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Effects of high CO_2 and light quality on the growth of the seagrass *Cymodocea nodosa* (Ucria) Ascherson

André Tavares Silva, 44831

Dissertation for Master's degree in Marine Biology

Advisors:

Professora Doutora Isabel Barrote (FCT)

Doutora Irene Olivé Samarra (CCMAR)

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Declaração de autoria de trabalho

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

André Tavares Silva

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To the best family in the world…

Abstract

The ocean is facing the acidification of seawater (Ocean Acidification), one of the major threats to marine ecosystems that affect water properties, changing the proportions of the inorganic carbon species in seawater and reducing pH. This process is suggested to benefit carbon dioxide (CO_2)-limited species such as seagrasses, which play important roles in marine and coastal ecosystems worldwide. However due to climate change and coastal degradation, which also affect light availability, these valuable systems are in decline. While it may be expected that ocean acidifcation would result in an increase of biomass and productivity in seagrasses, the few experiments published so far are inconclusive. In addition, most studies on seagrasses do not consider the effect of light quality. The effects of CO_2 (present (REF) vs high (CO_2) concentrations) under distinct light intensity and quality scenarios (high light (HL), low light (LL) and low blue light (LLB)) were assessed on the seagrass *Cymodocea nodosa* through a mesocosm experiment. Morphological aspects and dynamic rates related to growth, such as leaf appearance rate (LAR), leaf elongation rate (LER), shoot appearance rate (SAR) and rhizome elongation rate (RER) were evaluated. Biochemical analyses (C:N ratio, non-structural sugars and soluble protein) were also determined. Under high-CO_2, the abovegroung:beloweground ratio pointed to an increase of investment in belowground tissues. Morphological parameters and dynamic growth rates (SAR and RER) tended to decrease in REF plants with light deprivation but this tendency was attenuated or even disappeared in CO_2 plants. Sucrose concentration in tissues was relatively higher than starch. CO_2 plants displayed significantly higher ($p<0.05$) sucrose foliar content in low light conditions. Light deprivation coupled with high inorganic carbon availability revealed a higher accumulation of starch at rhizomes. In conclusion, light was the main driver in *C. nodosa* response, but high CO_2 somehow buffered the light effect.

Keywords: Climate change; CO_2; light quality and quantity; growth; *Cymodocea nodosa*

Resumo

A atividade antropogénica é responsável pela libertação de grandes quantidades de dióxido de carbono (CO_2) para a atmosfera, potenciando alterações climatéricas globais. A concentração de CO_2 na atmosfera aumentou cerca de 35 – 45% desde a revolução industrial, para os valores atuais de 400 partes por milhão (ppm) e, de acordo com alguns modelos, está previsto um aumento para valores superiores a 1000 ppm até ao final do século. Atualmente, um terço da

quantidade total de CO_2 presente na atmosfera é absorvido pelo oceano e, embora contrabalance o nível de CO_2 atmosférico, resulta, por outro lado, na diminuição do pH e na perda da capacidade tampão da água do mar, levando ao atual problema da acidificação do oceano. Até ao fim do século, as projeções apontam para um decréscimo de 0.2 a 0.5 nas unidades de pH, modificando a química dos carbonatos devido às alterações nas proporçoões de carbono inorgánico (Ci) dissolvido na água do mar. Segundo a literatura, estas alterações deverão ter um grande impacto negativo nos ecosistemas marinhos. Contudo, espécies que se encontram limitadas pelo carbono inorgânico dissolvido na água, como é, possivelmente, o caso de algumas espécies de ervas marinhas, poderão ser beneficiadas com o aumento da concentração de CO_2. As pradarias de ervas marinhas cobrem cerca de 0.1 a 0.2% do oceano e são um dos ecossitemas costeiros mais produtivos no planeta. Contudo, são também um dos ecossitemas mais ameaçados. O facto de as ervas marinhas serem consideradas plantas limitadas para a atual concentração de CO_2, leva a crer que num futuro onde as concentrações de Ci na água do mar forem muito superiores, a fotossíntese, o crescimento, a produtividade e a acumulação de energia sob a forma de carbohidratos nestes organismos, seja superior também. Contudo o crescimento e a sobrevivência das ervas marinhas depende também da quantidade de luz disponível para o processo fotossintético e para o crescimento. Mais recentemente, o papel da qualidade da radiação fotossinteticamente ativa (PAR) que atinge a superfície das plantas foi também considerado um fator importante para estes organismos. Portanto, a resposta das ervas marinhas é dependente das condições de luz que podem ser também afetadas pelas alterações globais. Assim, esta tese teve como principal objetivo a avaliação dos efeitos combinados do aumento de carbono inorgânico e diferentes condições luminosas (quantidade e qualidade) em *Cymodocea nodosa*, através de uma experiência de curta duração, num ambiente de mesocosmo controlado. Para tal, o crescimento foi avaliado através de aspetos morfológicos (comprimento e peso das folhas e rizomas) e dinâmicos (taxas de alongamento das folhas e dos rizomas, e taxas de aparecimento de novos *shoots* e folhas). Os aspectos fisiológicos das folhas e rizomas foram também avaliados através da determinação da proporção carbono:azoto (C:N), alocação de açúcares não estruturais e concentração de proteina solúvel. Para tal recolheram-se amostras de *C. nodosa* (rizomas com o *shoot* terminal) numa pradaria marinha na baía de Cádiz (Espanha) que foram posteriormente transportadas para a Estação Experimental do Ramalhete (CCMar, Faro, Portugal). As plantas foram distribuídas aleatoriamente por 24 tanques montados num circuito aberto e sujeitas a diferentes concentrações de CO_2 e quantidades/qualidades de luz. Após um período de aclimatação com a duração de 8 dias, deu-se início ao período experimental, com uma duração de duas semanas. Os parâmetros fisico-

quimicos foram medidos diariamente e a cada 3 dias foram retiradas amostras de água para a caraterização do sistema. Os aspetos morfológicos de *C. nodosa* foram determinados através da medição e do peso do tecido novo (folhas, bainha, rizomas e raiz) formado durante o período em que decorreu a experiência. As taxas dinâmicas foram determinadas com base nos parâmetros obtidos relativamente às marcas colocadas no início da experiência. O conteúdo de carbono e azoto foi determinado através de um analisador elementar e a concentração de açúcares solúveis (sacarose), amido e proteínas foi obtida por espectrofotometria, para rizomas e folhas. As plantas que cresceram em condições de condições de luz mais ambundante tenderam a evidenciar um maior peso, sendo as plantas em condições actuais de CO_2 negativamente afetadas pela redução na luz disponível. As plantas que cresceram em condições de elevado CO_2 tenderam a investir em biomassa de reserva e expansão, contudo a luz azul parece ter reduzido tal tendência. De uma maneira geral, os parâmetros morfológicos peso e comprimento de *shoots* e rizomas, foi afetado negativamente pela redução de luz. Contudo, nos tratamentos com elevada concentração de CO_2 esse efeito parece ter sido atenuado. A redução da luz tendeu a reduzir (taxas de aparecimento de novas folhas, LAR e alongamento foliar, LER) ou induziu a diminuição significativa das taxas dinâmicas (taxas de aparecimento de novos *shoots*, SAR e de elongamento do rizoma, RER) nos tratamentos onde as plantas de se encontravam em condições normais de CO_2, sendo que o mesmo não se verificou para os tratamentos com elevado CO_2. As análises bioquímicas revelaram uma manutenção da proporção C:N em todos os tratamentos. A sacarose foi encontrada em maior quantidade do que o amido, principalmente nos rizomas. A diminuição de luz pareceu também ter um efeito negativo na quantidade de sacarose e amido presente nas folhas. No caso do amido, a interação entre o efeito de menor luz disponível e maior disponibilidade de Ci levou ao aumento da concentração deste hidrato de carbono de reserva. A concentração de proteina solúvel revelou padrões inversos. As plantas de *C. nodosa*, quando não limitadas pela luz ou Ci pareceram investir nos orgãos de reserva e expansão. No entanto, quando a luz foi reduzida, as plantas pareceram investir na manutenção do tecido fotossintético. A interpretação dos resultados de proteina requer um maior conhecimento da proteómica para obter um maior detalhe do que realmente está presente nos tecidos e evitar interpretações incorretas. A resposta da *Cymodocea nodosa* foi afetada pelos dois fatores, luz e CO_2, sendo a luz o factor mais condicionante. A elevada concentração de CO_2 e a qualidade da luz tiveram um menor impacto nas respostas observadas, sendo que em condições de elevado CO_2 os efeitos da restrição da luz são atenuados. Os efeitos da restrição de luz parecem ter sido reforçado pelos tratamentos de luz azul.

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Abbreviation

ANOVA	-	Analysis of variance
ATP	-	Adenosine triphosphate
BSA	-	Bovine serum albumin
C	-	Carbon
Ca^{2+}	-	Calcium ion
$CaCO_3$	-	Calcium carbonate
CH_4	-	Methane
Ci	-	Inorganic carbon
CO_2	-	Carbon dioxide
CO_3^{2-}	-	Carbonate ion
DIC	-	Dissolved inorganic carbon
DTT	-	Dithiothreitol
DW	-	Dry weight
EDTA	-	Ethylenediaminetetraacetic acid
Es	-	Surface irradiance
Frutose-6P	-	Frutose-6-phosphate
GDH	-	Glucose-6P-dehydrogenase
Gluconate-6P	-	Gluconate-6-phosphate
Glucose-6P	-	Glucose-6P-phosphate
H^+	-	Hydrogen ion
H_2O	-	Water
HCO_3^-	-	Bicarbonate ion
HK	-	Hexokinase
INV	-	Invertase
LAR	-	Leaf appearance rate
LER	-	Leaf elongation rate
N	-	Nitrogen
N_2O	-	Nitrous oxide
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	-	Sodium hydroxide
NSC	-	Non-structural carbohydrate
P	-	Phosphorous
PAR	-	Photosynthetically active radiation
pCO_2	-	Partial pressure of carbon dioxide
PGI	-	Phosphoglucoisomerase
PMSF	-	Phenylmethylsulfonyl fluoride
PVPP	-	Polyvinyl-polypyrrolidone
RER	-	rhizome elongation rate
RuBisCO	-	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SAR	-	Shoot appearance rate
TA	-	Total alkalinity

1. Introduction

Anthropogenic activities such as fossil fuel burning, deforestation and industrial/agricultural gas emissions are responsible for the release of great quantities of heat-trap greenhouse gases, such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), altering the atmospheric composition and enhancing global climate changes (Seinfeld and Pandis, 2006; Doney et al., 2012). CO_2 is the main greenhouse gas that results from human activities, and its increase is arguably the most important factor contributing to climate change (National Research Council, 2011; Doney et al., 2014). The atmospheric concentration of CO_2 has increased by 35-45% since the Industrial Revolution, recently reaching 398.82 ppmv (August of 2015, in Manoa Loa, Hawai) (Sabine et al., 2004; Doney et al., 2009; Gattuso and Hansson, 2011; National Research Council, 2011; NOAA-CCGG, 2015) and this increase is expected to continue to values of (~) 420– 940 ppm, or even higher, until the end of the 21st century if mitigation measures are not taken (Plattner et al., 2008; Pörtner et al., 2014).

1.1 Ocean Acidification

The oceans occupy almost three-quarters of the planet's surface, which has a vast impact on life (Pörtner et al., 2014). They control temperature changes (Calvo et al., 2011; Hoegh-Guldberg et al., 2014) and regulate the atmospheric gases content by decreasing the CO_2 concentration in atmosphere (National Research Council, 2011). Approximately 30% of the anthropogenic CO_2 present in the atmosphere is absorbed by the oceans, increasing the partial pressure of CO_2 (pCO_2) in seawater and leading to ocean acidification (Sabine et al., 2004; Le Quéré et al., 2009; Ocean Studies Board, 2010; National Research Council, 2011).

The uptake of elevated amounts of CO_2 by the oceans causes unprecedented changes in the seawater chemistry, decreasing its pH and altering fundamental chemical balances, which disrupts the buffering capacity of seawater (Doney et al., 2009; Ocean Studies Board, 2010; Calvo et al., 2011; Gattuso and Hansson, 2011; Kaiser et al., 2011). Currently, at seawater pH of 8.1 (Gattuso and Hansson, 2011), bicarbonate (HCO_3^-) represents 90% of total dissolved organic carbon (DIC), carbonate (CO_3^{2-}) is (~) 9% and CO_2 (~) 1% (Doney et al., 2009). These ions constitute the principal components of DIC balance, which is pH dependent (Gattuso and Hansson, 2011). The projections for the end of the 21st century point to a pH decrease of 0.2 to 0.5 units, and thus to changes in the DIC balance, according to the different models used (Cao and Caldeira, 2008; Plattner et al., 2008; Pörtner et al., 2014).

Hence, the changes in seawater chemistry are large enough to affect other chemical, biological and exchange processes, such as ocean biogeochemical dynamics, leading to great impacts on the marine ecosystems (Doney et al., 2009; Ocean Studies Board, 2010; Calvo et al., 2011). Calcifying organisms, such as corals, mussels, coccolithophores and pteropods, that biomineralize CO_3^{2-} and calcium (Ca^{2+}) ions to form calcium carbonate ($CaCO_3$) skeletal or shell structures will be negatively affected (Guinotte and Fabry, 2008; Calvo et al., 2011; Kaiser et al., 2011). However, little is known about the effects of ocean acidification on carbon-limited primary producers such as seagrasses, where photosynthesis may be promoted by the increment on CO_2 concentration (Doney et al., 2012).

1.2 Seagrasses

Seagrasses are monocot plants, mostly dioecious species, capable of both clonal and sexual reproduction, with underwater pollination and dispersal (Hemminga and Duarte, 2000; Kaiser et al., 2011). Seagrasses are constituted by a consecutive repetition of a unit (ramet; Harper, 1977) created during the clonal growth, composed of below and aboveground parts, with morphological differences from species to species (Hemminga and Duarte, 2000; Kuo and den Hartog, 2006; Kaiser et al., 2011). The belowground part is constituted by the rhizome (responsible for the horizontal growth and the linkage between adjacent ramets), and the roots that grow vertically into the sediment. The aboveground part is composed by the strap-like or laminate leafs (or even rounded in the genus *Halophila*), which emerge from the rhizome and grow from the leaf sheath. Depending on the time of the year, flowers and fruits can also be observed (Hemminga and Duarte, 2000; Kuo and den Hartog, 2006; Kaiser et al., 2011).

Seagrasses cover approximately 0.1 to 0.2% of the global ocean (Duarte, 2002). These plants are fully adapted to its submerged existence and can be found worldwide, except along the Antarctic coast (Hemminga and Duarte, 2000; Coles et al., 2011; Kaiser et al., 2011). Seagrass meadows are considered one of the most productive ecosystem on Earth (Orth et al., 2006; Guinotte and Fabry, 2008; Costanza et al., 2014), being ecologically and economically important as they provide a great variety of ecosystem services (Hemminga and Duarte, 2000; Duarte, 2002; Marbà et al., 2006; Waycott et al., 2009; Cullen-Unsworth et al., 2014; Kachelriess et al., 2014; Pergent et al., 2014; Unsworth et al., 2014). However, these meadows are amongst the most threatened ecosystems on earth (Orth et al., 2006; Waycott et al., 2009), experiencing a global decline of 7% each year (Waycott et al., 2009).

1.3 Ocean Acidification impacts on seagrass meadows

Seagrasses are considered CO_2-limited plants (Hellblom et al., 2001; Invers et al., 2001; Palacios and Zimmerman, 2007) due to the relatively low concentration of CO_2 in seawater and the inefficient use of HCO_3^- as inorganic carbon source (Invers et al., 2001; Doney et al., 2009). Therefore, the predicted increase of CO_2 (up to 250%; Koch et al., 2013) and consequently higher inorganic carbon (Ci) availability is expected to benefit seagrass photosynthesis, productivity, biomass, leaf growth rate and accumulation of carbohydrates (Zimmerman et al., 1997; Palacios and Zimmerman, 2007; Jiang et al., 2010; Doney et al., 2012; Russell et al., 2013). Although in a few studies the CO_2 increase appeared to have negative or no effect on seagrass growth, the authors explain it with the possible limitation by other factors such inorganic substrates or the different inter-species responses to ocean acidification (Alexandre et al., 2012; Apostolaki et al., 2014). According to Liebig's law of the minimums, the primary producers are only limited by the one factor that is less available for use. In the case of seagrass meadows, light and nutrients are these most common factors (Touchette and Burkholder, 2000; Fourqurean et al., 2005; Johnson et al., 2006; Romero et al., 2006).

Nitrogen (N) and phosphorous (P) are nutrients generally considered growth limiting (Invers et al., 2001). N and P are found in a specific balance with C in the proportion of 550:30:1 (C:N:P) in plant tissues (Atkinson and Smith, 1983). As verified in terrestrial plants (Goufo et al., 2014), a higher CO_2 concentration is expected to have a positive increase in the seagrasses C:N:P ratio (Jiang et al., 2010; Arnold et al., 2012), which Jiang et al. (2010) explained with the dilution processes due to the active CO_2 uptake by seagrasses.

The synthesis of organic molecules, such as carbohydrates and proteins, requires nutrients (Creighton, 1993; McKee and McKee, 2003). Carbohydrates, organic material produced by photosynthesis (McKee and McKee, 2003; Gattuso and Hansson, 2011), are the most abundant biomolecules in nature and important for numerous metabolic pathways, as cells energy sources and acting as structural building blocks (McKee and McKee, 2003). During photosynthesis, carbon dioxide is reduced to triose phosphates that are further used to produce other carbohydrates such as sugars (soluble carbohydrates), mainly sucrose (up to 90%), fructose and glucose, in proportions that vary within species (Kraemer and Alberte, 1995; Larkum et al., 2006). Sucrose moves throughout the entire plant, transporting the energy to fulfil the demands of the plant, and is found in higher concentration in the tissues during fast growth periods (McKee and McKee, 2003; Burkholder et al., 2007). When produced in greater quantities than those required, carbohydrates are stored as starch (non-soluble carbohydrate used as energy

reservoir of plant cells), mainly in rhizomes, both to minimize carbon loss from herbivory and to be used in environmental strain situations (Touchette and Burkholder, 2000).

Proteins primary structure is made of amino acids (major form of nitrogen storage and essential constituents for all organisms; McKee & McKee, 2003; Romero et al., 2006) whose synthesis mainly requires carbon skeletons provided by carbohydrates, and nitrogen. Proteins are involved in diverse functions, such as serving as structural materials in membranes, metabolic regulation, transport, defence and catalysis (McKee and McKee, 2003). As cited above, the expected CO_2 increase will lead to a greater carbohydrate availability inside tissues and thus, under a non-limiting nutrient concentration, mainly N, it might be expected an increase in the protein synthesis to sustain the predictable higher growth rate.

Although evidence suggests that in a near future seagrasses may benefit from the increase of CO_2 in seawater, it is necessary to consider its interaction with other factors influencing the seagrasses growth and productivity, such as light availability (Kaiser et al., 2011; Chartrand et al., 2012; Pörtner et al., 2014).

1.4 Light quality and intensity

Seagrasses have high minimum light requirements, up to 37% of surface irradiance, Es, for growth and survival (Lee et al., 2007; Ralph et al., 2007). These characteristic high light requirements are not fully understood, and might be linked to the low photosynthetic efficiency due to the inefficient use of HCO_3^- (Zimmerman et al., 1997) and/or to maintain the respiratory demands of the belowground biomass (Orth et al., 2006).

Seagrasses photosynthetic activity is dependent on the red and blue wavelengths of the photosynthetic active radiation (PAR), as the photosynthetic absorption is inefficient at green and yellow wavelengths (Chartrand et al., 2012). Thus, growth and depth distribution of the seagrasses (between the shallow estuaries and up to 70m in clear water; Connolly, 2012), are limited by the quality and intensity of PAR reaching the cells. Light intensity is attenuated along the water column (Fig. 1) and by the growth of epiphytes on the leaves. The level of light attenuation and the optical quality of the light reaching the plants depend on factors such as depth and suspended sediments, dissolved organic matter and phytoplankton, whose presence and characteristics rely on natural and anthropogenic processes such as run-offs, dredging activities and altered river flows (Ralph et al., 2007; Coles et al., 2011).

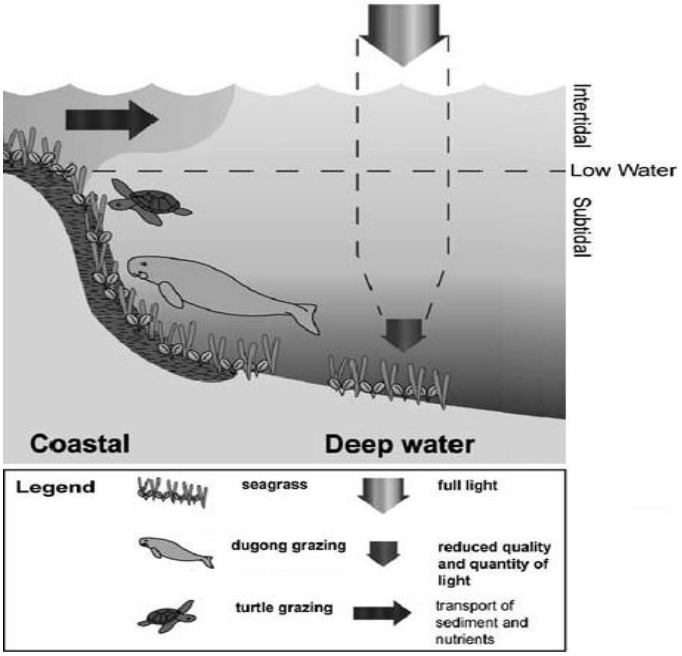


Figure 1. Scheme of light attenuation in ocean. At coastal and shallow waters there is a great impact of anthropogenic processes and at deep waters, the water column may play a greater impact on the attenuation of light. *Adapted from*: Waycott et al., 2005.

Therefore, global climate effects can potentially modify light conditions and thus the quality and intensity of PAR reaching seagrasses. On one hand, extreme events (frequency and intensity of storms) and rising sea-level lead to a loss of water quality due to effects as large land runoffs, mainly in coastal zones (Orth et al., 2006; Bellard et al., 2012; Garrido et al., 2013; Saunders et al., 2013). On the other hand the environmental changes in CO_2 concentration, temperature and other extreme events as dryer periods may lead to a stratified water column and thus to an increase in light availability for seagrasses (Raven et al., 2011).

Seagrasses are predominantly found down to 10 meters in soft bottoms of coastal waters worldwide, growing in the intertidal and subtidal of the shallow marine and estuarine environments, protected from strong water movement (Hemminga and Duarte, 2000; Spalding et al., 2003; Waycott et al., 2009; Coles et al., 2011; Kaiser et al., 2011). The addition of the global climate changes to the threats that seagrasses are already facing might lead to an increase in the events (eutrophication and sea level rising) that decrease the amount of light reaching the bottom (McPherson et al., 2011; Shiu et al., 2012; Doney et al., 2014). The first response of seagrasses to changes in light intensity and quality is at metabolic and physiological level and precede morphological changes (Collier et al., 2009, 2012). Thus, a predicted reduction in water quality will affect the photosynthetic activity, disturbing the important metabolic processes for the plant growth and survival. During low-light periods, the production of carbohydrates

decreases, and the sucrose transport to the tissues is reduced, leading to the use of starch reserves for maintenance processes (Burke et al., 1996; Olesen et al., 2002).

Nevertheless, the response of different seagrass species to light changes and CO_2 increase is dependent on the intrinsic processes and morphology that might vary between species (Ralph et al., 2007; Silva et al., 2013; Apostolaki et al., 2014). Furthermore other factors such as the increase of CO_2, may lower seagrasses light requirements, which turns the relationship with climate changes even more complex (Zimmerman et al., 1997; Jiang et al., 2010).

1.5 Main objectives

The thesis main aims were to evaluate the combined effects of high Ci and light deprivation (quality and quantity) on seagrasses growth, through a short-term mesocosm experiment, using *Cymodocea nodosa* as model species. In order to understand the response of *C. nodosa*, growth was evaluated by morphological-biometric aspects (AG:BG ratio, total shoot and rhizome biomass) and dynamic rates (leaf elongation rate, new shoots and leaves appearance rate, rhizome elongation rate). Biochemical aspects were also evaluated in leafs and rhizomes through the determination of the C:N ratio, allocation of non-structural sugars and concentration of soluble protein.

2. Material and Methods

2.1 Model species

Seagrasses are a polyphyletic group of monocotyledons, belonging to four families that contain about 50 - 60 species: Zosteraceae, Cymodoceaceae, Posidoniaceae that only comprehend truly marine plants and Hydrocharitaceae (Hemminga and Duarte, 2000; Spalding et al., 2003; Janssen and Bremer, 2004; den Hartog and Kuo, 2006; Orth et al., 2006; Kaiser et al., 2011). Although the taxonomy and the origin of seagrasses are not fully resolved, according to their characteristics it is thought that their ancestor is an ancient coastal plant (e.g. salt marshes, mangroves) or a freshwater hydrophyte (Hemminga and Duarte, 2000).

The species *Cymodocea nodosa* (Ucria) Ascherson is a tropical warm water, dioecious seagrass species that belongs to the Cymodoceaceae family (Hemminga and Duarte, 2000). It is distributed throughout the Mediterranean basin and in the North Atlantic Ocean, extending

its range from central Portugal to Cap d'Arguin in Senegal (Alberto et al., 2005; den Hartog and Kuo, 2006). *C. nodosa* can also be found in the Canary Archipelago and Madeira Islands. This species has an extensive morphological plasticity, growing on both sand and mud substrates, and can be found from the upper subtidal limits to depths of more than 30m, forming extensive meadows, either monospecific or mixed stands, in association with other seagrasses such *as Posidonia oceanica* (L.) Delile and *Zostera noltii* Horneman, in shallow and sheltered areas such as lagoons (Cancemi et al., 2002; Alberto et al., 2003; Orfanidis et al., 2007; Garrido et al., 2013).

Cymodocea nodosa exhibits both sexual reproduction and a fast clonal propagation through rhizome elongation (up to $2m \cdot year^{-1}$) (Ruggiero et al., 2004; Alberto et al., 2005). It is ecologically considered a coloniser species with high metabolic growth rate and greater morphological and functional plasticity when compared to other species, which confer a higher fitness to environmental variability and a more direct response to variations in light, temperature and nutrient load (Cancemi et al., 2002; Alberto et al., 2005; Garrido et al., 2013; Apostolaki et al., 2014; Sandoval-Gil et al., 2014). All these facts make it an ideal species to study the biological effects of the predicted changes in environmental factors (Garrote-Moreno et al., 2014). Accordingly, *C. nodosa* was the chosen species for this experiment. Specimens were collected in the Cádiz Bay for three motives: (i) The genetic diversity present of *C. nodosa* population in Ria Formosa is low, as it is predominantly constituted by a single large genet (Alberto et al., 2001) and in Cádiz Bay it has great genetic diversity (Alberto et al., 2005); (ii) *C. nodosa* can easily be transported and used in studies in facilities near Ria Formosa, as is autochthonous seagrass of Ria Formosa (Alberto et al., 2001); (iii) It is one of the most abundant seagrass species in Mediterranean (Marbà et al., 1996) and has high adaptive capacity under changing environmental condition, as referred above.

2.2 Model species collection

Cymodocea nodosa was collected during the 20th and 21th of March 2015, in a seagrass meadow located in Santibañez salt marsh (Fig. 2), in the inner bay of Cádiz Bay Natural Park, south-west coast of Spain (36°28'0N - 06°15'0W) in the Atlantic Ocean (Grignon-Dubois et al., 2012; Olivé et al., 2013). The Cádiz bay is subdivided into the inner bay and the outer bay, with a maximum depth of 20m at seaward edge and it has a semidiurnal tidal regime with 1.30 to 3.50m amplitude (neap and spring tides, respectively) (Alvarez et al., 1999; Grignon-Dubois et al., 2012; Olivé et al., 2013). The *C. nodosa* meadow is located in the inner bay and extends

from 0.5 to 3m depth (Alberto et al., 2005). It grows in a sand-muddy sediment, susceptible to temperature and salinities variations of 12 – 26ºC and 32 – 42 PSU, respectively (de la Rosa et al., 2006), and surface irradiances following the typical sinusoidal pattern for temperate latitudes, varying between 10 – 60 mol photons·m-2·d^{-1} (irradiance values values at year 2004 and 2005, data provided by the Spanish Meteorological Agency (AEMET), I. Olivé, pers. comm.).

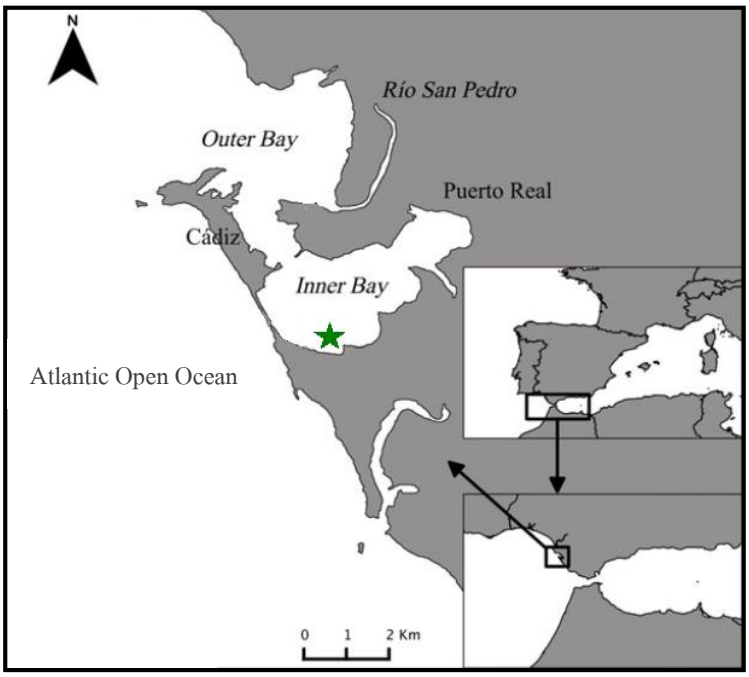


Figure 2. Map of Cádiz Bay. The green star (★) marks the sampling site, Santibañez. *Adapted from:* de los Santos et al., 2013

Cymodocea nodosa plants were carefully collected to ensure the integrity of the belowground part and stored in seawater in dark conditions and then transported within 48h to Ramalhete field station, Algarve. In between the sampling days, the plants were kept with air circulation in seawater in the installations of the Cádiz University.

2.3 Experimental design

Cymodocea nodosa plants were distributed in 24 tanks in an open circuit mesocosm at Ramalhete field station. Specimens were planted in sand and tanks filled with (~) 240 L of seawater. Two different water CO_2 concentrations (**REF-** air at ambient CO_2 concentration; **CO_2** – range of values of CO_2 air concentration predicted for 2100 by the Intergovernmental Panel on Climate Changes – IPCC; Pörtner et al., 2014) and 3 different light treatments encompassing both different qualities and quantities of light (**HL** – high light treatment,

receiving total natural irradiance; **LL** – low light treatment, receiving low natural irradiances; **LLB** – low light blue treatment, receiving also low natural irradiances with a selective filter to exclude irradiances with wavelengths above the blue region of the spectrum) were applied, resulting in six different treatments (**REF-HL**, **REF-LL**, **REF-LLB**, **CO_2-HL**, **CO_2-LL** and **CO_2-LLB)** with 4 replicates each, as shown in the scheme below (Fig. 3A).

Water from Ria Formosa was collected, filtered (sand filter, small particle filtre and UV filter) and directed to each head tank, where seawater was bubled with air (normal or CO_2-enriched) prior to entering the treatment tanks were plants were plotted.

CO_2 concentrations established in the head tanks were 370.81 (SD ±10.57) ppm and 1034.19 (SD ±41.51) ppm (for **REF** and **CO_2** treatments, respectively). For high CO_2 water, the air bubbled was previously enriched at (~) 1600 ppm, in a container with compressed CO_2, controlled by a partial pressure-driven solenoid valve. Subsequently, water flowed from the head tanks to the treatment. Finally, the water flowed out into the Ria Formosa.

For the irradiance treatments, all tanks were covered with a greenhouse plastic, 95% permeable to light (Solplast, S.A, Appendix I) to maintain the atmospheric conditions inside each tank. In order to manipulate the light quality, the tanks were covered with different light filters: **HL** tanks were covered with the greenhouse plastic; **LL** tanks were covered with the greenhouse plastic plus a 0.6 neutral density filter (Lee filters, ref. 210); **LLB** tanks were covered with greenhouse plastic plus a blue filter (Special Steel Blue, Lee filters, ref. 354), to absorb wavelengths greater than (~) 600 nanometres, coupled to a 0.15 neutral density filter (Lee filters, ref. 298) in order to obtain a light attenuation similar to that of LL tanks (see Appendix III). Neutral filters were used to reduce the light quantity but did not interfere with light quality.

At each tank, 34 plants (24 plants with 4 or more shoots and 10 with 3 shoots) were planted and 4 of them were tagged for growth estimation. Plants were acclimated under the greenhouse plastic and normal CO_2 water during one week. After the acclimation period, the air enriched in CO_2 started to be delivered to half of the tanks in order to allow the system stabilization and the light filters were placed. The experimental period lasted for 16 days (Fig. 3B).

Figure 3. **A)** Scheme and **B**) picture of mesocosm tanks' design during the experimental period. R: actual pCO_2 conditions; C: high pCO_2; HL: high light treatment; LL: low light treatment; LLB: low blue light treatment. Circumferences represent head tanks and rectangles the treatment tanks.

2.4 Physico-chemical monitoring

The water flow at the head tanks was daily measured and adjusted to ~ 42 $L.h^{-1}$ (0.7 $L.min^{-1}$). Water temperature (HOBO Pendant® Temperature/Alarm Data Logger 64K - UA-001-64, ONSET ®), irradiance reaching plants (Irradiance ODYSSEY - PAR sensor, Odyssey Light logger) and the dissolved oxygen concentration in the water (Optode - Oxygen optode, MiniDO2T Logger, PME, EUA) were measured throughout the experiment. 12 HOBO's were used in 12 tanks and changed to the other 12 tanks at the half of measuring period. One OPTODE and one ODYSSEY sensors were used in REF and CO_2 tanks.

A non-dispersive Infrared Gas Analyser (IRGA: WMA, PP Systems, UK) was coupled to the CO_2 enrichment system, measuring the CO_2 concentration continuously, to ensure that it

remained stable during the experimental period. pCO_2 was monitored in the water of the experimental tanks by another IRGA (EGM-4, PP Systems, UK) coupled to a gas-exchange column (Mini-Module membrane contractor, Celgard, USA) in (~) 24h cycles.

Each day (11h – 13h), pH (Thermo Scientific™ Orion™ Star A221 pH Portable Meter), temperature (CheckTemp1, Roth) and salinity (VWR, CO310) were registered for all tanks. Subsequently, OPTODES, ODYSSEYS and IRGA (EGM-4) were changed for different tanks (OPTODEs and ODYSSEYs were kept in the same treatment tanks and IRGA was moved each day to different tanks alternating CO2 and REF treatments). Water samples for DIC analyses were collected every 3 days, one from a REF treatment tank and another one from a CO_2 treatment tank.

The alkalinity of the water samples collected during the experiment was analysed through potentiometric titration method. Titration is based on the addition of small quantities of HCl to the sample until the endpoint pH is reached (pH=3). The pH change is monitored and ensured that the equivalence point (calculated by a Gran linearization method; Gran, 1988) is passed (approximately, pH 4.2) (Ohrel and Register, 2002). Then, total alkalinity, pH, temperature and salinity of the sample were used to calculate the carbonate system parameters of seawater, such as DIC concentration, using an updated version of the original Excel-based program CO_2SYS.XLS (Lewis and Wallace, 1998).

2.5 Growth estimation

Since the early 1970s, leaf and rhizome marking technique are recommended for direct short term growth rates measurements (Short and Duarte, 2001). Plant growth was estimated by the leaf elongation rate (LER), leaf appearance rate (LAR), shoot appearance rate (SAR) and rhizome elongation rate (RER). 100 plants equally randomly distributed among the tanks with 4 or more shoots (including the apical shoot) were selected for the growth experience and measured for morphometric characterization (internodes number and length, rhizome total length, shoot number, apical sheath length and apical leaves number and length). For growth estimation the rhizome was marked with a plastic tag tied in the nearest internode, as possible, of the apical shoot. The apical shoots of *C. nodosa* were also marked at the beginning of the experiment through the punching method (Short and Duarte, 2001). The mark was done by making a hole, on top of the sheath, with a sterilized syringe in order to create a scar in all leaves tissue in the bundle (Fig. 4).

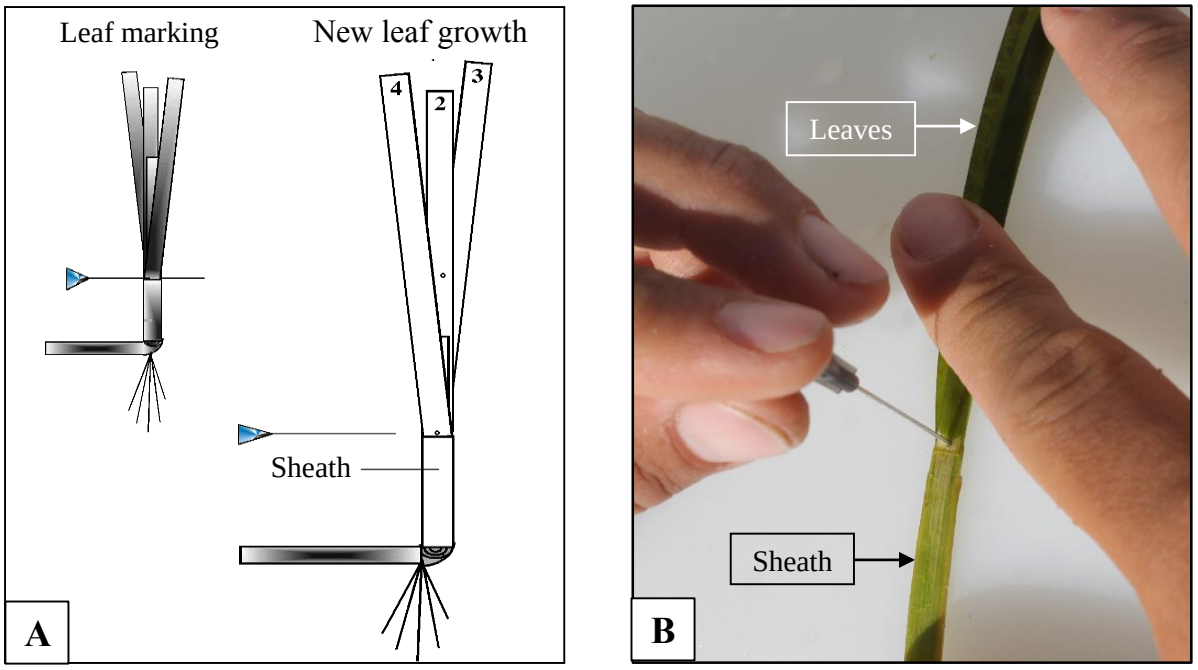


Figure 4. A) Scheme and **B**) picture of leaf marking technique to determine the leaf elongation rate in *C. nodosa*. Figure **A** *adapted from*: Short and Duarte, 2001.

At the end of the experiment the recovered plants (n= 92) were measured with mm precision (length of new leaves and sheaths, and length of total and new rhizomes), and the number of new leaves, shoots and new internodes was counted for morphometric characterization. The distance between the sheath and the scar was also measured in the marked leaves. The new material formed during the experiment was separated, frozen and transported to the laboratory where it was divided in rhizome, shoots (leaves and sheath) and roots, then dried at 60ºC until constant weight was attained.

The morphometric parameters evaluated during this thesis were total weight of new material (sum of, rhizome, shoots and roots); aboveground:belowground (AG:BG) ratio (calculated as the ratio of each individual plant, where leaves and sheath were considered as aboveground tissues and rhizomes as belowground tissues); new shoots length (sum of all leaves and sheath lengths); new rhizomes length; shoots weight (sum of leaves and sheaths) and rhizome weight. The data collected were also used for dynamic rates determination:

The leaf elongation rate (LER) evaluated the formation of new tissue within the leaf and was calculated as follows (*adapted from:* González, 2000):

$$\mathrm{LER} = \frac{\sum \mathrm{newFL}}{\Delta t}; \mathrm{cm} \cdot \mathrm{plant}^{-1} \cdot \mathrm{day}^{-1}$$

Where, **new FL -** represents the new foliar length between the sheath and the punching mark of leaves nº1, 2 and 3 (being the nº1 the youngest) at the final day and **Δt** – the time period of the experiment.

- The leaf appearance rate (LAR) evaluates the production of new leaves after leaf marking and was calculated as follows (*adapted from*: González, 2000):

$$LAR = \frac{\sum NL}{\Delta t}; \text{number of leaves} \cdot \text{plant}^{-1} \cdot \text{day}^{-1}$$

Where, **NL -** represents the number of new leaves grown during the experimental period and **Δt** – the time period of the experiment.

- The shoot appearance rate (SAR, new shoot·day^{-1}), which is the count of new shoots produced after the leaf marking (Short and Duarte, 2001).
- The rhizome elongation rate (RER, cm·day^{-1}), that is the daily increment on rhizome length (Marbà et al., 1996).

Each tank was an independent replicate (n=4) and plants within the same tank were considered as pseudo-replicates, thus the data obtained from each plant was averaged within each tank.

2.6 Biochemical analyses

Leaves and rhizomes of seagrasses were sampled between 12h and 17h, at seagrass collection site in Cádiz (n=5); at the beginning of experimental period (t0; n= 4 for leaves and n=3 for rhizomes) and at the end of the experimental period (t1; n= 24). At t0 and t1, leaves were also sampled at pre-dawn. Only the results obtained from t1 are presented in this thesis. Leaves and rhizomes were gently cleaned, frozen in liquid nitrogen and the stored in laboratory at -80ºC until analysis.

Laboratory analyses were conducted in "ALGAE: marine plant ecology research group" of the Centre of Marine Sciences (CCMAR), located at University of Algarve.

2.6.1 C:N ratio

The frozen samples of leaves and rhizomes were dried at 60ºC until constant weight was reached and ground to powder in a ball mill (TysseuLyser II) during 3 min at a 30s frequency. 2 to 3 mg of each sample were used to determine the quantity of C and N in a CNH Elemental Analyser.

2.6.2 Non-structural carbohydrates and starch

Non-structural carbohydrates (NSC) were extracted from freeze-dried samples of leaves and rhizomes. 10 mg dry weight were extracted in 10 ml of 80% ethanol at 80ºC during 30 min. The alcoholic extract was centrifuged at 5000 rpm (Thermo Scientific Heraeus, centrifuge model: Megafuge 16, rotor model: TX.4000) for 10 min, the supernatant was used to quantify the total soluble sugar and the pellet was stored at -20ºC for starch quantification (Burke et al., 1996). Before quantification, starch was hydrolysed to glucose in the presence of an enzymatic complex (14 U/ml amyloglucosidase and 1000 U/mg α-amylase per sample). The glucose equivalents were quantified by the phenol-sulphuric assay (DuBois et al., 1956), using glucose standards.

A protocol to quantify the commonly most abundant soluble sugars (sucrose, glucose and fructose), already used in terrestrial plants, was optimized to seagrasses. The alcoholic extract previously obtained from leaves and rhizomes was bleached with activated carbon and glucose, fructose and sucrose were determined according to Stitt et al. (1978, 1989) using the enzymatic method described by Jones et al. (1977). NSC determination was based on the indirect quantification using spectrophotometry to detect the alteration on the absorbance at 340nm due to the formation of NADPH. This method relies on the sequential addition of enzymes to induce sugar interconversion that leads to the increment on NADPH concentration in solution. The alcoholic extract was diluted in imidazole buffer containing the cofactors ATP and $NADP^+$. Hexokinase (HK) was added to catalyze glucose and fructose phosphorylation and turn them into the appropriate substrate for glucose-6P-dehydrogenase (GDH) and phosphoglucoisomerase (PGI) respectively. GDH oxidises glucose-6-phosphate (glucose-6P) to gluconate-6-phosphate (gluconate-6P) releasing NADPH to the reaction mixture. After the end of this first reaction, PGI was added to catalyze the isomerization of frutose-6-phosphate (frutose-6P) into glucose-6P. This glucose-6P was further oxidised by GDH to gluconate-6P releasing NADPH to the reaction mixture. Finally, invertase (INV) was added to hydrolyze sucrose to glucose and fructose that will be sequentially phosphorylated, isomerized (only fructose), and oxidised releasing NADPH to the reaction mixture. Glucose, fructose and sucrose were quantified following the evolution of NADPH given by the increment in absorbance during each enzymatic essay. The stoichiometry of the reactions is 1:1 to the hexoses fructose and glucose and 2:1 to sucrose. However, due to the lower glucose and fructose concentration present in the tissues, reliable values were only obtained for sucrose.

2.6.3 Soluble protein

Frozen leaf and rhizome samples (150 mg each) were ground in liquid nitrogen with PVPP (polyvinyl-polypyrrolidone) and extracted in 1.5ml of protein extraction buffer (100 mM Potassium phosphate, pH 7.8, 1 mM DTT, 1 mM PMSF, 2% (v/v) Triton-X). The extract was centrifuged at 14000 rpm (HERMLE centrifuge model: Z233MK-2, rotor model: 220.59VO4) during 10 mins and the supernatant was collected for the determination of soluble protein concentration by a dye-binding method, based on Bradford (1976). Protein quantification was done by comparison of the sample absorbance with a standard curve (using Bovine Serum Albumin – BSA) previously done. Coomassie Brilliant Blue G-250 dye was used and standards and samples we quantified spectrophotometrically at 595nm (Beckman-Coulter DU 650 spectrophotometer, Brea CA, USA).

2.7 Statistical analysis

Physico-chemical parameters measured all day long during the experiment (O_2, pCO_2 and irradiance) were integrated during each day (daily dose). O_2 and pCO_2 were statistically analysed using a t-test ($p<0.001$). Irradiance values violated the normality in the One-Way ANOVA test and thus were ranked and analysed using nonparametric test (Kruskal-Wallis One-Way Analysis of Variance on Ranks), with $p<0.001$ set as significant level. The data of pH, TA, DIC, pCO_2 and inorganic carbon forms used for system characterization between 11 and 13h, were analysed using a t-test ($p<0.001$).

The effects of CO_2 and light on *C. nodosa* plants were analysed considering pCO_2 and light as environmental factors and each tanks as a treatment replicate. Data from all plants within each tank were pooled to avoid pseudo-replication. Dead plants or without apex were not used. Two-Way ANOVA was used for the identification of significant effects of CO_2 and Light as well as their interaction in growth and biochemistry. The same statistical test was also used to identify significant effects of collection time (pre-dawn vs noon) and Light, and their interaction, in foliar sucrose and starch content within each CO_2 treatment. Whenever $p<0.05$, a SNK (Student-Newman-Keuls) test was applied for all pairwise comparisons. If data failed the tests for normality and variance homogeneity, a neperian logarithmic transformation was applied. In a few cases (i.e. Shoot Appearance Rate, Leaf Appearance Rate and Leaf Elongation Rate) data failed the Two-Way ANOVA assumptions even after transformation and the p-value

was lowered to 0.01 to minimize the risk of a Type 1 error (Underwood, 1997). The statistical results of growth and biochemical parameters are presented in the appendix (Appendix II).

The data collected during this thesis were analysed with the software "Sigmaplot" (© *Systat Software, Version 11.0*).

3. Results

3.1 Physico-chemical measurements

The characterization of the experimental conditions during the entire experimental period was guaranteed by the recording of physico-chemical parameters. The data was retrieved hourly and daily averaged (Fig. 5). Salinity was constant throughout the experimental period (36.8 ± 0.1) and temperature varied according to the natural conditions from 20.4 to 27.3ºC (Fig. 5A). The lowest temperature registered during the experimental period was 19.1ºC at 7:30h and the highest 30.4ºC at 16:20h at different days.

Plants experienced approximately a 14h photoperiod with sunrise at (~) 6:30h and the sunset at (~) 20:30h (Observatório Astronómico de Lisboa, 2015). The mean irradiance reaching the tanks during the experimental period followed the usual pattern at this latitude, being 0 during the night time, and increasing during the day to its maximum value, at 14h – 15h (Fig. 5B). In average, the daily dose reaching the greenhouse plastic covering the tanks was 52 mol photons$\cdot m^{-2} \cdot d^{-1}$, with a maximum mean value of 1791.5 µmol photons$\cdot m^{-2} \cdot s^{-1}$ during the day. Low Light (LL) and Low Light Blue (LLB) treatments received 32 – 33% and 24 - 26% of the irradiance reaching the High Light (HL) treatments, respectively. The light intensity of HL treatment was statistically different ($p<0.001$) from LL and LLB treatments and no statistically differences were found between low light treatments (LL and LLB). Thus, the differences in the growth and biochemical parameters between these two treatments can be attributed to the light quality.

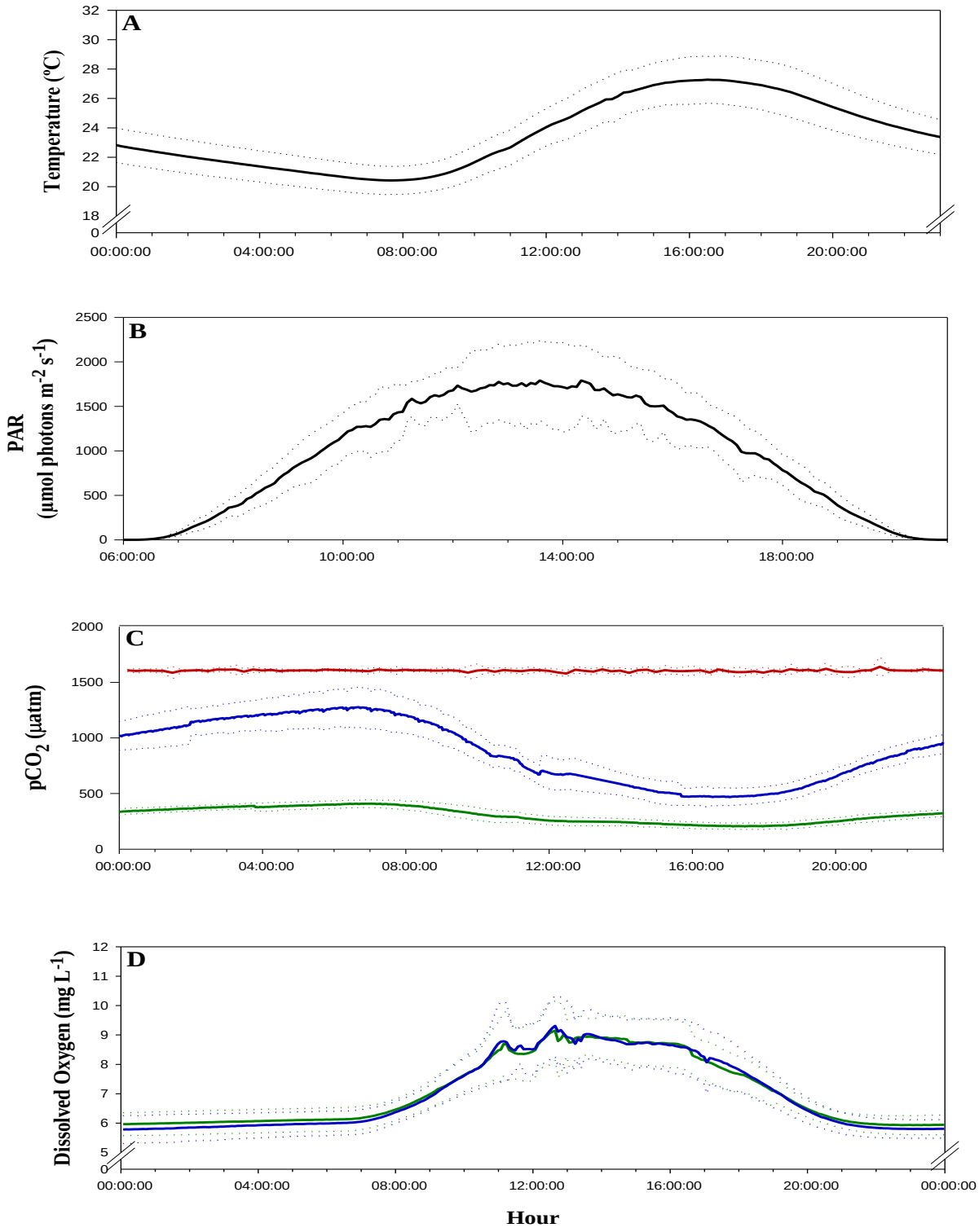


Figure 5. Daily means of **A**) Water temperature, expressed in ºC; **B**) Irradiance at the surface of the mesocosm (PAR, between 6 and 21h), expressed in µmol photons·m^{-2}·s^{-1}; **C**) Carbon dioxide partial pressure (pCO_2; expressed in µatm) at enrichment reservoir (red), high CO_2 treatments (blue) and reference CO_2 treatments (green); **D**) Dissolved Oxygen (DO; expressed in mg·L^{-1}) at high CO_2 treatments (blue) and reference CO_2 treatments (green). Solid lines represent the mean value of each parameter, dashed lines represents the standard deviation.

Throughout the experience, with exception of a few hours on the 30th of May (stabilization of the CO_2 at enrichment tank) and 13th of May (CO_2 source tank replacement), the partial pressure of CO_2 (pCO_2) at enrichment CO_2 tank was constant (1600 µatm). The daily variation of pCO_2 in water tanks was similar in both CO_2 treatments, higher values of pCO_2 at night and a decrease throughout the day until (~) 16h, followed by a further increase. As expected, statistically differences were recorded between the pCO_2 values in the different treatments (CO_2 and REF) and high pCO_2 treatment's tanks experienced a great daily variance throughout the experimental period (mean minimum and maximum values: 467.9 – 1276.3 µatm) when compared to the REF treatment (mean minimum and maximum values: 204.2 – 409.7 µatm) (Fig. 5C).

The concentration of dissolved O_2 (DO) followed the inverse pattern of pCO_2, increasing during the day and decreasing during the night (Fig. 5D). The DO values variation was similar for both treatments and thus no statistically differences ($p<0.001$) were found between different CO_2 treatments.

The pH mean (Table 1) was significantly lower in CO_2 treatments (7.9 ± 0.02) than in the REF tanks (8.3 ± 0.02) ($p<0.001$). Total alkalinity (TA) is a conservative parameter and was constant throughout the experiment on both treatments, while the pCO_2 was lower on REF (296 ± 31 µatm) than on CO_2 treatment (763 ± 56.9 µatm) ($p<0.001$). With the increase on pCO_2, DIC also increased significantly (Table 1) and thus the concentration of each inorganic carbon constituent has also changed. HCO_3^- and CO_2 concentrations increased (approximately 18% and 155%, respectively) and CO_3^{-2} decreased (45%) in high CO_2 treatments with respect to reference conditions.

Table 1. Carbonate system characterization. Means accounting all measures of each treatment (n=5) ± SD. **REF**: present water CO_2 partial pressure treatment; **CO_2**: high water CO_2 partial pressure treatment. Data are mean ±SE

CO_2 treatment	**REF**	**CO_2**	**Statistical difference ($p<0.001$)**
pH (NBS scale)	8.3 ± 0.03	7.9 ± 0.04	Yes
pCO_2 (µatm)	295.7 ± 31	763.1 ± 56.9	Yes
TA (µmol·kg^{-1} SW)	2580.8 ± 10.6	2586.3 ± 13.1	No
DIC (µmol·kg^{-1} SW)	2167.5 ± 27.95	2380.1 ± 25.4	Yes
HCO_3^- (µmol·kg^{-1} SW)	1862.9 ± 46.3	2196.1 ± 36.5	Yes
CO_3^{2-} (µmol·kg^{-1} SW)	295.7 ± 20.4	161.3 ± 14.5	Yes
CO_2 (µmol·kg^{-1} SW)	8.9 ± 1.0	22.7± 2.1	Yes

3.2 Growth Analysis

Plant growth was analysed at the end of the experimental period and the results only refer to the new material formed. Thus, the growth analysis is presented as gross rates.

The total new tissue (shoots, rhizomes and roots) weight tended to be higher in high light (HL) treatments (Fig. 6) and the lower mean value was observed in reference conditions under low light blue (LLB). No statistical differences were registered. However, light limitation had a negative effect on plants under reference conditions, as the mean weight of total new material decreased with light deprivation. This trend was not observed in plants under high CO_2 conditions.

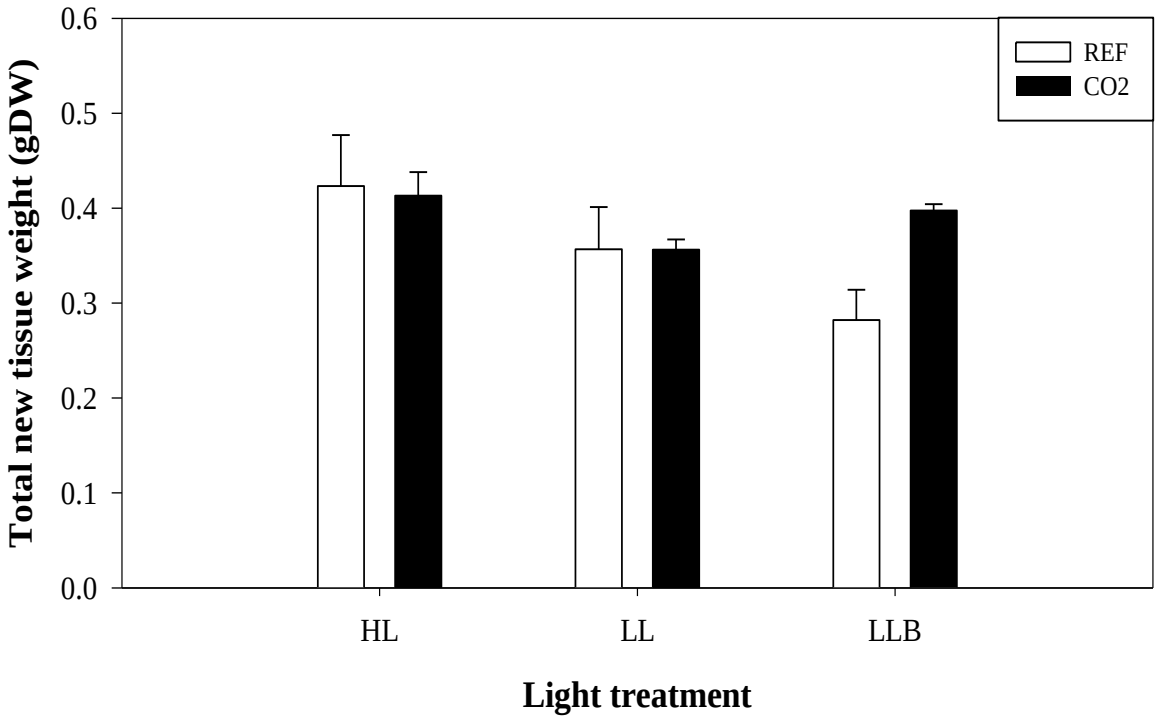


Figure 6. *Cymodocea nodosa* total (shoots, rhizomes and roots) new tissue weight, in grams of dry weight (gDW), produced after the imposition of the different light and CO_2 conditions. Blank columns represent reference conditions (REF, actual pCO_2), dark columns represent the high-CO_2 conditions (CO_2, (~) 1000 µatm). HL: high light treatment; LL: low light treatment; LLB: low light blue treatment. Columns represent mean ± SE (n=4).

The above:belowground (AG:BG) weight ratio (Fig. 7) tended to be lower in CO_2 plants particularly under low light intensity (LL) but blue light blurred this effect although no significant differences were found (p=0.053) .

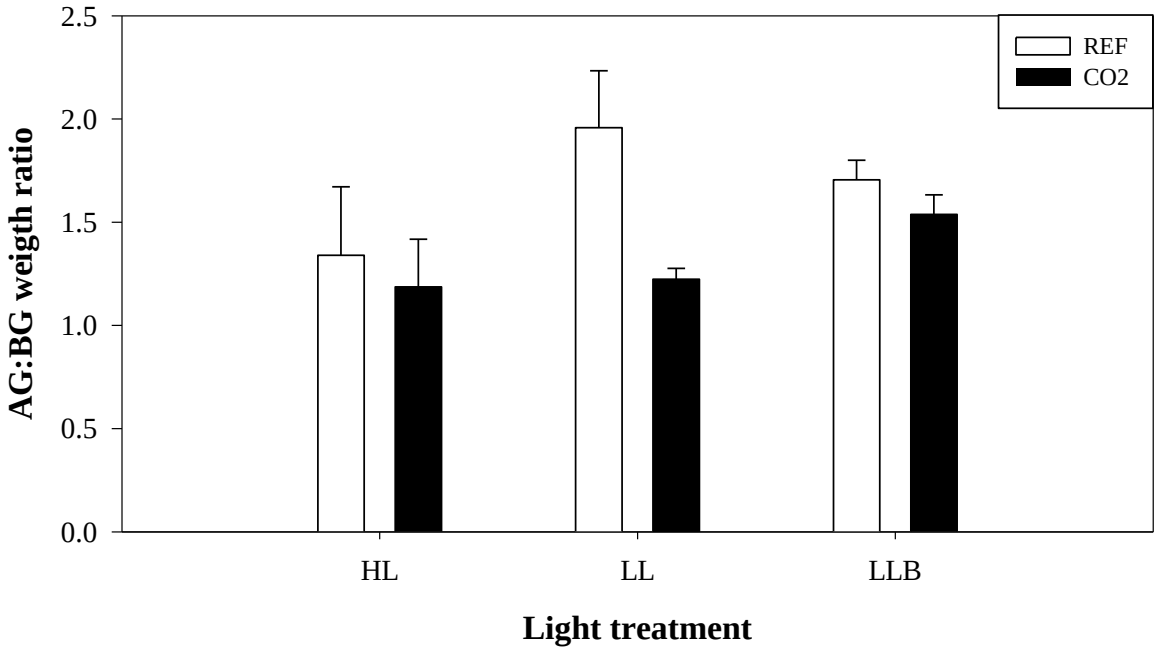


Figure 7. *Cymodocea nodosa* above:belowground (AB:BG) weight ratio. Blank columns represent reference conditions (REF, actual pCO_2), dark columns represent the high-CO_2 conditions (CO_2, (~) 1000 µatm). HL: high light treatment; LL: low light treatment; LLB: low light blue treatment. Columns represent mean ± SE (n=4).

New shoots (leaves and sheaths) and rhizomes total weight and length was compared to disentangle the responses of the above and belowground parts of the plants to CO_2 concentration and light regimes (Fig. 8). Light intensity/quality and CO_2 enrichment did not induce changes on shoot weight (Fig. 8A). However, a particular effect in LLB plants was observed suggesting an interaction of factors (light quality and CO_2) since under blue light both higher values (CO_2 plants) and lower values (REF plants) were registered. Rhizomes weight significantly decreased with light deprivation ($p=0.011$) being this decrease more pronounced for REF plants under low blue light (Fig. 8B). Regarding length, the shoots of CO_2 and REF plants under low blue light were the longer and the shorter, respectively (Fig. 8C). This changes were induced by an interaction effect of both factors (light and CO_2 enrichment; $p=0.030$). Rhizome length tended to decrease on REF light deprived plants while plants under enriched CO_2 conditions did not follow any trend (Fig. 8D). Although no significant differences were found, the pattern described by plants on rhizome length was similar to that already described for rhizome weight (Fig. 8B and D).

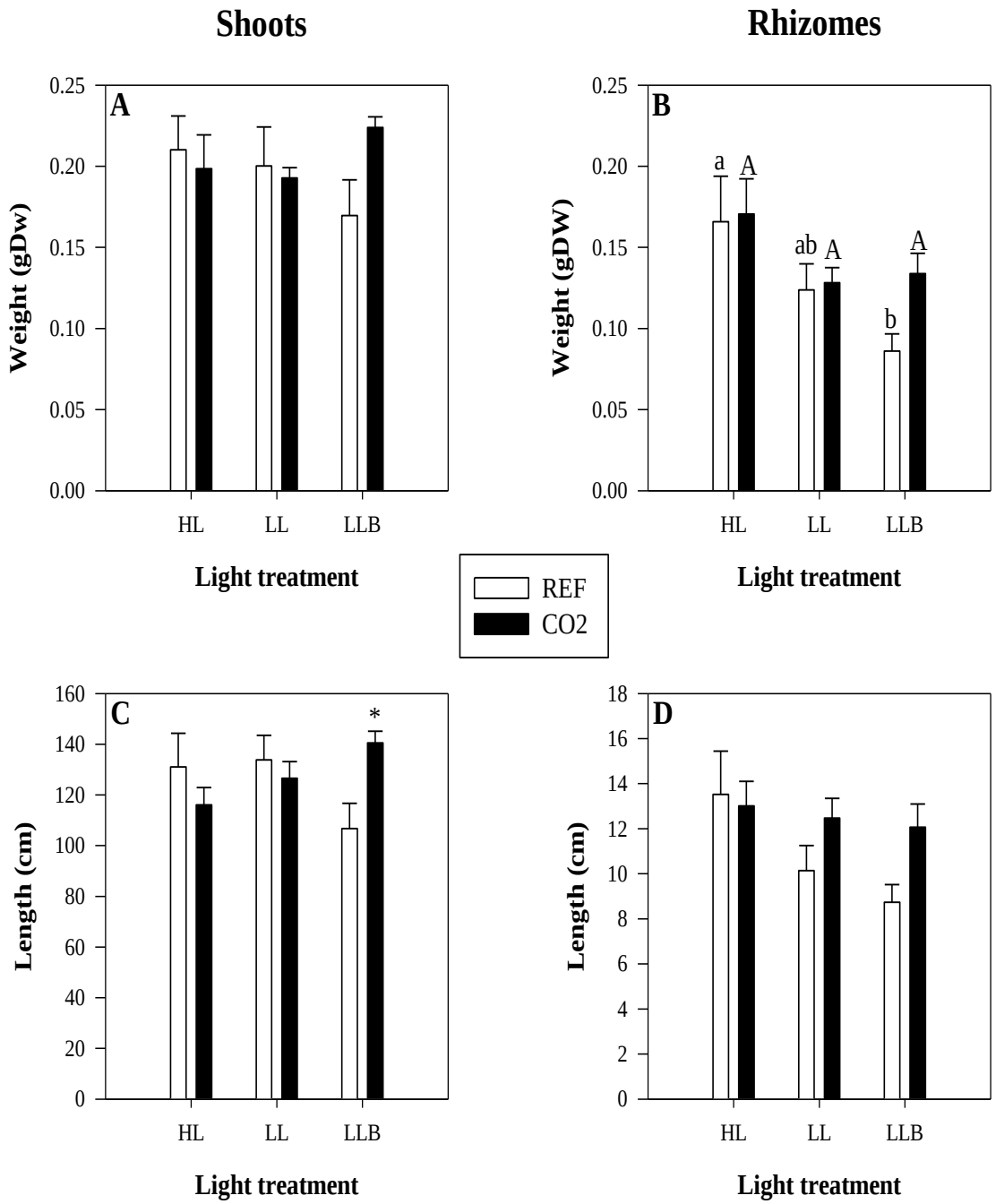


Figure 8. *Cymodocea. nodosa* **A**) total shoots weight of new aboveground material (leaves and sheaths) and, **B**) new belowground rhizome weight, expressed in grams of dry weigh. **C**) The sum of all shoot lengths of the new tissue and, **D**) new rhizome length, expressed in centimeters. Blank columns represent reference conditions (REF, actual pCO_2), dark columns represent the high-CO_2 conditions (CO_2, (~) 1000 µatm). HL: high light treatment; LL: low light treatment; LLB: low light blue treatment. Columns represent mean ± SE (n=4). Letters indicate statistical differences ($p<0.05$) between LIGHT treatments, lower case and capital letters for REF and CO_2 treatments, respectively. "*" indicate statistically differences ($p<0.05$) between REF and CO_2 treatments within LIGHT treatment.

No significant effects were found for SAR and RER (Fig. 9A and B), although light could have induced changes (p=0.032 and p=0.090 for SAR and RER, respectively). These two dynamic rates tended to decrease in REF plants with light deprivation. This tendency was attenuated (SAR) or disappeared (RER) in CO_2 plants.

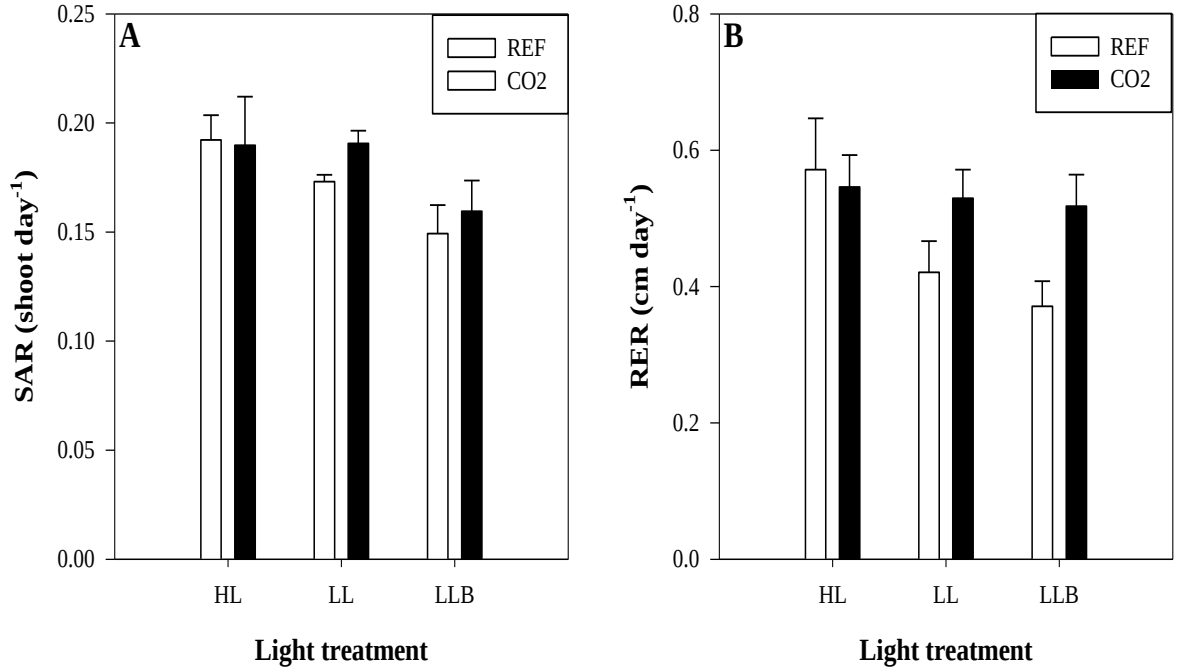


Figure 9. *Cymodocea nodosa* **A**) shoot appearance rate (SAR), expressed in shoot·day^{-1} and **B**) rhizome elongation rate (RER), expressed in cm·day^{-1}, of new tissue formed during the experimental period. Blank columns represent reference conditions (REF, actual pCO_2), dark columns represent the high-CO_2 conditions (CO_2, (~) 1000 µatm). HL: high light treatment; LL: low light treatment; LLB: low light blue treatment. Columns represent mean ± SE (n=4)

Leaf appearance rate (LAR) and leaf elongation rate (LER) were also determined in *C. nodosa* plants (Fig. 10A and B). Overall, LAR significantly decreased with light deprivation (p=0.006). To disentangle if this effect was significant for both CO_2 treatments, a Kruskal-Wallis One Way Analysis of Variance on Ranks was applied to each CO_2 treatment individually. Although LAR tended to decrease with light deprivation in both CO_2 treatments, it was only significant for REF plants (p<0.001). No significant differences were found in LER considering both the effect of light and CO_2 concentration although plants showed a decreasing trend with light deprivation.

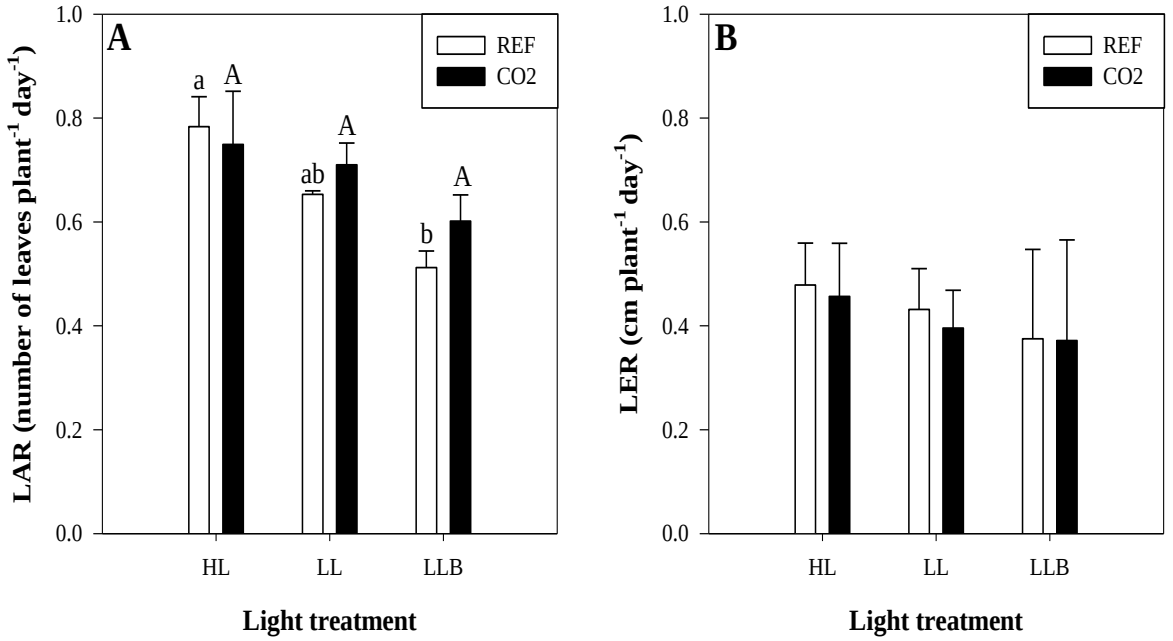


Figure 10. *Cymodocea nodosa* **A**) Leaf appearance rate (LAR), expressed in number of leaves·plant^{-1}·day^{-1} and, **B**) Leaf elongation rate (LER), expressed in cm.plant^{-1}·day^{-1} for different light treatments. Blank columns represent reference conditions (REF, actual pCO_2), dark columns represent the high-CO_2 conditions (CO_2, (~) 1000 µatm). HL: high light treatment; LL: low light treatment; LLB: low light blue treatment. Columns represent mean ± SE (n=4 for LAR and n=2 to n=4 for LER). Letters are used to indicate statistical differences ($p<0.01$) among light treatments, lower case and capital letter for REF and CO_2 treatments, respectively.

3.3 Biochemical Analyses

Carbon to nitrogen ratio (C:N) was determined for both leaves and rhizomes (Fig. 11). An effect of light ($p=0.037$) was found on leaves (Fig. 11A), where HL leaves evidenced a slight higher C:N when compared to LL and LLB leaves. These differences were due to a higher carbon content and lower nitrogen content inside plant tissues, under the high light treatment. No differences were found on leaf C:N between REF and CO_2 treatments. There were no significant differences on the rhizome C:N ratio neither for light nor for CO_2 concentration (Fig. 11B). Nonetheless, REF rhizomes under HL displayed a higher C:N ratio that reflects a slight lower N content found in rhizomes.

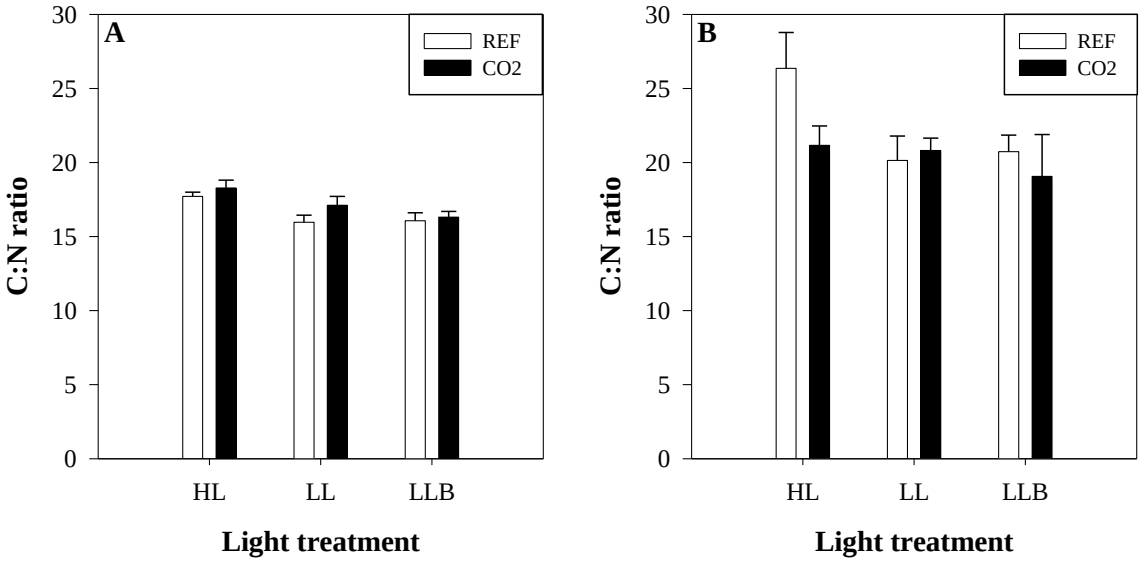


Figure 11. Carbon and nitrogen (C:N) ratio in **A)** leaves) and **B**) rhizomes of *C. nodosa* under high light (HL), low light (LL) and low intensity blue light (LLB) and actual (REF, actual pCO_2) and enriched CO_2 (CO_2, (~) 1000 µatm) conditions. Columns represent mean (n=4) ± SE.

Soluble carbohydrates and starch were determined both in leaves and rhizomes. Soluble sugars were discriminated into glucose, fructose and sucrose. Nonetheless only sucrose data are shown (Fig. 12) as glucose and fructose were present in very low concentrations to which the quantification method used lost resolution.

The light treatments did not affect the foliar content on sucrose at pre-dawn ($p=0.698$) (Fig. 12A). At noon, light significantly affected the plants' response ($p=0.003$), REF plants decreased the foliar sucrose content on both low light treatments (LL: $p=0.015$ and LLB: $p=0.017$). Contrastingly, CO_2 plants displayed a different pattern with a significantly higher ($p<0.05$) sucrose foliar content in LL plants, even higher than in REF plants. In REF plants, sucrose foliar content increased from pre-dawn to noon in HL plants but not in LL neither in LLB plants evidencing the negative effect of low irradiance on sucrose synthesis. Nevertheless, this low light effect disappeared at increased ambient CO_2 concentrations but only when plants were receiving the full light spectra (i.e. LL). Regarding the effect of CO_2 concentration in foliar sucrose content, at pre-dawn differences between CO_2 treatments tend to decreased with light deprivation ($p=0.059$). At noon, a significant effect of CO_2 ($p=0.005$) and interaction between both factors (light and CO_2; $p=0.008$) were verified and attributed to the large difference between CO_2 treatments under low light with the full spectra (LL) conditions.

Light induced a significant effect in rhizome sucrose content ($p=0.019$). Both low light treatments induced the decrease ($p<0.05$) in rhizome sucrose content in REF plants but no

significant differences were found in CO_2 plants. However, the effect of high CO_2 concentrations seemed to be more related to the decrease of sucrose induced by HL leaves than with significant changes in LL or LLB treatments (Fig. 12B).

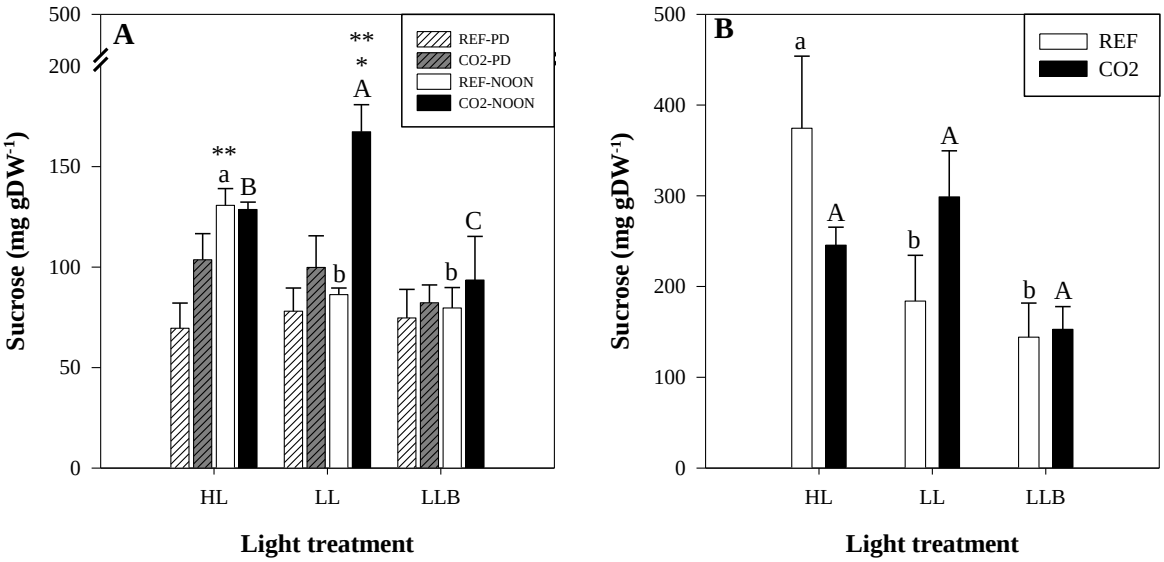


Figure 12. **A**) Foliar sucrose content and, **B**) rhizome sucrose content, expressed in mg·gDW^{-1} of *C. nodosa* exposed to high light (HL), low light (LL) and low blue light (LLB) under actual (REF, actual pCO_2) and enriched CO_2 (CO_2, (~) 1000 µatm) conditions. Columns represent mean (n=4) ± SE. Different letters indicate statistically significant differences among light treatments ($p<0.05$) within REF (lower case letters) and CO_2 (capital letters) treatments at the same hour (pd or noon); * indicates significant differences ($p<0.05$) between REF and CO_2 treatments within the same LIGHT treatment at each hour (pre-dawn (PD) or NOON); ** indicate significant differences ($p<0.05$) between PD and NOON treatments within the same LIGHT treatment for each CO_2 treatment (REF or CO_2).

Foliar starch content was not affected neither by light nor CO_2 treatments (Fig. 13A). Although no effect of sampling time (pre-dawn vs noon) was found ($p=0.078$), under CO_2 enrichment a decrease tendency between pre-dawn and noon was observed in HL and LL but blurred in LLB conditions. A significant interaction ($p=0.014$) between light and CO_2 treatments was detected for the rhizome starch content and attributed to the highest rhizome starch content measured in rhizomes of CO_2-LLB plants (Fig. 13B). Although no statistically significant differences were found, there was a contrasting pattern between REF and CO_2 plants with light, with a decreasing trend from high to low lights (LL and LLB) conditions in REF plants and an increasing one in CO_2 plants.

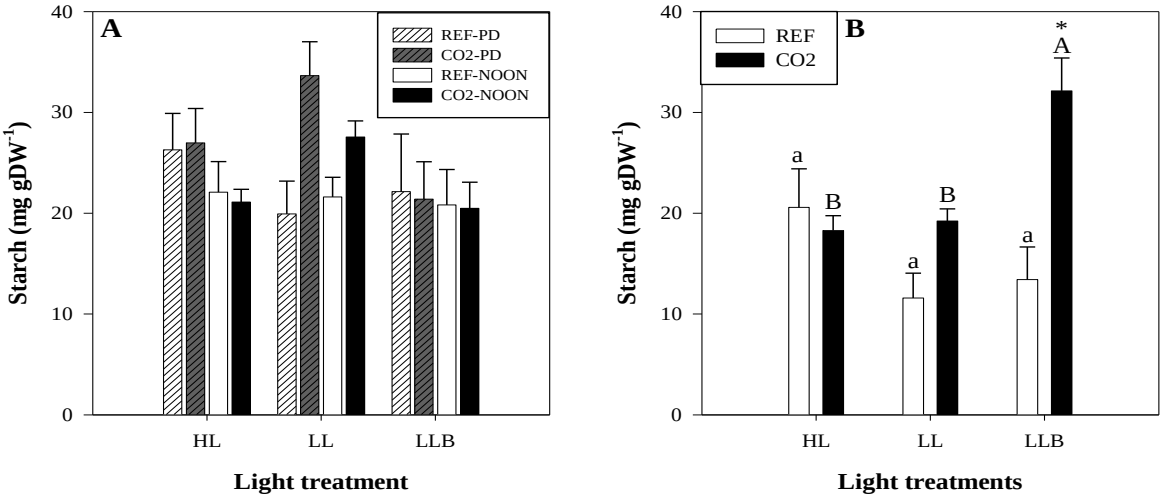


Figure 13. Starch content in **A**) leaves and **B**) rhizome starch content, expressed in mg·gDW^{-1} of *C. nodosa* exposed to high light (HL), low light (LL) and low blue light (LLB) under actual (REF, actual pCO_2) and enriched CO_2 (CO_2, (~) 1000 µatm) conditions. Columns represent mean (n=4) ± SE. Different letters indicate statistically significant differences among light treatments ($p<0.05$) within REF (lower case letters) and CO_2 (capital letters) treatments at the same hour (pre-dawn (PD) or noon or NOON); * indicates significant differences ($p<0.05$) between REF and CO_2 treatments within the same LIGHT treatment at each hour (PD or noon).

Leaf soluble protein content increased significantly in REF-LLB plants while in CO_2 low light treated (both LL and LLB) plants decreased, evidencing the interaction (p=0.004) between light quality and CO_2 concentration (Fig. 14A). An effect (p=0.002) and an interaction of light and CO_2 treatments (p=0.004) were detected on the concentration of soluble proteins, in leaves (Fig. 14A). In rhizomes (Fig. 14B) only an effect of the interaction of both factors (p=0.002) was detected.

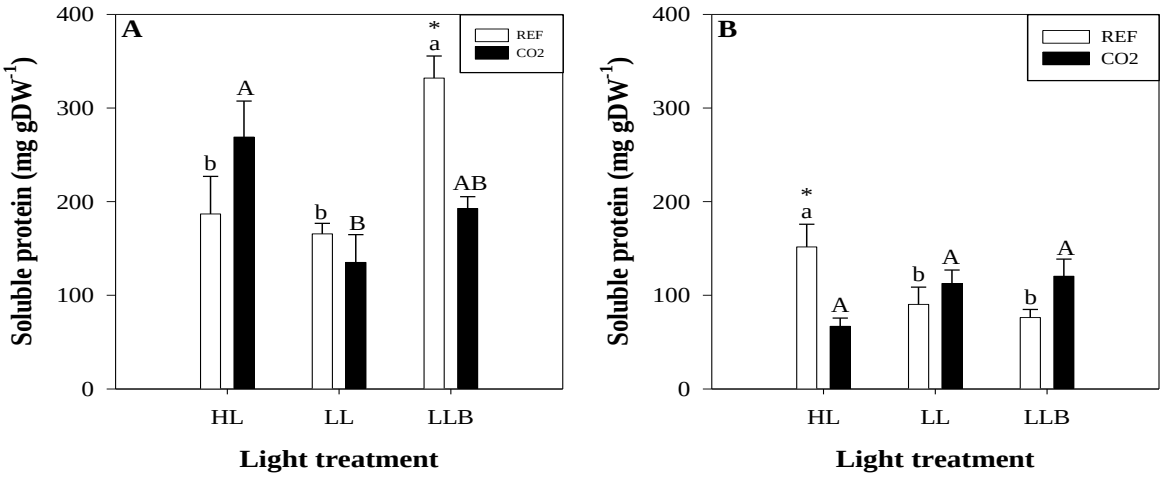


Figure 14. Soluble protein in **A**) leaves and **B**) rhizomes, expressed in mg·gDW^{-1} of *C. nodosa* exposed to high light (HL), low light (LL) and low blue light (LLB) under actual (REF, actual pCO_2) and enriched CO_2 (CO_2, (~) 1000 µatm) conditions. Columns represent mean (n=4) ± SE.Different letters indicate statistically significant differences among light treatments ($p<0.05$) within REF (lower case letters) and CO_2 (capital letters); * indicates significant differences ($p<0.05$) between REF and CO_2 treatments within the same LIGHT treatment.

4. Discussion

The present study evaluated, through a short-term mesocosm experiment, the response of the growth of the seagrass *Cymodocea nodosa* to changes on light quantity and quality together with the expected increase in Ci. The main conclusion is that both CO_2 and light affect the morphological and biochemical responses of *C. nodosa*, and despite some interaction between the two factors, light seems to be the main factor driving the seagrass responses.

There is a wide diversity of responses to increased CO_2 described for seagrasses, from the photosynthetic and biochemical level to the whole community response level (Jiang at al., 2010, Ow et al., 2015). Although there are some studies concerning seagrasses' relationship with light (Ruiz and Romero, 2001; Silva et al., 2013; Costa, 2014), the growth-irradiance relationship had received less attention when compared to photosynthesis-irradiance relationships (Peralta et al., 2002).

At the moment, there are a few studies performed in laboratory, mesocosm or *in situ* testing the effect of high-CO_2 (Zimmerman, 1997; Jiang et al., 2010; Ow et al., 2015) or the effect of irradiance through shading on seagrasses (Collier et al., 2009; 2012; Mazzuca et al., 2009; Silva et al., 2013). To the best of our knowledge, there is only one *in situ* study encompassing the photosynthetic response to both factors (high CO_2 and quantity of light) (Schwarz et al., 2000) and one mesocosm study that evaluated the growth response of *Zostera marina* (L.) to high CO_2 under two different natural light conditions (Palacios and Zimmerman, 2007). Regarding the light quality, only Mvungi et al. (2012) conducted an experiment assessing the effect of light quality on *Z. marina*. The present study goes further on the current knowledge of the effects of CO_2 and irradiance on seagrass growth and biochemistry. Thus, it represents a significant contribution to seagrass research.

4.1 Growth response

Regarding the total growth responses of *C. nodosa* to the factors tested, light had a greater effect modulating the growth of *C. nodosa*, while CO_2 seems to play an effect only at reduced light availability. At the whole plant level, total weight and AG:BG weight ratio did not show significant responses, however some trends were observed. Thus, high CO_2 conditions seem to prevent the weight loss at lower light intensity observed in plant at current CO_2 levels. The relative proportion of tissue produced, evaluated as AG:BG ratio, appears to respond to both light and CO_2 levels. Under HL conditions, the AG:BG ratio was similar regardless CO_2 conditions (1.2 – 1.3, for CO_2 and REF treatments, respectively) and close to the usual annual

mean value of 0.9 reported for *C. nodosa* (Pérez-Lloréns et al., 2014). Under REF conditions the AG:BG ratio increased in both lower light conditions. These results are in agreement with those reported by Fyfe (2004), who stated that plants exposed to higher irradiances allocate more carbon in the structure involved in nutrient uptake (BG biomass), which leads to lower values of AG:BG biomass ratios. However, in high CO_2 treatments, the AG:BG ratio only increased in LLB conditions. Thus, the higher CO_2 availability seems to buffer the ratio increase in LL treatment (Fig. 7), which means that under high CO_2 conditions *C. nodosa* seems to investment more in organs of reserve and expansion (rhizomes) than in production organs (leaves).

Regarding leaves, Palacios and Zimmerman (2007) observed that the CO_2 enrichment did not affect the leaf biomass. However the higher Ci available could explain the energetic investment of *C. nodosa* on increasing the leaves lengths and weight (Fig. 8C and D), which could be attributed to an increase in leaf width, as is seen in *C. nodosa* plants at greater depths (Olesen et al., 2002). Concerning rhizomes, the RER measured (Fig. 9) are in accordance with those reported by Pérez-Lloréns et al. (2014) for this species while the rates measured in HL treatments reached the maximum values registered by Marbà et al. (1998).

The morphologic parameters (length and weight) and dynamic growth rates (SAR, RER, LER, LAR) of *Cymodocea nodosa* presented some significant responses to light (i.e. total new rhizome weight and LAR) and interaction between factors, LIGHT x CO_2 (i.e. total length of leaves and sheaths at each plant) (Appendix II/ Table 3). The significant changes detected in LAR and the trends observed in the dynamic rates (SAR, RER and LER) of plants under low light (LL and LLB) evidenced the negative effect of light deprivation, which is in agreement with the observations of Peralta et al. (2002). The high pCO_2 seemed to reduce the negative effects of light deprivation both for morphologic parameters and dynamic rates. This effect was stronger, even significant, in LLB treatments.

During this study, plants might have responded differently within the CO_2 treatment. Plants subjected to REF conditions seemed to limit their investment in growth with the increasing light deprivation, reducing shoots and rhizome production. Rhizome growth was proportionally more reduced than shoots, which resulted in a higher proportion of aboveground tissues and a higher AG:BG ratio (Fig. 7). At high pCO_2, the rhizome elongation rate seemed to be maintained similar among light treatments (Fig. 9), although rhizomes showed less weight (probably due to the reduction in the rhizome width) in light limited treatments (Fig. 8B). Under severe light limitation plant growth is limited, but with higher availability of Ci, plants invested in the maintenance of the aboveground tissues, as shown by the slight increase in the AG:BG

ratio, shoots weight and length towards reduced light availability (Fig. 7, 8A, 8C). These results show that *C. nodosa* responses are affected by light than increasing pCO_2. However, high CO_2 seems to have a buffer effect attenuating negative effects of light limitation, which comes in agreement with other authors (e.g. Invers 1997, Zimmerman et al., 1997, Jiang et al., 2010; Ow et al., 2015) that consider that there is a decrease of seagrasses' light requirements due to high CO_2 concentrations, and thus plants with higher Ci available, during low light events could be less affected.

As a methodological comment, it is worthy to mention that the punching technique used during this experiment to calculate leaf growth (LAR and LER) presented some methodological limitations. The growth rate of the plants during the experiment was higher than expected, even considering it was the growing season. For this reason most of the leaves marked were lost due to leaf turnover rate or moved across new shoots making difficult the identification of marked leaves. This technique would be more useful if applied during a low growth season or in plants with lower growth rate. As a suggestion for future experiments conducted under similar conditions, a modified punching technique could be used such as the described in Peralta et al., (2000) where a plastic tag is tied to a chosen leaf (e.g. nº2). Although this technique reduces the number of leaves marked per shoot, it may allow a higher number of measures recovered.

4.2 Biochemical response

Biochemical analyses required great quantities of biomass for all the different analyses of the project that funded this work and thus it was necessary to harvest mixed pools of tissues formed during the experimental period, "new tissue", and also those that already existed before experimental period, "old tissue".

Morphological responses are a translation of responses and adaptations at the metabolic and photosynthetic level (Peralta et al., 2002 and references therein), and thus in a short-term study is expected to easily detect more differences at these levels. Overall, the tissue content analyses shows that the plants were not nutrient limited during the experimental study and, according to Short (1990), the plant presented the normal values of carbon (20-40%) and nitrogen (approximately 2%) of the composition for most seagrasses, although with lower values than those reported by Olesen et al. (2002) for *Cymodocea nodosa* (2.9 – 3.4%) in Mediterranean Sea. Disregarding the pCO_2, C:N ratio was affected by light, with higher values at higher intensities (Fig. 11). Likewise, Peralta et al. (2002) reported the higher values of C:N ratio at higher intensities (42 and 100% of Es), and also reported values almost 2-fold greater in

rhizomes. In our study, no effect of high CO_2 concentrations was found on this ratio, contrary to what was observed by Jiang et al. (2010) in *Thalassia hemprichii* (Ehrenb.) Aschers. The observed low sensibility of the elemental composition to changes in pCO_2 increase is expected for seagrasses that primarily use HCO_3^- as Ci source (Burkhardt et al., 1999). Mediterranean seagrass species are considered more efficient in the HCO_3^- use when compared to pacific species (Invers et al., 2001). The fact that *C. nodosa* is considered to be almost Ci saturated (91%) under present pCO_2 conditions (Invers et al., 1999; Schwarz et al., 2000) might explain the lower effect of high Ci availability in water.

Carbohydrates are a sink of both carbon and energy that can be used in growth or stored and used when conditions are less favourable (Alcoverro et al., 2001). The reserves of carbohydrates are crucial for seagrass survival, mainly during episodes of low light availability (Inver et al., 2004). We tried to quantify the three main soluble sugars present in seagrasses (i.e. glucose, fructose and sucrose) in both leaves and rhizomes. Unfortunately, only sucrose was present in sufficient quantity on tissues (Drew 1983; Larkum et al., 2006) allowing its reliable determination.

Touchette and Burkholder (2000) reported total carbohydrates mean values for leaf, root and rhizomes, of ca. 100, 135 and 275 $mg \cdot g^{-1}$ dry weight (DW), respectively. Higher sucrose concentration, a proxy of total soluble sugars concentration, was found at rhizomes (Fig. 12) in accordance with other studies (Drew 1983; Marbà et al., 1996; Peralta et al., 2002; Jiang et al., 2010, Silva et al., 2013), which indicates an effective translocation of sucrose from above to belowground tissues (Jiang et al., 2010; Campbell, 2012). High CO_2 conditions prevented the decrease of sucrose content in both LL leaves and rhizomes, contrary to what was observed in plants under current CO_2 level. However, even with greater Ci availability, sucrose content at reduced light spectra decreased. Regarding the leaves, the increase in Ci also seemed to increase their sucrose content. Starch accumulation in leaves was not influenced by the factors considered (light, CO_2 and time of collection – pre-dawn or noon). However, under attenuated full spectra of light, plants tended to increase the accumulation of starch at leaves. At rhizomes, CO_2 induced starch accumulation under the more restricted light conditions (LLB). Plants growing under actual pCO_2 showed a tendency towards starch depletion with light deprivation.

Sucrose values found in this study for *C. nodosa* are in agreement with those found for Ria Formosa (Silva et al., 2013) and slightly higher, mainly in rhizomes, than the reported for Mediterranean Sea, in Italy (Costa, 2014). On the other hand, starch values were in agreement with the values reported for Italy (Costa, 2014) and slightly higher and lower at leaves and rhizomes respectively, than the values measured by Silva et al. (2013). According to the results

of Silva et al. (2013), *Cymodocea nodosa* has lower starch content than sucrose. The lower starch reserves found by Burke et al. (1996), and more recently by Silva et al. (2013) in *Zostera marina*, is explained by a possible use of a more readily available energy source to save inter-conversion energy, as sucrose. This corroborates the statement of Malta et al. (2006), that starch plays a minor role than sucrose as a carbon reserve. During our experiment it is likely that sucrose was accumulated at greater light and/or inorganic carbon availability to be further used as an easy access storage carbohydrate for rapid utilisation depending on plant demands, as it was suggested by Costa (2014). With greater availability of inorganic carbon, plants stored the starch in the rhizome, in accordance with Jiang et al. (2010) and Campbell (2012). The decline in sugar and starch concentrations in REF *C. nodosa* plants under low light is in agreement with Fyfe (2004) that explains that fact with the decrease of net photosynthesis. Still, high concentrations of starch and sucrose in leaves even at lower light conditions may be an adaptation of seagrass to sustain the cost of harvesting more light (Pollard and Greenway, 1993).

Regarding the light effect on carbohydrates content, an opposite trend was found for *Zostera noltii* leaves (Peralta et al., 2002) with a light effect in the leaves but not in rhizomes. Differences among studies can be explained by the species-specific responses. However, even intra-specific differences can be found due to the time of the year when the experiment is conducted (Lavery, 2009).

Although high CO_2 had attenuated the reduction of growth caused by the light deprivation, growth still tended to decrease. Probably this decrease was a consequence of changes on the pattern of carbon investment by plants, decreasing the amount of carbon used in growth activity and redirecting it to be accumulated in rhizomes. Although, a low response of *C. nodosa* to light qualities was found (Mvungi et al., 2012), the effect of light quality must be addressed in the future, to unravel the real effects of different light spectra in seagrasses. As a methodological comment for biochemical analyses, rhizomes at pre-dawn should have been collected. However, sampling at that time of the day would destroy the plants required for the sampling at noon and great changes were not predicted in rhizomes during the short period between pre-dawn and noon. Nonetheless, significant differences may occur that could allow a more accurate vision of what is happening inside plants, and thus in a next opportunity it may worth the effort.

The synthesis of proteins is strongly dependent on C and N. Consequently, a reduction on carbohydrates production is likely to lower protein synthesis rate, as it was already verified in roots by Burke et al., 1996. In the present experiment, the soluble protein of *Cymodocea nodosa*

displayed significant responses to the single effect of light and pCO_2 (leaves) and there was an interaction between the two factors in analysis (Light x CO_2) in rhizomes. Regarding leaves, the concentration of soluble protein increased with light deprivation in plants growing under actual pCO_2 and the inverse pattern occurred for high CO_2 plants. In rhizomes, an inverse pattern of that above described for leaves was registered, although the increase in soluble proteins concentrations in CO_2 plants has been very slight. High CO_2 concentrations resulted in lower leaf soluble protein values for plants growing under low light, as Costa (2014) also found in Mediterranean sea, Italy. Actual ambient CO_2 together with high light resulted in a significant higher content of proteins at rhizomes.

Generally, is considered that ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) accounts for 50% or more of the total leaf protein (Taiz et al., 2015). Thus, variations on foliar soluble protein content are frequently associated to RuBisCO content. According to Costa (2014) and references therein, RuBisCO content decreases under high CO_2, and this is accompanied by the accumulation of sugars in the leaves, which was not the case in our study. However other proteins participating in other processes such the degradation/accumulation of starch/sucrose may influence the proteins amount. Mazzuca et al. (2009), verified the reduction of the proteins present during low light conditions, such as RuBisCO, and the increase of other proteins such as proteasome 26 S (a component of the proteolysis machinery mediated by ubiquitin) and cleavage enzymes (1-fructose-bisphosphate aldolase and beta-amylase). Those findings suggest a light effect on the processes involving protein turnover and on the biochemical pathways of carbon assimilation (Mazzuca et al., 2009). In the future, *Cymodocea nodosa* and seagrass proteomics must be address for a better understanding on soluble protein variations.

5. **Conclusions**

To frame the results and conclusions of this experiment, some points must be considered:

1. This study was performed in the peak *Cymodocea nodosa* growth period (Pérez-Lloréns et al., 2014).
2. *Cymodocea nodosa* presents a high natural morphological and growth variability (Marbà et al., 1996 & 1998; Olesen et al., 2002; Olivè et al., 2013).
3. This was a short-term experiment, and the morphological and biochemical responses must be dealt with in this framework.
4. The low number of replicates may not be enough to offset intraspecific variability, which influences the variance within treatment and thus, the statistical analysis.

Seagrasses morphological and biochemical descriptors are shaped by the changes in the natural environment, such as light and pCO_2. *Cymodocea nodosa* responses were mainly conditioned by light availability. High CO_2 and light quality played a secondary role attenuating or reinforcing, respectively, the light induced pattern. High-CO_2 conditions may buffer the effect of the light restriction due to the higher concentration of Ci. Low light blue treatments tend to reinforce the effect of light deprivation. Extrapolation of these results must be done with caution. Season, location and species-specific responses must be considered.

6. References

Alberto, F., Correia, L., Billot, C., Duarte, C. M., & Serrão, E. (2003). Isolation and characterization of microsatellite markers for the seagrass *Cymodocea nodosa*. *Molecular Ecology Notes, 3*, 397 – 399.

Alberto, F., Gouveia, L., Arnaud-Haond, S., Pérez-Llorèns, J. L., Duarte, C. M., & Serrão, E. A. (2005). Within-population spatial genetic structure, neighbourhood size and clonal subrange in the seagrass *Cymodocea nodosa*. *Molecular Ecology, 14*, 2669 – 2681.

Alberto, F., Mata, L., & Santos, R. (2001). Genetic homogeneity in the seagrass *Cymodocea nodosa* at its northern Atlantic limit revealed through RAPD. *Marine Ecology Progress Series, 221*, 299 – 301.

Alcoverro, T., Manzanera, M., & Romero, J. (2001). Annual metabolic carbon balance of the seagrass *Posidonia oceanica*: the importance of carbohydrate reserves. *Marine Ecology Progress Series, 211*, 105–116.

Alexandre, A., Silva, J., Buapet, P., Björk, M., & Santos, R. (2012). Effects of CO_2 enrichment on photosynthesis, growth, and nitrogen metabolism of the seagrass *Zostera noltii*. *Ecology and Evolution, 2*(10), 2620 – 2630.

Alvarez, O., Izquierdo, A., Tejedor, B., Mañanes, R., Tejedor, L., & Kagana, B. A. (1999). The Influence of Sediment Load on Tidal Dynamics, a Case Study: Cádiz Bay. *Estuarine, Coastal and Shelf Science, 48*, 439 – 450.

Apostolaki, E. T., Vizzini, S., Hendriks, I. E., & Olsen, Y. S. (2014). Seagrass ecosystem response to long-term high CO_2 in a Mediterranean volcanic vent. *Marine Environmental Research, 99*, 9 – 15.

Arnold, T., Mealey, C., Leahey, H., Miller, A. W., Hall-Spencer, J. M., Milazzo, M., & Maers, K. (2012). Ocean Acidification and the Loss of Phenolic Substances in Marine Plants. *PLoS ONE, 7*(4), e35107.

Atkinson, M. J., & Smith, S. V. (1983). C:N:P ratios of benthic marine plants. *Limnology and Oceanography, 28*(3), 568 – 574.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W., & Courchamp, F. (2012). Impacts of climate change on the future of biodiversity. *Ecology Letters, 15*, 365 – 377.

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry, 72*, 248 – 254.

Burke, M. K., Dennison, W. C., & Moore, K. A. (1996). Non-structural carbohydrate reserves of eelgrass *Zostera marina*. *Marine Ecology Progress Series, 137*, 195 – 201.

Burkholder, J. M., Tomasko, D. A., & Touchette, B. W. (2007). Seagrasses and eutrophication. *Journal of Experimental Marine Biology and Ecology, 350*, 46 – 72.

Calvo, E., Simó, R., Coma, R., Ribes, M., Pascual, J., Sabatés, A., Gili, J. M., & Pelejero, C. (2011). Effects of climate change on Mediterranean marine ecosystems: the case of the Catalan Sea. *Climate Research, 50*, 1 – 29.

Campbell, J. E. (2012). *The Effects of Carbon Dioxide Fertilization on the Ecology of Tropical Seagrass Communities*. PhD thesis. Florida International University.

Cancemi, G., Buia, M. C., & Mazzella, L. (2002). Structure and growth dynamics of *Cymodocea nodosa* meadows. *Scientia Marina*, *66*(4), 365 – 373.

Cao, L., & Caldeira, K. (2008). Atmospheric CO_2 stabilization and ocean acidification. *Geophysical Research Letters*, *35*, L19609.

Chartrand, K. M., Rasheed, M., Petrou, K., & Ralph, P. (2012). Establishing tropical seagrass light requirements in a dynamic port environment. *In: Proceedings of the 12th International Coral Reef Symposium* (15B Seagrasses and seagrass ecosystems). Cairns, Australia.

Coles, R., Grech, A., Rasheed, M., McKenzie, L., Unsworth, R., & Short, F. (2011). Seagrass Ecology and Threats in the Tropical Indo-Pacific Bioregion. *In:* R. S. Pirog (Ed.), *Seagrass: Ecology, Uses and Threats* (pp. 225 – 240). Hauppauge, New York: Nova Sciences Publishers.

Collier, C. J., Lavery, P. S., Ralph, P. J., & Masini, R. J. (2009). Shade-induced response and recovery of the seagrass *Posidonia sinuosa*. *Journal of Experimental Marine Biology and Ecology*, *370*, 89 – 103.

Collier, C. J., Waycott, M., & Ospina, A. G. (2012). Responses of four Indo-West Pacific seagrass species to shading. *Marine Pollution Bulletin*, *65*, 342–354.

Connolly, R. (2012). Seagrass. *In*: E. S. Poloczanska, A. J. Hobday, & A. J. Richardson (Eds.), *Marine Climate Change Impacts and Adaptation Report Card for Australia 2012 Report Card* (pp. 177 – 186). School of Environment, and Australian Rivers Institute – Coast and Estuaries Griffith University. Queensland, Australia.

Costa, M. M. (2014). *Effect of high CO_2 and ocean acidification on photosynthesis and response to oxidative stress in seagrasses*. PhD thesis. Universidade do Algarve.

Costanza, R., de Groot, R., Sutton, P., van der Ploeg, S. S. J. A., Kubiszewski, I., Farber, S., & Turner, R. K. (2014). Changes in the global value of ecosystem services. *Global Environmental Change*, *26*, 152 – 158.

Creighton, T. E. (1993). *Proteins: Structures and Molecular Properties* (2nd ed.). New York: W. H. Freeman and Company.

Cullen-Unsworth, L. C., Nordlund, L. M., Paddock, J., Baker, S., McKenzie, L. J., & Unsworth, R. K. F. (2014). Seagrass meadows globally as a coupled social–ecological system: Implications for human wellbeing. *Marine Pollution Bulletin*, *83*, 387 – 397.

de la Rosa, I. L., Rodríguez, A., & Raso, J. E. G. (2006). Seasonal variation and structure of a decapod (Crustacea) assemblage living in a *Caulerpa prolifera* meadow in Cádiz Bay (SW Spain). *Estuarine, Coastal and Shelf Science*, *66*, 624 – 633.

de los Santos, C. B., Brun, F. G., Vergara, J. J., & Pérez-Lloréns, J. L. (2013). New aspect in seagrass acclimation: leaf mechanical properties vary spatially and seasonally in the temperate species *Cymodocea nodosa* Ucria (Ascherson). *Marine Biology*, *160*, 1083 – 1093.

den Hartog, C., & Kuo, J. (2006). Taxonomy and Biogeography of Seagrasses. *In:* A. W. D. Larkum, R. J. Orth, & C. M. Duarte (Eds.), *Seagrass: Biology, Ecology and Conservation* (pp. 1 – 23). Dordrecht: Springer Netherlands.

Doney, S. C., Bopp, L., & Long, M. C. (2014). Historical and future trends in ocean climate and biogeochemistry. *Oceanography*, *27*(1), 108 – 119.

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean Acidification: the other CO_2 problem. *Annual Review of Marine Sciencee, 1*, 169 – 192.

Doney, S. C., Ruckelshaus, M., Duffy, J. E., Barry, J. P., Chan, F., English, C. A., Galindo, H. M., Grebmeier, J. M., Hollowed, A. B., Knowlton, N., Polovina, J., Rabalais, N. N., Sydeman, W. J., & Talley, L. D. (2012). Climate change impacts on marine ecosystems. *Annual Review of Marine Science, 4*, 11 – 37.

Drew, E. A. (1983). Sugars, cyclitols and seagrass phylogeny. Aquatic Botany, 15, 387 – 408.

Duarte, C. M. (2002). The future of seagrass meadows. *Environmental Conservation, 29*(2), 192 – 206.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry, 28*(3), 350 – 356.

Fourqurean, J. W., Escorcia, S. P., Anderson, W. T., & Zieman, J. C. (2005). Spatial and Seasonal Variability in Elemental Content, $d^{13}C$, and $d^{15}N$ of *Thalassia testudinum* from South Florida and Its Implications for Ecosystem Studies. *Estuaries, 28*(3), 447 – 461.

Fyfe, S. K. (2004). *Hyperspectral studies of New South Wales seagrasses with particular emphasis on the detection of light stress in Eelgrass Zostera capricorni*. PhD thesis. School of Earth and Environmental Sciences/ School of Biological Sciences, University of Wollongong.

Garrido, M., Lafabrie, C., Torre, F., Fernandez, C., & Pasqualini, V. (2013). Resilience and stability of *Cymodocea nodosa* seagrass meadows over the last four decades in a Mediterranean lagoon. *Estuarine, Coastal and Shelf Science, 130*, 89 – 98.

Garrote-Moreno, A., Fernández-Torquemada, Y., & Sánchez-Lizaso, J. L. (2014). Salinity fluctuation of the brine discharge affects growth and survival of the seagrass *Cymodocea nodosa*. *Marine Pollution Bulletin, 81*, 61 – 68.

Gattuso, J.-P., & Hansson, L. (Eds.). (2011). *Ocean Acidification*. New York: Oxford University Press.

González, G. P. (2000). *Estudios sobre el crecimiento en Zostera noltii Hornem.: dinámica estacional y aspectos ecofisiológicos*. PhD thesis. Facultad de Ciencias del Mar, Universidad de Cádiz.

Goufo, P., Pereira, J., Moutinho-Pereira, J., Correia, C. M., Figueiredo, N., Carranca, C., Rosa, Eduardo A. S., & Trindade, H. (2014). Rice (*Oryza sativa* L.) phenolic compounds under elevated 2 carbon dioxide (CO_2) concentration. *Environmental and Experimental Botany, 99*, 28 – 37.

Gran, G. (1988). Equivalence volumes in potentiometric titrations. *Analytica Chimica Acta, 206*, 111 – 123.

Grignon-Dubois, M., Rezzonico, B., & Alcoverro, T. (2012). Regional scale patterns in seagrass defences: Phenolic acid content in *Zostera noltii*. *Estuarine Coastal and Shelf Science, 114*, 18 – 22.

Guinotte, J. M., & Fabry, V. J. (2008). Ocean Acidification and Its Potential Effects on Marine Ecosystems. *Annals of the New York Academy of Sciences, 1134*, 320 – 342.

Harper, J. L. (1977). *Population Biology of Plants*. London: Academic Press.

Hellblom, F., Beer, S., Björk, M., & Axelsson, L. (2001). A buffer sensitive inorganic carbon utilisation system in *Zostera marina*. *Aquatic Botany, 69*, 55 – 62.

Hemminga, M. A., & Duarte, C. M. (2000). *Seagrass Ecology*. Cambridge, United Kingdom: Cambridge University Press.

Hoegh-Guldberg, O., Cai, R., Poloczanska, E. S., Brewer, P. G., Sundby, S., Hilmi, K., Fabry, V. J., & Jung, S. (2014). The Ocean. *In:* V. R. Barros, C. B. Field, D. J. Dokken, M. D. Mastrandrea, K. J. Mach, T. E. Bilir, Chatterjee, M., Ebi, K. L., Estrada, Y. O., Genova, R. C., Girma, B., Kissel, E. S., Levy, A. N., MacCracken, S., Mastrandrea, P. R., & L. L. White (Eds.), *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (pp. 1655 – 1731). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Invers, O., Kraemer, G. P., Pérez, M., & Romero, J. (2004). Effects of nitrogen addition on nitrogen metabolism and carbon reserves in the temperate seagrass *Posidonia oceanica*. Journal of Experimental Marine Biology and Ecology, 303, 97 – 114.

Invers, O., Pérez, M., & Romero, J. (1999). Bicarbonate utilization in seagrass photosynthesis: role of carbonic anhydrase in *Posidonia oceanica* (L.) Delile and *Cymodocea nodosa* (Ucria) Ascherson. Journal of Experimental Marine Biology and Ecology, 235, 125–133.

Invers, O., Romero, J., & Pérez, M. (1997). Effects of pH on seagrass photosynthesis: a laboratory and field assessment. *Aquatic Botany, 59*(3-4), 185–194.

Invers, O., Zimmerman, R. C., Alberte, R. S., Pérez, M., & Romero, J. (2001). Inorganic carbon sources for seagrass photosynthesis: an experimental evaluation of bicarbonate use in species inhabiting temperate waters. *Journal of Experimental Marine Biology and Ecology, 265*, 203 – 217.

Janssen, T., & Bremer, K. (2004). The age of major monocot groups inferred from 800+ *rbcL* sequences. *Botanical Journal of the Linnean Society, 146*, 385 – 398.

Jiang, Z. J., Huang, X.-P., & Zhang, J.-P. (2010). Effects of CO_2 Enrichment on Photosynthesis, Growth, and Biochemical Composition of Seagrass *Thalassia hemprichii* (Ehrenb.) Aschers. *Journal of Integrative Plant Biology, 52*(10), 904–913.

Johnson, M. W., Heck, K. L., & Fourqurean, J. W. (2006). Nutrient content of seagrasses and epiphytes in the northern Gulf of Mexico: Evidence of phosphorus and nitrogen limitation. *Aquatic Botany, 85*, 103 – 111.

Jones, M. G. K., Outlaw, W. H., & Lowry, O. H. (1977). Enzymic Assay of 10^{-7} to 10^{-14} Moles of Sucrose in Plant Tissues. *Plant Physiology, 60*, 379 – 383.

Kachelriess, D., Wegmann, M., Gollock, M., & Pettorelli, N. (2014). The application of remote sensing for marine protected area management. *Ecological Indicators, 36*, 169 – 177.

Kaiser, M. J., Attrill, M. J., Jennings, S., Thomas, D. N., Barnes, D. K. A., Brierley, A. S., Hiddink, J. G., Kaartokallio, H., Polumin, N. V. C., & Raffaelli, D. G. (2011). *Marine Ecology: Processes, Systems, and Impacts* (2nd ed.). New York: Oxford University Press.

Koch, M., Bowes, G., Ross, C., & Zhang, X.-H. (2013). Climate change and ocean acidification effects on seagrasses and marine macroalgae. *Global Change Biology, 19*, 103 – 132.

Kraemer, G. P., & Alberte, R. S. (1995). Impact of daily photosynthetic period on protein synthesis and carbohydrate stores in *Zostera marina* L. (eelgrass) roots: implications for

survival in light-limited environments. *Journal of Experimental Marine Biology and Ecology*, *185*, 191 – 202.

Kuo, J., & den Hartog, C. (2006). Seagrass Morphology, Anatomy, and Ultrastructure. In A. W. D. Larkum, R. J. Orth, & C. M. Duarte (Eds.), *Seagrass: Biology, Ecology and Conservation* (pp. 51 – 87). Dordrecht: Springer Netherlands.

Larkum, A. W. D., Drew, E. A., & Ralph, P. J. (2006). Photosynthesis and Metabolism in Seagrasses at the Cellular Level. *In:* A. W. D. Larkum, R. J. Orth, & C. M. Duarte (Eds.), *Seagrasses: Biology, Ecology and Conservation* (pp. 323 – 345). Dordrecht: Springer Netherlands.

Lavery, P. S., McMahon, K., Mulligan, M., & Tennyson, A. (2009). Interactive effects of timing, intensity and duration of experimental shading on *Amphibolis griffithii*. *Marine Ecology Progress Series*, *394*, 21–33.

Le Quéré, C., Raupach, M. R., Canadell, J. G., Marland, G., Bopp, L., Ciais, P., Conway, T. J., Doney, S. C., Feely, R. A., Foster, P., Friedlingstein, P., Gurney, K., Houghton, R. A., House, J. I., Huntingford, C., Levy, P. E., Lomas, M. R., Majkut, J., Metzl, N., Ometto, J. P., Peters, G. P., Prentice, I. C., Randerson, J. T., Running, S. W., Sarmiento, J. L., Schuster, U., Sitch, S., Takahashi, T., Viovy, N., van der Werf, G. R., & Woodward, F. I. (2009). Trends in the sources and sinks of carbon dioxide. *Nature Geoscience*, *17*, 831 – 836.

LEE Filters. (n.d.). Colour Information and Spectral Charts for LEE Lighting Filters. Retrieved September 14, 2015, from http://www.leefilters.com/lighting/colour-list.html.

Lee, K.-S., Park, S. R., & Kim, Y. K. (2007). Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: A review. *Journal of Experimental Marine Biology and Ecology*, *350*, 144 – 175.

Lewis, E., & Wallace, D. W. R. (1998). Program Developed for CO_2 System Calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

Malta, E.-J., Brun, F. G., Vergara, J. J., Hernández, I., & Pérez-Lloréns, J. L. (2006). Recovery of *Cymodocea nodosa* (Ucria) Ascherson photosynthesis after a four-month dark period. *Scientia Marina*, *70*(3), 413 – 422.

Marbà, N., & Duarte, C. M. (1998). Rhizome elongation and seagrass clonal growth. *Marine Ecology Progress Series*, *174*, 269–280.

Marbà, N., Cebrián, J., Enríquez, S., & Duarte, C. M. (1996). Growth patterns of Western Mediterranean seagrasses: species-specific responses to seasonal forcing. *Marine Ecology Progress Series*, *133*, 203–215.

Marbà, N., Holmer, M., Gacia, E., & Barrón, C. (2006). Seagrass Beds and Coastal Biogeochemistry. *In:* A. W. D. Larkum, R. J. Orth, & C. M. Duarte (Eds.), *Seagrasses: Biology, Ecology and Conservation* (pp. 135 – 157). Dordrecht: Springer Netherlands.

Mazzuca, S., Spadafora, A., Filadoro, D., Vannini, C., Marsoni, M., Cozza, R., Bracale, M., Pangaro, T., & Innocenti, A. M. (2009). Seagrass light acclimation: 2-DE protein analysis in *Posidonia leaves* grown in chronic low light conditions. *Journal of Experimental Marine Biology and Ecology*, *374*, 113 – 122.

McKee, T., & McKee, J. R. (2003). *Biochemistry: the molecular basis of life* (3rd ed.). New York: The McGraw-Hil Companies.

McPherson, M. L., Hill, V. J., Zimmerman, R. C., & Dierssen, H. M. (2011). The Optical Properties of Greater Florida Bay: Implications for Seagrass Abundance. *Estuaries and Coasts*, *34*, 1150 – 1160.

Mvungi, E. F., Lyimo, T. J., & Björk, M. (2012). When *Zostera marina* is intermixed with *Ulva*, its photosynthesis is reduced by increased pH and lower light, but not by changes in light quality. *Aquatic Botany*, *102*, 44–49.

National Research Council. (2011). *Climate Stabilization Targets: Emissions, Concentrations, and Impacts over Decades to Millennia*. Washington, DC: National Academies Press.

NOAA-CCGG. (2015). National Oceanographic and Atmospheric Administration Carbon Cycle Greenhouse Gases Group, Dr. Pieter Tans, NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/) and Dr. Ralph Keeling, Scripps Institution of Oceanography (scrippsco2.ucsd.edu/). *Retrieved* September 11, 2015, from http://www.esrl.noaa.gov/gmd/ccgg/trends/

Observatório Astronómico de Lisboa. (2015). Dados de 2015 | Observatório Astronómico de Lisboa. *Retrieved* September 22, 2015, from http://oal.ul.pt/publicacoes/almanaques/dados-de-2015/

Ocean Studies Board. (2010). *Ocean Acidification: A National Strategy to Meet the Challenges of a Changing Ocean*. Washington, D.C.: The National Academies Press.

Ohrel, R. L., & Register, K. M. (Eds.). (2002). *Volunteer Estuary Monitoring A Methods Manual* (2nd ed.). The Ocean Conservancy.

Olesen, B., Enríquez, S., Duarte, C. M., & Sand-Jensen, K. (2002). Depth-acclimation of photosynthesis, morphology and demography of *Posidonia oceanica* and *Cymodocea nodosa* in the Spanish Mediterranean Sea. *Marine Ecology Progress Series*, *236*, 89–97.

Olivé, I., Vergara, J. J., & Pérez-Lloréns, J. L. (2013). Photosynthetic and morphological photoacclimation of the seagrass *Cymodocea nodosa* to season, depth and leaf position. *Marine Biology*, *160*, 285 – 297.

Orfanidis, S., Papathanasiou, V., & Gounaris, S. (2007). Body size descriptor of *Cymodocea nodosa* indicates anthropogenic stress in coastal ecosystems. *Transitional Waters Bulletin*, *2*, 1 – 7.

Orth, R. J., Carruthers, T. J. B., Dennison, W. C., Duarte, C. M., Fourqurean, J. W., Heck, K. L., Hughes, A. R., Kendrick, G. A., Kenworthy, W. J., Olyarnik, S., Short, F. T., Waycott, M., & Williams, S. L. (2006). A Global Crisis for Seagrass Ecosystems. *BioScience*, *56*(12), 987 – 996.

Ow, Y. X., Collier, C. J., & Uthicke, S. (2015). Responses of three tropical seagrass species to CO_2 enrichment. *Marine Biology*, *162*(5), 1005–1017.

Palacios, S. L., & Zimmerman, R. C. (2007). Response of eelgrass *Zostera marina* to CO_2 enrichment: possible impacts of climate change and potential for remediation of coastal habitats. *Marine Ecology Progress Series*, *344*, 1–13.

Peralta, G., Pérez-Lloréns, J. L., Hernández, I., & Vergara, J. J. (2002). Effects of light availability on growth, architecture and nutrient content of the seagrass *Zostera noltii* Hornem. *Journal of Experimental Marine Biology and Ecology*, *269*, 9–26.

Peralta, G., Pérez-Lloréns, J. L., Hernández, I., Brun, F., Vergara, J. J., Bartual, A., Gálvez, J. A. & García, C. M. (2000). Morphological and physiological differences between two morphotypes of *Zostera noltii* Hornem. from the south-western Iberian Peninsula. *Helgoland Marine Research, 54*, 80 – 86.

Pérez-Lloréns, J. L., Vergara, J., Olivé, I., Mercado, J., Conde-Álvarez, R., Pérez-Ruzafa, Á., & Figueroa, F. (2014). Autochthonous Seagrasses. *In:* S. Goffredo & Z. Dubinsky (Eds.), *The Mediterranean Sea* (pp. 137–158). Springer Netherlands.

Pergent, G., Bazairi, H., Bianchi, C. N., Boudouresque, C.-F., Buia, M.-C., Calvo, S., Clabaut, P., Harmelin-Vivien, M., Mateo, M. A., Montefalcone, M., Morri, C., Orfanidis, S., Pergent-Martini, C., Semroud, R., Serrano, O., Thibaut, T., Tomasello, A., & Verlaque, M. (2014). Climate change and Mediterranean seagrass meadows: a synopsis for environmental managers. *Mediterranean Marine Science*, *15*(2), 462 – 473.

Plattner, G.-K., Knutti, R., Joos, F., Stocker, T. F., von Bloh, W., Brovkin, V., Brovkin, V., Cameron, D., Driesschaert, E., Dutkiewicz, S., Eby, M., Edwards, N. R., Fichefet, T., Hargreaves, J. C., Jones, C. D., Loutre, M. F., Matthews, H. D., Mouchet, A., Muller, S. A., Nawrath, S., Price, A., Sokolov, A., Strassman, K. M., & Weaver, A. J. (2008). Long-term climate commitments projected with climate-carbon cycle models. *Journal of Climate*, *21*(12), 2721 – 2751.

Pollard, P. C., & Greenway, M. (1993). Photosynthetic Characteristics of Seagrasses (*Cymodocea serrulata*, *Thalassia hemprichii* and *Zostera capricorni*) in a Low-light Environment, with a Comparison of Leaf-marking and Lacunal-gas Measurements of Productivity. *Australian Journal of Marine & Freshwater Research*, *44*, 123–139.

Pörtner, H.-O., Karl, D. M., Boyd, P. W., Cheung, W. W. L., Lluch-Cota, S. E., Nojiri, Y., Schmidt, D. N., & Zavialov, P. O. (2014). Ocean systems. *In:* C. B. Field, V. R. Barros, D. J. Dokken, K. J. Mach, M. D. Mastrandrea, E. Bilir, M. Chatterjee, K. L. Ebi, Y O. Estrada, R. C. Genova, B. Girma, E. S. Kissel, A. N. Levy, S. MacCracken, P. R. Mastrandrea, & .L. L. White (Eds.), *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (pp. 411–484). Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Ralph, P. J., Durako, J. M., Enríquez, S., Collier, C. J., & Doblin, M. A. (2007). Impact of light limitation on seagrasses. *Journal of Experimental Marine Biology and Ecology*, *350*, 176 – 193.

Raven, J. A., Giordano, M., Beardall, J., & Maberly, S. C. (2011). Algal and aquatic plant carbon concentrating mechanisms in relation to environmental change. *Photosynthesis Research*, *109*, 281 – 296.

Romero, J., Lee, K.-S., Pérez, M., Mateo, M. A., & Alcoverro, T. (2006). Nutrients Dynamics in Seagrass Ecosystems. *In:* A. W. D. Larkum, R. J. Orth, & C. M. Duarte (Eds.), *Seagrasses: Biology, Ecology and Conservation* (pp. 227 –254). Dordrecht: Springer Netherlands.

Ruggiero, M. V., Reusch, T. B. H., & Procaccini, G. (2004). Polymorphic microsatellite loci for the marine angiosperm *Cymodocea nodosa*. *Molecular Ecology Notes*, *4*, 512 – 514.

Ruiz, J. M., & Romero, J. (2001). Effects of in situ experimental shading on the Mediterranean seagrass *Posidonia oceanica*. *Marine Ecology Progress Series*, *215*, 107 – 120.

Russell, B. D., Connell, S. D., Uthicke, S., Muehllehner, N., & E., Katharina Fabricius Hall-Spencer, J. M. (2013). Future seagrass beds: Can increased productivity lead to increased carbon storage? *Marine Pollution Bulletin, 73*, 463 – 469.

Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R., Wong, C. S., Wallace, D. W. R., Tilbrook, B., Millero, F. J., Peng, T.-H., Kozyr, A., Ono, T., & Rios, A. F. (2004). The Oceanic Sink for Anthropogenic CO_2. *Science, 305*, 367 – 371.

Sandoval-Gil, J. M., Ruiz, J. M., Marín-Guirao, L., Bernardeau-Esteller, J., & Sánchez-Lizaso, J. L. (2014). Ecophysiological plasticity of shallow and deep populations of the Mediterranean seagrasses *Posidonia oceanica* and *Cymodocea nodosa* in response to hypersaline stress. *Marine Environmental Research, 95*, 39 – 61.

Saunders, M. I., Leon, J., Phinn, S. R., Callaghan, D. P., O'Brien, K. R., Roelfsema, C. M., … Mumby, P. J. (2013). Coastal retreat and improved water quality mitigate losses of seagrass from sea level rise. *Global Change Biology, 19*, 2569 – 2583.

Schwarz, A.-M., Björk, M., Buluda, T., Mtolera, M., & Beer, S. (2000). Photosynthetic utilisation of carbon and light by two tropical seagrass species as measured in situ. *Marine Biology, 137*(5-6), 755–761.

Seinfeld, J. H., & Pandis, S. N. (2006). *Atmospheric Chemistry and Physics: From Air Pollution to Climate Change* (2nd ed.). Hoboken, New Jersey, EUA: John Wiley & Sons.

Shiu, C.-J., Liu, S. C., Fu, C., Dai, A., & Sun, Y. (2012). How much do precipitation extremes change in a warming climate? *Geophysical Research Letters, 39*, L17707.

Short, F. T., & Duarte, C. M. (2001). Methods for the measurement of seagrass growth and production. *In:* F. T. Short & R. G. Coles (Eds.), *Global Seagrass Research Methods* (pp. 155 – 182). Amsterdam: Elsevier Science B.V.

Short, F.T., 1990. Primary elemental constituents, *in*: Phillips, R.C., McRoy, C.P. (Eds.), Seagrass Research Methods. UNESCO, pp. 105–109.

Silva, J., Barrote, I., Costa, M. M., Albano, S., & Santos, R. (2013). Physiological Responses of *Zostera marina* and *Cymodocea nodosa* to Light-Limitation Stress. *PLoS ONE, 8*(11), e81058.

Spalding, M., Taylor, M., Ravilious, C., Short, F., & Green, E. (2003). The distribution and status of seagrasses. *In:* E. P. Green & F. T. Short (Eds.), *World Atlas of Seagrasses* (pp. 5 – 26). Berkeley and Los Angeles, California: University of California Press.

Stitt, M., Bulpin, P. V., & Rees, T. A. (1978). Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum*. *Biochimica et Biophysica Acta, 544*, 200 – 214.

Stitt, M., Lilley, R. M., Gerhardt, R., & Heldt, H. W. (1989). Metabolite level in specific cells and subcellular compartments of plant leaves. *Methods in Enzymology, 174*(32), 518 – 552.

Taiz, L., Zeiger, E., & Møller, I. M. (2015). *Plant Physiology and Development* (6th ed.). Sinauer Associate.

Touchette, B. W., & Burkholder, J. M. (2000). Overview of the physiological ecology of carbon metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology, 250*, 169 – 205.

Underwood, A. J. (1997). *Experiments in ecology: their logical design and interpretation using analysis of variance*. Cambridge, United Kingdom: Cambridge University Press.

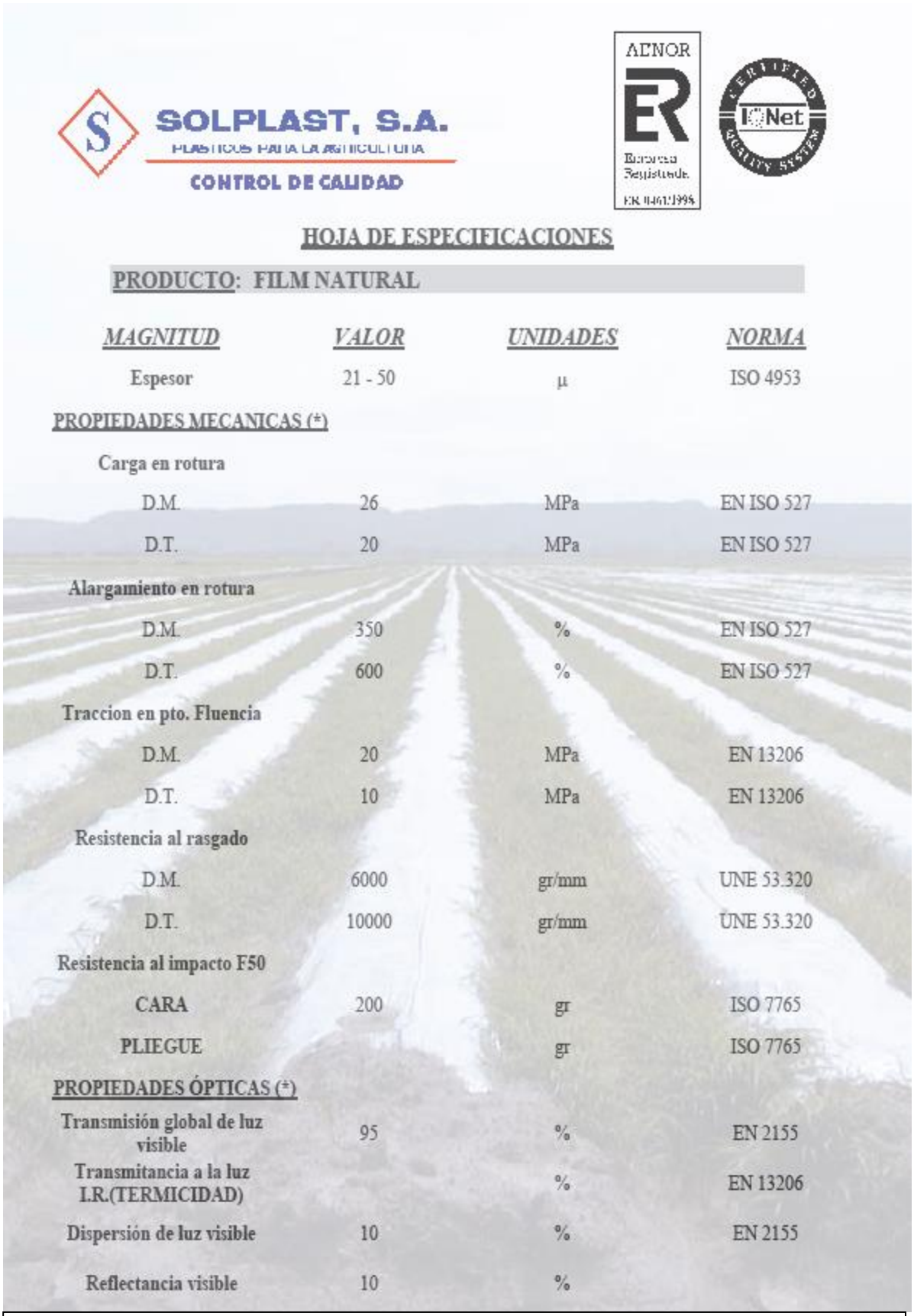
Unsworth, R. K. F., van Keulen, M., & Coles, R. G. (2014). Seagrass meadows in a globally changing environment. *Marine Pollution Bulletin*, *83*(2), 383–6.

Waycott, M., Duarte, C. M., Carruthers, T. J. B., Orth, R. J., Dennison, W. C., Olyarnik, S., Calladine, A., Fourqurean, J. W., Heck, K. L., Hughes, A. R., Kendrick, G. A., Kenworthy, W. J. Short, F. T., & Williams, S. L. (2009). Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *PNAS*, *106*(30), 12377 – 12381.

Waycott, M., Longstaff, B. J., & Mellors, J. (2005). Seagrass population dynamics and water quality in the Great Barrier Reef region: A review and future research directions. *Marine Pollution Bulletin*, *51*, 343 – 350.

Zimmerman, R. C., Kohrs, D. C., Steller, D. L., & Alberte, R. S. (1997). Impacts of CO_2 Enrichment on Productivity and Light Requirements of Eelgrass. *Plant Physiology*, *115*, 559–607.

7. Appendix



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PRODUCTO: FILM NATURAL

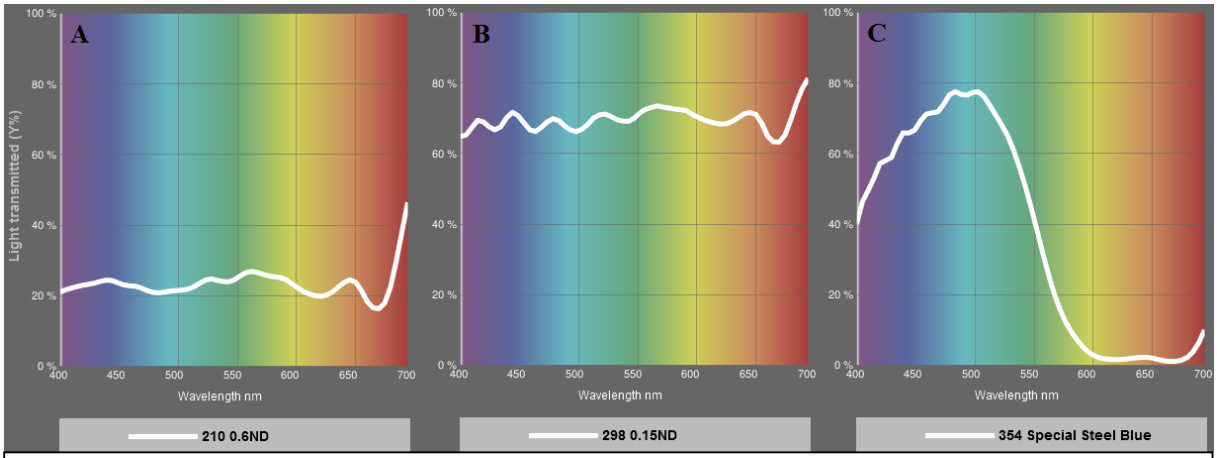
MAGNITUD	*VALOR*	*UNIDADES*	*NORMA*
Espesor	21 - 50	µ	ISO 4953
PROPIEDADES MECANICAS (*)			
Carga en rotura			
D.M.	26	MPa	EN ISO 527
D.T.	20	MPa	EN ISO 527
Alargamiento en rotura			
D.M.	350	%	EN ISO 527
D.T.	600	%	EN ISO 527
Traccion en pto. Fluencia			
D.M.	20	MPa	EN 13206
D.T.	10	MPa	EN 13206
Resistencia 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

Appendix II. Statistical results of the two-way-ANOVA analysis examining the effect of pCO_2, light and the interaction of these two factors in Growth and Biochemical parameters. In a few cases, was also examined the effect of the collection time (Pre-dawn, PD and Noon, NOON) and light within the different CO_2 treatments. * identifies the statistical differences considering $p<0.05$ and ** identifies the statistical differences considering $p<0.01$ for the exception cases (SAR, LAR and LER).

Variable	Factor	*df* treatment/*df* total	*MS*	*F*	*p*
GROWTH					
New tissue weight (total)	CO_2	1/23	0.00731	1.651	0.215
	LIGHT	2/23	0.0136	3.08	0.071
	CO_2xLIGHT	2/23	0.00974	2.198	0.14
AG:BG wieght ratio	CO_2	1/23	0.742	4.271	0.053
	LIGHT	2/23	0.316	1.82	0.191
	CO_2xLIGHT	2/23	0.22	1.267	0.306
New shoots total weight	CO_2	1/23	0.000823	0.613	0.444
	LIGHT	2/23	0.000159	0.118	0.889
	CO_2xLIGHT	2/23	0.00273	2.033	0.16
New shoots total length	CO_2	1/23	89.868	0.281	0.603
	LIGHT	2/23	115.906	0.362	0.701
	CO_2xLIGHT	2/23	1378.042	4.302	0.03*
New rhizome tissue weight	CO_2	1/23	0.00216	1.733	0.205
	LIGHT	2/23	0.00723	5.796	0.011*
	CO_2xLIGHT	2/23	0.00125	0.998	0.388
New rhizome total length	CO_2	1/23	17.739	3.108	0.095
	LIGHT	2/23	17.143	3.004	0.075
	CO_2xLIGHT	2/23	7.928	1.389	0.275
SAR	CO_2	1/23	0.000429	0.621	0.441
	LIGHT	2/23	0.00289	4.189	0.032
	CO_2xLIGHT	2/23	0.000205	0.296	0.747
RER	CO_2	1/23	0.0352	3.469	0.079
	LIGHT	2/23	0.028	2.759	0.09
	CO_2xLIGHT	2/23	0.0164	1.618	0.226
LAR	CO_2	1/23	0.00835	0.655	0.429
	LIGHT	2/23	0.0888	6.972	0.006**
	CO_2xLIGHT	2/23	0.00821	0.644	0.537
LER	CO_2	1/15	0.00574	1.328	0.276
	LIGHT	2/15	0.00815	1.888	0.202
	CO_2xLIGHT	2/15	0.0107	2.477	0.134

BIOCHEMICAL ANALYSES					
Variable	**Factor**	***df* treatment/*df* total**	***MS***	***F***	***p***
Leaves C:N ratio	CO_2	1/23	0.0483	2.694	0.118
	LIGHT	2/23	0.0718	4.001	0.037*
	CO_2xLIGHT	2/23	0.00876	0.488	0.622
Rhizomes C:N ratio	CO_2	1/23	25.638	1.908	0.184
	LIGHT	2/23	34.608	2.575	0.104
	CO_2xLIGHT	2/23	17.476	1.3	0.297
Leaves sucrose PD	CO_2	1/23	2684.24	4.068	0.059
	LIGHT	2/23	241.641	0.366	0.698
	CO_2xLIGHT	2/23	351.245	0.532	0.596
Leaves sucrose NOON	CO_2	1/23	5453.912	10.126	0.005*
	LIGHT	2/23	4557.287	8.462	0.003*
	CO_2xLIGHT	2/23	3517.123	6.53	0.008*
Leaves sucrose REF	PDvsNOON	1/23	3692.02	8.181	0.010*
	LIGHT	2/23	1169.808	2.592	0.103
	LIGHTxCO_2	2/23	1986.346	4.401	0.028*
Leaves sucrose CO_2	PDvsNOON	1/23	6816.843	8.978	0.008*
	LIGHT	2/23	3994.035	5.26	0.017*
	LIGHTx CO_2	2/23	1558.322	2.052	0.159
Rhizomes sucrose	CO_2	1/23	18.978	0.00197	0.965
	LIGHT	2/23	49021.548	5.08	0.019*
	LIGHTx CO_2	2/23	27444.343	2.844	0.086
Leaves starch PD	CO_2	1/23	124.536	2.01	0.173
	LIGHT	2/23	65.492	1.057	0.368
	LIGHTx CO_2	2/23	127.013	2.05	0.158
Leaves starch NOON	CO_2	1/23	14.373	0.598	0.449
	LIGHT	2/23	33.622	1.398	0.273
	LIGHTx CO_2	2/23	29.315	1.219	0.319
Leaves starch REF	PDvsNOON	1/23	980.452	0.18	0.676
	LIGHT	2/23	2591.175	0.476	0.629
	LIGHTx CO_2	2/23	1726.45	0.317	0.732
Leaves starch CO_2	PDvsNOON	1/23	11024.066	3.49	0.078
	LIGHT	2/23	19499.25	6.174	0.009*
	LIGHTx CO_2	2/23	1727.34	0.547	0.588
Rhizomes starch	CO_2	1/23	61.385	2.496	0.134
	LIGHT	2/23	100.694	4.095	0.037*
	LIGHTx CO_2	2/23	137.454	5.59	0.014*
Leaves TSP	CO_2	1/23	5110.083	1.598	0.222
	LIGHT	2/23	26298.374	8.222	0.003*
	LIGHTx CO_2	2/23	24500.968	7.66	0.004*
Rhizomes TSP	CO_2	1/23	221.674	0.206	0.655
	LIGHT	2/23	254.527	0.237	0.792
	LIGHTx CO_2	2/23	9531.447	8.869	0.002*



Appendix III. Light transmitted (Y%) for each PAR wavelength by different filters used. **A**) 0.6 neutral density filter (reference: 210); **B**) 0.15 neutral density filter (reference: 298); **C**) Special Steel Blue filter (reference: 354). *Adapted from*: LEE Filters.com