover, PSII effective quantum yield and relative electron transport rate of red-light maintained cultures was also the lowest with respect to the use of blue light (Figures 1.7 and 3.9B), since red-light caused a higher electron transport chain saturation level (Figure 3.11B). Most of these patterns have also been observed in *P. cruentum,* despite some differences with respect to green algae are noticeable. Furthermore, the results have shown that cultivation of this species with monochromatic light is possible, but the choice of the light composition is a sensitive variable that will make a difference for the biochemical composition of the culture.

*P. cruentum* was grown in batch mode with a moderate amount of initial nitrate in the culture medium, 4 mM $L^{-1}$, since the focus of the study aimed for the subjection of *P. cruentum* to a severe nitrogen starvation and continuous illumination, which would originate a highly viscous culture due to the accumulation of EPS in the culture medium and the synthesis of a gelatinous envelope around the cells (Ramus, 1972), as it was confirmed on day 3 by microscopic examination of the cultures. Moreover, cell composition under such conditions should be high in intracellular and cell-bound carbohydrates (Köst *et al*., 1983; Arad *et al*., 1985), and FA composition should be high in ARA (Cohen, 1990).

The results have shown that, within the light sources that have been used to study this effect, monochromatic blue light should be the preferred choice with respect to the use of monochromatic red light alone. In fact, cultivation under monochromatic red light manifested the more obvious signs of photoinhibition during stationary phase. Cultures maintained under red light showed the fastest increase in biomass concentration in nitrogen sufficiency, between days 0 and 3, achieving twice the dry weight than in blue-light cultures (Figure 4.1A), but once cultures reached stationary phase and nitrogen limitation, no further increase in biomass concentration was observed, which contrasts very much to the increase that was observed in blue-light and red-blue light cultures that increased dry weight more than two-fold with respect to day 3 (Figure 4.1A). Within the three light sources, blue-light maintained cultures reached the highest biomass concentration on day 7, at about 4 g $L^{-1}$ although differences with red-blue were not significant ($p > 0.05$). The increase in biomass concentration once the cultures reached nitrogen starvation must be associated mainly with an increase in intracellular and cell-bound carbohydrates and lipid bodies, as it has been shown in other studies that observed that *P. cruentum* accumulated mostly carbohydrates in stationary phase (Köst *et al*., 1983; Arad *et al*., 1985).

The minimal carbon fixation observed in red-light correlates with the drop observed in dissolved oxygen measurements during nitrogen starvation, from days 3 to 7. On day 3, treatments that contained red light showed higher dissolved oxygen concentrations than blue-

light cultures, in line with the increase in dry weight during the first days of experiment (Figure 4.1A). The opposite trend was observed with time until the end of the cultivation period, when a higher concentration was measured in treatments that contained blue light on day 7 (Figure 4.1B). For every treatment, dissolved oxygen decreased with culture time. However, the drop was steeper in red-light, that was reduced by 10 mg $L^{-1}$, compared with the other two treatments that were reduced only by 3.5 and 5.5 mg $L^{-1}$ (Figure 4.1B). Nevertheless, it is worth to mention that due to the increase of medium viscosity caused by the dissolution of polysaccharides into the liquid, it is plausible that gas exchange and therefore measurements like dissolved oxygen might be influenced by this to a certain extent.

Red-light maintained cultures showed clear signs of chlorosis with respect to cultures submitted to blue-light. Red-light cultures showed the largest decrease in chlorophyll, carotenoid, and phycoerythrin concentration (Figures 4.2D-F) that is in line with the observations made by Coward et al. (2016) that also reported the lowest chlorophyll and carotenoids content under red light. This was already noticeable from the physical appearance of the cultures, since cultures maintained with monochromatic red light looked yellow in contrast with the red colour observed in blue-light (Figure 4.3B). Yellow-looking cultures have been observed by other authors in dense cultures under conditions of high-light in contrast with low-light maintained cultures that kept the red colour (Arad *et al*., 1985). The authors attributed this difference to the differences in protein content, mainly. Despite they did not mention if nitrogen had been depleted from culture medium in the high-light maintained cultures, it is probable that these phenomena were co-occurring. Zhao et al. (2016) have also reported that cultures of *P. cruentum* maintained under non-monochromatic PAR light turned yellow during nitrogen starvation. They directly correlate the shrinkage in phycobilisome size with time under nitrogen limitation, since they hypothesize that phycobilisomes can serve not only as light-harvesting pigments but also as nitrogen storage, which would be movilized when cells are submitted to starvation. Moreover, they studied phycobilisome recovery after severe nitrogen limitation showing how the yellow colour of the cultures turned red again after the addition of nitrogen.

In the present study, nitrogen was exhausted from the culture medium of every treatment by day 3. The irradiance used for every light composition was equal, and cultures reached a density where all the photons were absorbed following Beer-Lambert's law. Therefore, observed differences must be driven by the responses to each light wavelength. In this respect, the level of electron transport chain saturation was different among treatments (Figure 4.3A). This was reflected in chlorophyll fluorescence transients measurements, which showed the same pattern throughout the experiment, being the saturation level directly correlated with the amount of red light in the light source (Figure 4.3A). This can be interpreted as if the growth under red-light would be similar to the growth under a higher irradiance, which would also cause a higher electron transport chain saturation level. Furthermore, the lower degree of photoprotection against red light, all of which is absorbed by the small amount of chlorophyll maintained under these light conditions (Figure 4.2A) and the growth restriction imposed by nitrate starvation, would be causing the obvious photodamage that was also revealed by the low $Fv/Fm$ maintained in this treatment, 0.2 (Figure 4.4). Results for red-light cultures are in line with recent studies that have shown a decrease in $Fv/Fm$ under nitrogen limitation (Zhao *et al.*, 2017). On the contrary, despite blue photons are more energetic than red photons, the cells maintained a higher and a more adequate pigmentation (Figure 4.2) to capture this energy and channel it to the photosynthetic apparatus, not only and not directly,

via chlorophyll but also carotenoids and through phycoerythrin, allowing the photosystems to keep functioning bypassing signs of photoinhibition (high $Fv/Fm$; Figure 4.4) that would be reflected in a higher $CO_2$ fixation, which is translated into a higher increase in dry weight (Figure 4.1A) and a higher polysaccharide content (Figure 4.6).

Photosynthetic response was also characterized by an increasing trend of $qP$ and a decreasing trend of $qN$ during nitrogen starvation, from days 3 to 7 (Figure 4.5). This is, once again, in line with Zhao *et al*. (2017) that explained the phenomena by the reduced light-harvesting ability due to the degradation of the phycobilisomes under nitrogen starvation, resulting in a decrease of the light energy transferred to PSII. These observations, together with a possible higher oxygen uptake rate in red-light cultures that has been observed in other microalgae species (Xu and Harvey, 2019a) may be ultimately reflected in the apparent restriction of carbon fixation evidenced by the non-increase in dry weight from days 3 to 7 (Figure 4.1A).

Blue-light cultures achieved the highest biomass concentration by the end of the experiment (Figure 4.1A) and maintained the highest pigment content (Figure 4.2), which is in line with other studies that cultivated *P. cruentum* under blue light (Coward *et al.*, 2016). Differences in phycoerythrin content might be the most significant among treatments (Figure 4.2C), and were obvious from the physical appearance of the cultures that maintained the characteristic red colour (Figure 4.3B). The difference was larger when pigment concentration was computed, being the concentration in blue-light three-fold the concentration in red-light (Figure 4.2F). Increase of phycoerythrin concentration with high intensity blue light was already described by Brody and Emerson (1959) in actively growing cultures of *P. cruentum*. The results of the present work show that the same effect might be valid in conditions of nitrogen starvation, where high blue-light irradiance prevents phycobilisome degradation to a larger extent than red-light. This evidences that, in *P. cruentum*, phycobilisome degradation is not only regulated by nitrogen limitation but the spectrum of incident irradiance must also play an important role. On the contrary to the studies of Zhao *et al*. (2017), who described a photosynthetic response in nitrogen limited cultures of *P. cruentum* similar to the response we show in the present work with respect to PSII quantum yield, $qP$ and $qN$, the photosynthetic response of blue-light cultures showed an opposite behaviour. PSII quantum yield remained the highest (Figure 4.4) and even showed a slight increase during the experiment, while electron transport chain saturation level remained the lowest (Figure 4.3A). Moreover, while $qP$ was maintained high showing a similar trend with respect to red-light, $qN$ showed a complete different pattern with respect to treatments where red light was present, showing a constant upward trend (Figure 4.5). Following Zhao *et al*. (2017) hypothesis, this phenomena may have to do with the maintenance of a higher light-harvesting ability due to the higher phycoerythrin content with respect to the remaining treatments that would allow a higher energy transfer to PSII resulting in the need for some thermal dissipation capacity. These results reflect the effective adaptation of the culture to these conditions, which must be related to the difference in pigment content and composition.

Despite the red-blue cultures achieved a biomass concentration similar to blue-light cultures by the end of the experiment (Figure 4.1A), and showed the highest dissolved oxygen concentration throughout the experiment (Figure 4.1B), in the remaining parameters that were monitored the values fall in between those of blue-light and red-light cultures. Both PSII quantum yield and chlorophyll fluorescence transients showed intermediate values with respect to the other two treatments (Figures 4.3A and 4.4), and $qP$ showed no significant

differences with respect to blue-light while $qN$ followed the same behaviour as red-light (Figure 4.5).

As for the production of EPS, the soluble carbohydrate fraction of the supernatants was measured by phenol-sulfuric method with glucose as standard. The results have shown that, by the end of the cultivation period, the EPS soluble carbohydrates content was the highest in blue-light maintained cultures, with 580 mg $L^{-1}$ (Figure 4.6). This value was about 20 % higher than the content that was found in red and red-blue light cultures ($p < 0.05$). The result is in accordance with You and Barnett (2004) who concluded that monochromatic blue light could be used to increase polysaccharide production with respect to a mixed light spectrum. Other studies reported similar results, with blue light producing slightly higher EPS concentrations than red light (Sun *et al*., 2008), while others did not report a difference between these wavelengths with respect to total EPS but a difference in the soluble carbohydrates fraction of total EPS that appeared slightly higher in blue light (Coward *et al.*, 2016). However, these works have also studied broader spectrums of light such as the addition of green light and, despite the differences with respect to monochromatic lights seem small, it looks that this approach could have potential to improve EPS productivity.

To the best of our knowledge, no other study compared the photosynthetic response of *P. cruentum* to different light composition using chlorophyll fluorescence techniques. The results evidenced that *P. cruentum* response to conditions of nitrogen starvation and continuous illumination was strongly influenced by light composition. We have found that the response measured in red-light cultures is the one that stands in consistency with what is described in the literature for broad-spectrum illuminated cultures of *P. cruentum* during nitrogen starvation (Zhao *et al.*, 2017). Under this light condition the cultures suffered the most marked chlorosis to the point that cultures looked yellow (Figure 4.3B) together with a drop in PSII effective quantum yield (Figure 4.4) and a characteristic $qP$ and $qN$ behaviour (Figure 4.5). On the other hand, the photosynthetic response of *P. cruentum* to blue-light under the conditions aforementioned was described for the first time. Pigment content also decreased in these cultures as a response to nitrogen starvation (Figures 4.2A-C). However, pigment degradation and especially phycoerythrin degradation was very low in comparison to red-light (Figures 4.2C and 4.2F), despite the severe nitrogen limitation. For this reason, the culture maintained the characteristic red colour of nitrogen saturated conditions (Figure 4.3B). Moreover, the photosynthetic efficiency remained high during the experiment (Figure 4.4) and $qP$ and $qN$ showed a complete different pattern that the one observed in red-light and red-blue cultures (Figure 4.5). This was reflected in higher EPS (Figure 4.6) and biomass concentrations (Figure 4.1A).

Despite further investigations should be carried out in order to analyse total EPS fraction, sulfatation degree, biochemical composition, and the effect of different ratios of red and blue together with different wavelengths like green, the results might have potential direct industrial application for those interested in producing stationary phase metabolites from *P.cruentum*. It looks evident that by the use of blue-light, cultures are more stable and not only a higher EPS fraction could be produced, but presumably also a higher ARA and total carbohydrate productivity, together with a more valuable protein fraction, due to the maintenance of a high PE content, could be achieved.

# GENERAL CONCLUSIONS / CONCLUSIONES GENERALES / CONCLUSIÓNS XERÁIS

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1. Monochromatic lights or a combination of monochromatic wavelengths can be used for mass cultivation of microalgae. However, the correct choice of the light composition is essential since differences in the photosynthetic response, growth, and pigment content, among others, can be significant. In this respect, differences in other biochemical characteristics are expected and should be investigated in the future.

2. Under conditions of nitrogen saturation, biomass concentration was enhanced with the use of red light in all species tested, while the lowest increase in dry weight was observed in the treatments that use blue light. When nitrogen is depleted from the culture medium, the consistency of this response is less clear. However, it seems that in the species belonging to the phylum Chlorophyta (*H. pluvialis*, *D. salina*, and *D. tertiolecta*) this trend is maintained over stationary phase. *P. cruentum*, a Rhodophyta, seemed to require blue light to sustain the increase in dry weight once nitrogen was absent from the culture medium.

3. In all species, chlorophyll to carotenoid ratio decreased under nitrogen starvation, regardless of the capacity of the species to massively synthesize carotenoids.

4. In all species, the use of red light is detrimental for total pigment content (chlorophyll and carotenoids, and phycoerythrin in *P. cruentum*), while monochromatic blue light treatments always present the highest pigment content.

5. In all species, oxygen production decreased during stationary phase, indicating a lower photosynthetic activity.

6. Wavelength does not seem to have any influence in oxygen production, regardless of the differences in the increase of dry weight, and therefore carbon fixation, that are observed among treatments.

7. The two carotenogenic species used in this work, *H. pluvialis* and *D. salina*, showed a similar response to conditions of nitrogen starvation and continuous illumination of different wavelengths. In both, carotenoid content was enhanced by the use of blue light and chlorophyll content was inversely correlated to carotenoid content. However, the use of a mix of red and blue wavelengths has been shown to balance biomass concentration and carotenoid content, maximizing carotenoid productivity in both species.

8. Moreover, the accumulation of secondary carotenoids protected the photosynthetic apparatus against blue light, mainly, which was reflected in the maintenance of the highest photosynthetic efficiency and chlorophyll content compared to red light, which in both species caused a marked decrease in these parameters.

9. In *P. cruentum*, long-term nitrogen starvation under continuous illumination with different wavelengths has also produced marked changes in the pigment profile of the biomass. It seemed like phycobilisome degradation is not only regulated by nitrogen deprivation but the light spectrum must play an important role, since the cultures maintained under blue light preserved the characteristic red colour of this species, which was reflected in a higher phycoerythrin content. Cultures of *P. cruentum* grown under blue light also maintained a higher chlorophyll and carotenoid content, and photosynthetic efficiency, and produced the highest biomass and soluble carbohydrates concentration compared to red and a mix of red and blue.

10. Optimum temperature for vegetative growth varies from that to maximize carotenogenesis in *H. pluvialis*. Cell division and nitrogen consumption were maximal at 20 ºC. Under inductive conditions, a temperature of 30 ºC produced a carotenoid content that was twice as high as that with 20 ºC, and a productivity three time higher than the one reached at 20 ºC. Production of *H. pluvialis* above 30 ºC was not viable.

11. It has been confirmed that carotenoid content in *H. pluvialis* is directly correlated with irradiance and inversely correlated with nitrogen availability.

12. Photobioreactors for the cultivation of microalgae at medium to industrial-scale require designs that maximize light distribution, reflected in high illuminated $S/V$. During this work, the importance of light distribution with respect to maximum irradiance has been evidenced. This is closely related with the photosynthetic capacity characteristic of each species but also with the conditions under which the respective species is growing.

13. The importance of laboratory-scale optimization and prototype testing prior to upscaling has been shown to have an upmost importance to maximize the results that can be achieved at industrial-scale. In this respect, the results in astaxanthin content and productivity have improved in the industrial-scale with respect to the lab bench.

14. It seemed that, in green-stage *H. pluvialis*, continuous illumination taxed cellular division with respect to the use of a circadian light: dark cycle.

15. Saturation point of photosynthesis ($Ek$) and maximum photosynthesis ($P_{\max}$), as well as the remaining characteristic points of a P-I curve change with respect to culture conditions and cellular state, and were correlated with division rate in green *H. pluvialis* cultures.

16. Chlorophyll fluorescence techniques are proven to be powerful tools to optimize microalgal growth. P-I curves generated using this method were crucial for the successful upscale of *H. pluvialis* pilot-scale photobioreactors. This was evidenced by the improvement of the pilot-scale results with respect to laboratory-scale optimization.

17. *D. tertiolecta* results under conditions of continuous illumination and nitrogen starvation reflected the existence of a different protective mechanism against oxidative stress. The response was characterized by a clear increase of non-photochemical fluorescence quenching, which correlated to a high concentration of lutein/ zeaxanthin. The photosynthetic apparatus response of this species was the only that appeared the same irrespective of the light wavelength that was used, despite differences in culture's dry weight were significant.

1. Las luces monocromáticas, o la combinación de luces monocromáticas con diferentes longitudes de onda pueden ser usadas para el cultivo de microalgas. Sin embargo, la elección de la composición lumínica es un factor esencial ya que afectará a parámetros del cultivo importantes como la respuesta fotosintética, cremiento, y pigmentación, entre otros. Diferencias en composición bioquímica son por lo tanto esperables y deben ser estudiados en futuras investigaciones.

2. En condiciones de saturación de nitrógeno, la luz roja favorece el aumento en peso seco en todas las especies investigadas en este trabajo. La luz monocromática azul genera lo contrario. En condiciones de limitación por nitrógeno, la respuesta es menos clara. Sin embargo, en las Clorófitas (*H. pluvialis*, *D. salina*, y *D. tertiolecta*) la tendencia parece mantenerse en fase estacionaria, mientras que la Rodófita *P. cruentum* parece necesitar luz azul para mantener el crecimiento en peso seco durante esta fase.

3. En todas las especies estudiadas, el ratio clorofila carotenoide disminuyó en condiciones de limitación por nitrógeno, independientemente de su capacidad carotenogénica.

4. Para todas las especies estudiadas la luz roja parece pesar en contra de el contenido total de pigmentos (clorofilas, carotenoides, y ficoeritrina para *P. cruentum*), mientras que los cultivos que se mantienen con luz azul muestran el contenido más alto.

5. La producción de oxígeno disminuye en todas las especies estudiadas durante la fase estacionaria, evidenciando una menor actividad fotosintética.

6. La longitud de onda parece no influenciar la producción de oxígeno, independientemente de las diferentes tasas de crecimiento en peso seco que sí se registran en las diferentes longitudes de onda.

7. Tanto *H. pluvialis* como *D. salina*, las dos especies carotenogénicas que han sido estudiadas, muestran respuestas similares en condiciones de limitación por nitrógeno e iluminación continua con longitudes de onda diferentes. Para ambas, el contenido en carotenoide total se incrementa con luz azul y el contenido de clorofila desciende con el aumento en carotenoides. Sin embargo, el uso de luz azul y roja combinada maximiza la productividad de carotenoides ya que genera un aumento en peso seco y en contenido de carotenoide más balanceado que cada longitud de onda por separado.

8. La acumulación de estos carotenoides secundarios ofrece protección al aparato fotosintético, principalmente contra el exceso de luz azul. Este efecto se refleja en los datos de eficiencia fotosintética y contenido en clorofila, ambos más altos en luz azul y disminuidos en luz roja.

9. El mantenimiento prolongado de *P. cruentum* en condiciones de limitación por nitrógeno e iluminación continua con diferentes longitudes de onda provocó cambios en la pigmentación de la biomasa. Parece que, en estas codiciones, no solo la limitación por nitrógeno, sino también la calidad de luz recibida juega un rol importante en la regulación de la degradación de los ficobilisomas. Los cultivos mantenidos en luz azul preservaron el color rojo característico de esta especie, manteniendo unos niveles de ficoeritrina mayores que en el resto de longitudes de onda. Por el contrario, en los cultivos en luz roja la degradación de pigmento fué mucho mayor y los cultivos presentaban un color anaranjado o amarillento. Además, los cultivos en luz azul también fueron los que presentaron un mayor aumento en peso seco y los que generaron la mayor concentración de carbohidratos solubilizados en el medio.

10. En *H. pluvialis*, el mayor crecimiento vegetativo se midió a una temperatura de 20 ºC, mientras que la temperatura optima para favorecer la carotenogénesis se encontró a 30 ºC. Por encima de 30 ºC, el cultivo de fase roja no fué viable.

11. Se confirmó que el contenido en carotenoides de *H. pluvialis* está directamente relacionado con la irradiancia, así como está inversamente relacionado con la disponibilidad de nitrógeno.

12. El diseño de un fotobioreactor para el cultivo masivo de microalgas debe maximizar la disponibilidad lumínica. Este factor se refleja en el ratio $S/V$, es decir, el ratio superficie iluminada - volumen. En este trabajo, se evidencia que una buena distribución lumínica debe de ser priorizada sobre la irradiancia total. Esto está relacionado con como se vé modificada la capacidad fotosintética de una célula respecto a la irradiancia que recibe.

13. Los resultados en cuanto a porcentaje de carotenoide total y productividad de pigmento en *H. pluvialis* fueron mejorados a escala industrial (piloto) con respecto a la escala de laboratorio. Este fenómeno no es baladí, y refleja la importancia que tiene la optimización a escalas menores que la industrial en cuanto al desarrollo y a la transferencia de procesos a escala industrial de manera exitosa.

14. En *H. pluvialis* la división celular se ve afectada por el fotoperíodo. Condiciones de iluminación continua son perjudiciales para la tasa de división celular, que fué menor que bajo un ritmo circadiano de 12 h luz 12 h oscuridad.

15. Tanto el punto de saturación de la fotosíntesis ($Ek$), como el punto de fotosíntesis máxima ($P_{\max}$), como el resto de parámetros derivados de una curva de fotosíntesis irradiancia (curva P-I), cambian respecto a las condiciones de cultivo y el estado celular. Estos puntos muestran una relación directa con la tasa de división celular en *H. pluvialis*.

16. Se ha demostrado que las técnicas derivadas de la medición de la fluorescencia de la clorofila son métodos con un gran potencial para la optimización de los cultivos microalgales. Las curvas P-I que se generaron usando estas técnicas fueron cruciales para el exitoso escalado del proceso de producción de *H. pluvialis*, tal y como evidencian los resultados a la escala piloto.

17. Los resultados derivados de la exposición de *D. tertiolecta* a las codiciones experimentales reflejan la existencia clara del funcionamiento, en esta especie no carotenogénica, de un mecanismo protector contra el estrés oxidativo generado por la iluminación continua en condiciones de limitación por nitrógeno. La respuesta se caracterizó por el aumento de procesos de fluorescencia no fotoquímica. El aumento de estos valores está en línea con una alta concentración de pigmentos relacionados con uno de los ciclos de xantofilas característico de las Clorófitas. En este caso, aunque sí se observaron diferencias claras en cuanto al aumento de peso seco, la respuesta fotosintética fue invariable con respecto a las distintas longitudes de onda.

1. As luces monocromáticas, ou a combinación de luces monocromáticas con diferentes lonxitudes de onda poden ser empregadas pra o cultivo de microalgas. Sen embargo, a elección da composición lumínica é un factor esencial xa que afectará os parámetros do cultivo importantes coma a resposta fotosintética, o crecemento, e a pigmentación, entre outros. Diferencias en composición bioquímica son, por tanto, esperábles e deben ser estudadas en futuras investigacións.

2. En condicións de saturación de nitróxeno, a luz vermella favorece o aumento en peso seco en tódalas especies investigadas neste traballo. A luz monocromática azul xera o contrario. En condicións de limitación por nitróxeno, a resposta é menos clara. Sen embargo, nas Clorófitas (*H. pluvialis*, *D. salina*, y *D. tertiolecta*) a tendencia parece manterse na fase estacionaria, mentre que a Rodófita *P. cruentum* parece requirir da luz azul para manter o crecemento en peso seco durante este estadio.

3. En tódalas especies estudadas, o ratio clorofila: carotenoide diminuíu en condicións de limitación por nitróxeno, independentemente da sua capacidade carotenoxénica.

4. Para tódalas especies estudadas a luz vermella parece actuar na contra do contido total de pigmentos (clorofilas, carotenoides, e ficoeritrina en *P. cruentum*), mentres que os cultivos mantidos con luz azul mostran o contido máis alto.

5. A producción de osíxeno disminuíu en tódalas especies estudadas durante o estadio estacionario, evidenciando unha menor actividade fotosintética.

6. A lonxitude de onda non parece influenciar a producción de osíxeno, independentemente das diferentes taxas de crecemento en peso seco que sí foron rexistradas cas diferentes lonxitudes de onda.

7. Tanto *H. pluvialis* coma *D. salina*, as dúas especies carotenoxénicas que foron estudadas, mostran respostas similares en condicións de limitación por nitróxeno e iluminación continua con diferentes lonxitudes de onda. Para ambas, o contido de carotenoide total incrementouse con luz azul e o contido de clorofila diminuiu co aumento de carotenoides. Sen embargo, o uso de luz azul e vermella combinadas maximiza a productividade de carotenoides xa que xera un aumento no peso seco e no contido de carotenoide máis balanceado que a luz azul ou vermella por separado.

8. A acumulación destes carotenoides secundarios ofrece protección ao aparato fotosintético, principalmente contra o exceso de luz azul. Este efecto reflícte nos datos de eficiencia fotosintética e no contido de clorofila, que son máis altos en luz azul e están diminuidos en luz vermella.

9. O mantemento prolongado de *P. cruentum* en condicións de limitación por nitróxeno e iluminación continua con diferentes lonxitudes de onda provocou cambios na pigmentación da biomasa. Parece que, baixo estas condicións, non só a limitación por nitróxeno, senón tamén a calidade de luz recibida xoga un papel importante na regulación da degradación dos ficobilisomas. Os cultivos mantidos en luz azul preservaron a cor vermella característica desta especie, mantendo uns nivéis de ficoeritrina maiores dos que o resto de lonxitudes de onda. Polo contrario, nos cultivos mantidos con luz vermella a degradación de pigmento foi moito maior e os cultivos presentaron unha cor alaranxado ou amarelado. Amais, os cultivos en luz azul tamén foron os que presentaron un maior aumento en peso seco e os que xeraron a maior concentración de carbohidratos solubilizados no medio.

10. En *H. pluvialis*, o maior crecemento vexetativo foi rexistrado a unha temperatura de 20 ºC, mentres que a temperatura óptima para favorecer a carotenoxénese foi de 30 ºC. Por enriba desta temperatura o cultivo da fase vermella non fói viable.

11. Confirmouse que o contido en carotenoides de *H. pluvialis* está directamente relacionado coa irradiancia e inversamente relacionado coa disponibilidade de nitróxeno.

12. O deseño de un fotobioreactor para o cultivo masivo de microalgas debe maximizar a dispoñibilidade lumínica. Este factor reflicte no ratio $S/V$, e dicir, a superficie iluminada respecto ao volumen total. Neste traballo evidénciase que unha boa distribución lumínica a de ser priorizada sobre a irradiancia total. Isto está relacionado con como vese modificada a capacidade fotosintética dunha célula respecto a irradiancia recibida.

13. Os resultados en canto ao porcentaxe de carotenoide total e a productividade de pigmento en *H. pluvialis* foron mellorados a escala industrial (piloto) con respecto a escala de laboratorio. Este fenómeno reflicte a importancia dos traballos de optimización a escalas menores ca industrial en canto ao desenvolvemento e a transferencia de procesos a gran escala con éxito.

14. En *H. pluvialis* a división celular vese afectada polo fotoperiodo. Condicións de iluminación continua son perxudiciáis para a taxa de división celular, que foi menor ca baixo ciclos de luz e escuridade.

15. Tanto o punto de saturación da fotosíntese ($Ek$) coma o punto de máxima fotosíntese ($P_{\max}$), coma o resto de parámetros derivados dunha curva de fotosíntese-irradiancia (curva P-I), cambian respecto as condicións do cultivo e ao estado celular. Estes puntos amosan unha relación directa coa taxa de división celular en *H. pluvialis*.

16. Demostróuse que as técnicas derivadas da medición da fluorescencia da clorofila son métodos cun gran potencial para a optimización dos cultivos microalgáis. As curvas P-I que foron xeradas usando estas técnicas foron cruciáis para o éxito no escalado do proceso de producción de *H. pluvialis*, tal e como evidencian os resultados obtidos nesa escala.

17. Os resultados derivados da exposición de *D. tertiolecta* as condicións experimentáis reflexan a existencia clara do funcionamento, nesta especie non carotenoxénica, dun mecanismo protector contra o estrás oxidativo xerado pola resposta celular a iluminación continua en condicións de limitación por nitróxeno. A resposta caracterizouse polo aumento de procesos de fluorescencia non-fotoquímica. O aumento destos valores está en linea cunha alta concentración de pigmentos relacionados cun dos ciclos de xantofilas característico das Clorófitas. Neste caso, aínda que se observaron diferencias claras en canto ao aumento de peso seco, a resposta fotosintética foi invariable con respecta as distintas lonxitudes de onda.

# SUMMARY / RESUMEN / RESUMO

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Mass cultivation of microbes is not simple. However, it is a crucial area in industrial microbiology and biotechnology, and therefore key in human industry, since mass cultivation of microbes is required in a broad range of applications; from the treatment of wastewater and bioremediation, generation of ethanol and biogas, feed of farmed animals, production of enzymes, foods, or beverages, and for the production of compounds with current and potential pharmacological use.

The cultivation of heterotrophs is currently well developed. Microbiologists can isolate wild-type strains suitable for each respective application and overproduce them for commercial purposes. Furthermore, genetic modification of heterotrophs for its use as vectors to produce compounds that do not naturally synthesize, such as insulin, is a relatively advanced field. High-value compounds are produced from heterotrophs in a relatively cost-effective way, since bacteria can grow in simple and cheap growth mediums and have a very fast life cycle when compared to an eukaryotic microorganism, yielding high productivities. Moreover, scalability is easier since the technology is well developed and standardized for the mass cultivation of these microorganisms, consisting on large volume, stainless steel fermentors, that allow precise adjustment of culture conditions, and most important, complete sterilization, essential for the successful scale up of these productions.

Identically to heterotrophs, microalgae, are characterized by a fast life cycle compared to other phototrophs. Microalgae show an important biochemical plasticity and the capacity to produce high-value compounds. In many ways, the work with microalgae requires identical procedures and techniques as the work with bacteria. However, industrial cultivation of these has not reached the level of heterotrophs for a number of reasons ranging from a more difficult scalability that leads to a more costly, less stable production and quality, and a smaller market volume reflected in the existing few microalgae-derived products regulated by food safety agencies and other authorities. Only species that are somehow extremophiles such as *Spirulina* spp., carotenogenic halophile *Dunaliella* spp., and *H. pluvialis*, or species that can grow well heterotrophically such as *Chlorella* spp. or the heterotroph *Schizochytrium* spp., generally included within the microalgal group since its taxonomy relates to certain algae, have been in the market for a significant period of time and have reached an industrialized cultivation level for their use to produce high-value compounds such as fatty acids and pigments, or for their introduction in the food market due to their rich nutritional value. Despite in a lower volume, in the last years a couple of other microalgae-based ingredients derived from species such as *Tetraselmis chuii* or *Phaeodactylum tricornutum* have made it to the human market, denoting some progress in the industry. Furthermore, although in smaller facilities and not as a direct product for the consumers market, microalgae has established well in the aquaculture industry, where they have been extensively grown to feed farmed-aquatic animals, since microalgae are essential in the aquaculture chain for the growth of molluscs, early-development of crustaceans, or the enhancement of the nutritional value of supporting species such as *Artemia* spp. or *Brachionus* spp. for marine fish larvae cultivation.

Besides new ingredients regulation, the diversification of the cultivatable microalgal species is hindered by basic problems derived from scalability such as contaminations, lack of technical understanding of the principles of mass microalgal growth, or an incomplete understanding of its biology. This makes that, in many cases, the industry looks like a branch of agriculture, lacking the pure industrial microbiology approach. Therefore, the improvement

of current systems, techniques, procedures, and strategies for microalgae cultivation, together with a more profound understanding of the physiological responses of the species to different conditions, can lead to the discovery, isolation, and development of new unique metabolites, unlocking the real possibilities of this field and positioning the industry in a level closer to that of industrial fermentation.

This work pivots on the topics aforementioned. LEDs, a relatively new illumination technology, have been used as light sources creating different light compositions, to grow a number of species with current industrial interest: *H. pluvialis*, *D. salina*, *D. tertiolecta*, and *P. cruentum,* in conditions of nitrogen starvation and continuous illumination, which are favourable for the production of secondary metabolites. One of the goals was to try to understand the response triggered by these conditions and evaluate potential biotechnological implications related to the use of different light wavelengths. The generalization on the use of LEDs in mass microalgae cultivation with artificial light will reduce the production costs in these facilities, since LEDs have a higher energy to photon conversion ratio than any other artificial light source. Moreover, LEDs allow the optimization of the light spectrum according to the action spectra of the cultivated species, a new dimension in light optimization that can lead to the use of different wavelengths to induce physiological responses in the culture with potential industrial interest. During this work, which has been carried out in an industrial environment, *H. pluvialis* cultivation was further optimized taking into consideration temperature, irradiance and photobioreactor design. Then, the scalability of the results was demonstrated in a pilot-scale cultivation unit built as a proof of concept of the growth strategies that were proposed during laboratory-scale. Considerations and constraints about the upscale process of *H. pluvialis*, but also applicable for general mass microalgal cultivation, are discussed together with a brief insight on the economical aspects of *H. pluvialis* industry.

In Chapter one, data from *H. pluvialis* culture conditions optimization are shown. As it was expected, from a biotechnological point of view that aims to the development of a two-stage cultivation of *H. pluvialis* for the production of astaxanthin, vegetative green-stage and non-vegetative red-stage growth demand significantly different culture conditions. *H. pluvialis* green cells grew well at 20 to 25 ºC. Nitrogen consumption was slightly faster at 20 ºC. Moreover, the use of red monochromatic light produced the highest growth rate compared to monochromatic blue, and a mix of red and blue in different red: blue ratios. Interestingly, in the combinations that were tested, which ranged from 25 % to 75 % red content, no differences in growth rate were observed as long as both wavelengths were present. Dry-weight during red-stage *H. pluvialis* growth was also highest with red light. Astaxanthin content was minimal under this light condition. Highest astaxanthin contents were observed when blue light was used alone or mixed in a 1: 1 ratio with red light. However, the highest pigment productivity was reached with a combination of both wavelengths that produced a more balanced increase of dry weight and astaxanthin synthesis. The combination of red light (660 nm) with blue light of different wavelengths (420, 450, and 470 nm) showed a significant effect of the blue wavelength on biomass concentration, while barely no effect was observed in astaxanthin synthesis. Chlorophyll content dropped once massive carotenogenesis was triggered after nitrogen depletion, and after 7 days both the content and concentration in cultures maintained under blue light was the highest, while red light produced the largest drop on chlorophyll content. Besides, temperature had a large effect on *H. pluvialis* carotenogenesis that was maximal at 30 ºC. Under this temperature, productivity was three-

fold than that at 20 ºC. Production of *H. pluvialis* was not viable over 30 ºC, since cellular lysis was observed in cultures maintained at 35 and 40 ºC. Moreover, astaxanthin content was directly correlated with irradiance. The accumulation of carotenoids protected *H. pluvialis* cells against blue photons, mainly, which was denoted in the maintenance of a higher PSII quantum yield than the treatments under red light. A higher photochemical fluorescence quenching and a lower non-photochemical fluorescence quenching were measured under blue light, while the opposite was observed under treatments containing red light. Moreover, PSII quantum yield showed an inverse correlation with irradiance and time under inductive conditions. Dissolved oxygen concentration dropped after nitrogen depletion, once primary metabolism was reduced.

Chapter two continues with the optimization of *H. pluvialis* culture conditions and its integration into a proof of concept pilot-scale production. Optimal irradiance for both stages was investigated in flat-plate photobioreactors. Chlorophyll fluorescence techniques were used to determine this factor during green-stage growth. The data showed how photosynthesis saturation point ($Ek$) is a dinamic value with regards to *H. pluvialis* cellular state, showing maximum values that correlated directly with division rate, and were about 500 μmol photon $m^{-2}$ $s^{-1}$. On the other hand, under the inductive conditions that were defined in chapter one, *H. pluvialis* reached a saturation of astaxanthin productivity around 750 μmol photon $m^{-2}$ $s^{-1}$, which was defined as optimum for this stage. Additionally, the light qualities and temperatures that were proposed as optimal for each of the two cultivation stages were proven to scale with success using a fermenter-type photobioreactor that was characterized by a low illuminated surface to volume ratio ($S/V$). It was this parameter, $S/V$, the one that was hindering the achivement of similar results to those at laboratory-scale. $S/V$ has to do with the capacity to maintain similar light conditions to those used at lab-scale and defines light distribution in a photobioreactor. The importance of a large $S/V$ was evidenced in this chapter, where two equal systems receiving the same amount of photons, distributed differently, were compared. In the system where photons were more concentrated, a lower $S/V$, growth was dimished and energy dissipation mechanisms were more active. Whereas when photons were better distributed, with a larger $S/V$, growth was higher and photosynthesis was more efficient. Furthermore, continuous cultivation of green-stage *H. pluvialis* was optimized in order to reach a high renewal rate with a minimized remaining nitrogen load. Three cultivation modes were compared, and the one working similar as a chemostat, which reached a biomass productivity of about 0.9 g $L^{-1}$ $day^{-1}$, was chosen to be optimal despite the less efficient cellular productivity than that running on semicontinuous mode. It seemed that continuous illumination taxed cellular division with respect to the use of a circadian light: dark cycle. The integration of all the optimized parameter aforementioned allowed the achievement of pigment content up to 6 % in dry weight and pigment productivities of around 50 mg $L^{-1}$ $day^{-1}$, about twice as high as those achieved at laboratory-scale.

The effect that light quality had on growth, pigment content, and photosynthetic response under conditions of nitrogen starvation and continuous illumination of two *Dunaliella* species, the carotenogenic *D. salina* and the non-carotenogenic *D. tertiolecta*, is described on Chapter three. The aim here was to characterize the response of this two species that, due to their carotenogenic and non-carotenogenic nature, was expected to be very different beforehand. The data evidenced the difference, which was obvious in photosynthetic response and pigment content. With regards to growth, both species showed a similar behaviour in

nitrogen-saturated conditions, during the first days of cultivation, showing no differences in cellular density among treatments and a clear difference in biomass concentration, which was the highest in red light and the lowest in blue light, evidencing that carbon assimilation and allocation may vary regardless of the cell division with respect to wavelength quality. During nitrogen starvation, cellular densities maintained the pattern observed in nitrogen saturation. The same happened in *D. tertiolecta*, while in *D. salina* red light and a mixed red and blue light ended up reaching the same biomass concentration. With regards to pigment content, *D. tertiolecta* did not show differences in the ratio chlorophyll: carotenoid, although total pigment content was the highest in cultures maintained under blue light. On the other hand, *D. salina* synthesized carotenoids massively, especially in blue light where accumulation rate was also the fastest. As in *D. tertiolecta*, total pigment content remained the highest in blue light, which maintained about twice the content of chlorophyll by the end of the cultivation period compared to the treatments were red light was present, under severe nitrogen limitation and with a high carotenoid content. Differences in the ratio chlorophyll to carotenoid were more evident in *D. salina* and this was obvious from the physical appearance of the cultures, that looked greenish in blue light despite the highest carotenoid content, and more orange in red and mixed light. Nevertheless, in *D. salina*, the highest carotenoid productivity was found with a mix of red and blue light since, as in *H. pluvialis*, this light condition generated a more balanced increase in dry weight and carotenoid content than the monochromatic wavelenghts that enhanced either one paramenter or the other. The pigment profile of *D. salina* was proven to be complex since not all the peaks could be identified with high performance liquid chromatography (HPLC). However, thin-layer chromatography (TLC) evidenced that a carotene was the main pigment accumulated by this microalgae that produced a clear, thick orange band close to the solvent's front and coincided with the absorption spectra of β-carotene in pure acetone once that band was eluted. β-carotene was also present in *D. tertiolecta*. However, lutein was identified as the major pigment in this alga, and was clearly observed with both HPLC and TLC methods. In this respect, xanthophyll cycle minor pigments were identified for both species, but the relative amount with respect to total carotenoids must be higher in *D. tertiolecta* than in *D. salina*, where carotene was obviously the major pigment. We hypothesize that the photosynthetic response that was characterized using chlorophyll fluorescence techniques depends on the pigment composition just described. PSII effective quantum yield was equal for every treatment in *D. tertiolecta,* dropping after nitrogen depletion being accompained by a decrease of photochemical fluorescence quenching and a clear increase of non-photochemical fluorescence quenching, denoting the activation of energy dissipation mechanisms, probably a xanthophyll cycle. The response of *D. salina* reminds more that of *H. pluvialis*, described in Chapter one, where the accumulation of carotenoids protect the cells against blue light, evidenced by PSII quantum yield. However, contrary to *H. pluvialis*, marked differences between treatments in photochemical and non-photochemical fluorescence quenching were not observed.

Finally, Chapter four studied the effect that light quality had on growth, pigment content, and photosynthetic response under conditions of nitrogen starvation and continuous illumination of the Rhodophyta *P. cruentum*. The alga showed a similar behaviour during nitrogen saturation to the other species used in this work, where maximal increase in dry weight was reached with red light. Then, during nitrogen starvation, growth under red light seemed to be restricted, since only the treatments containing blue light showed an increase of dry weight, being monochromatic blue light the treatment where biomass concentration was the highest by the end of the cultivation period. As in the carotenogenic species, *H. pluvialis* and *D.*

*salina*, large differences in pigment content were observed in *P. cruentum* with respect to light composition. Red light produced the largest drop in pigment content and concentration with respect to the initial conditions once the stationary phase was achieved, followed by mixed red and blue, and blue light that maintained the highest total pigment content throughout nitrogen starvation. Especially obvious was the effect on phycoerythrin content, which concentration remained about three-fold higher than in red light. The differences in pigment content were obvious in the physical appearance of the cultures, which looked red, yellow, and orange for blue, red, and mixed light, respectively. The photosynthetic response of this species, despite not being characterized for the accumulation of carotenoids, was as well more effective against blue light. Blue-light cultures maintained a higher PSII quantum yield and a lower non-photochemical fluorescence quenching than the treatments where red light was present that showed a response more similar to that characterized in the literature for a full light spectrum. *P. cruentum* is known for the production of polysaccharides, which are compounds with a thickening capacity and with pharmacological potential. Results showed that blue light enhanced exopolysaccharide production, reaching a concentration about 150 mg $L^{-1}$ higher than that with red or mixed light.

In summary, the data reflects a species-dependant response to the tested conditions. Carotenogenic species (*H. pluvialis* and *D. salina*) showed similarities in their response, which had an obvious direct industrial application, since light composition largely affected pigment production. Especially the use of a mixture of red and blue wavelengths is recommended, where the increase in dry weight and pigment content were more balanced. Astaxanthin productivity in *H. pluvialis* maintained under red light reached only 60 % of that under a mixture of red and blue wavelengths. A similar response was observed in *D. salina*. In *P. cruentum*, profound differences between wavelengths were also observed. Blue light cultures maintained the highest pigment abundance with respect to red and a mix of red and blue light. The difference was obvious in phycoerythrin content and concentration, and denotes that phycobilisome degradation must be affected not only by nitrogen deprivation but also by light quality to a certain extent. In *D. tertiolecta*, total pigment content was maintained higher in blue light with respect to red and mixed light. However, chlorophyll to carotenoid ratio did not differ between treatments. The photosynthetic response was equal regardless of the wavelength used and was characterized by a clear increase of non-photochemical fluorescence quenching, indicating the activation of energy dissipation mechanisms. The carotenoids accumulated in *H. pluvialis* and *D. salina* clearly acted as a screen protecting against blue photons, denoted by the maintenance of a higher PSII photosynthetic efficiency and low non-photochemical fluorescence quenching. The photosynthetic response of *P. cruentum* reminded that of the carotenogenic species, but in this case it must be explained by the maintenance of a higher phycoerythrin content in blue light. Moreover, the synthesis of soluble carbohydrates was enhanced under blue light. In terms of growth, all the species responded in a similar way between days 0 and 3, when nitrogen was still available in the culture medium. All the species achieved the onset of stationary phase with a higher biomass concentration in red light. During stationary phase, the response of the species belonging to the Chlorophyta group (*H. pluvialis*, *D. salina*, and *D. tertiolecta*) showed a similar pattern to that observed during nitrogen saturation, though differences diminished between treatments. On the contrary *P. cruentum* achieved the highest dry weight increase with blue light, while red light did not produce any gain during nitrogen starvation. The data that illustrates the scale up of a two-stage cultivation of *H. pluvialis* in 150 L pilot-scale photobioreactors evidenced that light distribution has a higher impact than irradiance on microalgae cultivation. Overall,

the results reflect that a high level of optimization, together with the design of cultivation systems that focus on photosynthetic requirements and allow the integration of such optimization, have a huge impact in microalgae production by improving current cultivation techniques and strategies. Ultimately, this may unlock new commercial applications and have important economic implications.

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El cultivo masivo de microoganismos no es un proceso simple. Sin embargo, es un área crucial en el ámbito de la microbiología industrial y la biotecnología, y por lo tanto un área clave para la industria humana por su gran rango de aplicaciones, desde el tratamiento de aguas residuales y ténicas de bioremediación, generación de etanol y biogas, a ser clave para la producción de alimento animal y humano, así como para la producción de enzimas, alimentos, bebidas, y compuestos con potencial farmacológico.

El cultivo de heterótrofos es una industria altamente desarrollada. Hoy en día, un microbiólogo puede aislar una cepa salvaje útil para una determinada aplicación y sobreproducirla a escala comercial. Además, la modificación genética de bacterias para su uso como vectores para la síntesis de compuestos que no pueden producir de manera natural, como la insulina, es un campo que goza de un amplio conocimiento y su desarrollo es avanzado. Los microorganismos heterótrofos tienen la capacidad de producir compuestos de alto valor de manera relativamente baja en costes, ya que estos organismos pueden crecer en medios simples y baratos y, además, poseen unos ciclos de vida muy rápidos comparados con otros microorganismos eucariotas que hacen que las productividades de estos compuestos sea alta. Por otro lado, su escalabilidad es sencilla ya que la tecnología usada para producirlos está bien desarrollada y estandarizada para su cultivo masivo. Los sitemas de cultivo consisten, normalmente, en fermentadores de acero inoxidable que permiten un ajuste preciso de las condiciones de cultivo y lo que es más importante, permiten su completa esterilización, factor esencial para el escalado de estos microorganismos.

Al igual que los heterótrofos, las microalgas se caracterizan por tener un ciclo de crecimiento rápido cuando los comparamos con otros fotótrofos. Las microalgas muestran una plasticidad bioquímica importante y, por tanto, la capacidad de producir metabolitos de alto valor. En general, el trabajo con microalgas requiere el uso de las mismas técnicas y procedimientos usados en el trabajo con bacterias. Sin embargo, el cultivo a escala industrial de microalgas todavía no ha llegado al mismo nivel de desarrollo que el cultivo industrial de heterótrofos. Esto puede deberse a una serie de motivos que van desde una escalabilidad más dificil y más costosa, que da producciones menos estables y más problemas derivados del mantenimiento de un estándar de calidad. A esto se suma un volumen de mercado más pequeño que está reflejado en los escasos productos derivados de estos microorganismos que han sido regulados por las autoridades competentes. En este sentido, tan solo especies que son de alguna manera extremófilas como *Spirulina* spp., las carotenogénicas *D. salina* y *H. pluvialis*, o especies que pueden ser fermentadas como algunas *Chlorella* spp. o el heterótrofo *Schizochytrium* spp., que normalmente se incluye en el grupo de las microalgas por su cercana relación taxonómica, han estado en el mercado el tiempo suficiente como para que hoy en día su capacidad de producción se haya desarrollado hasta un nivel claramente industrial para producir compuestos de alto valor como acidos grasos y pigmentos, o para su uso en alimentación humana por su valor nutricional. No obstante, y aunque en un volumen mucho menor, nuevos ingredientes derivados de microalgas han llegado al mercado en los últimos años, derivados de especies como *Tetraselmis chuii* o *Phaeodactylum tricornutum*, lo cual demuestra un avance en la industria. Otro de campo donde el cultivo de microalgas se ha establecido con claridad es en la industria de la acuicultura, ya que son organismos esenciales en la cadena de producción de moluscos, el desarrollo temprano de crustáceos, o para el enriquecimiento del valor nutricional de *Artemia* spp. o *Brachionus* spp. que son clave para el desarrollo de larvas de peces marinos.

Más allá de regulaciones de nuevos ingredientes derivados de estos microorganismos, la dificultad de diversificación del cultivo de microalgas a nivel industrial está muy relacionado con los problemas derivados de su escalabilida, como las contaminaciones en los cultivos, la falta de conocimiento de los principios del crecimiento microalgal, o el desconocimiento de una parte esencial de la biología del microorganismo. Esto hace que, en muchos casos, la industria de producción de microalgas se asemeje más a una rama de la agricultura, y que la aproximación más biotecnológica pierda protagonismo.

Este trabajo pivota sobre los temas comentados anteriormente. Los LEDs constituyen una tecnología de iluminación relativamente nueva con la cual se pueden crear calidades de luz diferentes dependiendo de la combinación de las longitudes de onda usadas. Hemos sometido a *H. pluvialis*, *D. salina*, *D. tertiolecta*, y *P. cruentum*, cuatro especies con interés industrial, a iluminación continua de diferente composición en condiciones de limitación por nitrógeno. Estas condiciones favorecen la producción de metabolitos secundarios. Uno de los objetivos fué entender la respuesta desatada por esas condiciones y evaluar el potencial biotecnológico del uso de diferentes longitudes de onda para la producción masiva de microalgas con luz artificial. La generalización en el uso de luces LED reducirá los costes de producción en los sistemas de cultivo que usen luz artificial tan solo por el hecho de que los LED presentan una conversión de corriente a fotones mucho más eficiente que otras fuentes de luz. Además, la optimización de la composición lumínica con respecto al espectro de acción de la composición pigmentaria puede dar lugar a respuestas fisiológicas con potencial industrial, de tal manera que se añade una nueva dimensión al campo de la optimización de la luz. Además, en este trabajo, que se ha llevado a cabo en un ambiente industrial, el cultivo de *H. pluvialis* se ha optimizado considerando otros factores como temperatura, irradiancia, y diseño de fotobioreactor. Tras la optimización a escala de laboratorio, el escalado de los resultados se ha demostrado en una escala piloto construida a modo de "prueba de concepto". Durante el trabajo, se comparten consideraciones sobre el aspecto económico y el proceso de escalado de esta microalga, que son aplicables a cualquier otra especie.

En el Capítulo uno se muestran y discuten datos de la optimización de *H. pluvialis* a escala de laboratorio. Como cabría esperar desde un punto de vista biotecnológico, que tiene como objetivo maximizar la producción de astaxantina en un proceso difásico donde la fase verde y roja del cultivo se producen por separado, los parámetros óptimos para producir cada una de las fases cambia drásticamente. La temperatura óptima para el crecimiento de la fase verde se encontró de 20 a 25 ºC. Finalmente se fijó a 20 ºC ya que a esta temperatura el consumo de nitrógeno era ligeramente más rápido. Además, en esta fase el uso de luz monocromática roja produjo las tasas de crecimiento más altas, mientras que la luz azul las más bajas. De manera interesante, no se encontraron diferencias en la tasa de crecimiento cuando ambas longitudes de onda estaban presentes en los ratios que fueron testados, que fueron en el rango de 25 a 75 % de luz roja o azul. El peso seco se comportó de manera idéntica. Por otro lado, el contenido en astaxantina con luz roja fué el menor, mientras que los mayores contenidos se observaron en luz azul o en mezcla de roja y azul (1: 1). La productividad más alta se encontró en la mezcla ya que las dos longitudes de onda combinadas produjeron un aumento en peso seco y una acumulación de pigmento más balanceada. Adicionalmente, la combinación de luz roja (660 nm) con diferentes longitudes de onda de luz azul (420, 450, 470 nm) mostraron diferencias significativas, especialmente en el aumento del peso seco. Por otro lado, el contenido de clorofila disminuyó una vez activada la síntesis masiva de carotenoides tras el agotamiento del nitrógeno en el medio de cultivo, pero el contenido de este pigmento se

mantuvo más alto en los cultivos iluminados con luz azul. Asimismo, se demostró el enorme efecto de la temperatura sobre la síntesis de carotenoides, donde la productividad de pigmento fué máxima a 30 ºC, alrededor del triple que a 20 ºC. Por encima de 30 ºC, la producción de *H. pluvialis* no es viable y se observa lisis celular. Además, la síntesis de carotenoides está directamente relacionada con la irradiancia recibida. Los carotenoides acumulados por esta microalga protegen a las células, principalmente, del exceso de fotones azules, que se refleja en el mantenimiento de una eficiencia fotosintética y de una fluorescencia fotoquímica ($qP$) mayor que en los tratamientos donde la luz roja está presente. Asimismo, la eficiencia fotosintética muestra una relación inversa con la irradiancia y el tiempo en condiciones inductivas. Por otro lado, la concentración de oxígeno disuelto disminuye tras el agotamiento del nitrógeno y está relacionado con la reducción del metabolismo primario.

El Capítulo dos continúa con la optimización de las condiciones de cultivo de *H. pluvialis,* así como muestra su integración en una "prueba de concepto" a escala piloto. En este capítulo se investiga la irradiancia óptima en fotobioreactores planos, o sistemas tipo "flat-plate". Para determinar este factor en la fase de crecimiento verde, se usaron técnicas de fluorescencia de clorofila. Los datos muestran como el punto de saturación de la fotosíntesis ($Ek$) constituye un valor dinámico que depende del estado celular de *H. pluvialis* y que presenta máximos que se relacionan directamente con la tasa de crecimiento. En este caso, el valor más alto de $E_k$ se midió alrededor de 500 μmol photon $m^{-2}$ $s^{-1}$. Por otro lado, bajo las condiciones de cultivo que se definieron a escala de laboratorio en el Capítulo uno, la tasa de producción de astaxantina por *H. pluvialis* llega al punto de saturación alrededor de 750 μmol photon $m^{-2}$ $s^{-1}$, que se definió como óptimo para la fase roja. Además, el escalado de los parámetros de calidad de luz y temperatura que se propusieron en el Capítulo uno se probaron en dos tipos de fotobioreactores diferentes: fermentadores con luz interna y fotobioreactores planos tipo "flat-plate". El fermentador se caracteriza por tener un ratio $S/V$ bajo, que se indentificó como el principal parámetro dificultando el logro de unos resultados similares a los obtenidos a escala de laboratorio. La importancia de el ratio $S/V$ queda evidenciada en este capítulo, comparando dos sistemas idénticos iluminados con idéntica cantidad de fotones distribuidos de manera diferente, es decir, ratios $S/V$ diferentes en cada reactor. En el sistema donde el $S/V$ era más bajo, es decir, donde los fotones están más concetrados, el crecimiento fué menor mientras que los valores de disipación de energía aumentaron con respecto al sistema con un $S/V$ más alto, donde se observó un crecimiento mayor y una tasa fotosintética más eficiente. Igualmente, en el Capítulo dos se optimiza el cultivo continuo de *H. pluvialis* en fase verde con el objetivo de alcanzar tasas de dilución altas junto con la minimización de la concentración de nitrógeno residual. Para ello se compararon tres modos de producción continua o semi-continua. El modo puramente continuo, con una similitud mayor a un quimiostato de bacterias, alcanzó las productividades más altas en cuanto a producción de biomasa, alrededor de 0.9 g $L^{-1}$ $día^{-1}$ y, aunque el crecimiento celular fué menos eficiente con respecto al cultivo semi-continuo y el uso de ciclos luz/ oscuridad, se escogió como el modo de producción más adecuado para su integración a escala industrial. Finalmente, la integración de todos los parámetros optimizados en la escala piloto propiciaron el alcance de contenidos de pigmento en torno al 6 % en peso seco y productividades de unos 50 mg $L^{-1}$ $día^{-1}$, superiores a las alcanzadas a escala de laboratorio,

En el Capítulo tres se describe el efecto de la composición lumínica sobre el crecimiento, pigmentación y respuesta fotosintética en condiciones de limitación por nitrógeno e iluminación continua, en dos especies del género *Dunaliella*: la carotenogénica *D. salina* y la

no-carotenogénica *D. tertiolecta*. El objetivo principal fue la caraterización de la respuesta de ambas especies según su naturaleza carotenogénica y no-carotenogénica, que se conocía de antemano. Los datos evidencian la diferente respuesta, que fué obvia desde el punto de vista de respuesta fotosintética y en pigmentación. Con respecto a crecimiento, ambas especies mostraron una respuesta similar durante los primeros días de cultivo, en condiciones de saturación de nitrógeno, caracterizada por no mostrar diferencias en densidad celular pero sí en peso seco. La luz roja generó una concentración de biomasa más alta que la luz azul. Estos datos muestran como la asimilación y distribución intracelular de carbono puede variar con respecto a la longitud de onda independientemente del crecimiento celular. En condiciones de limitación por nitrógeno, la densidad celular mantuvo el mismo patrón que en saturación de nitrógeno para ambas especies, mientras que el peso seco aumentó en *D. salina*, especialmente con la mezcla de luz roja y azul. En *D. tertiolecta,* las diferencias en peso seco entre tratamientos también se mantuvieron constantes en fase estacionaria. Con respecto a la pigmentación, en *D. tertiolecta* no se observaron diferencias en el ratio clorofila: carotenoide, a pesar de que el contenido de pigmentos totales fué mayor en luz azul. Por otro lado, en *D. salina* se observó una síntesis masiva de carotenoides, especialmente en los cultivos de luz azul. Al igual que en *D. tertiolecta*, la cantidad total de pigmento también fue mayor en luz azul, donde el contenido en clorofila se mantuvo alrededor del doble en comparación con los tratamientos iluminados con luz roja o mezcla. Las diferencias observadas en el ratio clorofila: carotenoide en *D. salina* se reflejaron en la apariencia física de los cultivos que mostraron un tono verdoso en luz azul, a pesar del mayor contenido de carotenoide, y un tono más anaranjado en luz roja y mezcla de luz. De cualquier modo, como en *H. pluvialis*, la productividad de carotenoides fué máxima en los cultivos de *D. salina* cultivados en mezcla de luz roja y azul ya que esta condición generó un aumento más balanceado del peso seco y la síntesis de carotenoides. Además *D. salina* presenta un perfil de pigmentos complejo, donde no todos los picos generados mediante análisis con cromatografía líquida de alta eficacia HPLC fueron identificados. Los resultados del HPLC, combinados con cromatografía en capa fina (TLC) y posterior elución e identificación espectrofotométrica mostraron, como era esperado, un abundante contenido de β-caroteno. Este pigmento también se detectó, en menor medida, en *D. tertiolecta*. Para esta especie, luteina fué el pigmento identificado como mayoritario, claramente observado tanto en HPLC como en TLC. Pigmentos minoritarios relacionados con un ciclo de xantofilas fueron identificados en ambas especies. Sin embargo, la cantidad relativa de estos repecto a los carotenoides totales es mayor en *D. tertiolecta* que en *D. salina*. Por este motivo, hipotetizamos que esta composición pigmentaria caracteriza la respuesta fotosintética que se estudió mediante técnicas de fluorescencia de clorofila. De este modo, la eficiencia fotosintética de *D. tertiolecta* cayó tras el agotamiento de nitrógeno, acompañado por un claro aumento de mecanismos de disipación de energía reflejados en valores elevados de fluorescencia no-fotoquímica posiblemente derivados de la activación de un ciclo de xantofilas. La respuesta de *D. salina* recuerda a la descrita para *H. pluvialis* en el Capítulo uno, donde la acumulación de carotenoides protege a las células contra luz azul, manteniendo una eficiencia fotosintética alta. Sin embargo, al contrario que en *H. pluvialis*, no se observaron diferencias claras en valores de fluorescencia fotoquímica y no-fotoquímica.

Para finalizar, en el Capítulo cuatro se estudió el efecto de la composición lumínica sobre el crecimiento, pigmentación, y respuesta fotosintética en condiciones de limitación por nitrógeno y luz continua en la Rodófita *P. cruentum*. Esta especie mostró un comportamiento similar en saturación de nitrógeno al resto de especies estudiadas en este trabajo, con un mayor aumento del peso seco en luz roja. Sin embargo, en condiciones de limitación de

nitrógeno, el aumento en peso seco parece restringirse en esta condición de luz ya que solamente se mide aumento en peso seco en los tratamientos de luz azul y mezcla de luz roja y azul. Al final del periodo de cultivo, la luz azul generó el mayor aumento en peso seco. Como en las especies carotenogénicas *H. pluvialis* y *D. salina*, en *P. cruentum* se observan grandes diferencias en pigmentación con respecto a la longitud de onda. Luz roja produjo la mayor degradación de pigmento total, mientras que la degradación fué menor en luz azul, que conservó el contenido más alto en condicioned de limitación por nitrógeno. Los efectos sobre el contenido en ficoeritrina, al ser este el principal pigmento en esta especie, fueron los más obvios. En luz azul, la concentración de ficoeritrina se mantuvo unas tres veces superior a la concentración en luz roja. Estas diferencias se reflejaron a simple vista en la apariencia de los cultivos, que conservaron el color rojo en luz azul y se mostraron anaranjados y amarillentos en mezcla de luz y luz roja, respectivamente. La respuesta fotosintética, a pesar de no estar caracterizada por la acumulación de carotenoides, la eficiencia fotosintética se mantuvo más alta en luz azul, donde también se observaron unos valores de fluorescencia no-fotoquímica menores que en los tratamientos con luz roja, que mostraron una respuesta similar a la descrita en la literatura para un espectro de luz completo. *P. cruentum* es una especie estudiada por su capacidad para producir polisacáridos que poseen cualidades espesantes que levantan interés en la industria alimentaria, así como potencial farmacológico. Los resultados obtenidos en esta investigación muestran como la concentración de estos en el medio también se ve afectada por la composición lumínica siendo más abundantes con luz azul, donde se produjeron alrededor de 150 mg $L^{-1}$ más que en luz roja y mezcla.

En resumen, los datos presentados en este trabajo reflejan las respuestas específicas a las condiciones de cultivo experimentales. Las especies carotenogénicas (*H. pluvialis* y *D. salina*) muestran una respuesta similar que tiene una aplicabilidad industrial directa, ya que la composición lumínica afecta claramente a la producción de pigmento. A partir de los resultados observados, se recomienda el uso de una mezcla de luz roja y azul ya que, con esta condición, se consigue un aumento del peso seco y de la acumulación de pigmento más balanceada, logrando productividades máximas. En *H. pluvialis*, las productividades alcanzadas en luz roja solo alcanzan un 60 % de las alcanzadas en mezcla de luz roja y azul. *D. salina* muestra una respuesta similar. En *P. cruentum*, también se observan diferencias entre longitudas de onda, donde en luz azul la biomasa conserva una contenido de pigmento mayor. Las diferencias más obvias se observan en el contenido de ficoeritrina, lo cual evidencia que el proceso de degradación de los ficobilisomas debe de estar afectado también por la composición de luz y no solo por la disponibilidad de nitrógeno. En *D. tertiolecta*, el contenido de pigmento total también se mantuvo más alto en luz azul. Sin embargo, no se observaron diferencias en el ratio clorofila: carotenoide entre distintas longitudes de onda. En esta especie la respuesta fotosintética fue independiente de la calidad lumínica y se caracterizó por un claro aumento de los valores de fluorescencia no-fotoquímica que están relacionados con el aumento de mecanismos de disipación de exceso de energía. Por otro lado, los carotenoides acumulados en *H. pluvialis* y *D. salina* actúan como una pantalla protectora principalmente contra la luz azul, reflejado en el mantenimiento de una eficiencia fotosintética alta en ambas especies. La respuesta fotosintética de *P. cruentum* muestra similitudes con las especies carotenogénicas pero, en este caso, la respuesta debe ser explicada por el mantenimiento de un contenido de ficoeritrina más alto en luz azul. Además, la síntesis de carbohidratos solubles también fué la más alta en luz azul. Con respecto al crecimiento, todas las especies estudiadas responden de una manera similar entre los días 0 y 3, que corresponden a condiciones de disponibilidad de nitrógeno. En todas las especies, el

mayor incremento en peso seco a día 3 se observa en luz roja. En fase estacionaria, la respuesta de las Clorófitas (*H. pluvialis*, *D. salina* y *D. tertiolecta*) es similar al observado en saturación de nitrógeno, aunque las diferencias disminuyen ligeramente. Al contrario, en *P. cruentum* no se registra aumento en peso seco durante la fase estacionaria en luz roja, mientras que el mayor aumento en peso seco se alcanza con luz azul. Asimismo, los datos presentados ilustran el escalado de una producción de *H. pluvialis* para la producción de astaxantina en dos fases, en fotobioreactores planos de 150 L. En estos sistemas quedó evidenciado el mayor benficio de una buena distribución lumínica sobre la irradiancia absoluta en el cultivo. Los resultados reflejan que un alto grado de optimización, junto con el diseño de sistemas de cultivo que permitan la integración de los parámetros optimizados centrándose en los factores que mayor influencia tienen sobre la fotosíntesis, tiene un gran potencial para el desarrollo de nuevas técnicas y estrategias de producción o para mejorar las existentes. Además, esto puede contribuir a desentrañar nuevas aplicaciones comerciales de estos microorganismos.

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O cultivo masivo de microorganismos non é un proceso sinxelo. Sen embargo, é un campo crucial no ámbito da microbioloxía industrial e a biotecnoloxía e, por tanto, clave para a industria humana polo amplo rango de aplicacións, dende o tratamento de augas residuáis e o seu uso en técnicas de bioremediamento, xeración de etanol e biogás, a ser esencial para a producción de alimento animal e humano, asi coma para a producción de enzimas, alimentos, bebidas, e compostos con potencial farmacolóxico.

O cultivo de heterótrofos é unha industria altamente desenvolta. Hoxe en día, un microbiologo pode aislar unha cepa salvaxe útil para unha determinada aplicación e sobreproducila a escala comercial. Amais, a modificación xenética de bacterias para o seu uso coma vectores para a síntese de compostos que non poden producir de maneira natural, coma a insulina, é un campo que goza de un amplo coñecemento e o seu desenvolvemento é avanzado. Os microorganismos heterótrofos teñen a capacidade de producir compostos de alto valor de xeito relativamente baixo en custos, xa que estos organismos crecen en medios simples e baratos e, ademais, posúen ciclos de vida moi rápidos comparados cos microorganismos eucariotas. Isto fai que as productividades sexan altas. Por outra banda, a escalabilidade é sinxela xa que a tecnoloxía empregada para a producción destes microorganismos está ben desenvolta e estandarizada para o cultivo masivo. Os sistemas de cultivo consisten en fermentadores de aceiro inoxidable que permiten o axuste preciso das condicións de cultivo e o que é máis imporante, permiten a sua esterilización completa, que é esencial para o escalado déstes microorganimos.

O igual ca os heterótrofos, as microalgas caracterízanse por ter un ciclo de crecemento rápido, cando son comparadas con outros fotótrofos. As microalgas amosan unha plasticidade bioquímica importante e, por tanto, a capacidade de producir metabolitos de alto valor. En xeral, o traballo con microalgas require o uso das mesmas técnicas e procedementos usados no traballo con bacterias. Sen embargo, o cultivo a escala industrial de microalgas todavía non chegóu o memo nivel de desenvolvemento ca o cultivo industrial de heterótrofos. Isto pode deberse a unha serie de motivos que van dende unha escalabilidade máis comprexa e máis custosa, que xera producción menos estables e máis problemas derivados do mantemento dun estándar de calidade. A isto se suma un volumen de mercado máis pequeno, proxectado nos escasos produtos derivados déstes microorganismos regulados polas autoridades competentes. Neste sentido, tan só as especies que son de algunha maneira extremófilas coma *Spirulina* spp., as carotenoxénicas *H. pluvialis* e *D. salina*, ou especies fermentábeis coma algunhas *Chlorella* spp. ou o heterótrofo *Schizochytrium* spp., incluído normalmente no grupo das microalgas pola súa cercana relación taxonómica, levan no mercado tempo dabondo como para que, hoxe, a súa capacidade de producción fora desenvolvida ata un nivel claramente industrial para a producción de compostos de alto valor coma ácidos graxos e pigmentos, ou para o seu uso en alimentación humana polo seu valor nutricional. Non obstante, aínda que nun volumen moito menor, nos últimos anos novos ingredientes derivados de microalgas coma *Tetraselmis chuii* ou *Phaeodactylum tricornutum* chegaron ó mercado, o cal é demostrativo dun avance na industria. Outro campo onde o cultivo de microalgas esta ben establecido é na industria da acuiculture, xa que as microalgas son microorganismos esenciáis na cadena de producción de moluscos, no desenvolvemento de crustáceos, ou para o enriquecemento do valor nutricional de *Artemia* spp. ou *Brachionus* spp., claves para o desenvolvemento larvario de peixes marinos.

Máis aló de regulacións de novos ingredientes derivados destes microorganismos, a dificultade de diversificación no cultivo de microalgas a nivel industrial está moi relacionada cos problemas derivados da súa escalabilidade, coma as contaminacións nos cultivos, a falta de coñecemento dos principios de crecemento microalgal, ou o descoñecemento dalgunha parte esencial da bioloxía do microorganismo. Isto fai que, en moitos casos, a industria de producción de microalgas aseméllase máis a unha rama da agricultura, e que a aproximación máis biotecnolóxica perda protagonismo.

Este traballo pivota sobre os asuntos comentados anteriormente. Os LEDs constitúen unha tecnoloxía de iluminación relativamente nova coa cal pódense crear calidades de luz diferentes dependendo na combinación das lonxitudes de onda empregadas. Neste traballo sometemos a *H. pluvialis*, *D. salina*, *D. tertiolecta*, e *P. cruentum*, catro especies cun interese industrial, a iluminación continua de diferente composición en condicións de limitación por nitróxeno. Estas condicións favorecen a producción de metabolitos secundarios. Un dos obxectivos foi entender a resposta desatada por estas condicións e evaluar o potencial biotecnolóxico do uso de diferentes lonxitudes de onda para a producción masiva de microalgas con luz artificial. A xeneralización do uso de luces LED reducirá os costes de producción dos sistemas de cultivo que utilizan luz artificial tan so polo feito de que os LEDs presentan unha conversión de corrente en fotóns máis eficiente ca outras fontes de luz. Amais, a optimización da composición lumínica respecto ó espectro de acción da composición pigmentaria pode dar lugar a respostas fisiolóxicas con potencial industrial. Desta maneira se engade unha nova dimensión no campo da optimización da luz. Ademais, neste traballo, levado a cabo nun ambiente industrial, o cultivo de *H. pluvialis* optimizouse considerando outros factores coma a temperatura, irradiancia, e o deseño do fotobioreactor. Trala optimización a escala de laboratori, o escalado dos resultados demostrouse a escala piloto, que se construíu a modo de "proba de concepto". Durante o traballo, compártense consideracións sobre o aspecto económico e o proceso de escalado desta microalga, aplicábeis a calquera outra especie.

No Capítulo un mostramos e discutimos datos da optimización de *H. pluvialis* a escala de laboratorio. Como cabría esperar dende un punto de vista biotecnolóxico, que ten como obxectivo a maximización da producción de astaxantina nun proceso difásico onde a fase verde e vermella do cultivo se producen por separado, os parámetros óptimos para cada unha das fases cambia de maneira drástica. A temperatura óptima para o crecemento en fase verde foi entre 20 e 25 ºC. Finalmente, elixiuse 20 ºC xa que a esta temperatura o consumo de nitróxeno foi lixeiramente máis rápido. Amais, nesta fase o uso de luz monocromática vermella producíu as taxas de crecemento máis altas, mentres que a luz azul producíu as máis baixas. De maneira interesante, non se atoparon diferenzas na taxa de crecemento cando ámbalas dúas lonxitudes de onda estaban presentes nos ratios testados, no rango de 25 a 75 % de luz vermella ou azul. O peso seco comportóuse de maneira idéntica. Respecto o contido en astaxantina, foi baixo en luz vermella e alto en luz azul e mestura das luces vermella e azul (1: 1). A productividade máis alta atopouse na mestura de luz xa que a combinación das dúas lonxitudes de onda produciu un aumento en peso seco e acumulación de pigmento máis balanceado. Adicionalmente, a combinación de luz vermella (660 nm) con diferentes lonxitudes de onda de luz azul (420, 450, 470 nm) amosaron diferenzas significativas, especialmente no aumento do peso seco. Por outra banda, o contido de clorofila diminuíu unha vez activada a síntese masiva de carotenoides tralo esgotamento de nitróxeno no medio de cultivo, aínda que o contido deste pigmento mantívose máis alto nos cultivos iluminados

con luz azul. Así mesmo, demostrouse o enorme efecto da temperatura sobre a síntese de carotenoides, onde a productividade de pigmento foi máxima a 30 ºC, ao redor do triplo ca 20 ºC. Por riba de 30 ºC, a producción de *H. pluvialis* non é viable e se observa lisis celular. Amais, a síntese de carotenoides está directamente relacionada coa irradiancia percibida. Os carotenoides acumulados por esta microalga protexen as células do exceso de fotóns azules, principalmente, e isto se reflexa no mantemento dunha eficicencia fotosintética e dunha fluorescencia fotoquímica ($qP$) maior ca a dos tratementos onde a luz vermella está presente. Así mesmo, a eficiencia fotosintética amosa unha relación inversa coa irradiancia e mais o tempo en condicións inductivas. Por outra banda, a concentración de osíxeno disolto reduciuse tralo esgotamento do nitróxeno e isto relaciónase coa reducción do metabolismo primario.

O Capítulo dous continúa coa optimización das condicións de cultivo de *H. pluvialis*, e amosa a integración destas nunha "proba de concepto" a escala piloto. Neste capítulo investígase a irradiancia óptima en fotobioreactores planos, ou sistemas tipo "flat-plate". Para determinar este factor en fase de crecemento verde, usáronse técnicas de fluorescencia de clorofila. Os datos amosan coma o punto de saturación da fotosíntese ($Ek$) constitúe un valor dinámico que depende do estado celular de *H. pluvialis* e que presenta máximos relacionados directamente coa taxa de crecemento. Neste caso, o valor máis alto de $E_k$ se mediu ao redor de 500 µmol photon $m^{-2}$ $s^{-1}$. Por outra banda, baixo as condición de cultivo definidas a escala de laboratorio no Capítulo un, a taxa de producción de astaxantina alcanzou a saturación ao redor de 750 µmol photon $m^{-2}$ $s^{-1}$, que se definiu coma óptimo para a fase vermella. Amais, o escalado dos parámetros de calidade de luz e temperatura propostos no Capítulo un ratificáronse en dous tipos de fotobioreactores diferentes: fermentadores con luz interna e fotobioreactores planos tipo "flat-plate". O fermentador caracterízase por ter un ratio $S/V$ baixo. Isto identificouse coma o principal parámetro dificultando o logro duns resultados similares os da escala de laboratorio. A importancia deste ratio quedou evidenciada neste capítulo, onde se comparan dous sistemas de cultivo idénticos, irradiados cunha indéntica cantidade de fotóns, mais distribuidos de maneira diferente, é dicir, diferentes ratios $S/V$ en cada reactor. No reactor co ratio máis baixo, onde os fotóns estan máis concentrados, o crecemento foi menor e os valores de disipación de enerxía aumentaron con respecto a o sistema con ratio $S/V$ maior onde a taxa de crecemento e a eficiencia fotosintética foron maiores. Neste Capítulo dous tamén se optimiza o cultivo continuo de *H. pluvialis* en fase verde co o obxectivo de alcanzar taxas de dilución elevadas xunto coa minimización da concentración de nitróxeno residual. Para isto compararónse tres modos de producción continua ou semi-continua. O modo puramente continuo, o máis similar a un quimiostato bacteriano, alcanzou as productividades máis altas en producción de biomasa, chegando ao redor de 0.9 mg $L^{-1}$ $día^{-1}$ e, aínda que o crecemento celular foi menos eficiente respecto a o modo semi-continuo e a o uso de ciclos de luz/ escuridade, escolleuse coma o modo de producción máis apropiado para a súa integración a escala industrial. Finalmente, a integración de tódolos parámetros optimizados na escala piloto propiciaron alcanzar contidos de pigmento ao redor de 6 % en peso seco e productividades arredor de 50 mg $L^{-1}$ $día^{-1}$, superiores as alcanzadas a escala de laboratorio.

No Capítulo tres descríbese o efecto da composición lumínica no crecemento, a pigmentación, e a resposta fotosintética en condicións de limitación por nitróxeno e iluminación continua, en dous especies do xénero *Dunaliella*: a carotenoxénica *D. salina* e a non-carotenoxénica *D. tertiolecta*. O obxectivo principal foi a caracterización da resposta de ámbalas dúas especies

segundo a súa natureza carotenoxénica e non-carotenoxénica, que se coñecía de antemán. Os datos evidencian a diferente resposta, que foi obvia dende o punto de vista da resposta fotosintética e na pigmentación. Respecto a o crecemento, ambas especies amosaron unha resposta similar durante os primeiros días de cultivo, en condicións de saturación de nitróxeno, caracterizada por mostrar diferenzas en peso seco pero non en crecimento celular. A luz vermella xerou unha concentración de biomasa maior ca luz azul. Estes datos amosan como a asimilación e posterior distribución intracelular de carbono pode variar con respecto a lonxitude de onda, independentemente do crecemento celular. En condicións de limitación por nitróxeno, a densidade celular mantivo o mesmo patrón ca en saturación de nitróxeno en ambas especies, mentres que o peso celular aumentou en *D. salina* iluminada coas luces azul e coa mestura de luz azul e vermella. En *D. tertiolecta*, as diferenzas en peso seco entre os tratamentos tamén se mantiveron constantes na fase estacioria. Respecto a pigmentación, en *D. tertiolecta* non se observaron diferenzas no ratio clorofila: carotenoide, a pesar de que o contido de pigmentos totales foi maior en luz azul. Por outra banda, en *D. salina* observouse síntese masiva de carotenoides, especialmente nos cultivos en luz azul. O igual que en *D. tertiolecta*, a cantidade total de pigmento tamén foi maior en luz azul, onde o contido en clorofila mantívose ao redor do dobre en comparación cos cultivos iluminados con mestura de luz e con luz vermella. As diferenzas observadas no ratio clorofila: carotenoide en *D. salina* reflctiron na apariencia física dos cultivos, que amosaron tonalidades verdosas en luz azul, a pesar do maior contido en carotenoides, e tonalidades alaranxadas en luz vermella e mestura de luz azul e vermella. O igual que en *H. pluvialis*, a productividade más alta de carotenoides observouse nos cultivos de *D. salina* iluminados con mestura de luz, que producíu o aumento máis balanceado de peso seco e síntese de carotenoides. Ademais, os análises de cromatografía líquida de alta eficacia (HPLC) e de cromatografía en capa fina (TLC) e posterior elución e identificación espectrofotométrica, revelaron a complexidade do perfil de pigmentos destas especies, especialmente de *D. salina*. Como era de esperar, β-caroteno foi identificado coma o pigmento maioritario en *D. salina*, mentres que a luteina foi indentificado coma maioritario en *D. tertiolecta*. En canto aos pigmentos minoritarios, pigmentos relacionados con ciclos de xatofilas foron identificados en ámbalas dúas especies. Sen embargo, a cantidade relativa destos respecto a os carotenoides totales é maior en *D. tertiolecta* que en *D. salina.* Por este motivo, hipotetizamos que é a diferente composición pigmentaria a que caracteriza a resposta fotosintética estudada mediante técnicas de fluorescencia de clorofila. De este modo, a eficiencia fotosintética de *D. tertiolecta* caeu tralo esgotamento de nitróxeno xunto a o aumento de mecanismos de disipación de enerxía reflictidos en valores elevados de fluorescencia non-fotoquímica ($qN$) derivados da activación dun ciclo de xantofilas. A resposta de *D. salina* asemella a descrita para *H. pluvialis* no Capítulo un, onde a acumulación de carotenoides protexe as células da luz azul, mantendo unha eficiencia fotosintética alta. Sen embargo, ao contrario que *H. pluvialis*, non se observaron diferenzas claras en valores de fluorescencia fotoquímica ($qP$) e non-fotoquímica ($qN$).

Para rematar, no Capítulo cuatro se estuda o efecto da composición lumínica no crecemento, pigmentación, e a resposta fotosintética en condicións de limitación por nitróxeno e luz continua na Rodófita *P. cruentum.* Esta especie amosou un comportamento similar en condicións de saturación de nitróxeno o resto das especies estudadas neste traballo, cun maior aumento do peso seco en luz vermella. Sen embargo, en condicións de limitación por nitróxeno, o aumento do peso seco parece restrinxido os tratamentos de luz azul e mestura de luz azul e vermella. O final do periodo de cultivo, a luz azul xerou o maior aumento en peso

seco. Coma nas especies carotenoxénicas *H. pluvialis* e *D. salina*, en *P. cruentum* observáronse grandes diferenzas en pigmentación respecto a lonxitude de onda. A luz vermella produciu a maior degradación de pigmento total, mentres os cultivos en luz azul preservaron o contido máis alto en condicións de limitación por nitróxeno. Os efectos sobre a ficoeritrina, o principal pigmento nesta especie, foron os máis obvios. En luz azul, a concentración de ficoeritrina se mantivo arredor do triplo ca en luz vermella. As diferenzas foron apreciables a simple vista na apariencia física dos cultivos, que preservaron a cor vermella característica de esta especie en luz azul, mentres que a cor tornou alaranxada e amarelada en mestura de luz e en luz vermella, respectivamente. Aínda que a resposta fotosintética non estivo caracterizada polo incremento no contido de carotenoides totales, a eficiencia fotosintética se mantivo máis alta en luz azul, xunto a uns valores menores de fluorescencia non-fotoquímica respecto a os tratamentos onde a luz vermella está presente, que amosaron un comportamento similar o descrito na literatura para un espectro de luz máis completo. *P. cruentum* é unha especie amplamente estudada pola súa capacidade para a producción de polisacáridos, que posúen cualidades espesantes que xeran interese na industria alimentaria, asi coma potencial farmacolóxico. Os resultados acadados na presente investigación amosan como a concentración de estos polisacáridos no medio tamén vese afectada pola composición lumínica sendo máis abundantes con luz azul, onde se produciron arredor de 150 mg $L^{-1}$ máis ca en luz vermella e mestura de luz.

En resumo, os datos presentados neste traballo amosan as respostas específicas as condicións experimentadas. As especies carotenoxénicas (*H. pluvialis* e *D. salina*) amosan unha resposta similar que ten aplicabilidade industrial directa, xa que a composición lumínica afecta claramente a producción de pigmento. A partir dos resultados observados, aconséllase o uso dunha mestura de luz azul e vermella, que foi a condición onde se conseguiu a maior productividade debido a un aumento de peso seco e a síntese de pigmento máis balanceado. En *H. pluvialis*, a productividade acadada en luz vermella e tan só dun 60 % respecto a mestura de luz. *D. salina* amosa unha resposta similar. En *P. cruentum* tamén se observan diferenzas entre lonxitudes de onda, onde a biomasa preserva un contido maior en pigmento total con luz azul. As diferenzas máis obvias se observan no contido de ficoeritrina, evidenciando que a degradación dos ficobilisomas debe estar afectada pola composición de luz e non só pola dispoñibilidade de nitróxeno. En *D. tertiolecta*, o contido de pigmento total tamén foi maior en luz azul. Sen embargo, non se observaron diferenzas no ratio clorofila: carotenoide entre as distintas lonxitudes de onda. A resposta fotosintética nesta especie foi independente da calidade lumínica e caracterizouse por un claro aumento dos valores de fluorescencia non-fotoquímica, relacionados co aumento de mecanismos de disipación de exceso de enerxía. Por outra banda, os carotenoides acumulados en *H. pluvialis* e *D. salina* actúan coma unha pantalla protectora contra a luz azul, principalmente, traducido no mantemento dunha eficiencia fotosintética alta en ámbalas dúas especies nesta condición. A resposta fotosintética de *P. cruentum* amosa similitudes coas especies carotenoxénicas pero, neste caso, a resposta debe ser explicada polo mantemento de un contido de ficoeritrina maior en luz azul. Amais, a síntese de carbohidratos solubles tamén foi a máis alta en luz azul. Respecto a o crecemento, tódalas especies estudadas responden de maneira similar entre os días 0 e 3, correspondentes a condicións de dispoñibilidade de nitróxeno. En tódalas especies, o maior incremento en peso seco a día 3 observouse en luz vermella. Na fase estacionaria, a resposta das Clorófitas (*H. pluvialis*, *D. salina* e *D. tertiolecta*) foi similar o observado en saturación de nitróxeno, aínda que as diferencias diminuiron lixeiramente. O contrario, en *P. cruentum* non se observa aumento en peso seco en luz vermella e limitación de nitróxeno,

mentres que isto sí acontece en luz azul. Así mesmo, os datos presentados ilustran o escalado dunha producción de *H. pluvialis* para a producción de astaxantina en dúas fases, en fotobioreactores planos de 150 L. Neste sistema quedou evidenciado o maior beneficio dunha boa distribución lumínica por enriba dos valores de irradiancia absolutos. Os resultados reflicten que un alto grado de optimización, xunto co deseño dun sistema de cultivo que permita a integración dos parámetros optimizados co foco nos factores que maior influencia teñen na fotosíntese, ten un gran potencial para o desenvolvemento de novas técnicas e estatexias de producción así como para a mellora das xa existentes. Isto pode contribuir a desentrañar novas aplicacións comerciales de estes microorganismos.

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# ANNEX: PUBLICATIONS

**Does Haematococcus pluvialis need to sleep?**

A. Domínguez a, S. Pereira a , b, A. Otero a ,
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**Effect of light quality on carotenogenic and non-carotenogenic species of the genus Dunaliella under nitrogen deficiency**

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Effect of light quality on carotenogenic and non-carotenogenic species of the genus *Dunaliella* under nitrogen deficiency

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SUPPLEMENTARY MATERIAL

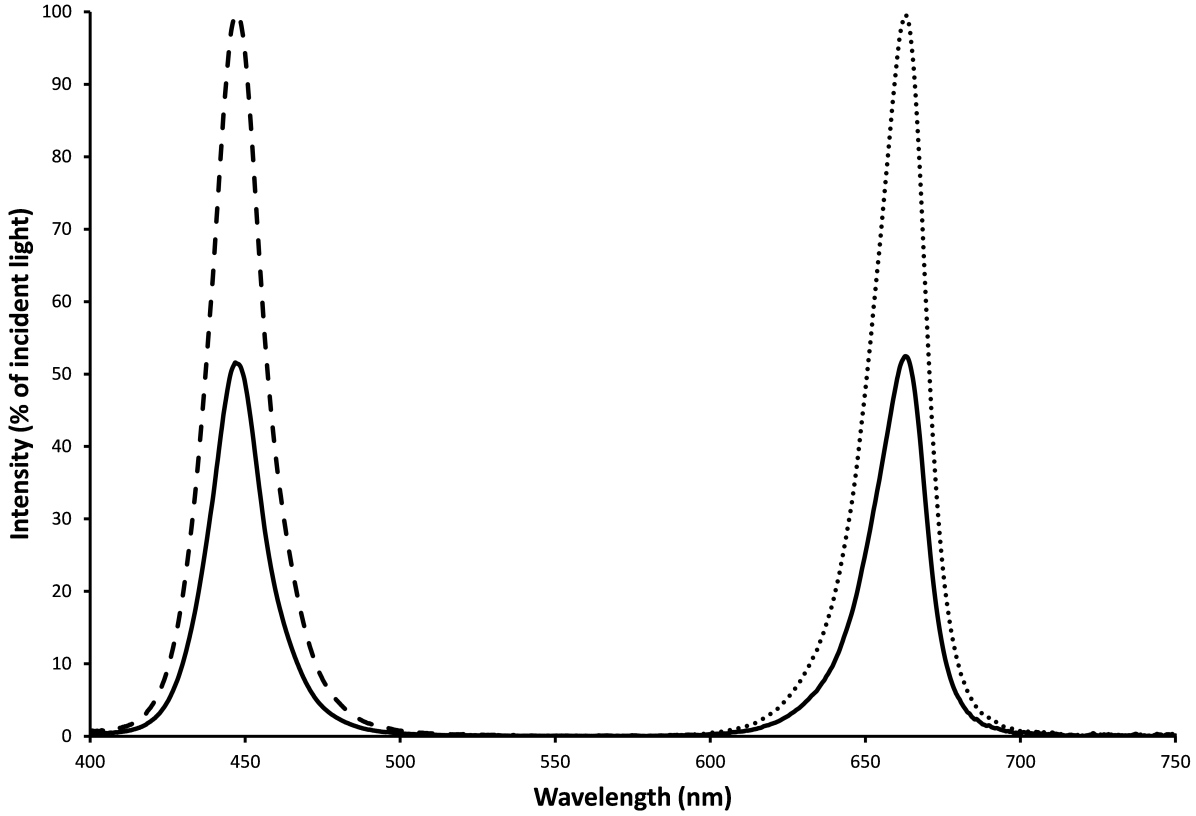



Figure S1. Spectral characteristics of the LED light sources. RB – Mix of red (660 nm) and blue (450 nm) (continuous line); B – Monochromatic blue (450 nm) (discontinuous line); R – Monochromatic red (660 nm) (dotted line).

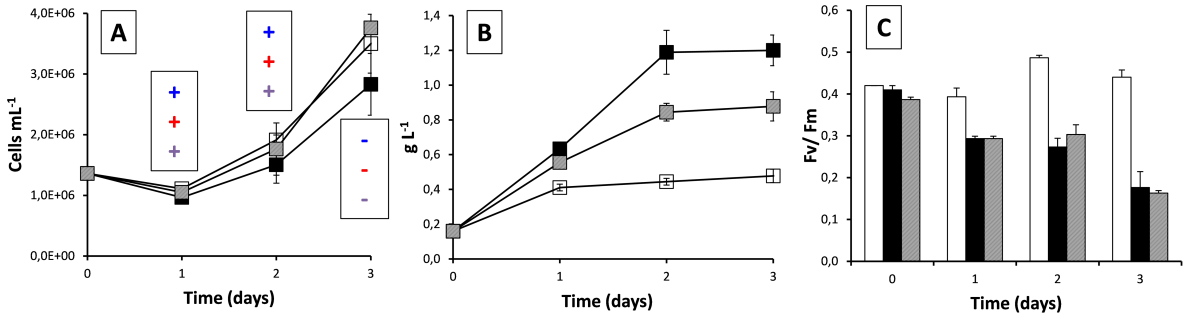



Figure S2. Cell density (A), biomass concetration (B), and PSII effective quatum yield of batch cultures of *D. salina* until nitrogen depletion (days 0 to 3) maintained under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (white symbols), red (black symbols), and mix of red and blue (grey symbols). Inserts in A represent the presence (+) or absence (-) of nitrogen in the culture medium for each of the light qualities per day. Vertical bars represent S.D. (*n = 3*).

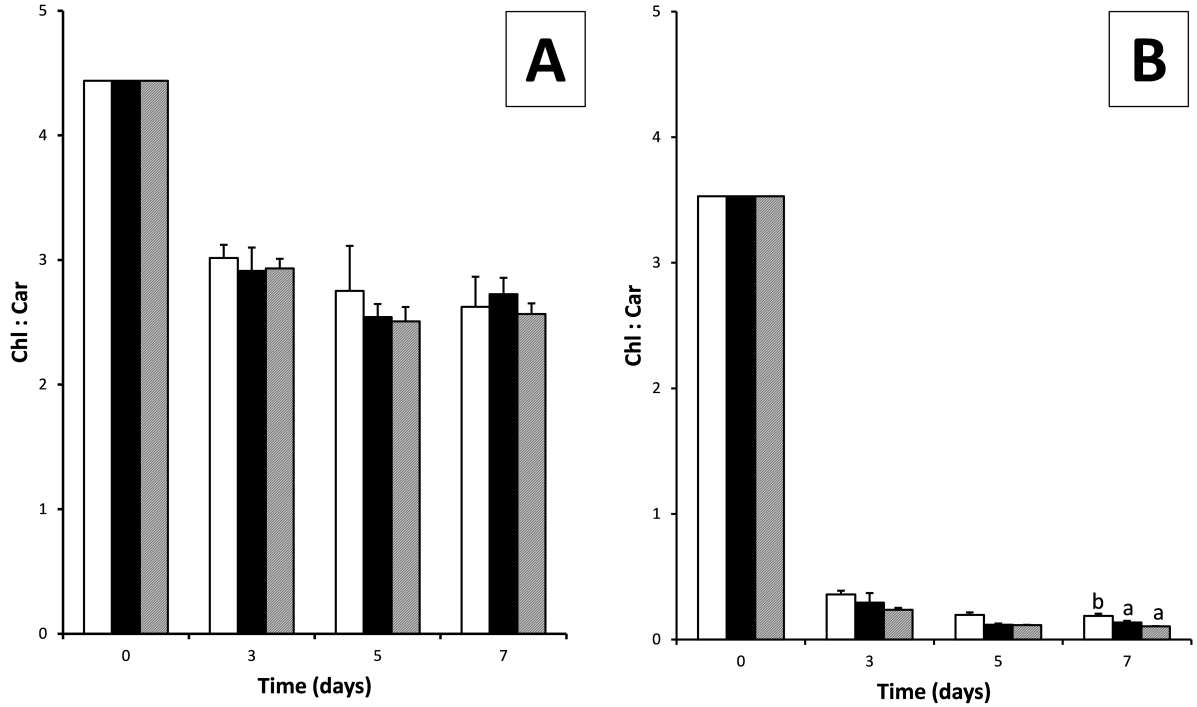



Figure S3. Chlorophyll to carotenoid ratio in batch cultures of *D. tertiolecta* (A) and *D. salina* (B) under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (white bars), red (black bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. Vertical bars represent S.D. (*n = 3*).

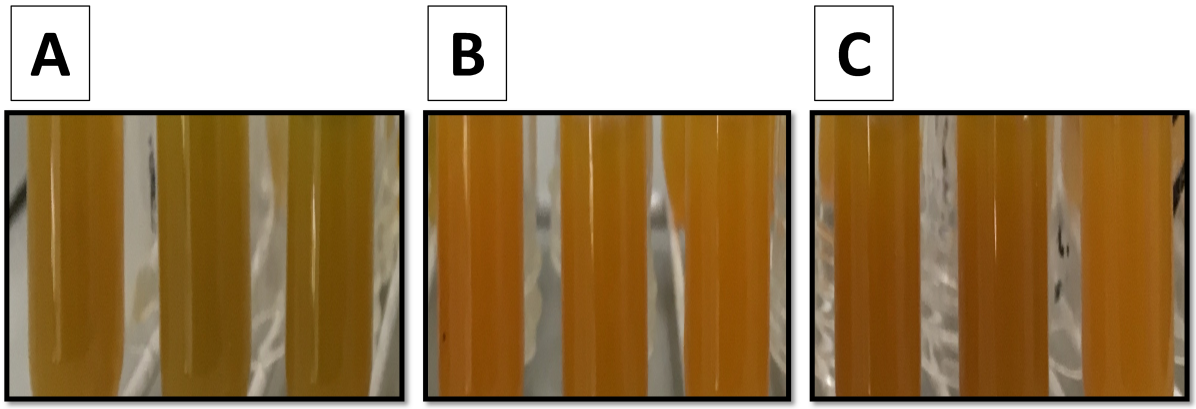



Figure S4. Physical appearance of stationary phase cultures of *D. salina* grown under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (A), red (B), and mix of red and blue (C).

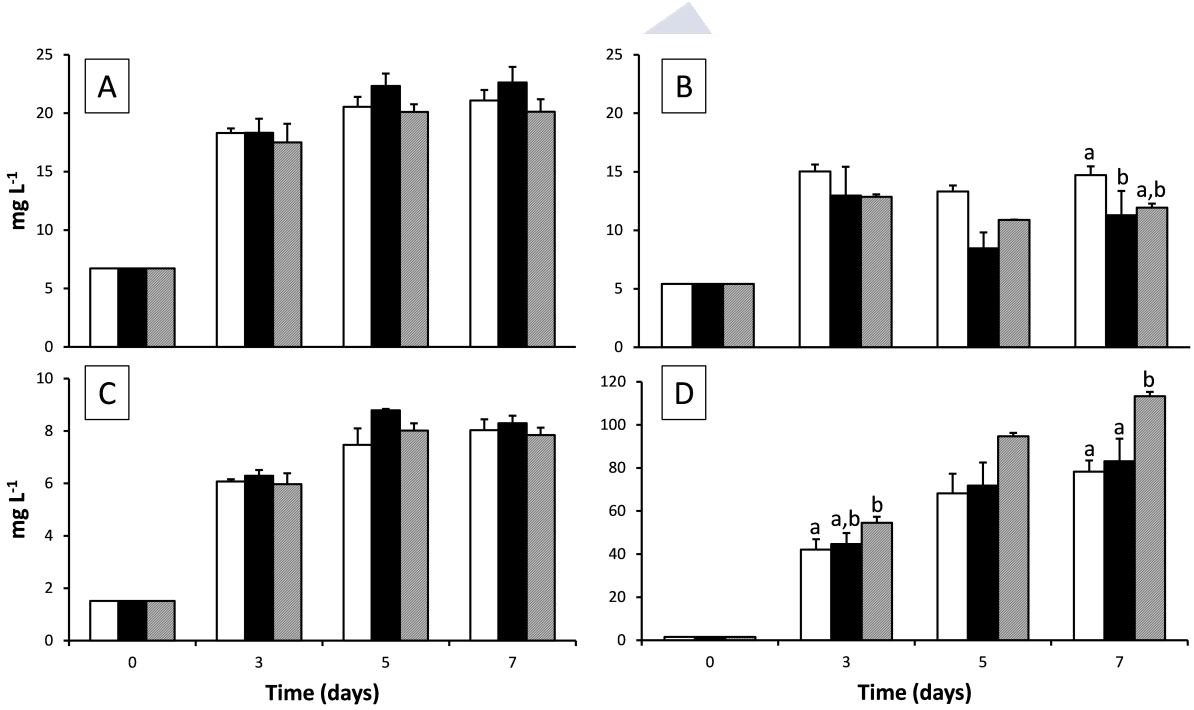



Figure S5. Chlorophyll and carotenoid concentrations in batch cultures of *D. tertiolecta* and *D. salina* under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (white bars), red (black bars), and mix of red and blue (grey bars). Nitrogen was absent for all treatments in day 3. A and C represent chlorophyll and carotenoid concentration for *D. tertiolecta*; B and D represent chlorophyll and carotenoid concentration for *D. salina*. Vertical bars represent S.D. (*n = 3*).

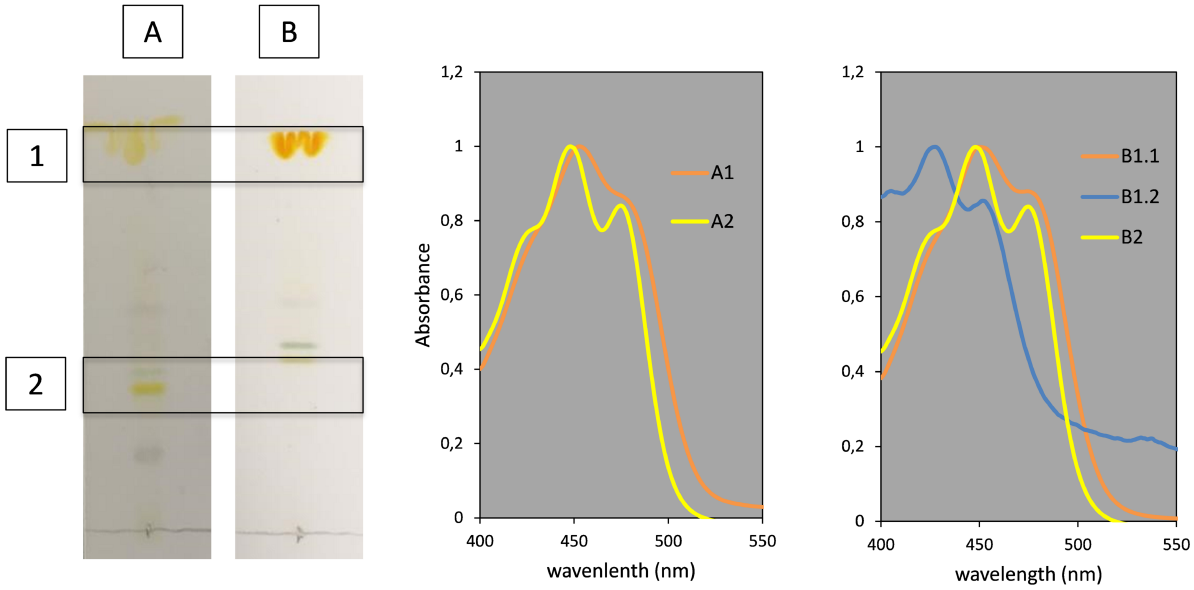



Figure S6. TLC analysis and absorption spectra after elution with pure acetone of the detected carotenoids for *D. tertiolecta* (A) and *D. salina* (B) by the end of the experiments (day 7).

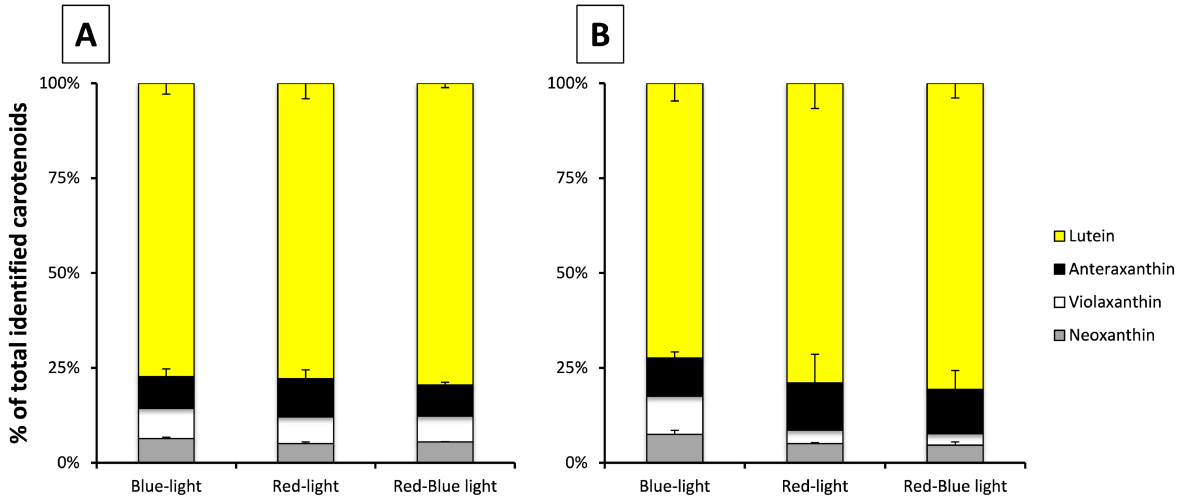



Figure S7. Carotenoid composition in percentage of total identified carotenoids in day 7 of batch cultures of *D. tertiolecta* (A) and *D. salina* (B) maintained under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$. Vertical bars represent S.D. (*n* = 2).

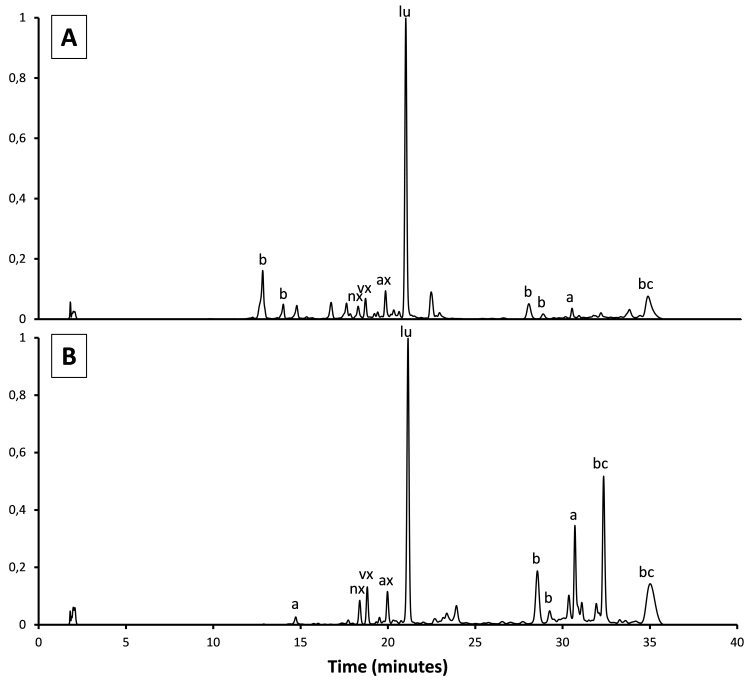



Figure S8. Representative HPLC chromatograms of *D. tertiolecta* (A) and *D. salina* (B) by day 7 of the cultivation period. Superscript letters indicate identified peaks. a: Chlorophyll *a*; b: Chlorophyll *b*; nx: neoxanthin; vx: violaxanthin; ax: anteraxanthin; lu: lutein; bc: β-carotene.

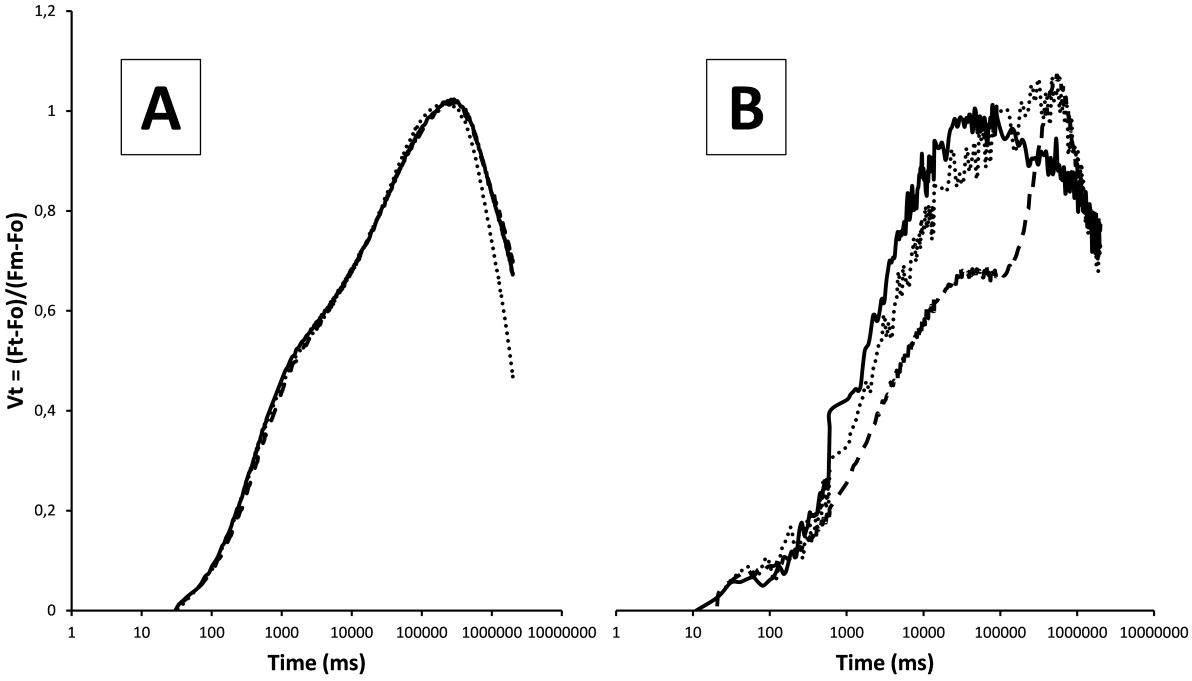



Figure S9. OJIP curves of a representative stationary phase culture of *D. tertiolecta* (A) and *D. salina* (B) under different light qualities at 300 µmol photon $m^{-2}$ $s^{-1}$: Blue (discontinuous line), red (dotted line), and mix of red and blue (continuous line). X axis is reresented in log-scale.

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