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Compared physiological performances of *Caulerpa prolifera* and native seagrasses of Ria Formosa

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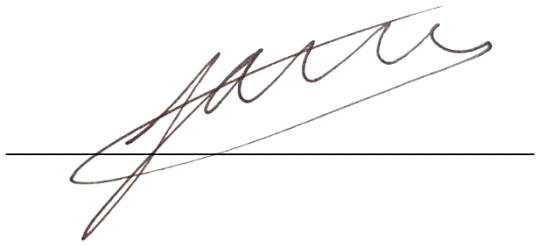
Jan Dirk Hofman



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“Nothing is too wonderful to be true if it is consistent with the laws of nature”

– Micheal Faraday

Abstract

Seagrasses are among the most important and also mostly threatened ecosystems in the marine environment. Seagrass loss can occur in their competition with other macrophytes, like invasive macroalgae. These pose a serious threat and present numerous consequences to their new environment. Among those that can affect seagrasses, *Caulerpa* sp. are one of the most recognized genus. Different mechanisms can grant them a competitive advantage over seagrasses, resulting in partial or even complete replacement. Therefore, knowing how *Caulerpa* sp. functions and interacts with a new environment is crucial, especially in a system like Ria Formosa, where three of the four European seagrass species can be found. We aimed to study and compare different photo-physiological components of the subtidal seagrasses *Cymodocea nodosa* and *Zostera marina* and of the macroalgae *Caulerpa prolifera* along diel cycles, while at the same time identifying possible consequences of their interaction, using a mesocosm experiment. The first experiment was performed over the course of two days (48 hour cycle), collecting samples for biochemical analysis at pre-dawn and solar noon at the end. The mesocosm experiment involved planting *Z. marina* and *C. prolifera* separately and mixed. After 4 weeks, photosynthetic performance was tested using photosynthesis-irradiance curves and rapid light curves, and samples for biochemical analysis were collected. The seagrasses revealed higher effective quantum yield and non-photochemical quenching, mainly related to their xanthophyll pigments, while *C. prolifera* displayed a typical shade-adapted response. Furthermore, it displayed a different carbohydrate usage regime, which was related to its higher respiration rates. Although no different overall photosynthetic performance was detected in the interaction of the species, starch content in *Z. marina* rhizomes was significantly lower when *C. prolifera* was present. This work gives initial insight on the physiological performance of *C. prolifera* in Ria Formosa.

Keywords

Cymodocea nodosa, Zostera marina, Macroalgae, *Caulerpa prolifera*, Photophysiology, Ria Formosa

Resumo

Ervas marinhas são plantas angiospérmicas adaptadas ao ambiente marinho que formam pradarias extensivas em zonas costeiras a nível mundial, com exceção dos polos. Estas pradarias formam um habitat complexo e diverso, apresentando uma alta produtividade e prestando serviços ecossistémicos importantes, como o sequestro e armazenamento de carbono, reciclagem de nutrientes e proteção costeira. No entanto, apesar da sua importância, são um dos habitats marinhos mais ameaçados. A maior causa de perda de pradarias de ervas marinhas é a atividade antropogénica. Dragagens, eutrofização, descargas de nutrientes e desenvolvimento costeiro são exemplos de atividades que diminuem a qualidade da água e afetam diretamente as pradarias. Além disso, ocorrem também perdas naturais das pradarias, por exemplo, pela competição com outros macrófitos, como é o caso das algas que podem ocupar o mesmo espaço e utilizar os mesmos recursos. A sua colonização pode afetar não só o ecossistema, mas também a abundancia de espécies endémicas e a sua diversidade. Diferentes macroalgas verdes já provaram ter um elevado potencial invasor, entre as quais se destingem espécies do género *Caulerpa.* Este género inclui algumas das algas mais invasoras conhecidas atualmente. *Caulerpa taxifolia* e *Caulerpa racemosa* são alguns exemplos de algas que invadiram o Mar Mediterrâneo e que afetaram negativamente várias pradarias de ervas marinhas. Além de competirem por recursos como nutrientes e luz, competem também pelo mesmo substrato, previamente ocupado com ervas marinhas, não permitindo que elas recuperem. Fisiologicamente, ervas marinhas e algas, mais especificamente do género *Caulerpa*, são limitadas pela luz. As primeiras apresentam uma maior necessidade de luz, mas isso não impede que várias espécies tenham capacidade de sobreviver quando a irradiância é baixa. Vários mecanismos de defesa, que variam de espécie para espécie, determinam a sua resiliência a estas condições. Por outro lado, a exposição a luz excessiva também pode ser prejudicial para as plantas, nomeadamente induzindo stress foto-oxidativo. Para defesa contra o excesso de luz (fotoproteção), as plantas possuem pigmentos secundários, os carotenóides, capazes de transformar o sistema de captura de luz num sistema de dissipação de energia excessiva na forma de calor. O género *Caulerpa* não só apresenta uma elevada plasticidade a nível morfológico mas também ao nível dos seus pigmentos. Tal como as ervas, as algas possuem carotenóides capazes de dissipar energia excessiva. Além disso, a sua capacidade para tolerar altas intensidades de luz depende dos

nutrientes disponíveis, que podem obter não só da coluna de água, mas também do sedimento, havendo assim competição entre as ervas marinhas e as algas em ambos os meios. Com o aparecimento da *Caulerpa prolifera* em zonas menos profundas da Ria Formosa, onde ocorrem três das quatro espécies de ervas marinhas a nível Europeu, surge a necessidade de perceber o seu funcionamento fisiológico comparativamente com as ervas e avaliar o potencial para interações fisiológicas. Para tal, analisou-se a performance circadiana de *Zostera marina, Cymodocea nodosa* e *C. prolifera* ao longo de um ciclo de 48 horas numa sistema seminatural. Foram medidos parâmetros relacionados com a fluorescência da clorofila *a*, medindo a eficiência quântica e recolhendo amostras para analise bioquímica. Numa outra experiência, para estudar possíveis consequências da interação entre ervas e algas, *Z. marina* e *C. prolifera* foram recolhidas no campo e plantadas em tanques, quer individualmente quer em conjunto, em cinco replicados, recebendo sempre a mesma intensidade de luz com um fotoperíodo de treze horas, durante quatro semanas. Após as quatro semanas, foi avaliada a resposta da fotossíntese à luz de curvas em ambas as espécies e recolhidas amostras para posterior analise bioquímica. Foram analisados os conteúdos em açúcares solúveis e amido, proteína solúvel, compostos adenilados e pigmentos fotossintéticos nas três espécies. Os açúcares e amido foram analisados pelo método fenol-sulfúrico e as proteínas solúveis pelo método de Bradford. Os compostos adenilados foram analisados por cromatografia liquida de alta eficiência (HPLC). Os pigmentos fotossintéticos foram analisados primeiro por espectrofotometria para identificar concentrações de clorofila, complementando com cromatografia (HPLC) para a identificação dos carotenoides. As ervas apresentaram níveis de eficiência quântica real e potencial e dissipação de energia sob a forma de calor mais elevadas comparativamente com a alga, e índices de desepoxidaçao dos pigmentos do ciclo das xantofilas mais elevados, mostrando estar mais protegidas contra intensidades de luz mais elevadas. *C. prolifera*, pelo contrário, apresentou uma resposta típica de espécies adaptadas a baixas intensidades de luz, com razões de clorofila *a*/*b* mais baixas do que as ervas e índices de desepoxidação do ciclo das xantofilas muito baixos. Apresentou ainda taxas fotossintéticas ligeiramente mais baixas, uma taxa de respiração mais elevada e uma maior resposta foto-inibitória a intensidade de luz mais alta, comparado a *Z. marina*. O conteúdo de açúcar solúvel da alga foi significativamente mais baixo do que o das ervas, enquanto que as suas reservas de amido foram muito mais elevadas, revelando um regime de

utilização de hidratos de carbono bastante distinto. A interação de *Z. marina* com *C. prolifera* não mostrou ter influência nas capacidades fotossintéticas e teores de pigmentos fotossintéticos de ambas as espécies. No entanto, verificou-se um esgotamento das reservas de amido nos rizomas de *Z. marina*. Uma vez que não foram observadas alterações nas taxas de respiração, colocamos a hipótese que os hidratos de carbono foram utlizadas como base para síntese de moléculas com funções alelopáticas. Desta forma, a alga parece induzir um efeito fisiológico nesta erva marinha, ao nível da utilização dos hidratos de carbono. Esta é a primeira vez que é reportado um efeito fisiológico deste tipo. Apesar de apresentar adaptações para ambientes de baixa intensidade luminosa, o avanço observado de *C. prolifera* em zonas menos profundas da Ria Formosa pode estar relacionado com a sua plasticidade morfológica e a sua resistência a intensidades mais elevadas desde que os nutrientes não são o fator limitante. Este estudo apresenta uma introdução às diferenças fotofisiológicas entre *C. prolifera* e as ervas marinhas nativas da Ria Formosa, mas deverá ser completado eventualmente com uma análise sazonal e de crescimento, para uma compreensão mais detalhada de como a *C. prolifera* interage com as ervas marinhas, tanto a nível ecológico como também a nível bioquímico.

Index

Abbreviation list

(AZ)/(VAZ): de-epoxidation index of the violaxanthin cycle pigments

(V+A+Z)/Chl: pigments of the xanthophyll cycle to chlorophyll ratio

(V+A+Z)/Lut: pigments of the xanthophyll cycle to lutein ratio

(V+A+Z): Violaxanthin + Antheraxanthin + Zeaxanthin

A: Antheraxanthin

ADP: Adenosine diphosphate

AEC: Adenylate energy charge

AL: Actinic light

AMP: Adenosine monophosphate

ATP: Adenosine triphosphate

BSA: Bovine serum albumin

Chl *a*/*b*: Chlorophyll *a*/*b* ratio

Chl *a*: Chlorophyll *a*

Chl *b*: Chlorophyll *b*

Chl T: Total chlorophyll

DW: Dry weight

F'_m: Maximum fluorescence in light acclimated leaves/fronds

F_0: Basal fluorescence

F_m: Maximum fluorescence

F_t: Steady-state level of fluorescence

F_v/F_m: Maximal quantum efficiency

HPLC: High performance liquid chromatography

I_c: Light compensation point

I_k: Half-saturating irradiance

I_{max}: Minimum photosynthetic irradiance that supports P_{max}

L: Lutein

Lx: Lutein epoxide

ML: measuring light

NPQ: Non-photochemical quenching

PAR: Photosynthetic active radiation

P_{max}: Maximum photosynthetic rate

PS_{II}: Photosystem II

PVPP: Polyvinylpolypyrrolidone

Rd: Dark respiration

rETR: Relative electron transport rate

$rETR_{max}$: Relative maximum electron transport rate

RLC: Rapid Light Curve

SP: Saturating pulse

TEA: Triethylamine

V: Violaxanthin

Z: Zeaxanthin

α: Photosynthetic efficiency

$\Delta F/F'_m$: Effective quantum yield

1. Introduction

1.1. Seagrasses: a key element in the marine environment

Seagrasses are marine flowering plants (angiosperms) that are fully adapted to the marine environment and form extensive meadows in shallow coastal waters all around the world, with exception to the polar seas (Green & Short, 2003; Orth et al., 2006). These meadows form highly complex and diverse habitats, providing important ecosystem functions, presenting high primary productivity and are the basis of many food webs, be it through herbivoral or detrital pathways (Short et al., 2011). Their high productivity has also been found to support other adjacent ecosystems such as coral reefs, mangroves and even terrestrial ecosystems, through energy and material transfers (Heck et al., 2008). Furthermore, they play an important role as nurseries for juvenile fish assemblages (Björk et al., 2008; Blandon and Zu Ermgassen, 2014), bury and store large amounts of organic carbon (Duarte, Middelburg & Caraco, 2004; Fourqurean et al., 2012), export O_2 from their leaves to their rhizomes and roots (oxygenating the sediment and stimulating microbial activity and nutrient recycling), while also promoting wave attenuation (Christianen et al., 2013) and preventing coastal erosion (Björk et al., 2008).

Despite being such an important element in the marine environment, a global decline of seagrasses has been observed. A review of 215 studies revealed that seagrass habitats declined at a rate of 110 km^2 yr^{-1} between 1980 and 2006 (Waycott et al., 2009). This rapid loss makes them one of the most threatened habitats in the world (Orth et al., 2006; Waycott et al., 2009). The only known exceptions to this alarming trend are the recently reported signs of seagrass stabilization and recovery in Europe (de los Santos et al., 2019). Seagrass loss can occur both naturally or due to anthropogenic disturbances, which are among the main causes of seagrass loss. Dredging, eutrophication, nutrient loading, land reclamation and shoreline development are examples of human activities that, besides increasing seawater turbidity, negatively affect seagrass meadows (Duarte, 2002; Erftemeijer & Lewis, 2006; Waycott et al., 2009). Indirect impacts, affecting seagrasses globally include: i) sea level rise, ii) increased wave action and storms and iii) increased sea temperature (Duarte, 2002). Natural losses of seagrasses can also be caused by other macrophytes that compete with them for light and nutrients. Replacement of slower growing seagrass species, like *Posidonia*

oceanica, by faster growing species like *Cymodocea nodosa* has already been observed in the Mediterranean sea (Burgos et al., 2017). The same applies for fast growing algae as for example, species of the genus *Caulerpa*, which are known to rapidly propagate in areas occupied by seagrasses (Lloret et al., 2005; Holmer et al., 2009; García-Sánchez et al., 2012; Tuya et al., 2013).

1.2. Invasive marine macroalgae

Invasive species are species that due to their high dispersion capability have a negative effect in the environment in which they are introduced (William & Smith, 2007). But before it can be considered invasive, the species has to be capable of reproducing in large numbers and show potential to spread over large distances (Richardson, Pyšek & Carlton, 2011). Their ecological role can be debated, as their dominance can be due to their highly interactive, competitive and limiting nature ("driver" model) or depend on non-interactive factors (e.g. environmental changes) which are less constraining on the invasive species, hence their domination (MacDougall & Turkington, 2005).

In the marine environment, invasive species pose a serious threat, affecting and altering entire ecosystems using a variety of mechanisms and leading to a wide range of consequences (Ehrenfeld, 2010). Among the many invasive species that can threaten the marine environment, a number of them are opportunistic macroalgae. Their colonization can affect the surrounding environment and lead to a significant loss of native species and diversity (Stæhr et al., 2000; Sanchez et al., 2005; Scheibling & Gagnon, 2006; William & Smith, 2007). Increased pressure exerted by certain macroalgae can negatively affect seagrass meadows as they compete for the same resources such as space, light and nutrients. For example, the green algae *Enteromorpha radiata* spread through subtidal *Zostera marina* and *Zostera noltii* meadows in northern Europe, and their decrease was assumed to be caused by either shading or anoxic conditions caused by the algae matts (Den Hartog, 1994). Another example is *Codium* sp., which have proven to be very successful invaders, exhibiting high levels of competition with not only native seaweed species (Scheibling & Gagnon, 2006; Williams & Smith, 2007) but also with seagrasses as *Codium* sp. is able to attach epiphytically to seagrasses, potentially disrupting them (Drouin, McKindsey, and Johnson,

2012). Also within the Chlorophyta we can find the genus *Caulerpa*, containing some of the most notorious invasive species that affect seagrass meadows. They can rapidly propagate in areas occupied by seagrasses and compete with them on different levels, threatening seagrass meadow stability (Lloret et al., 2005; García-Sánchez et al., 2012; Tuya et al., 2013).

Caulerpa taxifolia, for example, is a notorious, fast-spreading invasive algae that successfully invaded the Mediterranean Sea after an accidental introduction (Meinesz & Hesse, 1991), among other marine environments (Schaffelke, Murphy & Uthicke, 2002). It spread along native *Posidonia oceanica* meadows, directly affecting them by limiting light resources (de Villèle & Verlaque, 1995). *Caulerpa racemosa* also successfully invaded the Mediterranean Sea where it was found along *Cymodocea nodosa* meadows (Raniello et al., 2004) and certain *P. oceanica* meadows, depending on its state and integrity (Klein & Verlaque, 2008; Checcherelli et al., 2014). Furthermore, invaded assemblages containing encrusting algae were especially affected by *C. racemosa* (Piazzi & Balata, 2009), related to the wide mats created by their stolons, limiting light, trapping and reducing the lower vegetative habitat (Piazzi et al., 2007).

Furthermore, *Caulerpa* sp. can indirectly affect seagrass meadows by occupying space previously occupied by the latter. In other words, seagrass regression can consequently lead to the replacement of seagrass meadows by macroalgae such as *Caulerpa* sp. (Lloret et al., 2005; Pérez-Ruzafa et al., 2012). This has been recorded, for example, in Mar Menor lagoon (Spain), where reduction and restriction of *C. nodosa* onto shallower sandy patches occurred (Pérez-Ruzafa et al., 2005; García-Sánchez et al., 2012;), having almost three-quarters of the area been replaced by *C. prolifera* (García-Sánchez et al., 2012). This can result in the deterioration of the seafloor, with the accumulation of organic matter that induces anoxic sediment conditions. In turn, this inhibits the settlement of other flora and fauna, negatively influencing species composition and diversity, with a broad range of ecological and economic implications, namely by affecting local fishing activities (Lloret et al., 2005; Pérez et al., 2006; York et al., 2007).

1.3. Seagrasses versus algae: A physiological comparison

In a generic physiologically comparison between seagrasses and macroalgae, it is apparent that, although the distribution of both groups is limited by light availability (Häder et al., 1996; Kenworthy & Fonseca 1996; Lloret et al., 2005), seagrasses have higher minimum light requirements compared to that of macroalgae (Abal et al., 1994; Duarte, 1995), varying between species (Abal et al., 1994; Campbell et al., 2008). Seagrasses can nevertheless survive in low light conditions or even light deprivation, but their survivability, i.e. the ability to use non-structural accumulated carbon, reducing growth rates and increasing their photosynthetic efficiency, depends on the species and ambient conditions (O'Brien et al., 2018). The increase in photosynthetic efficiency is related to the ability of macrophytes, including seagrasses, to adjust their pigment levels and ratios (Riechert & Dawes, 1986; Silva et al., 2013). Their efficiency and resilience, i.e. the capacity to undergo disturbance without the loss of key structures and functions, varies among species (Silva et al., 2013; O'Brien et al., 2018). Furthermore, if light levels fall below the required threshold, severe seagrass loss may be observed (Collier, Waycott, & McKenzie, 2012).

On the opposite end, seagrass photo-protection mechanisms enable these plants to also withstand high light levels. This ability is related to the xanthophyll cycle, in which the concentration of three pigments, violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) (VAZ pigments), are adjusted according to environmental light conditions. Higher light intensities can induce photo-oxidative stress, caused by the formation of reactive oxygen species. The reversible de-epoxidation of the VAZ cycle pigments, mainly V into Z through A, play an important role in the dissipation of excess excitation energy as heat and are involved in the conversion of the light harvesting state of photosystem II (PS_{II}) into an energy dissipating state (Jahns, Latowski & Strzalka, 2009; Jahns & Holzwarth, 2012). The amount of VAZ pigments and the ability to interconvert them under different light intensities depends on the individual plant adaptation (Adams & Demmig-Adams, 1996; Larkum, Drew & Ralph, 2006).

The morphology and photosynthetic performance of species of the genus *Caulerpa* depend largely on the environmental conditions (Collado-Vides & Robledo, 1999). They present remarkable plasticity regarding photosynthetic traits, modifying their pigment pool

in order to adjust the photosynthetic efficiency (Raniello et al., 2004). Just as in seagrasses, VAZ pigments can be found in the genus *Caulerpa*, however the amount of each individual pigment is species and light dependent (Raniello et al., 2006; García-Sánchez et al., 2012). Additionally, the genus *Caulerpa* presents two additional carotenoids: siphonaxanthin and siphonein, the former presenting a role in the adaptation to deeper and darker environments in *C. racemosa* (Raniello et al., 2006; García-Sánchez et al., 2012). *C. prolifera* has been proven to act as a shade-adapted algae, presenting a high quantum efficiency (α) and a low saturation irradiance (Terrados & Ros, 1992). Additionally, it was found that in the same environment, *C. prolifera* had a lower maximum Electron Transport Rate (ETR_{max}), yet almost double the photosynthetic efficiency (α) when compared to *C. nodosa* (García-Sánchez et al., 2012). Furthermore, it has the capability to acclimate to different light environments due to a photoprotective mechanism that was found to depend on nutrient availability (Malta et al., 2005). Being a rhizophitc algae, however, *Caulerpa* sp. has access to nutrients not only from the water column, but also from the sediment porewater, thus competing with seagrasses not only above, but also below the substrate (William, 1984). The combination of high growth rates, low light requirements, various photoprotective mechanisms and the capability of occupying different substrates makes *Caulerpa* sp. strong seagrass competitors.

Recognizing the invasive potential held by species of the genus *Caulerpa* and the effect they may have on native species, the need emerges to understand how they will act in different environments and the impact they may have. *Caulerpa prolifera* was "re-discovered" in Ria Formosa (Cunha et al., 2013), and has since spread all over the system, occupying deeper channels and slowly spreading toward shallower subtidal areas, where it possibly interferes with native seagrass meadows. While the distribution and physiological performance of the seagrasses has been extensively studied, little to no information exists regarding the physiology of *Caulerpa prolifera* in Ria Formosa.

1.4. Main objectives

The main goal of this thesis is to investigate the compared physiological performance of *Caulerpa prolifera* and the native seagrasses *Z. marina* and *C. nodosa* in Ria Formosa, in

order to understand its different photophysiological and biochemical traits, while also elucidating the potential inter-specific physiological interactions.

2. Material and Methods

C. nodosa, Z. marina and *C. prolifera* co-occur in shallow subtidal ranges of Ria Formosa. The physiological performance of these three species was analyzed under the identical conditions and the interaction between *C. prolifera* and *Z. marina* was also investigated in a mesocosm experiment in controlled conditions.

In a semi-field experiment, ten leaves of each species were marked, and photosynthetic performance was evaluated during 48-hour using chlorophyll fluorescence. Plant tissue samples were collected at 4:00 (pre-dawn) and 12:30 (solar noon) for biochemical analysis (photosynthetic pigments, soluble carbohydrates, soluble protein and ATP, ADP and AMP).

To analyze the interaction between *C. prolifera* and *Z. marina*, a mesocosm experiment was conducted at the Ramalhete Experimental Station (CCMAR). Individuals of both species were collected and placed in tanks under ambient temperature and controlled light conditions. Each species was planted both separately, 30 shoots of *Z. marina* and patches of *C. prolifera*, and mixed, 21 shoots of *Z. marina* and smaller patches of *C. prolifera*, in 15 different tanks of 65L each (n=5). Chlorophyll *a* fluorescence measurements were done daily to monitor the performance of *Z marina* and *C. prolifera*. After 4 weeks in the mesocosm, leaf/frond and rhizome samples were taken for biochemical analysis (photosynthetic pigments, soluble carbohydrates, soluble protein and ATP, ADP and AMP). Rapid light curves were performed and leaf /frond samples were also collected for photosynthesis-irradiance (P-I) curves.

2.1. Site characterization

The Ria Formosa coastal lagoon, located in the south of Portugal, is delimited by a highly dynamic barrier-island system. It is connected to the Atlantic Ocean through a series of natural and artificial inlets. Although located along the Atlantic Ocean, Ria Formosa has a Mediterranean climate. However, in contrast to Mediterranean lagoons and their microtidal regime, Ria Formosa displays a mesotidal regime, with amplitudes ranging from 1.35m (neap

tides) to 3m (spring tides) (Newton & Mudge, 2003). The open coastal seawater temperature varies between 12-26ºC, while the inner lagoon water can reach up to almost 30ºC during the summer. Salinity varies seasonally, with values recorded as low as 26.8 in mid-autumn, and as high as 36.15 in the summer, with higher values recorded at the saltpans (Newton & Mudge, 2003). The lagoon is characterized by its shallow channels, mudflats and salt marches (Cunha et al., 2013) and is a highly productive system, supporting different habitats and a wide variety of fauna and flora (Falcão & Vale, 1990; Newton & Mudge, 2003). Three of the four European seagrass species can be found in Ria Formosa: *Zostera noltii*, *C. nodosa* and *Z. marina* (Cunha et al., 2011). *Z. noltii* mainly resides in intertidal areas but can also inhabit subtidal flats (Borum et al., 2004; Moore & Short, 2006). No information has been found regarding *C. prolifera* occupying intertidal seagrass meadows, therefore *Z. noltii* was not used as a model species in this work.

2.2. Species: a brief introduction

2.2.1. *Cymodocea nodosa*

C. nodosa is widely distributed across the Mediterranean (Mascaró et al., 2009), including Southern Portugal (Cunha and Duarte, 2007; Cunha et al., 2011) and part of the adjacent Eastern Atlantic Ocean, including the Macaronesian archipelagos of Madeira and the Canaries (Mascaró et al., 2009; Tuya et al.,2013). It can be found in depths ranging from the shallow subtidal to deeper waters (50-60m). *C. nodosa* shoots contain 2-5 leaves that can reach up to 50 cm in length. Each shoot is connected to the horizontal rhizome via a vertical segmented rhizome. These rhizomes can grow meters per year, and this species can therefore be considered a pioneer, capable of colonizing vast areas of the sea floor (Borum et al., 2004). In Ria Formosa, *C. nodosa* extends through the edges of the main and secondary channels, covering an area of almost 1 km^2 (Cunha et al. 2011). Since *C. nodosa* is often nutrient limited (Pérez et al., 1994), the increased supply of nutrients in Ria Formosa (Falcão & Vale, 1990) allows high growth and production rates. Nevertheless, declines in *C. nodosa* meadows can occur, mainly due to increases in water turbidity and successive occupation by macroalgae (Lloret et al., 2005; García-Sánchez et al., 2012; Tuya et al., 2013).

2.2.2. *Zostera marina*

Z. marina is distributed along the coasts of the Northern Hemisphere (Green & Short, 2003), including colder Norwegian waters and can also be found in the Mediterranean (Borum et al., 2004). Although it mainly forms mono-specific meadows, it can co-occupy substrate with other seagrasses, e.g. *Z. noltii* and *C. nodosa*. Its vertical distribution ranges from its predominant shallow subtidal habitat to 10-15 meters in depth, depending on water clarity (Borum et al., 2004; Cunha et al., 2011). *Z. marina* shoots contain 3-7 leaves, generally 30-60 cm length that can grow up to 150 cm if certain conditions are met. The leaves grow from a terminal shoot, that is connected to the horizontal rhizome. For each new leaf produced, a new internode is formed and two bundles of roots grow from the node between each segment (Borum et al., 2004). In Ria Formosa, it occupies the least area when compared with the other two seagrass species (0.05km^2) and its patches seem to be declining (Cunha et al., 2011), maybe due to its high susceptibility to light reduction (Silva et al., 2013).

2.2.3. *Caulerpa prolifera*

Caulerpa spp., including *C. prolifera*, are one the most differentiated single-celled organisms, as no cell wall or membrane separates the many nuclei and cytoplasm (Jacobs, 1994). *C. prolifera* (Forsskål) J. V. Lamouroux is present in most of the Mediterranean (Cunha et al., 2013; Mateu-Vicens et al., 2010; Tuya et al., 2013), and distributed among the tropical and subtropical Atlantic Ocean areas (Cunha et al., 2013; Tuya et al., 2013). Its growth and distribution are temperature limited (Mateu-Vicens et al., 2010; García-Sánchez et al., 2012) and it prefers sheltered areas, with reduced hydrodynamics and water renewal with a considerable amount of organic matter available (Sánchez-Moyano et al., 2001). It usually forms dense meadows in shallow waters (1-20 meters) on both soft bottoms, such as sand or mud, and on hard substrates like rock (Terrados and Ros 1995; Sánchez-Moyano et al., 2001). It forms fronds, stems and rhizoids, that contrarily to other multicellular plant organisms, elongate at a constant rate. The roots and the fronds are formed at regular intervals, the latter forming more often than the former (Jacobs, 1994).

2.3. Diel photophysiology of *Zostera marina, Cymodocea nodosa* and *Caulerpa prolifera*

The diel cycle experiment was done, in May 2019, with *C. prolifera*, *Z. marina* and *C. nodosa*, as these are the two seagrass species that can be affected by *C. prolifera* in Ria Formosa. *Z. noltii* was not selected as up to today there is no record of *C. prolifera* interfering with this species, mainly due to its intertidal distribution. *Z. marina*, *C. nodosa* and *C. prolifera* were collected near Ilha da Culatra (37°00'07.8"N 7°49'22.6"W). The individuals were carefully collected and transported to the Ramalhete field station (CCMAR) in the dark in tanks filled with water from Ria Formosa. Three cylindrical tanks were placed into 5 larger tanks as shown in figure 2.1A, resulting in a total of 15 cylindrical tanks. The bottom of each cylindrical tank was covered with ca. 7 cm of sand and filled with water from the Ria Formosa, previously filtered with sand and UV-filters.

Seagrasses and algae were then randomly assigned to each cylindrical tank, in which they were then carefully planted in the sand as follows (Fig. 2.1A):

A. *Z. marina*: 21 shoots evenly spread in each tank;
B. *C. nodosa*: 25 shoots evenly spread in each tank;
C. *C. prolifera*: 2-3 patches each tank, gently pressed against the sand to bury rhizoids, almost completely covering the available sediment.

After one day of acclimation, two leaves (mature zone of the second and third leaf of the shoot) an two healthy fronds were selected in each tank and marked with a clamp, to ensure that the same section was measured every time (Fig. 2.1B). A 48-hour cycle was performed during which fluorescence data was collected to access the diel cycle of effective quantum yield ($\Delta F/F'_m$) and non-photochemical quenching (NPQ). Chlorophyll *a* fluorescence measurements were done hourly from 14:00 to 21:00 of day 1, and after 3:00 until 14:00 of day 2 (maximal fluorescence during the night and effective quantum yield when there was light). All measurements were done with a Diving-PAM (Underwater Fluorometer Pulse-

Amplitude-Modulated (PAM), Heinz Walz GmbH, Germany) under ambient irradiance and temperature.

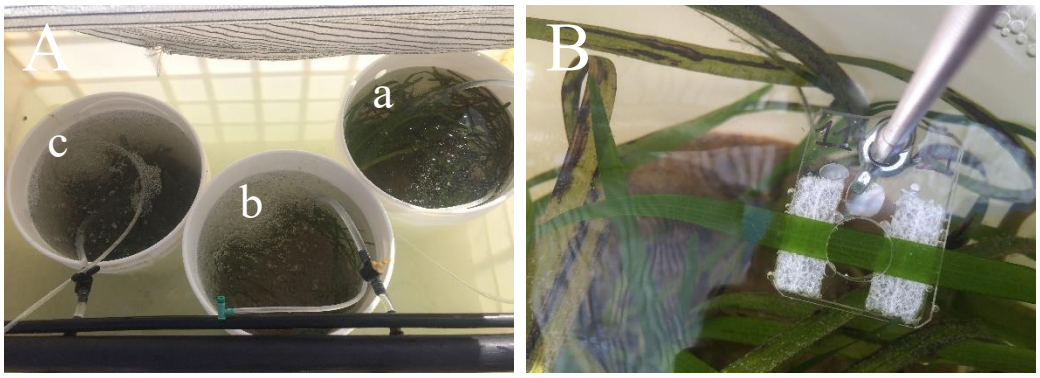

Figure 2.1: (A) Tanks setup during the diel cycle of (a) Z. marina, (b) *C. nodosa* and (c) *C. prolifera*; (B) Clamp designed to ensure the correct measurements of the chlorophyll *a* fluorescence parameters

Samples were taken for biochemical analysis (photosynthetic pigments, soluble carbohydrates, soluble protein and ATP, ADP and AMP) at 4:00 (pre-dawn) and 12:30 (solar noon). Samples were taken from the same kind of tissue that was being used for fluorescence measurements, i.e., the middle mature part of the second and third leaf of seagrasses and healthy fronds of *C. prolifera*. Seagrass rhizomes were also sampled after roots removal. *C. prolifera* fronds were removed at the stolon, and then rapidly processed to avoid loss of cellular content. All samples were quickly washed in distilled water, dry blotted, frozen in liquid nitrogen and stored at -80°C until analysis.

2.4. *Z. marina* and *C. prolifera*: An interaction mesocosm experiment

Z. marina and *C. prolifera* individuals were collected near Ilha da Culatra, Faro (37°00'07.8"N 7°49'22.6"W) in the end of May 2019. Leaf/frond samples were taken from both species (Field samples) rinsed in distilled water, blotted dry, frozen in liquid nitrogen and stored at -80°C for future analysis. The individuals were carefully collected and transported, in the dark in tanks filled with water from Ria Formosa, to the Ramalhete field station (CCMAR) where, after carefully cleaned, were planted in an open-mesocosm system. The bottom of fifteen tanks (65L) were covered with 8cm of sand and filled with water from Ria Formosa after filtering with sand and UV-filters.

The 15 tanks were set up side by side (Fig. 2.2), and each treatment was randomly assigned:

A. *Z. marina*: 32 shoots evenly spread in each tank;
B. *C. prolifera*: enough biomass to cover the bottom of the tank but avoiding overcrowding;
C. *Z. marina* + *C. prolifera:* 20 shoots of *Z. marina* mixed with patches of *C. prolifera.*

Figure 2.2: Partial view of the mesocosm system in the Ramalhete field station (CCMAR).

The plants were subjected to a constant light intensity (± 210 $\mu mol_{photons}\, m^{-1}s^{-}1$), for 13 hours per day (8:00-21:00). The water was continuously pumped from Ria Formosa into cylindrical head tanks with at a flow of 50 liters per hour. In the head tanks, the water was mixed with air before entering the tanks where the seagrasses/algae were planted. Each tank had its own head tank. In order to avoid epiphyte accumulation, one treatment (5 tanks) was cleaned per day. Both species spent a total of 4 weeks in these tanks during which maximum quantum efficiency (F_v/F_m) was measured daily to monitor the physiological performance of the seagrasses/algae. At the end of the experiment, two leaves/fronds from each tank were selected to perform rapid light curves (RLC). Furthermore, leaf and frond samples were taken for photosynthesis-irradiance (P-I) curves. Finally, samples were collected for biochemical analysis. The middle mature part of the second and third leaf of *Z. marina* were collected, quickly washed in distilled water, dry blotted, frozen in liquid nitrogen and stored at -80°C until analysis. Seagrass rhizomes were also sampled and, after roots removal, cleaned and processed the same way as the leaves. *C. prolifera* fronds were collected in small patches,

cleaned, washed in distilled water, carefully blot dried, cut at the stolon, frozen in liquid nitrogen and stored at -80°C until analysis.

2.5. Chlorophyll fluorescence – Basic concepts

Chlorophyll fluorescence parameters were measured using a Diving-PAM (Underwater Chlorophyll Fluorometer Pulse-Amplitude-Modulated (PAM), Walz, Germany). These parameters allowed us to monitor and assess the photosynthetic performance of the different species, determining the effective quantum yield of photosystem II ($\Delta F/F'_m$) and their respective non-photochemical quenching (NPQ), and allowed us to develop rapid light curves (RLC).

Chlorophyll fluorescence has been widely used to study photosynthetic organisms. Its use relies on basic principles related to the mechanisms of chlorophyll excitation and de-excitation and fluorometers such as the Pulse-Amplitude-Modulated (PAM) -fluorometers have been used to study the chlorophyll responses to light. In the photosynthetic apparatus, the chlorophyll *a* of the reaction centers becomes excited after the absorption of incident photons and the transfer of their excitation energy by the antenna pigments to the reaction centers. After excited, chlorophyll relaxes by three different pathways: 1) photochemical, i.e. the use of the excitation energy for photochemistry, 2) thermal dissipation, where the excitation energy is dissipated as heat, and 3) chlorophyll fluorescence, where the excitation energy is re-emitted as light of a higher wavelength than that captured (fluorescence). The principle of conservation of energy states that energy is neither created, nor destroyed, rather, conserved and thus these pathways constantly balance with each other, meaning that if the efficiency of one of them increases, it will be at the cost of the others (Roháček & Barták, 1999; Maxwell & Johnson, 2000).

When the photosynthetic apparatus is exposed to a saturating flash (0.8 s, 2000–3000 $\mu mol_{photons}\ m^{-2}s^{-1}$), fluorescence yield peaks and then slowly declines. Fluctuations in fluorescence signals are known as the "Kautsky curve" (Fig. 2.3) and various points along this curve allow the insight on dynamic changes in photosynthesis or photokinetics (Larkum, Drew & Ralph, 2006).

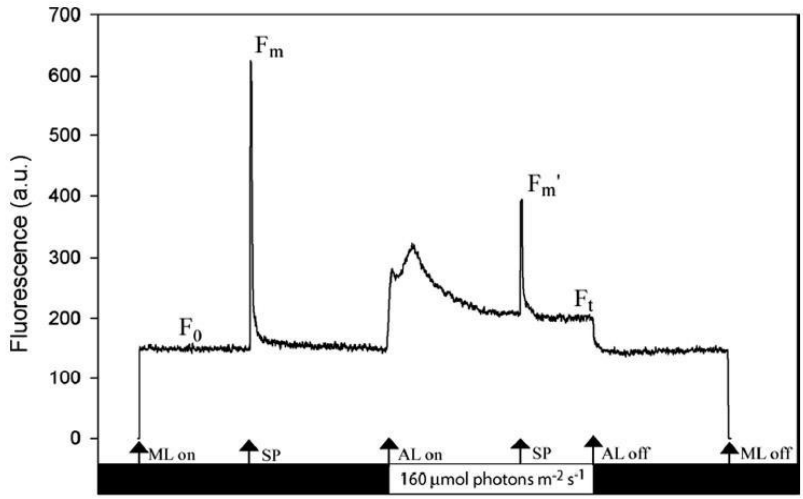

Figure 2.3: Kautsly curves are the representation of the chlorophyll fluorescence induction kinetics as measured in light and dark-adapted seagrass leaves. ML: measuring light, SP: saturating light pulse, AL: actinic light, F_o and F_m: minimum and maximum chlorophyll fluorescence (dark-adapted leaves) induced by ML and SP, respectively, F'_m: maximum chlorophyll fluorescence in light-adapted leaves (induced by SP after AL on); F_t: chlorophyll fluorescence level induced by non-saturating irradiation (adapted from Larkum, Drew & Ralph, 2006).

The maximum quantum efficiency (F_v/F_m) is determined in dark adapted photosynthetic apparatuses after obtaining F_0 (basal fluorescence) and F_m (maximum fluorescence). After being in the dark for a certain period of time, that should be long enough to guarantee that all the reaction centers are open. In this situation, the electron transport chain is oxidized, any photoprotective mechanism (such as the xanthophyll cycle) is relaxed and there is the depletion of the *trans*-thylakoid gradient. Fluorometers emit a measuring light (ML), which induces fluorescence but is not strong enough to start the electron transport chain. The resulting fluorescence is called the basal fluorescence (F_0). Next, a pulse of saturating light is applied for a short period of time, allowing the measurement of the maximal level of fluorescence (F_m) (Ralph & Gademann, 2005). F_v/F_m is determined from the following equation:

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m}$$

Equation 1: Maximal photochemical efficiency of PS_{II} **(F_v/F_m). F_m:** maximum chlorophyll fluorescence in the dark-adapted state, induced by a saturating-light pulse; **F_0:** basal chlorophyll fluorescence in the dark adapted state, induced by weak red pulses (measuring light); **F_v:** variable chlorophyll fluorescence (F_m-F_0) (adapted from Larkum, Drew and Ralph, 2006).

Estimating the effective quantum yield ($\Delta F/F'_m$) requires that the photosynthetic apparatus is adapted to light. The saturating light pulse is applied allowing the measurement of the plant's maximum fluorescence in light (F'_m). Prior to the saturating light pulse application and after quenching, fluorescence presents a steady state value (F_t) (Larkum, Drew & Ralph, 2006). Effective quantum yield of PS_{II} ($\Delta F/F'_m$) is calculated as in equation 2:

$$\frac{\Delta F}{F'_m} = \frac{F'_m - F_t}{F'_m}$$

Equation 2: Effective quantum yield of PS_{II} ($\Delta F/F'_m$). **F'_m:** maximum chlorophyll fluorescence induced by a saturating-light flash, in the light adapted state; **F_t:** steady state level of chlorophyll fluorescence induced by ambient light in the light adapted state; **ΔF:** fluorescence spike on top of the actinic light-induced fluorescence kinetic (F'_m -F_t) induced by a saturating-light flash; (adapted from Larkum, Drew and Ralph, 2006).

Non-Photochemical Quenching (NPQ) is calculated as in Equation 3, and is related to the photoprotective mechanisms that are used to dissipate excess excitation energy (Larkum, Drew & Ralph, 2006). NPQ calculation is done using pre-dawn values of F_m, obtained after several hours of darkness (Maxwell & Johnson, 2000).

$$NPQ = \frac{F_m - F'_m}{F'_m}$$

Equation 3: Non-photochemical quenching (**NPQ**). **F_m:** Maximum chlorophyll fluorescence in the dark-adapted state, induced by a saturating-light pulse; **F'_m:** maximum chlorophyll fluorescence induced by a saturating-light flash, in the light adapted state; (adapted from Maxwell & Johnson, 2000).

In this work, all the chlorophyll fluorescence measurements were done with a diving-PAM (Underwater Chlorophyll Fluorometer Pulse-Amplitude-Modulated (PAM), Walz, Germany)

2.6. Light response curves

2.6.1. Photosynthesis-Irradiance curves

Photosynthesis-irradiance (P-I) curves (Fig. 2.4), are the graphical representation of the relationship between photosynthesis and irradiance and provide valuable information about the photo-physiological fitness of photosynthetic organisms. These curves allow the determination of several important photosynthetic parameters such as the photosynthetic efficiency (α), measured at the initial slope of the light response where light is limiting for photosynthesis, the maximum photosynthetic rate at saturating irradiance (P_{max}), the half-saturating light intensity (I_k), at which the onset of saturation occurs, and the light compensation point (I*c*), at which net photosynthesis is zero (Gilbert, Wilhelm & Richter 2000).

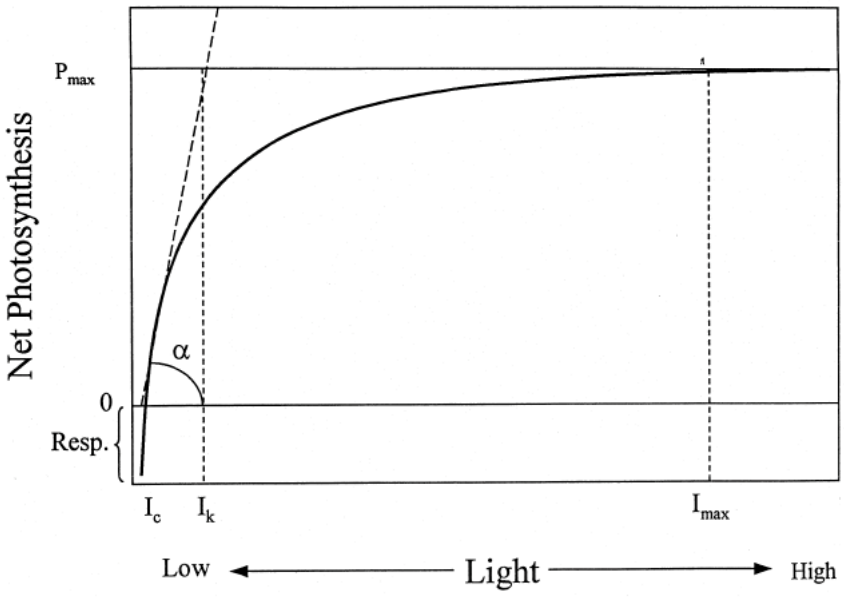

Figure 2.4: Theoretical photosynthesis–irradiance (P–I) curve, where P_{max} is the maximum photosynthetic rate, I_{max} is the minimum photosynthetic irradiance that supports P_{max}, I_k is the half-saturation irradiance, I_c is the light compensation point, and α is the photosynthetic efficiency (adapted from Touchette & Burkholder, 2000)

Photosynthetic rates and dark respiration were measured in cleaned leaf segments of *Z. marina* and fronds of *C. prolifera* (ca. 50 cm^2). Second or third leaves of *Z. marina* were selected and cut into smaller section in order to fit in the incubation chamber. Because *C. prolifera* is a single-celled organism, fronds could not be cut and had to be selected according to size, selecting the largest fronds that would still fit the chamber. Fronds were removed

from the stolon, and a tourniquet was placed at the point where the frond meets the stolon to prevent any loss of internal structures and organelles (Jacobs, 1994). 70 ml of filtered water were taken from the same tanks where the leaves/fronds came from, and placed in incubation chambers filling the chamber completely. Foliar tissue/fronds were put into the water and the incubation chamber was carefully closed to avoid the formation of any gas bubble. Oxygen microsensors were placed on the lid of each chamber, with easy access for the oxygen probe. Oxygen concentration in the water was determined with a PreSens Precision Sensing Microx 4 Microsensor Oxygen meter and probe (PreSens Precision Sensing GmbH, Regensburg, Germany). After being in the dark for, at least, 30 min, the leaves/fronds dark respiration was measured. Then, leaves/fronds photosynthesis was measured under ten increasing light intensities, ranging from 7 to 1340 $\mu mol_{photons}\, m^{-2}s^{-1}$, for approximately 10 min each. Light was provided by a halogen light source, and the different light intensities were obtained using combinations of neutral density filters. After being exposed to the highest light intensity, leaves were again placed in the dark for at least 10 min to measure dark respiration. During all this procedure the water temperature in the chambers was maintained at 22ºC.

Photosynthetic/respiration rates were determined as follows:

$$X = \frac{\frac{\text{Final } [O_2] - \text{Initial } [O_2]}{t \times 60} \times V}{DW}$$

Equation 4: Calculation of the photosynthetic/respiration rates. **X:** Photosynthetic/respiration rate ($\mu molO_2\ gDW^{-1}\ h^{-1}$); **Final [$O_2$]:** dissolved O_2 concentration (µmol/L) measured at the end of the incubation time **t** (minutes); **Initial [O_2]:** dissolved O_2 concentration (µmol/L) measured at the beginning of the incubation; **DW:** leaf or frond dry weight (g); V: volume of water in the chamber (L)

The maximum photosynthetic rate (P_{max}) and the efficiency of light utilization (α) (Ralph et al., 2002) were calculated from the Smith and Talling model (Smith, 1936; Talling, 1957) which gave the best fit from all the mathematical models tested (Henley, 1993; Platt et al. 1980). Half-saturating irradiance (I_k) was obtained from the ratio between P_{max} and α.

2.6.2. Rapid light curves

Rapid light curves (RLC) plot the relative electron transport rate (rETR) (i.e. the rate at which electrons are pumped through the photosynthetic electron transport chain) as a function of the photosynthetically active radiation (PAR). rETR is calculated using the following equation (Ralph & Gademann, 2005):

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

Equation 4: Calculation of the relative electron transport rate (**rETR**). **$\Delta F/F'_m$:** effective quantum yield; **PAR:** photosynthetically active radiation.

RLCs were done on healthy and undamaged previously marked second or third leaves of *Z. marina* and larger fronds of *C. prolifera* that presented a homogenous cellular distribution, i.e. that did not have any white or transparent sections (n=10). Leaves/fronds were exposed to nine consecutive light levels: 6, 22, 51, 97, 146, 204, 310, 421 and 560 $\mu mol_{photons}.m^{-2}.s^{-1}$. Effective quantum yield was measured (Diving-PAM, Underwater Chlorophyll Fluorometer Pulse-Amplitude-Modulated (PAM), Walz, Germany) and rETR was calculated, for each light level, as in Equation 4.

The maximum relative electron transport rate ($rETR_{max}$), and the efficiency of light utilization (α) (Ralph et al., 2002) were calculated from the Platt, Gallegos & Harrison (1980) model which gave the best fit from all the mathematical models tested (Henley, 1993; Platt, Gallegos & Harrison, 1980) as it is one of the only models that accounts for photo-inhibition. I_k was calculated as the ratio between $rETR_{max}$ and α.

2.7. Biochemical analysis

2.7.1. Soluble sugars and starch

Soluble sugars and starch were extracted from 65 mg of both leaf/frond and rhizome samples, grounded in liquid nitrogen. Sugars were extracted in 10 mL of ethanol 80% at 80ºC during 30 minutes. The extract was then centrifuged (5 minutes, 2000 rpm, Thermo Fisher Scientific Heraeus Megafuge, 16R, U.S.A). The supernatant was used to quantify total soluble sugars and the pellet was used to quantify starch.

Total soluble sugars were quantified by the phenol-sulfuric assay, using glucose as a standard (adapted from Dubois et al., 1956). 1 mL of the supernatant was carefully mixed with 1mL of 5% phenol and 5 mL of sulfuric acid (95%) This mixture was then agitated and cooled down to room temperature and read at 490 nm and 750 nm (Beckman-Coulter DU650 spectrophotometer, U.S.A)

Starch was determined in the pellet which was previously washed in three successive resuspensions in 1 ml Mili-Q water and centrifugations (12000 rpm, 2 minutes, LabNet hermle Z233 MK Refrigerated Microcentrifuge, MA, U.S.A). After the last cycle of resuspension/centrifugation, the supernatant was removed, 1mL of water was added to the pellet, and this suspension was incubated in a water bath at 100˚C during 5 minutes. Then, 100 µL of the suspension were added to 500 µL of an enzymatic suspension containing α-amilase (4 U/mL, Roche 102 814) and amiloglucosidase (2.8 U/mL, Roche 102 857). This mixture was centrifuged (12000 rpm, 2 minutes, LabNet hermle Z233 MK Refrigerated Microcentrifuge, MA, U.S.A) and the resulting suspension was then processed as described above for the soluble sugars.

2.7.2. Soluble protein

Soluble protein was determined by a semiquantitative method where the protein concentration in the samples is calculated from a calibration curve made with known concentrations of Bovine Albumin Serum (BSA) where Coomassie Brilliant Blue G-250 (Bio-Rad) is added to both the samples and BSA standards (Bradford, 1976).

A stock solution of BSA (10 mg mL^{-1}) was prepared in Mili-Q water and diluted to obtain standards with 0, 2, 4, 6 and 8 µg.mL^{-1} of BSA. 200 µL of Coomassie Brilliant Blue G-250 was added to 800 µL of each standard.

Soluble protein was extracted from 150 mg (fresh weight) of leaf/frond or rhizomes that were ground in liquid nitrogen and polyvinylpolypyrrolidone (PVPP). 1500 µL of extraction buffer (potassium phosphate 100mM pH 7.8, triton-x 2%, DTT 1mM, PMSF 1mM) was then added and the extract was homogenized in a vortex. The homogenate was centrifuged (12000 rpm during 1 min, at 4˚C, LabNet hermle Z233 MK Refrigerated Microcentrifuge, MA,

U.S.A), and 4 µL of the supernatant were added to 796 µL of Mili-Q water and 200 of Coomassie Brilliant Blue-250 (Bio-Rad).

After 10-30 minutes of incubation, both the standards and the samples were read at 750 nm and 595 nm (Beckman - Coulter DU650 spectrophotometer, U.S.A). The difference of the absorbances at 595 and 750 nm was calculated to discard the absorbance due turbidity. A linear regression equation obtained from the standards was used to calculate the concentration of soluble protein in each sample.

2.7.3. Adenylate compounds and adenylate energy charge (AEC)

ATP (adenosine triphosphate), ADP (adenosine diphosphate) and AMP (adenosine monophosphate) were quantified by High Performance Liquid Chromatography (HPLC) according to Padinha et al. (2000) and Coolen et al. (2008). 500 mg of leaves/fronds or rhizomes were extracted in 10 mL of perchloric acid ($HClO_4$) 0.6M. After homogenization, the extracts were heated in a water bath at 100ºC during 10 minutes and then cooled in ice during 1 minute and centrifuged (4600xg, 4ºC, 30 minuntes) (Thermo Fisher Scientific Heraeus Megafuge, 16R, U.S.A). The pH of the supernatant was then adjusted to 6.5, with potassium hydroxide (KOH) 1.0M. The final volume was measured and the extracts were placed in an ice bath during 30 min, to ensure the precipitation of all the potassium perchlorate ($KClO_4$) formed. After the precipitation was concluded, 2 ml of the extract were filtered through a 0.22µm porosity hydrophobic PTFE filter and the concentrations of ATP, ADP and AMP in the samples were determined at 254 nm by HPLC (Alliance Waters separation module 2690, MA Milford, U.S.A, and Alliance Waters Photodiode Array Detector). ATP, ADP and AMP in the samples were quantified after the HPLC calibration with known concentrations of the pure commercially available compounds.

Adenylate energy charge (AEC), which reflects the availability of chemical energy, was calculated as in Equation 5 (Larcher, 2003):

$$\mathrm{AEC} = \frac{[\mathrm{ATP}] + \frac{1}{2}[\mathrm{ADP}]}{[\mathrm{AMP}] + [\mathrm{ADP}] + [\mathrm{AMP}]}$$

Equation 5: Calculation of the adenylate energy charge (**AEC**). **ATP, ADP, AMP:** ATP, ADP and AMP concentration, respectively

2.7.4. Photosynthetic pigments

Frozen frond/leaf samples of *C. prolifera*, *C. nodosa* and *Z. marina* (0.20g – 0.24g) were ground in liquid nitrogen and with sodium ascorbate to avoid pigment degradation. The photosynthetic pigments were then extracted using 5mL of 100 % acetone buffered with calcium carbonate, and the extract was filtered using a 0.45 µm hydrophobic PTFE filter followed by a 0.22 µm hydrophobic PTFE filter. The filtered extracts were then stored in the dark at -20˚C until analysis.

All the extracts were analyzed spectrophotometrically at three different wavelengths: 470 nm, 644.8 nm and 661.6 nm. Chlorophyll *a*, *b* and the bulk of carotenoids were quantified using the equations described by Lichtenthaler and Buschmann (2001):

6. Chl *a* (µg/mL) = 11.24 $A_{661.6}$ – 2.04 $A_{644.8}$

7. Chl b (µg/mL) = 20.13 $A_{644.8}$ – 4.19 $A_{661.6}$

8. Carotenoids (x + c) (µg/mL) = (1000 A_{470} – 1.90 c_a – 63.14 c_b) /214

Equation 6: Chlorophyll *a* (Chl *a*);

Equation 7: Chlorophyll *b* (Chl *b*);

Equation 8: Carotenoids ((x+c); xanthophylls and carotenes);

As the absorption peaks of the different carotenoids are too close to accurately identify and quantify each one using the spectrophotometric method, each carotenoid (α- and β-carotene, lutein, neoxanthin, lutein epoxide, violaxanthin, antheraxanthin and zeaxanthin) was separated and quantified by HPLC as described in Silva et al. 2013 after Larbi et al.

(2004) and de las Rivas, Abadía & Abadía (1989).

Liquid chromatography analysis was performed in an Alliance Waters 2695 separation module (Milford MA, USA), with a Waters 2996 photodiode array detector and a Waters 21 Novapak C18 radial 86100 mm compression column (4 mm particle size). Prior to sample injection, the column was equilibrated, and the samples were injected together with two different organic solvents (filtered and sonicated before use: R1: 879.5 mL acetonitrile, 117 mL methanol and 3.5 mL triethylamine (TEA); Eluent R2: 434 mL acetonitrile, 59.6 mL methanol, 7 mL TEA, 496 ml ethyl acetate and 2.5 mL of Milli-Q water). These organic solvents act as the mobile phase during the separation process. Each carotenoid in the samples was quantified after the HPLC calibration with known concentrations of commercially available pure pigments.

The de-epoxidation index of the violaxanthin xanthophyll cycle pigments (AZ/VAZ) was calculated as in Equation 6 (Niyogi, Grossman & Björkman, (1998)).

$$\frac{\mathrm{AZ}}{\mathrm{VAZ}} = \frac{\mathrm{A} + \mathrm{Z}}{\mathrm{V} + \mathrm{A} + \mathrm{Z}}$$

Equation 9: Calculation of the de-epoxidation index of the xanthophyll cycle pigments (**AZ/VAZ**). **V, A, Z:** violaxanthin, antheraxanthin and zeaxanthin concentrations, respectively

2.8. Data analysis

All data was statistically analyzed with the software "Sigmaplot" (Copyright © 2008 Systat Software, Inc. Germany, Sigmaplot for Windows Version 14.0). Before analysis, all data was tested for normality (Shapiro-Wilk tests) and homogeneity of variances and transformed when necessary.

Differences of the maximum quantum yield (F_v/F_m), effective quantum yield of photosystem II ($\Delta F/F'_m$), and non-photochemical quenching (NPQ) between species were tested using a One-Way Analysis of Variance (ANOVA) ($p<0.05$). Whenever a significant effect was detected, a Student-Newman-Keuls test was applied.

One-Way ANOVA was also applied to detect differences in the parameters obtained from the light response curves (P-I curves and RLCs) ($p<0.05$). Whenever a significant effect was

detected, a Student-Newman-Keuls test was applied. Dark Respiration was tested with a Two-Way ANOVA to detect any effect caused by the interaction between *Z. marina* and *C. prolifera*.

Biochemical parameters were statistically tested depending on the experiment. Diel cycle samples were tested with a One-Way ANOVA ($p<0.05$) for differences between pre-dawn and solar noon and also between species. Whenever significant differences were detected, a Student-Newman-Keuls test was applied. The interaction experiment was tested with Two Way ANOVA ($p<0.05$) to detect whether or not the presence of *C. prolifera* affected *Z. marina*. If significant differences were detected, a Holm-Sidak test was applied. Whenever equality of variance failed, data were ln transformed. If equality of variance continued to fail after transformation, a One-Way ANOVA ($p<0.05$) was applied between treatments, as it was the case for foliar starch content. Rhizomes data were tested with One-Way ANOVA ($p<0.05$) and complement with Student-Newman-Keuls test if significant differences were detected. If normal distribution failed, data were ln transformed. Whenever data transformation failed, a non-parametric Kruskal-Wallis test (One-Way Analysis of Variance on Ranks) ($p<0.05$) was applied, as was the case of the soluble sugar content of rhizomes.

3. Results

3.1. Diel photophysiology of *Zostera marina, Cymodocea nodosa* and *Caulerpa prolifera*

3.1.1. Chlorophyll fluorescence

The effective quantum yield ($\Delta F/F'_m$) displayed the same pattern in all the three species: it increased as the day ended and light conditions slowly faded, and decreased as the morning of the next day arrived and the irradiance slowly increased (Fig. 3.1). Non-photochemical quenching (NPQ) is positively related with the photoprotective mechanisms from which results the dissipation of excess energy of excitation in photosynthesis (Larkum, Drew & Ralph, 2006), and is inversely related to the effective quantum yield, decreasing as effective quantum yield increases, as shown in Figure 3.1.

Seagrasses revealed to have significantly higher F_v/F_m measured at 4:00 when compared to *C. prolifera* (Table 3.1). *Z. marina* presented the highest value, followed by *C. nodosa.* $\Delta F/F'_m$ measured at 14:00 was also significantly higher in seagrasses, with *Z. marina* using light more efficiently then *C. nodosa* and both seagrasses being more efficient than *C. prolifera*. On the other hand, seagrasses also had higher NPQ than *C. prolifera* (Table 3.1 and Figure 3.1) revealing a higher photoprotective capacity.

Table 3.1: Maximum quantum yield (F_v/F_m) measured at 4:00 h on dark-adapted leaves, effective quantum yield ($\Delta F/F'_m$) and non-photochemical quenching (NPQ) measured on light-adapted leaves at 14:00 h. Values are means ± SE (n=10). Different letters indicate significant differences between species ($p<0.05$)

Species	F_v/F_m	$\Delta F/F'_m$	NPQ
Z. marina	0.803 ± 0.002^a	0.393 ± 0.011^a	3.193 ± 0.318^a
C. nodosa	0.782 ± 0.007^b	0.315 ± 0.009^b	3.496 ± 0.262^a
C. prolifera	0.732 ± 0.007^c	0.263 ± 0.002^b	1.778 ± 0.297^b

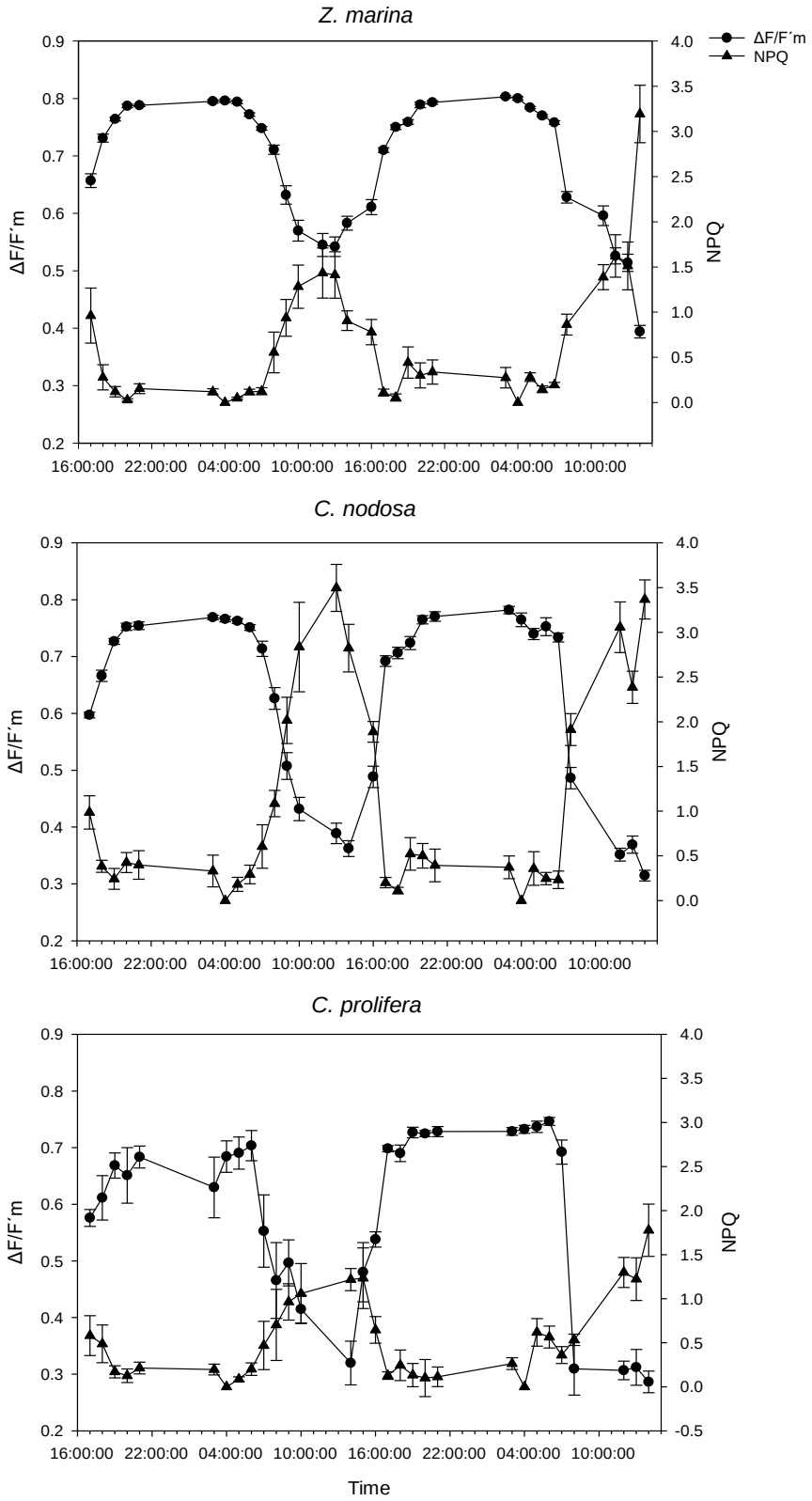

Figure 3.1: Quantum yield ($\Delta F/F'_m$) and non-photochemical quenching (NPQ) of *Zostera marina*, *Cymodocea nodosa* and *Caulerpa prolifera* along a 48 hour chlorophyll fluorescence cycle. Values are means ± SE, n=10.

3.1.2. Biochemical Analysis

3.1.2.1. Soluble Sugars and starch

While the foliar concentration of soluble sugars significantly increased from pre-dawn to solar noon in both seagrass species, the same cannot be said for *C. prolifera´*s fronds, where soluble sugar content did not change (Fig. 3.2A). When comparing species, they all presented significantly different soluble sugar content at pre-dawn, where *C. prolifera* had the lowest concentration. This difference however only persisted for *C. prolifera* at solar noon, as both seagrasses no longer present a significant difference in their soluble sugars content (Fig. 3.2A).

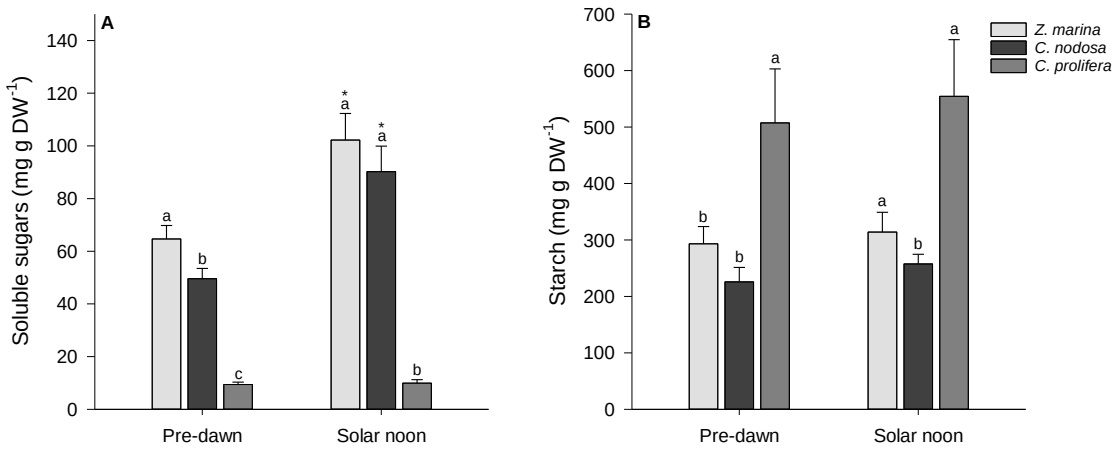

Figure 3.2: Soluble sugars (A) and starch (B) content of *Z. marina*, *C. nodosa* leaves and *C. prolifera* fronds at pre-dawn and solar noon. Values are mean ± SE (n = 10, except for *Z. marina* starch at solar noon, n = 9); different letters indicate significant differences among species, (*) indicates significant differences pre-dawn and solar noon ($p<0.05$).

Foliar/frond starch content did not vary between pre-dawn and solar noon in any of the three species, and *C. prolifera´*s starch content is approximately twice that of both seagrasses independently of the hour of the day (Fig. 3.2B).

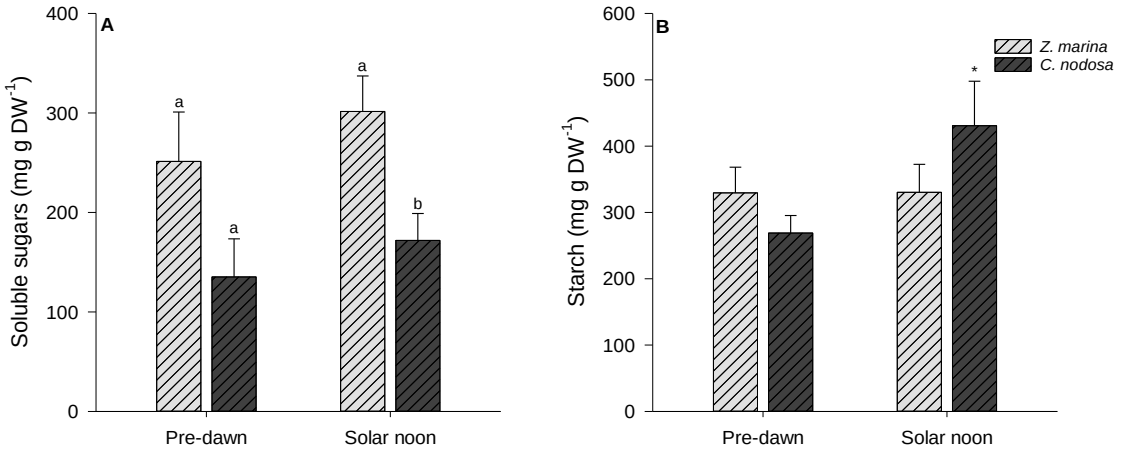

Figure 3.3: Soluble sugars (A) and starch (B) content of *Z. marina* and *C. nodosa* rhizomes at pre-dawn and solar noon. Values are mean ± SE (n = 10, except for *C. nodosa* starch at pre-dawn, n = 8); different letters indicate significant differences among species, (*) indicates significant differences between pre-dawn and solar noon ($p<0.05$).

Soluble sugars content didn't change in the rhizomes of the seagrasses from pre-dawn to solar noon. *Z. marina* rhizomes appear to contain more soluble sugars than those of *C. nodosa*, but that difference between the two species was significant only at solar noon (Fig. 3.3A). Starch content in *Z. marina* rhizomes was identical at pre-dawn and solar noon, while in *C. nodosa* it increased significantly during the day (Fig. 3.3B).

3.1.2.2. Soluble proteins

Foliar soluble protein of all species showed a tendency to increase between pre-dawn and solar noon. However, no significant differences were found neither between species nor between pre-dawn and solar noon (Fig. 3.4A). The same results were obtained for rhizomes of both seagrass species (Fig. 3.4B)

3.1.2.3. Adenylate energy charge (AEC)

Foliar AEC increased from pre-dawn to noon in *Z. marina*, but no differences were found in *C. nodosa* and *C. prolifera.* (Fig. 3.5A). At pre-dawn, *Z. marina* leaves had a significantly lower AEC than the other two species, but at solar noon this difference was not observed and

there were no significant differences among species (Fig. 3.5A). In rhizomes, the AEC of both seagrasses did not change significantly from pre-dawn to solar noon and there were no differences between species (Fig. 3.5B)

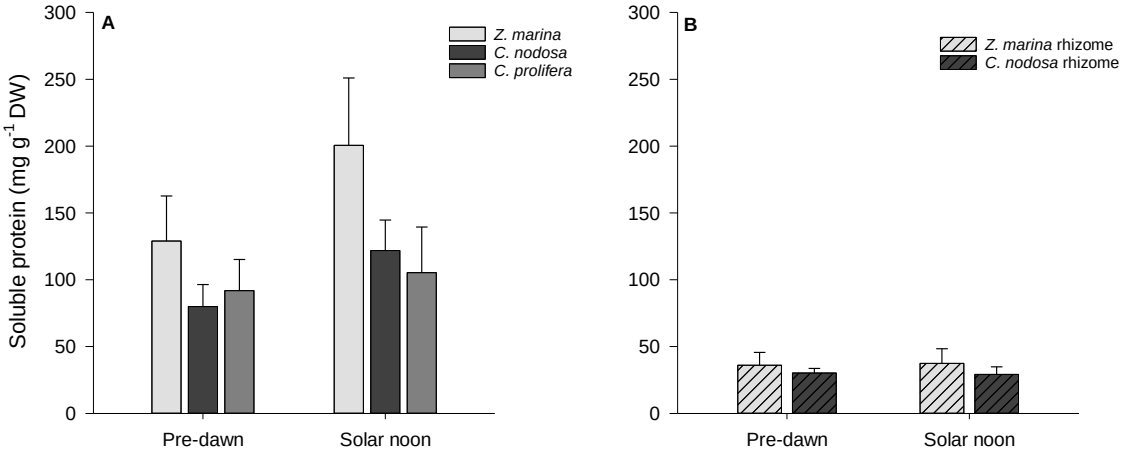

Figure 3.4: Soluble protein content in *Z. marina* and *C. nodosa* leaves and *C. prolifera* fronds (A), and seagrasses rhizomes (B) at pre-dawn and solar noon. Values are mean ± SE (n = 5) ($p<0.05$).

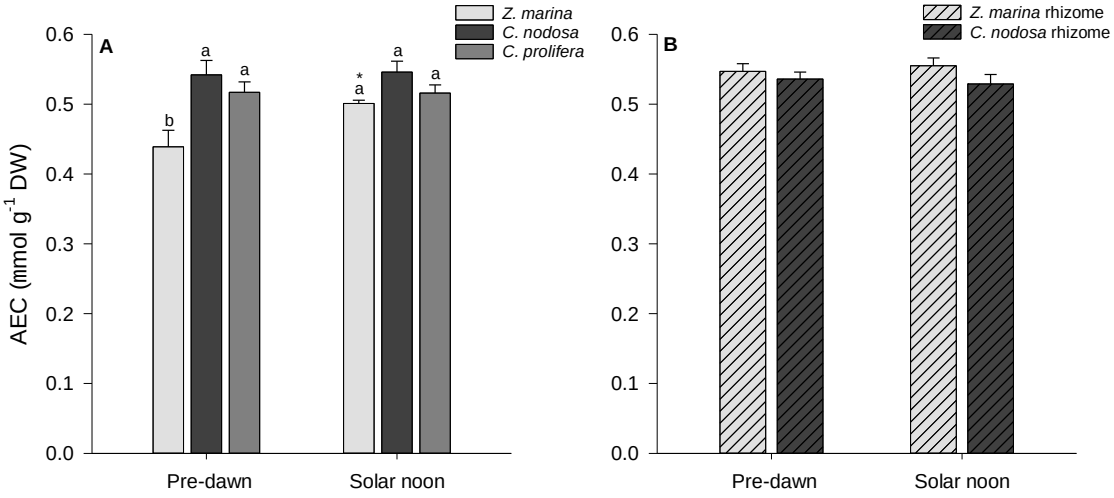

Figure 3.5: Adenylate energy charge (AEC) of *Z. marina* and *C. nodosa* leaves and *C. prolifera* fronds (A) and *Z. marina* and *C. nodosa* rhizomes (B) at pre-dawn and solar noon. Values are mean ± SE (n = 5, except for *Z. marina* (pre-dawn), *Z. marina* rhizomes (pre-dawn) and *C. nodosa* rhizomes (pre-dawn) where n=4); different letters indicate significant differences among species($p<0.05$); (*) indicates significant differences between pre-dawn and solar noon ($p<0.05$).

3.1.2.4. Photosynthetic pigments

Chlorophylls *a* and *b*, and eight carotenoids (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein epoxide, lutein and α- and β-carotene) were quantified in leaf/frond extracts of *Z. marina*, *C. nodosa* and *C. prolifera*. α-carotene was identified in *C. prolifera* but it was absent from any of the seagrasses (Table 3.2).

In all three species there was a tendency for total chlorophyll (Chl T), neoxanthin, lutein, and β-carotene concentrations to increase from pre-dawn to solar noon, however no significant differences were found (Table 3.2).

At solar noon, lutein epoxide concentrations were significantly higher in *C. prolifera* while lutein was significantly lower, when compared to the seagrasses. (V+A+Z)/ChlT was not significantly different between species or time of the day, although there was a slight tendency to increase at solar noon. The (V+A+Z)/Lutein ratio however, was found to be significantly higher in *C. prolifera*, due to its lower lutein concentrations. This ratio is over ten times greater than that of seagrasses (Table 3.2).

Violaxanthin (V) concentration was significantly higher in *C. prolifera* than in seagrasses. During pre-dawn, antheraxanthin (A) concentrations were significantly higher in *C. prolifera* than in seagrasses but, while in seagrasses anteraxanthin concentration increased from pre-dawn to solar noon, the same did not happen in *C. prolifera*. On the other hand, zeaxanthin (Z) content was significantly lower in *C. prolifera* than in seagrasses during pre-dawn. Similarly to antheraxanthin, *C. prolifera* zeaxanthin content did not change from pre-dawn to solar noon while it significantly increased in seagrasses. These changes on the xanthophyll cycle pigments (V,A and Z) were reflected on the de-epoxidation index ((AZ)/(VAZ)) that significantly increased from pre-dawn to solar noon in seagrasses but not in *C. prolifera* whose de-epoxidation index was kept very low during solar noon. Considering seagrasses, *C. nodosa* demonstrated a higher photoprotective response than *Z. marina* (Table 3.2, Fig. 3.6).

Table 3.2:. Total chlorophyll (Chl T), chlorophyll a/b ratio (Chl a/b), Neoxanthin, Violaxanthin, Antheraxanthin, Zeaxanthin, Lutein epoxide, Lutein, α- and β-carotene, Violaxanthin + Antheraxanthin + Zeaxanthin (V+A+Z), de-epoxidation index of the pigments of the xanthophyll cycle (AZ/VAZ), (Violaxanthin + Antheraxanthin + Zeaxanthin)/ total chlorophyll ((V+A+Z)/Chl T) and (Violaxanthin + Antheraxanthin + Zeaxanthin)/lutein ((V+A+Z)/lutein) ratios determined for *Z. marina*, *C. nodosa* and *C. prolifera* at pre-dawn and solar noon. Pigments are presented in µmol g DW^{-1}, with exception of the ratios. Values are means ± SE (n = 5); different letters indicate significant differences among species; (*) indicates significant difference between pre-dawn and solar noon ($p<0.05$).

	Zostera marina		***Cymodocea nodosa***		***Caulerpa prolifera***	
	Pre-dawn	**Solar noon**	**Pre-dawn**	**Solar noon**	**Pre-dawn**	**Solar noon**
Chl T	2.70± 0.76	2.78 ± 0.54	2.23 ± 0.26	3.58 ± 0.71	3.48 ± 0.55	3.71 ± 0.87
Chl *a*/*b*	1.91 ± 0.22	2.15 ± 0.27	2.16 ± 0.07	1.80 ± 0.18	1.50 ± 0.10	1.53 ± 0.06
Neoxanthin	0.14 ± 0.03	0.17 ± 0.03	0.15 ±0.01	0.27 ± 0.07	0.24 ± 0.04	0.24 ± 0.06
Violaxanthin	0.28 ± 0.05	0.30 ± 0.02^{a}	0.29 ± 0.02*	0.16 ± 0.02^{b}	0.47 ± 0.09	0.58 ± 0.14^{a}
Antheraxanthin	0.009 ± 0.002^{b}	0.08 ± 0.009^{*a}	0.01 ± 0.001^{b}	0.11 ± 0.01^{*a}	0.03 ± 0.007^{a}	0.03 ± 0.007^{b}
Zeaxanthin	0.02 ± 0.01^{a}	0.08 ± 0.01^{*b}	0.03 ± 0.007^{a}	0.19 ± 0.02^{*a}	0.004 ± 0.002^{b}	0.004 ± 0.002^{c}
Lutein epoxide	0.007 ± 0.002	0.005 ± 0.002^{b}	0.006 ± 0.003	0.002 ± 0.002^{b}	0.02 ± 0.006	0.02 ± 0.005^{a}
Lutein	0.38 ± 0.09^{a}	0.48 ± 0.05^{a}	0.37 ± 0.03^{a}	0.49 ± 0.09^{a}	0.04 ± 0.003^{b}	0.06 ± 0.001^{b}
α-carotene	n.d.	n.d.	n.d.	n.d.	0.10 ± 0.03^{a}	0.11 ± 0.04^{a}
β-carotene	0.30 ± 0.06	0.40 ± 0.03	0.40 ± 0.09	0.45 ± 0.05	0.29 ± 0.06	0.32 ± 0.09
V+A+Z	0.31 ± 0.06	0.46 ± 0.03*	0.33 ± 0.03	0.46 ± 0.04*	0.53 ± 0.10	0.62 ± 0.15
(AZ)/(VAZ)	0.06 ± 0.01^{b}	0.35 ± 0.04^{*b}	0.14 ± 0.01^{a}	0.65 ± 0.02^{*a}	0.06 ± 0.01^{b}	0.05 ± 0.003^{c}
(V+A+Z)/Chl T	148.64 ± 37.71	189.32 ± 30.22	127.11 ± 15.86	139.41 ± 17.86	153.11 ± 16.57	166.28 ± 12.30
(V+A+Z)/Lutein	0.92 ± 0.15^{b}	0.95 ± 0.002^{b}	0.77 ± 0.08^{b}	0.98 ± 0.07^{b}	14.91 ± 2.16^{a}	13.12 ± 0.78^{b}

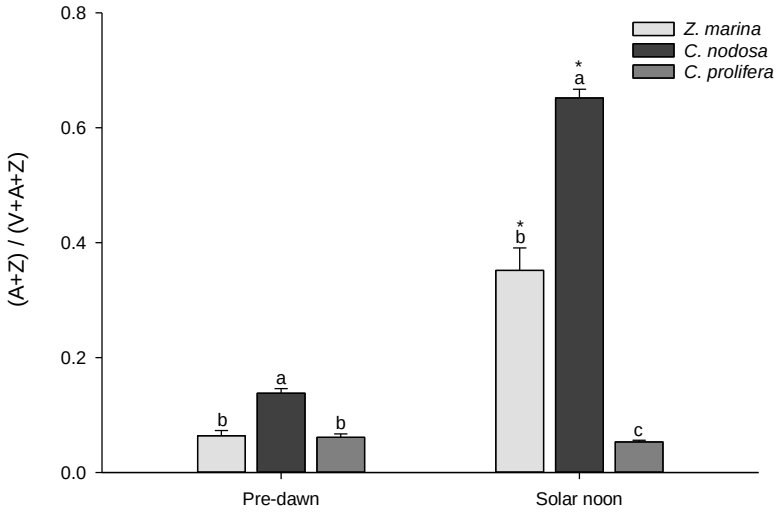

Figure 3.6: De-epoxidation index in leaves/fronds of *Z. marina*, *C. nodosa* and *C. prolifera* at pre-dawn and solar noon. Values are mean ± SE (n = 5), different letters indicate significant differences among species; (*) indicate significant difference between pre-dawn and solar noon ($p<0.05$).

3.2. Z. *marina* and *C. prolifera*: An interaction mesocosm experiment

3.2.1. Photosynthetic light response curves

Both *Z. marina* and *C. prolifera* displayed the typical hyperbolic response of photosynthesis to increasing irradiance. Photosynthetic rates increased linearly with the lower limiting irradiances until approximating their maximum (P_{max}), at half-saturating irradiances (I_k) (Fig. 3.7).

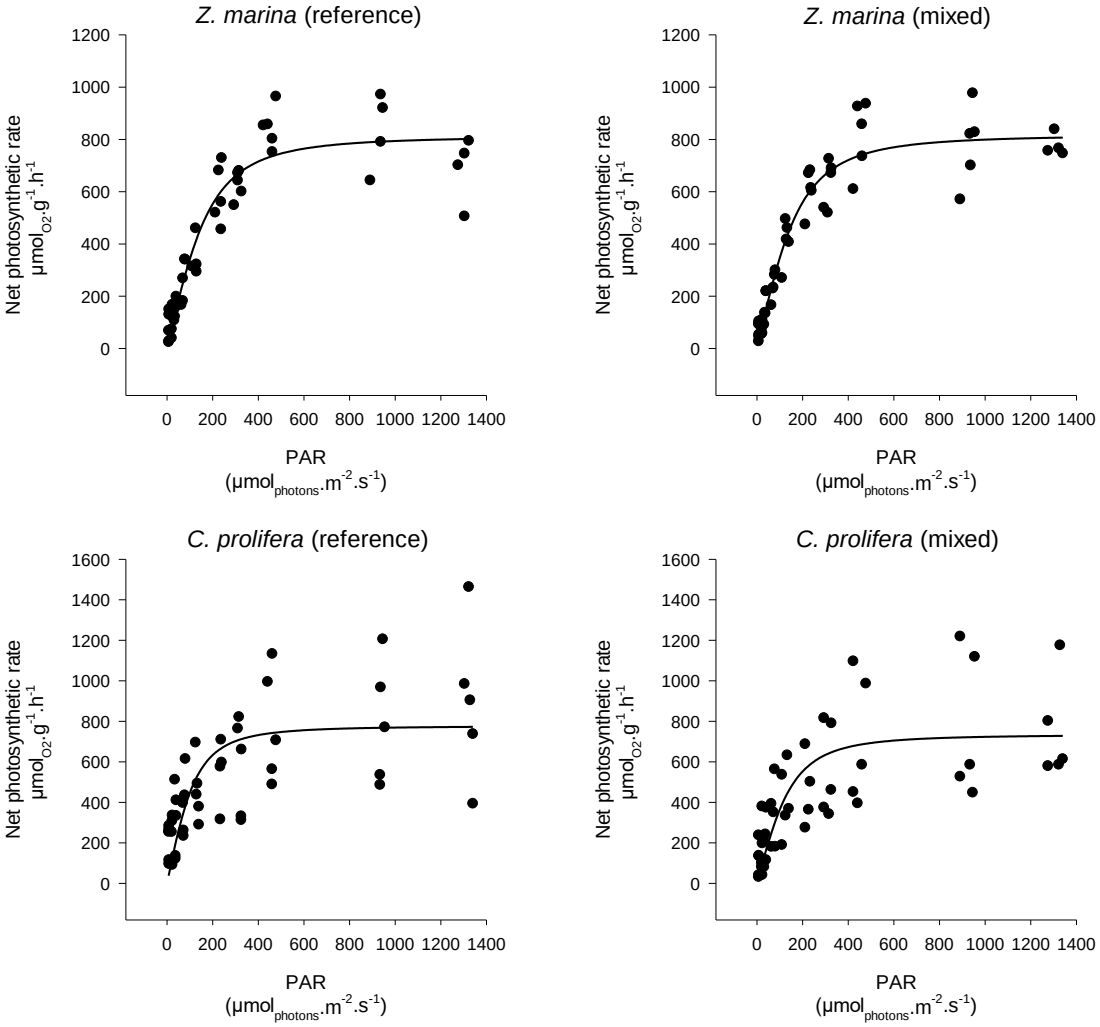

Figure 3.7: Photosynthetic light response curves of *Z. marina* and *C. prolifera* (n=5), 4 weeks after being planted separately (reference) and together (mixed**).** Curves were fitted using the Smith and Talling (Smith (1936), Talling (1957)) model.

Dark respiration, photosynthetic quantum efficiency (α), maximal photosynthesis (Pmax), and half-saturation irradiance (I_k) were calculated for both species from the P-I

curves after applying the mathematical model of Smith and Talling (Smith, 1936; Talling, 1957). The results obtained show no differences between species, and also that neither species was significantly affected when mixed with each other. Although not significant, P_{max} and I_k appear to be higher in *Z. marina* while photosynthetic quantum efficiency (α) appears to be higher in *C. prolifera* (Table 3.3).

Table 3.3: Maximum photosynthetic rate (P_{max}, $\mu molO_2.g^{-1}.s^{-1}$), photosynthetic quantum efficiency at limiting irradiances (α, $\mu mol_{O2}/\mu mol_{photons}$), half-saturation irradiance (I_k, $\mu mol_{photons}.m^{-2}.s^{-1}$) and the coefficient of the model adjustment to the data (r^2) of Smith and Talling (Smith, 1936; Talling, 1957) of *Z. marina* and *C. prolifera* 4 weeks after being planted separately (reference) and together (mixed). Values are means ± SE (n=5).

Treatment	P_{max}	α	I_k	r^2
Z. marina				
Reference	812.27 ± 31.56	3.77 ± 0.34	215.46 ± 21.16	0.8972
Mixed	817.27 ± 25.55	3.82 ± 0.28	210.63 ± 16.56	0.9265
C. prolifera				
Reference	777.23 ± 58.37	5.47 ± 1.21	142.09 ± 33.19	0.4730
Mixed	734.69 ± 58.16	4.20 ± 0.89	174.92 ± 39.56	0.5178

Dark respiration of both species was not significantly affected by mixing but *C. prolifera* seems to respire at a higher rate than *Z. marina* (Fig. 3.8).

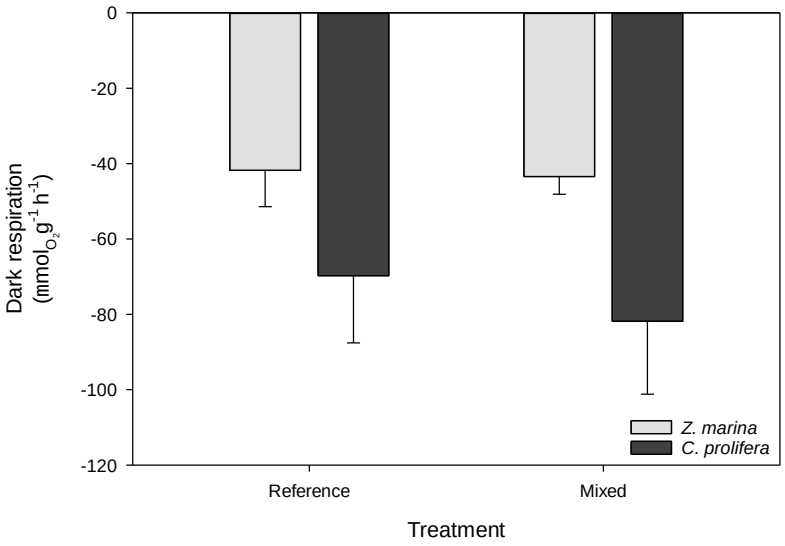

Figure 3.8: Dark respiration of *Z. marina* and *C. prolifera*, 4 weeks after being planted separately (reference) and together (mixed). Values are Mean ± SE (n=5).

3.2.2. Rapid light curves

Rapid light curves (RLC) allow the analysis of the response of the relative electron transport rate (rETR) to irradiance. The response to lower irradiances was similar for both species, with increasing rETR as increasing low light intensities activated the electron transport chain. However, at irradiances higher than ca. 150 $\mu mol_{photons} m^{-2} s^{-1}$ *C. prolifera* showed its shade adapted nature (García-Sánchez et al., 2012), revealing a distinct and more pronounced photoinhibitory response to higher light intensities, four times more pronounced than that of *Z. marina*. No differences in $rETR_{max}$, α, and I_k were detected between species or treatments (reference and mixed) (Fig. 3.9, Table 3.4).

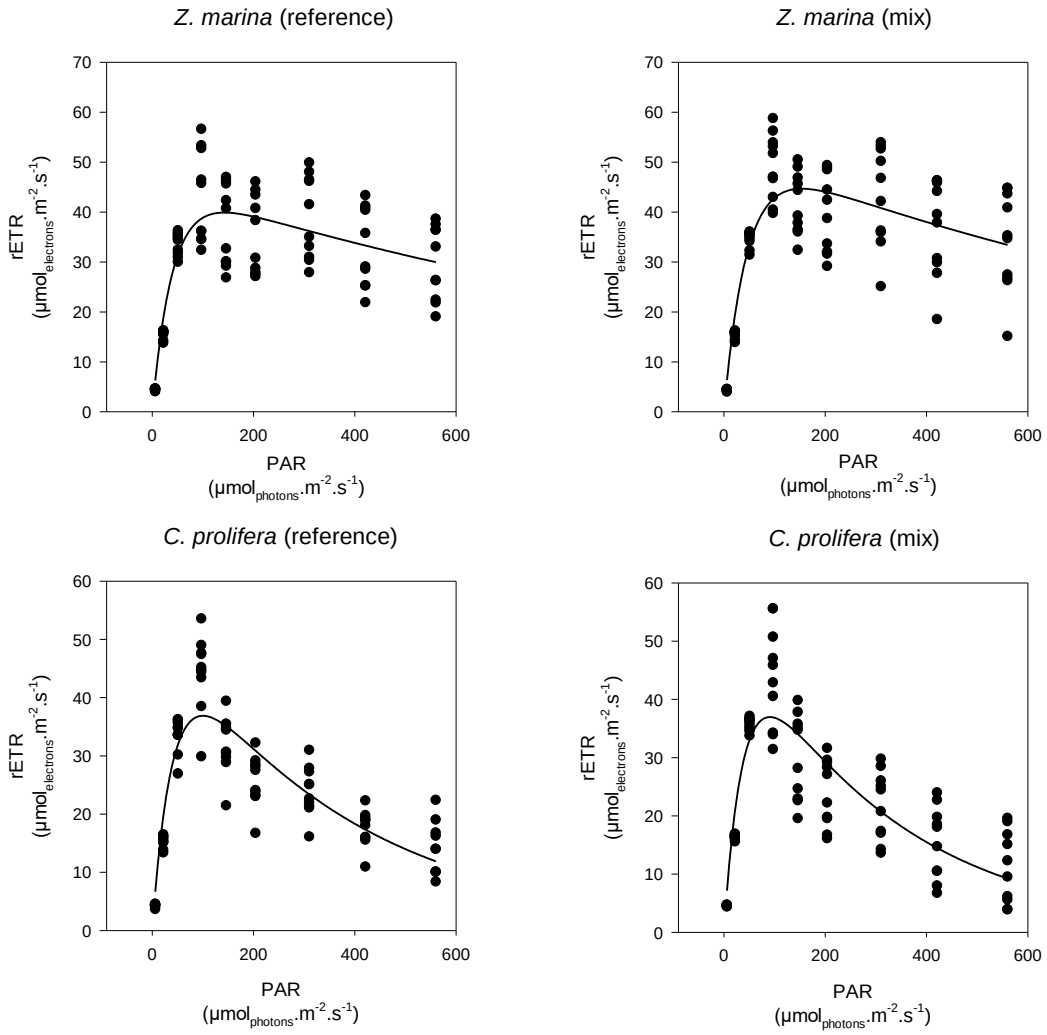

Figure 3.9: Rapid light curves of *Z. marina* and *C. prolifera* (n=10), 4 weeks after being planted separately (reference) and together (mixed). Curves were fitted using the Platt, Gallegos & Harrison (1980) model.

Table 3.4: Maximum relative electron transport rate ($rETR_{max}$, $\mu mol_{electrons} m^{-1} s^{-1}$), initial slope ($\alpha$), inhibition term ($\beta$), saturation irradiance (I_k, $\mu mol_{photons} m^{-1} s^{-1}$) and the coefficient of determination of the model adjustment to the data (r^2). Photosynthetic parameters obtained from the adjustment of the model equation of Platt, Gallegos & Harrison (1980) to the observed rapid light curve data for *Z. marina* and *C. prolifera*, 4 weeks after being planted separately (reference) and together (mixed**)** Values are means ± SE (n=10); Different letters indicates differences between species ($p<0.05$).

Treatment	$rETR_{max}$	α	β	I_k	r^2
Z. marina					
Reference	45.827 ± 3.179	1.130 ± 0.125	0.035 ± 0.012[a]	40.541 ± 5.295	0.7286
Mixed	52.442 ± 3.844	1.136 ± 0.118	0.042 ± 0.014[a]	46.180 ± 5.875	0.7592
C. prolifera					
Reference	54.266 ± 4.405	1.205 ± 0.101	0.147 ± 0.027[b]	45.053 ± 5.253	0.8049
Mixed	56.592 ± 5.898	1.304 ± 0.131	0.183 ± 0.041[b]	43.409 ± 6.293	0.7454

3.2.3. Biochemical analysis

The biochemical analysis included the quantification of soluble sugars, starch, soluble proteins, AMP, ADP and ATP in order to obtain the adenylate energy charge (AEC), and finally photosynthetic pigments (chlorophylls *a* and *b*, and the carotenoids α- and β-carotene, neoxanthin, lutein, lutein epoxide, violaxanthin, anteraxanthin and zeaxanthin). Field samples were first compared to reference samples from the mesocosm experiment (Tables 3.5 and 3.6). Of all the components analyzed and displayed in Table 3.5, only starch appeared to be significantly higher in *Z. marina* reference, while there were no significant differences among the rest.

Most photosynthetic pigments did not vary between field and reference in neither species. However, it is important to mention that total chlorophyll (Chl T) in field samples presented to be extremely low (Table 3.6). This could be associated to pigment degradation occurred during procedures. Significant differences were found in violaxanthin, antheraxanthin and zeaxanthin concentrations of both species between Ria Formosa and mesocosm samples. Furthermore, neoxanthin concentration in *Z. marina* reference, and the de-epoxidation index were significantly higher in the field when compared to the reference tanks (Table 3.6) but this may have to do most probably with the plants acclimation to the mesocosm light

conditions in which irradiance was lower than that in the field (± 210 $\mu mol_{photons}\, m^{-1}s^{-1}$) The same might have happened in *C. prolifera* in which lutein concentrations were significantly higher in the field samples.

Table 3.5: Soluble sugars, starch, soluble protein (mg g DW^{-1}) and adenylate energy charge (AEC) of *Z. marina* leaves and *C. prolifera* fronds from field samples and samples taken from the reference tanks 4 weeks after being planted for the mesocosm experiment. Values are means ± SE (n=10 for soluble sugar and starch, n=5 for soluble protein and AEC); (*) indicates difference among Field and Reference ($p<0.05$).

	Zostera marina		***Caulerpa prolifera***	
	Field	**Reference**	**Field**	**Reference**
Soluble sugar	78.22 ± 7.21	85.34 ± 14.40	13.12 ± 0.50	8.931 ± 1.46
Starch	274.94 ± 15.06*	201. 28 ± 6.89	514.26 ± 41.43	510.28 ± 91.90
Soluble protein	95.26 ± 22.70	64.31 ± 14.10	141.37 ± 39.28	110.74 ± 26.06
AEC	0.48 ± 0.007	0.50 ± 0.013	0.52 ± 0.015	0.57 ± 0.017

Table 3.6: Total chlorophyll (Chl T), chlorophyll a/b ratio (Chl a/b), Neoxanthin, lutein epoxide, Lutein, α- and β-carotene, Violaxanthin + Antheraxanthin + Zeaxanthin (V+A+Z), de-epoxidation index (AZ)/(VAZ), (Violaxanthin + Antheraxanthin + Zeaxanthin)/total chlorophyll ratio and (Violaxanthin + Antheraxanthin + Zeaxanthin)/lutein ratios were determined for *Z. marina* and *C. prolifera* field samples and samples taken from reference tank at the end of the mesocosm experiment. Pigments are presented in μmol g DW^{-1}, with exception of the ratios. Values are means ± SE (n=5); different letters indicate significant difference among species of the same treatment; (*) indicates significant difference between treatments ($p<0.05$).

	Zostera marina		***Caulerpa prolifera***	
	Field	**Reference**	**Field**	**Reference**
Chl T	1.81 ± 0.50[b]	3.21 ± 0.71	4.96 ± 1.10[a]	4.48 ± 1.64
Chl *a*/*b*	2.10 ± 0.22	1.79 ± 0.22	1.64 ± 0.04	1.68 ± 0.16
Neoxanthin	0.09 ± 0.02[b]	0.17 ± 0.02*	0.36 ± 0.10[a]	0.28 ± 0.08
Violaxanthin	0.10 ± 0.02[b]	0.26 ± 0.04*[b]	0.78 ± 0.13[a]	0.57 ± 0.18[a]
Antheraxanthin	0.04 ± 0.008*	0.02 ± 0.003	0.04 ± 0.008*	0.01 ± 0.004
Zeaxanthin	0.10 ± 0.02*[a]	0.02 ± 0.004[a]	0.02 ± 0.005*[b]	0.002 ± 0.004[b]
Lutein epoxide	0.01 ± 0.008	0.005 ± 0.002	0.02 ± 0.007	0.008 ± 0.003
Lutein	0.30 ± 0.07[a]	0.45 ± 0.08[a]	0.08 ± 0.001[b*]	0.02 ± 0.005[b]
α-carotene	n.d.	n.d.	0.39 ± 0.15[a]	0.19 ± 0.05[a]
β-carotene	0.24 ± 0.05	0.36 ± 0.06	0.54 ± 0.14	0.44 ± 0.11
V+A+Z	0.24 ± 0.05[b]	0.29 ± 0.04	0.89 ± 0.19[a]	0.58 ± 0.18
(AZ)/(VAZ)	0.57 ± 0.02[a*]	0.11 ± 0.02[a*]	0.07 ± 0.003[b*]	0.03 ± 0.006[b*]
V+A+Z/Chl T	148.85 ± 21.61	100.94 ± 13.45[b]	182.30 ± 4.68	148.90 ±15.26[a]
V+A+Z/Lutein	0.81 ± 0.07[b]	0.68 ± 0.04[b]	11.61 ± 154[a]	18.19 ± 3.97[a]

3.2.3.1. Soluble sugars and starch

Soluble sugar content of *Z. marina* leaves was not affected by the presence of the macroalgae. The same can be said for *C. prolifera* fronds which, however, had a significantly lower concentration of soluble sugars when compared to *Z. marina* (Fig. 3.10A). Conversely, *C. prolifera* had approximately twice the starch content of *Z. marina* which increased when mixed with the macroalgae. *Caulerpa* starch content was unaffected by the presence of the seagrass (Fig. 3.10B). Taking a closer look at the seagrass rhizomes reveals that the presence of the macroalgae negatively affected its soluble sugar content that was almost four times lower than the reference. Starch content however, remained unaffected, as no significant differences were detected (Fig. 3.11).

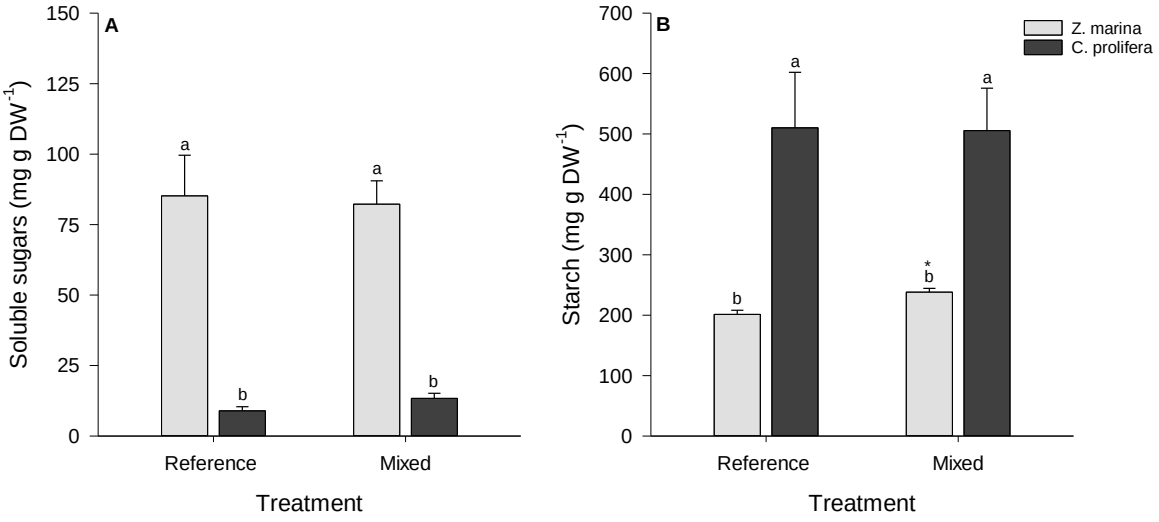

Figure 3.10: (A) Soluble sugars and (B) starch content of *Z. marina* leaves and *C. prolifera* fronds 4 weeks after being planted separately (reference) or together (mixed) for the mesocosm experiment. Values are mean ± SE (n=10); (*) indicates significant differences between reference and mixed plants/algae ($p<0.05$).

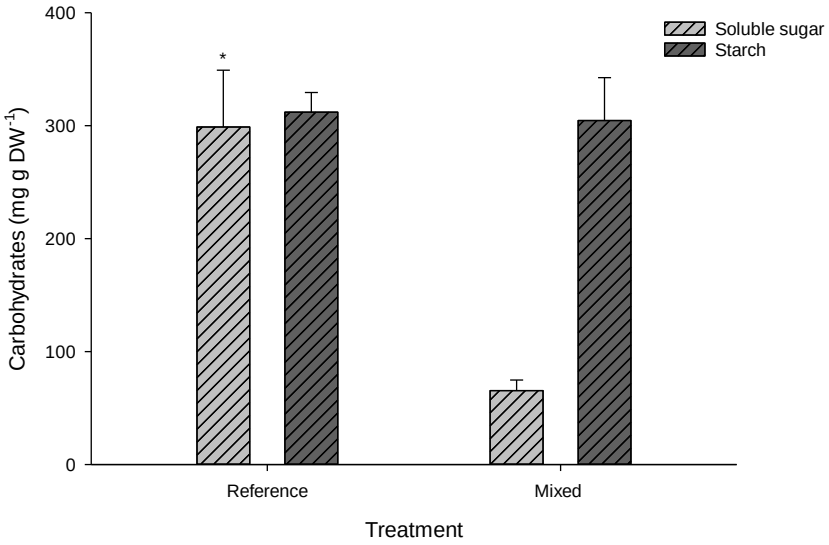

Figure 3.11: Soluble sugars and starch content of *Z. marina* rhizomes 4 weeks after being planted separately (reference) or together with *C. prolifera* (mixed) for the mesocosm experiment. Values are mean ± SE (n=8 for sugar samples, n=10 for starch); (*) indicates significant difference between reference and mixed plants ($p<0.05$).

3.2.3.2. Soluble proteins

Foliar soluble protein content of *Z. marina* showed no significant change in the presence of the macroalgae. On the other hand, protein content in the fronds of *C. prolifera* slightly increased, enough to be significantly higher than *Z. marina´*s content, a trend not observed in the reference treatments (Fig. 3.12A). Furthermore, soluble protein content in the rhizomes of *Z. marina* did not seem to be affected by the presence of the macroalgae (Fig. 3.12B).

3.2.3.3. Adenylate energy charge (AEC)

Z. marina and *C. prolifera* AEC was not affected by mixing the two species, but in *Z. marina* rhizomes AEC tended to decrease in the presence of *C. prolifera*. *C. prolifera* fronds had a significantly higher AEC than *Z. marina* leaves no matter the two species were separated or mixed (Fig. 3.13A and 3.13B).

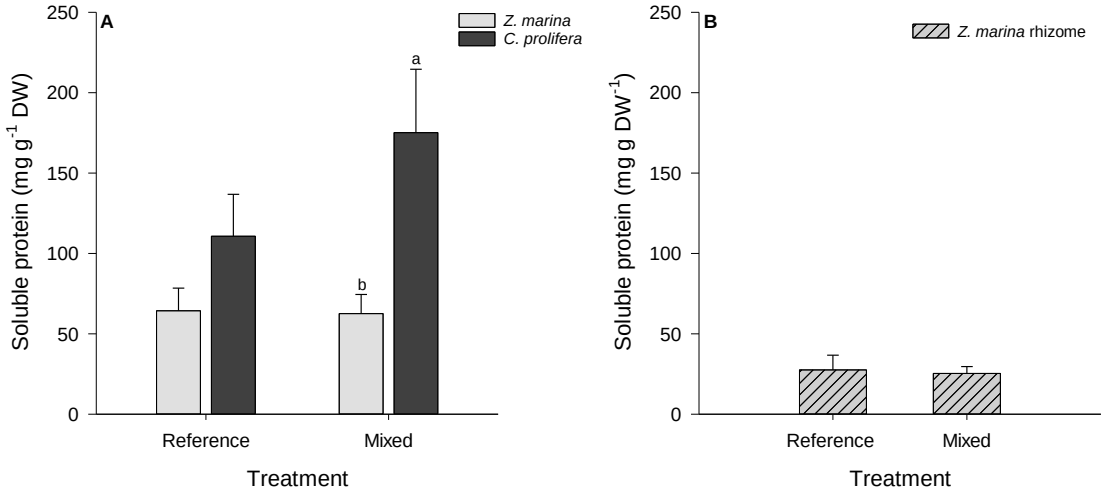

Figure 3.12: Soluble protein content of (A) *Z. marina* leaves, *C. prolifera* fronds and (B) *Z. marina* rhizomes, 4 weeks after being planted separately (reference) or together (mixed). Values are Mean ± SE (n=5); different letters indicate significant differences among species ($p<0.05$).

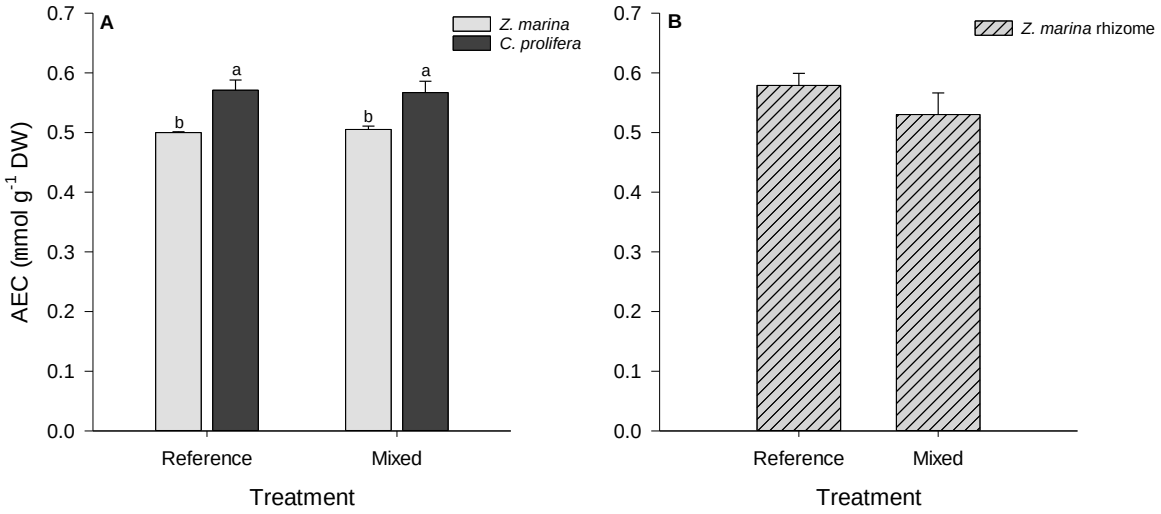

Figure 3.13: Adenylate energy charge of (A) *Z. marina* leaves and *C. prolifera* fronds and (B) *Z. marina* rhizomes 4 weeks after being planted separately (reference) or together (mixed). Values are Mean ± SE (n=5); different letter indicate significant difference among species ($p<0.05$).

3.2.3.4. Photosynthetic pigments

The foliar/frond photosynthetic pigments concentration was not affected by mixing *Z. marina* and *C. prolifera*. There were however, some significant differences among species. *Z. marina* contained significantly more lutein and a significantly higher de-epoxidation

index, while *C. prolifera* contained α-carotene which was absent in *Z. marina* and a xanthophyll pigment/lutein ratio significantly higher (Table 3.7).

Table 3.7: Total chlorophyll (Chl T), chlorophyll a/b ratio (Chl a/b), Neoxanthin, Violaxanthin, Antheraxanthin, Zeaxanthin, Lutein epoxide, Lutein, α- and β-carotene, Violaxanthin + Antheraxanthin + Zeaxanthin (V+A+Z), de-epoxidation index (AZ)/(VAZ), (Violaxanthin + Antheraxanthin + Zeaxanthin)/ total chlorophyll ratio and (Violaxanthin + Antheraxanthin + Zeaxanthin)/lutein ratios were determined for *Z. marina*, *C. nodosa* and *C. prolifera* 4 weeks after being planted separately (reference) or together (mixed). Pigments are presented in µmol g DW^{-1}, with exception of the ratios. Values are means ± SE (n=5); different letters indicate significant difference among species of the same treatment ($p<0.05$).

	Zostera marina		***Caulerpa prolifera***	
	Reference	**Mixed**	**Reference**	**Mixed**
Chl T	3.21 ± 0.71	2.79 ± 0.83	4.48 ± 1.64	5.13 ±1.17
Chl *a*/*b*	1.79 ± 0.22	1.93 ± 0.18	1.68 ± 0.16	1.51 ± 0.07
Neoxanthin	0.17 ± 0.02	0.35 ± 0.07[a]	0.28 ± 0.08	0.14 ± 0.09[b]
Violaxanthin	0.26 ± 0.04	0.21 ± 0.05[b]	0.57 ± 0.18	0.57 ± 0.15[a]
Antheraxanthin	0.02 ± 0.003	0.02 ± 0.006	0.01 ± 0.004	0.009 ± 0.004
Zeaxanthin	0.02 ± 0.004[a]	0.01 ± 0.001[a]	0.002 ± 0.002[b]	0.002 ± 0.001[b]
Lutein epoxide	0.005 ± 0.002	0.004 ± 0.002	0.008 ± 0.003	0.011 ± 0.008
Lutein	0.45 ± 0.08[a]	0.44 ± 0.12[a]	0.02 ± 0.005[b]	0.01 ± 0.002[b]
α-carotene	n.d.	n.d.	0.19 ± 0.05[a]	0.20 ± 0.09[a]
β-carotene	0.36 ± 0.06	0.32 ± 0.06	0.44 ± 0.11	0.56 ± 0.09
V+A+Z	0.29 ± 0.04	0.23 ± 0.05	0.58 ± 0.18	0.58 ± 0.15
(AZ)/(VAZ)	0.11 ± 0.02[a]	0.13 ± 0.02[a]	0.03 ± 0.006[b]	0.02 ± 0.005[b]
(V+A+Z)/Chl T	100.94 ± 13.45[b]	97.74 ± 14.06	148.90 ±15.26[a]	112.40 ± 9.64
(V+A+Z)/Lutein	0.68 ± 0.04[b]	0.59 ± 0.07[b]	18.19 ± 3.97[a]	27.74 ± 5.26[a]

4. Discussion

The photosynthetic production of *C. prolifera* was slightly lower compared to that of *Z. marina*, while dark respiration almost doubled that of the seagrass. *C. prolifera* presented similar results in terms of photosynthetic efficiency and saturating irradiance in Ria Formosa as in other comparable coastal lagoons (Collado-Vides & Robledo, 1999). Rapid light curves of *Z. marina* and *C. prolifera* showed similar responses to lower light levels, with *C. prolifera* using the light more efficiently. However, the rapid light curves indicate that photoinhibition occurs in both species, but significantly more in *C. prolifera*. As the VAZ xanthophyll cycle

is not as effective in *C. prolifera*, photoinhibition presents itself as dependent on other photoprotective mechanisms in higher irradiances that allow the dissipation of energy, through the depression of photosynthetic rates and the impairment of electron transport and photophosphorylation (Touchette and Burkholder, 2000), or as the result of photodamage (Niyogi, 1999). Together with its distinct pigment content, compared to seagrasses, photoinhibition at higher irradiances points towards the fact that *C. prolifera* in Ria Formosa maintains its well-described shade-adapted characteristics (Terrados & Ros, 1992; Robledo & Freile-Peligrin, 2005; García-Sánchez et al., 2012).

Comparison of the diel cycles revealed significantly lower maximal quantum yield (F_v/F_m), minimal effective quantum yield ($\Delta F/F'_m$) and non-photochemical quenching (NPQ) in *C. prolifera* when compared to seagrasses. Malta et al. (2005) found similar F_v/F_m in *C. prolifera* submitted to high light and high nutrients, which could relate to its capacity to survive higher light intensities, if nutrients are not limiting. In another study, *C. nodosa* and *C. prolifera* of Mar Menor lagoon were compared, revealing a similar response (García-Sánchez et al., 2012). *C. nodosa´*s and *Z. marina´*s higher F_v/F_m were not found early in the evening during the 48-hour cycle but rather during late night (around 4:00), with NPQ values being lower at this same period. This is caused by the fact the antennae need to relax and that epoxidation of zeaxanthin into violaxanthin takes longer then the inverse de-epoxidation.

Higher values of NPQ indicate the presence of photoprotective mechanisms and can be related to xanthophyll cycles (Demmig-Adams & Adams, 1996). Excessive light energy is dissipated in the antennae through reversible de-epoxidation of certain xanthophyll pigments. This is caused by an increase of the pH in the thylakoid lumen, resulting of exposure to excessive light (Demmig-Adams et al., 1999; Müller, Li & Niyogi, 2001). Sun-adapted macrophytes have shown to contain larger pools of xanthophyll cycle pigments and possess a greater ability to convert these pigments under high light conditions (Adams & Demmig-Adams, 1996; Larkum, Drew & Ralph, 2006). However, differences between sun-adapted species exist (Silva et al., 2013; Marín-Guirao et al., 2015). One of them is how differently seagrasses react to excess light. The differences in NPQ between species are related to the individual capacity of each species to dissipate excess energy (Enríquez & Borowitska, 2002). This was visible when comparing the 48-hour chlorophyll fluorescence cycle. As can

be seen in the results, the first day of measurements was a darker and cloudier day, which is probably the reason behind the lower NPQ in *Z. marina* during the first day, being a lot higher during the second, when it was sunny. *C. nodosa,* however, revealed similar NPQ response during both days, revealing that its photoprotective mechanisms are activated even under lower irradiances. In this study, *C. nodosa* presented a higher zeaxanthin concentration and de-epoxidation index, which points towards a highly photoprotective response to light, converting more violaxanthin into zeaxanthin (Adams & Demming-Adams, 1992). A similar response was found elsewhere for *Z. marina* (Ralph et al., 2002), which also increased its zeaxanthin concentration and de-epoxidation index, but not to the same extent as *C. nodosa* in this study.

*C. prolifera´*s lower NPQ values during the 48-hour cycle reveals that, although some excess energy is dissipated, it is nowhere near that of seagrasses. Additionally, it showed little to no change in its antheraxanthin or zeaxanthin concentrations at solar noon. This does not occur in seagrasses, with both species demonstrating a significant rise of the de-epoxidized xanthophylls. Higher antheraxanthin content in the morning in *Caulerpa* sp. has been recorded before and was related to low light photo-oxidative stress that can occur in these species (Raniello et al., 2006). This reveals that exposure to low light after longer dark conditions (night) may cause photo-oxidative stress.

The accumulation of lutein epoxide (Lx) has been related to the improvement on light harvesting efficiency of PS_{II} antennae and its commonly found in higher foliar concentrations in deeply shaded species/individuals (García-Plazoala, Matsubara & Osmond, 2007). Lutein epoxide de-epoxidation into lutein transforms an efficient light harvesting system into a photoprotective one, under excess light (Müller, Li & Niyogi, 2001). In addition to the VAZ xanthophyll cycle and working in parallel, Lx cycle aid in the dissipation of excess energy, further improving photoprotection by lutein, or improve light capture if lutein epoxide concentration is high. The Lx cycle, however, has only been identified in higher plants (García-Plazoala, Matsubara & Osmond, 2007). Solar noon samples of *C. nodosa* and *Z. marina* appear to contain more lutein and less lutein epoxide, which indicates that seagrasses may use this cycle for additional photoprotection only. However, to the best of our knowledge, this cycle has not yet been described in macroalgae. It cannot be excluded that

the higher Lx values in *C. prolifera* may be associated to a constitutive shade adapted behavior, and its need for a higher light harvesting capacity in low light environments.

The amount of light that can be absorbed by a leaf/frond depend on its pigment content. Modification of this content as, for example, the increase of chlorophylls content and modification of their ratios can enhance (or decrease) light harvesting capacity and the efficiency of absorption (Marín-Guirao et al., 2015). Chlorophyll content in macroalgae tends to be higher when compared to seagrasses. Although not significant, the results of both experiments reveal that *C. prolifera* total chlorophyll content is slightly higher than that of seagrasses. *C. prolifera* seems to present a lower Chl *a/b* ratio, when compared to both *C. nodosa* and *Z. marina*. Lower Chl *a*/*b* ratios are generally found in shade-adapted species, while light-adapted species are characterized by higher Chl *a*/*b* ratio (Robledo & Freile-Peligrin, 2005; Rosenburg & Ramus, 1982). These results seem to corroborate the idea that *C. prolifera* in Ria Formosa performs like a shade-adapted species (Terrados & Ros, 1992; García-Sánchez et al., 2012) while, in comparison, the seagrasses behave as light-adapted species.

At night, seagrass leaves contained significantly lower soluble sugar values than during the day. *C. nodosa* and *Z. marina* have been shown to contain less soluble sugar during lower or no irradiance (Zimmerman et al., 1995; Silva et al., 2013). Comparatively, *C. prolifera* revealed no significant change of its soluble sugar content. Similar results were obtained during the mesocosm experiment, with soluble sugar being significantly higher in *Z. marina* than in *C. prolifera*, while starch was significantly higher in *C. prolifera*. This leads us to believe that *C. prolifera* has a different carbohydrate regime compared to seagrass. Lower soluble sugar content could be linked to *C. prolifera´s* higher respiration rate. Plant respiration produces a large amount of chemical energy and components necessary for biosynthesis and cellular maintenance (Atkins et al., 2005). Thus, under the same conditions as *Z. marina*, *C. prolifera´*s higher adenylate energy charge could be a result of a higher respiratory activity compared to *Z. marina.* Furthermore, starch content was still rather high (May) when compared to findings by other authors (Terrados & Ros, 1992; Robledo & Freile-Peligrin, 2005; Vergara et al., 2011). This was linked with an increase in protein content and higher productivity and recruitment of new fronds. In Ria Formosa, *C. prolifera*

total carbohydrate content is relatively high, which leads us to believe that starch content remains untouched, while soluble sugars are enough to support the various biochemical processes. Further analysis of the annual carbohydrate usage of *C. prolifera* in Ria Formosa may help elucidate its global carbohydrate management strategy, including production, use and storage.

Z. marina revealed limited flexibility in its capacity to allocate carbon and carbohydrates, presumably to save inter-conversion energy, and to maintain a possible energy source (Burke, Dennison & Moore, 1996; Silva et al., 2013). This would also explain why *Z. marina* shows a lower adenylate charge during the morning, allocating less carbon to maintain its energy levels. Silva et al. (2013) showed that *C. nodosa* possesses a more advantageous strategy, capable of maintaining similar energy levels at night or during the day. Nevertheless, this was tested using an *in situ* shading experiment, and not under an invasive pressure caused by an algae. The significant decrease of *Z. marina* soluble sugars in the rhizomes, when mixed with *C. prolifera*, was accompanied by a significant increase of starch in the leaves. However, the magnitude of the increase in leaf starch content is much lower than the decrease in soluble sugars in the rhizomes. This significant decrease of carbohydrates content in *Z. marina* while maintaining a similar energy charge could be related with the increase in dark respiration. However, dark respiration was kept at the same rate, not indicating the use of extra carbohydrates. We hypothesize whether *Z. marina* may use carbohydrates as building blocks for the synthesis of molecules with allelopathic functions such as aminoacids, fatty acids, phenolic compounds and others (Gniazdowska & Bogatek, 2005) as a response to *C. prolifera*, explaining this way their decrease. The reduction of the sediment quality may induce stress in *Z. marina*, with a corresponding physiological response. This could involve the activation of detoxifying mechanisms, antioxidant systems (Gniazdowska & Bogatek, 2005), and production of secondary metabolites, like flavonoids and phenolic acids, which have a potential algicidal effect (Laabir et al., 2013).

C. prolifera is a rhizophitic and nitrophilic algae, capable of using both sediment and water column nutrients, actively foraging for nutrients (Malta et al., 2005). In *C. prolifera*, as in most species, the nutrients are used to produce aminoacids, proteins such as Rubisco (which accounts for up to 50% of the soluble protein of leaves) (Parry et al., 2003), and

secondary metabolites. In the presence of *Z. marina*, the nutrients in the sediment must be shared between both species. An increase in soluble protein may be the result of *C. prolifera* actively foraging for more nutrients in order to sustain its productivity, especially in the presence of neighboring species, more so than *Z. marina*. Furthermore, recent research in Ria Formosa demonstrated that *C. prolifera* absorbs most of its nutrients by the belowground thallus, with absolute rates that are much higher than those of *Z. marina* (Alexandre, pers. comm.).

Different mechanisms that contribute to *Caulerpa* sp. successful invasions have been suggested. Among these, the allelopathic use of metabolites has been described in *Caulerpa racemosa* (Raniello et al., 2007). The mesocosm experiment revealed that *C. prolifera* has no effect on the overall photosynthetic performance of *Z. marina*. Furthermore, no differences in foliar/frond soluble sugars, soluble proteins and adenylate energy charge and pigments were found. Beneath the sediment, however, a significant decrease in starch content in *Z. marina*'s rhizomes reveals that the presence of *C. prolifera* may affect this seagrass. Checcherelli et al. (2014) showed that *C. racemosa* can cover both substrate and seagrass rhizomes, depending on the state the meadow. Invasive species can have a significant impact on the fitness of native plants, namely by alterations of the ecosystem processes above or below ground (Oduor, 2013). Our observation appears to be the first record of *C. prolifera* rhizoids found among *Z. marina* rhizomes (Fig. 4.1). This allows hypothesizing that *C. prolifera* could affect the substrate around *Z. marina* rhizomes, namely by increasing organic matter pools and microbial activity but also increasing sulfide, which is particularly harmful for seagrasses (Holmer et al., 2009).

Figure 4.1: *Z. marina* collected in Ria Formosa, illustrating *C. prolifera* rhizoids entangled around the seagrass rhizome.

Another trait described by Collado-Vides (2002) is *C. prolifera´*s remarkable morphological plasticity, with big differences between shade and light fragments. We can´t

exclude the possibility that *C. prolifera* of Ria Formosa is capable of such morphological change, depending on environmental conditions. In fact, during sampling for both experiments, *C. prolifera* samples were rather compact, and blades were short, in contrast with those observed in deeper waters. This would indicate that *C. prolifera* growing in shallower regions of Ria Formosa may be light-adapted fragments. Furthermore, *C. prolifera* appears to present different growth rates, depending on the environmental conditions (Malta et al., 2005; Gab-Alla, 2007). The regression of seagrasses meadows of Ria Formosa may lead to their replacement by these light-adapted fragments. *C. nodosa,* which presents marked differences in its seasonal leaf production rates in Ria Formosa (Cunha & Duarte, 2007), may provide the chance for these macroalgae to advance and replace parts of these meadows.

5. Conclusion

Based on the chlorophyll *a* fluorescence response and its photosynthetic pigments, *Caulerpa prolifera* of Ria Formosa displays a shade-adapted response, while seagrasses were more light-adapted. The algae demonstrated differences in its carbohydrate proportions, using more soluble sugars but storing more starch compared to the seagrasses. This lower soluble sugar content was connected to its higher respiration rates. The interaction of *Z. marina* and *C. prolifera* revealed to have no effect on photosynthetic performance or pigment concentrations. The algae does however appear to have a physiological effect on the seagrass, which drastically decreased its rhizome starch content. We hypothesize that, instead of accumulating extra carbohydrates as starch, *Z. marina* may be using them as a base for the synthesis of molecules with allelopathic functions. This is the first record of this response. Due to its morphological and physiological plasticity, further studies are required to fully comprehend *C. prolifera´*s ecological and biochemical processes in Ria Formosa on a seasonal scale.

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