University of Algarve

Faculty of Sciences and Technology

Effect of elevated CO_2 concentrations and irradiance on the photosynthetic performance of the seagrass *Cymodocea nodosa*

Cátia José Abreu Freitas

Dissertation for Master's degree in Marine Biology

Advisors: Isabel Barrote, FCT
João Silva, CCMar

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Cátia José Abreu Freitas

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Abstract

Currently, the large quantity of the anthropogenic carbon dioxide (CO_2) emitted to the atmosphere is leading to ocean acidification (OA). Seawater pH is lowered as compared to pre-industrial era and a further decline is expected. Seagrasses are one of the most productive and important coastal and marine ecosystems on Earth. Understanding how these marine plants will respond to CO_2 enrichment is decisive to future coastal ecosystems management. This experiment aimed to investigate how the photosynthetic ecophysiology of the seagrass *Cymodocea nodosa* is affected by high CO_2 concentrations, under different light intensity and quality. This work was conducted in a mesocosm controlled system during two weeks. Photosynthetic responses were quantified using an oxygen electrode and rapid light curves were performed with a Diving-PAM. Chlorophylls and carotenoids were accessed by spectrophotometry and high-pressure liquid chromatography. In low light intensity treatments, the increase in CO_2 partial pressure (pCO_2) resulted in a higher maximum photosynthetic rate (Pmax). The relative maximum electron transport rate (rETRmax) and the saturation irradiance (Ik) were significantly reduced in light limiting circumstances, regardless the pCO_2. In contrast, the light utilization coefficient (α) increased. The concentration of Chl a and b as well as Chl a/b ratio were not affected by the different conditions of light and/or CO_2. In general, distinct light conditions were likely to induce greater differences in *C. nodosa* responses than the increase in pCO_2. Distinct spectrum wavelengths reaching seagrasses may play a key role in the photosynthetic process in low light conditions. Ratios between xanthophyll pigments and total chlorophyll revealed an increased investment on the xanthophyll pool and the triggering of the xanthophyll cycle as a response to high light intensity. All plant responses to changing environmental patterns should be interpreted carefully, once they may be highly species specific.

Keywords

Photosynthesis, Light, *Cymodocea nodosa*, Seagrass, High Carbon Dioxide

Institutions

The laboratory work was conducted in the “Algae: marine plant ecology research group” at the Centre of Marine Sciences (CCMar), located at Universidade do Algarve; field work was conducted at the Ramalhete Experimental Station (CCMar).

Resumo

Durante 800000 anos a concentração de dióxido de carbono (CO_2) na atmosfera permaneceu entre 172-300 partes por milhão (ppm). No entanto, após a era industrial, a concentração de CO_2 atmosférico sofreu um aumento sem precedentes para os níveis atuais de aproximadamente 391 ppm de CO_2, consequência da atividade antropogénica, como a queima de combustíveis fósseis. Atualmente, cerca de 30 % da totalidade de CO_2 emitido para a atmosfera é absorvido pelo oceano, levando à diminuição do pH e alterando a química dos carbonatos na água, processo que resulta na acidificação do oceano. Apesar dos previstos impactos potencialmente negativos da acidificação do oceano nos ecossistemas e organismos marinhos, as diferentes espécies não serão identicamente afetadas. As pradarias de ervas marinhas, angiospérmicas que se adaptaram a uma existência totalmente submersa, constituem um dos habitats marinhos e costeiros mais produtivos do planeta. São responsáveis pelo melhoramento da qualidade da água e promovem o aumento da biodiversidade disponibilizando habitat para outras espécies. No entanto, até à data, o efeito da acidificação do oceano na biologia e ecologia destas plantas marinhas é pouco conhecido, ainda que esteja prevista a estimulação positiva das taxas fotossintéticas e de crescimento com o aumento do CO_2. Compreender como as pradarias de ervas marinhas serão afetadas pelo enriquecimento de CO_2 na água será decisivo para a criação de futuras medidas de conservação e gestão de ecossistemas costeiros. O principal objetivo deste estudo foi o de investigar o efeito de elevadas concentrações de CO_2 na ecofisiologia da fotossíntese de *Cymodocea nodosa* sob diferentes intensidades e qualidades de luz em condições ambientais controladas. Amostras de *C. nodosa* foram recolhidas na Baía de Cádiz, em Espanha e transportadas para a Estação de Experimental do Ramalhete (CCMar), Faro, Portugal, onde se desenvolveu toda a experiência. Rizomas terminais de *C. nodosa* foram aleatoriamente distribuídos em diferentes tanques contendo substrato arenoso. A experiência compreendeu um período de aclimatação que precedeu o período experimental, este último com duração de duas semanas. Os parâmetros físico-químicos e os parâmetros fotossintéticos derivados da fluorescência da clorofila a foram monitorizados diariamente. Diferentes processos fisiológicos foram estudados de forma a elucidar como a estrutura e o funcionamento dos sistemas de captura de energia luminosa variam em ambientes luminosos diferenciados. Foram realizadas curvas de resposta da fotossíntese à luz com base na libertação/consumo de oxigénio e com base na taxa de transporte de eletrões através de um elétrodo de oxigénio tipo Clark e de um fluorómetro Diving-PAM, respetivamente. As clorofilas foram quantificadas espectrofotometricamente e os carotenoides foram quantificados por cromatografia líquida de

alta eficiência (HPLC). Mediu-se também a eficiência fotoquímica máxima do fotossistema II (Fv/Fm). Em tratamentos com baixa intensidade luminosa o aumento da pressão parcial de CO_2 resultou em maiores taxas fotossintéticas máximas (Pmáx), no entanto este aumento em Pmáx não se fez acompanhar pelo aumento da eficiência fotossintética (α). Perante níveis atuais de CO_2 atmosférico e entre tratamentos com baixa intensidade luminosa o valor de Pmáx foi superior quando a qualidade espectral foi limitada ao comprimento de onda da luz azul. Os níveis elevados de CO_2 na água aumentaram também as taxas de respiração no escuro quando comparado com as mesmas taxas a níveis de CO_2 de referência, no entanto este aumento foi apenas significativo para plantas em tanques sem qualquer atenuação luminosa. A máxima taxa relativa de transporte de eletrões (rETRmáx) e a irradiância de saturação (Ik) foram significativamente reduzidas em condições de baixa intensidade luminosa, independentemente da concentração de CO_2. Em tratamentos sem atenuação luminosa a rETRmáx foi alcançada apenas quando a intensidade da luz foi aproximadamente 150 $\mu moles_{fotões}.m^{-2}.s^{-1}$, ao contrário dos tratamentos onde a intensidade luminosa foi atenuada, em que a rETRmáx foi alcançada a baixas irradiâncias (a partir de 74.4 $\mu moles_{fotões}.m^{-2}.s^{-1}$) resultando num coeficiente da utilização da luz (α) significativamente superior. A eficiência fotoquímica máxima do fotossistema II foi significativamente superior em plantas aclimatadas a baixas intensidades luminosas. A intensidades luminosas naturais, o rácio Fv/Fm foi superior em plantas com elevada disponibilidade de CO_2 na água. Relativamente ao pigmentos fotossintéticos, as concentrações de clorofila a (Chl a), b (Chl b) e clorofila total (Chl T) foram significativamente superiores em Cádiz do que após o período experimental, no entanto não se registaram alterações significativas no rácio de clorofila a/b (Chl a/b). Nos tratamentos sem atenuação da luz, a taxa de desepoxidação dos pigmentos do ciclo das xantofilas, violaxantina (V), anteraxantina (A) e zeaxantina (Z) - (AZ/VAZ) - foi significativamente superior do que nos tratamentos com baixas intensidades luminosas. O rácio entre os pigmentos do ciclo das xantofilas e a clorofila total [(VAZ)/ChlT] foi menor em plantas aclimatadas a baixa disponibilidade luminosa, independentemente do tratamento de CO_2. De uma forma geral, os resultados obtidos sugerem que distintas condições luminosas induziram diferenças mais acentuadas na ecofisiologia da fotossíntese de *C. nodosa* do que o aumento da pCO_2. RuBisCO é a enzima que catalisa a fixação primária do CO_2 na fotossíntese, no ciclo de Calvin, em plantas C_3. A maioria das espécies de ervas marinhas são classificadas como plantas C_3, no entanto, há estudos que apontam para a ocorrência do metabolismo fotossintético C_4 (ex. *Cymodocea serrulata*). No estado atual do conhecimento, em plantas C_4 o aumento do CO_2 não resulta em alterações na resposta fotossintética, uma vez que a fixação primária de carbono é catalisada pela enzima fosfoenolpiruvato carboxilase (PEPC'ase), que ao contrário da RuBisCO não é afetada pela concentração de O_2, minimizando a fotorrespiração e tornado a fotossíntese saturada para a atual concentração de carbono inorgânico (Ci). No entanto, e contrariamente ao que é ocasionalmente assumido, *C. nodosa* não apresentou respostas que indiquem um estado de saturação relativamente à atual concentração de carbono inorgânico uma vez que neste estudo o aumento do CO_2 resultou em maiores taxas fotossintéticas máximas em ambientes com baixa intensidade luminosa. Os resultados obtidos indicam ainda que em condições luminosas limitantes, a qualidade da luz poderá ter um papel-chave na resposta fotossintética da planta. A redução nas taxas relativas de transporte de eletrões, a diminuição da irradiância de saturação e o aumento do coeficiente de utilização de luz são típicos indicadores de plantas adaptadas a baixas intensidades luminosas. Adaptações fisiológicas de plantas de *C. nodosa* em diferentes locais e ambientes marinhos podem resultar em alterações na concentração dos pigmentos fotossintéticos, sem implicar uma perda de performance fotossintética ou uma alteração no seu estado fisiológico. Os rácios entre pigmentos fotossintéticos revelam o investimento na *pool* dos pigmentos do ciclo das xantofilas e a ativação do seu ciclo de desepoxidação/epoxidação como respostas a elevadas intensidades luminosas. As respostas de diferentes espécies de ervas marinhas à mudança nos padrões ambientais devem ser interpretadas cautelosamente uma vez que podem ser altamente específicas para cada espécie.

Contents

Abbreviations list

(AZ) / (VAZ) - Xanthophyll de-epoxidation index
β-car - β-carotene
[(VAZ)/ChlT] – Xanthophyll cycle pigments to total chlorophyll ratio
ΔF - Proportion of open reaction centers
ΔF/F'm - Effective quantum yield
A - Antheraxanthin
C (x + c) – Carotenoids (xanthophylls and carotenes)
$C_6H_7NaO_6$ - Sodium ascorbate
$CaCO_3$ - Calcium carbonate
Chl a - Chlorophyll a
Chl a/b - Chlorophyll a/b ratio
Chl b - Chlorophyll b
Chl T - Total chlorophyll
Ci - Inorganic carbon
CO_2 - Carbon dioxide
CO_3^{2-} - Carbonate ion
D - Heat
DIC - Dissolved inorganic carbon
DO - Dissolved oxygen
F - Fluorescence
F'm – Maximum fluorescence in the light
F0 – Basal fluorescence
Fm – Maximum fluorescence
Ft – Steady-state level of fluorescence
Fv/Fm - Maximal photochemical efficiency of PSII
H^+ - Hydrogen ion
H_2CO_3 - Carbonic acid
H_2O - Water
HCl - Hydrochloric acid
HCO_3^- - Bicarbonate ion

KCl – Potassium chloride
L - Lutein
N - Neoxanthin
N_2 - Nitrogen
O_2 - Oxygen
OA – Ocean acidification
P - Photochemistry
PAR - Photosynthetic active radiation
pCO_2 - Partial pressure of carbon dioxide
PEPC’ase - Phosphoenolpyruvate carboxylase
Pmax – Maximum photosynthetic rate
PSII - Photosystem II
Rd – Dark respiration
rETR – Relative electron transport rate
rETRmax - Relative maximum electron transport rate
RuBisCO - Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
T - Taraxanthin
TA - Total alkalinity
V - Violaxanthin
VAZ - Violaxanthin (V) + Anteraxanthin (A) + Zeaxanthin (Z)
Z - Zeaxanthin

1. Introduction

The ocean covers about 71 % of the Earth's surface and supports the greatest biodiversity in the planet. It regulates climate and weather systems, plays a key role in the global carbon cycle and is recognized as one of the humanity's most important natural resources (Costanza, 1999; Raven, 2005). Knowing how rapid changes on climate events are disturbing the whole marine ecosystems is crucial in order to predict severe risks involved and limit further negative impacts of anthropogenic activities (Hoegh-Guldberg and Bruno, 2010). One of the climate complications that currently is under focus concerns the increase of CO_2 in the atmosphere, leading to a host of additional changes such as ocean acidification (OA) (Brierley and Kingsford, 2009; Doney *et al.*, 2012).

During 800000 years the carbon dioxide (CO_2) concentration in the atmosphere varied between 172-300 parts per million (ppm), but since the Industrial Revolution, the human activities are responsible for releasing gigatons of carbon to the Earth's atmosphere annually, increasing those values (Lüthi *et al.*, 2008; Brierley and Kingsford, 2009). It is predicted that the continuous and significant increase in the atmospheric concentration of CO_2 will conduce to ocean acidification (Brewer, 2009). Significant changes in biodiversity and marine ecosystems function are expected to be major problems due to ocean acidification.

The influence of these climatic changes on seagrasses, organisms that are often labeled as *"ecosystem engineers"*, is a topic of study as interesting as necessary these days. Seagrasses are flowering plants that can form dense meadows. They play essential key multifunctional roles providing food and habitat, stabilizing sediments and improving water clarity, thus constituting one of the most productive coastal and marine ecosystems on Earth (Valentine and Duffy, 2006; Short *et al.*, 2007; Unsworth and Cullen, 2010). However, at a global level, 29 % of seagrasses communities are already lost or degraded (Waycott *et al.*, 2009).

1.1. Ocean Acidification

The ocean and the atmosphere exchange considerable amounts of CO_2. Oceans used to be a source of CO_2 to the atmosphere, but over the past 250 years and as a consequence of human activity such as the burning of fossil fuels (Cao *et al.*, 2007), agricultural labor (e.g. deforestation) and cement production (Sabine *et al.*, 2004; Feely *et al.*, 2009; Duarte *et al.*, 2013), atmospheric CO_2 levels have experienced a large and rapid increment (Fig.1).

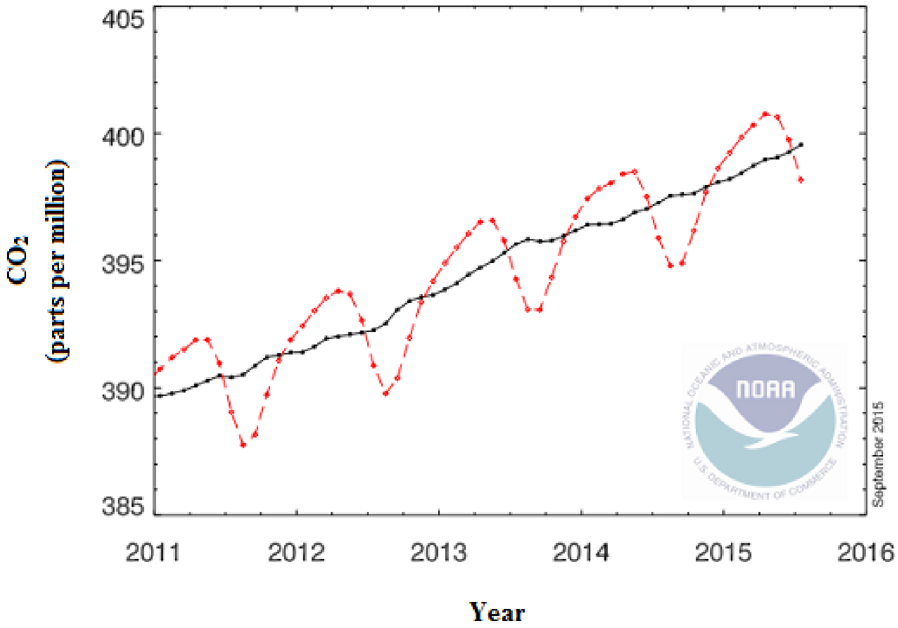

Figure 1: Monthly levels of CO_2 globally averaged over marine surface sites since 2011. The dashed red line represents the monthly mean values, centered on the middle of each month. The black line represents the same, after correction for the average seasonal cycle. (*Adapted from*: Ed Dlugokencky and Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/).

Atmospheric CO_2 values recorded range from roughly 278 parts per million (ppm) at pre-industrial era (Cao *et al.*, 2007) to an annual average of 391 ppm in 2011 (Stocker *et al.* 2013). Accordingly to Intergovernmental Panel on Climate Change (IPCC) business-as-usual forecasts, these atmospheric levels can achieve 936 ppm by 2100 in the worst case scenario (Stocker *et al.* 2013).

Currently, the ocean takes up large quantities of anthropogenic CO_2 (nearly 30 %) (Feely *et al.*, 2004; Arnold *et al.*, 2012) and although this helps limiting the level of CO_2 in the atmosphere it is also responsible for the modification of the carbon chemistry balance both in the mixed layer and in the deep ocean (Sabine and Feely, 2007; Hofmann *et al.*, 2010; Gattuso and Hansson, 2011).

When CO_2 is absorbed by the ocean, it reacts with water molecules changing the proportions of carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). In aqueous medium, CO_2 is slowly hydrated to H_2CO_3 which, at seawater pH, rapidly ionizes to HCO_3^- and CO_3^{2-}.

This process increases the concentration of hydrogen ion (H^+) leading to the decrease of the seawater pH (Ishimatsu *et al.*, 2005), according to the equation (Branch *et al.*, 2013):

$$\boxed{CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}}$$

Seawater pH has already decreased 0.1 units since the Industrial Revolution (Caldeira and Wickett, 2003, 2005; Orr *et al.*, 2005; Doney *et al.*, 2009). A few models scenarios anticipate the decrease of further 0.3-0.4 pH units until the end of the 21st century (Brierley and Kingsford, 2009; Gattuso and Lavigne, 2009), probably at a rate 100 times higher than any time over the past hundreds of millennia (Raven, 2005). Despite the evidence of chemical principles and research studies supporting the potentially negative impacts of ocean acidification (Riebesell *et al.*, 2000; Seibel and Walsh, 2001; Ishimatsu *et al.*, 2005; Orr *et al.*, 2005; Dupont *et al.*, 2010), undoubtedly not all marine species and habitats will be similarly affected and the impact of high-CO_2 water condition in its physiology and biochemistry is scarcely understood.

1.2. Effects of Ocean Acidification in Seagrasses

According to Beer *et al.* (2002), seagrasses appear to be photosynthetically limited by the current availability of CO_2 concentration due to its low solubility and slow diffusion rates in seawater (4 to 5 orders of magnitude lower than in the air) (Zou and Gao, 2010). So, it is expectable that seagrasses meadows will become competitively advantageous with increased growth rates and productivity in acidified environments (Polunin, 2008; Doney *et al.*, 2012; Koch *et al.*, 2013). Seagrasses possess organs and tissues identical to the terrestrial plants (Kuo and den Hartog, 2006). However, given the environment in which they are inserted, the biochemistry of photosynthesis will be distinctly affected by elevated CO_2 levels (Koch *et al.*, 2013). Several seagrass species are also photosynthetically ineffective using HCO_3^- because bicarbonate is not directly usable by RuBisCO (Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase) to convert it into organic carbon in the Calvin Cycle (Palacios and Zimmerman, 2007). Moreover, HCO_3^- represents 90 % of the total dissolved inorganic carbon

(DIC), at normal seawater pH of ~8.1, while CO_2 and CO_3^{2-} represent only 1 % and 9 % respectively (Feely *et al.*, 2009; Branch *et al.*, 2013).

RuBisCO is the enzyme involved in the primary carbon fixation in C_3 plants and catalyzes the first step of the Calvin cycle in photosynthesis. In terrestrial C_3 plants, RuBisCO activity seems to be decreased or not affected by elevated levels of CO_2 (Pérez *et al.*, 2005; Pandurangam *et al.*, 2006). Despite seagrasses have globally been regarded as C_3 plants, photosynthetic pathways are not well defined for some species. It has been suggested that C_4 metabolism probably occurs in some seagrasses. However this is not widely accepted mainly because the results obtained regarding the distinction between C_3 and C_4 metabolisms in seagrasses have to be interpreted with caution as they may reflect particular effects related to the aquatic environment (Touchette and Burkholder, 2000). Albeit the controversy surrounding the C_3/C_4 classification of seagrasses, *Cymodocea nodosa* (the study model specie in this thesis) has been proposed to be a C_4 species (Beer *et al.*, 1980).

The increase in CO_2 levels will promote C_3 marine plants photosynthetic pathways since RuBisCO is not substrate-saturated at the current CO_2 concentration (Koch *et al.*, 2013). However, in C_4 photosynthetic metabolism the primary carbon fixation is catalysed by phosphoenolpyruvate carboxylase (PEPC'ase), which is not affected by O_2 thus minimizing photorespiration and making photosynthesis saturated at actual atmospheric CO_2 concentration.

1.3. Light, the Key Driver of Photosynthesis

When compared to terrestrial plants, seagrasses are exposed to significantly lower light regimes that are more similar to those of macrophytic algae (Casazza and Mazzella, 2002). Seagrasses vertical distribution ranges from the intertidal to more than 70 m deep (Duarte, 1991). Underwater light availability decreases with depth, limiting the vertical distribution of seagrasses that cannot survive below their light compensation point. Also, the spectral composition of light varies with the properties of water and depth. In general, red light is extinguished above 10 m, orange is extinguished above 30 m and only yellow, violet, green and blue reach higher depths (Kirk, 2011). Hence, light availability and quality have to be considered when studying the effects of ocean acidification on seagrasses photosynthesis.

Light drives photosynthesis and it is one of the major drivers of seagrasses growth (Ralph *et al.*, 2007). Seagrasses light requirements comprise up to 37 % of surface irradiance and determine their limits of distribution both geographically and in depth (Zimmerman, 2003). For the photosynthetic process to be successful, light has to reach leaf epidermis and be captured by the photosynthetic apparatus. However, natural and anthropogenic processes driving water eutrophication, are diminishing the penetration of light in water column and changing its optical properties resulting in ineffective CO_2 assimilation and limited growth, particularly at the deepest edge of the meadow (Via *et al.*, 1998; Duarte *et al.*, 2007; Ralph *et al.*, 2007). Unlike terrestrial plants, seagrasses leaves are devoid of specific tubular cells (that help with the light entrance into the leaves) and spongy mesophyll (that increases the optical path length). Nonetheless seagrasses possess effective light harvesting capacities (Cummings and Zimmerman, 2003; Ralph *et al.*, 2007), located in the epidermis of the leaf blade which is the major site of photosynthesis with high concentrations of chloroplasts (Kuo and den Hartog, 2006). Pigment content, chloroplast location, morphology and physical properties of the leaf regulate its light absorbance (Ralph *et al.*, 2007).

Chlorophylls a and b absorb in the red region of the spectra (650-700 nm), where they have maximum photosynthetic efficiency, and in the blue region of the spectra (400-500 nm), whereas green and yellow (500-600nm) are inefficient at driving photosynthesis (Ralph *et al.*, 2007; Gallegos *et al.*, 2009; Chartrand *et al.*, 2012). In eutrophic and turbid waters, the photosynthetically active radiation (red and blue) is mainly removed by phytoplankton chlorophyll that tends to absorb the same wavelengths as requested by plants (Gallegos *et al.*, 2009). After a long literature review, Duarte *et al.* (2007) conclude that plant communities living in the deeper limits of the meadows but in clear waters have fewer light requirements (until threefold less) than those closest to the surface in turbid waters. These differences in light requirements may be associated with light quality, considering that high-energy blue light travels further through water column in clear waters, while in shallow and turbid waters the smaller blue wavelengths are quickly attenuated (Gallegos *et al.*, 2009) and scattered by suspended and dissolved components that also play an important role in light attenuation (McPherson *et al.*, 2011).

Apart from chlorophylls a and b, Casazza and Mazzella (2002) and Costa (2014) quantified the carotenoid pool constituted by β-carotene (β-car) and the xanthophylls lutein (L) neoxanthin (N), taraxanthin (T), violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) in *C. nodosa*. Besides their structural functions in light harvesting complexes, carotenoids are precursors of vitamins and phytohormones, participate in light capturing and have important photoprotective functions (Cazzonelli, 2011). Xantophylls (V, A and Z) play a protective role against excess light by dissipating the excess energy as heat and thus preventing oxidative stress and photosystem damage, through the de-epoxidation of V to A and Z in the xanthophyll cycle (VAZ cycle) (Jahns *et al.*, 2009).

Under low light conditions, plants become limited by both energy and reducing power and consequently less carbon is fixed by seagrasses. Therefore, understanding how seagrasses cope with changes in light quantity and quality will allow a more accurate and detailed assessment of carbon budget adjustments. To the best of our knowledge, there is no published data regarding how the effects of elevated CO_2 will affect seagrass photosynthetic performance under different light intensities and qualities. In spite of the putative consequences of light spectral quality on seagrass photosynthetic carbon acquisition, no experimental evidence has so far been obtained to clarify such relationship.

Ocean acidification rapidly became a global concern, especially because it's biological, ecological and biochemical effects are largely unknown and hardly predictable for marine biota (Blackford, 2010). The inconsistency of the results obtained so far evidence that there is a substantial lack of knowledge about the structure and function of seagrasses photosynthetic apparatus regarding metabolic pathways and physiological processes, leading to the need for further specific research studies. Understanding the effects of elevated availability of $CO_{2,}$ both at the physiological level and on the ecological function and distribution of seagrasses, will provide a better insight and the improvement of techniques for seagrass preservation and restoration.

1.4. Main Objectives

This thesis main goal was to investigate and understand the effect of high CO_2 concentrations on the photosynthetic ecophysiology of the seagrass *Cymodocea nodosa* under different light intensities and spectral conditions in a mesocosm controlled environment. Essential physiological processes were evaluated in order to elucidate how the structure and functioning of the photosynthetic light harvesting apparatus fits to the distinct light environments.

2. Material and Methods

Cymodocea nodosa specimens were randomly collected on the 21st of April 2015, during low tide, from the shallower inner bay of the Bay of Cádiz, Spain. Intact shoots with horizontal rhizomes were excavated from the sediment, stored in seawater and in the dark, and transported to Ramalhete Experimental Station (CCMar) where the experiment was held in a mesocosm facility. A total of approximately 34 plant rhizomes were randomly distributed through each 24 different tanks and planted in sandy substrate. Each tank was supplied with water from Ria Formosa, continuously flowing through an open system. *Cymodocea nodosa* was maintained under current atmospheric and water conditions during 8 days (acclimation period) from the 22nd to the 29th of April, 2015. The experimental period began with the imposition of different combinations of light intensity/quality and CO_2 partial pressure during 16 days (from the 30th April to the 15th May).

During both acclimation and experimental period, CO_2 concentrations, water physical-chemical parameters (such as temperature, pH, oxygen and salinity), irradiance, and chlorophyll-fluorescence-derived photosynthetic parameters were monitored on a daily basis.

Leaf samples were collected for pigments analysis at the time of collection, in Cádiz, at pre-dawn (PD) and noon after the acclimation period (T0) and at PD and noon at the end of the experiment (T1). Photosynthetic light response curves and rapid light curves (RLC) were done at the end of the experiment (T1).

2.1. Model Species: *Cymodocea nodosa*

Seagrasses are aquatic monocot angiosperms, worldwide distributed in most shallow and sheltered coastal waters. They consist of a unique group of flowering plants that can be found in the sea, where they are adapted to full submersion (Orth *et al.*, 2006a).

The model species of this thesis, *Cymodocea nodosa* (Ucria) Ascherson, is a dense and highly productive seagrass species that belongs to Cymodoceaceae family which includes 5 genus: *Amphibolis, Cymodocea, Thalassodendron, Syringodium* and *Halodule* (Orth *et al.,* 2006b). Four species of the genus *Cymodocea* are spread over the tropical and temperate seas: *C. angustata, C. rotundata,* and *C. serrulata* in the Indo-Pacific Ocean, and *C. nodosa* throughout the Mediterranean Sea and in the North Atlantic from South Portugal to Senegal as well as in

Madeira Island and Canary archipelago (Fig.2) (den Hartog, 1970; Green and Short, 2003; Alberto *et al.*, 2005; Sandoval-Gil *et al.*, 2012).

Figure 2: Global distribution of *Cymodocea nodosa* (Ucria) Ascherson (Cymodoceaceae) (*Adapted from*: IUCN 2014).

Cymodocea nodosa is a perennial, fast-growing and dioecious tropical seagrass. Although usually found in a broad range of marine environments, *C. nodosa* does not occur as often in the sea, where some meadow fragments can be found, as in coastal lagoons (Hemminga and Duarte, 2000; Procaccini *et al.*, 2003; Espino *et al.*, 2008; Boudouresque *et al.*, 2009). *Cymodocea nodosa* is an abundant species in the Mediterranean Sea and also in the remaining area where it is distributed, and often appears mixed with other phanerogams such as *Zostera noltii* and *Z. marina* (Ayala, 2010). It is considered an opportunistic, colonizer and pioneer species, due to its wide vertical distribution, from upper subtidal limit to 50 m depth, high rates of clonal growth through horizontal vegetative propagation, which can reach several meters per year colonizing bare areas, and low demanding habitat requirements (Templado and Calvo, 2002; Marbá *et al.*, 2004; Borum *et al.*, 2004).

Given its high capacity to tolerate and adapt to several changes in the habitat conditions, *C. nodosa* is the ideal species to study the effect of changes in environmental factors (Cancemi *et al.*, 2002; Balestri and Lardicci, 2013) such as elevated CO_2 and different light conditions on the photosynthetic performance, as it was done in this study. Moreover, *C. nodosa* has also been one of the most threatened seagrasses in the last 30 years, having its abundance significantly diminished due to coastal and industrial devastation and consequent increased water turbidity, reduced light penetration and lower photosynthetic rates (Luque and Templado, 2004; Tuya *et al.*, 2002; 2013).

Despite of the several scientific publications regarding the effects of reduced irradiance on the growth and photosynthesis of *C. nodosa* (Olesen *et al.*, 2002; Silva *et al.*, 2013; Cabello-Pasini *et al.*, 2015) there is a lack of knowledge regarding the effects of ocean acidification together with irradiance and light quality changes.

2.2. Sampling Site

The Bay of Cádiz (Fig. 3) is a semi-enclosed water body (Periáñez *et al.*, 2013) located in southwest of Iberian Peninsula, Spain, between 36º23' to 36º37'N and 6º08 to 6º15'W (Morris *et al.*, 2009) with approximately 28.5 km long and 13.5 km wide (Achab, 2013) and is divided in two basins: (i) a deeper outer bay (surface ≈ 118 km^2) opened to the Atlantic Ocean, and (ii) a shallower inner bay (surface ≈ 40 km^2), under limited external marine influence with maximum depth of 11 m (Rueda and Salas, 2003; Achab, 2013). The inner bay is composed essentially of fine sand and mud where *C. nodosa* occurs in patches dispersed throughout the extensive intertidal areas and it's also a habitat of singular relevance for local biodiversity (López de la Rosa *et al.*, 2006; Morris *et al.*, 2009). The water temperature in the Bay of Cádiz varies along the year, reaching a maximum of 26ºC in summer and a minimum of 12ºC in winter. Regarding salinity, the lower values of 32 psu (practical salinity units) are reached during the wet season while the higher values of 42 psu during the dry season (López de la Rosa *et al.*, 2006).

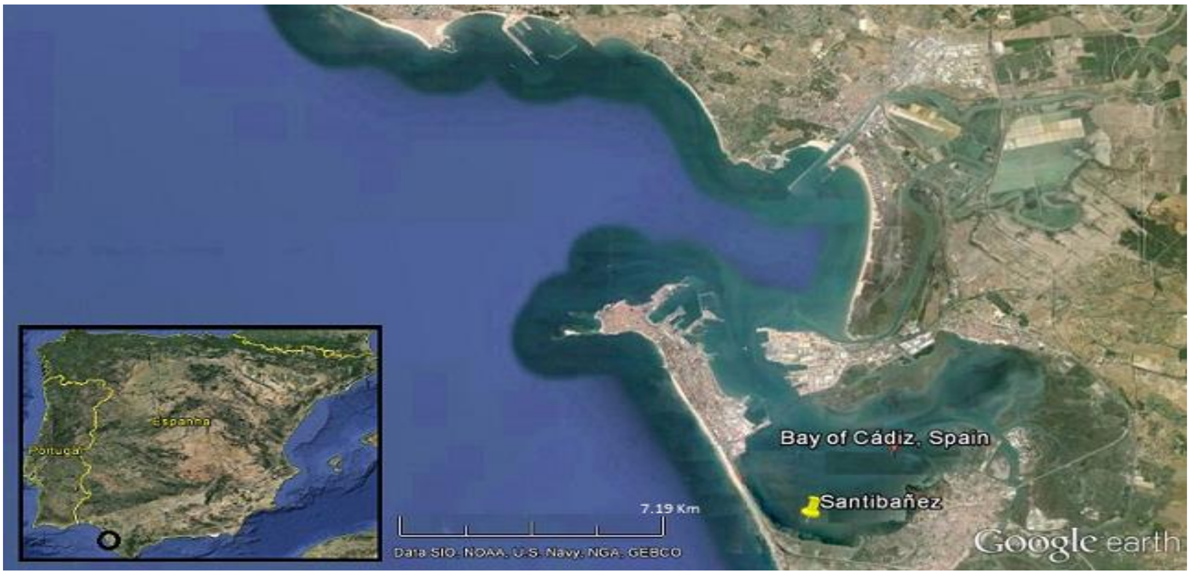

Figure 3: Bay of Cádiz, Spain. The yellow label on the map indicates the sampling site (Santibañez) (*Adapted from*: Google™ earth).

Although *C. nodosa* is the second dominant seagrass specie in the Mediterranean Sea, this species has very low genetic diversity in the Portuguese coast, unlike *C. nodosa* meadows in the Bay of Cádiz (Ayala, 2010). Seagrass meadows with high genetic diversity have higher adaptive capacity under changing environmental conditions (Reynolds *et al.*, 2012), which made *C. nodosa* specimens from Cádiz more appropriate for this experiment.

2.3. Experimental System (Mesocosm)

A controlled open system (Fig. 4) was built at Ramalhete field station to maintain *C. nodosa* rhizomes and shoots under manipulated CO_2 and light conditions that allowed the study of the combined effects of high-CO_2/low pH and light intensity and quality on seagrass physiology. After the acclimation period, *C. nodosa* plants were kept under different carbon dioxide partial pressures (pCO_2). Half of the tanks worked with a high pCO_2 ($pCO_2 \approx 1034.19 \pm 23.96$ µatm) by adding commercial CO_2 (named as "CO_2" treatments), from a main reservoir with compressed CO_2, metered by a partial pressured controlled solenoid valve. Thus it was simulated the water conditions predicted for the end of the century, without additional mitigation efforts, according to IPCC (IPCC Climate Change, 2014) report. The other half worked with an ambient pCO_2 ($pCO_2 \approx 370.81 \pm 6.10$ µatm), without any CO_2 addition (named as "REF" treatments).

Figure 4: Global view of the experimental system at Ramalhete Experimental Station (CCMar).

The same tanks were also maintained under different light intensities and qualities using different light filters (for both REF and CO_2 treatments):

(i) High Light intensity treatments (HL): plants received 95 % of total daily irradiance available, both in CO_2 and REF tanks. Tanks were covered with a greenhouse plastic, 95 % permeable to light (Solplast, S.A, Appendix I).

(ii) Low Light intensity treatments (LL): plants received ≈ 32-33 % of total daily irradiance reaching the HL treatments, both in CO_2 and REF tanks. Plus the greenhouse plastic, these tanks were also covered with a 0.6 neutral density filter to reduce light intensity (2 stops) but not quality (LEE Filters, reference 210; Fig. 5 A).

(iii) Low Light intensity selective to specific Blue light wavelength treatments (LLB): plants received ≈ 24-26 % of total daily irradiance reaching the HL treatments, both in CO_2 and REF tanks. Besides the greenhouse plastic, these tanks were also covered with a special steel blue filter (LEE Filters, reference 354; Fig.5 B), selective to specific blue light wavelength and a 0.15 neutral density filter that reduce light intensity (1/2 stop) (Lee Filters, reference 298; Fig.5 C).

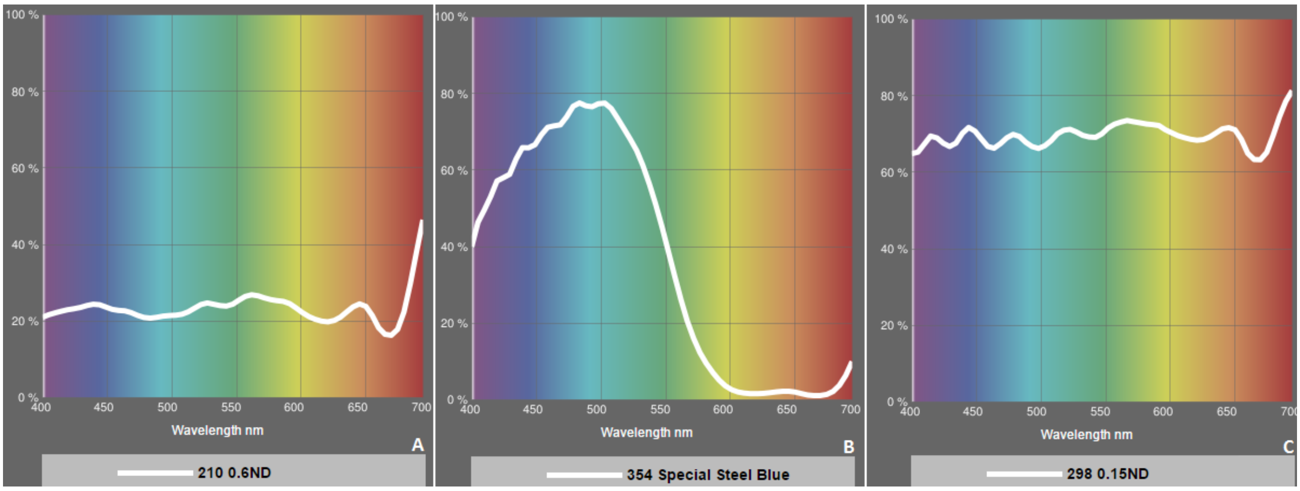

Figure 5: Light transmitted (Y %) for each colour wavelength. A - 0.6 neutral density filter (LEE Filters, reference: 210); B - special steel blue filter (LEE Filters, reference: 354); C - 0.15 neutral density filter (LEE Filters, reference: 298). *Adapted from*: LEE Filters.com;

In a total of 24 tanks, six different treatments were performed (REF- HL, REF- LL, REF – LLB, CO_2 – HL, CO_2 – LL and CO_2 – LLB), four tanks each, randomly distributed (Fig.6).

The water from Ria Formosa was filtered with sand and ultraviolet light and pumped into cylindrical headtanks, with a flow of approximately 42 liters per hour. The mixture between water and air or CO_2 was done in these headtanks in order to obtain the desired pCO_2 in each tank. Then, the mixed water was pumped to the HL, LL and LLB treatment tanks (with approximately 240 L of seawater each) containing *C. nodosa* plants. During the experimental period, *C. nodosa* leaves were all cleaned on a daily basis to avoid epiphyte accumulation.

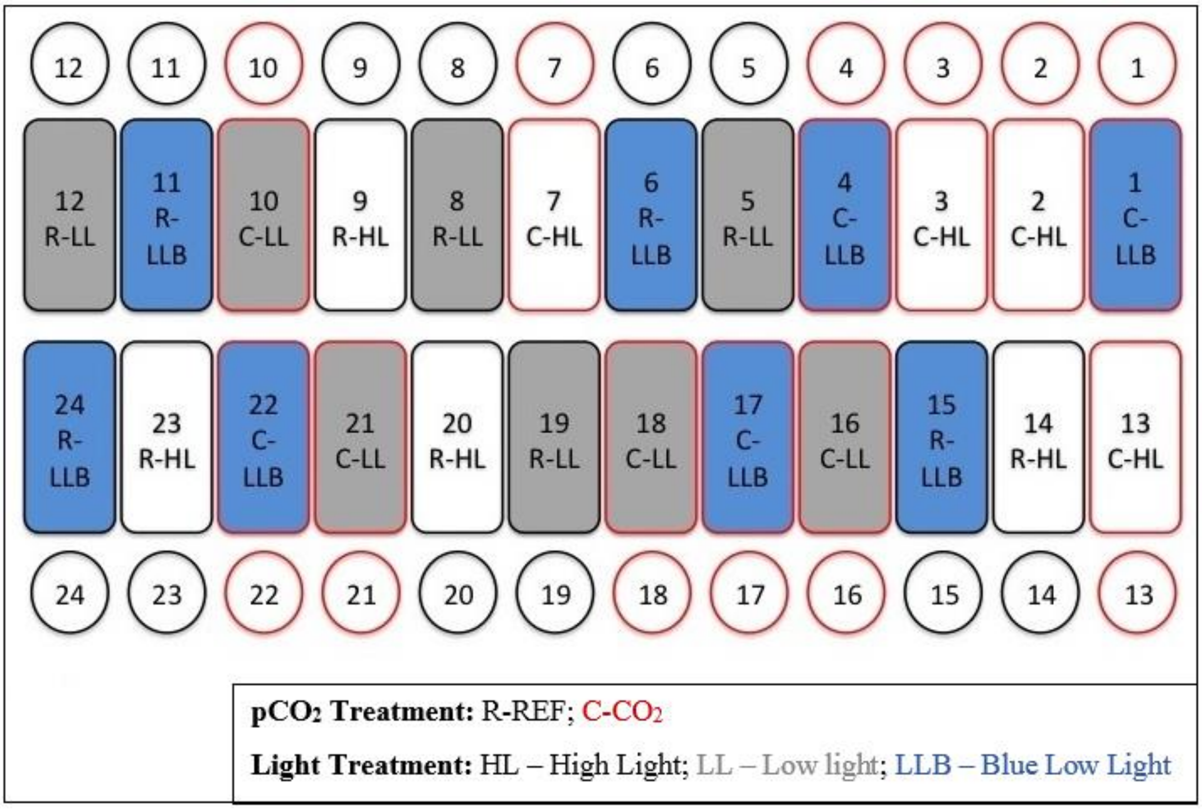

Figure 6: Study experimental design. Random distribution of the different light and pCO_2 treatments for 24 tanks at Ramalhete field station. Rectangles represent the treatment tanks containing *C. nodosa*. Circles represent the headtanks where mixture between water and air or CO_2 occurred.

2.4. Physical-Chemical Parameters

Water temperature (1), oxygen concentration (2), irradiance (3), and pH (4) were measured daily during both acclimation and experimental periods. 4 different sensors were used: (1)Hobo - HOBO Pendant® Temperature/Alarm Data Logger 64K - UA-001-64, ONSET ®; (2)Optode - Oxygen optode, MiniDO2T Logger, PME, EUA; (3)Irradiance ODYSSEY - PAR sensor, Odyssey Light logger; (4) Thermo Scientific™ Orion™ Star A221 pH Portable Meter. The ambient irradiance was recorded, near to the experimental site using an Irradiance PAR meter sensor - Irradiance LICOR (Li-192SA, LiCor, EUA). Water samples for dissolved inorganic carbon analysis were taken every 3 days from each tank between 11a.m. and 1p.m.

To ensure the stability of pCO_2, a Non-dispersive Infrared Gas Analyzer (IRGA: WMA-4, PP Systems, UK), was coupled to the system, in order to control the pCO_2 in the CO_2 main reservoir. A second IRGA (EGM-4, PP Systems, UK) coupled to a gas-exchange column (Mini-Module membrane contractor, Celgard, USA) was used each day in a different treatment tank (during ca. 24 h) to measure and monitor the pCO_2 in the tank water's.

2.5. Alkalinity

According to Dickson (1981), the total alkalinity of a seawater sample is defined as "the number of moles of hydrogen ion equivalent to the excess of proton acceptors over proton donors in 1 kg sample". In other words, alkalinity is a chemical measurement of the water's capacity to neutralize acids (Ohrel and Register, 2002; Emerson and Hedges, 2008).

Water samples from each of the tanks were collected for dissolved inorganic carbon (DIC) description trough pH and total alkalinity (TA) determination. Alkalinity was measured by potentiometric titration. The potentiometric titration method works by the addition of small quantities of hydrochloric acid (HCl) to the sample while monitoring the change in the pH until it reaches the endpoint pH (pH=3), which is already known to be after the equivalence point (pH ≈ 4.2) (Ohrel and Register, 2002). The equivalence point of the titration was calculated by employing a Gran linearization method (Gran, 1988). Below the equivalence point, all the alkaline compounds are unable to neutralize the acid and there is a linear relationship between the amount of acid added to the sample and the change of pH. Carbonate system parameters, as DIC concentration, were determined from total alkalinity, pH, temperature, and salinity of the sample through the Excel-based program CO_2SYS.XLS 1.0 (Lewis and Wallace, 1998).

2.6. Photosynthetic Light Response Curves

Photosynthetic and respiratory rates of *C. nodosa* leaves were measured in a Clark type oxygen electrode system (DW3/CB1, Hansatech Instruments, Norfolk, United Kingdom; Fig.7).

Figure 7: Clark type oxygen electrode. Connection of incubation chambers to light sources.

The oxygen electrode works coupled to an incubation chamber and is composed by a platinum cathode and a silver anode mounted on an epoxy resin disc. A teflon or polyethylene oxygen permeable membrane embedded in a saturated electrolyte solution (e.g. KCl) is placed to establish an electrolyte bridge between the cathode and the anode. The electrolyte is uniformly distributed by a paper spacer placed under the membrane. The polarization of the platinum cathode takes place by applying a small voltage. When this voltage is increased, occurs the reduction of the dissolved oxygen at the cathode and an electric current of low intensity is generated. This current is registered by a plotter fed with continuous paper flowing at a previously fixed velocity and is linearly related to the oxygen concentration in the solution, allowing its determination (Walker, 1987).

2.6.1. Principle of operation

Inside the plant, in specialized structures known as chloroplasts, the light energy captured by the pigment molecules in the antenna complexes of photosystems I and II is converted into chemical energy and reducing power that is used to obtain carbohydrates by the CO_2 reduction in the Calvin Cycle. In this process part of the light energy is used to split the water molecule (water oxidation) into electrons that are used in the photosynthetic electron transport chain, protons that stay in the lumen of the thylakoids, and releasing oxygen (Lambers *et al.* 1998). The rate of oxygen evolution is proportional to the increase of current registered and is used to calculate the photosynthetic rate after the calibration of the oxygen electrode. As well, dark respiration (Rd) can be measured considering the rate of oxygen uptake in the absence of light, by covering the entire electrode with an opaque material.

2.6.2. Calibration

The calibration of the electrode was made by removing all the dissolved oxygen from the seawater by bubbling nitrogen (N_2) into the incubation chamber. A baseline was recorded after the oxygen concentration reached zero, and the seawater from the incubation chamber was replaced by another 15 ml of O_2 saturated filtered seawater. The saturation line was registered until stabilization. The calibration height is the difference between the base line and the line for oxygen saturated water and is related to the amount of oxygen that saturates the water at a certain temperature and salinity (Benson and Krause 1984).

Photosynthetic rates were calculated from the following equation (1):

$$\text{A} = \frac{\frac{[O_2]\,(\text{mg/l})}{1000\,\text{ml}} \times 15\,\text{ml}}{C_1\,(\text{cm})} \times tg\alpha \times \frac{30\,(\text{cm})}{3600\,(\text{s})} \times \frac{1}{\text{U}\,(\text{cm}^2\ \text{ou g})} \times \frac{1}{Exp}$$

Equation 1: Equation for calculating the oxygen evolution/uptake. A – Oxygen release/consumption rate per area unit or per weight unit (mg O_2/s/cm^2 or g); [O_2] – Oxygen concentration (mg/l); C_1 – Calibration height (cm); α - Angle of the registration line of oxygen release/consumption; 30/3600 – Paper speed in the plotter (cm/s); U – area (cm^2) or weight (g) of the photosynthetic material; Exp – signal amplification.

Photosynthetic and respiratory rates of *C. nodosa* leaves were measured for each of the 6 different treatments (n=4 replicates, except for HL (REF and CO_2) treatments to which n=3). For each photosynthetic light response curve (P-I curve), the electrode incubation chamber was filled with 15 ml of seawater from the corresponding treatment tank and a magnetic stirrer was used to ensure homogenization. Seawater was replaced whenever the light was changed, thus providing the renewal of CO_2 for photosynthesis and avoiding oxygen supersaturation. The water temperature was maintained at 22 ºC by using an external thermostatic water-bath with a recirculation system (Julabo HC Julabo Labortechnik, Seelbach, Germany). A slide projector (Pradovit 150, Leica, Germany) equipped with a halogen lamp (Osram XENOPHOT 150W) was used as actinic light source. Neutral density filters (HL and LL leaves) and blue light filters (LLB leaves), the same used to manipulate light intensity and quality inside the treatment tanks, were combined and mounted on slide frames to provide different light quality and the appropriate range of PAR intensities inside the incubation chambers. Two leaf segments of each replicate were clipped and attached vertically inside the incubation chamber, one next to the other for an equal exposure to the incident light. Leaf samples were submitted to increasing PAR intensities ranging between 17 to 2198 µmol$_{photons}$.m^{-2}.s^{-1} (HL) 7 to 2198 µmol$_{photons}$.m^{-2}.s^{-1} (LL) and 6 to 772 µmol$_{photons}$.m^{-2}.s^{-1} (LLB). Each light level was imposed for the time necessary to generate a straight line with a consistent slope in the oxygen recording system. This slope was used to calculate the oxygen evolution/uptake (equation 1).

After incubation, the length and width of each fragment leaf was measured in order to normalize the photosynthetic rates to the leaf area.

Different mathematical models were applied to each P-I curve to determine which one adjusted better to our results. The best fitted model was chosen to calculate the maximal photosynthetic rate (Pmax, µmolO$_2$.m^{-2}.s^{-1}), the photosynthetic quantum efficiency at limiting irradiances (α, µmolO$_2$/µmol$_{photons}$) and the saturation irradiance (Ik, µmol$_{photons}$.m^{-2}.s^{-1}) calculated as the ratio between Pmax and α for each treatment incorporating error propagation.

2.7. Chlorophyll Fluorescence

Chlorophyll fluorescence parameters were measured with a Diving-PAM (Underwater Chlorophyll Fluorometer Pulse-Amplitude-Modulated (PAM), Walz, Germany) to both monitor the plants photosynthetic performance through the determination of their maximal photochemical efficiency of PSII (Fv/Fm) and construct the rapid light curves (RLC).

2.7.1. Fluorescence Parameters for Assessment of Photosynthetic Performance

Fluorometers are widely used to evaluate the photosynthetic performance trough parameters such as maximal photochemical efficiency (Fv/Fm) of photosystem II (PSII) (Beer *et al.*, 2001; Schreiber, 2004). The decrease of Fv/Fm is associated to stress and to the decrease of the photosynthetic performance. After excitation, chlorophyll relaxes emiting fluorescence (F), releasing energy as heat (D) using photoprotective mechanisms such as the xanthophyll cycle, and using the excitation energy in photochemistry (P) (Krause and Weiss, 1991). The sum of this processes is 1 (F+D+P=1) and the increment in one of them is done at the expenses of the other(s) (Falbel *et al.*, 1994; Maxwell and Johnson 2000). After dark-adaptation, all the reaction centers of the photosynthetic tissue are open, the components of the electron transport chain are oxidized and the photoprotective heat dissipating mechanisms (xanthophyll cycle) are relaxed. At this point, a weak measuring light (0.15 $\mu mol_{photons}.m^{-2}.s^{-1}$) is applied on the leaf tissue with a PAM fluorometer. This weak light makes chlorophyll to fluoresce but is not strong enough to induce the electron transport chain. This fluorescence emission is called minimum or basal fluorescence (F0). F0 is normally quite stable, and represents the fluorescence emission when all the reaction centers are open, without beginning the photosynthetic process. Then, a short pulse of saturating light (>10,000 $\mu mol_{photons}.m^{-2}.s^{-1}$ during 0.4-0.8 s) is applied. At this point all the reaction centers close, but the saturating light pulse doesn't last for enough time to induce photochemical processes (P=0) and the fluorescence yield increases rapidly to its maximum (Fm), after what it slowly declines (Ralph and Gademann, 2005). The saturating light pulse also activates the heat dissipating mechanisms and this why the decrease in Fm is frequently considered to be related with the increase of D (as P=0) and results on the decrease of Fv/Fm. Fv/Fm is determined from the following equation (2) (Larkum *et al.*, 2006).

$$\frac{Fv}{Fm} = \frac{Fm - F0}{Fm}$$

Equation 2: Maximal photochemical efficiency of PSII. Fv - variable fluorescence; Fm - maximum fluorescence; F0 - basal fluorescence.

For each tank, two mature leaves from different shoots were marked to the daily measure of fluorescence parameters. Dark adaptation was done by fitting a dark adaptation clip to the leaf. After one hour and a half of dark acclimation fluorescence measuremnents took place.

2.8. Rapid Light Curves

Productivity can be estimated using rapid light curves, where the relative maximum electron transport rate (rETRmax, yy axis) is plotted as a function of photosynthetically active radiation (PAR, xx axis). These curves of rapid light response have been used in a wide range of seagrass studies (Ralph and Gademann 2005; Saroussi and Beer 2007).

In light-adapted tissues, the effective quantum yield (ΔF/F'm) is measured by emitting a short-saturating pulse of light to light adapted leaves. It measures the efficiency of the photosystem II (PSII) photochemistry and gives information about the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry (i.e. PSII quantum yield in the light) (Mvungi, 2011) by the following equation (3) (Larkum *et al.*, 2006):

$$\frac{\Delta F}{F'm} = \frac{F'm - Ft}{F'm}$$

Equation 3: Effective Quantum Yield; ΔF - proportion of open reaction centers; F'm – maximum fluorescence in the light; Ft – steady-state level of fluorescence.

ΔF/F'm can be used to calculate the linear relative electron transport rate (rETR) by the following equation (4) (Silva *et al.*, 2009):

$$rETR = \frac{\Delta F}{F'm} \times PAR$$

Equation 4: Relative electron transport rate. ΔF/F'm – effective quantum yield; PAR – photosynthetically active radiation.

Eight consecutive light levels of 13, 43, 92, 158, 234, 320, 464 and 628 $\mu mol_{photons}.m^{-2}.s^{-1}$ were applied. The relative electron transport rate of *C. nodosa* leaves were measured in replicates (n=8, except for LL (REF and CO_2) treatments, to which n=7) out of each of the 6 different treatments. Shoots chosen for RLC measurements were representative of the shoots observed at each tank.

Different mathematical models were applied to each RLC to determine which one adjusted better to the results obtained. The best fitted model was chosen to calculate the maximal relative electron transport rate (rETRmax, $\mu mol_{electrons}.m^{-2}.s^{-1}$), the light utilization coefficient (α, $\mu mol_{electrons}/\mu mol_{photons}$) and the saturation irradiance (Ik, $\mu mol_{photons}.m^{-2}.s^{-1}$) calculated as the ratio between rETRmax and α for each treatment incorporating error propagation.

2.9. Mathematical Models: Photosynthetic Light Response Curves and Rapid Light Curves

Some mathematical models were tested to determine the best fit to the set of gross photosynthetic rates-irradiance pairs in each of the treatments tested. The application of these mathematical models to the results obtained with the oxygen electrode involves the use of gross photosynthetic rates (net photosynthetic rate measured plus dark respiration) as one of their limitations is that they do not work with negative values. The mathematical models tested were developed by: Smith (1936), Jassby and Platt (1976), Platt *et al* (1980) and Bannister (1979). The same models were applied to rapid light curves. The selection of the mathematic model that best fit the overall responses in each treatment was determined according to the value of the correlation coefficient (R). Still, when the correlation coefficient was identical for more than one of the models tested, it was also used a Durbin-Watson statistic for autocorrelation, which function is to test the independence of residual values within each other. The Durbin-Watson parameter is equal to 2 when there is no autocorrelation. The deviation from this value indicate the degree of correlation among the residuals.

Curves were fitted using “SigmaPlot” (Copyright © 2008 Systat Software, Inc. Germany, Sigmaplot for Windows Version 11.0).

2.10. Photosynthetic Pigments Analysis

C. nodosa frozen leaf samples (0.19 g – 0.22 g) were ground to powder with sodium ascorbate ($C_6H_7NaO_6$), to avoid pigments degradation, in liquid nitrogen. Photosynthetic pigments were extracted under dim light, in 100 % acetone buffered with calcium carbonate ($CaCO_3$) (Abadía and Abadía, 1993). The extracts (5 ml) were filtered with LS 5.0 µm membrane and hydrophobic PTFE 0.2 µm filters.

The spectrophotometer measures the absorption of light at a particular wavelength by shining a beam of electromagnetic radiation onto a sample. It is a common and easy way to quantify several substances and is commonly used to quantify chlorophylls a and b and carotenoids present in a mixture (Dudek *et al.*, 2014). Chlorophylls a and b and total carotenoids extracts were read spectrophotometrically, at three different wavelengths: 470 nm, 644.8 nm and 661.6 nm (Beckman-Coulter DU 650 spectrophotometer, Brea CA, USA). Quantification was done using the following equations (5, 6, and 7) of Lichtenthaler and Buschmann (2001):

$$5)\ \text{Chl a } (\mu g/ml) = 11.24\, A_{661.6} - 2.04\, A_{644.8}$$

$$6)\ \text{Chl b } (\mu g/ml) = 20.13\, A_{644.8} - 4.19\, A_{661.6}$$

$$7)\ C\ (x{+}c)\ (\mu g/ml) = (1000\, A_{470} - 1.90\, c_a - 63.14\, c_b)\ /214$$

Equation 5: Chlorophyll a (Chl a);
Equation 6: Chlorophyll b (Chl b);
Equation 7: Carotenoids (C (x+c); xanthophylls and carotenes);

However, when the amount of pigments to be analyzed is higher than 3, the estimation of individual pigment concentration through spectrophotometry is less efficient (Dudek *et al.*, 2014), once chlorophylls and carotenoids have similar spectra and the absorption peaks of the different carotenoids are very close making it impossible to accurately identify each carotenoid by spectrophotometry (Dudek *et al.*, 2014). Carotenoids (β-carotene, lutein, neoxanthin, taraxanthin, violaxanthin, antheraxanthin and zeaxanthin) were separated and quantified by high performance liquid chromatography (HPLC), as described in Larbi *et al.* (2004) after de las Rivas *et al.* (1989).

Liquid chromatography analysis was performed in an Alliance Waters 2695 separation module (Milford MA, USA), with a Waters 2996 photodiode array detector and a Waters

Novapak C18 radial 86100 mm compression column (4 mm particle size). The basic principle of liquid chromatography application relies on the fact that, under the same circumstances each component in a mixture usually interacts with its environment distinctively from all other components (Bidlingmeyer, 1992). The extracted pigments were dissolved in a mixture of appropriate organic solvents (filtered and sonicated prior to use, R1: acetonitrile, methanol and triethylamine (TEA); and R2: acetonitrile, methanol, Milli-Q, acetate ethyl and TEA; mobile phase) and pumped through a column, in precise volumes (20 µl) regulated by an injector. In the course of the injection, extracts were maintained at 5 ºC and the column was kept at a constant temperature of 25 ºC. The mobile phase was forced through the packing material, and the pigments separation occurs as a function of their affinity for both the mobile and stationary phases. A detector recorded the concentration of each pigment that migrates at different rates, having different retention times within the column (Bidlingmeyer, 1992). The chromatogram is the output of the HPLC and shows detector responses indicating the presence of the components as "peaks" whose area corresponds to a certain concentration after calibration (Malviya *et al.*, 2009). For photosynthetic pigments quantification, the device was previously calibrated for each pigment by injecting known amounts of commercially available pure pigments (CaroteNature, Lupsingen, Switzerland) (de las Rivas *et al.* 1989).

2.11. Data Analysis

All data was statistically analyzed with the program "Sigmaplot" (Copyright © 2008 Systat Software, Inc. Germany, Sigmaplot for Windows Version 11.0). Before analyses, all data were tested for normality (Shapiro-Wilk tests) and homogeneity of variances and transformed when necessary.

A statistical t-test ($p<0.001$) was applied to detect differences in the physico-chemical data of pH, TA, DIC, pCO_2 and inorganic carbon forms (CO_2, HCO_3^- and CO_3^{2-}), obtained from DIC analysis and used for system characterization.

Oxygen (optode) and pCO_2 (IRGA) data obtained along the day were daily integrated for each day (daily dose) and analyzed with a t-test ($p<0.001$) to detect differences between REF and CO_2 treatments. Differences in the irradiance reaching *C. nodosa* leaves in distinct light levels treatments (HL, LL and LLB) were analyzed by a non-parametric Kruskal-Wallis test (One Way Analysis of Variance on Ranks), with $p<0.001$, because data were not normality distributed.

One Way Analysis of Variance tests for each CO_2 treatment were applied to detect differences in the fluorescence RLCs (rETRmax, α and Ik) and photosynthetic P-I curves (Pmax, α, Ik and Rd) derived parameters among light treatments ($p<0.05$). In case of significant effect detected, a posthoc Student-Newman-Keuls test was applied. In the case of Fv/Fm a two-way ANOVA test was applied. Once data failed the Two-Way ANOVA assumptions even after transformation, the p-value was lowered to 0.01 to minimize the risk of a Type 1 error (Underwood, 1997).

Differences in the concentration of chlorophylls (Chl a, Chl b and Chl T) and chlorophyll a/b ratio (Chl a/b) in *C. nodosa* leaves among the three sampling periods (Cádiz, T0 and T1) were tested with a one-way ANOVAs. Student-Newman-Keuls post-hoc test was used whenever significant differences were detected by one-way ANOVA. Since plants' collection in Cádiz was at noon, only T0-noon and T1-noon data were considered in this analysis. Differences between T0 and T1 in plants harvested at pre-dawn were tested with a t-test.

A two-way ANOVA tests were conducted to detect significant effects of CO_2, light and sampling period (pre-dawn and noon) on chlorophyll (Chl a, Chl b and Chl T) and xanthophyll at the end of the experiment (T1). Whenever the variance test failed (for AZ/VAZ ratio), a t-test was used to detect differences between pre-dawn and noon sampling period for different light treatments. When $P<0,050$ the effect tested was considered statistically significant.

3. Results

3.1. Experimental Physical-Chemical Parameters

The concentration of CO_2 and HCO_3^- increased in the high pCO_2 tanks while CO_3^{2-} concentration decreased, when compared to the reference treatments. As expected, pH was significantly lower in the high pCO_2 tanks. Total alkalinity was not significantly different among treatments (Table 1). On contrary, dissolved inorganic carbon was significantly different between treatments, as projected in experimental design, ensuring the differences between distinct pCO_2 conditions imposed.

Table 1. Carbonate system parameters during acclimation and experimental periods; Statistical differences were determined in the experimental period between Reference and CO$_2$ treatments. Values are mean ± SD; *n* = 5;

Period	**Acclimation**	**Experiment**		**Statistical difference (p<0.001)**
pCO$_2$ treatment		**Reference**	**CO$_2$**	
pH (NBS scale)	8.3 ± 0.02	8.3 ± 0.03	7.9 ± 0.04	Yes
TA (µmol.kg^{-1} SW)	2568.6 ± 26.7	2580.8 ± 10.6	2586.3 ± 13.1	No
DIC (µmol.kg^{-1} SW)	2175.2 ± 17.2	2167.5 ± 27.9	2380.1 ± 25.4	Yes
pCO$_2$ (µatm)	460 ± 121	295.7 ± 31	763.1 ± 56.9	Yes
HCO$_3^-$ (µmol.kg^{-1} SW)	1879.4 ± 17.3	1862.9 ± 46.3	2196.1 ± 36.5	Yes
CO$_3^{2-}$ (µmol.kg^{-1} SW)	284.9 ± 9.8	295.7 ± 20.4	161.3 ± 14.5	Yes
CO$_2$ (µmol.kg^{-1} SW)	10.9 ± 1.4	8.9 ± 1.0	22.7± 2.1	Yes

Water salinity remained constant at 36 psu throughout the experiment and between different treatments. The maximum and minimum mean water temperature registered was 27.3 and 20.4 ºC, respectively (Fig. 8A). Daily averaged integrated PAR at the surface of the mesocosm was 52 mol$_{\text{photons}}$.m^{-2}.d^{-1} with ca. 14 hours of light photoperiod (Observatório Astronómico de Lisboa, 2015) and a maximum mean value of 1791.5 µmol$_{\text{photons}}$.m^{-2}.s^{-1} (Fig. 8B). Despite the fluctuations, the irradiance values exhibited a typical diurnal pattern with low irradiances in the morning, highest irradiances from midday until the early afternoon and a decrease in irradiances as the day progressed. Low light (LL) and low blue light (LLB) treatments received 32 – 33% and 24 - 26% of the irradiance reaching the high light (HL) treatments, respectively. The light intensity reaching HL treatment was statistically different (p<0.001) from the light intensity reaching both LL and LLB treatments. On the other hand, no statistically differences were found in the intensity of light between both low light treatments (LL and LLB). Thus, the differences between these two treatments can be attributed to the light quality.

The daily fluctuation in pCO$_2$ values in the experimental tanks followed the expected trend with higher values at night, when respiration surpasses photosynthesis, and the opposite during the day, when photosynthesis is higher than respiration. CO$_2$ partial pressure decreased ca. 50% on the reference treatments and ca. 63% on CO$_2$ enriched treatments (Fig. 8C). As the CO$_2$ and O$_2$ evolution related with photosynthesis and respiration are opposite, pCO$_2$ and dissolved oxygen (DO) varied inversely along the day, with higher values between 11 a.m. and 6 p.m. DO values were similar in both treatments throughout the day and no significant differences between the different pCO$_2$ treatments (REF and CO$_2$) were found.

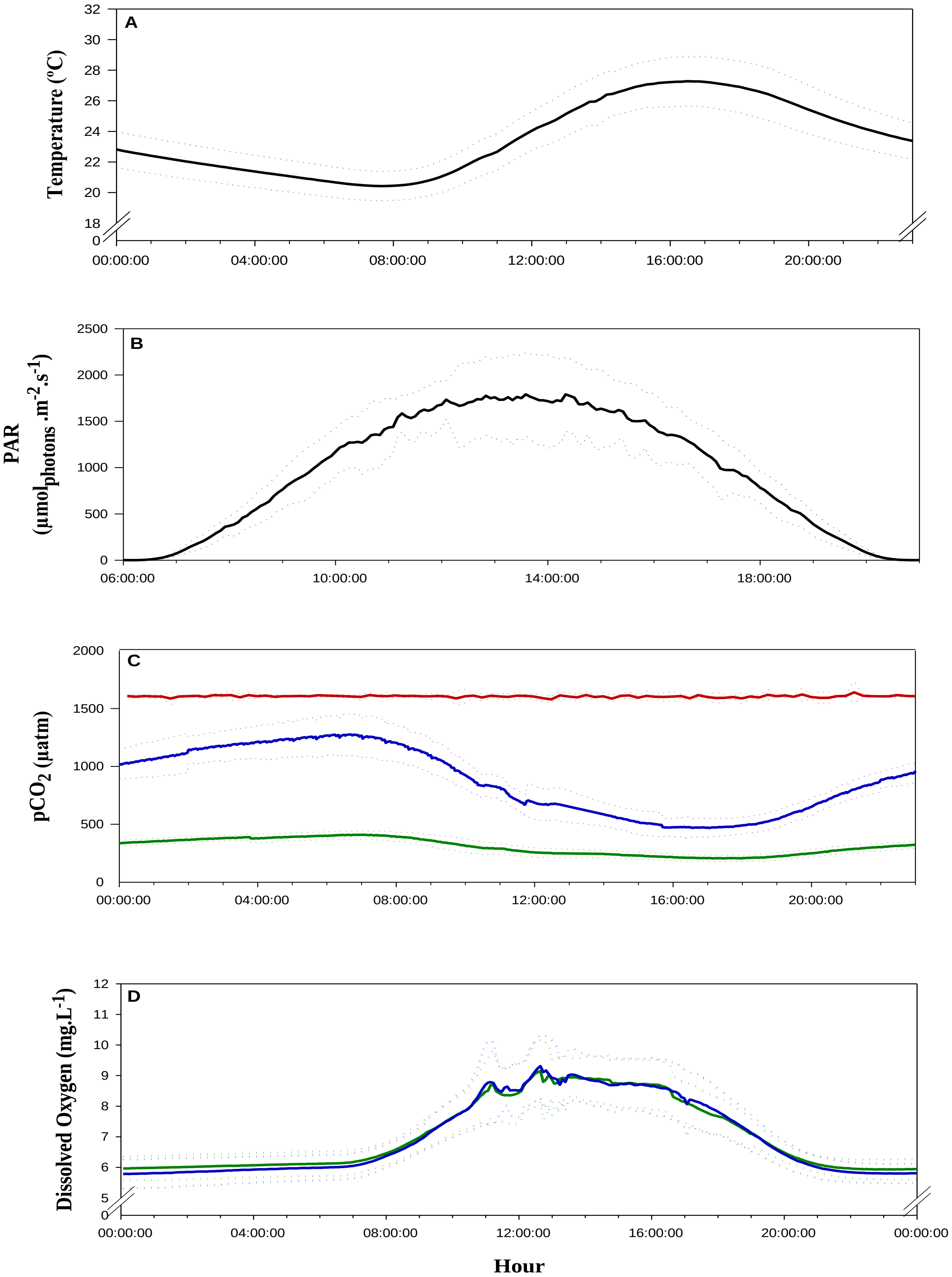

Figure 8: Daily measured parameters: **(A)** Mean water temperature and **(B)** Mean irradiance at the surface of the mesocosm (PAR, between 6 a.m. and 9 p.m). Solid lines represent the mean value of each parameter, dashed line represents the standard deviation; **(C)** Mean carbon dioxide partial pressure (pCO_2) and (**D**) Mean dissolved oxygen (DO): red solid line corresponds to pCO_2 at main reservoir, blue and green solid lines correspond to pCO_2 and DO at high and reference pCO_2 treatments, respectively. Colored dashed lines represents the respectively solid colored line's standard deviation.

3.2. Photosynthetic Light Response Curves

Cymodocea nodosa photosynthesis exhibited the typical hyperbolic response to light in all the treatments imposed (Fig. 9). Under limiting irradiances, photosynthetic rates increased linearly with increasing light and reached its maximum at saturating irradiance. Among the tested mathematical models, Smith (1936) model gave the best fit to the overall responses for each treatment (table 2) in P-I curves. Fitted curves according to Smith (1936) are plotted with a solid line (Fig. 9).

Table 2. Coefficient of determination (R^2) and statistical Durbin Watson values obtained after the application of the different mathematical models to the photosynthetic light response made with plants acclimated to high light (HL), low light (LL) and low blue light (LLB) under high (CO_2) and current atmospheric (REF) pCO_2.

	Treatment	**Models**							
		Bannister (1979)		Jassby and Platt (1976)		Platt *et al* (1980)		Smith (1936)	
		R^2	**Durbin Watson**	**R^2**	**Durbin Watson**	**R^2**	**Durbin Watson**	**R^2**	**Durbin Watson**
T1	**REF - HL**	0.6072	2.5658	0.6111	2.5818	0.6138	2.6336	0.6035	2.5256
	REF - LL	0.7565	2.2363	0.7491	2.1037	0.7603	2.2313	0.7531	2.1672
	REF - LLB	0.8335	2.2547	0.8314	2.1891	0.8409	2.2858	0.8324	2.2238
	CO_2 - HL	0.5874	1.9503	0.5495	1.9358	0.5976	2.1398	0.5674	2.0043
	CO_2 - LL	0.8623	2.1835	0.7521	1.2863	0.8267	1.7976	0.7810	1.4309
	CO_2 - LLB	0.9181	1.8985	0.8337	1.0497	0.8917	1.4187	0.8543	1.1703

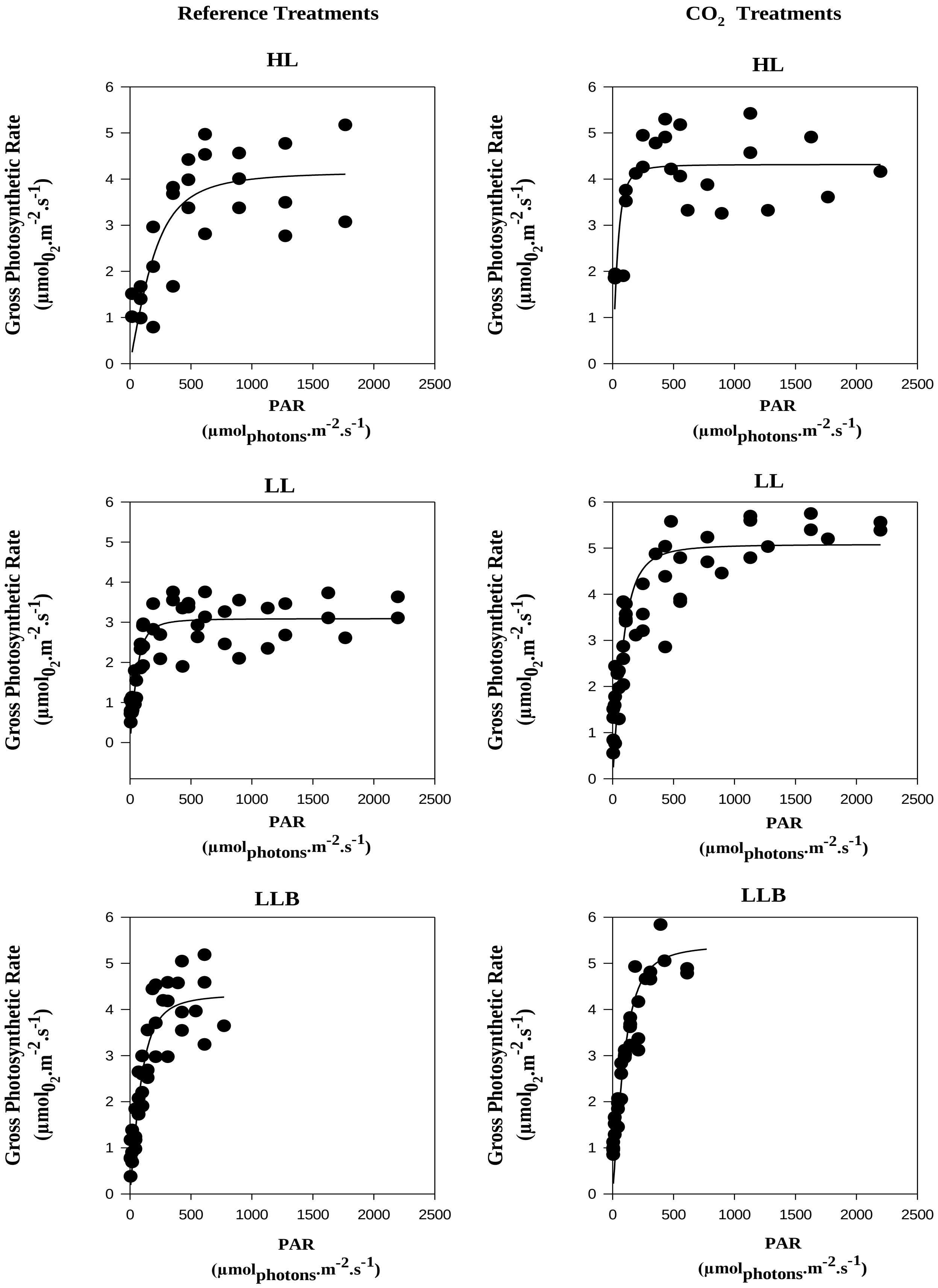

Figure 9: Light response curves of *C. nodosa* acclimated to high light (HL), low light (LL) and low blue light (LLB) under high (CO_2) and current (REF) pCO_2. Curves were fitted according to Smith (1936) mathematical model.

All the photosynthetic parameters (Pmax, Ik, α and dark respiration) derived from the P-I curves were analyzed for both pCO_2 (reference and high pCO_2) and light (HL, LL and LLB) treatments.

Maximum photosynthesis (Pmax) in CO_2 enriched treatments was significantly higher in low light treatments (both LL and LLB), compared to high light ones (Fig. 10). Yet, the treatments with current atmospheric CO_2 levels, only a significant decrease in Pmax from HL to LL levels was found, while LLB showed a Pmax value similar to HL acclimated leaves.

In low light intensity treatments (LL and LLB), Pmax values were significantly higher in enriched (CO_2) than in current (REF) CO_2 levels. However, in HL no significant differences between CO_2 treatments were found.

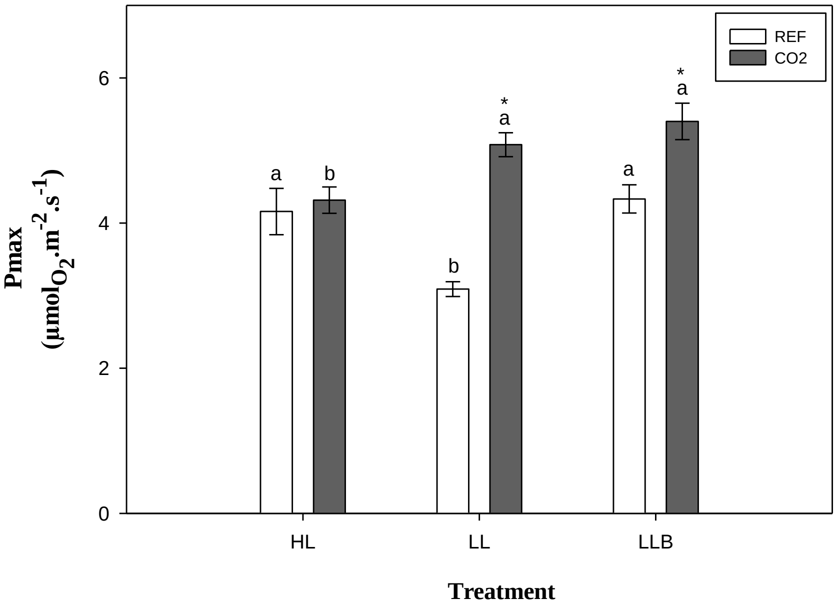

Figure 10: Maximum photosynthetic rate (Pmax) of *C. nodosa* leaves after the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current atmospheric (REF) pCO_2; Different letters indicate significant differences among light levels (for CO_2 and REF treatments, independently); * indicates significant differences between CO_2 and REF treatments, for the same light level; Values are mean ± SE, n=4 (except for HL (REF and CO_2) treatments, to which n=3).

The photosynthetic quantum efficiency (α) was significantly higher in plants acclimated to high levels of pCO_2 than in those acclimated to ambient pCO_2, in HL treatments (Fig. 11A). Regarding different light levels, a significant increase in photosynthetic efficiency occurred from high to low irradiance treatments but only for the plants under present-day pCO_2 (REF). An opposite trend was found in plants under high levels of pCO_2 (CO_2), although the differences were not significant.

Low light significantly decreased the saturation irradiance (Ik) of the plants under current atmospheric pCO_2. However, under high atmospheric pCO_2 the opposite was verified with significantly higher Ik in low light irradiances (LL and LLB) treatments (Fig. 11B). Within the same light treatment Ik value was significantly lower in HL enriched-CO_2 treatments but no differences between CO_2 treatments were found on the other two low light treatments.

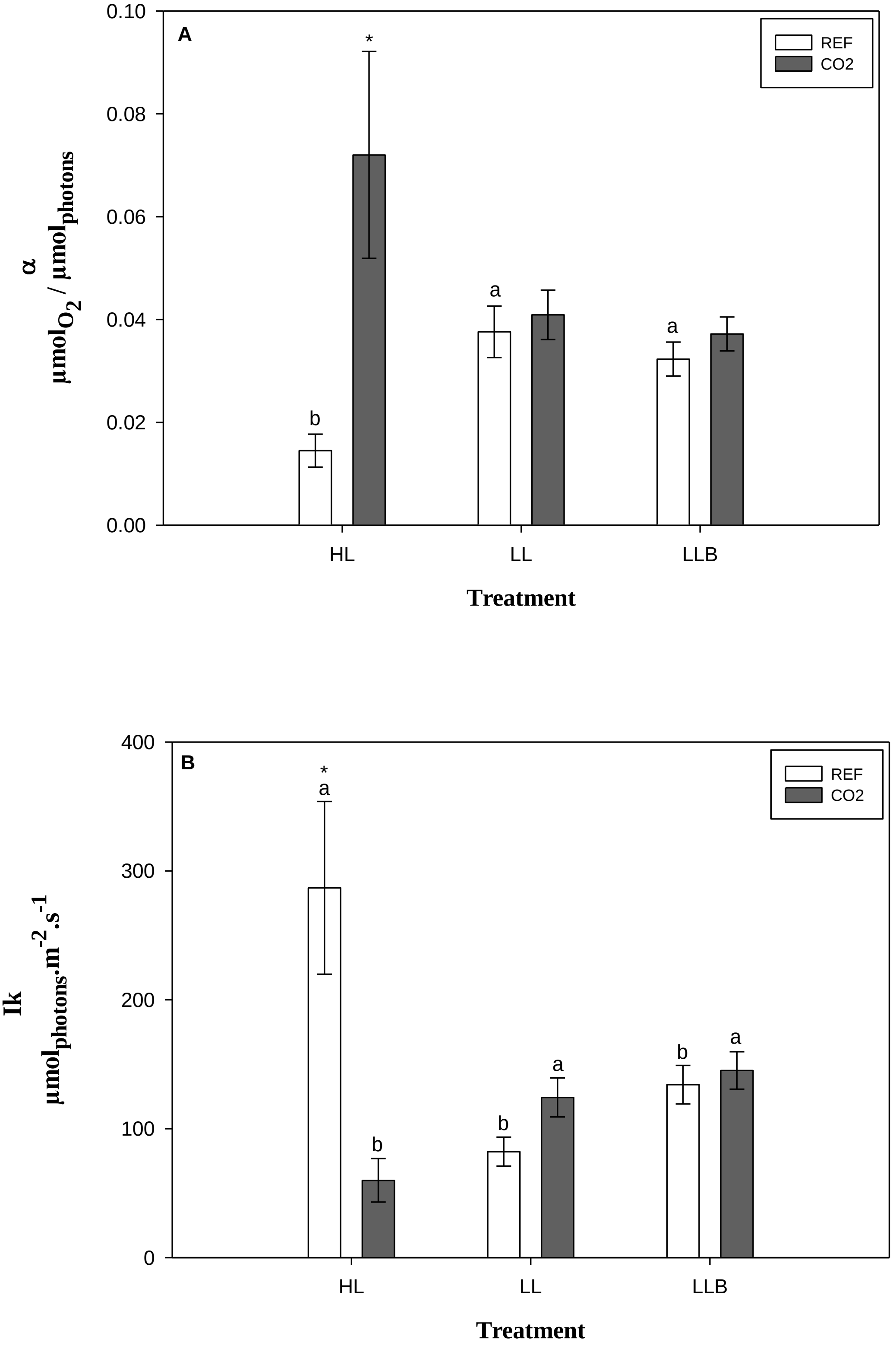

Figure 11: **(A)** Photosynthetic quantum efficiency (α) and **(B)** saturation irradiance (Ik) of *C. nodosa* leaves after experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current atmospheric (REF) pCO_2. Different letters indicate significant differences between light levels (for CO_2 and REF treatments, independently); * indicates significant differences between CO_2 and REF treatments, for the same light level; Values are mean ± SE, n=4 (except for HL (REF and CO_2) treatments, to which n=3).

3.3. Dark Respiration

High CO_2 partial pressures induced higher respiration rates when compared with current CO_2 levels but differences were significant only for HL plants (Fig. 12). There was a tendency for decrease respiratory rates with decreasing light intensity in both pCO_2 treatments, although it was more evident in plants under high pCO_2.

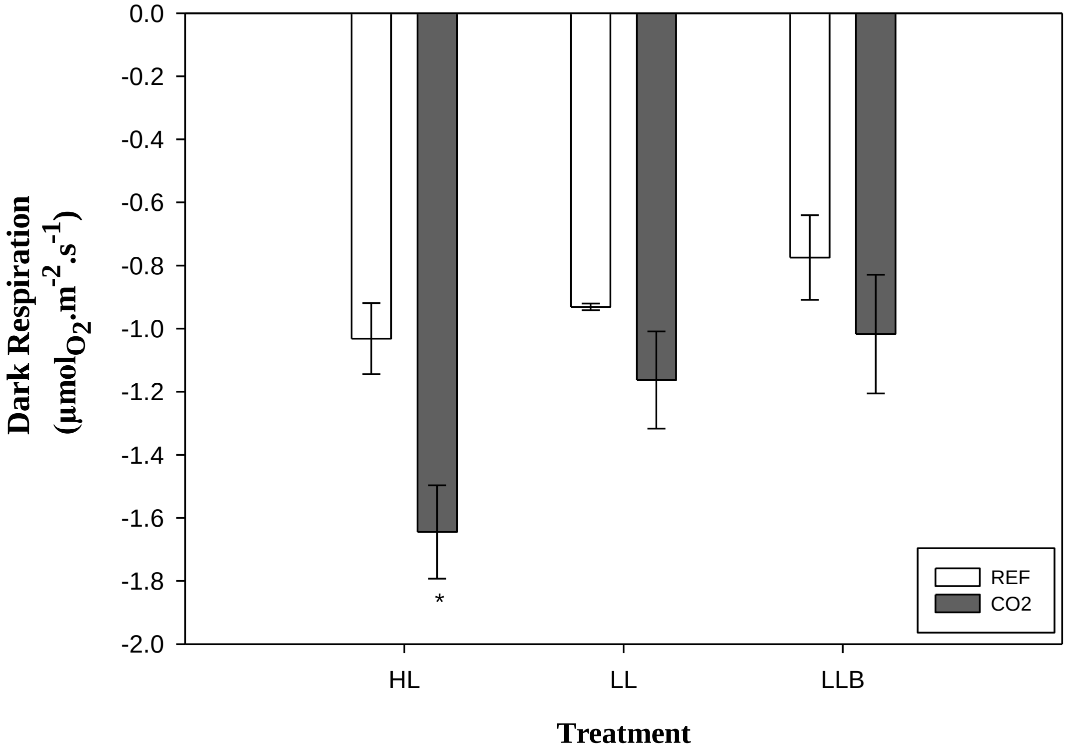

Figure 12: Dark respiration rates (Rd) of *C. nodosa* leaves after the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current atmospheric (REF) pCO_2; * indicates significant differences between CO_2 and REF treatments, for the same light level; Values are mean ± SE, n=4 (except for HL (REF and CO_2) treatments, to which n=3).

3.4. Rapid Light Curves

Rapid light curves (RLC) were generated from the calculated relative electron transport rate (rETR) in response to the irradiances applied. Among the tested mathematical models, Smith (1936) gave the best fit to the overall responses of rapid light curves for each treatment (Table 3).

Table 3. Coefficient of determination (R^2) and statistical Durbin Watson values obtained after the application of the different mathematical models to the rapid light curves made with plants acclimated to high light (HL), low light (LL) and low blue light (LLB) under high (CO_2) and current atmospheric (REF) pCO_2.

	Treatment	**Models**							
		Bannister (1979)		Jassby and Platt (1976)		Platt *et al* (1980)		Smith (1936)	
		R^2	**Durbin Watson**	**R^2**	**Durbin Watson**	**R^2**	**Durbin Watson**	**R^2**	**Durbin Watson**
T1	**REF - HL**	0.9657	0.5369	0.9655	0.5433	0.9636	0.5981	0.9643	0.5543
	REF - LL	0.9283	0.4067	0.9257	0.4051	0.9307	0.3851	0.9164	0.4709
	REF - LLB	0.889	0.4307	0.8849	0.4495	0.8948	0.4225	0.8735	0.5033
	CO_2 - HL	0.9575	0.4776	0.9566	0.4851	0.9552	0.5207	0.9527	0.548
	CO_2 - LL	0.9699	0.6514	0.9673	0.5864	0.9726	0.6247	0.9586	0.6573
	CO_2 - LLB	0.9628	0.664	0.9567	0.6869	0.9692	0.4825	0.9437	0.7803

Rapid light curves of HL, LL and LLB acclimated *C. nodosa* plants under CO_2 and REF treatments are shown in Fig. 13. Curves fitted according to Smith (1936) are plotted as a solid line.

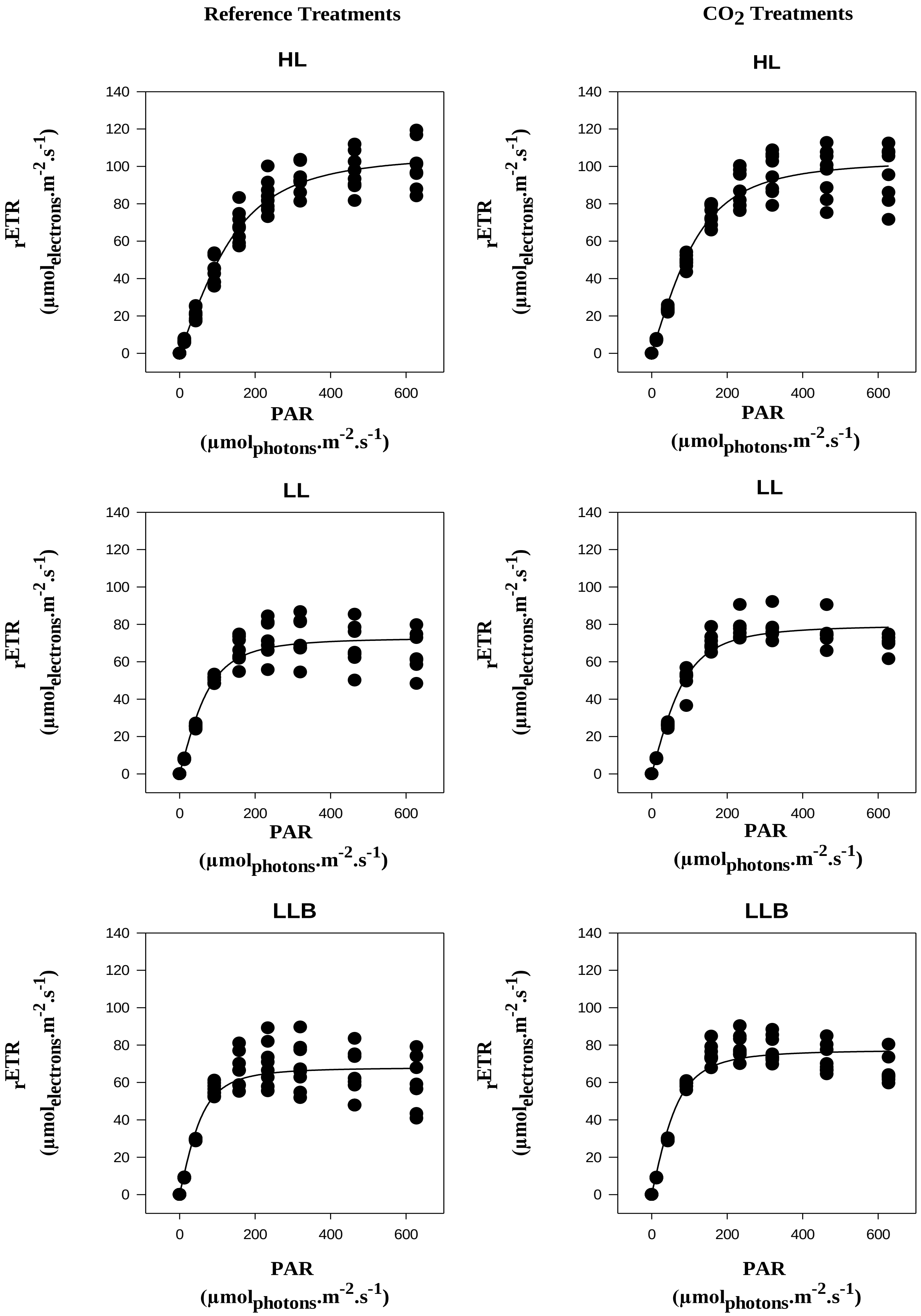

Figure 13: Rapid light curves of *C. nodosa* acclimated to high light (HL), low light (LL) and low blue light (LLB) under high (CO_2) and current (Reference) pCO_2. Curves were fitted according to Smith (1936) mathematical model.

The maximum relative electron transport rate (rETRmax) was significantly higher (more than 26%) in HL leaves, regardless the CO_2 treatment, than low light treatments (LL and LLB) (Fig. 14). Regarding different CO_2 treatments, LL and LLB leaves significantly displayed higher rETRmax under high pCO_2.

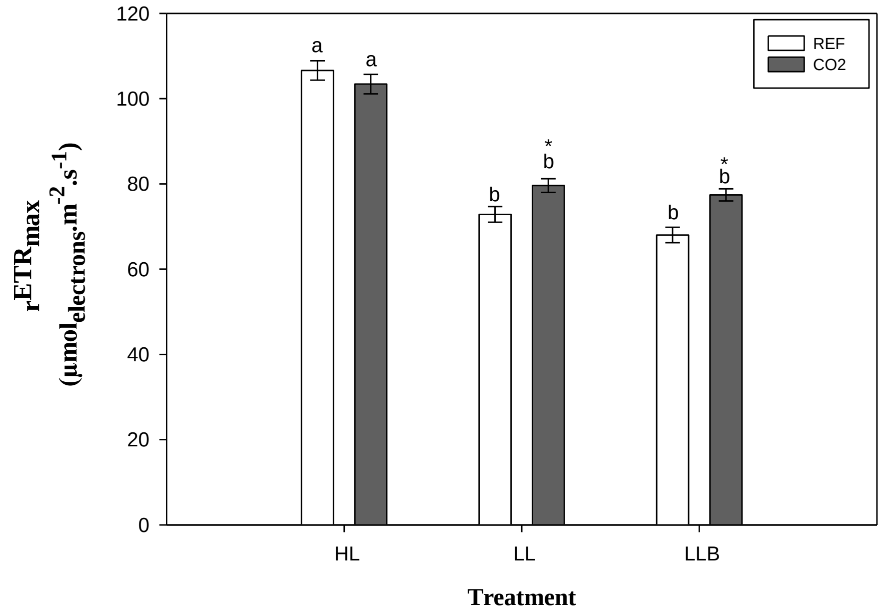

Figure 14: Maximum relative electron transport rate (rETRmax) of *C. nodosa* leaves after the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current atmospheric (REF) pCO_2; Values are mean ± SE, n=8 (except for LL (REF and CO_2) treatments, to which n=7).

The light utilization coefficient (α) for fluorescence was significantly higher in LL and LLB than in HL leaves at current atmospheric pCO_2 (Fig. 15A). In enriched-pCO_2 plants, only the α value of LLB plants was significantly higher. Regarding leaves under distinct pCO_2 conditions, in HL conditions, the light utilization coefficient significantly increase in plants growing with high CO_2 availability. On the other hand, no significant differences were detected between CO_2 and REF leaves under both low light conditions (LL and LLB).

The saturation irradiance (Ik) followed the inverse tendency of α and a similar trend as the rETRmax (regarding light levels) although more pronounced. At high pCO_2 conditions, considering the quality of light, it must be noted that Ik value was significantly lower in LLB than in LL (Fig. 15B). Regardless pCO_2 treatment, HL leaves displayed significantly higher Ik value when compared to LL and LLB. About the pCO_2, Ik was significantly higher in REF treatments only in HL conditions, meaning that rETR saturates at lower irradiance in plants acclimated to high pCO_2. However this was not observed for LL and LLB treatments.

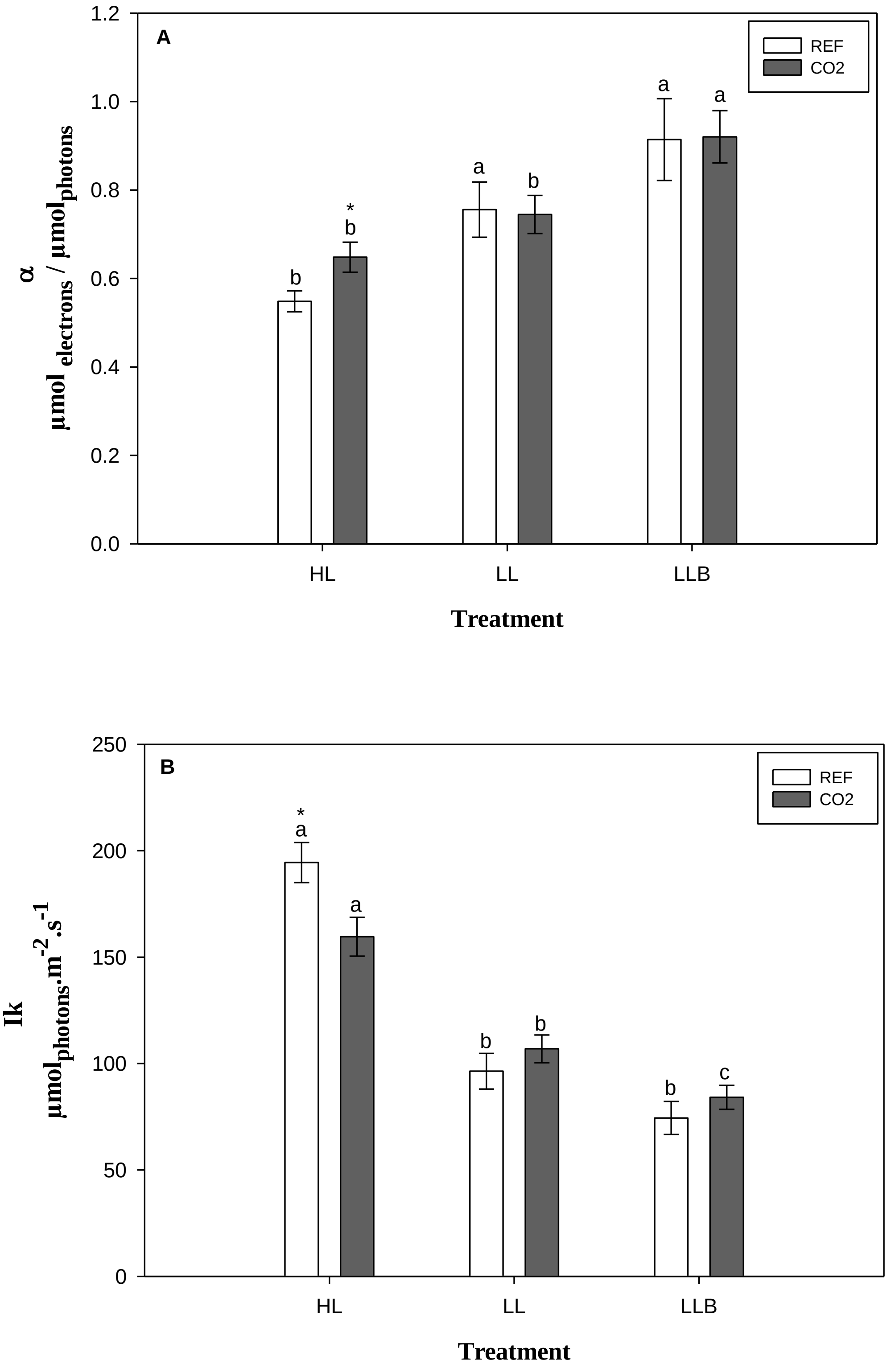

Figure 15: **(A)** Light utilization coefficient (α) and **(B)** saturation irradiance (Ik) of *C. nodosa* leaves after the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current (REF) pCO_2; Values are mean ± SE, n=8 (except for LL (REF and CO_2) treatments, to which n=7).

3.5. Maximal Photochemical Efficiency of PSII

The maximal photochemical efficiency (Fv/Fm) of the photosystem II evidenced similar patterns of fluctuation throughout the experiment (Fig. 16). HL leaves (both CO_2 and REF) displayed the higher Fv/Fm fluctuations and the lower Fv/Fm values, with a significant decrease in REF (14%) and CO_2 (8%) when compared to LL and LLB leaves within which there were no significant differences. Regarding the CO_2 effect, Fv/Fm was significantly lower in HL-REF than at HL-CO_2 leaves.

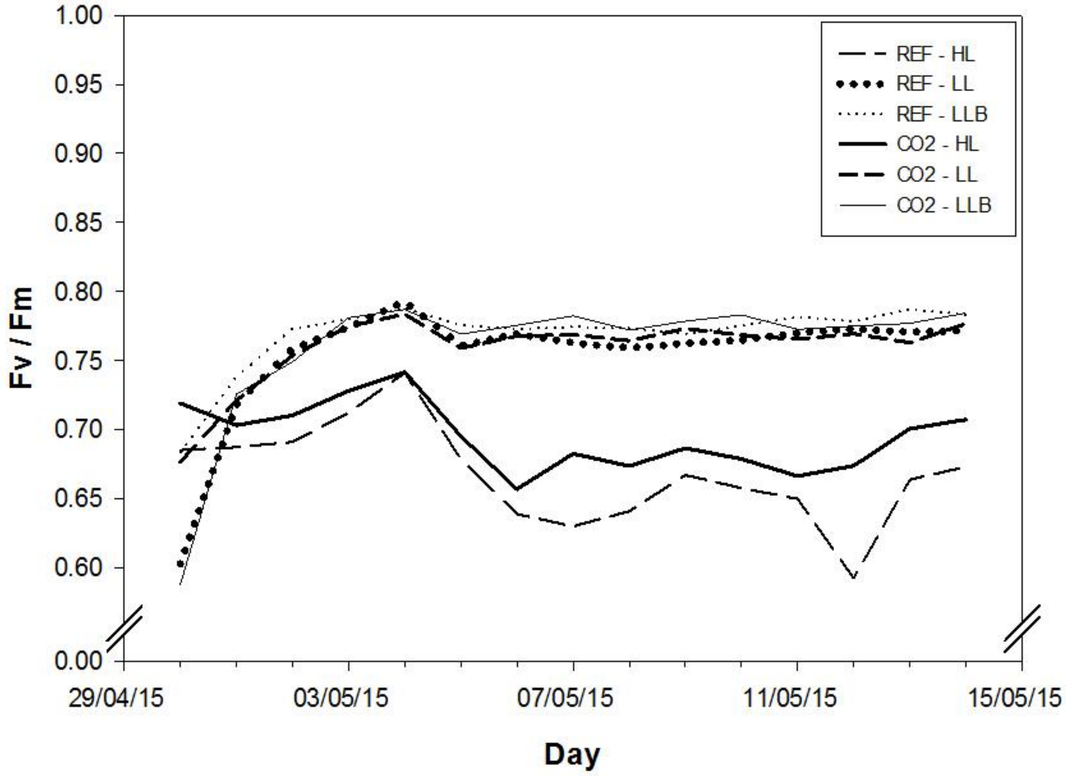

Figure 16: Maximal photochemical efficiency (Fv/Fm) of photosystem II daily average of *C. nodosa* leaves under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current atmospheric (REF) pCO_2, during the experimental period.

3.6. Photosynthetic Pigments

The concentration of chlorophyll a, chlorophyll b, and total chlorophyll content was significantly higher in *C. nodosa* samples collected in Cádiz than in leaves collected at the end of the acclimation period, T0 (noon) (Table 4). The concentrations of these pigments continued to decline over the experimental period, and were significantly lower in plants harvested at the end of the experiment, T1 (noon) than at T0 (noon). This decrease pattern in Chl a, Chl b and Chl T concentration per unit area, from T0 to T1, was also observed in plants collected at pre-dawn. However, chlorophyll a/b ratio remained similar in all of these sampling periods (table 4).

Regarding T1 sampling period, although not significant, Chl T tended to increase in leaves under low light intensity and current ambient conditions of pCO_2. Concerning different CO_2 treatments, Chl a, Chl b, Chl T and Chl a/b ratio were not significantly affected.

Table 4. Concentrations of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl T) and chlorophyll a/b ratio (Chl a/b) in *Cymodocea nodosa* leaves at the time of collection, in Cádiz, after acclimation to the mesocosm (T0) and at the end of the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current (REF) pCO_2; Values are mean ± SE, n=4 (except for T0 Pre-dawn, T0 Noon and T1 Pre-dawn (REF-LL) to which n=3).

	Treatment	**Chl a (µmol/m^2)**	**Chl b (µmol/m^2)**	**Total Chl (µmol/m^2)**	**Chl a/b**
Cádiz		120.9 ± 6.4	48.3 ± 3.4	169.2 ± 9.8	2.5 ±0.1
T0 Pre-dawn		71.3 ± 11.9	27.4 ± 5	98.7 ± 16.6	2.6 ± 0.2
T0 Noon		67.5 ± 12.4	26 ± 4.9	93.5 ± 17.2	2.6 ± 0.1
T1 Pre-dawn	**REF - HL**	22.2 ± 5.4	8.3 ± 2.1	30.5 ± 7.5	2.7 ± 0.1
	REF - LL	25.7 ± 4.7	11 ± 2.6	36.6 ± 7.3	2.4 ± 0.2
	REF - LLB	38.4 ± 9.4	15.9 ± 4.1	54.2 ± 13.5	2.5 ± 0.1
	CO_2 - HL	34.3 ± 11.9	12.6 ± 4.3	46.9 ± 16.2	2.7 ± 0.1
	CO_2 - LL	30.3 ± 6.2	11.7 ± 2.5	42 ± 8.7	2.6 ± 0
	CO_2 - LLB	36 ± 8.3	14.6 ± 3.2	50.7 ± 11.5	2.5 ± 0.1
T1 Noon	**REF - HL**	25.8 ± 2.8	10.9 ± 1.6	36.6 ± 4.4	2.4 ± 0.1
	REF - LL	30.3 ± 3.4	12.8 ± 3.1	40.1 ± 8.8	2.2 ± 0.1
	REF - LLB	41.1 ± 11.1	17 ± 8.1	53 ± 21.5	2.4 ± 0.2
	CO_2 - HL	35.8 ± 9.3	15.1 ± 3.9	50.9 ± 13.1	2.4 ± 0.1
	CO_2 - LL	29.3 ± 3	13.3 ± 2.4	42.6 ± 5.4	2.3 ± 0.1
	CO_2 - LLB	33.5 ± 10.6	14.1 ± 4.3	47.6 ± 14.9	2.4 ± 0.2

Ten different lipossoluble photosynthetic pigments were detected and quantified by HPLC in *C. nodosa* leaves, with a clear separation of all pigments (Fig. 17). Chlorophyll *a*, chlorophyll *b*, neoxanthin, violoxanthin, lutein, zeaxanthin and β-carotene were common to all different treatments. However antheraxanthin and taraxanthin were not detected in 4 (at pre-dawn) and 3 (at noon) samples, respectively. One unknown peak was detected in all treatments.

Total carotenoids were ca. 41% and 38% of the total photosynthetic pigment content in *C. nodosa*, at T1: pre-dawn and noon, respectively.

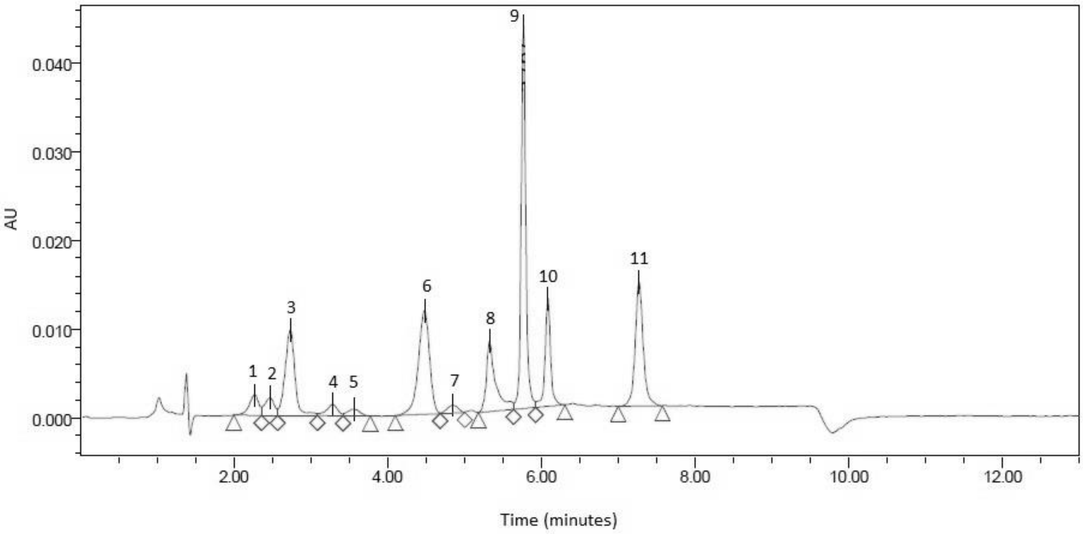

Figure 17: HPLC chromatographic profile of *C. nodosa* photosynthetic pigment extracts. Peak identification: 1 - Neoxanthin 1; 2 - Neoxanthin 2; 3 - Violaxanthin (V); 4 - Taraxanthin; 5 - Anteraxanthin (A); 6 - Lutein; 7 - Zeaxanthin (Z); 8 - unknown peak; 9 - Chlorophyll b; 10 - Chlorophyll a; 11 - β-carotene.

The de-epoxidation index of the xanthophyll cycle pigments, anteraxanthin (A), violaxanthin (V) and zeaxanthin (Z) (AZ/VAZ) (Fig. 18) and the ratio between xanthophyll pigments and total chlorophyll [(VAZ)/ChlT] (Fig. 19) in *Cymodocea nodosa* leaves were also determined.

The AZ/VAZ ratio was significantly higher at noon than at pre-dawn independently of the light treatment. Within the same light treatment there were no significant differences between the CO_2 partial pressures tested, both at pre-dawn and noon.

Regarding the distinct light intensities, there was a significantly higher increase of AZ/VAZ (ca. 59%) from PD to noon in HL leaves, when compared to those acclimated to low light intensities. Both at pre-dawn and noon the AZ/VAZ ratio was significantly higher in HL plants. Blue light didn't affect significantly this ratio.

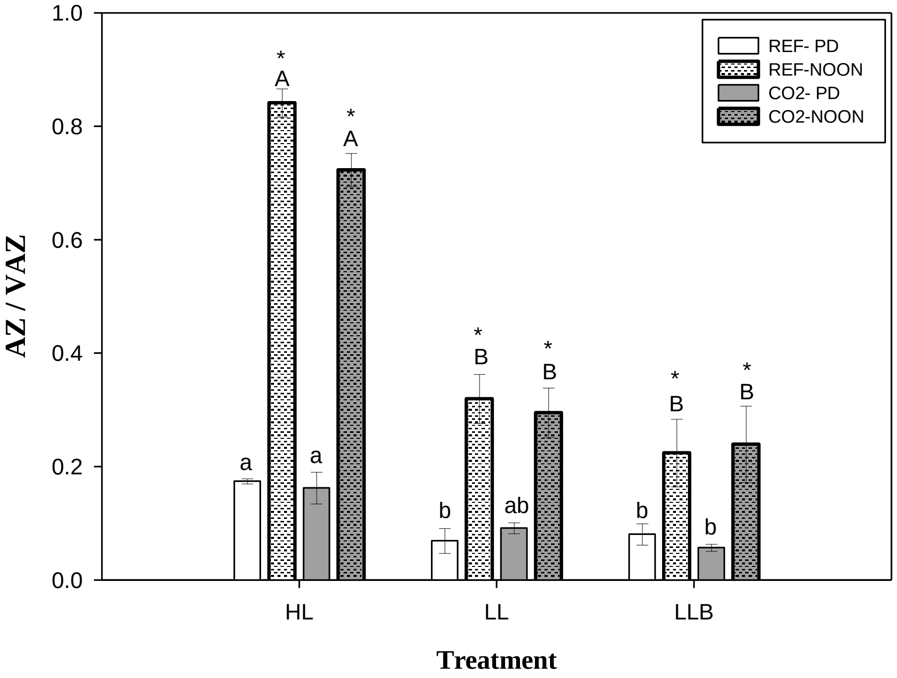

Figure 18: De-epoxidation index of the xanthophyll cycle pigments anteraxanthin (A), violaxanthin (V) and zeaxanthin (Z) **(**AZ/VAZ) in *Cymodocea nodosa* leaves after the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current (REF) pCO_2; Lowercase and uppercase letters indicate significant differences between light levels within pre-dawn (PD) and midday (Noon), respectively (for CO_2 and REF treatments, independently); * indicates differences between PD and Noon for the same light treatment (for REF and CO_2, independently). Values are mean ± SE, n=4 (except for T1 Pre-dawn (REF-LL) to which n=3).

In general, there was a significant decrease in VAZ/Chl T ratio in plants acclimated to low light intensities when compared to high light acclimated plants, no matter the CO_2 partial pressures tested (Fig. 19). In HL acclimated plants VAZ/Chl T ratio was significantly lower in enriched-CO_2 treatments either in pre-dawn or noon (35% and 33%, respectively).

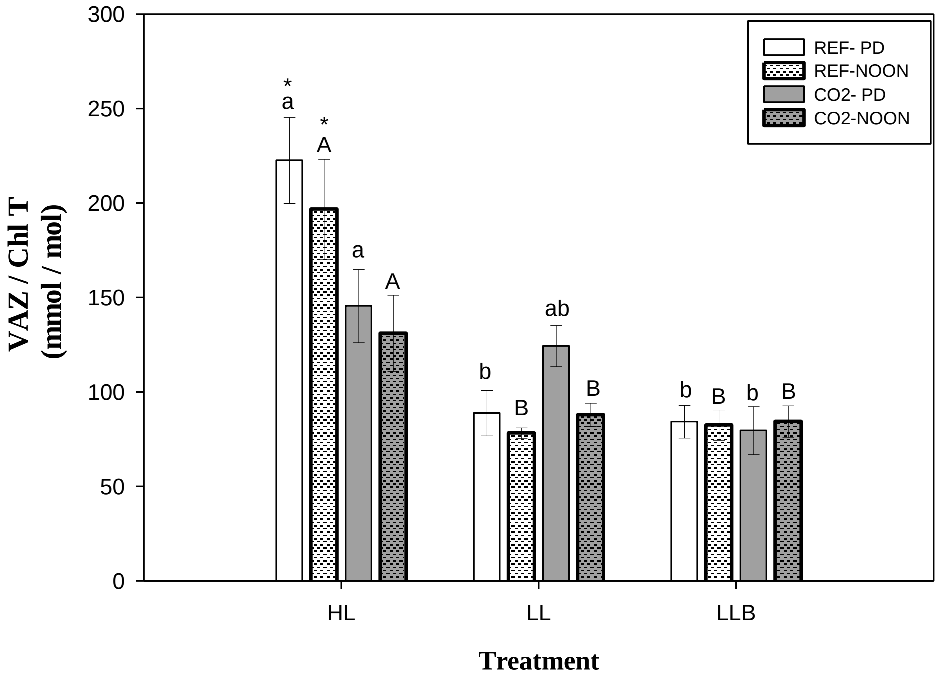

Figure 19: Xanthophyll cycle pigments (V – violaxanthin, A – anteraxanthin and Z – zeaxanthin) to total chlorophyll (Chl T) ratio [(VAZ)/ChlT] in *Cymodocea nodosa* leaves after the experimental period (T1) under high light (HL), low light (LL) and low blue light (LLB) and high (CO_2) or current (REF) pCO_2; Lowercase and uppercase letters indicate significant differences between light levels within pre-dawn (PD) and midday (Noon), respectively (for CO_2 and REF treatments, independently); * indicates differences between CO_2 and REF for the same light treatment (for PD and Noon, independently). Values are mean ± SE, n=4 (except for T1 Pre-dawn (REF-LL) to which n=3).

4. Discussion

Most experiments on seagrass acclimation to high pCO_2 resulted on the increment of photosynthetic and growth rates and place seagrasses as "winners" in the future scenario of high ambient CO_2 concentrations (Palacios and Zimmerman, 2007; Fabricius *et al* 2011; Russell *et al.*, 2013) which might become a critical environment to many species, mainly for calcifying organisms. Apart the few similarities between this study and the one conducted by Schwartz *et al.* (2000), this is the first experiment in mesocosm combining the effects of light intensity and quality and different pCO_2 on seagrass physiological responses. The results of this study evidence that distinct light conditions are likely to induce greater differences in *C. nodosa* plants responses than the increase in pCO_2.

Considering previous experiments on the effects of ocean acidification (Koch *et al.*, 2013; Ow *et al.*, 2015), increased photosynthetic rates were expected. In this study, the greater CO_2 availability resulted in higher Pmax values than those obtained at current atmospheric pCO_2, but only in those plants under low light irradiance (both LL and LLB). These results are in accordance with the hypothesis of Jiang *et al.* (2010), supported by Ow *et al.* (2015), that high pCO_2 conditions could potentially increase the tolerance of seagrasses to low light conditions. However, it is necessary to take into account that bellow the saturation irradiance, is the alpha (α) that informs about the plant photosynthetic efficiency and, in this experiment, the efficiency of photosynthesis in *C. nodosa* leaves under LL and LLB conditions was similar for both reference and enriched-CO_2 treatments.

In HL acclimated leaves, there were no significant differences in Pmax between the different pCO_2 treatments. This may be explained by the significant higher rate of dark respiration of CO_2-HL acclimated leaves, as the metabolic balance changes because a large fraction of the carbon fixed by photosynthesis was remineralized and released as CO_2, the main product of mitochondrial respiration (Martin *et al.*, 2006). Regarding distinct light patterns, Silva *et al.* (2013) found a significant reduction of Pmax in shaded *C. nodosa* plants. In our experiment, the same occurred to LL plants at current atmospheric pCO_2 levels, while Pmax in LLB treatments, not only was higher than in LL as it was similar to that found in HL. This outcome may suggest that in environments where light is less available, the quality of light that reaches seagrass leaves may be imperative, once, according to Chartrand *et al.* (2012), the seagrasses photosynthetic process is sensitive to the spectral quality of light.

Indeed, shading caused variations not only on Pmax but also on α and Ik. In reference treatments, such shading conditions allowed a significant increase in photosynthetic efficiency (α), compared to high light acclimated plants. The same result was obtained by Ruiz and Romero (2001) for *Posidonia oceanica*. Silva *et al.* (2013) obtained a similar result for *C. nodosa* and the opposite one for *Z. marina*, thus emphasizing the fact that the plant response to changes in environmental parameters can be strongly species specific. However, there were no significant differences in photosynthetic efficiency between distinct light treatments in plants exposed to elevated pCO_2. Regarding the effect of different pCO_2, the photosynthetic quantum efficiency of HL acclimated leaves was significantly higher when there was no CO_2 limitation. This is in agreement with the results obtained by Ow *et al.* (2015) who found an increase in photosynthetic quantum efficiency in high pCO_2 levels across all species tested.

The saturation irradiance (Ik) was negatively affected by shading in REF treatments, with values at least 2 times greater in HL than in LL and LLB as it was previously found by Silva *et al.* (2013) for *C. nodosa*. Interestingly, high CO_2 concentrations induced the opposite effect, as the light level required to reach maximum photosynthetic rates was found to be significantly lower for HL than for LL and LLB. This result points to a future situation where *C. nodosa* might be limited by light as, according to current projections, the increment on pCO_2 is likely to be accompanied by the increase on seawater turbidity (Orth *et al.*, 2006a). In the present study, Ik was significantly lower in plants under high pCO_2 and high light, when compared to unenriched treatments, contrary to the results obtained for *Z. noltii* (Alexandre *et al.*, 2012), *Z. marina* (Zimmerman *et al.*, 1997), in which Ik was 3-fold higher under CO_2 enrichment, and *C. nodosa* (Apostolaki *et al.*, 2014), in which a low pH also induced higher Ik. However, a careful interpretation must be done since the above-mentioned experiments not only were performed under different environmental conditions but also, in some cases, in different species. Zimmerman *et al.* (1997) and Alexandre *et al.* (2012) exposed *Z. marina* and *Z. noltii* under high CO_2 conditions during 45 days and 5 months, respectively. In turn, Apostolaki *et al.* (2014) carried out its experiment in a submarine volcanic vent that ejects mainly CO_2, reducing pH to 5.5 close to the main emission point. On the other hand, Ow *et al.* (2015) found no significant differences in Ik values at high CO_2 conditions.

Despite the assumed similarities between P-I curves and RLCs, the parameters obtained from both did not always show the same results. In general, the effect of light was greater than the effect of CO_2 levels on the relative rate of electron transport through photosystem II. rETRmax significantly decreased with shading both in reference and high-CO_2 treatments. Based on literature reviewed by Dattolo *et al.* (2014), the real capacity to move electrons along the electronic chain is likely to be lower in low-light adapted plants. Similarly to Pmax values, in LL and LLB treatments, a greater availability of CO_2 also significantly increased rETRmax. However, while rETRmax decreased in both REF and CO_2-LL and LLB plants, Pmax unexpectedly increased in CO_2-LL and LLB plants suggesting the loss of electrons out of the electron transport chain of these plants.

As expected, the decrease in rETRmax was accompanied by the increase of light use coefficient (α) at low light intensities treatments (not significant for CO_2 – LL). The significantly higher light utilization coefficient in LLB compared to LL acclimated leaves, under high pCO_2 conditions, suggests again that the quality of light can, under certain circumstances, have an active role in the performance of the plant. Nonetheless, both low light treatments (LL and LLB) were not significantly affected by different pCO_2. The same applies for saturation irradiance values (Ik) in low light treatments, unaffected by high CO_2 values. On the other hand, in treatments with no light limitation, α and Ik, were, as in P-I curves, significantly higher and lower, respectively, in enriched-CO_2 conditions. Jiang *et al.* (2010), found the opposite Ik pattern, with higher light level required to reach maximum photosynthetic rates under large CO_2 enrichment treatment.

A reduced rETRmax and Ik and high α values at low irradiances are typical photoacclimation indicators of low-light acclimated seagrasses (Collier *et al.*, 2009). Such outcomes have also been observed by Campbell *et al.* (2003), in response to light intensity gradients. In HL treatments, the maximum rETR was only achieved when the light intensity was on average more than 150 $\mu mol_{photons}.m^{-2}.s^{-1}$, unlike what happened in low light treatments, where the maximum rETR was reached at lower light intensities (from 74.4 $\mu mol_{photons}.m^{-2}.s^{-1}$), resulting in the high light utilization efficiency (α) observed. According to Silva *et al.* (2013), light harvesting systems have the ability to regulate its operational efficiency to the light environment, i.e., they are highly efficient in environments where light is limiting and less efficient when there is too much light. Maintaining this balance comes from their ability to manage carbon supply and respiratory demands (Collier *et al.*, 2012), resulting in lower minimum light requirement for photosynthesis (Olesen *et al.*, 2002).

RLCs cannot be considered exactly the same as typical P-I curves. RLCs must be interpreted differently since they reflect the short-term light history and the actual state of photosynthesis (Uthicke, 2006). Furthermore, based on the literature, Ralph *et al.* (2007) explained that gradients of light within the seagrass canopy may be so different that the tip and base of a unique leaf blade could be exposed to a light environment that oscillate by as much as three orders of magnitude. These differences can be detected in the electron transport rate but do not have an effect on photosynthetic rate.

This pattern of reduction in rETRmax and Ik and increase in α values in low-light adapted plants may be accomplished by an increase in chlorophyll concentrations in marine angiosperms (Ralph *et al.* 2007), which is in agreement with the results obtained by Collier *et al.* (2012), although in this experiment, regarding only T1 sampling period, total chlorophyll content and chl a/b ratio were not significantly affected either by light or CO_2. Similar to these results were the ones obtained by Ow *et al.* (2015) that did not find differences in chlorophyll content at higher pCO_2. Also Jiang *et al.* (2010) results showed a chlorophyll concentration decrease with decreasing pH, while chlorophyll a/b ratio was not affected.

The significant differences in chl a, chl b and total chlorophyll concentrations, between Cádiz, T0 and T1 sampling periods, may be explained by physiological adaptation of plants to the distinct conditions imposed from Cádiz to the acclimation period and then to the experimental period. It should not be associated with a loss of photosynthetic performance, since there was no significant change in chlorophyll a/b ratio between sampling periods. In addition, Fv/Fm values during the experimental time show that the plants maintained a satisfactory physiological state. Also for the same seagrass species, *C. nodosa*, Costa (2014) found a considerable variation in total chlorophyll content in different locations. In Ria Formosa, Portugal, the recorded value was 56.29 ± 7.07 µmol $Chl.m^{-2}$ (July), while in Vulcano island, Italy, the recorded value was 157.9 ± 23.6 and 95.51 ± 25.11 µmol $Chl.m^{-2}$, for pre-dawn and midday (May), respectively. Thus, total chlorophyll content may vary depending on a set of features imposed by plant location.

According to Ralph *et al.* (2002) and Collier *et al.* (2008), pigments other than the chlorophylls can respond to changing light intensity, including the photoprotective pigments. VAZ/Chl T and AZ/VAZ ratios are widely accepted measures of plant stress (Fyfe, 2004). In general, *C. nodosa* exhibited a lower VAZ/Chl T ratio in LL and LLB treatments, compared to HL treatments. AZ/VAZ values also decreased with decreasing light intensity which is in agreement with Fyfe (2004), who observed a decline in protective pigments in response to decreasing daily irradiances.

The xanthophylls (violaxanthin, antheraxanthin and zeaxanthin) form the only portion of the carotenoids which are capable of inducing rapid and reversible changes in its concentrations in response to variations in light intensities (Demmig-Adams and Adams, 1994). Thayer and Bjorkman (1990), claimed that in terrestrial plants an increase up to 400% can be observed in the VAZ pool relative to the pool of total chlorophylls in plants exposed to high irradiances. This agrees with the significant higher VAZ pool found in *C. nodosa* leaves from HL treatments, as well as with low α values of both light response curves (P-I and RLCs), since seagrasses are generally considered shaded plants (Larkum *et al.*, 2006).

Ralph *et al.* (2002) also stated that the increase in VAZ pool could be a relatively fast process. His results showed an increment in the VAZ content with increasing light intensity on the course of a single day, in *Z. marina*. This VAZ pool observed by Ralph *et al.* (2002) was 120-150 mmol VAZ per mol Chl T, which is in line not only with the values normally found in terrestrial sun-acclimated plants (100 to 200 mmol VAZ/ mol Chl T; *in*: Demmig-Adams *et al.*, 1999), but also with the values obtained in this experiment for plants exposed to HL treatments. In both LL and LLB treatments, no differences regarding the CO_2 treatment were observed in VAZ/Chl T, contrary to what occurred in HL plants, where the increase in CO_2 availability did significantly decreased this ratio, suggesting a higher capacity to use light, advantageous in environments where the excess of irradiance can lead to photoinhibition.

In periods where exposure to light is very low or even zero (as in predawn), most of the xanthophyll pool is converted into violaxanthin (epoxidation process). On the other hand, in periods of high solar exposure (at noon) the reverse process occurs and violaxanthin is de-epoxidised into antheraxanthin and finally in zeaxanthin (this de-epoxidation process is responsible for dissipating excess excitation energy) (Adams and Demmig-Adams, 1992; Esteban *et al.*, 2007). These processes justify the higher AZ/VAZ ratio at noon in all light treatments.

According to Fernández-Marin *et al.* (2011), regardless of the factor responsible for the V de-epoxidation induction, the resultant A+Z molecules will lead to the down-regulation of the maximal photochemical efficiency of PSII (Fv/Fm), as a result of shunting the excess of captured energy. Decreases in this ratio have been associated with environmental stressors that directly affect PSII effectiveness such as high light intensities (Fyfe, 2004, Ralph *et al.*, 2005). This down-regulation process that establishes a balance between the energy used to maintain the carbon assimilation and the excessive energy that is dissipated before it induces photooxidative damage (in PSII reaction centers) is then called photoprotection, normally inferred from a decline in Fv/Fm (Ralph *et al.*, 2002; Walters, 2005). The VAZ pool size and the degree of de-epoxidation determine the amount of excessive light that needs to be dissipated (Fyfe, 2004). Since the reference Fv/Fm value for unstressed seagrasses is 0.79 (Malta *et al.*, 2006), the lower Fv/Fm (between 0.6-0.75) observed in HL treatment may indicate plant stress and photosystem II down-regulation, which is common when the amount of light energy exceeds the capacity for energy utilization and frequently results in dissipation of excess excitation energy as heat in photosystem II (Gévaert *et al.*, 2003). In contrast, the higher Fv/Fm values (above 0.75) observed in LL and LLB treatments suggests that the probability of photosystem damage is lower.

In a general way, the blue light had less impact on the photosynthetic responses of *C. nodosa* than originally expected. Still, the Pmax values from the blue light treatments, (under actual conditions of pCO_2) and the higher photosynthetic light utilization efficiency in LLB compared to LL acclimated leaves (from RLCs, under high pCO_2 conditions), indicate that the quality of light should be taken into account in future studies. However, and apart from the differences between Mvungi *et al.* (2012) experiment and this one, this authors claimed that changes in light quality had little effects on the seagrass photosynthetic rate. A future research focused only on the quality of light may clarify this issue.

Based on the literature, the type of responses of different seagrass species to enriched-CO_2 conditions appear to be highly species-specific (Campbell and Fourquean, 2013; Apostolaki *et al.*, 2014). Some seagrass species (such as C. *serrulata*) seem to be already saturated at the current concentration of inorganic carbon (Ci) and therefore no differences were verified when the availability of CO_2 was increased (Schwartz *et al.*, 2000). Invers *et al.* (2001), also state that Pacific seagrass species seemed to be more reliant on CO_2 while Mediterranean species (as *C. nodosa*) appear to be more efficient in HCO_3^- utilization. In Beer *et al.* (1980) experiment, *C. nodosa* was classified as a C_4 plant, however, some results obtained in this study were more characteristic of a C_3 plant since the increase in CO_2 promoted the maximum photosynthetic rate in plants adapted to low light intensities (although this increase in Pmax has not been accompanied by an increase in photosynthetic efficiency, α). This reinforces the fact that the studies conducted to classify the seagrass as C_3 or C_4 must be interpreted with caution.

5. Conclusions

In general, changes in light environment seemed to induce greater differences in the physiological response of *C. nodosa* than high CO_2 conditions. However, accordingly to these experiment results, and contrary to what is occasionally assumed, *C. nodosa* photosynthesis does not seem to be already saturated at the current concentration of inorganic carbon. This study also revealed that the quality of light reaching seagrasses may play a key role in the photosynthetic process in low light conditions. The concentration of Chl a and b as well as Chl a/b ratio were not affected by the different conditions of light and/or CO_2. Ratios between xanthophyll pigments and total chlorophyll revealed an increased investment on the xanthophyll pool and the triggering of the xanthophyll cycle as a response to high light intensity. All plant responses to changing environmental patterns should be interpreted carefully, once it may be highly specie specific.

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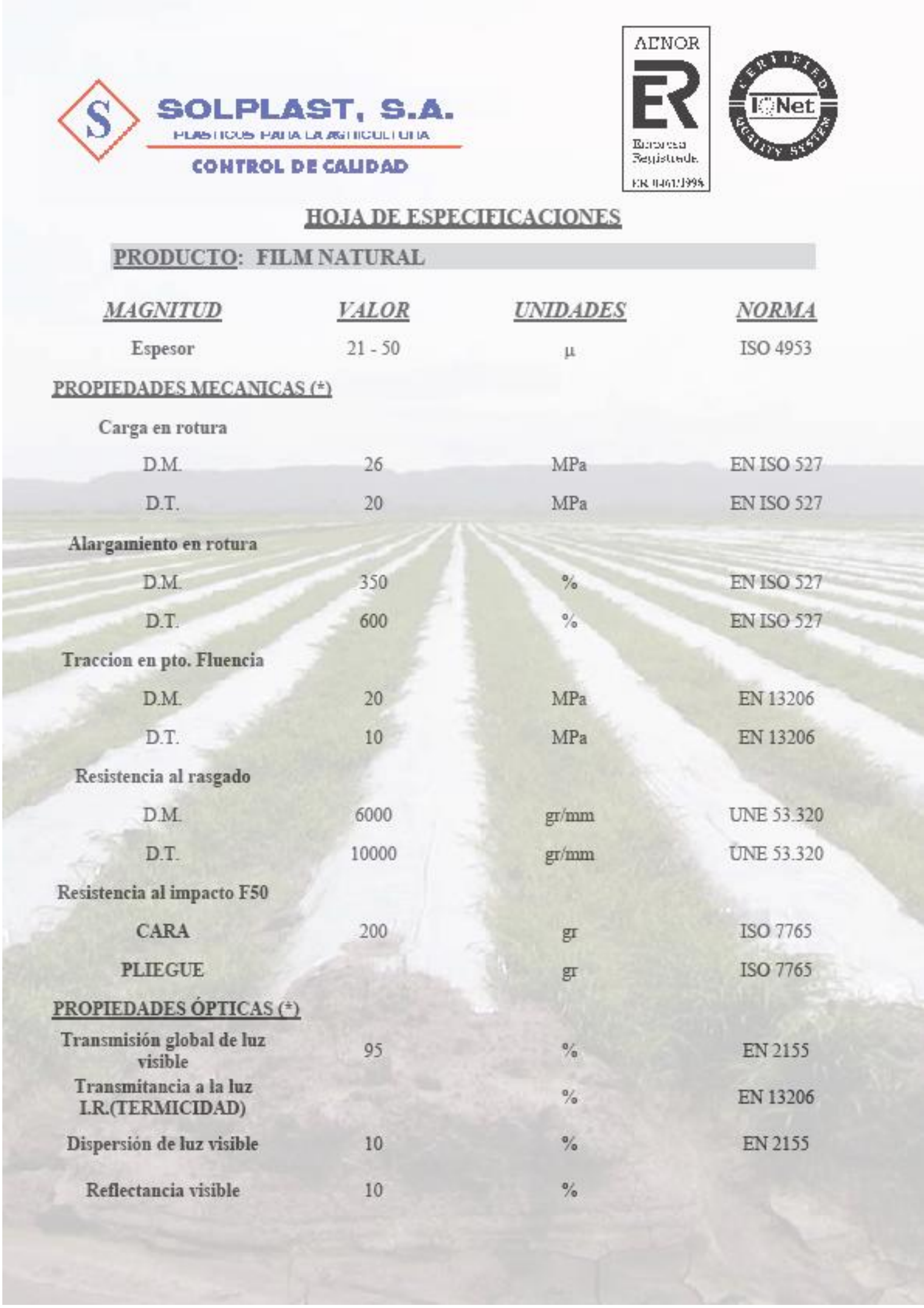
7. Apprendix



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HOJA DE ESPECIFICACIONES

PRODUCTO: FILM NATURAL

MAGNITUD	*VALOR*	*UNIDADES*	*NORMA*
Espesor	21 - 50	µ	ISO 4953
PROPIEDADES MECANICAS (*)			
Carga en rotura			
D.M.	26	MPa	EN ISO 527
D.T.	20	MPa	EN ISO 527
Alargamiento en rotura			
D.M.	350	%	EN ISO 527
D.T.	600	%	EN ISO 527
Traccion en pto. Fluencia			
D.M.	20	MPa	EN 13206
D.T.	10	MPa	EN 13206
Resistencia al rasgado			
D.M.	6000	gr/mm	UNE 53.320
D.T.	10000	gr/mm	UNE 53.320
Resistencia al impacto F50			
CARA	200	gr	ISO 7765
PLIEGUE		gr	ISO 7765
PROPIEDADES ÓPTICAS (*)			
Transmisión global de luz visible	95	%	EN 2155
Transmitancia a la luz I.R.(TERMICIDAD)		%	EN 13206
Dispersión de luz visible	10	%	EN 2155
Reflectancia visible	10	%	

Appendix I: Greenhouse Plastic Specifications, Soldplast, S.A.