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The Effects of Natural and Anthropogenic Drivers on Seagrasses

Los Efectos de Factores Naturales y Antropogénicos en Fanerógamas Marinas

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Resumen

Las fanerógamas marinas son monocotiledóneas que recolonizaron los mares hace cien millones de años formando áreas de gran cobertura llamadas “praderas marinas”. Las praderas marinas son consideradas ecosistemas clave alrededor del mundo, ya que proporcionan una gran biodiversidad e importantes servicios ecosistémicos a las costas y estuarios donde habitan. Entre otras funciones, las fanerógamas marinas destacan porque son altamente productivas, son fuente de alimento y de refugio para numerosos organismos acuáticos, juegan un papel clave en el mantenimiento de la estructura y calidad de las costas, e incluso disminuyen los efectos del cambio climático. Sin embargo, a pesar de su importancia, estas especies están siendo seriamente amenazadas debido al incremento de las actividades humanas de las últimas décadas, las cuales ya han ocasionado el declive del 29% de su superficie global.

Desde la Revolución Industrial, las actividades humanas han acelerado las emisiones de dióxido de carbono a la atmósfera, provocando, entre otros, el calentamiento global y la acidificación oceánica. Esto producirá a finales de siglo un incremento de las temperaturas superficiales de 2 a 4ºC y una disminución de 0.06 a 0.35 unidades del pH superficial de los océanos. La combinación de estos fenómenos con las numerosas actividades humanas que hoy en día afectan a las praderas marinas desembocará en un complejo escenario de múltiples factores de estrés en estos importantes ecosistemas marinos. Es por ello, que la experimentación y simulación de futuros escenarios que tengan en cuenta la combinación de varios factores estresantes sobre las fanerógamas marinas, son hoy en día altamente necesarios para tomar medidas en su manejo y conservación.

Generalmente, las praderas marinas responden de dos maneras diferentes a las perturbaciones o cambios ambientales en el medio ambiente en el que habitan. Las praderas que son dominadas por especies cuya presencia es perdurable y su abundancia y dominancia de especies no varía drásticamente después de las perturbaciones, son consideradas “praderas marinas persistentes”. Por el contrario, las praderas que están formadas por especies que disminuyen su cobertura cuando las condiciones ambientales no son favorables y cuya dominancia de especies es variable con el tiempo, son consideradas “praderas marinas transitorias”. A su vez, esta respuesta a las perturbaciones determina la morfología, ecología y estrategias en la reproducción sexual de las fanerógamas marinas. Las praderas persistentes suelen estar compuestas por especies de fanerógamas marinas de gran porte cuyos rizomas tienen una tasa de crecimiento lenta. Estas especies se caracterizan por formar frutos voluminosos y flotantes que pueden ser transportados por la superficie marina largas distancias. Sin embargo, las praderas marinas transitorias suelen ser especies con pequeños rizomas de crecimiento rápido y que producen pequeños y numerosos frutos que son enterrados en el sedimento formando bancos de semillas. Un claro ejemplo de praderas persistentes son las formadas por la especie *Posidonia oceanica* (L.) Delile en el Mar Mediterráneo. Mientras que las praderas mixtas de las especies *Halodule wrightii* Asch. y *Ruppia maritima* L. en el Golfo de México, son un claro ejemplo de praderas marinas transitorias.

La principal vía de expansión de las fanerógamas marinas es mediante la clonación asexual de sus rizomas. Pero la formación de frutos y semillas por reproducción sexual permite que las fanerogamas marinas puedan colonizar nuevos ambientes o recuperar praderas que han sido previamente impactadas. Además, la formación de frutos y semillas aumenta la variabilidad genética de las especies, incrementando las posibilidades de desarrollar características resistentes a los factores estresantes naturales y antropogénicos. Sin embargo, es también en esta etapa cuando las fanerógamas marinas cuentan con el mayor número de cuellos de botella en su crecimiento, especialmente durante el asentamiento y el anclaje de las semillas sobre el fondo marino. Esto se debe a que, para tener una colonización exitosa, las fanerógamas marinas tienen que desarrollar rápidamente un fuerte sistema radicular que sea capaz de adherirse al sustrato y de resistir los movimientos del sedimento provocados por las olas o corrientes marinas. Los cuellos de botella en las primeras etapas de desarrollo de las fanerógamas marinas también implican problemas en el anclaje de los trasplantes al sedimento en los programas de restauración ambiental de estas especies. Por lo tanto, aumentar nuestro conocimiento sobre las respuestas fisiológicas de las fanerógamas marinas frente a los factores ambientales, especialmente al tipo de sustrato durante la germinación, puede ser altamente útil para aumentar el reclutamiento sexual de las praderas marinas y, a su vez, para mejorar el anclaje de estas especies en los programas de restauración ambiental. Estos conocimientos son esenciales para mantener el éxito reproductivo de las fanerógamas marinas y así, mejorar la variabilidad genética de las especies y su conservación y resiliencia frente a futuros cambios ambientales.

El objetivo general de esta tesis es incrementar el conocimiento sobre las estrategias en el reclutamiento sexual y sobre los efectos de los factores naturales y antropogénicos en el desarrollo y fotosíntesis de fanerógamas marinas. Para llevar a cabo este objetivo, se realizaron experimentos con enfoques diferentes, en distintas etapas de desarrollo y especies de fanerógamas marinas; cuyos objetivos y resultados quedan recopilados en los cinco capítulos siguientes:

En el **Capítulo 1** se estudia el reclutamiento sexual de la fanerógama marina *P. oceanica*. El reclutamiento sexual de esta especie es poco conocido debido a la dificultad que supone el seguimiento de sus frutos y semillas sobre la superficie marina. En este capítulo se evalúan las adaptaciones que desarrolla *P. oceanica* desde su dispersión en el interior de los frutos hasta su asentamiento y colonización del sustrato. Para ello, se realizan una serie de observaciones histológicas, análisis ultra-estructurales y experimentos de mesocosmos en frutos y semillas recolectados en costas de la Región de Murcia. A partir de los resultados obtenidos y de conocimientos previos, se concluye que el reclutamiento sexual de *P. oceanica* puede ser dividido en tres etapas principales: (I) *Dispersión del fruto*, (II) *Adhesión de la semilla* y (III) *Anclaje de la plántula*. Estas tres etapas comparten dos objetivos esenciales en la colonización de esta fanerógama marina: el aumento de la fotosíntesis de la semilla y la mejora del anclaje del sistema radicular en los diferentes sustratos. Las adaptaciones que desarrollan las semillas de *P. oceanica* en este estudio son similares a las producidas por otras fanerógamas marinas durante el reclutamiento sexual, como en especies de los géneros *Thalassia sp.* y *Enhalus sp.* que, al igual que en Posidonia, producen semillas sin latencia y en el interior de frutos membranosos. Estas coincidencias sugieren que las praderas marinas que presentan este mecanismo de dispersión han evolucionado de una manera convergente a las mismas estrategias de desarrollo durante el reclutamiento sexual.

En el **Capítulo 2** se estudia la influencia de la consolidación del sedimento y del enterramiento de la semilla en el desarrollo de plántulas de *P. oceanica*. Para ello, las plántulas fueron previamente germinadas durante un mes en un sustrato duro (sobre cristal) y en un sustrato blando (sobre arena) y fueron posteriormente trasplantadas en arena con tres niveles distintos de enterramiento de la semilla. Los resultados muestran que las plántulas que previamente crecieron en arena tenían una mayor longitud de raíz pero una menor longitud foliar que las que crecieron sobre un sustrato duro. No obstante, después del primer mes de germinación, las raíces de las plántulas que crecieron sobre el sustrato duro no se desarrollaron completamente. Sin embargo, después del trasplante de las plántulas en arena con diferentes niveles de enterramiento de semilla, las hojas de las plántulas crecidas en los distintos sustratos fueron equilibradas en cuanto a la superficie foliar, pero las raíces de las plántulas que crecieron en sustrato duro apenas crecieron. Además, el enterramiento de la semilla no afectó al desarrollo de las plántulas. Por lo tanto, se concluye que una previa germinación de las plántulas en arena puede mejorar considerablemente la supervivencia de las plántulas en programas de restauración ambiental, ya que al aumentar el tamaño de las raíces, permiten que las plántulas sean más resistentes a las corrientes marinas y al oleaje.

En el **Capítulo 3**, con el objetivo de determinar los efectos de los eventos extremos derivados del calentamiento global en los primeros estadios de desarrollo de *P. oceanica*, se cultivaron plántulas de cinco meses de edad a las actuales y futuras temperaturas extremas superficiales que se esperan que se alcancen mediante olas de calor en el Mediterráneo (27ºC, 29ºC, 31ºC y 33ºC). Los resultados demuestran como temperaturas por encima de 27ºC, las cuales están siendo alcanzadas actualmente durante los meses más calurosos del verano, limitan el crecimiento de las plántulas debido a fallos en el sistema fotosintético. Las plántulas que crecieron a temperaturas superiores a 27ºC mostraron una reducción en el crecimiento de las hojas y, en algunos casos, sufrieron senescencia foliar y mortalidad. Estos resultados implican que la capacidad de colonización de esta fanerógama marina puede verse seriamente amenazada en las próximas décadas. La limitación de la colonización podría tener importantes efectos en cascada en los ecosistemas, peligrando la supervivencia de los organismos marinos que viven entre las praderas de Posidonia y disminuyendo la variabilidad genética de la especie.

En el **Capítulo 4**, para comprobar los efectos de las olas de calor sobre las plántulas de *P. oceanica* en combinación de otros factores ambientales como el sobrepastoreo o el enterramiento de las semillas por sedimentación, se cultivaron plántulas en el mismo gradiente de temperaturas que se usó en el capítulo anterior y simulando estos dos factores estresantes. Los resultados muestran como los aumentos en la temperatura derivados de las olas de calor tienen un mayor efecto sobre el crecimiento y la fotosíntesis de las plántulas que los otros dos factores estresantes. Mientras que el enterramiento no afecto al desarrollo de las plántulas, la simulación del sobrepastoreo redujo considerablemente el crecimiento de las hojas. Además, cuando ambos factores fueron simulados simultáneamente tuvieron un mayor efecto sobre el desarrollo de las plántulas que por sí solos. En este estudio queda demostrado como la acumulación de factores estresantes en los ecosistemas marinos pueden provocar efectos negativos sinérgicos en el desarrollo de plántulas de *P. oceanica*. Además, los eventos extremos de temperatura pueden tener mayores efectos negativos en las praderas que la combinación de otros factores ambientales, limitando la colonización de esta importante especie.

En el **Capítulo 5** se estudia la influencia de la acidificación oceánica en praderas marinas homoespecíficas y heteróspecificas de dos especies que coexisten en los estuarios del Golfo de México, *H. wrightii* y *R. marítima*. Se prevé que el incremento de CO_2 debido a la acidificación oceánica beneficie el desarrollo, productividad y reproducción sexual de las fanerógamas marinas. Esto se debe a que en condiciones ambientales normales las fanerógamas marinas están limitadas en cuanto a disponibilidad de carbono y a que estas especies presentan mecanismos para aprovechar productos derivados de la reacción de carbono en el agua para realizar la fotosíntesis. Sin embargo, se desconoce cómo puede influir un aumento de CO_2 a las fanerógamas marinas de ambientes estuarinos, donde las condiciones ambientales de luz, salinidad y temperatura están cambiando continuamente debido a grandes descargas de agua dulce. Por lo tanto, lechos marinos de ambas especies de fanerógamas marinas fueron expuestos en combinación y por separado a un tratamiento de aguas marinas en condiciones ambientales y a otro tratamiento de aguas marinas con una alta concentración de CO_2. Los resultados muestran como en ecosistemas dinámicos como los estuarios del Golfo de México, la acidificación oceanica no tiene efectos positivos sobre el crecimiento y productividad de *H. wrightii* y *R. maritima*. Estos resultados implican que, a corto plazo, las fluctuantes condiciones ambientales de los estuarios pueden provocar en las fanerógamas marinas diferentes respuestas que las que se esperan que ocurran bajo un escenario de acidificación oceánica.

Los resultados de esta tesis doctoral nos permiten completar la información acerca de la reproducción, desarrollo y coexistencia de las praderas marinas bajo la influencia de distintos factores ambientales y perturbaciones. Estos resultados son esenciales para realizar estrategias de mantenimiento y conservación de las praderas marinas y a su vez, para la mejora de los procedimientos de anclaje en los programas de restauración ambiental. La investigación acerca de la ecología, fisiología y reproducción sexual en las praderas marina es fundamental para determinar qué ecosistemas serán más vulnerables a los futuros cambios ambientales y, por lo tanto, para asegurar la conservación de estos importantes ecosistemas en el futuro.

1 General Introduction

Seagrass meadows as key marine habitats

Seagrasses are a group of marine monocotyledons which re-colonized the sea about 100 million years ago (Den Hartog 1970). Seagrasses form extensive three-dimensional areas of great foliar coverage called "seagrass meadows". This structure allows seagrasses to be considered habitat-forming species as it confers ecosystems high biodiversity.

Seagrass meadows are ideal habitats for hiding from predators, which means they act as effective shelters and attractive feeding areas for many marine organisms (Jackson et al. 2001). The high canopy of seagrass beds also reduces the strength of marine currents by stabilizing sediments and modifying irradiance conditions, which also makes seagrasses perfect nursery areas for numerous larvae or juvenile stages, many of them come from commercial, recreational and subsistence fish and shellfish (Watson et al. 1993, Hemminga and Duarte 2000, Unsworth et al. 2014). The leaf surface of seagrasses are often encrusted by several families of epiphytes, which provide a major food source for large marine organisms which, in turn, are threatened species, including turtles or manatees (Green and Short 2003).

Despite seagrasses representing less than 2% of all flowering plant species (Cook 1990, Les and Philbrick 1993), they are considered one of the most productive ecosystems worldwide as they contribute about 1.13% of the global marine primary production. Unlike other photosynthetic organisms, seagrasses store most of their primary production in sediments (Duarte and Cebrián 1996). This leads to the fact that seagrasses are considered one of the most effective carbon sinks of oceans as they reduce the impacts of global warming and ocean acidification (Björk et al. 2008, Russell et al. 2013, Duarte et al. 2013). Old leaves, rhizomes and roots of seagrasses often accumulate on shores and form large surfaces created by accumulated seagrass beach-casts that protect coasts from the erosion of waves and maintain their geo-morphology (Koch 2001, Manca et al. 2012). In addition, the leaves of seagrasses can filter and clean seawater from suspended sediments and toxic trace elements by controlling water quality in ecosystems (Hemminga and Duarte 2000, Sanz-Lázaro et al. 2012).

These numerous ecological services that seagrass meadows provide marine ecosystems with are important sources of human benefits in socio-economic terms (Costanza et al. 2006, Barbier et al. 2011, Vassallo et al. 2013, Unsworth and Cullen-Unsworth 2018). Yet despite its distinguished importance, in recent decades human activities and climate change have led to a 29% decline in seagrass beds worldwide (Orth et al. 2006, Waycott et al. 2009).

Disturbances to seagrasses

Seagrasses usually inhabit overpopulated areas which are constantly subjected to numerous anthropogenic disturbances, such as coastal or estuarine systems. Among others, the construction of infrastructures, fishing or aquaculture activities or increasing tourism and transport on coasts alter the chemical composition of seawater, and affect seagrass development and productivity (Hemminga and Duarte 2000). Besides the disturbances that occur *in situ*, rivers and water inputs frequently flow to coasts and estuaries, and unload nutrients or pollutants that derive from human activities in upstream areas (Vitousek et al. 1997). These substances can be harmful for seagrasses or degrade ecosystems' water quality to cause the loss or fragmentation of seagrass meadows.

Seagrasses are vulnerable to many natural and anthropogenic factors, but most reported declines are related to human activities, especially those that reduce light availability for photosynthesis, such as burial and eutrophication (Short and Wyllie-Echeverria 1996). Anthropogenic drivers degrade seagrass meadows at a rate of 110 km^2 per year (Waycott et al. 2009). Natural causes can also negatively influence the development of seagrass beds by either physical habitat alterations or other organisms interacting. For example, one of the biggest disasters to have occurred in seagrasses was due to parasitic proliferation that caused high mortality and large-scale declines in the seagrass populations of *Zostera marina* L. along both European and North American coasts (Rasmussen 1977). Geological and meteorological events, such as storms, cyclones, hurricanes, floods or earthquakes, can lead to seagrasses being uprooted or buried which, in turn, promotes negative effects on meadows, among others (Poiner et al. 1989, Preen et al. 1995, Larkum and West, 1990). However, burial of seagrasses is produced principally by human activities. Building infrastructures on coasts (Burdick and Short 1999, Ruiz and Romero 2003) and excess siltation from changes in land uses or dredging (Onuf 1994, Badalamenti et al. 2006) usually lead in sediment mobilization, which results in the leaves of seagrasses being buried, which limits their light availability. A lowering light incidence on the leaves of seagrasses can alter photosynthesis performance and, thus, their development declines (Cabaço et al. 2008).

Other common disturbances to seagrass beds are due the nutrients or toxic elements that derive from fishing, aquaculture or industrial activities, which can alter sediment, and deteriorate water quality or eutrophication. Eutrophication is produced by the intensive accumulation of nutrients and fertilizers on coasts (Nixon 1995). This causes epiphytic and benthic macroalgae to proliferate (Rabalais and Nixon 2002) by outcompeting and shading leaf surfaces and limiting seagrass productivity (Cambridge et al. 1986, Short et al. 1995). Aquaculture activities can affect the development of seagrass beds because feed waste and fish excretions accumulate in both, seawater and sediment (Ruiz et al. 2001, Cancemi et al. 2003). These products increase seawater turbidity and the concentrations of organic matter in sediments, which enhances microbial activity and oxygen deficit and, thus, deteriorates seagrass meadows (Borum et al. 2004). Increasing organic matter in seawater can also increase the proliferation of epiphytes on the surfaces of seagrasses by increasing their palatability for marine grazers (Vergés et al. 2007, Prado et al. 2010).

Increasing industrial and human activities on coasts also promote the accumulation of chemical substances in seawater or invasion by exotic species, which can be directly toxic for seagrasses or cause large-scale declines in seagrasses meadows, respectively (Meinesz and Hesse 1991, Short and Wyllie-Echeverria 1996). Fishing activities can also lead to the overfishing of coastal predators and can have cascading effects for seagrass communities by increasing grazers' consumption rates (Valentine and Duffy 2006). In addition, the mechanical damage caused by boats can cause seagrass meadows to fragment and destabilize, especially in shallow areas (Walker et al. 1989, Chartón et al. 1993, Creed and Filho 1999).

Ever since the Industrial Revolution, human activities have accelerated carbon dioxide (CO_2) emissions to the atmosphere which cause climate change and ocean acidification (Watson et al. 1995, Caldeira and Wickett 2003). One of the most remarkable effects of climate change is the global warming caused by an increase in greenhouse gases to the atmosphere (Watson et al. 1995, Cai et al. 2014). Greenhouse gases (CO_2, methane, nitrous oxide and chlorofluorocarbons) are radioactively active, and can absorb and reradiate back the heat emitted from the Earth's surface. This action results in higher global temperatures, a situation that is likely to result in a global warming from 2 ºC to 6 ºC by the end of the century (IPCC 2001, 2007). Increments in temperature can be especially detrimental in closed basins, such as the Mediterranean Sea, because its confined nature makes it more sensitive to rising temperatures than open oceans (Giorgi and Lionello 2008, Vargas-Yáñez et al. 2008). Increases in temperature above the temperature thresholds of seagrasses can have serious consequences for their productivity by mainly affecting their reproduction and development (Diaz-Almela et al. 2007, Massa et al. 2008, Olsen et al. 2012, García et al. 2013, Thomson et al. 2015, Ruiz et al. 2017).

However, recent studies have suggested that the most devastating climate change effects will occur by extreme events, which makes the values of important meteorological variables extreme; for example, high temperatures (heat waves), strong winds (hurricanes) or precipitation (heavy rainfall) (Stephenson 2011). The occurrence and intensity of such events, which play a major role by structuring biological assemblages compared to the continuous and gradual impacts of global warming (Jentsch et al. 2007, Sanz-Lazaro 2016, Mitchell et al. 2018), are expected to gradually increase with climate change (IPCC 2007, Watson et al. 1995).

Increasing atmospheric CO_2 also has altered the balance of inorganic carbon compounds in oceans by increasing the concentrations of bicarbonate (HCO_3^-), carbonic acid (H_2CO_3), and hydrogen ions (H^+) (Elderfield et al. 2005). These products have dropped the average sea surface pH by 0.1 units, which is expected to further decline by 0.06-0.32 units by the end of this century (IPCC, 2013). Ocean acidification is thought to alter marine ecosystems, when one organism benefits more than another which, thus, alters marine ecosystems (Guinotte and Fabry 2008, Hall-Spencer et al. 2008). Seagrasses are able to use both CO_2 and HCO_3^- for photosynthesis, and their production is generally limited by carbon (Koch et al. 2013). So they are expected to benefit from ocean acidification in the next decades. Under ocean acidification conditions, the productivity (Durako 1993, Zimmerman et al. 1997, Invers et al. 2002), vegetative growth (Zimmerman et al. 1997, Jiang et al. 2010, Russell et al. 2013, Martínez-Crego et al. 2014) and flowering frequency (Palacios and Zimmerman 2007) of seagrasses can rise. However, seagrasses adopt different carbon allocation strategies, which suggests differential growth responses to high carbon availability (Ow et al. 2015). So, seagrasses can benefit from this extra carbon availability to different extents, which may alter seagrass meadows' current species composition and structure.

The numerous human activities that can disturb seagrass beds have led to a complex scenario of multiple stressors that affect the marine environment. Under these conditions, stressors can act independently of one another (additive effects), or can act combined, which leads to interactions that can enhance (synergy) or diminish (antagonism) their effects compared to them acting individually (Darling and Côté 2008). Thus the effects of natural drivers and their combination on long-term scales can produce unexpected complex responses in ecosystems (Heugens et al. 2002, Sih et al. 2004, Crain et al. 2008). Among others, the coupling of multiple stressors could reduce seagrasses' resilience to future global-scale environmental changes, such as, climate change or ocean acidification (Connell et al. 2013). Therefore, the experiments that simulate scenarios with multiple stressors are necessary to design future environmental management measures, especially in the habitat-forming species that live in coastal areas with high anthropogenic pressures like seagrass meadows.

Life-history strategies and clonal development in seagrasses

Seagrasses belong to four families of submerged monocotyledons (Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, Ruppiaceae, and Zosteraceae) and are found in a wide variety of coastal habitats, tidal flats, littoral zones and estuaries from sublittoral, circumpolar, tropical, subtropical and temperate regions (Short et al. 2007). In temperate regions, most seagrass meadows are monospecific (Hemminga and Duarte 2000), but in tropical and subtropical areas they are formed by multiple seagrass species, especially in indo-pacific regions with high diversity in species composition (Terrados et al. 1999).

Despite the wide ranging environmental conditions that they can tolerate as a group, as a species seagrasses show very marked geographical limitations as regards light, salinity and temperature (Drew 1979). The specific ranges of these environmental factors imply that seagrasses develop restricted life-history strategies that depend on their response to changes in environmental conditions. The meadows dominated by persistent species with minor seasonal variations in abundance after unfavorable periods are considered enduring seagrass meadows. In contrast, the meadows formed by species of seagrasses that disappear under unfavorable environmental conditions result in a dynamic composition of species dominance with time are considered transitory seagrass meadows (Kilminster et al. 2015).

The differential response to environmental changes of enduring *versus* transitory meadows spells marked differences in the size and velocity of seagrass species development. Enduring seagrass meadows are usually composed of species that produce large slow-growing ramets and form extensive and persistent meadows (Fig. 1), while transitory seagrass meadows are formed by species that produce small short-lived ramets and quickly developing rhizomes (Kilminster et al. 2015; Fig. 2). Ramets are the clonal growth units of seagrasses composed of a rhizome, a variable number of leaves and a root system (Figs. 1 and 2). Rhizomes can develop in both directions, horizontally (plagiotropic) or vertically (orthotropic). The majority of seagrasses display both development types in their rhizomes (dimorphic), but some seagrasses only present plagiotropic development (Beer 2001).

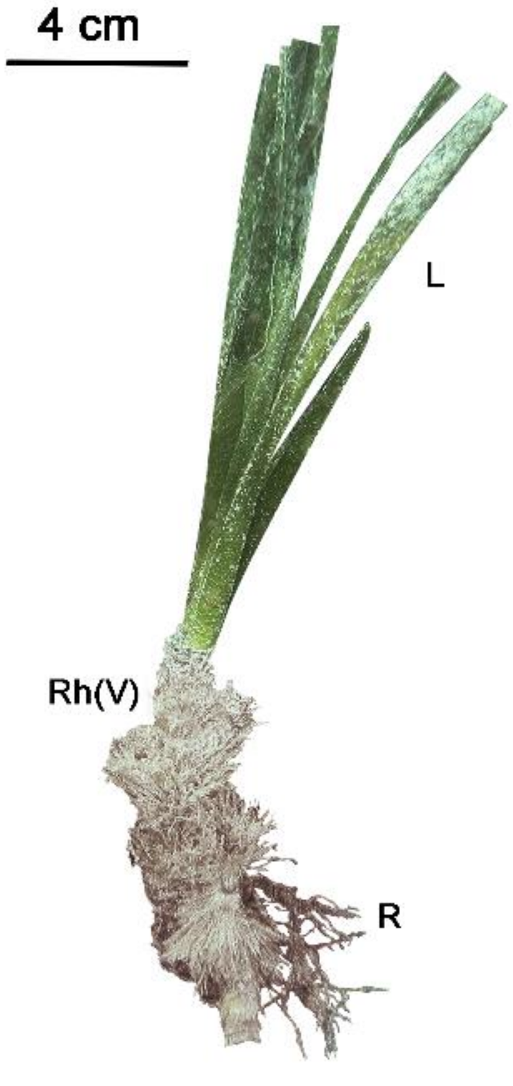

Fig. 1. Ramet of the seagrass *Posidonia oceanica.* L, leaves; Rh(V), vertical rhizome; R, root system.

Examples of enduring meadows are those formed by the seagrass *Posidonia oceanica* (L.) Delile on Mediterranean coasts (Fig. 1). This seagrass forms extensive meadows that occupy an area covering approximately 12,200 km^2 (Telesca et al. 2015). In the Mediterranean, *P. oceanica* forms three genetically differentiated groups as a result of its re-colonization after the closure and partial desiccations of the Mediterranean Sea during the "Messinian salinity crisis". Two of these groups are located in the west (north and south) and seem more genetically polymorphic than the other group located in the east of the basin (Boudouresque 2004, Waycott et al. 2006). *P. oceanica* grows in a several types of sediments and lives in zones between the surface to a maximum depth of 45 m (Green and Short 2003). This seagrass tolerates temperatures from 15 ºC to 25 ºC (Drew 1978) and only survives in small salinity fluctuations (from 1 psu to 2 psu above a mean ambient salinity of 37-38 psu; Sánchez-Lizaso et al. 2008). The rhizomes of this seagrass have very low growth rates, but can endure several decades (Marbá et al. 1996, Marbà and Duarte 1998). The vegetative growth of this seagrass occurs continuously throughout the year, but in spring, coinciding with meadow flowering, high seawater temperatures bring about increased leaf mortality (Díaz-Almela et al. 2009).

Clear examples of transitory seagrass beds are those composed of *Halodule wrightii* Asch. and *Ruppia maritima* L. in the estuarine waters of the Gulf of Mexico (Fig. 2). In this particular habitat, both seagrasses co-exist with differing seasonal peaks given their different ecological strategies. *H. wrightii* and *R. maritima* are, respectively, opportunistic and colonizing species because they take advantage of appropriate environmental conditions to grow rapidly. *H. wrightii* tends to grow all year long with maximum growth in summer and flowers in late spring. *R. maritima* grows at cool temperatures and undergoes senescence after flowering in spring (Pulich 1985, Cho and

Poirrier 2005). Both seagrasses prefer different salinity ranges as *H. wrighii* does not survey salinities lower than 6 psu, while *R. maritima* develops better between 10-30 psu (Adair et al. 1994, Doering et al. 2002). *R. maritima* is considered the most widely distributed seagrass in the world because it is present in all types of biogeographical regions, and often inhabits muddy sediments in estuaries or coastal lagoons. The distribution of *H. wrighii* is more narrower than in Ruppia, but is also distributed worldwide in temperate and tropical areas in the Atlantic, Pacific and Indic oceans (Green and Short 2003, Short et al. 2007).

Sexual reproduction: implications for seagrass conservation

The commonest form of expansion in seagrasses takes place by the clonal development of seagrass rhizomes. But, like monocotyledons, seagrasses can also form seeds and fruits by sexual reproduction, which allows seagrasses to colonize new areas or recover their meadows after perturbations (Kendrick et al. 2012, 2017). The formation of fruits and seeds provides the species' genetic recombination by increasing the probability of developing phenotypes that resist natural and anthropogenic stressors (Reusch et al. 2005, Reynolds et al. 2012, van Katwijk et al. 2016).

Sexual reproduction in seagrasses is also strongly influenced by the life-history strategies of species against environmental changes (Kilminster et al. 2015). Transitory meadows usually form numerous small seeds with hard pericarps that are buried under the sediments, forming seed banks (e.g. *Halophyla sp., Halodule sp., Cymodocea sp., Phyllospadix sp., Zostera sp., Ruppia sp.* and *Heterozostera sp.*; Kuo et al. 1990, Inglis 2000).

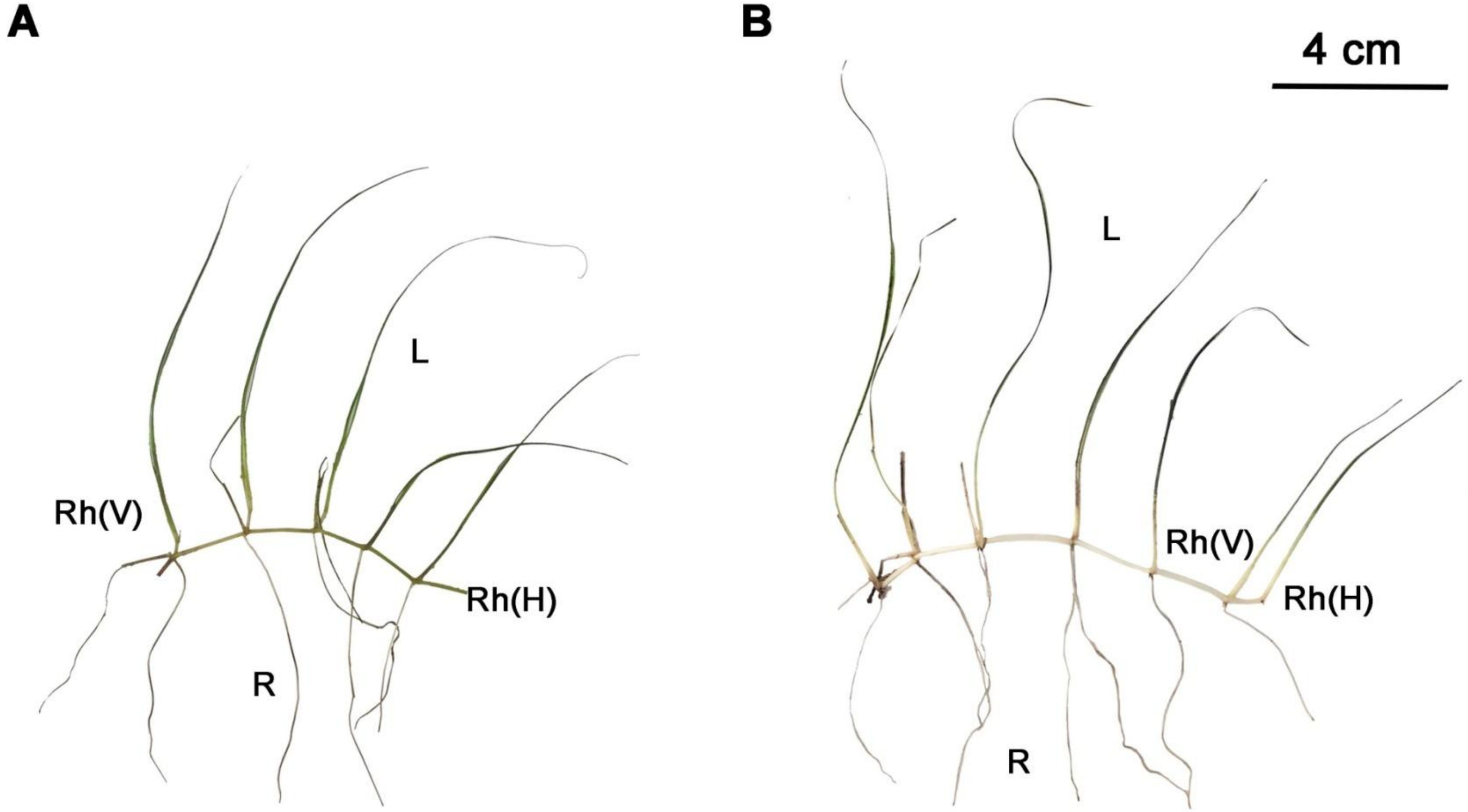

Fig. 2. Ramets of the seagrasses *Halodule wrightii* (A) and *Ruppia maritima* (B). L, leaves; Rh (V), vertical rhizome; Rh (H), horizontal rhizome and R, root system.

Seeds are dormant, but their germination activates once environmental conditions are adequate for seedling development. For example, species *H. wrightii* and *R. maritima* form seeds with a hard durable pericarp, and they also form seed banks in the sediments that are close to parental plants. The fruits of *H. wrightii* mature at the rhizome level under sediment, where can be stored for at least 4 years (McMillan 1981, 1985, 1991). The seeds of *R. maritima* can survive drought and excessive water salinity periods that can last up to 3 years under sediments. The dispersal distances in these seagrasses are very short, but they can colonize new habitats far away from parent plants, helped by the ingestion and dispersion of birds or fish (Kantrud 1991).

Persistent seagrass meadows can form either fleshy or viviparous seedlings that grow on the parent meadow (e.g. *Thalassodendron sp.* and *Amphibolis sp.*) or large fruits with membranous pericarps that can float long distances on the sea surface with seed inside (e.g. *Posidonia sp.*, *Enhalus sp.* and *Thalassia sp.*; Kuo and Kirkman 1996, Inglis 2000). The seeds of these seagrasses are non-dormant and start germination as soon as they are released from parent plants (Larkum et al. 2006). This is the case of the seagrass *P. oceanica* which forms large buoyant fruits with a single seed inside in spring (Fig. 3). *P. oceanica* fruits can be transported by floating hundreds of kilometers with seeds inside for at least 2 weeks (Buia and Mazzella 1991, Aliani et al. 2006, Micheli et al. 2010). The large supply of nutrients in *P. oceanica* seeds constitutes supply for growth for up to the first year of seedling development (Balestri et al. 2009). This seagrass' seeds perform photosynthetic activity (Celdrán and Marín 2011) and part of the energy that seeds obtain by photosynthesis is invested in developing leaves and roots during the first month of germination at least (Celdrán and Marín 2013). So in a few months, *P. oceanica* seedlings can develop a strong root system and longer leaves that permit their survival in seabed, as well as the formation of a new meadow on a long-term scale (Fig. 3C).

However, sexual reproduction in seagrasses often encounters certain bottlenecks, especially in the germination stage and when seedlings settle, when they have to quickly develop their root system to be able to anchor to a sustainable sediment environment (Harper 1977, van Katwijk et al. 2016) and to survive grazers and bioturbations (Statton et al. 2017). This has led to some seagrasses, such as *P. oceanica*, to develop adhesive root hairs on their primary roots to enhance their attachment to seabeds (Badalamenti et al. 2015).

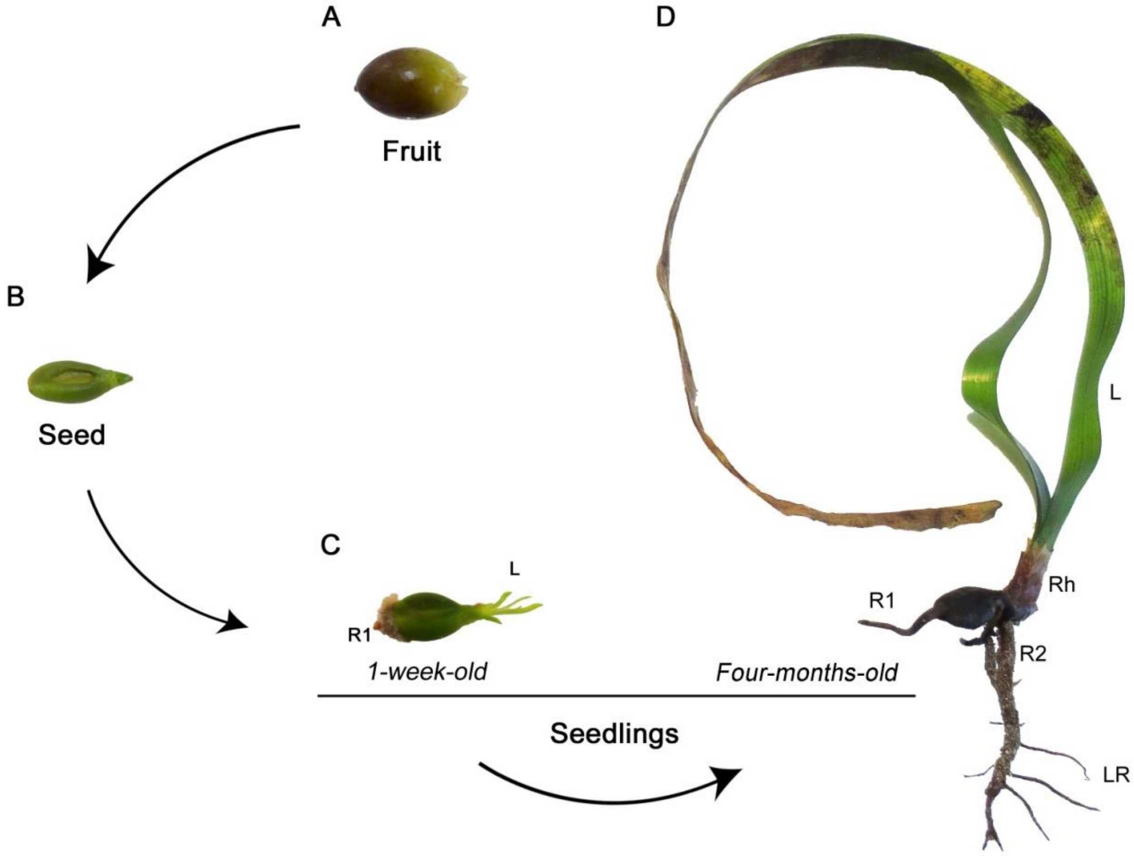

Fig. 3. The seagrass *Posidonia oceanica's* sexual recruitment cycle. The picture shows the buoyant fruit (A), newly-released seed (B) and seedling after 1 week (C) and 4 months of development (D). S: seed; L: leaves; Rh: Rhizome R1: primary root; R2: secondary roots and LR: lateral roots.

The advantages of sexual reproduction have attracted much interest in using seeds and seedlings for environmental restoration projects (Balestri et al. 1998, Terrados et al. 2013). Such projects can help seagrasses to recover after the perturbations that produce the partial loss or fragmentation of meadows. Restoration by using seeds and seedlings can increase the success of seagrass recruitment once perturbations have been eliminated. This is especially important in seagrasses with slow growth rates in their rhizomes and rare sexual reproduction, such as the species that form persistent seagrass meadows.

Seagrass seeds can be transplanted in the sea similarly to the way terrestrial seeds are employed in tree reforestation programs (Balestri et al. 1998, Terrados et al. 2013, Zarranz et al. 2012). However, this restoration type entails anchorage problems, especially in the seagrasses of the genus *Posidonia*. Even though seagrass seedlings are capable of remaining anchored to different substrates (Badalamenti et al. 2015, Balestri et al. 2015), their roots are not long enough to be firmly adhered (Balestri and Bertini 2003). Therefore, seedlings can be uprooted by waves and currents (Meinesz et al. 1993), which means relatively unsuccessful recruitment (Statton et al. 2017). Thus more knowledge on the physiological mechanisms which seagrass seedlings develop according to different environmental factors is necessary to improve our understanding of sexual recruitment and first seedling development stages and to, thus, increase the success of environmental restoration projects carried out on seagrass meadows.

Objectives

The different life-history strategies and the morphological variability in both clonal development and sexual reproduction that show seagrasses imply that the conservation of this important habitat-forming species is strongly linked to knowledge on not only their sexual recruitment strategies, but also their responses to natural and anthropogenic drivers. Understanding these approaches is extremely necessary to design environmental management strategies that consider the environmental requirements of each seagrass species for its successful sexual recruitment, and to promote the conservation, resilience and evolution of seagrass meadows in future scenarios. In order to increase our understanding in these areas, the general aim of this thesis is to shed light on sexual recruitment strategies, and also on the effects of natural and anthropogenic divers on the development and photosynthesis performance of seagrasses.

Different approaches, species and development stages of seagrasses were used to achieve this aim. This thesis was divided into five chapters that correspond to the following specific objectives:

Chapter 1. To evaluate the influence of natural drivers on seagrasses' sexual recruitment. The effects of light and substrata type on the productivity and development of *P. oceanica* seeds are tested to understand the sexual recruitment of the seagrasses that form non dormant seeds.

Chapter 2. To examine the influence of natural drivers on seagrasses' germination. The effects of substrate hardness and seed burial on the development of *P.*

oceanica seedlings are tested to evaluate the influence of sediment type and burial in the first months of this seagrass' development.

Chapter 3. To study the effects of anthropogenic drivers on seagrasses' germination. The effects of the present and forecasted extreme temperatures events produced by heat waves on *P. oceanica's* productivity and development are evaluated.

Chapter 4. To study the interaction of several anthropogenic drivers in seagrasses' germination. The effects of seed burial, grazing and heat waves on *P. oceanica's* productivity and development are evaluated.

Chapter 5. To evaluate the effects of anthropogenic drivers on co-occurring seagrass species. The influence of ocean acidification is tested in homospecific and heterospecific seagrass beds of *H. wrightii* and *R. maritima*.

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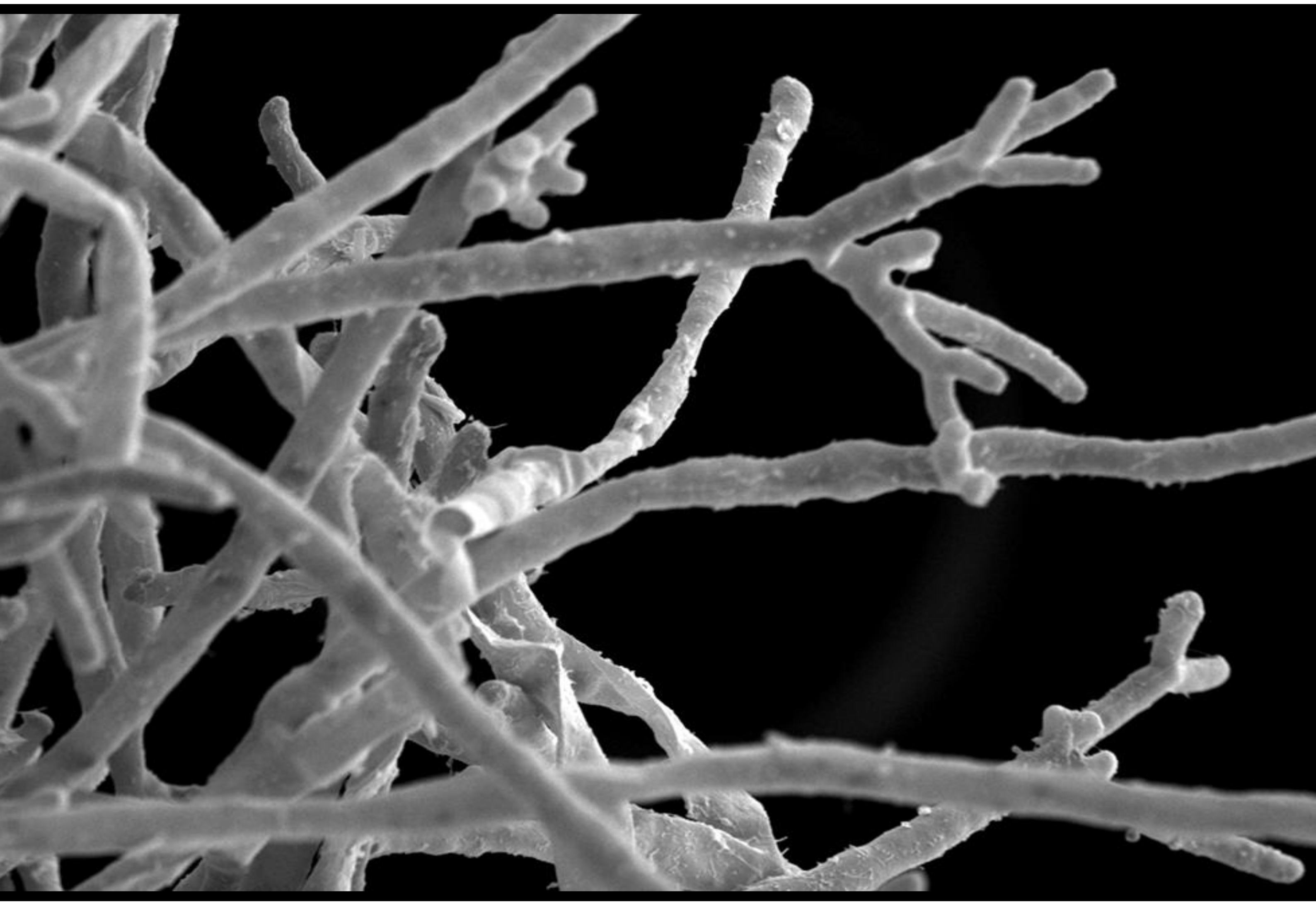
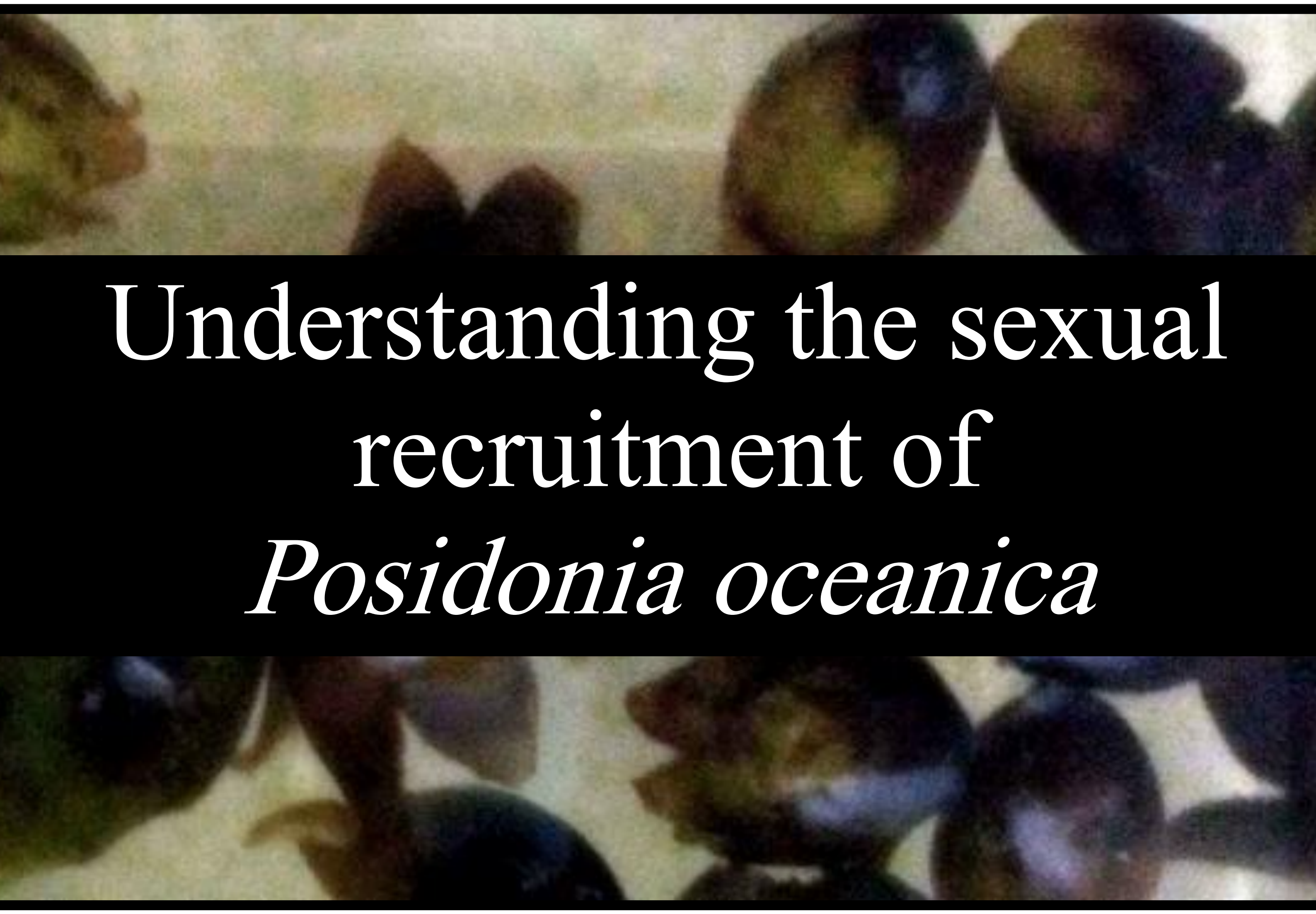
2 Chapters



Chapter 1



Understanding the sexual recruitment of *Posidonia oceanica*

Introduction

Seagrasses are formed by a polyphyletic group of monocotyledons (order Alismatales) which recolonised marine environments 80 million years ago (Les et al. 1997). Seagrasses are habitat-forming species because they are a source of food and shelter for a wide variety of fish and invertebrates, and perform relevant ecosystems services (Heck et al. 2003, Hughes et al. 2009). Despite their importance, seagrass populations are currently threatened by a variety of anthropogenic stressors (Waycott et al. 2009, Collier and Waycott 2014). The ability of seagrasses to cope with environmental perturbations depends, to some extent, on genetic variability, which is obtained through sexual recruitment (Reusch et al. 2005, Ehlers et al. 2008, Reynolds et al. 2012). By forming new individuals, seagrasses increase their genetic diversity and, thus, their ability to colonise new areas and to adapt to environmental changes (Orth et al. 2006, van Dijk et al. 2009, Kendrick et al. 2012, McMahon et al. 2014, Smith et al. 2016).

Seagrasses have contrasting colonisation strategies (Inglis 2000). Some seagrasses form seed banks of small seeds with hard pericarps that can remain in the dormancy stage for several months. These seagrasses are generally short-lived and can recover quickly from disturbances by not germinating far away from parent meadows (e.g. *Halophyla sp., Halodule sp., Cymodocea sp., Phyllospadix sp., Zoslera sp.* and *Heterozostera sp.*; Kuo et al. 1990, Inglis 2000). In contrast, other seagrasses form dispersal propagules. This strategy is typical of long-lived seagrasses that form buoyant fruits with large non-dormant seeds inside, such as genera *Posidonia sp.*, *Enhalus sp.* and *Thalassia sp.* (Inglis 2000, Kuo and Den Hartog 2006). Accordingly, the seeds of these latter type of seagrasses have an enormous dispersal capacity compared to the former type (Den Hartog 1970, Fonseca and Kenworthy 1987), which permits the evolution of species beyond unfavourable light conditions for the seedling development of parent meadows.

The seagrass *Posidonia oceanica* (L.) Delile is considered one of the oldest and largest species on Earth. An individual can form meadows measuring nearly 15 km wide and can be as much as 100,000 years old (Arnaud-Haond et al. 2012). *P. oceanica* has singular adaptations to increase its survival during recruitment. The large amounts of nutrient reserves contained in the seeds of this seagrass support shoot and root growth, even up to the first year of seedling development (Balestri et al. 2009). In the first months of germination, when leaf development is scarce, *P. oceanica* seeds perform photosynthetic activity, which increases its photosynthetic rates and, thus, maximises seedling establishment success (Celdrán and Marín 2011, 2013). Seedlings also show high morphology plasticity while their root systems develop (Balestri et al. 2015, Guerrero-Meseguer et al. 2017) by forming adhesive root-hairs to help anchorage to rocky sediments (Badalamenti et al. 2015, Tomasello et al. 2018). However, many issues about *P. oceanica's* sexual recruitment remain unknown, such as when photosynthesis in seeds is active, or understanding how seeds can remain anchored to and persist on the substrate until their root systems have completely developed. Increasing our knowledge about *P. oceanica* adaptations during its sexual recruitment is essential to design environmental policies that conserve threatened habitat-forming seagrasses with similar characteristics.

The objective of this chapter was to increase our understanding of the morphological and physiological adaptations involved in the dispersion and settlement of seagrasses which form non-dormant seeds and buoyant fruits. To do so, we performed a histological analysis and mesocosm experiments to evaluate the importance of light and substrata type in the first weeks of sexual recruitment using *P. oceanica* as a model of this type of seagrasses.

Materials and methods

P. oceanica's sexual recruitment was studied by distinguishing the dispersion and settlement stages, and by analysing the fruit pericarp, newly-released and 1-week-old seeds (Fig. 1). Seed development in the dispersion stage was evaluated by performing histological and light-transmission analyses, and by testing photosynthetic activity. The settlement stage was evaluated by performing ultrastructural analyses in the primary system of seed adherence, and two mesocosm experiments in which the influence of light and type of substrata on the development of both seeds and the primary root system was tested.

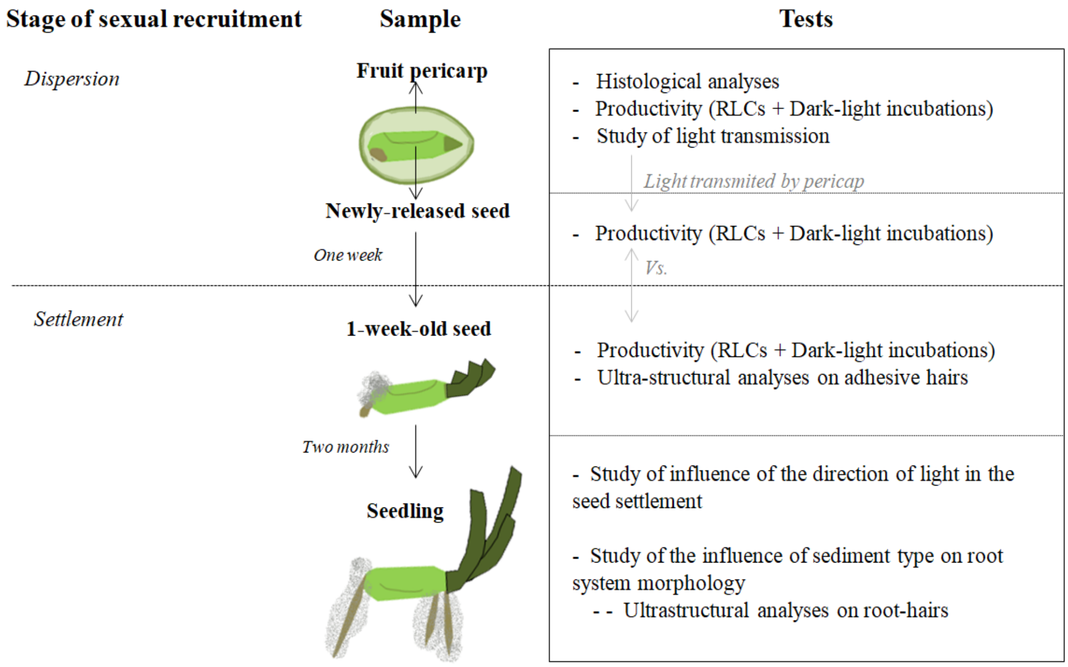


Fig. 1. Schematic representation of the experimental setup used in this study. The scheme indicates the recruitment stages of the *Posidonia oceanica* seeds (seed inside fruit pericarp, newly-released and 1-week-old seeds) and the tests used in each experiment.

The temperature and salinity of seawater used in all the experiments were 21ºC and 36, respectively. Temperature was monitored by dataloggers (HOBO, Bourne, MA, USA). Artificial seawater was prepared with bidistilled water and marine salt (Ocean Fish, PRODAC International, Cittadella, Italy). Aeration was provided by a system of tubes and capillaries connected to an air pump. In the experiments that lasted more than 1 month, a 14:10 (light:dark) photoperiod was applied in the environmental chambers. In all the other experiments, no photoperiod was applied because they lasted less than 1 day. Unless otherwise indicated, a sample size of n=5 was used in all the experiments.

Fruit collection and seed germination

P. oceanica fruits were collected on beaches in the Murcia Region (Spain) in May 2016. Fruits were immediately transported to the laboratory to avoid their pericarps from degrading, and were placed inside aquaria filled with artificial seawater and sufficient aeration to always maintain the oxygen concentration above 5.5 mg O_2/l^{-1}. Then healthy fruits were selected for the experiments. Fruits were dark green and ovoid (2.54±0.21 cm length and 1.60±0.05 cm wide) and their pericarps looked like a membranous coat over seeds (Fig. 2A). Fruit pericarp density was 716.9±64.1 $kg^{-1} \cdot m^{-3}$.

Seeds were extracted from fruits by longitudinally cutting the pericarp with a scalpel (newly-released seed). The newly-released seeds were 1.65±0.05, 0.98±0.03 and 0.57±0.02 cm length, wide and thick, respectively, and weighed 0.77±0.04 g (DW). They were green, ovoid and, on occasion, presented a short leaf and root primordial on the apical extremes (Fig. 2A). Newly-released seed density was 1,073.0±18.9 $kg^{-1} \cdot m^{-3}$. Some seeds were germinated for 1 week in individual glass jars filled with aerated artificial seawater to study the settlement stage (1-week-old seed, Fig. 2B). The 1-week-old seeds had 3-7 leaves measuring 1.68±0.12 length and 0.002–0.003 cm wide. The primary root of the 1-week-old seeds ranged from 0.001 cm to 0.005 cm length. Prior to primary root development, the base of the posterior extreme of the 1-week-old seeds developed a dense material of adhesive hairs that covered the primary root (Fig. 2B).

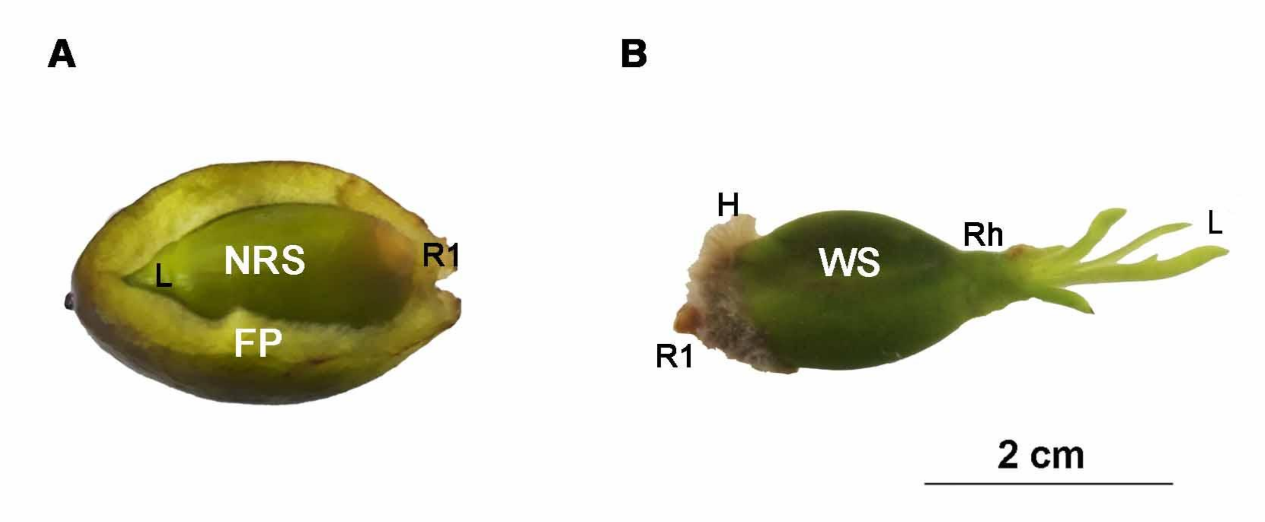


Fig. 2. Newly released seed inside a fruit (A) and 1-week-old seeds (B) of *Posidonia oceanica*. FP, fruit pericarp; NRS, newly-released seeds; WS, 1-week-old seeds; H, adhesive hairs; S, seed; R1, primary root; Rh, rhizome and L, leaves.

Histology analyses and light-transmission tests in fruit pericarp

Firstly, the cellular structure of the layers that composed the fruit pericarp was observed by optical microscopy. To do so, the fruit pericarp pieces were previously cut with a microtome (RM 2155 Leica, LeicaBiosistems, Wetzlar, Germany) in 10 µm-thick sections. These sections were stained with 0.05% toluidine blue and mounted in DPX medium (Agar Scientific, Stansted, UK). They were observed under an optical microscope (Leica DMLB, LeicaBiosistems, Wetzlar, Germany) and photographs were taken (Leica DC500, LeicaBiosistems, Wetzlar, Germany).

Subsequently, to test the light that was transmitted through the pericarp to the seed, the fruit pericarp fragments were exposed to a gradient of light irradiance (from 10 to 1,000 µE· $m^{-2} \cdot s^{-1}$, n=18). The light intensity capable of penetrating the pericarp was determined by placing pericarp segments (1.5 cm^2) over the photoradiometer datalogger probe (DO 9721, Delta OHM, Padova, Italy). Then pericarps were exposed to eight light intensities (10, 30, 50, 150, 300, 500, 750 and 1,000 µE· $m^{-2} \cdot s^{-1}$) by varying the distance from the light source (LED: 20W, 6,400K and 1,600 lumens daylight; Electro DH, Barcelona, Spain). To ensure a similar area of light availability in all the measurements, the probe was covered by a handmade mould made of dark plasticine, with a 1 cm-diameter gap left in the middle (Fig. 3). The employed light intensities were based on the average solar light radiation recorded in the month of fruit dispersion (May) on the Murcia Region coast (1,035.6±23.79 µE· $m^{-2} \cdot s^{-1}$; data download from the Agricultural Information System of the *Murcia Institute for Agricultural and Food Research and Development*, IMIDA; http://siam.imida.es/).

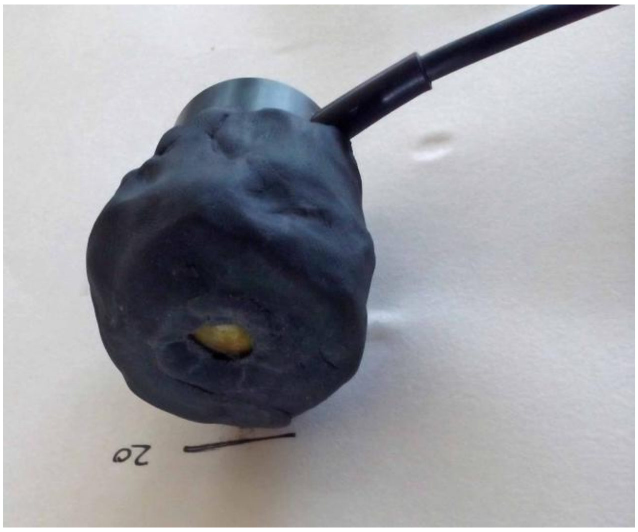


Fig. 3. Photoradiometer probe covered with the handmade mould used to evaluate the light transmitted by the fruit pericarp of *Posidonia oceanica* within the gradient of light intensity. In this image, the probe, with the fruit pericarp coupled inside it, was situated 20 cm from the light source.

Photosynthetic activity tests

Photosynthetic activity was tested in both fruits and seeds with their corresponding light intensities. Light-adapted yield and rapid light curves (RLCs) were also measured in the fruit pericarps and in both seed development stages by PAM fluorometry (MINI-PAM, Waltz, Effeltrich, Germany).

Net primary production (NPP) and respiration rates were tested by measuring the oxygen concentration (optical electrode; Portable Meter Hach HQ30d, HACH, Loveland, Colorado, USA) in the dark and light incubations of fruit pericarps, newly-released and 1-week-old seeds separately. Then gross primary production rates (GPP) were calculated by subtracting the respiration from the NPP rates. Incubations were carried out with airtight glass jars filled with artificial seawater at the light intensity average obtained during the *P. oceanica* fruit dispersion period (~1,000 $\mu E \cdot m^{-2} \cdot s^{-1}$) in fruits, and at the average light radiation that seeds reached after passing the pericarp (~10 $\mu E \cdot m^{-2} \cdot s^{-1}$).

RLCs were performed in fruit pericarps and seeds using a range of light intensities from 10 to 1,000 $\mu E \cdot m^{-2} \cdot s^{-1}$. Each light intensity was applied for 10 s, which was followed by a saturating pulse of 0.2 s. The *r*ETR values against light irradiances were fitting to the exponential model proposed by Platt et al. 1980. The derived parameters of RLCs, including photosynthetic efficiency (α), photoinhibition parameter (β), maximum electron transport rate ($r\mathrm{ETR}_{max}$) and saturation irradiance (E_k), were calculated following the equation of Ralph and Gademann 2005. Additionally, light-adapted yields were tested in seeds to verify if photosynthesis efficiency depended on prior adaptation to light (Genty et al. 1989).

Influence of light on the seed settlement stage

To evaluate the influence of the direction of light on the settlement stage, the 1-week-old seeds were incubated according to three different directions of light as regards the longitudinal seed axis (top, right or left) for 2 months. To determine if the grain size of substrata and the light direction had interactive effects on seedlings' responses, two types of substrata (sand and pebble) were used in the experiment.

The three directions of light were applied by using three environmental chambers: an environmental chamber with the light bulbs placed on the roof and two environmental chambers with the light bulbs positioned on walls. Seeds were placed individually in autoclaved glass jars filled with artificial seawater and substrate (2 cm). All the seeds were oriented with the apical extreme, where leaves came out and faced the back of the environmental chamber. The grain size of the sand substrate used in the experiment was 0.03% of gravel, 1.46% of very coarse sand, 85.1% of coarse sand, 13.4% of medium sand and 0.01% of fine sand. The grain size of the pebble substrate was 80.8% of pebble and 19.2% of gravel (Wentworth 1922). The light intensity on seeds was 100 $\mu E \cdot m^{-2} \cdot s^{-1}$.

After 2 months, the angle of orientation of seeds was measured by respecting the light direction: above ($\pi/2$ radians), right (2π radians) and left (π radians). To do this, the final angle of rotation of the seeds obtained in each treatment was subtracted from the initial angle of orientation ($\pi/2$ radians).

Ultrastructure of adhesive hairs

Prior to primary root development, the morphology of the adhesive hairs in the 1-week-old seeds was analysed by transmission and scanning electron microscopy.

For transmission electron microscopy (JSM 6100, Jeol, MA, USA), pieces of the basal surface of the 1-week-old seed and primary roots were fixed in 2.5% Milloning´s phosphate-buffered glutaraldehyde (pH 7.2-8.2) for 1 h. These pieces were washed in 2.5% $NaHCO_3$ (60 min at 25ºC) and post-fixed in a solution of 2% OsO_4 and1.25% $NaHCO_3$ for 1 h. Subsequently, pieces were dehydrated by ethanol series and embedded in an epoxy resin solution (Epon). Then ultra-thin transverse sections were cut with glass and diamond knives. Sections were stained in a solution of uranyl acetate and lead citrate before being observed under a microscope.

For the scanning electron microscope observations (JEOL-6100 Scanning Microscope; Oxford Instrument, Abingdon, UK), the pieces of the basal surface of seeds and the pieces of their primary roots were previously dehydrated in 96% absolute ethanol, and were then point-dried and sputter-coated with gold.

Influence of substrata type on the seed settlement stage

To evaluate the influence of substrata type on success of anchorage and ultrastructure of root system, the 1-week-old seeds were individually placed into glass jars filled with artificial seawater and substrata (sand, pebble, sand+pebble and fibreglass; n=8). The sand and pebble treatments were composed of the substrata used in the previous experiment. The sand+pebble treatment involved mixing 50% of the sand and 50% of the pebble of the previous treatment. The fibreglass treatment was used to evaluate the effects on the root system’s morphology of the fibrous substrate, such as a canopy of algae or a surface composed of seagrass beach-casts with no organic matter decomposition. Light intensity was 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ during daylight hours.

After the first month of the experiment, the success of root system anchorage and the presence of adhesive hairs were estimated by ranking them into percentage intervals depending on the number of roots anchored and hair density, respectively (Table 1). Finally after 2 experiment months, the root system of three samples per treatment was observed by scanning electron microscopy.

Table 1. Ranges of % used in the experiment of sediment type influence on the root system morphology to determine anchorage success and the density of adhesive hairs obtained after 1 month of seedling development.

Anchorage success of the root system		Density of adhesive hairs
0	The seedling was floating in seawater	The presence of hairs was null
25%	The seedling was anchored only to the sediment by one root	Hairs were hardly observable and whitish
50%	The seedling was anchored to the sediment by the primary root and at least by a secondary root	Hairs were clearly distinguishable and whitish
75%	The seedling was anchored by the primary root and more than half of the secondary root.	Hairs were very clearly distinguishable and yellowish
100%	The seedling used the primary root and all the secondary roots to anchor to sediment	Hairs were very dense and brownish

Data analysis

The gradient of light transmitted through the fruit pericarp was fitted to a regression model, which was chosen with the correlated Akaike information criterion test (AICc).

One-way ANOVAs were used to test the differences between treatments in the photo-physiological parameters (α, β, $r\mathrm{ETR}_{max}$, E_K and light-adapted yield) and photosynthetic activity (NPP, GPP and Respiration). Prior to the ANOVAs, data were tested for normality and homoscedasticity of variances by the Shapiro and Bartlett tests, respectively. Transformations were applied if data did not meet the assumptions. Statistical tests were conducted with a significance level of α=0.05. In those cases in which data did not meet the assumptions after being transformed, the significance level was lowered to α=0.01 (Underwood 1997). Tukey's test was used to examine the pairwise differences among levels when the main effects showed significant differences. The influence of the substrata type on seed anchorage success and the density of adhesive root hairs were tested by applying Kruskal-Wallis and Kramer (Nemenyi) tests.

To test the influence of the direction of light during seedling settlement, a Watson–William's test was used to determine whether the mean of the angles obtained in the seeds cultured under lateral light (right and left) differed from the mean of the angles obtained in the seeds grown with the light above in both the substrata types used in the experiment (sand and pebble). Rayleigh's tests for circular uniformity were previously tested to determine that data were unimodal and not diametrically bidirectional.

Statistical analyses were performed with the R statistical software (v. 3.2.5) using the packages "AICcmodavg","GAD" ,"PMCMR" and"CircStats" packages (R

Core Team 2016). The data results are reported throughout the manuscript as mean±standard error (SE).

Results

External morphology and fruit pericarp ultrastructure

The histological analyses showed that *P. oceanica's* fruit pericarps display the typical fruit covering structure, formed by a first layer of epidermis and a subsequent layer of mesophyll (Fig. 4A). The epidermis consisted in a single layer of thick-walled, rounded and relatively large epidermal cells (4,023.3±219.3 µm) covered by a cuticle (523.3±83.5 µm), with the cytoplasm showing numerous chloroplasts. The mesophyll was composed of two layers of different cell types, the hypodermis and the spongy mesophyll. The hypodermis (11,502.6 ± 1,990.5 µm) was composed of a compact coat of hexagonal-shaped cells with electro-dense material and chloroplasts distributed in the cytoplasm periphery (Fig. 4A). The spongy mesophyll (149,987.7±3,400.8 µm) comprised large cells containing central vacuoles or air lacunae that occupied the main cellular volume (Fig. 4A). The volume of the central vacuoles or air lacunae increased towards the internal mesophyll part (from 1 to 3.5 mm in diameter).

Light transmission in fruit pericarps

Light transmission through pericarps followed a significant linear trend according to light intensity exposure (Fig. 5A). The light transmission of pericarps ranged between 10.50±1.56 and 0.14±0.01 µE· $m^{-2} \cdot s^{-1}$ for light exposures of 10 to 1,000 µE· $m^{-2} \cdot s^{-1}$, respectively (Fig. 5A).

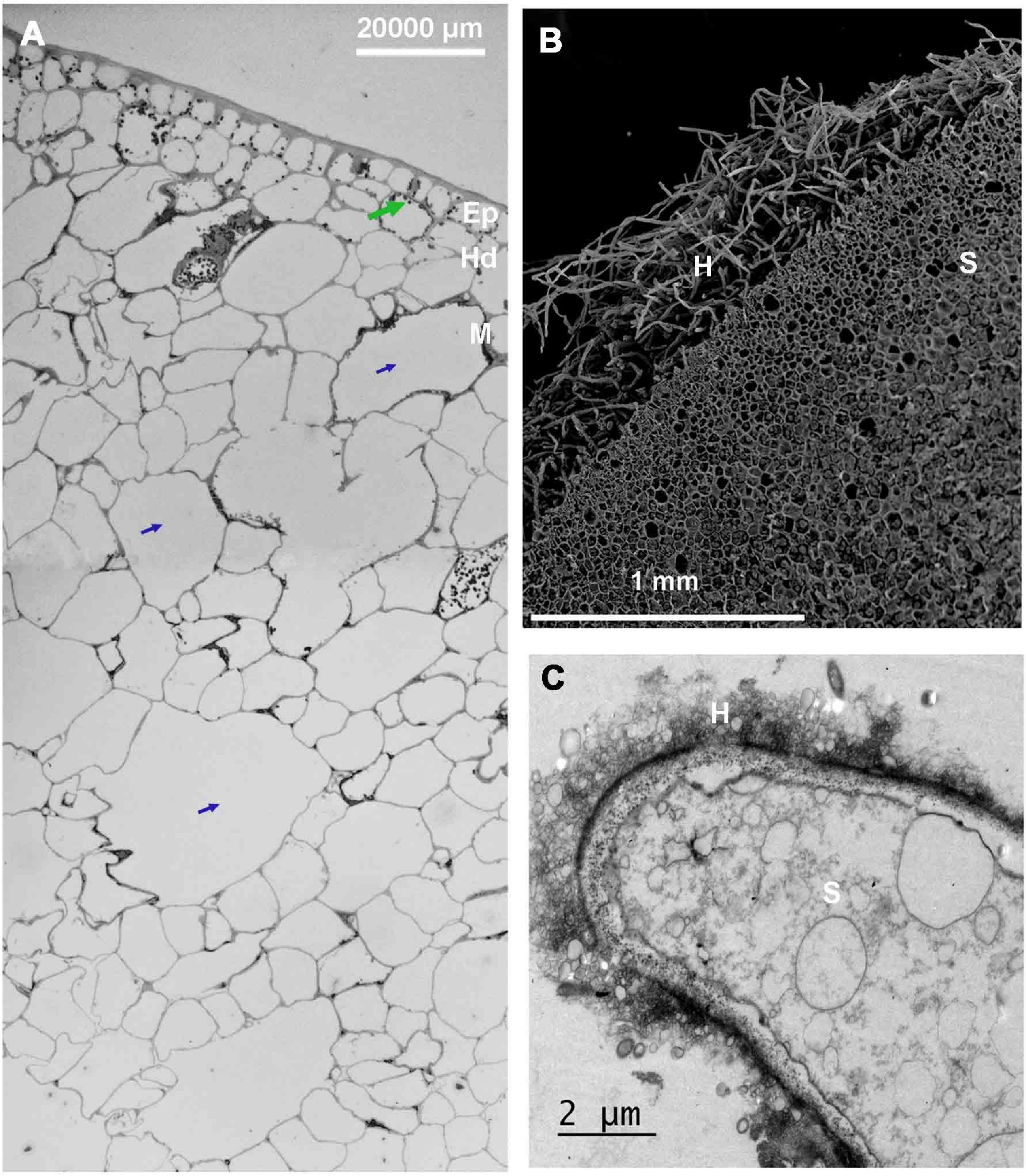


Fig. 4. Details of the histology, ultrastructure and morphology of the fruit pericarp and 1-week-old seeds of *Posidonia oceanica*. Images show the histological fruit pericarp sections (A) and the ultrastructure of the adhesive basal hairs of a 1-week-old seed (B and C). Green arrows indicate chloroplasts, while blue arrows denote air lacunae. Ed, epidermis; Hd, hypodermis; M, mesophyll; H, adhesive hairs and S, seed.

Photosynthetic activity in fruit and seed during dispersion

The RLCs showed significant differences in the parameters of α and $r\mathrm{ETR}_{max}$ among the fruit pericarps, the newly released seeds and the 1-week-old seeds (Table 2, Fig. 5B). The highest α values were found for the 1-week-old seeds (0.048 ± 0.005), while the fruit pericarps and the newly-released seeds gave similar values (0.016±0.001 and 0.017±0.002 in the fruit pericarps and the newly released seeds, respectively; Table 2, Fig. 5B). The newly released seeds and the 1-week-old seeds had significantly higher $r\mathrm{ETR}_{max}$ values (6.207±1.484 and 10.41±1.64, respectively) than those obtained in the fruit pericarps (2.905±0.496; Table 2, Fig. 5B). Notwithstanding, the β and E_K values between treatments were similar (Table 2). The fruit pericarps' light-adapted yield was significant lower than in the newly-released and the 1-week-old seeds (Fig. 5C). At a light intensity of 1,000 $\mu E \cdot m^{-2} \cdot s^{-1}$, fruit pericarps obtained negative values for NPP (-0.272±0.014 mg $O_2 \cdot g^{-1} \cdot h^{-1}$) and respiration rates (-0.234±0.007 mg $O_2 \cdot g^{-1} \cdot h^{-1}$), which gave a negative GPP rate (-0.039 ± 0.017 mg $O_2 \cdot g^{-1} \cdot h^{-1}$). At 10 $\mu E \cdot m^{-2} \cdot s^{-1}$ of light intensity, seeds gave similar GPP positive values ($p = 0.437$; Fig. 5D). In contrast, the newly-released seeds obtained significantly lower values for respiration and NPP than the 1-week-old seeds ($p < 0.05$; Fig. 5E and 5F).

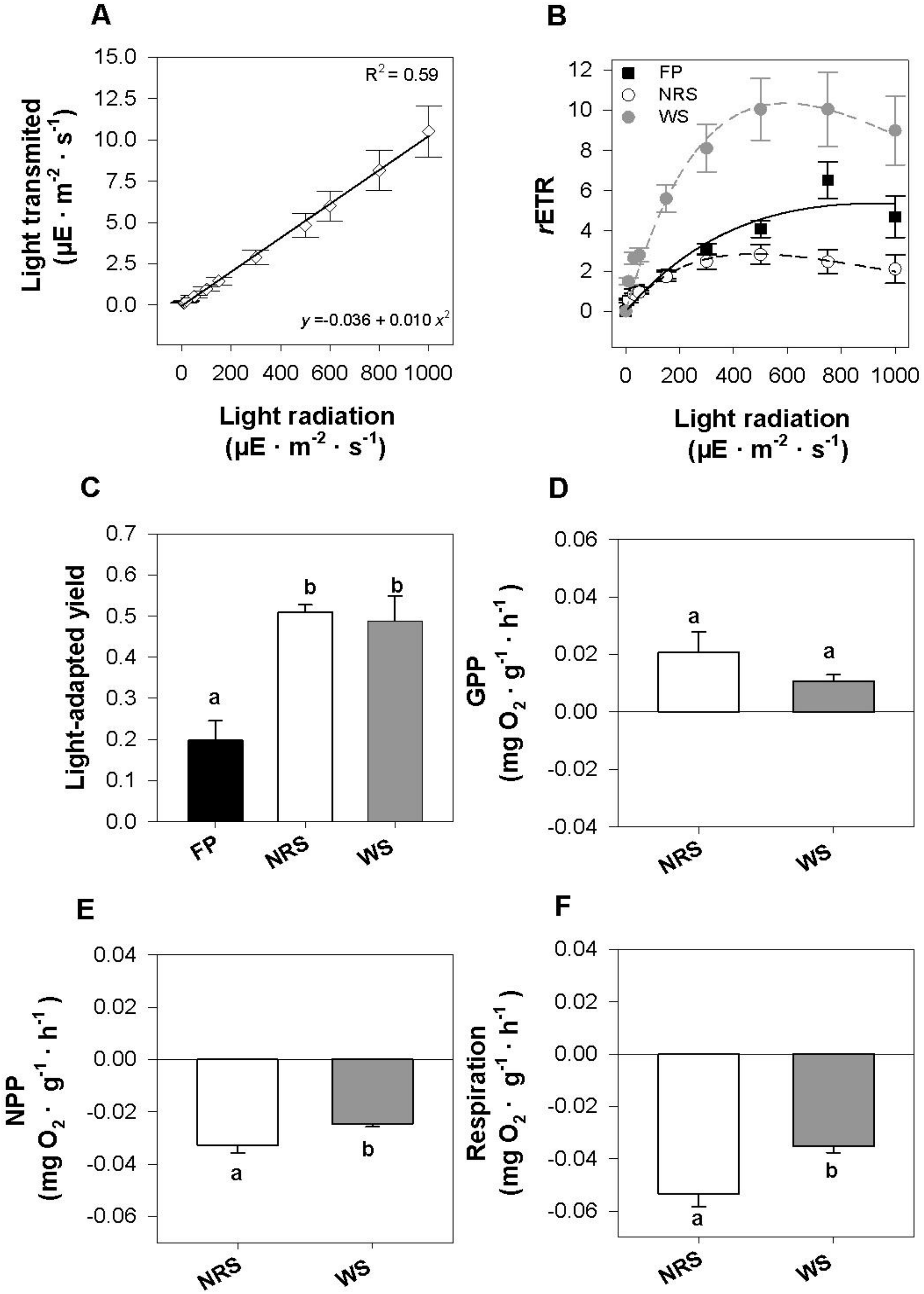


Fig. 5. Results obtained in the experiments performed to test the influence of light on the sexual recruitment of *Posidonia oceanica* (mean±SE; n=5): light transmitted by fruit pericarps in a gradient of light irradiance (A); RLCs (B); Light-adapted yields of fruit pericarp and seeds (C); GPP (D), NPP (E) and Respiration (F) obtained in newly-released (NRS) and 1-week-old seeds (WS) at a light intensity of 10 $\mu E \cdot m^{-2} \cdot s^{-1}$. Lines indicate significant regressions ($p < 0.01$) in fruit pericarps (black lines; $R^2 = 0.98$), newly released seeds (dotted black lines; $R^2 = 0.96$) and 1-week-old seeds (dotted grey lines; $R^2 = 0.97$). Letters indicate significant pairwise differences between treatments.

Table 2. Summary of the results obtained in the one-way ANOVAs and Tukey's HSD tests performed in the photophysiology parameters (α, β, *r*ETRmax, E_K and light-adapted yield) of fruit pericarps (FP), newly released (NRS) and 1-week-old seeds (WS) of *Posidonia oceanica* (n=5) . Numbers in bold indicate significant effects (α=0.05). An asterisk over the response variables indicates that data did not meet assumptions and a significance level of α=0.01 was applied.

	One-way ANOVA	***Pairwise comparisons***		
		FP *vs.* NRS	FP *vs.* WS	NRS *vs.* WS
Response variable	*p*	*p*	*p*	*p*
α	**< 0.01**	0.99	**< 0.01**	**< 0.01**
β	0.078	n.s.	n.s.	n.s.
$rETR_{max}$*	**< 0.005**	0.112	0.119	**< 0.005**
E_K	0.077	n.s.	n.s.	n.s.
Light-adaptedYield	**< 0.01**	0.088	0.155	0.933

Influence of the direction of light on seed orientation

The 1-week-old seeds showed a positive phototropism to the direction of light (Fig. 6). The angles of orientation of the seeds grown with light on walls significantly differed from those obtained in the seeds that developed with light that came from the top ($p< 0.01$). When light came from the top, seeds turned an average of 0.061±0.019 radians in relation to the initial position. Seeds rotated an average of 0.878±0.046 radians when the light came from the right and 2.242±0.041 radians when it came from the left (Fig. 6). However, the phototropism of seeds was not influenced by the sediment type in which seedlings were grown (sand and pebble; Fig. 6).

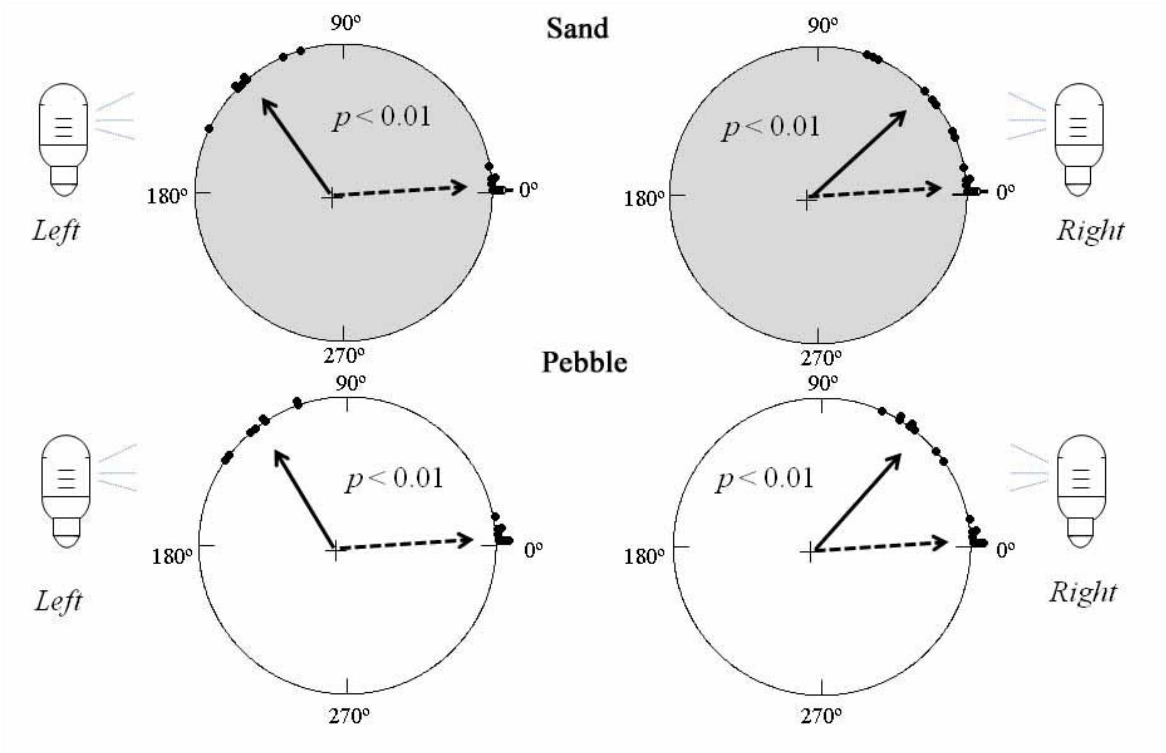


Fig. 6. Test of phototropism in the *Posidonia oceanica* seeds (n=5) grown in sand (grey circles) and pebble (white circles). Dashed lines indicate the angle of the orientation of the seeds obtained when light came from the top. Solid lines indicate the average angle of orientation of the seeds obtained in each treatment in the seedlings cultured with lateral lights (right: 180º; left: 0 º). Black points indicate the angle of orientation obtained in each sample. Significant differences between the top and lateral lights (right and left) were indicated as p <0.01 in each treatment.

Primary system of adherence in seeds

After about 1 week of development, a matrix of adhesive hairs appeared on the basal surface of seeds (Figs. 4B and 4C). In subsequent weeks, adhesive hairs concentrated on the most posterior seed part covering the primary root (Fig. 4B). After 1 month of development, all the seeds, primary and secondary roots had adhesive hairs all over their surfaces. Adhesive hairs disappeared from the surface of seeds after 2 months of development, but persisted in the primary and secondary roots for the rest of the experimental period (Fig. 7).

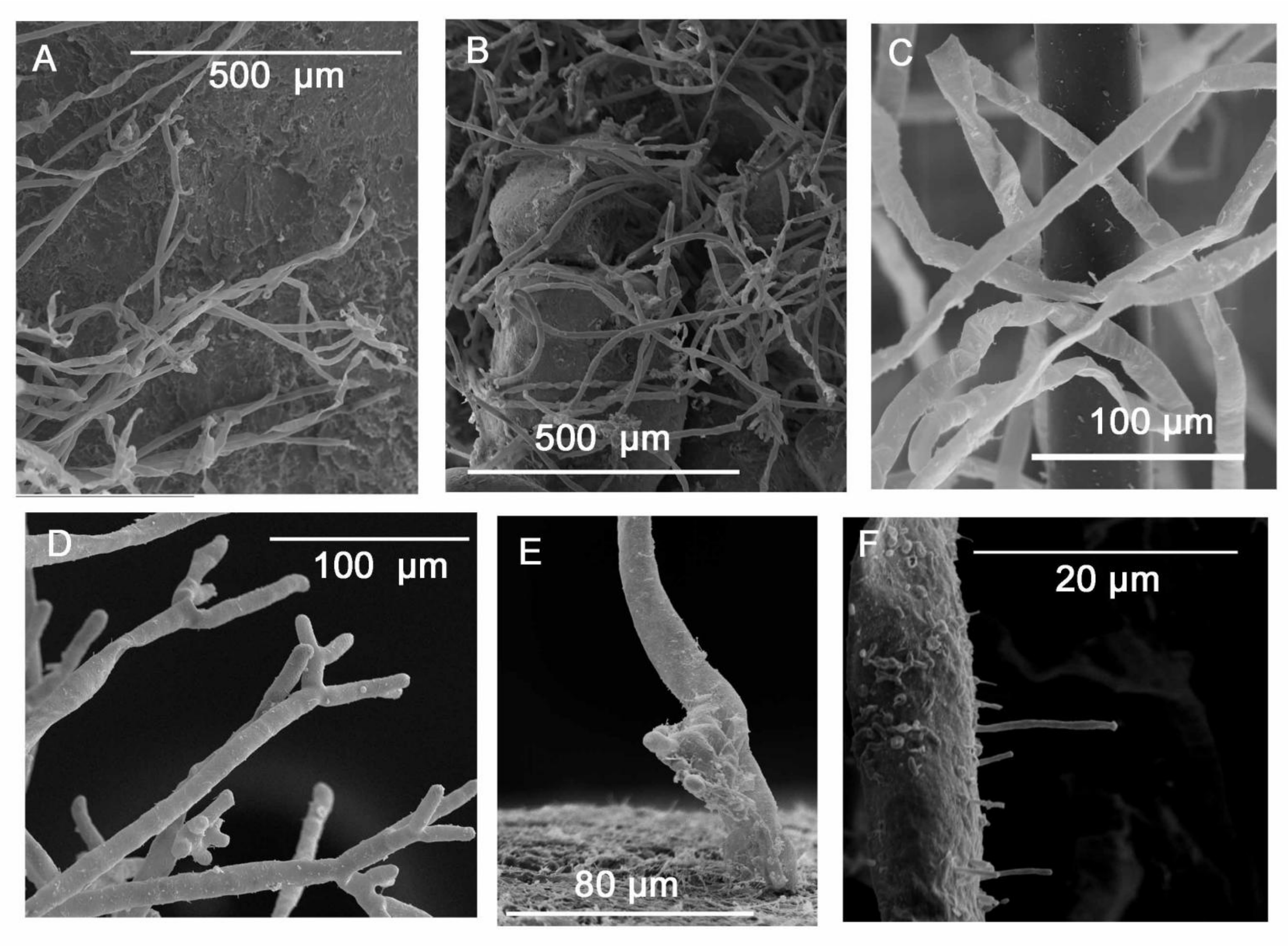


Fig. 7. Images obtained by the scanning electron microscopy of the root hairs of *Posidonia oceanica* seedlings grown in different types of sediment treatments: root hairs adhered by their basal extreme in the pebble treatment (A), root hairs surrounding the grains of sand in the sand treatment (B), root hairs that interwove a fibre in the fibreglass treatment (C), the branches edges of root hairs (D), the basal extreme of a root hair anchored to the pebble treatment (E) and the microtrichomes of the lateral surface of root hairs (F).

The adhesive hairs of seeds had the same structure as the adhesive hairs found in the primary and secondary roots of seedlings (Figs. 4B and 7). On both, adhesive hairs came from hypodermis cells, were tubular-shaped, and had a seed length that went from 5 to 10 µm and a seed width from 0.5 to 1 µm (Figs. 4B and 4C). In roots, the lengths of root hairs were not measured as they were too entangled. Root hairs were highly branched (Fig. 7D) and produced microtrichomes over their lateral sides (Fig. 7F).

Influence of substrata type on root system morphology

Despite the root system's anchoring success was significantly different between substrata types, no pairwise differences were found in the *post hoc* analyses (Table 3). The least anchorage success went to sand (45.0±14.6%), while the highest values went to the fibreglass, pebble and sand+pebble treatments (100±0.0%, 95.0±5.0% and 90.0±6.1%, respectively).

Sediment type did not influence the production of adhesive hairs in both seeds and roots (Table 3). The presence of hairs was clearly noted and formed a layer over seeds and primary roots, which was slightly greater in roots (56.0±6.1%) than in seeds (48.0±11.4%). Hair length was not measured in this experiment because hairs were totally incrusted or wrapped in sediment (Figs. 7A, 7B and 7C). The root hairs of the seedlings in the sand or fibreglass treatments were involved between their respective substrates (Figs. 7B and 7C). In contrast with the seeds grown with pebbles, root hairs were anchored by their edges to the surface of pebbles to form an amorphous adhesive matrix (Figs. 7A. and 7E). The seedling formed a tripod-like form with its root system, made up of elongated secondary roots, even in the seedlings that were not completely anchored to substrata (Fig. 8).

Table 3. Summary of the statistical results obtained in the Kruskal-Wallis and Kramer (Nemenyi) tests on the effect of substrate type influence on the root system morphology of *Posidonia oceanica* (n=5). Numbers in bold indicate significant effects. Data did not meet assumptions and a significance level of α=0.01 was applied. S: sand treatment, S+P: Sand+Pebble treatment, P: Pebble treatment and F: Fibreglass treatment.

	Kruskal-Wallis	**Pairwise comparisons**					
		S vs. S+P	**S vs. P**	**S vs. F**	**S+P vs. P**	**S+P vs. F**	**P vs. F**
	p	*p*	*p*	*p*	*p*	*p*	*p*
Anchorage success	0.02	0.38	0.18	0.07	0.97	0.83	0.97
Seed adhesive hairs	0.48	0.79	0.99	0.99	0.61	0.59	1.00
Root hairs	0.86	0.94	1.00	1.00	0.88	0.94	1.00

Fig. 8. Tripod-like formation of the roots of the *Posidonia oceanica* seedlings on a sand+pebble substrate 1 month after settlement.

Discussion

Our results shed light on the development of *P. oceanica's* seeds during sexual recruitment. Based on this and previous knowledge, we divided the process into three stages (Fig. 9): *(I) fruit dispersion*, where seeds displayed relevant photosynthetic activity inside fruits; *(II) seed adhesion*, where seeds developed adhesive hairs on their basal surface and primary roots; and *(III) seedling anchorage*, where seeds produced a tripod-like form with their primary and secondary roots, and oriented seeds to face light. These three development stages of sexual recruitment focus on two common colonisation strategies: maximisation of the photosynthesis of seeds and enhanced seed anchorage to substrate.

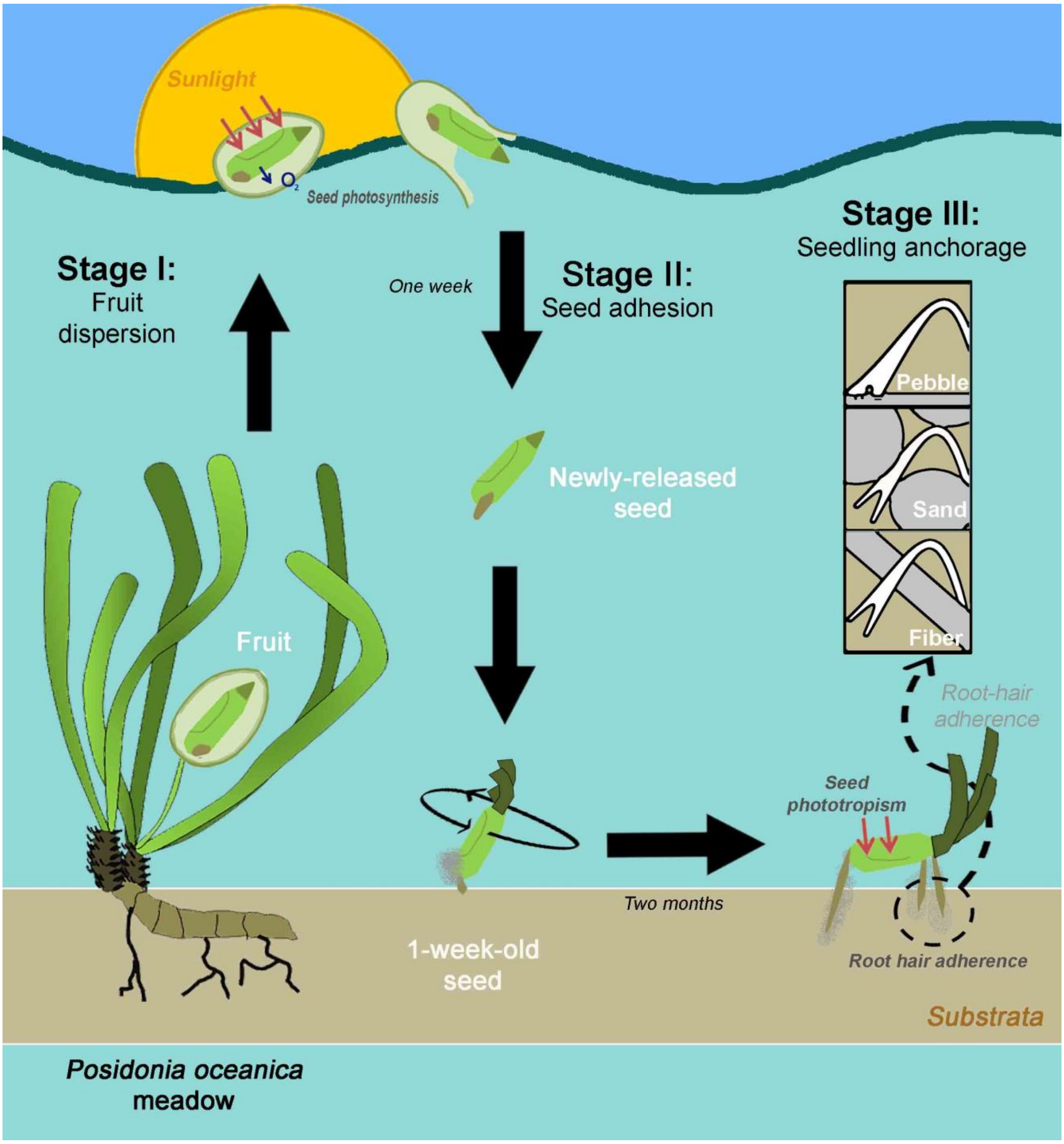


Fig. 9. Schematic representation of the three sexual recruitment stages of *Posidonia oceanica*, including dispersion and settlement.

Stage I: Fruit dispersion

P. oceanica seeds are formed inside large ovoid fruits composed of a spongy pericarp. The fruit pericarp confers buoyancy, while favouring light transmission to seeds. Seeds are also capable of performing relevant photosynthetic activity while transported to the fruit's interior (Fig. 9).

The chloroplasts in *P. oceanica* fruits are mainly distributed on outermost pericarp layers. These chloroplasts obtained high electron transport rates at the expected light intensities on the Mediterranean Sea's surface during *P. oceanica's* fruit dispersion period. This suggests that fruits can use the light that reaches the sea's surface to produce oxygen. In other plants, whose seeds also are dispersed by floating in aquatic environments, the oxygen produced by pericarps in the dispersion stage is essential to maintain fruit tissues fresh by maximising buoyancy in water and by, thus, favouring its dispersion (Spence et al. 1971). The respiration rates of *P. oceanica* fruits were very high, which caused negative values of GPP. This fact was also reflected by the low light-adapted yield values obtained in fruit pericarps, and indicates poor photosynthesis efficiency in photosystems. In contrast, seeds were able to produce a positive GPP, while showing high light-adapted yield values at low light intensities. In addition, the air lacunae of the pericarp (located near the seed position) increased in size in innermost mesophyll areas. So seeds' good photosynthesis activity, despite low light intensity, indicated that they were the main contributors to pericarp oxygenation.

In this stage, the newly-released seeds displayed a similar photosynthetic efficiency to that released from fruits for 1 week. However at the same light intensities, young seeds had lower electron transport rates than old ones. Respiration rates were higher in the newly-released seeds than in the 1-week-old ones, probably due to the low light intensity inside fruits. High respiration rates resulted in negative NPP rates, despite seeds producing positive GPP rates in both seed development stages. Therefore, the benefits of a positive GPP in seeds can be 2-fold, i.e., slowing down pericarp degradation and favouring seed dispersion, but can also enhance initial seed development inside fruits. Part of the energy that *P. oceanica* seeds obtain through photosynthesis is invested in root and leaf development in at least the first germination month (Celdrán and Marín 2013). Thus the ability shown by *P. oceanica* seeds to use low light intensities for photosynthesis could be a strategy to enhance seed development during dispersion. This could explain the advanced state of germination of seeds when released from fruits.

Stage II: Seed adhesion

When fruits open, seeds are released and fall to the seabed, helped by the geotropism that promotes early primary root formation. The seed forms adhesive hairs on its basal surface and primary roots, which improve adherence to substrata. In addition, seeds increase their range of light tolerance for photosynthesis by enhancing their photosynthetic activity (Fig. 9).

The high electron transport rates obtained in the RLCs of the 1-week-old seeds compared to those in the newly-released seeds indicated a clear photo-acclimation in *P. oceanica* seeds over time (Platt et al. 1980, Campbell et al. 2007). Inside fruits, light intensity is low and the newly-released seeds do not need to adapt to high light intensities. In contrast, the 1-week-old seeds need to adapt to the new light intensities inside fruits once released. Adult *P. oceanica* meadows also show marked adaptations to light according to their depth (Dattolo et al. 2013). Similarly to adult plants, *P. oceanica* seeds adapt their light tolerance range to optimise photosynthesis performance in different seed development stages by, therefore, enhancing seed germination during settlement.

As regards settlement, *P. oceanica* developed adhesive hairs on the basal surface of seeds. The formation of adhesive hairs also occurs in other seagrasses of genera

Thalassia sp. and *Enhalus sp.* (Brouns and Heijs 1986, Lacap et al. 2002, Kiswara et al. 2009, Soong et al. 2013), but seems scarce in other species of the genus *Posidonia sp.* (Kuo and Cambridge 1978, Hovey et al. 2012). Production of adhesive hairs helps seeds and primary roots to come into contact with sediment by increasing the possibilities of adhesion to substrate (Brouns and Heijs 1986, Lacap et al. 2002, Soong et al. 2013). Adhesive hair formation also occurs in seagrasses that form dormant seeds by helping seeds to bury under sediment and to form seed banks (Kuo and Kirkman 1992, Kuo et al. 1993, Stafford-Bell et al. 2015). In freshwater macrophytes such as wetland plants, adhesive basal hairs also perform important functions during seed development and water uptake before primary roots completely develop (Kaul 1978, Rutishauser et al. 1999, Robinson et al. 2008). Hence, the formation of adhesive hairs in seagrasses does not seem directly related with seed dormancy type, but appears to play a key role in initial seed adherence in aquatic environments prior to full primary root development.

The histological structure of the initially formed adhesive hairs in *P. oceanica* seeds is similar to that observed on the primary and secondary roots of seedlings after 1 month of development. In both cases, adhesive hairs were long, tubular and immersed in a gelatinous matrix. The adhesive hairs of both seeds and roots were formed from the differentiation of hypodermic cells, which also occurs in the genus *Thalassia sp.* (Soong et al. 2013). So the formation of adhesive hairs on the basal surface of *P. oceanica* seeds and roots could be an adaptation to overcome seedlings' anchorage difficulties in the first weeks of development. By forming adhesive hairs on the basal surface, the geotropism of seeds can be favoured by establishing contact between primary roots and sediment and by, thus, maximising the anchorage capacity of seedlings to the seabed.

Stage III: Seedling anchorage

During the first month of development, seeds develop secondary roots on their apical extreme, which are later covered by adhesive root hairs. As secondary roots elongate, with the primary root they form a tripod-like structure with a two-fold function by favouring the anchorage success of seedlings to substrate and orientating seeds towards the light source (Figs. 8 and 9). This formation is linked to the presence of substrata. Increasing light exposure of *P. oceanica* seeds while seedlings are anchored to substrate could increase photosynthesis rates in seeds by accelerating seedling development and, thus, settlement.

In our experiment, secondary roots developed in the seedlings grown in sand did not all completely enter substrate. In contrast, on the other tested substrata (pebble, sand pebble and fibreglass treatments) most seedlings anchored by introducing most of their roots into substrata. These results coincide with the good adherence capacity shown by the *P. oceanica* root system to rocks and substrata covered by algae (Alagna et al. 2013, Badalamenti et al. 2015). The anchorage capacity of the seeds grown in sand could be related with the different adherence strategies observed in root hairs. Our experiments showed that adhesive root hairs were produced in all the tested substrata types. Yet they seemed to use different anchorage strategies depending on substrata type. In agreement with the results of other studies (Badalamenti et al. 2015, Balestri et al. 2017), the adhesive root hairs of the seedlings grown in pebble adhered to the edge to substrate and formed a kind of adhesive button, even when pebbles were mixed with sand. Yet when the substrata were just sand or fibreglass, root hairs were not anchored by their edge to substrate, and hairs embraced particles instead. These different mechanisms of adherence seem to be related with the presence of microtrichomes on the sides of adhesive roothairs, which could act as small hooks by sticking to grains of sand and fibres. However, the images obtained from the microtrichomes of the root hairs in this study were not sufficient to clearly understand the mechanism of adherence of root hairs to sand and fibres. Future research in this area is needed to increase knowledge on the functionality and mechanisms of adherence of *P. oceanica* root hairs in different substrata types.

Regardless of adhesion type, the *P. oceanica* root system seemed more stable in hard and coarse substrata than in a fine type. This coincides with *P. oceanica's* greater sexual recruitment success on sheltered and rocky surfaces than on sand (Alagna et al. 2013, Badalamenti et al. 2015, Balestri et al. 2017). The high branching that showed root hairs in all sediment types could also reinforce this hypothesis. Branching increased the number of edges and the possibilities of root hairs adhering to a hard stable substrate. In contrast, coarse sand composition could cause grains of sand to move while roots penetrate substrata, which would increase the possibilities of uprooting seedlings during disturbance events. The seedlings of the seagrass *Posidonia australis* Hook. f., which usually recruits in sand, show high mortality rates during the first months of development due to grazing and bioturbation (Statton et al. 2017). This indicates that despite developing a well-adapted root system, the seagrasses of the genus *Posidonia sp.* are very vulnerable to disturbance events when grown in substrata with a fine particle size. Thus the high plasticity of roots to different substrata types suggests that the seedling's colonisation success depends more on the stability that the substrate provides roots with than on seedlings' adherence capacity.

P. oceanica seeds development strategies during sexual recruitment observed here in revealed a great similarity with other seagrasses which form non-dormant seeds and buoyant fruits, such as the species of genera *Thalassia sp.* and *Enhalus sp.*.

Similarly to *P. oceanica*, the seeds of these seagrasses are large and contain many nutrients (Hocking et al. 1980, Montaño et al. 1999, Balestri et al. 2009) which, along with their expected photosynthetic capacity, can explain the advanced state of germination of seeds when released from fruits (Den Hartog 1970, Brouns and Heijs 1986, Kuo et al. 1991, Kirkman 1999, Lacap et al. 2002, Rollon et al. 2003, Micheli et al. 2010). Seed photosynthetic activity has been demonstrated only in *P. oceanica* and *Thalassia testudinum* Banks ex König (Celdrán and Marín 2011, Celdran 2017), but the fruits and seeds of the other species of these genera display a bright green colour on the surface, which indicates plenty of chloroplast (Montaño et al. 1999, Soong et al. 2013, Statton et al. 2013, Boisset and Ferrer-gallego 2016). These three seagrass genera also form microscopic adhesive root hairs that enhance the anchorage of seeds and the primary root system to substrata (Lacap et al. 2002, Kiswara et al. 2009, Soong et al. 2013, Badalamenti et al. 2015). These seagrass genera also share a similar life history strategy by forming persistent extensive meadows with sporadic sexual reproduction (Hemminga and Duarte 2000, Larkum et al. 2006). Thus the numerous coincidences in the adaptations of such seagrasses indicate similar sexual recruitment strategies and seem to suggest convergent evolution. Accordingly, these facts allowed us to hypothesise that this type of seagrasses can have similar development stages for sexual recruitment as those described in the present study in *P. oceanica*. These strategies can be general for this type of seagrass to maximise its dispersal capacity.

The importance of light and substrate type during *P. oceanica's* sexual recruitment demonstrated herein are important processes that need to be taken into account for the environmental management of long-lived seagrass meadows. Management decisions can affect the survival of long-lived meadows, such as fishing and construction activities on coasts, which should be careful to not affect the sexual reproduction of seagrass meadows, especially during dispersion and settlement periods. The protection of possible recruitment areas for seagrasses with the necessary requirements for successful species colonisation is essential for the resilience of the seagrass populations against present and future anthropogenic stressors. The future indicates an environmental restoration of seagrasses by means of seeds, which should take into account species' preferences of substrate and environmental conditions during sexual recruitment. In *P. oceanica*, despite growing seeds in hard substrata providing shorter root development in roots than in sand (Guerrero-Meseguer et al. 2016), seedlings' anchorage success appears higher in the presence of pebbles or fibres. Such substrata types favour the adhesion and establishment of root hairs. Thus the addition of fibrous, coarse or hard substrata to the sandy substrate generally used for *in vitro* germination, such as filamentous algae, seagrass dead matte and fragments of rocks or pebbles, could enhance the seedling anchorage success of restoration projects. Although experimental testing is needed, these environmental management recommendations could be extended to other seagrasses which form fruits with membranous pericarps and non-dormant seeds.

According to our results, *P. oceanica's* sexual recruitment can be divided into three stages: fruit dispersion, seed adhesion and seedling anchorage. These three stages aim to maximise the recruitment success of this seagrass by promoting photosynthetic activity in seeds and enhancing seedlings' anchorage capacity to the seabed. These results also revealed the importance of environmental conditions, such as light and substrata type, for the sexual recruitment of seagrasses that form fruits with membranous pericarps and non-dormant seeds, such as *P. oceanica*. This knowledge should be taken into account when selecting conservation and protection areas as they may ensure the successful colonisation of seagrass populations. Conducting more research into the mechanisms of adherence of *P. oceanica* root hairs to different substrata types and seagrasses' sexual recruitment is absolutely necessary to improve the connectivity, genetic variability and recruitment of these important habitat-forming species.

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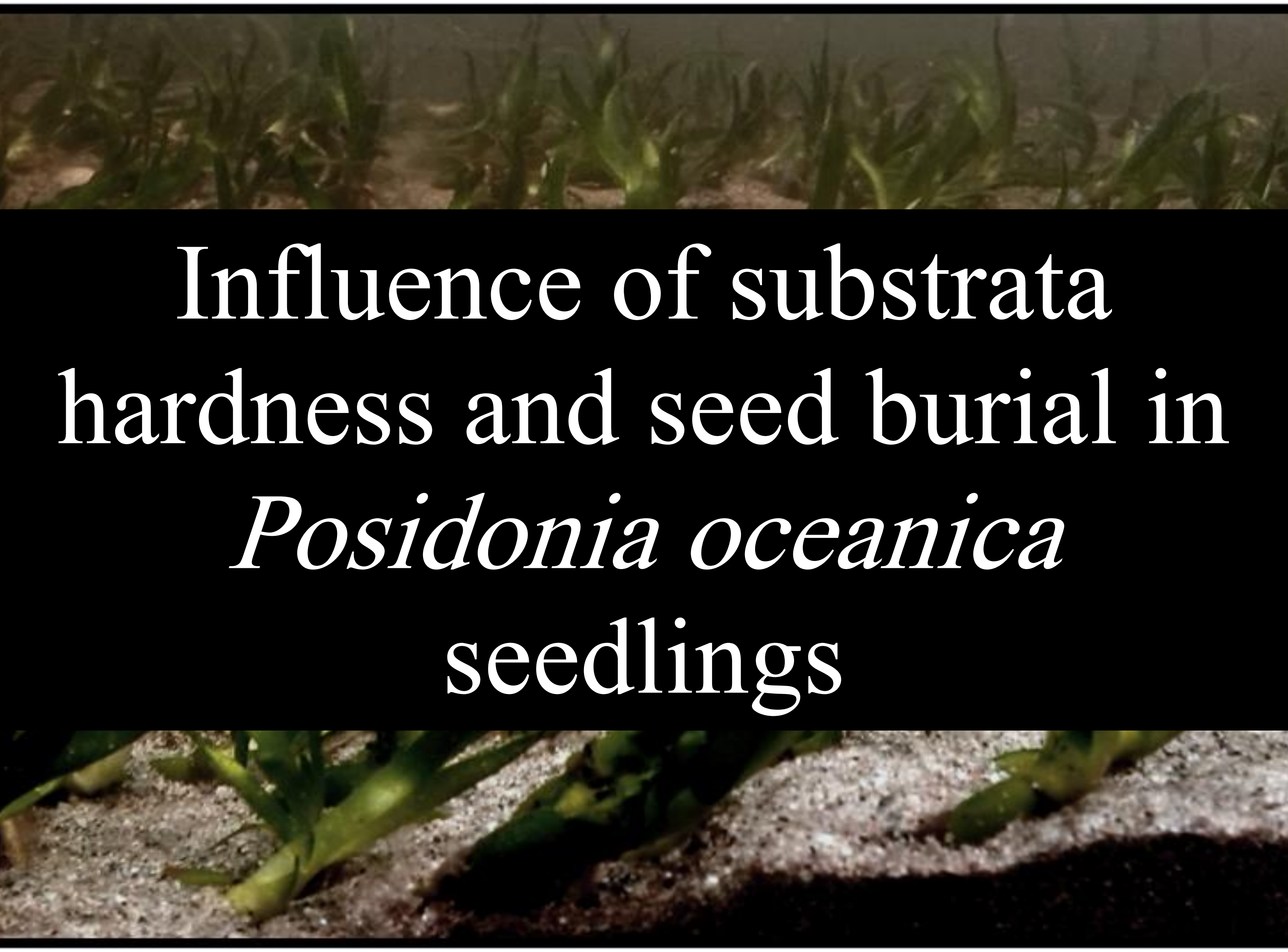
Chapter 2



Influence of substrata hardness and seed burial in *Posidonia oceanica* seedlings

Introduction

Posidonia oceanica (L.) Delile is one of the most important habitat-forming species in the Mediterranean Sea (Duarte and Chiscano 1999, Pergent et al. 1997). It has been selected as an indicator species to assess the ecological status of Mediterranean coastal water bodies (WFD, 2000/60/EC, Lopez y Royo et al. 2011) because it supports high biodiversity and plays a key role in several ecosystem functions (e.g. Molinier and Picard 1952, Koch et al. 2009, Duarte et al. 2010, Valle 2011, Sanz-Lázaro et al. 2012).

P. oceanica meadows are currently declining (Boudouresque et al. 2009, Marbà et al. 2014) due to pollution (Cancemi et al. 2003, Balestri et al. 2004) and a range of anthropogenic activities that alter sedimentation rates and consolidation of seabed substrates (Pasqualini et al. 2000, Ruiz and Romero 2003, Badalamenti et al. 2006, 2011, González-Correa et al. 2005, 2008). To mitigate the decline of *P. oceanica* meadows, environmental restoration projects have been undertaken. Recent projects have used laboratory-cultivated seedlings, which has the advantage of promoting genetic variability (Balestri et al. 1998, Terrados et al. 2013). However, this type of restoration has anchorage problems. Even though seedlings are capable of remaining anchored in different substrates (Alagna et al. 2013, Badalamenti et al. 2015, Balestri et al. 2015), their roots are not long enough to adhere firmly (Balestri and Bertini 2003), so seedlings can be uprooted by waves and currents (Meinesz et al. 1993).

Some authors argue that *P. oceanica* seedlings only persist on vegetated rocky substrates, while those in sand and gravel are unable to grow (Alagna et al. 2013, Balestri et al. 2017). Nevertheless, other studies confirm the establishment of seedlings in sandy bottoms (Balestri and Lardicci 2008, Balestri et al. 2015). In general, the seeds of seagrasses germinate within the sediment, which benefits their growth (Marba and Duarte 1994, Moore et al. 1993, Terrados 1997). The *P. oceanica* seeds have photosynthetic activity (Celdrán and Marín, 2011), which contributes to early development after germination (Celdrán and Marín 2013). It is not known how seed-burial could affect the development of seedlings. Increasing our knowledge of *P. oceanica* establishment on different substrates and effects of seed-burial is key to improving restoration of this plant and, consequently, supporting management strategies for its conservation.

The aim of this chapter was to test the effects of substrate hardness and seed-burial on the development of *P. oceanica*. Using microcosm experiments, we tested three interrelated hypotheses. First, substrate hardness could affect the root system development during the first months of germination. Second, the substrate where the seedlings germinate could influence their subsequent development upon transplantation. Third, shallow burial of the seed could decrease seedling development, possibly by limiting photosynthesis.

Materials and Methods

Culture of *P. oceanica* seedlings

P. oceanica buoyant fruits were collected in May 2013 from beaches in Ibiza Island (Spain, western Mediterranean Sea). Seeds were manually extracted and germinated in aquaria with artificial seawater prepared with sterilized bi-distilled water and marine salt (Ocean Fish, PRODAC International, Cittadella, Italy). Seawater had a salinity of 36 psu and a temperature of 21±1 ºC. The average photosynthetic photon flux density on the surface of seedling leaves was 100 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent lights, with a 14:10 h light:dark photoperiod.

Effects of substrate hardness on seedling development

To test the effects of substrate hardness on the early stages of growth of *P. oceanica* seedlings, seeds of similar size (1.65±0.2 cm length) were placed into replicate 10 L aquaria in plastic pots (9 x 9 x 10 cm) containing glass slides or sand (n=30).

The effects of sand were tested using pots filled to 10 cm depth with sand collected from an unpolluted area close to the marine reserve of Cabo de Palos-Islas Hormigas (Murcia, Spain). The sediment was composed of 32% coarse-sand, 67% of fine-sand according to the Wentworth (1992) scale of particle size, 0.75% organic matter and a C:N ratio of 13.9 : 3.67. To simulate hard substrate, glass microscope slides were placed over the sand in the pots. Glass was chosen as the hard substrate because it is an inert material of known chemical composition, with a constant roughness and structure.

The experiment was performed in a culture chamber room under controlled temperature, salinity and photoperiod conditions. Water level and salinity within aquaria were checked every three days, and aeration was adjusted to supply dissolved oxygen without disturbing the sediment. Aquaria were refilled to compensate for evaporation and maintain salinity of 36 psu. Aquaria were maintained at 21±1 °C, with a 14:10 h light: dark photoperiod and a light intensity of 100 μmol m^2 s^{-1}. The redox potential of the sand was measured with an Orion ORP 91-80 electrode prior to calibration with a redox buffer solution (220 mV at 25 ºC). Measurement of the sediment redox was performed by randomly taking four cores from the sediment-collection area and inserting the electrode to a depth of ca. 4 cm. The sand used as a unconsolidated substrate had a positive redox potential in all pots (+84.5±6.9 mV). Aquaria were aerated to avoid changes in redox potential during the experiment.

The lengths of leaves and roots (principals and laterals) were measured after four months.

Legacy of initial substrate on seedling development and responses to seed-burial

To evaluate the influence of shallow seed-burial and the legacy of substrate hardness, we simulated a restoration event: 60 four-month-old seedlings from the previous experiment were transplanted to sand. Seedlings were carefully removed from the initial substrates by hand, to avoid damaging roots.

We used a factorial design with two fixed factors, *initial substrate* (glass vs. sand) and *seed-burial level* (non-buried, half-buried and full-buried). For the "non-buried" treatment, the seedling was anchored on the sand only by their roots, so each seed was totally uncovered and exposed to light. For the "half-buried" treatment, the seed was partially covered (0.5 cm). In the "full-buried" treatment, the seed was covered by sand (1.5 cm) with only the leaves unburied. The sand over the seed reduced light intensity to 1.45±0.01 µmol m^2 s^{-1} (Fig. 1).

Seedlings were planted in plastic pots (9 x 9 x 10 cm) and placed in individual 10 L aquaria (n= 10), which were maintained in a culture chamber room with the same controlled conditions and sand characteristics as in the previous experiment. The lengths of seed, leaves and roots (principal and lateral) of each seedling were measured at the start of the experiment and again after one month (October 2013) to calculate the net growth of leaves and roots (principal and laterals) per seedling.

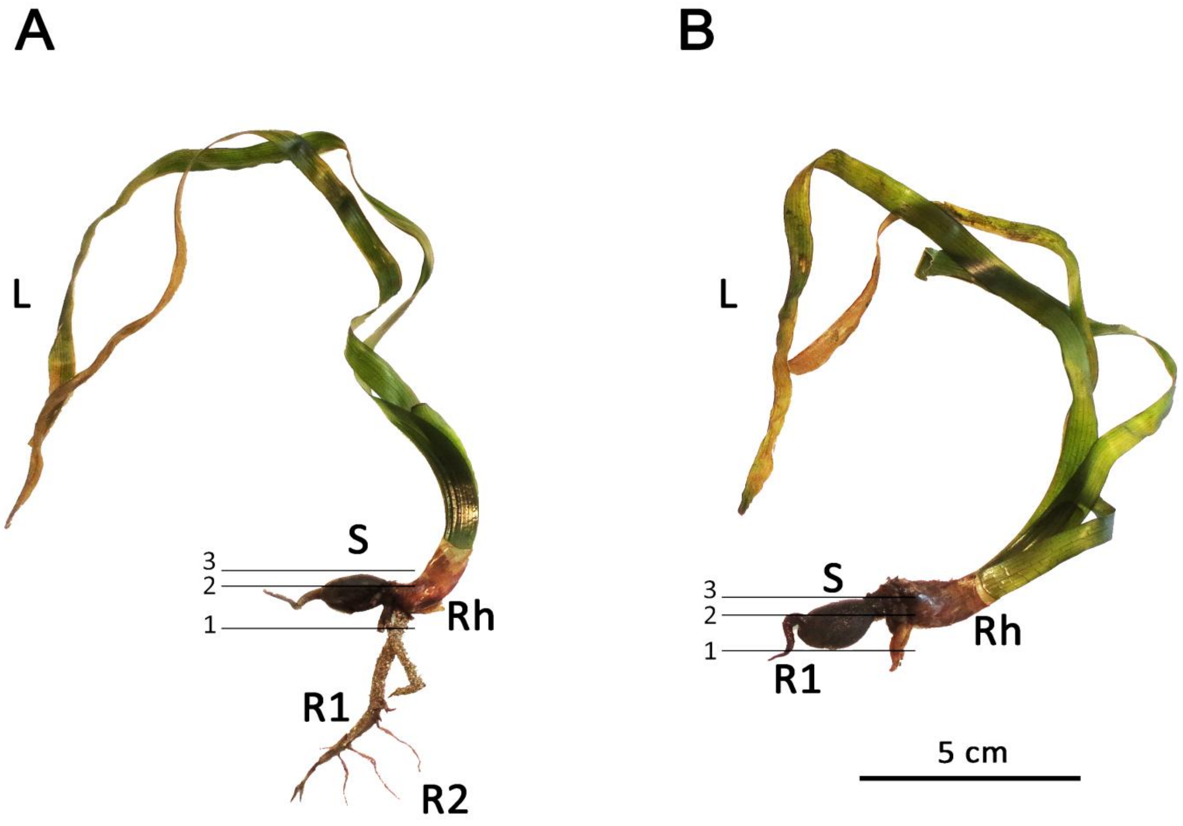


Fig. 1. *P. oceanica* seedlings grew in unconsolidated (A) and hard (B) substrates. Numbers indicate burial level of the seed used in the restoration simulation: 1: non-buried; 2: half-buried; 3: full-buried. L: leaves; S: seed; Rh: rhizome, R1: principal roots and R2: lateral roots.

Data analysis

Data normality and homogeneity of variance were tested using *P-P* plots and Levene's test, respectively. If data did not meet parametric assumptions, they were transformed [$Ln(x + 1)$], and re-tested. If data still did not meet homogeneity of variances, a significance threshold of $p<0.01$ was assigned, which is a conservative option considering the high number of total replicates (Underwood 1997, Benjamini et al. 1995). Otherwise, a significance threshold of $p<0.05$ was used.

To test the effects of substrate hardness on seedling development, a Student's *t*-test was carried out to evaluate the possible effects of the fixed factor *initial substrate* on the length of roots (principal and lateral) and leaves. A two-way factorial analysis of variance (ANOVA) was used to evaluate the effects of *initial substrate* and *seed-burial level*, and their possible interaction, on growth of leaves and principal and lateral roots. Additionally, linear regression analysis tested whether seed size influenced the growth of leaves and roots. Data are reported as mean±standard error (SE). All statistical analyses were carried out using R (v. 3.1.1).

Results

Effects of substrate on seedling development

There were significant differences between substrates (*p* <0.001) in the total length of principal roots and in the number and length of lateral roots. The roots of seedlings germinated on sand (mean = 17.0±5.4 cm) were up to five times longer than those germinated on glass (mean = 3.7±1.7 cm; Figs. 1 and 2).

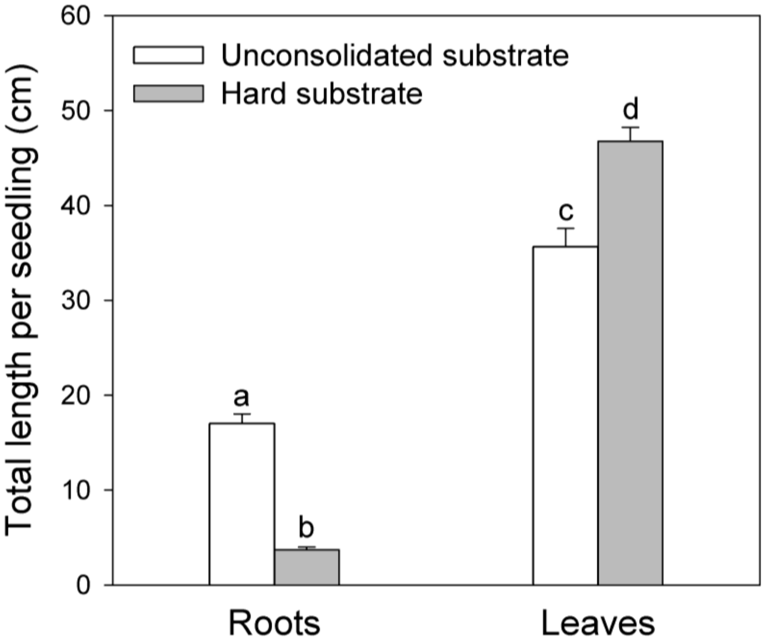


Fig. 2. Growth of principal roots and leaves per seedling for unconsolidated and hard substrates (mean±SE; n=30). Letters above the bars indicate significant differences between substrate types (Student's *t* -test, *p*<0.05).

Total leaf growth had the opposite trend: leaves of seedlings cultured on the glass averaged 46.8±7.9 cm per seedling, which was significantly greater than on sand (35.6±10.6 cm per seedling; Fig. 2). Seedlings germinated on glass did not have lateral roots; while on the sand lateral roots were found on 70% of seedlings. Lateral root length varied, ranging from 0.1 to 3.3 cm (Fig. 3).

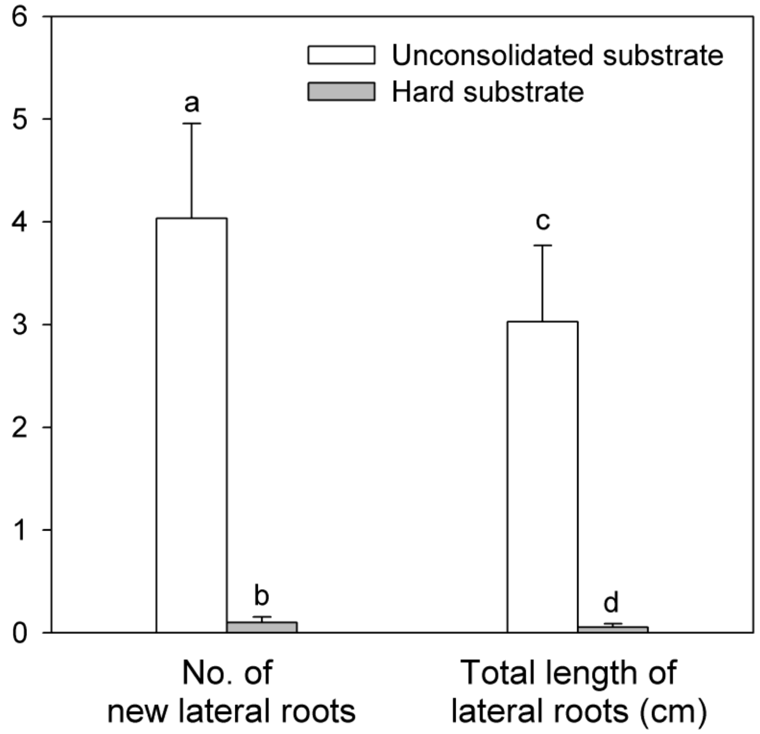


Fig. 3. Number of new lateral roots and total length of lateral roots per seedling for different substrates (mean±SE; n=30). Letters above the bars indicate significant differences between treatments (Student's *t*-test, $p<0.05$).

Legacy of initial substrate on seedling development and responses to seed-burial

The growth rates of leaves and roots were not influenced by seed size (p-value of the regression = 0.96 and 0.26, respectively). Shadow seed-burial did not affect leaf or root growth rate (p = 0.39 and 0.07, respectively; Table 1). However, seedling development appeared to be influenced by their initial substrate. After the restoration simulation, the growth rates of leaves and lateral roots were significantly higher ($p<0.01$) in seedlings that had originally developed in sand. In contrast, the growth rate of principal roots was not affected by the initial substrate ($p= 0.71$; Fig. 4).

Table 1. Summary of the Levene test and two-way analysis of variance (ANOVA) on the fixed factors "*Initial substrate*" (glass vs. sand) and "*Seed-burial level*" (non-buried, half-buried and full-buried). Significant results are in bold.

Source	**d.f.**	**MS**	***F***	**P**
Growth of leaves				
Initial Substrate (S)	1	7.37	10.35	**<0.01**
Seed-Burial level (B)	2	0.68	0.96	0.39
S x B	2	0.16	0.22	0.80
Residual	54	0.71		
Levene's test:	$p = 0.36$			
Transformation:	None			
Growth of principal roots				
Initial Substrate (S)	1	0.03	0.14	0.71
Seed-Burial level (B)	2	0.65	2.85	0.07
S x B	2	0.41	1.81	0.17
Residual	54	0.23		
Levene's test:	$p = 0.72$			
Transformation:	$Ln\ (x + 1)$			

Growth of lateral roots

Initial Substrate (S)	1	26.22	50.97	**<0.001**
Seed-Burial level (B)	2	1.29	2.51	0.09
S x B	2	0.95	1.85	0.17
Residual	54	0.51		

Levene's test: $p < 0.001$

Transformation: $Ln\ (x + 1)$

New lateral roots per seedling

Initial Substrate (S)	1	12.14	60.29	**<0.001**
Seed-Burial level (B)	2	0.33	1.64	0.20
S x B	2	0.08	0.38	0.69
Residual	54	0.20		

Levene's test: $p < 0.001$

Transformation: $Ln\ (x + 1)$

Leaves of seedlings germinated on sand grew 2.6±0.9 cm · leaf^{-1} · month^{-1}, while those on glass grew 1.9±0.6 cm · leaf^{-1} · month^{-1}. At the end of the experiment, the leaves of the seedlings that initially developed on glass were as long as those germinated on sand (9.1±1.4 and 9.3±1.9 cm, respectively). The growth rate of lateral roots on seedlings germinated on sand was four times greater than those germinated on glass (Fig. 4). The production of new lateral roots per seedling was significantly greater on sand than on glass ($p < 0.001$). At the end of both experiments, the sediments in all pots had a positive redox potential.

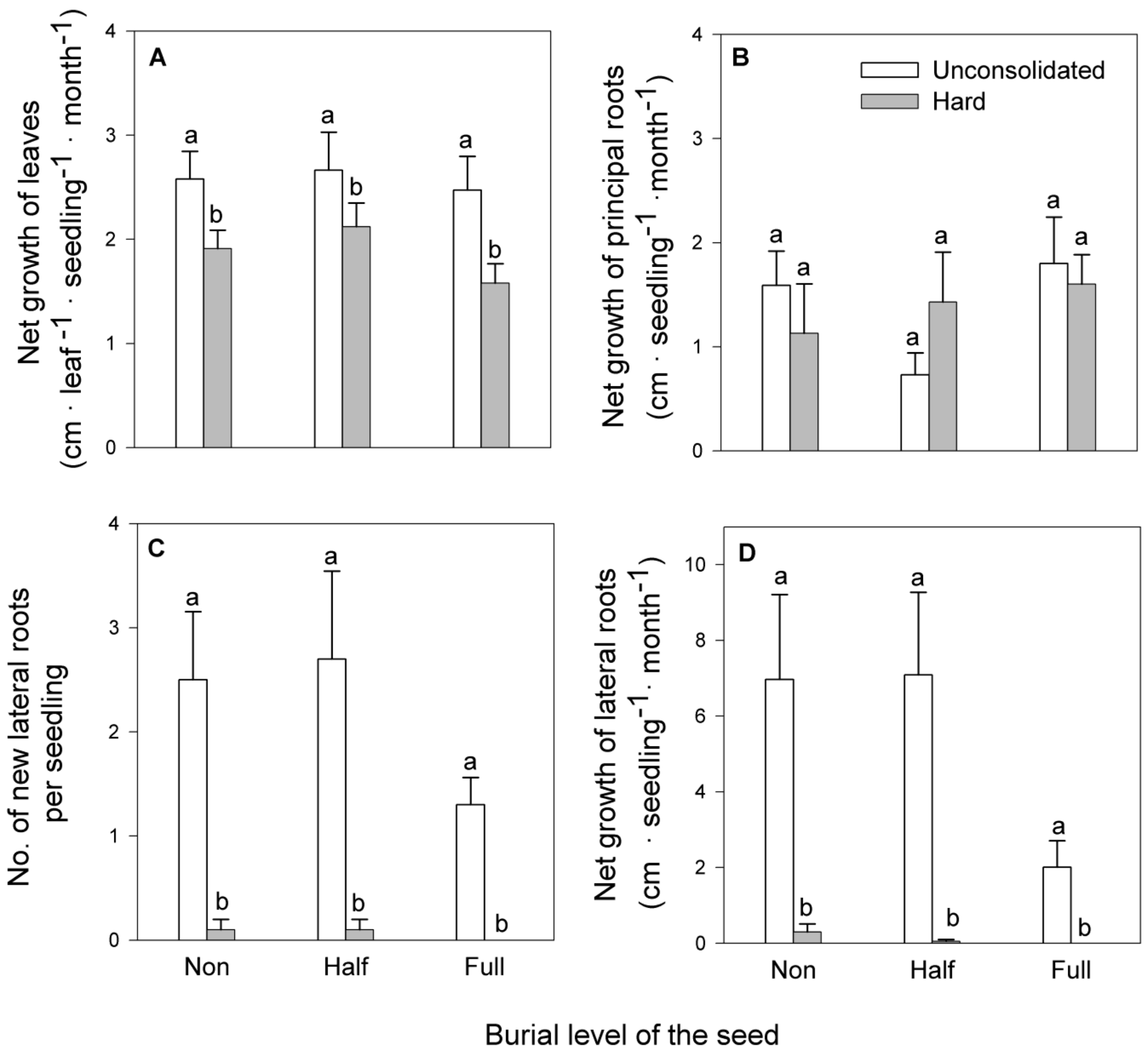


Fig. 4. Growth of leaves (A), growth of principal roots (B), number of new lateral roots (C) and growth of lateral roots per seedling (D) after of the restoration simulation for the treatments *Burial level of the seed* (non-buried, half-buried and full-buried) and *Initial substrate* (unconsolidated in empty and hard in grey; mean±SE; n=10). Letters above the bars indicate significant differences (two-way ANOVA, $p<0.05$).

Discussion

P. oceanica showed a high morphological plasticity to two substrates after germination. Sand seemed to promote the growth of principal and lateral roots during the initial four months of plant development. Moreover, the use of sand in germinating *P. oceanica* had legacy effects on subsequent leaf and root development after transplantation to sand. Extensive root growth on sand appeared to be at the expense of leaf development. A similar leaf-root trade-off was observed in *P. oceanica* growing on rubble mounds (Di Carlo et al. 2007). However, when seedling were transplanted to sand, all reached same length.

These results agree with field studies where sand promoted the formation of lateral roots, more than on rock (Balestri et al. 2015). The nutrient content and the unconsolidated structure of the sand could enhance root growth of *P. oceanica*, while the hard glass limited elongation and nutrient acquisition. *P. oceanica* seedlings have adhesive root hairs that facilitate anchorage on rocky substrates (Badalamenti et al. 2015). Likewise, roots on glass were fully adhered to the surface in our experiment.

P. oceanica seeds photosynthesize even after germination (Celdrán and Marín 2011), so it is possible that shallow seed-burial could negatively affect development. Nevertheless, growth of four-month-old seedlings was not influenced by seed-burial. It is likely that the leaves of young *P. oceanica* soon compensate for any reduced photosynthesis by seeds.

The general procedure for restoration of *P. oceanica* is to attach transplants to the substrate using non-biodegradable materials (Augier et al. 1996, Balestri et al. 1998, 2011, Meinesz et al. 1992, Meinesz et al. 1993, Molenaar et al. 1993, Molenaar and Meinesz 1992). A well-developed root system is expected to maximize anchoring capacity and nutrient acquisition (Balestri and Lardicci 2005, Schutten et al. 2005, Statton et al. 2014). Infantes et al. (2011) evaluated the substrate-anchoring capacity of *P. oceanica* seedlings for the hydrodynamic conditions of the Mediterranean and estimated the minimal root length needed by seedlings to prevent dislodgement from sand (0.35 times the square root of the leaf area). Using this equation, 100% of our seedlings on sand and 13% of those on glass would remain attached to a sandy seabed.

Germination of *P. oceanica* seedlings on sand enhanced the growth of principal root and had a legacy of enhanced leaf and lateral root growth after transplantation. However, shallow burial of the seed did not slow the development of five-month-old *P. oceanica* seedlings. Thus, we suggest culturing seedlings on sand before transplantation, to facilitate anchorage and avoid using non-biodegradable materials in seagrass restoration. Transplantation to sand should be limited to periods of calm weather and in bays that are protected from strong hydrodynamic events.

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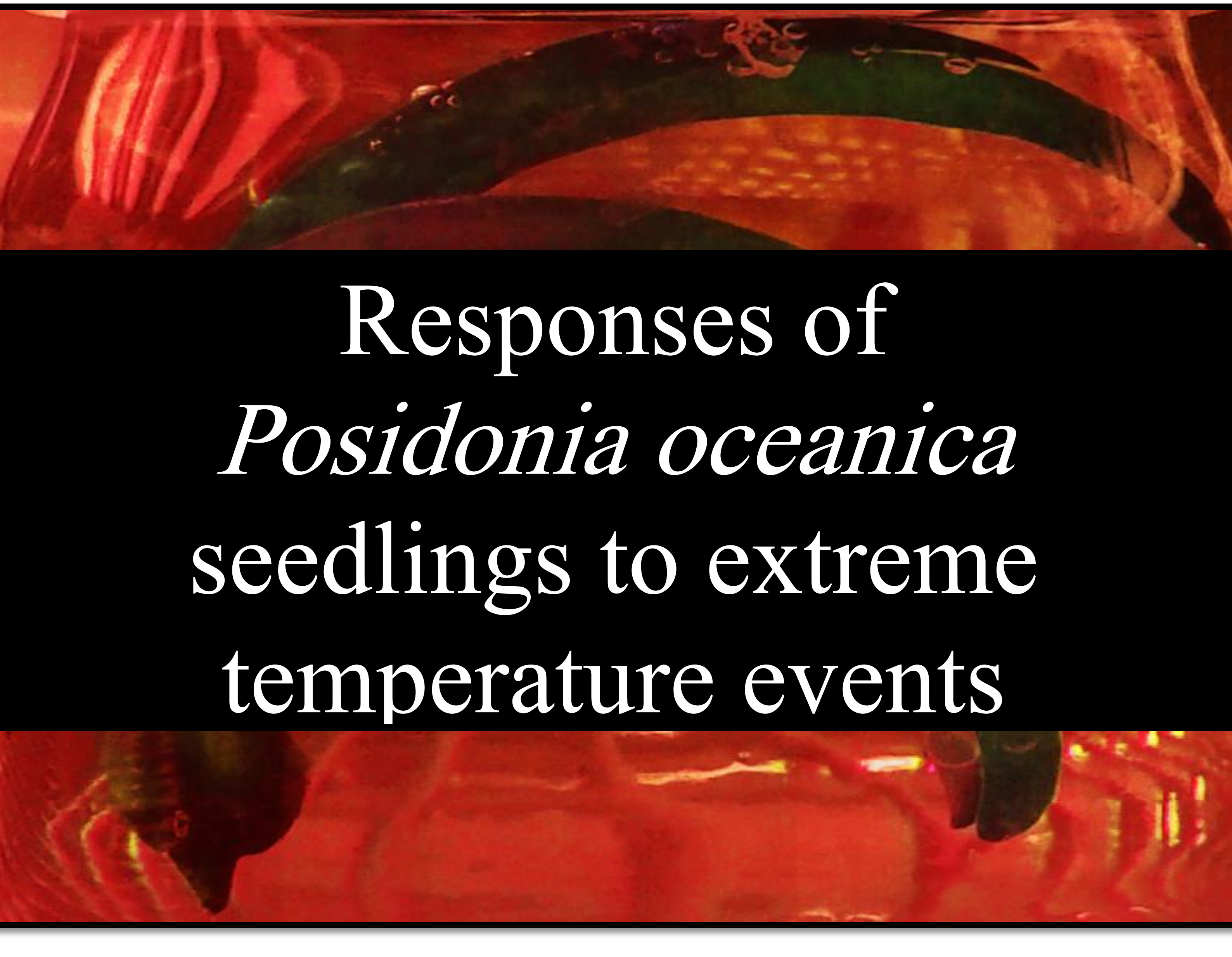
Chapter 3



Responses of *Posidonia oceanica* seedlings to extreme temperature events

Introduction

Climate change is a significant threat to global biodiversity, resulting in important social and economic impacts (EEA 2004). Most of the research on the effects of climate change has focused on gradual changes of the mean values of climatic variables. However, it is also important to consider the impact of abrupt changes in climatic parameters, such as extreme events (Jentsch et al. 2007).

Extreme events are climate events which have an unusually-high intensity (Easterling et al. 2000, Stephenson 2011). The abrupt nature of extreme events can cause significant ecological shifts (IPCC 2001, Sanz-Lázaro 2016), because species often have a lower capacity to adapt to abrupt events rather than to gradual changes (Wernberg et al. 2012, Smale and Wernberg 2013). The occurrence of extreme events is increasing due to climate change (IPCC 2012), so it is necessary to focus research efforts on this issue in order to predict the effects of climate change.

Heat waves are a type of extreme event that can cause abnormally warm seawater (Hegerl et al. 2011), leading to deleterious impacts on marine organisms (Garrabou et al. 2009, Smale and Wernberg 2013). The effects of heat waves can be especially detrimental in the Mediterranean Sea (Diffenbaugh et al. 2007), which are considered to be hotspots of global warming (Giorgi 2006), as their confined nature makes them more sensitive to temperature rises than open oceans (Vargas-Yáñez et al. 2008). The ecological consequences of heat waves can be particularly deleterious in habitat-forming species such as *Posidonia oceanica* (L.) Delile, which have a key role in structuring food webs, supporting diversity and ecological complexity (Hemminga and Duarte 2000, Lopez y Royo et al. 2011, Mazzella et al. 1992).

P. oceanica is the dominant seagrass in the Mediterranean Sea, covering an area of approx. 12,200 km^2 (Telesca et al. 2015). It is sensitive to high temperatures (Diaz-Almela et al. 2007, Marbà and Duarte 2009, Marín-Guirao et al. 2016), especially during the early stages of its development (Olsen et al. 2012). In the first months of development, *P. oceanica* depends not only on its leaves, but the seed also has an important contribution to the photosynthesis of the plant (Celdrán and Marín 2011, 2013). Photosynthesis can be disrupted by temperature rises (Bulthuis 1987, Ralph et al. 1998), which could cause a deficit in the carbon fixation capacity and the respiration rates of the plant (Berry and Bjorkman 1980), threatening its survival.

Photosynthetic parameters of seagrasses can be studied using pulse amplitude modulated (PAM) fluorometry (Ralph et al. 1998). This non-invasive technique allows the collection of quantitative measurements of the maximum photochemical quantum yield (F_V/F_M) and the minimum chlorophyll fluorescence (F_0) of photosystem II (PSII), which are surrogates of photosynthetic efficiency under heat stress situations (Havaux 1993).

Modelling of the predicted occurrence of extreme events can be a useful tool to understand the effects of temperature rises due to heat waves in ecosystems (Jentsch et al. 2007, Smith 2011), especially for habitat-forming species such as seagrasses, which have a key ecological role.

The aim of this chapter was to test the effects of heat waves in the Mediterranean Sea on the development and metabolism of *P. oceanica* seedlings. To do this, we carried out a mesocosm experiment exposing the seedlings to an increasing temperature gradient to evaluate the potential effects of current and predicted maximum sea-surface temperatures (SST). Our hypothesis is that the expected increases in respiration and reduction in photosynthetic efficiency will negatively affect the development and survival success of the recently settled *P. oceanica* seedlings.

Material and Methods

Simulation of extreme temperature events

Two mesocosm experiments were performed to test the effects of extreme temperature events in *P. oceanica*. A short- and long-term experiment were used to evaluate the effects of short and long periods of temperature rise on seedling photosynthesis and development, respectively.

The seedlings used in both experiments were germinated from seeds collected on the beaches of Mazarrón Bay (Murcia, Spain) in May 2013. Seedlings were grown in glass aquaria filled with seawater at 21±1 ºC and salinity of 36, continuously aerated by air pumps to ensure that oxygen was close to saturation. After five months, seedlings of similar sizes were randomly selected for the experiments (n=6).

Four temperatures were used in the experiments (27, 29, 31 and 33°C) based on current and forecasted maximum SST (Cruz et al. 2007) (IPCC 2007). Maximum SST of Mediterranean coast oscillated between 27 and 29 ºC in the years 2012-2015 (data from oceanographic buoys belonging to the Port Authority, Ministry of Development, Spain; Cabo de Palos, Barcelona II and Central Dragonera). A temperature of 29ºC was reached on some occasions, but temperatures in excess of 27ºC were maintained for several weeks during the summer (Fig. 1). Temperatures of 31 and 33ºC were selected according to climatic model predictions of temperature rises between 1 and 3.5 °C above the current maximum summer temperature for the rest of this century (Cruz et al. 2007, Watson et al. 1995) (IPCC 2007, Watson et al. 1996).

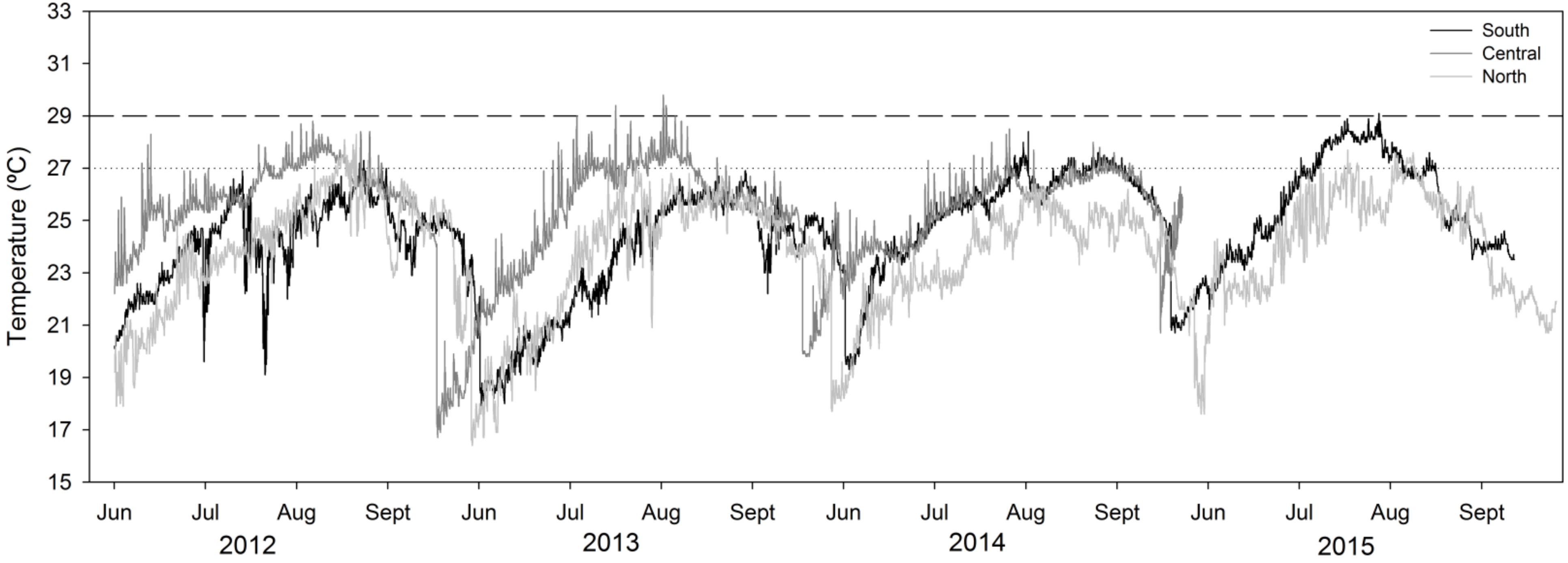


Fig. 1. Maximum SST (June-September; 2013-2015) registered by oceanographic buoys along the Western Mediterranean Sea in Spain. Southern, central and northern buoys were located at Cabo Palos, (0.33ºW, 37.65ºN), Dragonera Island (2.10ºE, 39.56ºN) and Barcelona (2.20Eº, 41.32ºN). Data from Dragonera Island 2015 was not available.

We controlled temperature using four environmental chambers (CLIMACELL-404, MMM Medcenter Einrichtungen GmbH, München, Germany). Each chamber was set at a fixed temperature and had the same photoperiod and photon flux light (light:dark 14:10, 100 µmol m^2 s^{-1}). The temperature of each environmental chamber was recorded continuously by data loggers to ensure that the temperatures were constant during the whole experiment.

Short-term exposure to high temperatures

To test the short-term effects of temperatures on the photosynthesis activity of *P. oceanica*, seedlings were incubated in individual airtight glass jars for three hours. One day before the incubations, seedlings and the artificial seawater necessary for the incubations were gradually acclimatised in the environmental chambers. To do this, seedlings were placed in an aerated glass jar filled with artificial seawater with a salinity of 36, prepared using bidistilled water and marine salt (Ocean Fish, PRODAC International, Cittadella, Italy). Aeration was maintained using an air pump attached to a system of plastic capillary tubes inside the glass jars to ensure that oxygen concentration was always above 5.5 mg O_2/l^{-1}. Light, temperature and chamber characteristics were as described above.

To perform the incubations airtight glass jars were filled with the acclimatised seawater in each chamber, ensuring the absence of air bubbles. Incubations had a duration of three hours. Oxygen production was estimated by measuring oxygen concentration in seawater in each glass jar before and after the incubation using an optical electrode (Portable Meter Hach HQ30d, HACH, Loveland, Colorado, USA).

Net leaf photosynthesis (NLP), net seed photosynthesis (NSP) and plant respiration (PR) was estimated by oxygen measurements, with incubations covering the leaves, seed and whole plant, respectively. To quantify net plant photosynthesis (NPP) the whole plant was exposed to light. The measurements were standardized to the weight of the seedlings, which was estimated by measuring their area and interpolating these data to the corresponding weight based on a regression of area-weight of previously-measured seedlings (n=20) collected at the same time and location.

Additionally, values of F_V/F_M and F_0 were measured using saturation pulse analysis by a Portable Chlorophyll Fluorometer (MINI-PAM, Waltz, Effeltrich, Germany), in leaves and seeds that had been previously scraped with a glass slide to remove epiphytes. Before the measurements, samples were dark-adapted by covering them with aluminium foil for 15 minutes. Dark-acclimation is necessary to ensure that the non-photochemical dissipation of excitation energy is minimal and all reaction centres of PSII are open. To take homogeneous samples, all the measurements were made at the base of the second leaf of each seedling and over the same zone of the seed.

The F_V/F_M value is an estimate of the fraction of absorbed quanta used for PSII photochemistry after the dark-acclimation, and is defined as:

$$\frac{F_V}{F_M} = \frac{F_M - F_0}{F_M}$$

where F_M corresponds to the maximum fluorescence level after saturation pulse, and F_0 corresponds to the minimum fluorescence level reached after a saturated pulse in dark-acclimated samples.

Long-term exposition to high temperatures

To evaluate the long-term effects of temperature on the development of *P. oceanica*, seedlings were incubated in individual (500 ml) aerated glass jars filled with artificial seawater for one month. Aeration and artificial seawater were maintained in the same manner as the short- term simulations. To prevent colonization of epiphytes and changes in water quality, the artificial seawater was replaced every week. Leaves were counted and measured (weight and length) at the beginning and end of the incubation to calculate leaf net growth per seedling, % of leaves dead and % of new leaves. At the end of the experiment F_0 was measured in both the leaf and seed.

Data analysis

We analyzed the trends of the measured parameters along a temperature gradient by adjusting the data to the best fitted regression models using correlated the Akaike information criterion (AICc; Table 2). Previously, normality and homoscedasticity of variances of the data was checked using *Q*-*Q* plots and Bartlett tests, respectively. Then, ANCOVA analysis was applied to compare the trend of the regressions against temperature among NPP, NLP, NSP and PR.

Statistical analyses were performed with R statistical software (v. 2.15.0) using the packages “ggplot2”, “AICcmodavg” and “stringr”. All data were reported as mean±standard error (SE). Statistical tests were conducted with a significance level of α=0.05.

Results

Short-term effects on seedling photosynthesis

The oxygen production of seedlings declined steeply above 27ºC, following a linear trend (Table 1). Leaves were the major contributor to the total oxygen production of the plant and were significantly affected by temperature. In contrast, the seed contributed a minor fraction to the total oxygen production and was less sensitive to temperature. Respiration did not seem to be markedly affected by temperature (Fig. 2, Table 2).

F_V/F_M and F_0 values declined significantly with temperature, both showing a minimum at 33ºC. The trend of F_0 with the rise in temperature was different between leaf and seed (exponential and second-degree polynomial, respectively), but both peaked at 31ºC. Values of F_V/F_M showed the same trend in leaf and seed, decreasing exponentially beyond 31ºC and with the minimum at 33ºC (Fig. 3).

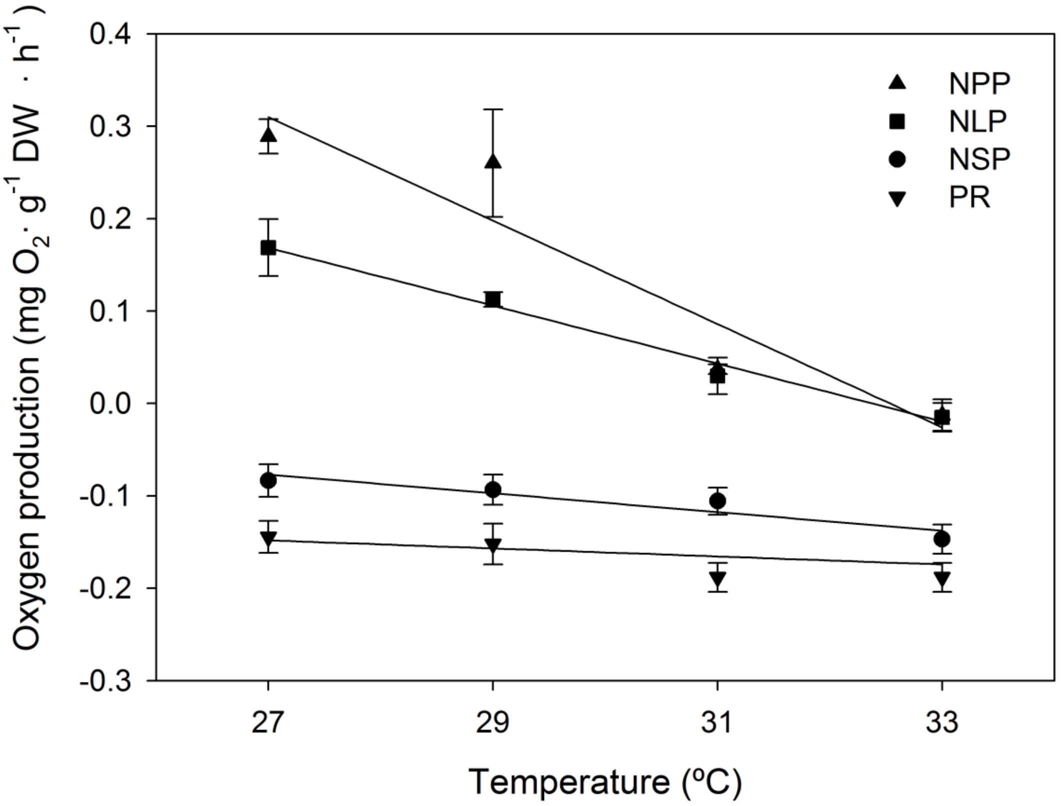


Fig. 2. Oxygen production (mean±SE; n=6) of *P. oceanica* seedlings across the temperature gradient: net plant production (NPP), net leaf production (NLP), net seed production (NSP) and plant respiration (PR). Lines show significant regressions across the temperature gradient.

Table 1. Summary of Akaike information criterion (AICc) results. Lower values (in bold) determine the model of regression which best fitted to regression models.

AICc

	Short-term experiment					Long-term experiment				
	Oxygen production	F_V/F_M		F_0		F_0		Leaf net growth	Leaf mortality	New Leaves
		Leaf	Seed	Leaf	Seed	Leaf	Seed			
$y_i = \beta_0 + \beta_1 x_i$	**-87.47**	-31.33	-43.64	351.7	366.0	323.0	327.1	24.46	239.2	192.1
$y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i^2$	-85.26	-37.48	-45.81	349.8	**361.8**	**318.4**	328.9	23.23	239.9	192.0
$y_i = \beta_0 + \beta_1 e^{x_i}$	-84.64	**-41.44**	**-47.51**	**346.5**	362.1	338.8	**326.8**	39.11	**238.2**	**191.8**
$y_i = \beta_0 + \beta_1 \frac{1}{x_i}$	-87.43	-30.15	-43.37	352.0	366.1	321.3	327.3	**23.20**	239.6	192.5

Long-term effects on seedling development

At 27 and 29ºC all the seedlings remained alive, but above 29ºC, 33% of the seedlings died. Above 29ºC, the plants that remained alive showed a deterioration of the leaves, suffering bleaching and with more than 60% falling after one month.

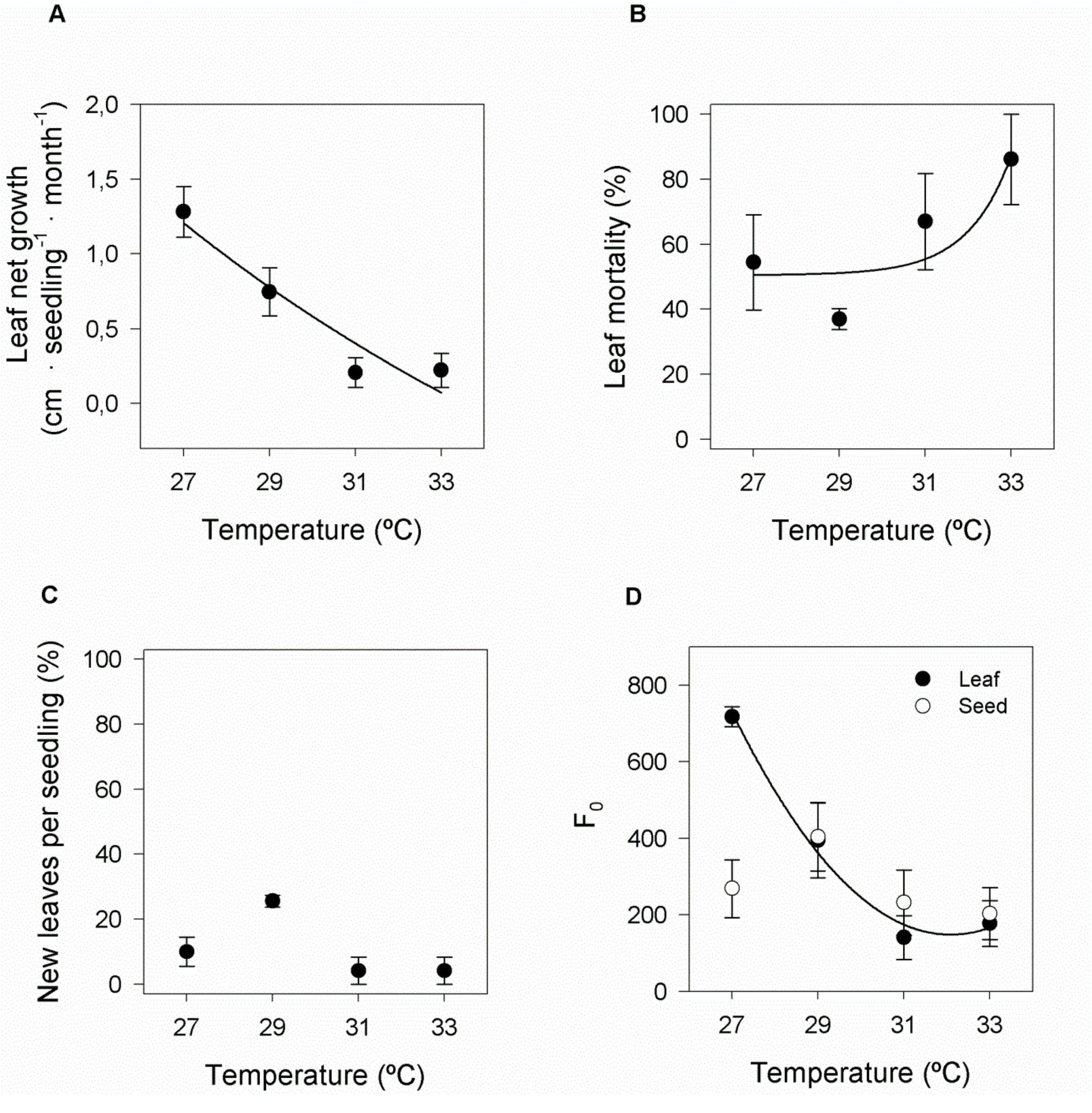


Fig. 4. Effects of the temperature gradient on leaf net growth (A), leaf mortality (B), new leaves (C), leaf and seed F_0 (D) (mean±SE; n=6). Lines show significant regression along the temperature gradient.

Leaf net growth had an inversely-significant trend against temperature (Fig. 4A, Table 1 and 3). Maximum leaf growth was at 27ºC (1.28±0.42 cm^{-1} ·leaf^{-1} ·month^{-1} per seedling). Above 29ºC the leaf growth rate decreased significantly from 0.75 ±0.40 to 0.21 ±0.24 and 0.22 ±0.28 cm^{-1} ·leaf^{-1} ·month^{-1} at 31 and 33ºC, respectively. Leaf mortality increased exponentially with temperature (Fig. 4B showing a minimum at 29ºC (36.9 ±7.93 %) and a maximum at 33ºC (86.1 ±34.0 %), Table 1 and 3). Conversely, temperature did not affect new leaf formation (Fig. 4C, Table 1 and 3). Leaf F_0 was also influenced by temperature: maximum values were reached at 27ºC (718.2 ±63.8), while above 29ºC F_0 decreased to values below 200. In contrast, seed F_0 was not affected by temperature (Fig. 4D, Table 1 and 3).

Table 3. Summary of the regression results of the effects of the temperature gradient in the leaves and seed F_0, net growth of roots, net growth of leaves, % of dead leaves and % of new leaves of *P. oceanica* seedlings.

		Coefficient	**± SE**	***p***
F_0 Leaf $(y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i^2)$	β_0	23301.8	-7292.0	**<0.001**
	β_1	-1443.2	488.2	
	β_2	22.49	8.133	
F_0 Seed $(y_i = \beta_0 + \beta_1 e^{x_i})$	β_0	309.4	49.02	0.259
	β_1	$-5.2 \cdot 10^{-13}$	$4.5 \cdot 10^{-13}$	
Root net growth	β_0	0.357	0.140	0.293
$(y_i = \beta_0 + \beta_1 e^{x_i})$	β_1	$-1.4 \cdot 10^{-15}$	$-1.3 \cdot 10^{-15}$	
Leaf net growth	β_0	-5.030	0.964	**<0.001**
$(y_i = \beta_0 + \beta_1 \frac{1}{x_i})$	β_1	168.4	28.66	

% Leaves dead	β_0	50.41	7.744	**0.025**
($y_i = \beta_0 + \beta_1 e^{x_i}$)	β_1	$1.7 \cdot 10^{-13}$	$7.1 \cdot 10^{-14}$	
% New leaves	β_0	14.12	2.948	0.075
($y_i = \beta_0 + \beta_1 e^{x_i}$)	β_1	$-5.1 \cdot 10^{-14}$	$2.7 \cdot 10^{-14}$	

Discussion

Our results show that recently-germinated seedlings of *P. oceanica* are vulnerable to the extreme temperature events that are predicted at the end of the 21st century. Temperatures above 27ºC significantly affected the photosynthetic rate of the seedlings, decreasing the production of oxygen and the performance of the PSII. One month exposure of the seedlings at temperatures above 29ºC produced serious alterations in their development, especially in leaf growth and mortality.

Short-term exposure of the seedlings to increasing temperatures produced a continuous decrease of oxygen production in leaves and seeds. This is related to the performance of the PSII, showing a critical temperature threshold in *Fv/Fm* at 31ºC, above which *Fv/Fm* drastically fell. This decrease was more pronounced in the leaves, which were the main contributors of the total seedling primary production. In contrast, leaf and seed F_0 peaked at 31ºC before dropping, while one-month exposure led to a continuous decline along the temperature gradient. Increases in F_0 indicate critical temperature thresholds that cause PSII deactivation (Havaux 1993, Yamane et al. 2000, Yamada et al. 1996). The differences in behaviour of F_0 between short- and long-term exposures may be because PSII has the ability to resist heat stress on the scale of hours in order to adapt to daily temperature variations of the environment (Havaux 1994), but this ability seems to be lost at longer time spans. Similar trends of PSII performance have been found in adult species in *P. oceanica* meadows (Marín-Guirao et al. 2016). A reduction in PSII is expected to inhibit photosynthesis, leading to a loss in primary production and a carbon imbalance, resulting in a limitation in the growth of the seedling.

After one month of exposure at 29ºC, the inhibition of photosynthesis could result in a decrease in the carbon-assimilation capacity of the plant (Berry and Bjorkman 1980, Feller et al. 1998), although the development of the seedling was not affected. Above this temperature, the leaves presented symptoms of deterioration, losing their green colour and increasing the rate of senescence and leaf fall. This resulted in a marked reduction of the leaf growth rate, leaf mortality above 60% and a seedling mortality of 33%. The limitation of carbon assimilation coincided with the loss of photosynthetic tissues and increased mortality. Similar behaviour occurs in other seagrasses, where high temperatures increase shoot mortality and affect seed production (Massa et al. 2008, Thomson et al. 2015).

Our data indicates that a heat wave that warmed seawater above 29ºC for a period of one month could significantly slow the growth rate of early stages of *P. oceanica*. Above this threshold there would be severe damage that may be irreversible, leading to high mortality rates. This experiment shows how extreme events can have detrimental effects due to their abrupt nature, as this can overcome the adaptive capacity of organisms (Smale and Wernberg 2013, Wernberg et al. 2012).

Extreme events, such as heat waves, are expected to increase in frequency in the coming decades (Easterling et al. 2000). In addition, the intensity of heat waves (i.e.

temperature) is also expected to increase. Previous heat waves in the Western Mediterranean Sea led to water temperatures close to 29ºC (28.5-28.8ºC) (Marbà and Duarte 2009, Luterbacher et al. 2004), and in the coming decades they are expected to reach higher values (Cruz et al. 2007, Giorgi and Lionello 2008). Therefore, according to our results, the early stages of *P. oceanica* could be threatened under the predicted scenario of greater occurrence of heat waves in the context of a continuously warming world.

This study demonstrates that future heat waves could have severe effects on seagrasses such as *P. oceanica*. Seagrasses are habitat-forming species that play key ecological roles for a range of other flora and fauna. Particularly, *P. oceanica* sustains communities with a high biodiversity and productivity (Hemminga and Duarte 2000), acts as a source of food and shelter for many marine pelagic animals, some of them of commercial interest (Calizza et al. 2013, Seytre and Francour 2014) and plays an important role in the sinking of carbon and element cycling (Gacia et al. 2002, Sanz-Lázaro et al. 2012). All of these functions support ecosystem services for society (Vassallo et al. 2013). However, seagrasses are subject to multiple stressors such as pollution, eutrophication and invasive species (Balestri et al. 2004, Marbà et al. 2014). The sublethal development-limiting effects of heat waves on *P. oceanica* may cause it to become more sensitive to the above-mentioned stressors, endangering the survival of the seedlings and thus, the colonization capacity of this seagrass.

The outcomes of this study have important ecological implications related to the loss of colonization capacity of *P. oceanica*. Taking into account that the seedlings of *P. oceanica* are more affected by temperature increases than adults, future heat waves are expected to partially decrease its recruitment. Where sexual reproduction is threatened, this species could lose its colonization capacity from seed dispersal. In addition, this limitation to sexual reproduction could reduce the genetic variability of seagrass populations, which may reduce the capacity of the plant to adapt to changes in its environment, thus endangering its natural resistance and resilience against environmental stressors (Ehlers et al. 2008). As in other habitat-forming species, the loss of this seagrass could have cascade effects on other flora and fauna, causing profound alterations on the diversity and structure of the associated communities (Hughes et al. 2009, Moore and Duffy 2016). Therefore, integral management policies that aim to maintain a good ecological status are necessary to mitigate the effects of global warming and cumulative effects with other stressors, especially in the case of habitat forming species such as seagrasses.

This chapter demonstrates that future heat waves are likely to negatively affect the development of early stages of *P. oceanica* by damaging its photosynthetic system. Negative effects on this seagrass are expected to occur above a threshold of 27ºC. Above this temperature there is likely to be a limitation in leaf growth, an increased rated of leaf senescence and, in some cases, plant mortality. The greater occurrences of extreme events that will be caused by temperature increases in the coming decades is expected to compromise the colonization ability and the genetic diversity of this plant. This situation could cause a decline in the range and condition of *P. oceanica* meadows. To prevent or mitigate these impacts, it is important to design integral management strategies that minimize the effects of global warming, and to preserve the resistance and resilience capacity of ecologically relevant habitats such as *P. oceanica*meadows. This will help to prevent additive or synergistic effects with other environmental stressors, thus helping to preserve this habitat-forming species.

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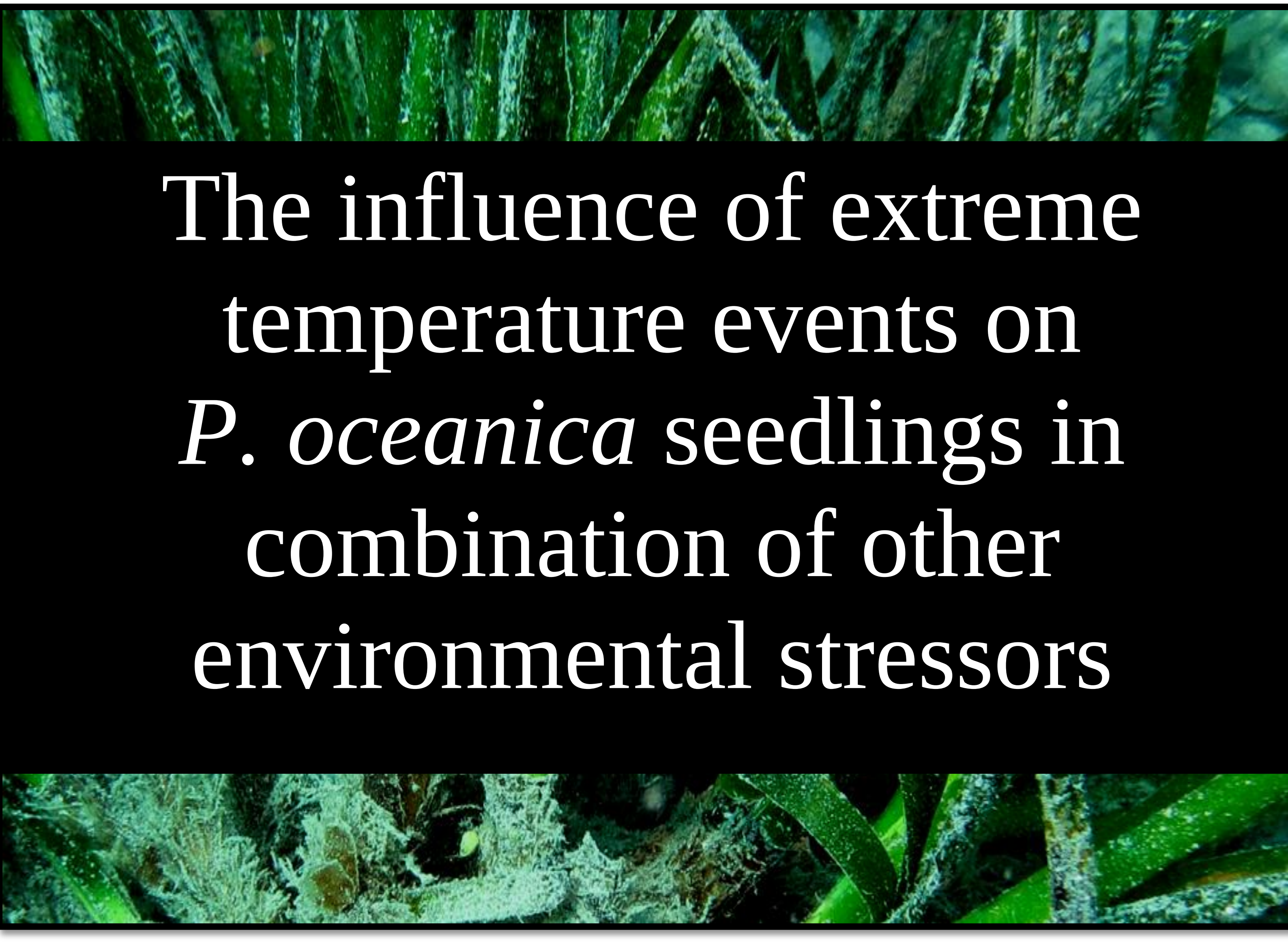
Chapter 4



The influence of extreme temperature events on *P. oceanica* seedlings in combination of other environmental stressors

Introduction

Habitat forming species promote habitat complexity and provide trophic resources, sustaining a large part of ecosystem biodiversity and production. Seagrasses are habitat forming species with key functions in coastal systems (Hemminga and Duarte 2000). They are a sink of carbon and nursery for fish, protect coasts from storms and play a relevant role in the cycling of many elements (Koch et al. 2009, Duarte et al. 2010, Sanz-Lázaro et al. 2012). *Posidonia oceanica* (L.) is the most abundant seagrass in the Mediterranean (Pasqualini et al. 2000). It forms meadows that are catalogued by the Habitat Directive of the European Union as a priority habitat for conservation. Many anthropogenic pressures are producing a regression in this seagrass (Orth et al. 2006, Waycott et al. 2009).

Coastal areas, due to their dense population, are accompanied by intense human-induced stressors compared to other less populated areas (Lotze 2006). Many environmental stressors threaten the meadows of *P. oceanica* and other seagrasses. Overgrazing can be caused by overfishing top predators, which increases grazer abundance through cascading effects (Short and Wyllie-Echeverria 1996, Orth et al. 2006). Another cause is related to eutrophication, which increases epiphyte density and thus, the palatability of the *P. oceanica* leaves (Mazzella et al. 1992). Another environmental stressor for *P. oceanica* is burial (Cabaço et al. 2008), which can be provoked by several activities such as dredging or increased sedimentation rates of particles due to increased erosion enhanced by deforestation.

Climate change is introducing new environmental stressors into already stressed ecosystems, which are becoming of increasing concern. To date, most of the research related to climate change has focused on the effects of the mean change of specific parameters (Jentsch et al. 2007). Nevertheless, extreme climate events are expected to increase their occurrence due to climate change (IPCC 2012). This type of events are generally rare and have an abnormally high intensity (Easterling et al. 2000, Stephenson 2011), having a key role in structuring ecosystems (Sanz-Lázaro 2016). Specifically, heat waves are expected to have deleterious effects on seagrasses such as *P. oceanica* (Olsen et al. 2012, Duarte et al. 2018, Guerrero-Meseguer et al. 2017). So, these new stressors are adding to the “traditional” ones that habitats, such as *P. oceanica* meadows, have been suffering in the last decades.

Nowadays, the numerous anthropogenic activities have led to a complex scenario of multiple stressors affecting the environment (Halpern et al. 2008). Under these conditions, stressors can act independently from each other (additive/cumulative effects) or combined, leading to interactions among them, which can result in an increase (synergy) or lowering (antagonism) of their effects if they were acting individually (Vinebrooke et al. 2004, Thrush et al. 2009, Kelly et al. 2017). Experiments simulating scenarios with multiple stressors, despite they are much more realistic, are rarely performed due to their complexity. Nevertheless, this information is crucial for environmental management, especially relevant in ecosystems under high anthropogenic pressure such as coastal areas.

The aim of this chapter is to study the effects of multiple environmental stressors on *P. oceanica*. To do so, we performed a manipulative experiment in the laboratory to study the single and combined effects of overgrazing, burial and heat waves on *P. oceanica* seedlings. The hypothesis were that (i) heat waves will foster the negative effects of overgrazing and burial on *P. oceanica*, and (ii) the combined effect of the all the stressors will be cumulative.

Materials and Methods

Experimental set-up

A mesocosm experiment was performed to test the effects of multiple environmental stressors on *P. oceanica* seedlings. The seedlings used in the experiment were germinated from seeds collected on the beaches of Mazarrón Bay (Murcia, Spain) in May 2013. Seedlings were grown in glass aquaria filled with seawater at 21±1 ºC and 36 of temperature and salinity, respectively; and continuously aerated by air pumps to ensure that oxygen was close to saturation. After five months, seedlings of similar sizes were randomly selected for the experiments.

Each seedling was incubated for one month in individual aerated glass jars (500 ml) filled with artificial seawater and sediment (constituting the experimental unit). Seawater (salinity of 36) was prepared using bidistilled water and marine salt (Ocean Fish, PRODAC International, Cittadella, Italy). Aeration was maintained using an air pump attached to a system of plastic capillary tubes (1 mm diameter) inside the glass jars to ensure that oxygen concentration was always above 5.5 mg O_2/l^{-1}. Glass jars were covered with a diffusible top to allow air exchange while minimizing changes in salinity and pH. Weekly variations were below the limit of detection (0.2) and 0.03±0.01 for salinity and pH, respectively. To prevent colonization of epiphytes and changes in water quality, the artificial seawater was replaced every week. We used environmental chambers (CLIMACELL-404, MMM Medcenter Einrichtungen GmbH, München, Germany) to control light intensity (100 µmol m^2 s^{-1}), photoperiod (light:dark 14:10) and temperatures.

The environmental stressors were set up by controlling temperature and simulating grazing and seed burial. Temperatures used (27, 29, 31 and 33°C) were based on current and expected maximum superficial seawater temperature reached by heat waves in the Mediterranean (IPCC 2007). To select these temperatures the same method described in the previous chapter of this thesis was used. The temperature of each environmental chamber was recorded continuously by data loggers (HOBO Onset Computer Corporation, Bourne, MA, USA) to ensure that the fixed temperatures were constant during the whole experiment.

Grazing was simulated by cutting the leaves of each seedling approximately two centimeters above the rhizome at the beginning of the experiment. Seed burial was done by totally burying the seed of the seedlings under small rock fragments than had been previously sterilized (size composition: 80.8 and 19.2% of pebble and gravel, respectively; Wentworth 1922). The leaves of the seedlings remained above the substrate. This type of substrate was chosen because it is not prone to suffer anaerobiosis and thus, no derived toxic by-products were expected to be produced. For each treatment, six seedlings were used as replicates (n=6).

Parameters measured

Leaves and roots, they were counted and measured (weight and length) at the beginning and end of the incubations to calculate net growth per seedling, % of dead leaves and % of new leaves. Then, net leaf production (%) was calculated by subtracting the % of dead leaves to the % of new leaves per seedling. At the end of the experiment minimum fluorescence after dark adaptation (F_0) was measured in both the leaf and seed by using saturation pulse analysis by a Chlorophyll Fluorometer (MINI-PAM, Waltz, Effeltrich, Germany). For the fluorescence measurements, the samples were previously cleaned of epiphytes by gently scraping the seed and leaves with a glass slide. Dark-adaptation consisted on covering plants with aluminum foil for 15 minutes. To take homogeneous measurements, all of them (two different points per leaf and seed on each plant) were made at the base of the second leaf of each seedling and over the same zone of the seed. The average of these two measurements was used as a replicate. Biomass production was calculated by wet weight difference of the seedling after and before the incubation.

To study possible changes in the mobilization of nutrients, carbon (C) and nitrogen (N) content was measured in the leaves of the seedlings. The leaves were separated at the end of the experiment and, after removing epiphytes, were stored frozen at -80ºC. Afterwards, they were freeze-dried using an Alpha 1-2 LD plus freeze dryer (Martin Christ, Osterode, Germany) to constant weight and then finely ground. POC (after a pre-treatment consisting of adding 1:1 HCl), and PON were determined using a Carlo Erba Inst. EA 1108 Elemental Analyser (Carlo Erba Strumentazione, Milan, Italy). Duplicate measurements were done of two subsamples of leaves. The average of these two measurements was used as a replicate.

Data analyses

We analyzed the trends of the measured parameters with increasing temperatures by adjusting the data to the best fitted regression models using the Akaike information criterion (AICc; supplementary Table 1). Previously, normality and homoscedasticity of variances of the data was checked using *Q-Q* plots and Bartlett tests, respectively. Then, we performed an ANCOVA analysis to compare the trend of the regressions among the different treatments on each measured parameter. Statistical analyses were performed with R statistical software (v. 2.15.0) using the packages “AICcmodavg” and “stringr” (R Core Team 2016). All data were reported as mean±standard error (SE). Statistical tests were conducted with a significance level of α=0.05.

Results

Impacts of grazing and burial

Physiological responses

The net growth of the leaves at 27ºC was significantly lowered by grazing but not burial (Fig. 1). When both stressors were combined they had a synergistic effect, decreasing the net growth of leaves a 69% and yielding to a net loss of 60% of their initial leaves (Table 1, Fig. 1).

The net leaf production was generally negative indicating that the seedlings loss more leaves than were produced (Fig. 2A). At 27ºC, *P. oceanica* had a net loss of leaves around 20%, that was comparable to the obtained when the seedlings had its seed buried (Fig. 2A, Table 1). When *P. oceanica* was affected by grazing or by the combination of both factors, the loss of leaves increased to 40 and 50%, respectively (Fig. 2A). As regards F_0 at 27ºC, both factors, burial and grazing, decreased the photosynthetic efficiency but non-significantly, yielding to additive effects when both stressors acted together, resulting in a reduction of the F_0 of 48% (Fig. 2B, Table 1). Biomass production did not seem to be affected by burial nor grazing, yielding very similar values. When both stressors occurred together, values within the range of the previous treatments showed that the effects of burial and grazing were additions for biomass production (Fig. 2C, Table 1).

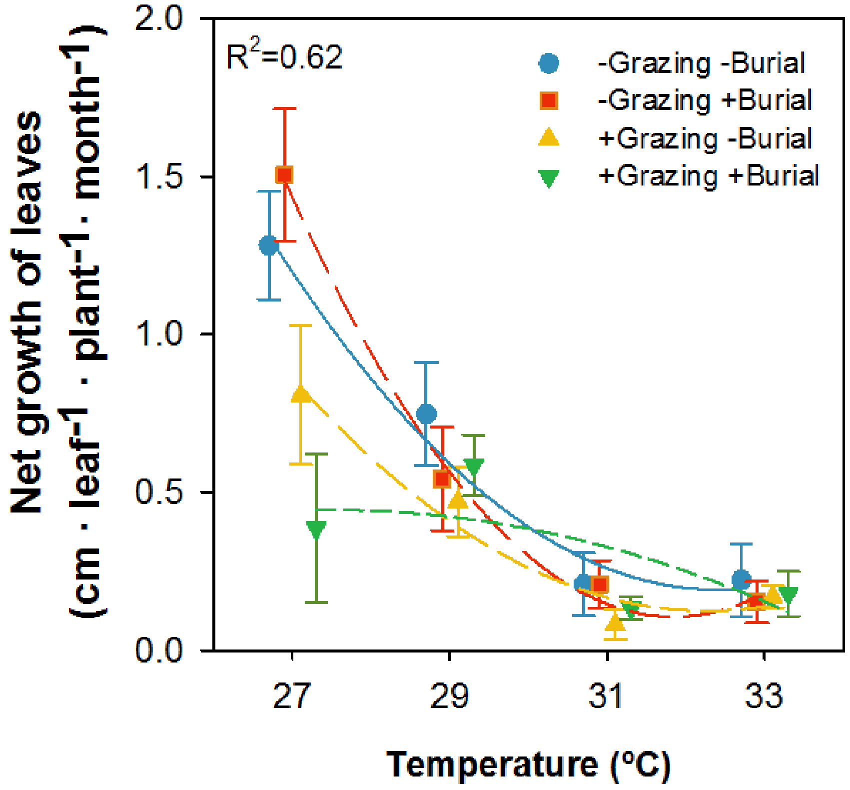


Fig. 1. The effects of overgrazing and seed burial (separately and together) under a gradient of temperature on the net leaf growth of *Posidonia oceanica* seedlings (n=6, mean ±SE). Lines indicate significant (p<0.05) second order polynomial regression. R^2 refers to the whole regression model. All treatments were tested at 27, 29, 31 and 33ºC, but treatments have been slightly moved from the temperature in the graph to favour visualization.

Table 1. Coefficients (mean±SE; intercept centred at 27ºC) of the regression models testing the effects of overgrazing and seed burial (separately and together) under a gradient of temperature for each measured parameter. Regression models can be first-order polynomial (a) or second-order polynomial (b) according to AICc (corrected Akaike information criterion; Table 2). Significant effects (p<0.05) are indicated in bold.

		Mean at 27ºC	**Temperature linear trend**	**Temperature quadratic trend**
Net growth of leaves [b]	Intercept	**1.32±0.13**	**-0.39±0.11**	**0.034±0.017**
	Grazing (G)	**-0.48±0.19**	0.12±0.15	-0.008±0.024
	Burial (B)	0.18±0.19	-0.17±0.15	0.022±0.024
	GxB	**-0.57±0.27**	**0.45±0.21**	-0.058±0.034

Leaf F_0 [a]	Intercept	**640.4±62.4**	**-93.1±16.6**	-
	Grazing (G)	-43.7±88.2	8.02±23.5	-
	Burial (B)	-20.3±88.2	23.8±23.5	-
	GxB	-135.6±124.8	15.4±33.3	-
Net leaf production [a]	Intercept	**-31.6±11.2**	**-7.49±2.99**	-
	Grazing (G)	-8.33±15.9	0.67±4.23	-
	Burial (B)	14.7±15.9	-2.89±4.23	-
	GxB	-16.5±22.4	1.99±5.98	-
Biomass [a]	Intercept	**0.36±0.054**	**-0.042±0.014**	-
	Grazing (G)	0.023±0.076	0.024±0.02	-
	Burial (B)	-0.05±0.076	0.002±0.02	-
	GxB	-0.013±0.11	-0.017±0.029	-
C:N in leaves [a]	Intercept	**11.3±2.14**	**2.14±0.57**	-
	Grazing (G)	-1.23±3.03	0.42±0.81	-
	Burial (B)	**7.66±3.03**	**-2.05±0.81**	-
	GxB	**-7.86±4.28**	**2.55±1.14**	-

Table 2. Summary of Akaike information criterion (AICc) results. Lower values (in bold) determine the best fitted regression model.

	Polynomial first-order $y_i = \beta_0 + \beta_1 x_i$	**Polynomial second-order** $y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i^2$	**Exponential** $y_i = \beta_0 + \beta_1 e^{x_i}$
Net growth of leaves	87.88	**79.78**	134.7
Leaf F_0	**1283.7**	1284.0	1317.4
Net leaf production	**978.0**	979.0	985.0
Biomass	**-70.79**	63.99	-63.76
C:N in leaves	**403.2**	410.5	408.2

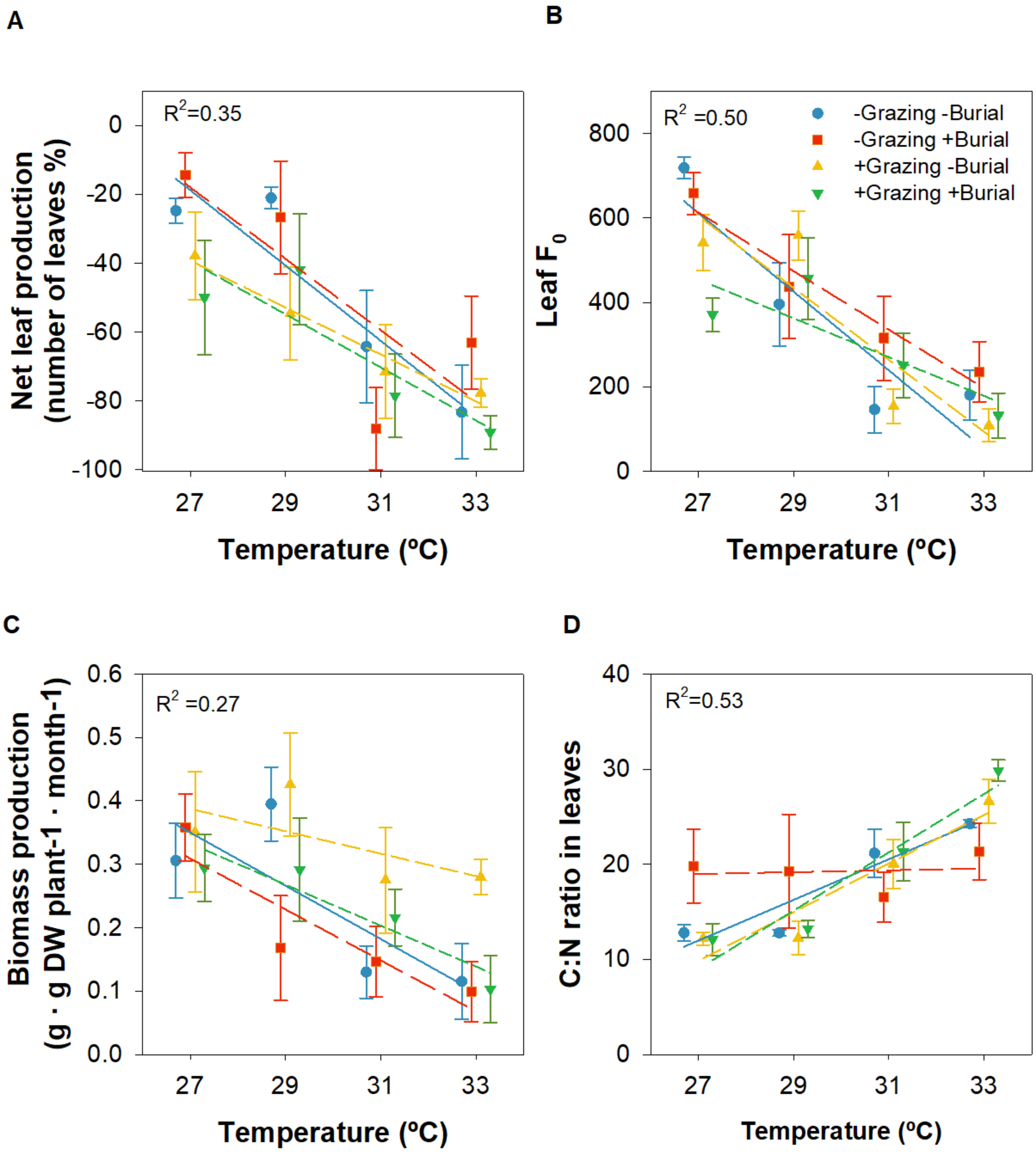


Fig. 2. The effects of overgrazing and seed burial (separately and together) under a gradient of temperature on the net leaf production (production - loss of leaves, A), leaf F_0 (B), biomass production (n=6, mean ±SE; C) and C:N in leaves (n=4, mean ±SE; D) of *Posidonia oceanica* seedlings. Lines indicate significant (p<0.05) first order polynomial regression. R^2 refers to the whole regression model. All treatments were tested at 27, 29, 31 and 33ºC, but treatments have been slightly moved from the temperature in the graph to favour visualization.

Influence in nutrient concentration

Focusing on the responses of nutrient concentration of the leaves at 27ºC, buried seedlings increased the C:N, while grazing had no marked effect as well as when both stressors co-occurred, producing an antagonistic effect (Fig. 2D, Table 1).

Interactions of grazing and burial with rising temperature

Physiological responses

The net growth of the leaves along the temperature gradient followed a significant second order polynomial regression being the trend significantly different when grazing and burial co-occurred (Tables 1 and 2). Differences among treatments gradually diminished with temperature, resulting in values very homogeneous and close to 0 at 33ºC (Fig. 1, Table 1). As regards F_0, there was a linear decrease with temperature irrespectively of the treatment (Fig. 2B, Table 1). Similarly, temperature had a negative effect on the percentage of net leaf production following all the treatments similar linear decreasing trends (Tables 1 and 2). At 33ºC, mean values were all below -60% (Fig. 2A). For biomass production, the same negative trend occurred among treatments in general, although grazing seemed to diminish to some extent the effect of temperature (Fig. 2C, Table 1).

Many plants at high temperatures presented symptoms of deterioration: leaves loosing green colour, fragile rhizome, hollow seeds and roots (Fig. 3). Mortality rates of the seedlings were 4, 8, 21 and 21% for plants grown at 27, 29, 31 and 33ºC, respectively.

Fig. 3. A seedling of *P. oceanica* grown at 33ºC. The picture shows the symptoms of deterioration in the leaves produced by the high temperature.

Influence in nutrient concentration

Temperature generally incremented the C:N of the leaves of *P. oceanica* seedlings in a linear trend, but in the case of the ones with buried seeds, temperature up to 33ºC did not have a marked effect on the C:N of the leaves (Fig. 2D, Table 1).

Discussion

The effect of multiple stressors in ecosystems is a major focus of research because to the current number of anthropogenic stressors, new ones are adding up derived from global change. In general, the expected effects among stressors are additive, synergistic or antagonistic. However, this study shows that a stressor linked to global change can override the effects of other stressors. This chapter shows that heat waves can have an overwhelming effect on *P. oceanica* seedlings compared to other environmental stressors such as overgrazing and seed burial. A temperature rise from 2 to 6ºC above the normal temperature in the warm season during one month can have a greater deleterious effect on *P. oceanica* than overgrazing and seed burial when occurring together, despite their synergistic effects.

Effects of grazing and burial

Seagrasses such as *P. oceanica* are key habitat forming species in coastal areas due to the relevant ecosystem functions they support and the ecosystem services that provide to society (Hemminga and Duarte 2000). Seagrasses can be threatened by several environmental stressors, especially, in the early stages of its development. Overgrazing is one of them and can be caused by several anthropogenic pressures such as excessive fishing effort on high trophic levels which generally feed on grazers (Short and Wyllie-Echeverria 1996, Orth et al. 2006). Another cause is the increment of epiphytes due to increase of nutrient inputs, which can be due to activities such as sewage or fish farming (Vergés et al. 2007, Prado et al. 2010). Burial can be another environmental stressor, which are produced by increasing sedimentation rates above natural levels (Onuf 1994, Cabaço et al. 2008, Badalamenti et al. 2011). Although total burial of the plant is unusual, partial burial is more likely to occur, resulting in the burial of the seed. This can lead to deleterious effects when the leaves of the seedling are not sufficiently growth, since at this stage of development, species such as *P. oceanica* are supported by the photosynthesis produced by the seed (Celdrán and Marín 2013).

When overgrazing and seed burial were the only occurring environmental stressors, overgrazing showed a larger magnitude effect than seed burial, greatly decreasing the net growth of leaves. This makes sense because at this stage of development, the area of the leaves to photosynthesize was greater than the size of the seed. But when overgrazing was simulated, a reduction of the leaf surface made than the contribution of the seed to the overall photosynthetic area of the plant was notably increased. Thus, the net growth of leaves was much more impaired when sediment burial co-occurred with overgrazing, indicating a synergistic effect. Likewise, other response variables related with the development of *P. oceanica* (net leaf production, leaf F_0 and biomass production), showed, to a lesser or greater extent, the lowest values when grazing and burial co-occurred. But in these cases, no synergistic effect was detected.

C:N in plants are related with their health status (Pirc and Wollenweber 1988). As regards C:N, plants with no stressors showed low values, indicating a good status, while stressors specifically increased C:Ns in the plants. The increase of C:N in the leaves indicates a lower mobilization of N in the seedlings which were stressed comparing with the seedling which growth without stressors. Regarding the negative effects in the net growth of the leaves in all the stressors tested, the decrease in the N mobilization also seems to be related with the increases of temperatures.

Interactions of grazing and burial with rising temperatures

Global change is introducing new anthropogenic stressors to ecosystems. The effects of these stressors are expected to become more dramatic as the causes that produce global change, such as CO_2 emissions, are increasing constantly. Although, the changes in the mean values of relevant parameters such as temperature are important, the increasing occurrence of extreme events due to global change is of great relevance since extreme events are major drivers of ecological change (Stephenson 2011). Seagrasses are sensitive to small variations of temperature (Marín-Guirao et al. 2016) and so, heat waves are expected to threaten their survival (Guerrero-Meseguer et al. 2017).

Our study shows that increases of temperature produced a much more marked effect in reducing the net growth of the leaves, irrespectively from other stressors. Heat waves overrode the effects of other stressors, leading to a homogenization among treatments. This effect is expected to be due to the impairment of photosynthesis caused by temperature rises above the natural high temperatures. As a consequence, the net growth of leaves notably decreases and then, the net leaf production is affected. In general terms, this lead to an increase in the C:N of the leaves, which is a typical response to stress in seagrasses (Pirc and Wollenweber 1988, Sandoval-Gil et al. 2014). Overall, this leads to a reduction in the biomass production. These reductions in biomass at such early stages of development can greatly endanger the survival of *P. oceanica*.

The effect of multiple stressors in the environment is a major focus of research because the number and intensity of stressors is constantly growing. The main research question to date has been if multiple stressors either have either cumulative or interactive effects, showing in many cases that interactions are wide-spread (Folt et al. 1999, Crain et al. 2008). Nevertheless, asymmetry among individual stressors can cause one to be dominant and override the effect of others (Kelly et al. 2017). This chapter is a clear example of heat wave as a dominant stressors compared to overgrazing or burial. Extreme events can act as dominant stressors when the parameter modified is critical due to their high intensity (Halpern et al. 2008).

The cumulative impact of multiple stressors is among the most needed research questions related with informing decisions for environmental agencies (Rudd 2014). The results of the present study show that a heat wave can have major deleterious effects on the seagrass *P. oceanica*. This strong effect of the simulated heat wave could be explained by the high intensity of extreme events. If the intensity of the extreme event is high enough, the extreme event can override the effects of other environmental stressors since the extreme event by itself is strong enough to produce the collapse of the species, in this case, *P. oceanica*. It is likely to think that other types of extreme events can also act as dominant stressors. If this is the case, extreme events could have a very marked negative consequences compared to other environmental stressors. Nevertheless, after suffering an extreme event, the resistance and resilience of a species or community maybe hampered if the other additional environmental stressors remain. Thus, specific experiments should be performed to test the effects with other types of extreme events, species and communities and the effect of the remaining environmental stressors after the extreme events has finished. These issues are relevant aspects that need to be studied so environmental agencies can increase their forecasting ability of scenarios of multiple stressors including extreme events, to do improve their management strategies.

In conclusion, our study demonstrates that an extreme such as heat waves, can override other environmental stressors in the habitat forming species *P. oceanica*. Further experiments testing the effects of different types of extreme events in species relevant for ecosystem functioning are mandatory to understand the consequences of climate change. This information is vital for environmental agencies to design preventive and mitigation measurements against climate change.

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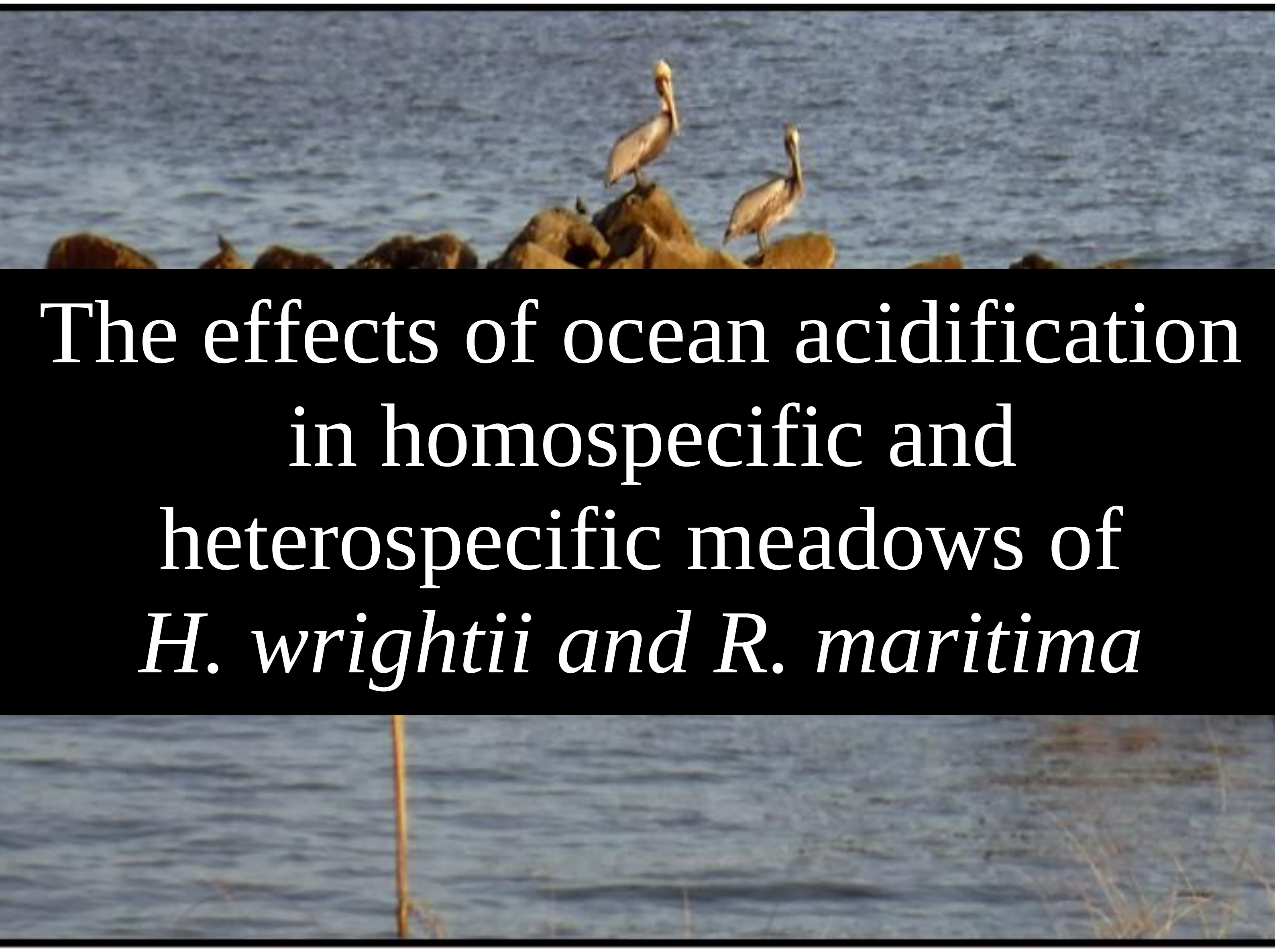
Chapter 5



The effects of ocean acidification in homospecific and heterospecific meadows of *H. wrightii and R. maritima*

Introduction

The exponential increase in atmospheric CO_2 since the industrial revolution has altered the equilibrium of inorganic carbon compounds in the ocean, increasing the concentrations of bicarbonate (HCO_3^-), carbonic acid (H_2CO_3), and hydrogen ions (H^+ (Elderfield et al. 2005). These changes, referred to as ocean acidification, have caused the average sea surface pH to drop by 0.1 units, and the pH is projected to further decline by 0.06-0.32 units by the end of this century (IPCC, 2013). Ocean acidification is known to have impacts on species physiologies to alter their interactions with cascading affects at the ecosystem level (Hall-Spencer et al. 2008).

Seagrass meadows are highly productive (Duarte and Cebrián 1996) and they provide food and refuge for many marine organisms (Hemminga and Duarte 2000). In addition, seagrasses play an important ecological role in coastal waters as carbon sinks (Duarte et al. 2010, Russell et al. 2013). Seagrasses are expected to benefit from ocean acidification because, they are carbon limited. In addition, seagrasses are able to use both CO_2 and HCO_3^- for photosynthesis, yet they tend to have a higher affinity for CO_2 (Koch et al. 2013). Indeed, previous reports have shown increases in seagrass productivity (Durako 1993, Zimmerman et al. 1997, Invers et al. 2002), vegetative growth (Zimmerman et al. 1997, Jiang et al. 2010, Russell et al. 2013, Martínez-Crego et al. 2014), and flowering frequency (Palacios and Zimmerman 2007) under lowered pH conditions.

Ocean acidification, however, is not the only driver shaping coastal ecosystems and physiological responses of aquatic plants. Coastal environments are highly dynamic in terms of fluctuating light, nutrients, and salinity, particularly in mesohaline estuaries. Estuaries commonly receive freshwater inputs that change the chemical and physical properties of the seawater (Aufdenkampe et al. 2011). Furthermore, high biological activity, hydrodynamic processes and excessive primary production and respiration rates in shallow areas, can result in highly variable pH and CO_2 environments with estuarine organisms experiencing daily incremental changes in pH already outside of those predicted for the open ocean within the next century (Duarte et al. 2013). Future climate conditions will intensify these changes (Hofmann et al. 2011, Waldbusser and Salisbury 2014). As a result of this dynamic environment, seagrasses which inhabit estuarine systems may not be limited by carbon availability (Frieder et al. 2014, Pacella et al. 2018). Therefore, to more clearly predict where acidification will shape seagrass ecosystems it become necessary to test for effects within a variety of environments that persist.

Ocean acidification also has the potential to shift competitive strengths between species (Connell et al. 2013, Russell et al. 2013, Takeshita et al. 2015). Due to inter-specific differences in HCO_3^- utilization efficiency, the response to lowered pH levels varies considerably among seagrass species (Invers et al. 2001, Campbell and Fourqurean 2013). Species which rely less on CO_2 and have efficient HCO_3^- use should be less sensitive to altered future carbonate chemistry and thus benefit less from ocean acidification (Gutow et al. 2014). Seagrasses also have different carbon allocation strategies, which further suggests differential growth responses to elevated partial pressure of CO_2 (pCO_2; Ow et al. 2015). For example, in terrestrial experiments, the direct positive effects of elevated CO_2 for plant species are at times outweighed by negative effects due to stimulation of the growth of other plant competitors (Poorter and Navas 2003). Indeed, a change in seagrass species composition has been observed near and removed from a volcanic CO_2 vent at Papua New Guinea. It was suggested that the species with taller blade-like leaves that were more dominant nearest the vent CO_2 source kept the smaller successional species from benefitting from the increased CO_2

availability (Takeshita et al. 2015). Despite these observations, there have been few investigations on the differential impacts of ocean acidification on co-habiting seagrass species, and how such impacts affect the competition between the species and meadow structure.

Halodule wrightii Asch. and *Ruppia maritima* L. are widespread seagrasses that co-exist in mixed beds along the mesohaline coast of the North-Central Gulf of Mexico (Linnaeus). These species have different growth cycles and seasonal peaks in biomass. *Halodule wrightii* grows throughout the year and typically reaches maximum biomass in late summer-early fall. *Ruppia maritima* grows during cool temperatures and undergoes senescence after flowering in spring (Pulich 1985, Cho and Poirrier 2005, Anton et al. 2011). Thus, the species may co-exist in mixed beds and show staggered biomass peaks, although in some occasions, *R. maritima* can outcompete and replace *H. wrightii* as the dominant species, seemingly under increased environmental stress (Christiaen et al. 2016). Few studies have evaluated the influence of ocean acidification on *H. wrightii* and *R. maritima*. It seems that both seagrasses may increase their productivity under elevated $pCO_{2,}$ but *R. maritima* production has been carbon saturated in natural settings (Sand-Jensen and Gordon 1984, Campbell and Fourqurean 2013, Koch et al. 2013). However, how elevated pCO_2 differently affects the two species, and the consequences on the structure of these mixed beds, has not been examined.

The objective of this chapter is to (1) evaluate the effects of ocean acidification on the productivity and vegetative growth of seagrasses in the mesohaline waters of the Northern Gulf of Mexico (2) test for potential shifts in species composition in mixed meadows of *H. wrightii* and *R. maritima* resulting from an increase in CO_2 availability. To do this, homo- and hetero-specific beds of *H. wrightii* and *R. maritima* were placed in an outdoor mesocosm under ambient and elevated pCO_2 (low pH) conditions. After at least five weeks of exposure to conditions, seagrass morphology and biomass, photo-physiology, and metabolism were measured. The hypotheses are that (i) the enhanced CO_2 availability will stimulate photosynthesis and benefit growth and production and (ii) because the two species have different physiologies and seasonal peaks in above-ground biomass, the increased CO_2 availability will alter their competition and mixed bed structure.

Materials and Methods

Seagrass bed collection

Sixty rectangular cores of seagrass beds (10 x 4 cm), 4 cm deep, were collected from single species patches of *H. wrightii* and *R. maritima* from approximately 1 m depth in Pointe-aux-Pins, Bayou la Batre (30°23'4.26"N, 88°18'42.73"W Northern Gulf of Mexico, Alabama, USA) on 27th February 2017. These seagrasses commonly coexist in this region to form homospecific patches that border each other. At the field, cores were introduced into 30 aquaria (21 x 13 x 13 cm) in pairs, such that there were 10 aquaria with two cores of *H. wrightii*, 10 aquaria with two cores of *R. maritima*, and 10 aquaria with a core of *H. wrightii* and a core of *R. maritima*. We butted the cores against each other to simulate homospecific beds of either species as well as the fringing area between adjacent beds of *H. wrightii* and *R. maritima*. The aquaria filled with cores were immediately brought back to Dauphin Island Sea Lab and kept in an outdoor experimental setup for seventy days (16 days of acclimation, 54 days of experimental manipulation, with final measures taken after at least 4.9 weeks of different CO_2 exposure, Fig. 1).

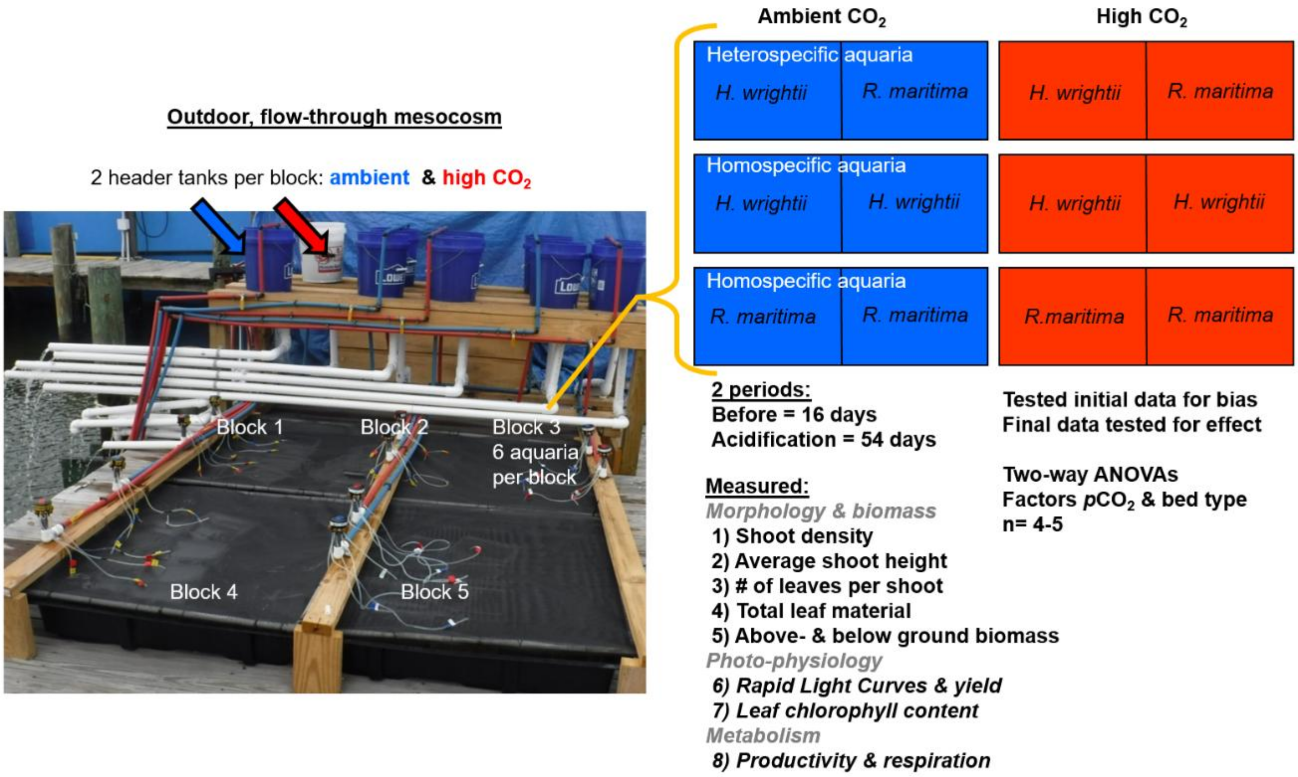


Fig. 1. Experimental setup applied in this study.

Experimental setup

Two aquaria of each seagrass bed type (*Halodule-Halodule*, HH; *Ruppia-Ruppia*, RR; and heterospecific, *Halodule-Ruppia*, HR) were randomly assigned to five experimental blocks in an outdoor flow through system (Fig. 1). Then, one of the two aquaria for each type within the block was assigned to the ambient CO_2 treatment (natural pCO_2/ pH), and the other to the high CO_2 treatment (high pCO_2/ low pH). Aquaria were arranged randomly within each block and covered with screen to prevent excess light stress (Fig.1; Cebrian et al. 2013). Seawater was pumped from the bay (1 m depth) into header tanks, from where it was channeled into the aquaria to overflow into surrounding water bath and released back into the bay. There were two header tanks per block, one for the ambient CO_2 aquaria and another for the high CO_2 aquaria, for 10 headers tanks in total and each tank feeding three aquaria (Fig. 1). The residence time of the seawater in each aquarium was around 30 minutes. The experiment had six treatments resulting from the crossing between seagrass beds types and CO_2 levels (i.e. HH/ambient; HH/high; RR/ambient; RR/high; HR/ambient; and HR/high), with five replicates per treatment. However, due to system failure and human error, replicate aquaria were reduced for some treatments.

A pH stat system (*IKS* Aquastar Germany) was used to control bubbling of CO_2 from a gas cylinder into the header tanks for the high CO_2 aquaria. For each block, the header tank bubbled withCO_2 was chosen at random from the two.

Environmental conditions in the aquaria were constantly monitored during the entire duration of the experiment, including the acclimation period. Water temperature was logged by HOBO pendantsusing1 logger per block (HOBO Onset Computer Corporation, Bourne, MA, USA). Surface photosynthetic active radiation (PAR) was downloaded from an environmental station maintained by the Dauphin Island Sea Lab (30°15.075'N, 88°04.670'W Dauphin Island, Alabama, USA; http://cf.disl.org/mondata/mainmenu.cfm) located within 0.1 miles from the outdoor flow-through system. Point measurements of salinity were obtained throughout the study duration using a hand-held YSI-85 conductivity probe (YSI, Yellow Springs, Ohio, USA)

pH was monitored at each aquarium and head tank approximately every three days with an in LabRoutine Pro calibrated glass electrode (Mettler Toledo, Ohio, USA). The pH was measured on the total scale (pH_T) using certified reference material provided by A. Dickson (Batch 30). In addition, water samples were collected, approximately once per week, from one of the ambient and high CO_2 treatment header tanks chosen at random to measure total alkalinity (A_T, 120 ml). Samples for A_T were filtered on combusted glass microfiber filters membranes and immediately inoculated with 72 µl of 33% saturated mercuric chloride (HgCl) solution and stored until analyzed. A standard provided by A. Dickson (Batch 157) was used to check precision and accuracy (A_T, 2229.9±0.05µmol kg^{-1}). The carbonate chemistry was assessed using pH_T, A_T, salinity and temperature using the R package "seacarb" (Gattuso et al. 2018).

The pCO$_2$ in the ambient treatment was ~350 µatm, which corresponded to the value found in local coastal waters. An offset of pH (approximately -0.3 to -0.4) was applied to the "high CO$_2$" treatment to mimic the extreme of pH variability expected by the end of this century based on IPCC scenario for 2100 (IPCC, 2013).

Morphology and biomass

Shoot density was determined during the acclimation period (day 2), and after 34 days of exposure to experimental conditions. Shoot density was measured for each core, and the two cores representing the same species were averaged for homospecific aquaria. On day 9 of the acclimation period and after 41 days of CO$_2$ perturbation, we haphazardly selected five shoots from each core in each aquarium. We counted the leaves on the shoots and measured the length of each leaf on the shoot. With these measurements, we calculated shoot height (average leaf length per shoot), leaf number per shoot, and summed the length of the leaf material per shoot. Then we calculated the average for the ten shoots in homospecific aquaria or the average of five shoots of each species in heterospecific aquaria. In combination, these measurements allowed us to infer whether, as a response to enhanced CO$_2$, shoots grew existing leaves longer, produced shorter and younger leaves, or a combination of both. For instance, the average number of leaves per shoot may not change, but shoots may show longer leaves (increased shoot height) and larger total leaf material, indicating shoots elongate their existing leaves, but do not produce more new leaves under enhanced CO$_2$. In contrast, higher number of leaves per shoot in combination with shorter shoot height and larger total leaf material per shoot would indicate a response to enhanced CO_2 centered in the production of new leaves. Plant biomass was only measured at the end of the study (54 days of CO_2 exposure) due to destructive sampling. Sediment was carefully rinsed off shoots and belowground materials (roots and rhizome). Above- and belowground materials were separated, dried at 60ºC, and the dry weight (DW) determined.

Photo-physiology

Photo-physiological measurements (dark- and light- adapted yield and rapid light curves) were done with a diving-pulse amplitude modulated fluorometer (diving-PAM, Waltz, Germany) eleven days into the acclimation period and after 43 days of exposure to experimental conditions. In each aquarium, measurements were done on two haphazardly selected leaves of the corresponding species in homospecific aquaria, or on two haphazardly selected leaves for each species in heterospecific aquaria. The leaves were placed side by side on the Waltz dark-adapted fiberoptic clip, so that the initial F' value would read above 200. Leaves for dark-adapted yield measurements were dark-adapted for five minutes, and leaves for light-adapted yield measurements were exposed to light conditions for at least ten minutes. Leaves were then exposed to a saturating light pulse. The intensity and width of the saturation pulse was adjusted to ensure a distinct plateau of maximum quantum yield at a set distance from the blade. Namely, for all samples a saturation intensity setting of 1 with a width of 0.8 was used in the initial measurements, and an intensity of 2 and a width of 0.8 in the final measurements (Genty et al. 1989).

The irradiances for rapid light curves (RLCs) ranged between 0 to 1700 µmol $\cdot$ $m^{-2} \cdot s^{-1}$ and were each applied for 10 s followed by a saturating pulse of 0.8 s. The absorption factor needed to calculate RLCs parameters was determined using the methods described in Beer and Björk 2000 and averaged to 0.84. The *r*ETR values were plotted against the light irradiances to produce a curve fitting the exponential model proposed by Platt et al. 1980. Derived parameters of RLCs include photosynthetic efficiency (α), dynamic photoinhibition parameter (β), relative electron transport rate maximum ($r\text{ETR}_{\text{max}}$) and the minimum saturation irradiance (E_k), which were all calculated following Ralph and Gademann 2005.

To better interpret the photo-physiological experiments, we also measured leaf chlorophyll *a* (Chl *a*) content, but only at the end of the experiment (54 days of exposure to experimental conditions) due to the destructive nature of this sampling. To do this, we haphazardly selected one shoot from each core (two shoots of the same species in the homospecific aquaria, and one shoot of each species in the heterospecific aquaria) and clipped the upper 5 cm section of the middle leaf on the shoot. Chlorophyll was extracted from that section in the dark in 90% acetone for 24 hours, and the extract measured in a fluorometer (Model TD-700 Turner Designs, California, USA, Welschmeyer 1994). The two values of Chl *a* content from the same species in homospecific aquaria were averaged to avoid pseudoreplication.

Metabolism

Net community productivity (NCP), and respiration rates were determined from the change in water dissolved oxygen content in two hour-long incubations using 10.2 x 5.7 x 5 cm clear (for NCP) or dark (for respiration) chambers placed onto both cores in each aquarium. Measurements were done seven days into the acclimation period and after 48 days of experimental exposure. At each sampling time, one clear and one dark chamber were placed at the exact same location on the core (i.e. the location of the chambers was marked in the first deployment and repeated for the second). Incubations were performed on clear days (mean PAR of 880 μmol photons m^2 s^{-1}in the first incubation and 1150 μmol photons m^2 s^{-1} in the final incubation). Dissolved oxygen content was measured with a Portable Meter Hach connected to probe with an optical sensor (HQ30d, Hach, Loveland, Colorado, USA). Rates of NCP and respiration were derived, and rates of gross primary production (GCP) from those rates, as explained in Cebrian et al. 2009. The two values of GCP were averaged in the homospecific aquaria to avoid pseudo-replication.

Data analysis

Data were analyzed by species, involving separate comparisons for each of the two species between the two beds (by itself or in the presence of the other species) and CO_2 levels (ambient or high CO_2). Thus, data were analyzed with two-way ANOVA separately for each species with seagrass bed type (homo- or hetero-specific) and treatment (ambient or high CO_2) as fixed factors. Comparisons were also done separately for data obtained during the acclimation period, to ensure homogeneous conditions among treatments before starting the actual CO_2 application, and for data obtained at the end of the experiment to test for the effects of the CO_2 application. Tukey’s multiple comparison tests were used to examine pairwise differences. Data were tested for normality using the Shapiro test and for homogeneity of variance using the Bartlett’s test, and transformed when necessary to comply with the assumptions of ANOVA. The statistical α was adjusted to 0.01 in order to account for the many tests and comparisons and avoid false positives (Benjamini and Hochberg 1995). For the same reason, the statistical α was adjusted to 0.005 for four parameters which could not be transformed to meet parametric requirements (Underwood 1997). All results are expressed as mean±standard error (SE) throughout this manuscript unless otherwise stated.

Results

Environmental conditions

Environmental conditions in aquaria were typical for seagrass beds in the region (see Anton et al. 2011, Christiaen et al. 2016). The mean (± SD) temperature logged by HOBO pendants was 23.0±0.6 ºC, ranging from 13.6 to 31.8 ºC. During daylight hours of the study, mean PAR (± SD) was 774.3±3.4 μmol photons m^{-2} s^{-1} and ranged from 10.0 as a minimum in morning and in twilight hours to a maximum of 2123.3 μmol photons m^{-2} s^{-1} at the peak of a sunny day. The salinity in aquaria over the study duration ranged from 4.3 to 30.7 (Fig. 2, Table 1).

The pH_T in aquaria was variable in both, ambient and high CO_2 treatment (Fig. 2), but the range of pH_T difference between the treatments was maintained between -0.29 to 0.44 along the experimental period (Fig. 2). Under the ambient treatment the pH_T averaged (± SD) 8.09±0.04, while in the high CO_2 treatment was 7.70±0.05 (Fig 2). The pH_T offset from ambient was similar between the three seagrass habitat types (HH, HR and RR), showing an average pH_T offset of -0.39±0.08 (Fig 2).

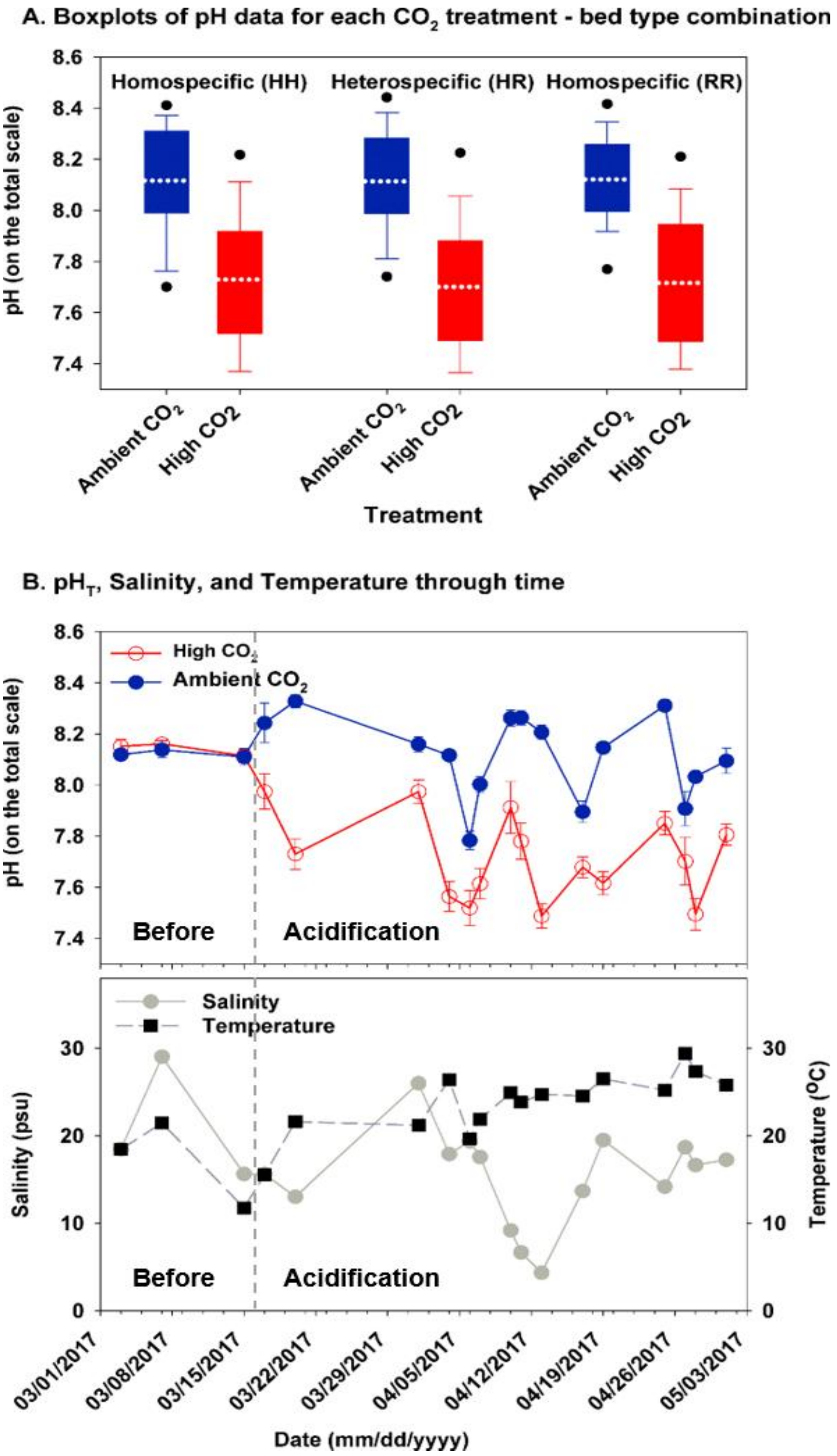


Fig. 2 The pH_T, salinity and temperature in the ambient and high CO_2 aquaria. Panel A is a boxplot of the all the discrete measures of pH_T presented by bed type (homo- or hetero-specific and CO_2 treatment (ambient or high). The dotted white line within the bar is the mean and the whiskers from the bars capture the 5th and 95th percentile. Panel B top, shows the evolution of pH_T (mean±SE, n = 27 aquaria) throughout the experiment as a function of (bottom) probed temperature and salinity (n = 5) used to calculate the carbonate chemistry. The dotted lines indicate the beginning of the perturbation.

Table 1. Temperature and salinity (mean±SD) measured per block during the experiment.

	Temperature		**Salinity**	
	MEAN	±SD	MEAN	±SD
Tank 1	23.31	2.290	15.48	6.201
Tank 2	23.22	2.248	16.25	5.829
Tank 3	23.13	2.577	16.43	5.993
Tank 4	23.28	2.353	16.37	5.892
Tank 5	21.90	3.936	16.36	5.842
Total	22.97	0.602	16.18	0.395

The pH_T in the header tanks during the experimental period was congruent with the values in the aquaria, varying in a range of 8 – 8.4 in the ambient treatment and of 7.2 – 8.0 in the high CO_2 treatment (Table 2, Fig. 2). Levels of A_T in the header tanks significantly fluctuated during the experimental period and it was related to changes in salinity (Table 2). In the ambient treatment header tanks, A_T ranged from 1443.7 to 1835.9 µmol kg^{-1} and from 1543.7 to 2069.9 µmol kg^{-1} in the high CO_2 treatment header tanks (Table 2). In ambient header tanks, pCO_2 and total dissolved inorganic carbon (C_T) ranged from 118.8 to 426.6 µatm and from 1268 to 1686 µmol kg^{-1}, respectively; while in the high CO_2 header tanks values ranged from 291.0 to 342.4 µatm and from 1504 to 2001 µmol kg^{-1}. The seawater in the ambient treatment was saturated with respect to both aragonite and calcite. In the high CO_2 treatment calcite and aragonite were under saturation most of the time, except after the storms on the 20th of March and 28th of April (Table 2).

Table 2. Environmental data and carbonate chemistry (as calculated from pH_T, A_T) in the header tanks of the ambient and high CO_2 treatments during the experimental period. Temperature (T, ºC); salinity (S); pH on the total scale (pH_T); total alkalinity (A_T, µmol kg^{-1}); partial pressure of CO_2 (pCO_2, µatm); dissolved inorganic carbon (C_T, µmol kg^{-1}) and saturation states with respect to aragonite (Ω_A) and calcite (Ω_C).

		Ambient								**High CO_2**							
Day	**Date**	**T**	**S**	**A_T**	**pH_T**	**pCO_2**	**C_T**	**Ω_A**	**Ω_C**	**T**	**S**	**A_T**	**pH_T**	**pCO_2**	**C_T**	**Ω_A**	**Ω_C**
1	17 March 2017	15.6	15.2	1443.7	8.4	118.8	1268	2.1	3.5	15.6	15.3	1586.4	8.0	342.4	1504	1.1	1.9
3	20 March 2017	19.0	12.9	1525.3	8.4	131.3	1342	2.3	4.0	18.9	12.7	1543.7	7.4	1824.8	1591	0.3	0.5
11	28 March 2017	23.3	20.5	1835.9	8.1	344.9	1670	2.1	3.4	23.8	20.3	1835.5	7.7	825.7	1771	1.1	1.7
15	1 April 2017	21.2	17.2	1829.2	8.1	337.5	1686	1.9	3.2	21.2	17.4	1905.5	7.7	954.4	1867	0.9	1.5
33	19 April 2017	24.6	21.4	1801.6	8.1	315.7	1617	2.3	3.7	24.5	22.3	2069.9	7.7	1010.7	2001	1.2	1.9
42	28 April 2017	26.0	16.9	1692.7	8.0	409.5	1568	1.7	2.8	26.1	16.9	1751.9	7.2	2910.4	1813	0.3	0.6
45	5 May 2017	25.2	17.2	1642.0	8.0	426.6	1530	1.5	2.5	25.5	17.3	1672.0	7.7	839.8	1623	0.9	1.5
Mean		22.1	17.3	1681.5	8.2	297.8	1526	2.0	3.3	22.2	17.5	1766.4	7.6	1244.0	1739	0.8	1.4
SD		3.8	2.9	154.2	0.2	124.5	162	0.3	0.5	3.9	3.1	185.9	0.3	856.7	174	0.4	0.6

Morphology and biomass

Morphology of *H. wrightii* and *R. maritima* did not differ among treatments before the pH manipulation (Table 3). After five weeks of pH manipulation, shoot and leaf development of *H. wrightii* and *R. maritima* did not appear to be affected by elevated $p\mathrm{CO}_2$ and plants also did not differ in morphology when grown in homo- or hetero-specific beds (Table 4, Fig. 3 and 4).

Table 3. Summary of two-way ANOVA results obtained in the initial measurements of morphology and biomass, photo-physiology, and metabolic parameters for *H. wrightii* and *R. maritima* in homospecific and heterospecificaquaria (n=5). Degrees freedoms were 1 for all analyses. No significant differences were found at a statistical α < 0.01.

Response variable	Species	Factors	F-stat	*p*-value
Morphology and biomass				
Shoot density	*H. wrightii*	*pH*	0.357	0.560
		Bed type	0.654	0.432
		pH x Bed type	0.226	0.642
	R. maritima	*pH*	0.005	0.946
		Bed type	0.003	0.960
		pH x Bed type	1.282	0.277
Number of leaves per shoot	*H. wrightii*	*pH*	8.401	0.012
		Bed type	0.535	0.477
		pH x Bed type	0.245	0.628
	R. maritima	*pH*	4.826	0.045
		Bed type	0.180	0.678
		pH x Bed type	0.356	0.560
	H. wrightii	*pH*	5.643	0.032

Total leaf material		*Bed type*	0.892	0.361
		pH x Bed type	0.014	0.908
	R. maritima	*pH*	0.011	0.916
		Bed type	0.270	0.611
		pH x Bed type	0.096	0.761
	H. wrightii	*pH*	0.500	0.491
Shoot height		*Bed type*	0.568	0.464
		pH x Bed type	0.907	0.357
	R. maritima	*pH*	3.405	0.086
		Bed type	0.669	0.427
		pH x Bed type	0.064	0.804
Photo-physiology				
	H. wrightii	*pH*	0.162	0.694
α		*Bed type*	3.078	0.103
		pH x Bed type	0.088	0.772
	R. maritima	*pH*	0.388	0.545
		Bed type	0.129	0.726
		pH x Bed type	0.388	0.545
	H. wrightii	*pH*	0.179	0.679
β		*Bed type*	0.254	0.622
		pH x Bed type	0.410	0.533
	R. maritima	*pH*	0.057	0.816
		Bed type	0.038	0.848
		pH x Bed type	0.766	0.399
	H. wrightii	*pH*	0.010	0.923
$r\mathrm{ETR}_{\mathrm{max}}$		*Bed type*	3.175	0.098
		pH x Bed type	0.238	0.634
	R. maritima	*pH*	0.844	0.376

		Bed type	0.302	0.593
		pH x Bed type	1.834	0.201
E_K	*H. wrightii*	*pH*	0.091	0.767
		Bed type	3.507	0.838
		pH x Bed type	0.388	0.544
	R. maritima	*pH*	0.120	0.735
		Bed type	0.058	0.813
		pH x Bed type	0.377	0.551
Dark-adapted yield	*H. wrightii*	*pH*	2.568	0.129
		Bed type	2.893	0.108
		pH x Bed type	0.003	0.955
	R. maritima	*pH*	0.021	0.888
		Bed type	1.505	0.238
		pH x Bed type	0.736	0.403
Light-adapted yield	*H. wrightii*	*pH*	1.622	0.224
		Bed type	2.672	0.124
		pH x Bed type	1.987	0.180
	R. maritima	*pH*	0.227	0.642
		Bed type	0.058	0.813
		pH x Bed type	0.025	0.876
Metabolism				
GCP	*H. wrightii*	*pH*	0.007	0.936
		Bed type	5.827	0.031
		pH x Bed type	1.282	0.278
	R. maritima	*pH*	0.013	0.931
	(squared root transformed)	*Bed type*	2.836	0.104
		pH x Bed type	1.629	0.224

NCP	*H. wrightii*	*pH*	0.443	0.517
		Bed type	1.337	0.268
		pH x Bed type	0.360	0.559
	R. maritima	*pH*	1.213	0.291
		Bed type	0.124	0.730
		pH x Bed type	2.703	0.124
Respiration	*H. wrightii*	*pH*	0.319	0.582
		Bed type	2.285	0.155
		pH x Bed type	0.691	0.421
	R. maritima	*pH*	3.693	0.077
		Bed type	5.613	0.034
		pH x Bed type	0.007	0.934

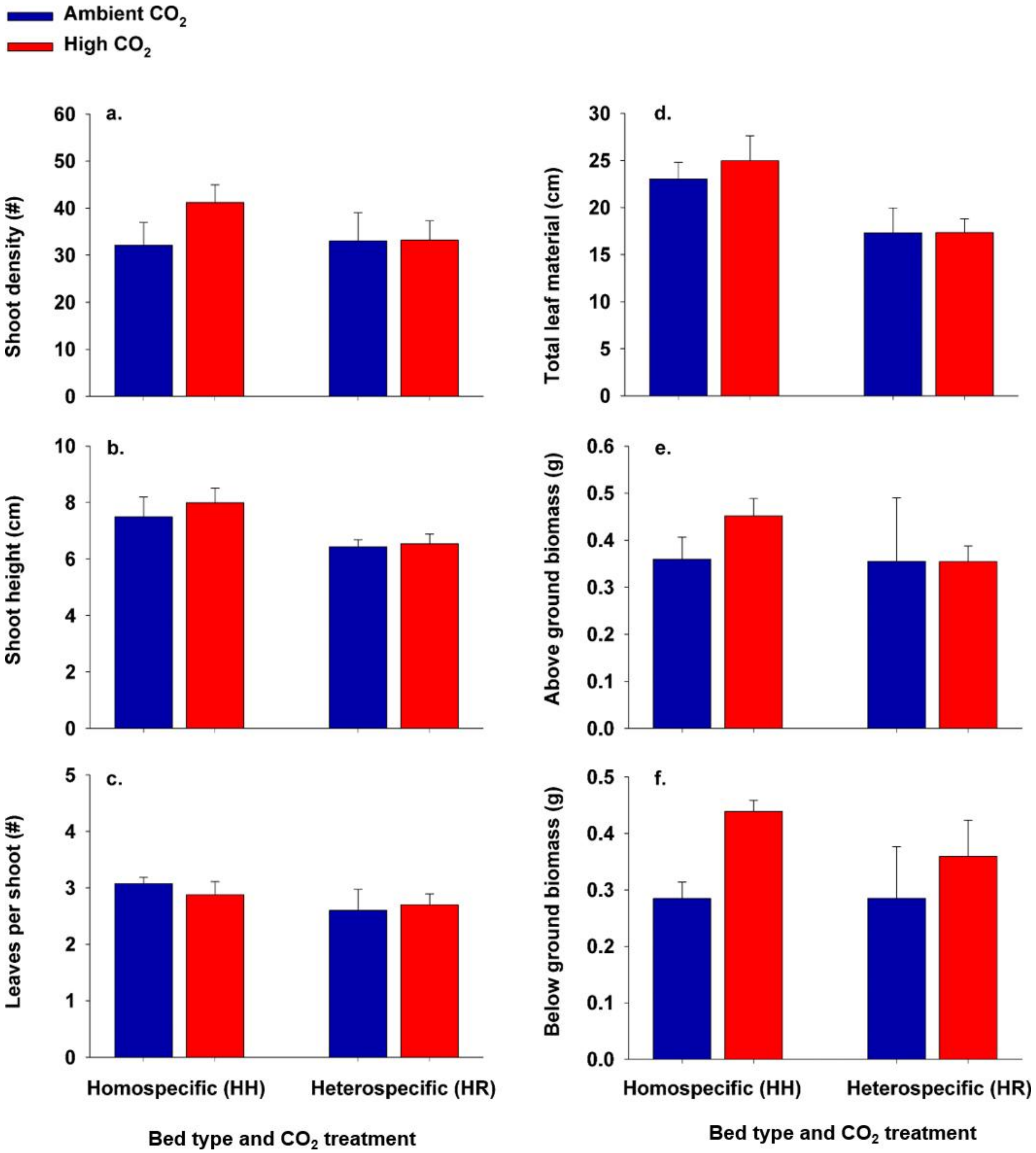


Fig. 3. *H. wrightii* (mean±SE) morphology (shoot density, a; shoot height, b; leaves per shoot, c; total leaf material, d) and above- and below ground biomass (e, f) after maintained for 34, 41, or 54 days at ambient (blue) and high CO_2 (red) treatments. *Halodule wrightii* was grown in homospecific (*H. wrightii* with *H. wrightii*, HH) and heterospecific (*H. wrightii* with *R. maritima*, HR) beds. No significant differences were found at a statistical $\alpha < 0.01$ and at an $\alpha = 0.005$ for two instances (noted with * in Table 1) where data did not meet parametric assumptions.

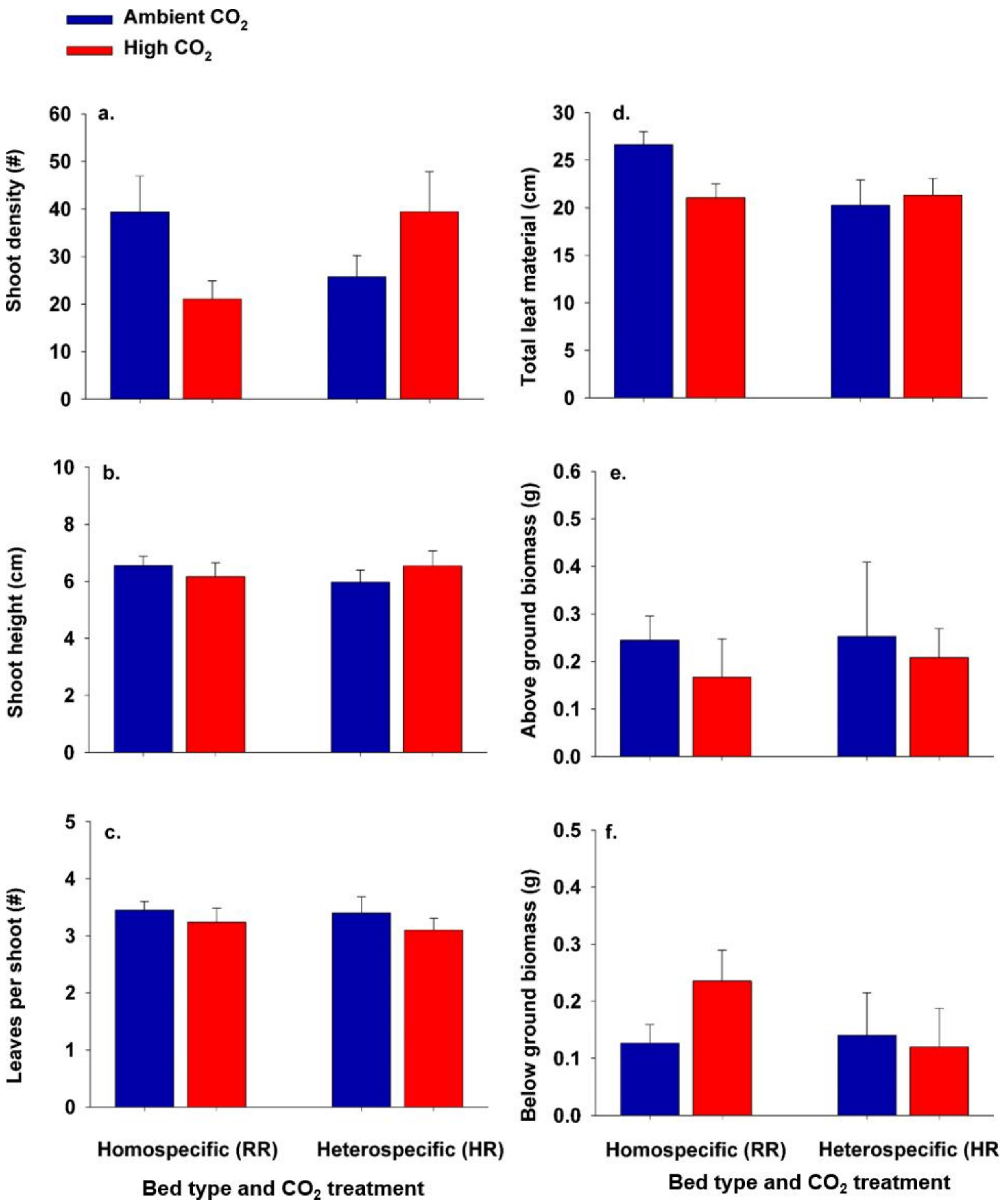


Fig. 4. *R. maritima* (mean±SE) morphology (shoot density, a; shoot height, b; leaves per shoot, c; total leaf material, d) and above- and below ground biomass (e, f) after maintained for 34, 41, or 54 days at ambient (blue) and high CO_2 (red) treatments. *Ruppia maritima* was grown in homospecific (*R. maritima* with *R. maritima*, RR) and heterospecific (*R. maritima* with *H. wrightii*, HR) beds. No significant differences were found at a statistical $\alpha < 0.01$ and at an $\alpha=0.005$ for two instances (noted with * in Table 1) where data did not meet parametric assumptions.

Table 4. Summary of two-way ANOVA final results testing for the effects of ambient and elevated pCO_2 on the morphology, photo-physiology, and metabolism of *H. wrightii* and *R. maritima* in homospecific and heterospecific aquaria (n= 4 to 5). Degrees freedoms were 1 for all analyses. No significant differences were found at a statistical $\alpha < 0.01$, and at an $\alpha = 0.005$ for four instances (noted with *) where data did not meet parametric assumptions.

Response variable	**Species**	**Factors**	**F-stat**	***p*-value**
Morphology and biomass				
Shoot density	*H. wrightii*	*pH*	0.997	0.335
		Bed type	0.589	0.456
		pH x Bed type	0.913	0.355
	R. maritima	*pH*	0.126	0.728
		Bed type	0.129	0.725
		pH x Bed type	6.005	0.028
Number of leaves per shoot	*H. wrightii*	*pH*	0.037	0.850
		Bed type	1.771	0.206
		pH x Bed type	0.359	0.559
	R. maritima	*pH*	1.213	0.291
		Bed type	0.153	0.702
		pH x Bed type	0.032	0.862
Total leaf material	*H. wrightii*	*pH*	0.184	0.675
		Bed type	8.418	0.012
		pH x Bed type	0.173	0.685
	R. maritima	*pH*	1.466	0.248
		Bed type	2.656	0.127
		pH x Bed type	3.163	0.099
Average shoot height	*H. wrightii*	*pH*	0.380	0.548
		Bed type	6.349	0.026
		pH x Bed type	0.154	0.701

	R. maritima	*pH*	0.392	0.542
		Bed type	1.146	0.304
		pH x Bed type	3.689	0.077
Above-ground biomass	*H. wrightii*	*pH*	0.445	0.516
		Bed type	0.546	0.472
		pH x Bed type	0.456	0.511
	R. maritima	*pH*	0.440	0.518
	(Ln(x) transformed)	*Bed type*	0.006	0.937
		pH x Bed type	1.086	0.315
Below-ground biomass	*H. wrightii**	*pH*	4.065	0.063
		Bed type	0.483	0.498
		pH x Bed type	0.496	0.493
	R. maritima	*pH*	0.001	0.976
	(Ln(x) transformed)	*Bed type*	0.544	0.473
		pH x Bed type	0.815	0.382
Photo-physiology				
Chlorophyll *a*	*H. wrightii*	*pH*	0.226	0.642
		Bed type	0.558	0.467
		pH x Bed type	0.186	0.673
	R. maritima	*pH*	0.379	0.548
		Bed type	0.776	0.393
		pH x Bed type	0.493	0.494
α	*H. wrightii*	*pH*	0.135	0.721
		Bed type	2.600	0.135
		pH x Bed type	0.575	0.464

	R. maritima	*pH*	1.942	0.189
		Bed type	0.282	0.605
		pH x Bed type	0.250	0.626
β	*H. wrightii*	*pH*	0.091	0.768
		Bed type	3.666	0.082
		pH x Bed type	0.734	0.410
	R. maritima	*pH*	0.377	0.551
		Bed type	0.173	0.685
		pH x Bed type	0.648	0.436
$r\mathrm{ETR}_{\mathrm{max}}$	*H. wrightii*	*pH*	0.031	0.864
		Bed type	1.478	0.249
		pH x Bed type	0.276	0.610
	R. maritima	*pH*	0.001	0.986
		Bed type	0.068	0.798
		pH x Bed type	0.518	0.485
E_{K}	*H. wrightii*	*pH*	0.026	0.874
		Bed type	3.528	0.087
		pH x Bed type	0.739	0.408
	R. maritima	*pH*	0.425	0.527
		Bed type	0.140	0.714
		pH x Bed type	0.510	0.489
Light-adapted yield	*H. wrightii*	*pH*	0.484	0.498
		Bed type	2.190	0.161
		pH x Bed type	0.073	0.791
	*R. maritima**	*pH*	0.001	0.980
		Bed type	0.143	0.711
		pH x Bed type	0.216	0.649
Dark-adapted yield	*H. wrightii**	*pH*	0.226	0.642
		Bed type	0.558	0.467

		pH x Bed type	0.186	0.673
	*R. maritima**	*pH*	0.379	0.548
		Bed type	0.776	0.393
		pH x Bed type	0.493	0.494
Metabolism				
NCP	*H. wrightii*	*pH*	0.023	0.882
	(Ln(x) transformed)	*Bed type*	3.258	0.093
		pH x Bed type	3.664	0.076
	R. maritima	*pH*	2.980	0.108
		Bed type	0.062	0.808
		pH x Bed type	0.496	0.494
Respiration	*H. wrightii*	*pH*	0.325	0.578
		Bed type	0.264	0.616
		pH x Bed type	3.107	0.100
	R. maritima	*pH*	1.501	0.242
		Bed type	0.823	0.381
		pH x Bed type	0.304	0.591
GCP	*H. wrightii*	*pH*	0.000	0.993
	(Ln(x) transformed)	*Bed type*	0.053	0.821
		pH x Bed type	0.678	0.424
	R. maritima	*pH*	0.541	0.475
		Bed type	0.055	0.818
		pH x Bed type	1.581	0.231

Over the course of the experiment, *Halodule wrightii* plants tended to increase shoot density (27.6±2.0 to 35.1±2.3 shoots per core), produce new leaves (2.4±0.1 to 2.8±0.1 leaves per shoot), and gain total leaf material (13.0±0.7 to 20.9± 1.3 cm). Shoot density of *R. maritima* did not change much over the course of the experiment (from 34.9±3.1 to 31.3±3.6 shoots per core), although plants produced new leaves (from 2.8±0.1 to 3.3±0.1), increased in total leaf material (from 12.4±0.5 to 22.2±1.0 cm) and average shoot height (from 4.6±0.2 to 6.5±0.22 cm).

The above-ground biomass was not significantly affected by $p\mathrm{CO_2}$ and nor by co-occurrence of other seagrass species (Table 4). Above ground biomass was 0.38±0.04 g DW in *H. wrightii* and 0.21± 0.04 g DW in *R. maritima*. The allocation of biomass to below ground also did not differ for seagrasses grown in homo- or hetero-specific beds and for seagrasses at the two pH treatments. The below-ground biomass for *H. wrightii* and *R. maritima* at the end of the experiment was 0.34±0.08 and 0.16±0.07 g DW, respectively (Table 4, Figs. 3 and 4).

Photo-physiology

There were no differences in photo-physiology measures prior to pH manipulation (Table 3). Both species shared similar parameters derived from the rapid light curves: α was 0.30±0.01 for *H. wrightii* and *R. maritima* leaves, β was 102.2±1.0 and 101.0±1.6, $r\mathrm{ETR_{max}}$ was 100.2±2.7 and 102.6±3.2 μmol electrons $\mathrm{m^{-2}\ s^{-1}}$, and the E_k was 332.4±6.4 and 340.7±10.8 μmol photon $\mathrm{m^{-2}s^{-1}}$ for *H. wrightii* and *R. maritima* leaves, respectively. Values for dark- and light- adapted fluorescence yields were similar for both species (0.72±0.01 and 0.59±0.02 for *H. wrightii* and 0.71±0.01 and 0.57±0.02 for *R. maritima*, respectively).

After pH manipulation, the parameters derived from the rapid light curves of *H. wrightii* did not differ between ambient and elevated $p\mathrm{CO}_2$ exposure and did not differ with bed type (Table 4, Fig. 5).

For example, the derived α for *H. wrightii* was 0.29±0.01 and 0.30±0.01 in homo-specific aquaria and 0.32±0.01 and 0.32±0.01 in hetero-specific aquaria after exposure to ambient and elevated $p\mathrm{CO}_2$ conditions, respectively. Furthermore, mean $r\mathrm{ETR}_{\mathrm{max}}$, E_{k}, and β values varied little among bed type and $p\mathrm{CO}_2$ condition for *H. wrightii* (mean±SD: $r\mathrm{ETR}_{\mathrm{max}}$ from 99.1±10.9 to 108.3±21.6 μmol electrons $\mathrm{m}^{-2}\ \mathrm{s}^{-1}$, E_{K} from 308.8±34.5 to 356.4±54.5 μmol photon $\mathrm{m}^{-2}\mathrm{s}^{-1}$, and β from 98.5± 8.4 to 105.9±5.6). There tended to be greater photoinhibition within the ambient, mixed bed treatment compared to the other treatments, however, this trend was not statistically significant at $\alpha=0.05$ or when the statistical α was adjusted to 0.01 for the many comparisons (Table 4).

After the pH manipulation, the parameters derived from the rapid light curves of *R. maritima* also did not differ between ambient and elevated $p\mathrm{CO}_2$ exposure and did not differ with bed type (Table 4, Fig. 5). This result is evident in the curves (Fig. 5) with the similar range of derived values of α, $r\mathrm{ETR}_{\mathrm{max}}$, and E_{k} regardless of growing condition (mean± SD: α from 0.29±0.02 to 0.32±0.02, $r\mathrm{ETR}_{\mathrm{max}}$ from 103.8±23.4 to 111.9±11.1 μmol electrons $\mathrm{m}^{-2}\ \mathrm{s}^{-1}$, E_{k} from 325.2±89.6 to 377.5±84.7 μmol photon $\mathrm{m}^{-2}\mathrm{s}^{-1}$). Similar to observations for *H. wrightii,* there tended to be greater photoinhibition for *R. maritima* plants within the ambient CO_2, mixed bed condition when compared to the other treatments. This trend was not statistically significant at $\alpha<0.05$ nor when the statistical α was adjusted to 0.01 for the many comparisons (Table 1, β ranged from 95.9±11.9 to 103.7±14.0).

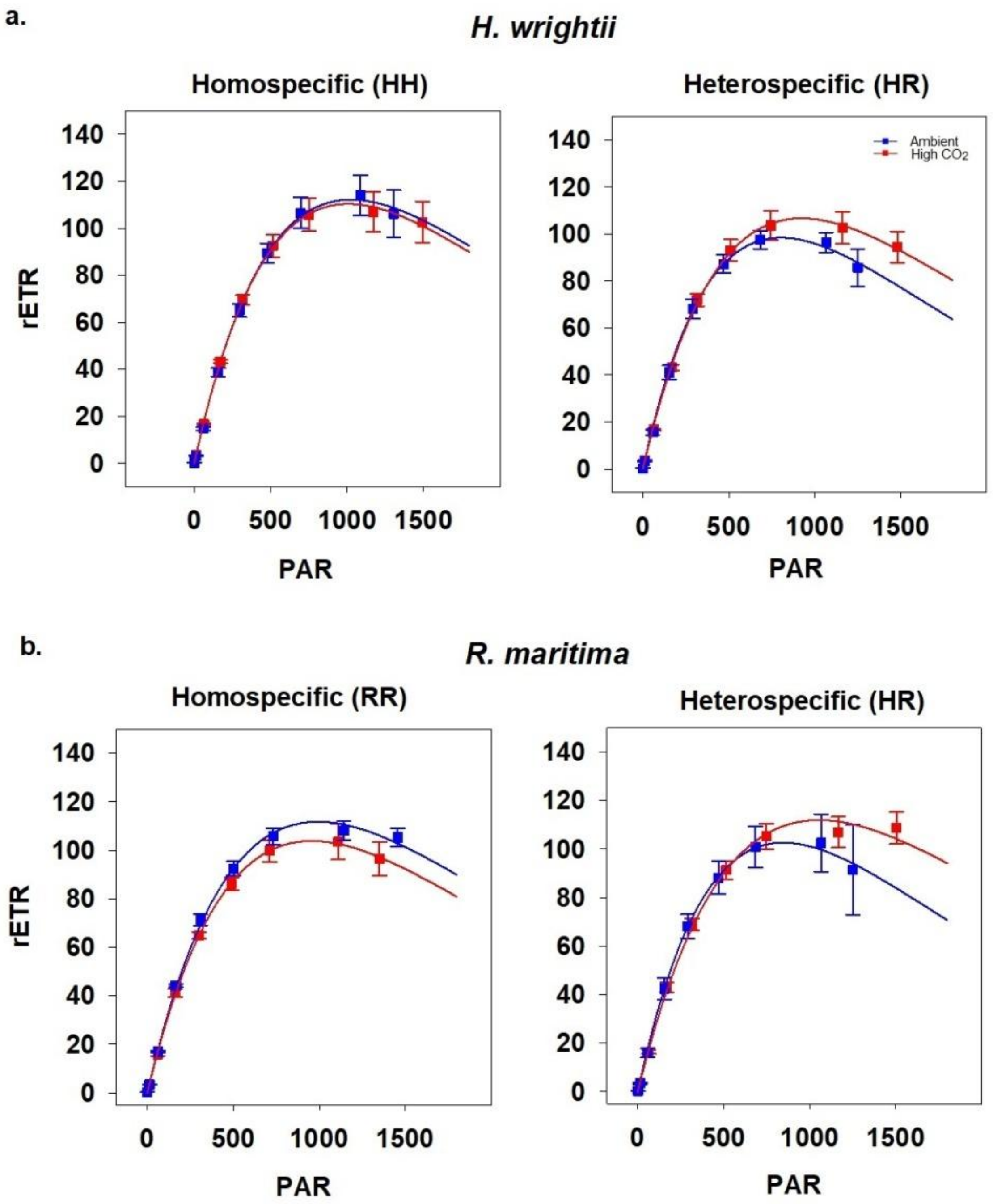


Fig. 5. Rapid light curves from *H. wrightii* (top, a) and *R. maritima*(bottom, b) placed within homospecific (left) and heterospecific (right) beds (*H. wrightii* with *H. wrightii*, HH; *R. maritima* with *R. maritima*, RR and *H. wrightii* with *R. maritima*, HR) after maintained for 43 days under ambient and high CO_2 treatments (continuous modeled lines). Modeled lines and *r*ETR (mean ±SE) values are based upon an average from 4 to 5 aquaria.

For both species, dark- and light-adapted yields at the end of the experiment did not differ with bed type nor $p\mathrm{CO}_2$ condition (Table 4). *H. wrightii* plants yielded 0.74±0.01 after the dark acclimation and 0.70±0.03 in the light. *R. maritima* plants yielded 0.76±0.02 and 0.69±0.02 after dark and light acclimation, respectively.

Leaf Chl *a* content was not affected by $p\mathrm{CO}_2$ nor by seagrass bed type (Table 4). The average of leaf Chl *a* content was 0.011±0.002 and 0.010±0.002 mg · cm^{-2} per leaf for *H. wrightii* and *R. maritima*, respectively.

Metabolism

H. wrightii and *R. maritima* metabolism (NCP, respiration, GCP in units of mg O_2 $\mathrm{m}^2\mathrm{hr}^{-1}$) was similar among bed type and assigned $p\mathrm{CO}_2$ condition during the acclimation period of experimentation (Table 3). In *H. wrightii* beds, the NCP was 0.56±0.10, respiration was -0.60±0.08, and GCP was 1.98±0.09. In *R. maritima* beds the NCP was 0.74±0.09, respiration was -0.56±0.08, and GCP was 1.32±0.12.

NCP, GCP and respiration (in units of mg O_2 m^2 hr^{-1}) did not statistically differ between ambient and elevated $p\mathrm{CO}_2$condition for either species, and rates did not differ when plants were grown in homo- or hetero- specific beds (Table 4, Fig. 6). It was noted that there was a lot of variation in some metabolic measures at the end of the study, particularly for *H. wrightii* beds in hetero-specific aquaria maintained under elevated $p\mathrm{CO}_2$ conditions.

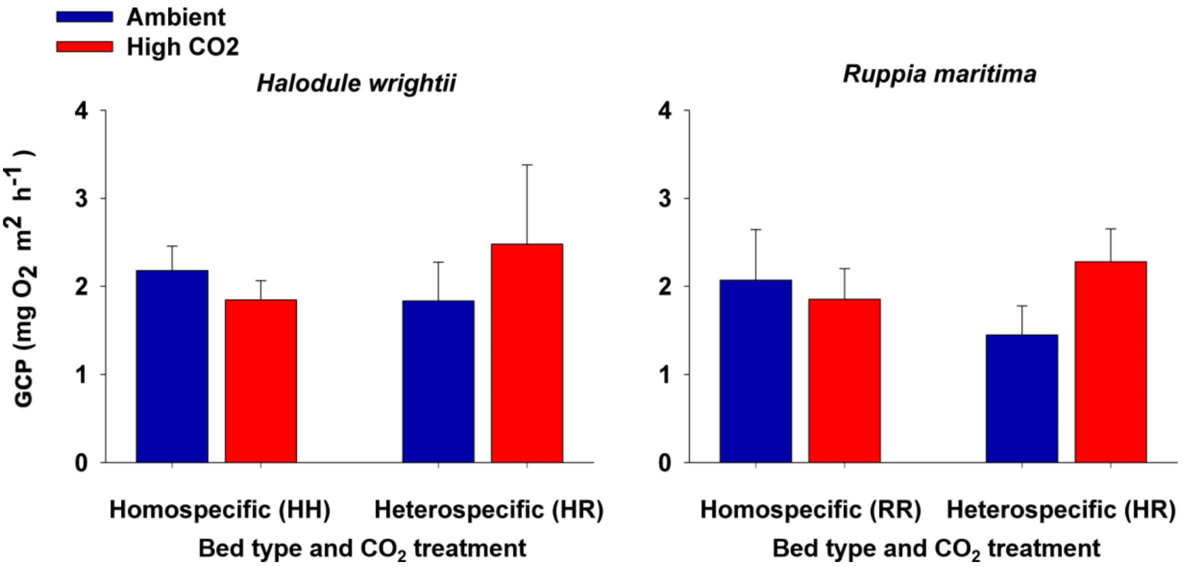


Fig. 6. Gross community productivity (GCP) obtained in *H. wrightii* (left) and *R. maritima* (right) placed within homospecific and heterospecific beds (*H. wrightii* with *H. wrightii*, HH; *R. maritima* with *R. maritima*, RR and *H. wrightii* with *R. maritima*, HR) after maintained for 48 days under ambient (blue) and high CO_2 treatments (red). No significant differences were found at a statistical α=0.01.

Discussion

Results do not support the hypothesis that seagrasses will benefit from ocean acidification in the coming decades. Furthermore, in the time period of 54 days, we did not observe a change in seagrass species composition within the hetero-specific aquaria, indicating little competition between the two species that did not change with elevated pCO_2 conditions.

We hypothesize that lack of CO_2 stimulation for plant growth and metabolism, found in this study, could be related to the mesohaline conditions of the Northern Gulf of Mexico. Environmental factors such as salinity, temperature and irradiance, are important controls in the seagrasses development (Livingston et al. 1998, Bortone and Turpin 2000, Koch 2001). In this study, the beds of *H. wrightii* and *R. maritima* were grown under highly variable environmental conditions, which are typical of these mesohaline estuarine habitats (Anton et al. 2009). During the second month of the experiment, heavy rainfall caused the average salinity in the Bay to decrease from 17 to 7 psu, reaching a minimum of 3.8 (see Fig. 1) and increased water turbidity. While both species are eury- to mixo-haline, low salinity water outside their preferred range can slow productivity and seawater below 6 can be lethal (Adair et al. 1994, Doering et al. 2002). Therefore, the environmental changes in salinity and turbidity during our experiment could be limiting factors for the productivity and development of *H. wrightii* and *R. maritima*, acting antagonistically to the positive effects of ocean acidification. Indeed, there are a growing number of studies on ocean acidification effects on seagrasses which show elevated $p\mathrm{CO}_2$ to have limited effect on productivity and biomass when tested in natural settings, or effects to be unable to counteract negative impacts of other stressors, such as warming or limiting light (Apostolaki et al. 2014, Cox et al. 2016, Pajusalu et al. 2016, Hendriks et al. 2017, Repolho et al. 2017).

The organisms living in estuarine systems encounter complexities in the seawater chemistry that can influence their resilience and adaptation to environmental changes in a different way than in other aquatic ecosystems (Hofmann et al. 2011, Arnold et al. 2017). Thus, it is not surprising that despite any stress or fluctuations in salinity, light, or sedimentation, both seagrass species increased their above-ground biomass during the course of study. The lack of a clear elevated $p\mathrm{CO}_2$ benefit for both species could be additionally related to the fact that they efficiently use HCO_3^- and exhibit less reliance on CO_2 than other seagrass species, such as *Thalassia testudinum* (Campbell and Fourqurean 2013). Although the internal inorganic carbon concentrations of *R. maritima* can be close to saturation under natural conditions, this saturation was observed when dissolved inorganic carbon to oxygen ratio was low from ecosystem productivity and as a consequence photorespiration occurred (Buapet et al. 2013, Koch et al. 2013). In this study, both seagrasses have a C3 fixation pathway and adding CO_2 to the seawater should have increased the ratio and reduced photorespiration. It is also noted in the estuarine waters of the Chesapeake Bay that elevated pCO_2 stimulated eelgrass production to eliminate the lethal effects of elevated temperature (Zimmerman et al. 2017). The contrast of results between studies could be explained by differences in estuarine conditions or perhaps from species differences. Northern Central Gulf of Mexico has six rivers that drain into it, thus it could be less suited for seagrass growth. For instance, in Zimmerman et al. (2017), eelgrass experienced a mean (± SD) salinity of 24±3 with punctuated drops in salinity. Seagrass in the present study experienced mean salinity of 16±6 SD with low salinity events persisting for several days. On the other hand, eelgrass is perennial with shoot heights at times reaching over a meter, thus it is likely to have greater carbon demands and greater storage in the roots and rhizomes.

Many of the organisms that colonize estuarine systems have transient life-history strategies, showing colonization strategies more ephemeral and opportunistic than the species which inhabit in more stable environments (Kilminster et al. 2015). This type of dynamic development implies that the responses to perturbations can depend on time of day, season or year, as a function of the environmental changes. For instance, in estuaries in Texas (USA), responses of submerged aquatic vegetation were specific to the moment, corresponding with the interaction of the salinity, temperature, water levels and water clarity that occurred in the water column (Adair et al. 1994). The present study was conducted over 70 (54 acidified) days, a relatively short-duration for seagrass. This duration was selected based on the annual growth of *R. maritima* in these beds and the seasonal co-occurrence with *H. wrightii*. The differing ecological strategies and physiological preferences of these two species, combined with yearly fluctuations in climatic events (which will subsequently be altered by climate change), could lead to different annual outcomes as environmental stressors and preferences vary.

The present study was conducted during time of year when *R. maritima* is reaching a peak biomass followed by flowering and senescence (Pulich 1985, Flores-Verdugo et al. 1988, Cho and Poirrier 2005). Whereas, the biomass of *H. wrightii* peaks later in summer months and the seagrass proliferates in environments with high light intensity (Stockwell 1993). These seasonal changes were noted in this study. Both species increased in above ground biomass, the shoot density of *H. wrightii* increased while *R. maritima* did not, and at the end of the present study, *R. maritima* became more vertical and branching as it began to form flowers. Initiation of flowering by *R. maritima* and early flower stages were noted in homospecific and heterospecific bedsunder ambient and high CO_2 conditions but, unlike effects reported for *Zostera marina* (Palacios and Zimmerman 2007), the onset of flowering was not more frequent at either $p\mathrm{CO}_2$ condition.

If a biological benefit from the high CO_2 condition did occur (and was not easily measured with a “snapshot” sampling), this could favor growth in the long-term. For instance, at a CO_2 vent in Italy only the seagrass species with high investment in below ground biomass had greater abundance nearest to the vent source (Russell et al. 2013). *Halodule wrightii* favors a greater allocation to the below ground biomass than *R. maritima* (Pulich 1985). In this study, we did not see any statistically significant differences in biomass allocation. However, we did not measure carbon storage in the rhizome or roots. These long-term hypotheses warrant further investigation.

Seagrasses provide important ecological functions in the estuarine systems. They slow water flow, add to nutrient and carbon cycling, and provide a shelter for many larvae and juvenile fish and shellfish (Heck et al. 2003, Larkum et al. 2006, Orth et al. 2006, Waycott et al. 2009). Seagrasses are essential component of many coastal ecosystems, but their populations have been lost due to localized anthropogenic perturbations and global climate change (Orth et al. 2006, Waycott et al. 2009), including in estuarine waters (Orth et al. 2010, Moore et al. 2012). Thus, as climate change continues, it is necessary to understand the influences of environmental variability on the ecology and physiology of seagrasses to determine which ecosystems are most susceptible to change. The outcome of this study, in context with literature, leads to the speculation that ocean acidification will shape seagrass ecosystems more strongly in optimal, stable light and saline environments with longer-lived seagrass species with greater carbon demands. Further experimentation is needed to refute or support this hypothesis.

Nevertheless, this study shows that, in this estuarine environment, short-term increased carbon dioxide availability had no clear benefit for seagrass production, which suggests that future changes in carbonate chemistry will not stimulate the vegetative growth of *H. wrightii* and *R. maritima* to alter seagrass composition. The absence of positive effects may be related to the variable environmental conditions and, albeit not measured by this study, the efficiency of these seagrasses to use $HCO3^{-}$ and their lower carbon demands. However, long-term studies should be undertaken to clearly understand this highly dynamic system. This research highlights the need to incorporate other environmental factors to clearly understand highly dynamic systems and how inherent heterogeneity will influence ocean acidification effects on seagrass persistence as dominant producers in shallow coastal ecosystems.

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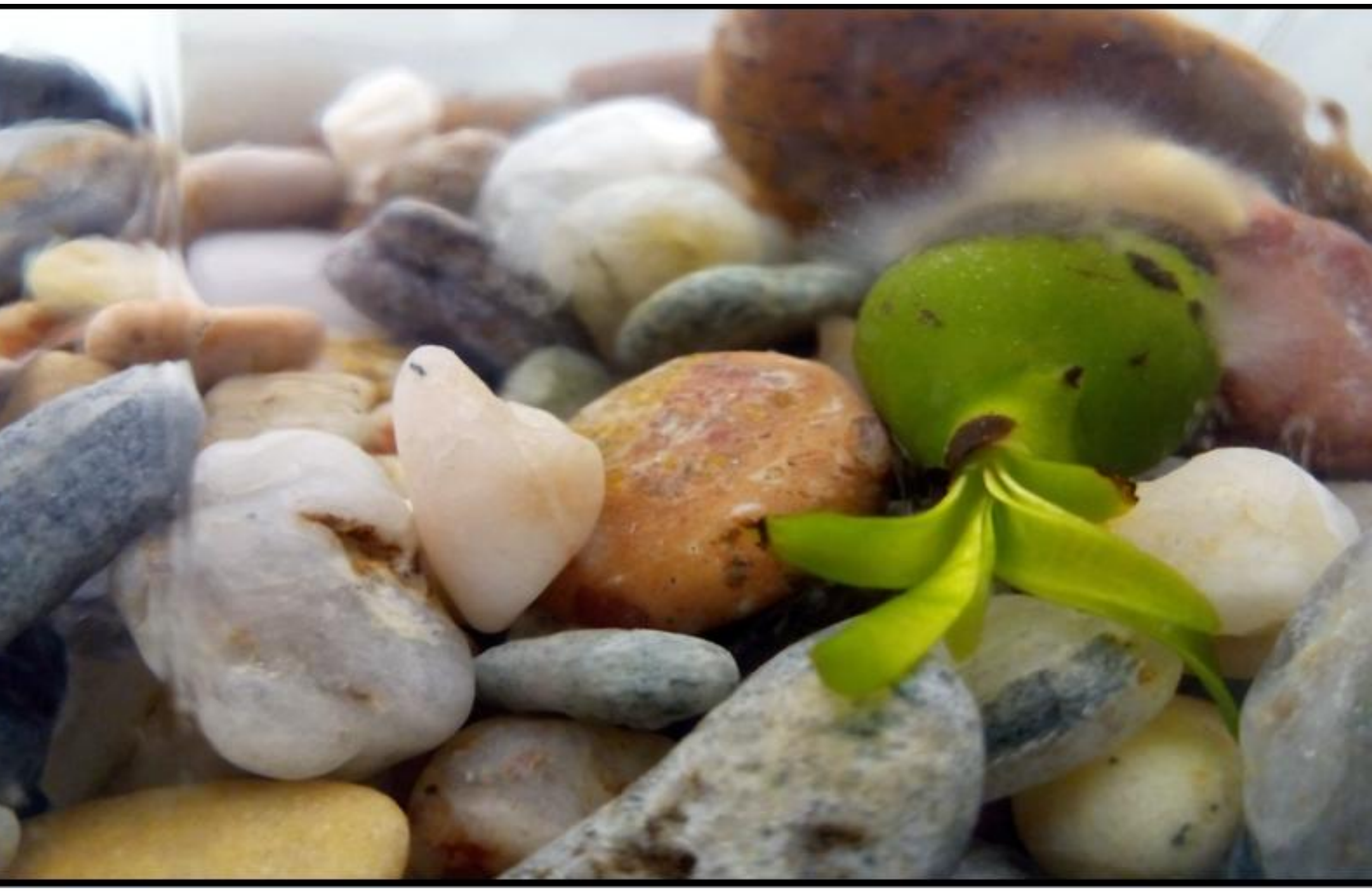
3

General Discussion



The information of this thesis bridges part of the gaps in knowledge on the ecophysiology and sexual reproduction of seagrasses. This thesis describes the sexual recruitment of the seagrasses that form non dormant seeds and the responses of this important habitat-forming species to natural and anthropogenic drivers, including heat waves and ocean acidification. This knowledge is essential for the management and conservation of seagrass meadows in an attempt to increase their resilience against present and future environmental pressures.

The vulnerability that the seedlings of *P. oceanica* show in this thesis implies that the conservation and management of this seagrass species need to exhaustively control its sexual recruitment. Laboratory studies about seagrass physiology in the earliest development stages, such as the information compiled in this thesis, compensate the scarce information available in this area given the difficulties of monitoring the sexual reproduction of *P. oceanica* in the Mediterranean. These studies can help us to identify the factors that benefit or limit the species' recruitment success. The localization, protection and conservation of the areas with the light availability and substrata conditions for successful seedling anchorage are necessary to maintain a good status for seagrass meadows. These findings, together with the use of effective methods to identify adequate recruitment areas of seagrass species, such us recruitment simulation models (Statton et al. 2017), can help us to detect the possible bottlenecks of seedling development in the future. This would allow us to improve the species' genetic variability and, therefore, its conservation and resilience against future environmental perturbations (Orth et al. 2006).

This thesis demonstrates the high plasticity that the root system of *P. oceanica* shows, which can help us to improve the environmental restoration projects of seagrass meadows.

Seagrass restoration projects normally use expensive transplantation methods with low recovery rates (Paling et al. 2009, van Katwijk et al. 2016, Statton et al. 2017). This is due mainly to problems in transplant uprooting and to poor knowledge of the physical conditions and possible perturbations in restoration areas (Statton et al. 2012, van Katwijk et al. 2016). In the last few decades, the interest shown in environmental restoration projects using laboratory-cultivated seedlings to mitigate the decline in seagrasses of the genus *Posidonia* has increased (Balestri et al. 1998, Terrados et al. 2013, Statton et al. 2013). In the seagrasses species where seed availability is high, this restoration type is less expensive than using adult shoots as transplants (Statton et al. 2017), but this method entails seedling anchorage problems (Meinesz et al. 1993, Balestri and Bertini 2003). Research studies about seagrass seedling development requirements are scarce in the literature, which implies poor knowledge about the bottlenecks of these species during settlement, which thus limits their recovery in restoration projects. In comparison, terrestrial restoration projects have advanced seed dispersal simulation models that identify seedling limitations with improved recovery rates in restoration projects (James et al. 2011, Dalgleish et al. 2011). Recent research into the restoration on *P. australis* seeds has demonstrated that this methodology type can be successfully applied to restore seagrasses (Statton et al. 2017). Thus future environmental restoration projects of *P. oceanica* could integrate the use of simulation models to identify adequate colonization areas and to lower failure rates during the recovery of transplants. Increasing knowledge about the substrate preferences of seagrasses, such as those cautiously reflected in this thesis, could help us to localize bottlenecks in seedling development. The use of adequate substrata for seedlings germination and the selection of the transplantation areas that respect the seagrass species' recruitment preferences could solve anchorage and uprooting problems and, thus, the survival of transplants. To detect any problems during sexual recruitment in combination with applying dispersal simulation models in seagrasses could improve the survival of transplants in future environmental restoration seagrass projects, which would enhance the conservation of these threatened ecosystems.

Activities in coastal zones, such as constructing and maintaining infrastructures, fishing or tourism, promote the burial and overgrazing of seagrass meadows, among others (Peterson et al. 2002, Valentine and Duffy 2006, Cabaço et al. 2008, Manzanera et al. 2011, Unsworth et al. 2018). The overgrazing simulation performed in this thesis had negative effects on the photosynthesis and development of *P. oceanica* seedlings. In addition, despite the partial burial of seedlings not showing negative effects during the first development months in this thesis, the burial of meadows above 5 m can have deleterious effects on the development of this seagrass (Gera et al. 2014). Thus the management of the activities performed on coasts that promote sediment mobilization, such as dredging, the construction of docks and ports, should control and prevent massive sedimentation over *P. oceanica* meadows. In addition, the overfishing of marine predators must be limited, among others, by increasing the control of illegal fishing and enforcing fishing activity regulations. This can strike a balance in the food chain to avoid the overgrowth of herbivores in marine ecosystems.

As discussed throughout this thesis, most ecological systems are exposed to multiple stressors and their combination can have synergic or antagonistic effects on organisms (Crain et al. 2008, Darling and Côté 2008). For example, the combination of the effects of extreme temperature events, along with other environmental perturbations, led to a much more marked negative effect on *P. oceanica* seedlings (synergy) than when they acted separately. However, a drop in salinity and turbidity after heavy storms caused antagonistic effects, like those that occur by ocean acidification in the estuarine coast seagrasses of the Gulf of Mexico, *H. wrightii* and *R. maritima*. The fact that these factors produce antagonistic or synergistic responses is closely related with the differences of action that both factors have on the physiology of seagrasses. Global warming is considered the severest climate change threat for seagrasses (Diaz-Almela et al. 2007, Moore et al. 2012, Repolho et al. 2017) because, in macrophytes, an increment in temperature above the tolerance threshold can alter the photosynthetic performance in molecular terms, and can damage photosynthetic tissues (Berry and Bjorkman 1980, Bulthuis 1987, Ralph et al. 1998). However, ocean acidification is likely to produce benefits for the growth, photosynthesis and reproduction of seagrasses (Zimmerman et al. 1997, Invers et al. 2002, Palacios and Zimmerman 2007, Martínez-Crego et al. 2014) because incremented carbon availability can have positive effects on seagrasses' productivity as they are often carbon-limited under current ocean-dissolved carbon conditions (Koch et al. 2013). Seagrasses have restricted tolerances to salinity, temperature and light availability. Thus alterations to these environmental conditions outside the species' vulnerability range can have negative effects on the photosynthesis and growth of plants. In the experiments performed in this thesis, these negative effects are added to those produced by heat waves, but limit the positive effects caused by an increase in CO_2 under ocean acidification simulation conditions. These results highlight the need to incorporate other environmental factors into the study of seagrasses, especially in high dynamic ecosystems such as estuaries, to clearly understand how the combination of environmental perturbations will influence global warming and ocean acidification effects. As global warming and ocean acidification will occur simultaneously in the next decades, future research into these species is required to know the effects of combining both factors of change in combination with other environmental perturbations. As demonstrated in this thesis, the combination of several factors in seagrasses can alter their photosynthesis and development differently to what is expected. Thus the control and consideration of the local factors that currently affect seagrass meadows are fundamental to acquire adequate knowledge about the effects of climate change on these important marine ecosystems.

The results obtained in this thesis suggest that as the effects of climate change can limit seagrass survival, this will imply major negative consequences in these marine ecosystems. On the one hand, loss of *P. oceanica* meadows in the Mediterranean can imply that other seagrass species, such as *Cymodocea nodosa* Asch. and *Halophila stipulacea* (Forssk.) Asch., could take advantage of this decline and would change the current species composition (Pergent et al. 2014). A decline in *P. oceanica* could also cause the loss of important ecological services, such as seawater cleaning and the maintenance of coastal geomorphology, with negative effects for coasts. Other dramatic consequences that can cause *P. oceanica* meadows to decline include the release of the vast long-term carbon stock that has accumulated between their rhizomes over millennia (Fourqurean et al. 2012). The return of the carbon stored in the environment comes in a form of CO_2, which can cause a negative feedback to accelerate climate change and ocean acidification.

On the other hand, despite ocean acidification not being able to alter the seagrass composition of *H. wrightii* and *R. maritima* in the Gulf of Mexico, other factors like global warming or nutrient enrichment can negatively affect the meadows of these species. High temperatures can also restrict seagrass growth and the distribution of *H. wrightii* and *R. maritima* (Onuf et al. 2003). Notwithstanding, the main threat of climate change to estuarine systems seems the exacerbation of current stressors, especially those which increase eutrophication (Howarth et al 1996, Vitousek et al. 1997). Changes in freshwater runoff, temperature, sea level and ocean exchanges will increase eutrophication in estuaries by decreasing filter feeders, altering phytoplankton abundance and reducing water clarity (Scavia et al. 2002). In the last few decades, climate change has brought about changes in precipitation rates by increasing nitrogen delivery in the Gulf of Mexico. This causes eutrophization and a massive hypoxia area on the coasts of Louisiana (Rabalais et al. 2002). Eutrophization can have several negative effects on seagrasses, especially due to factors like reduced light availability and hypoxia (Cambridge et al. 1986, Short et al. 1995).

The wide range of ecological services and biodiversity provided by seagrass meadows make them priority conservation habitats in many coasts and estuaries worldwide (Hemminga and Duarte 2000). For example, *P. oceanica* meadows are included in the “The European Union’s Habitat Directive” (Annex I, Dir 92/43/ CEE) and *P. oceanica* has been selected as a species responsible for the quality of the marine ecosystems in the Marine Strategy Framework Directive (MFSD) (Annex I, Dir 2008/56/EC). Thus management efforts should focus on maintaining seagrass meadows so that the ecological status of marine ecosystems is good. The control of the anthropogenic drivers that can cause a decline in seagrasses is fundamental to maintain their meadows in healthy conditions, especially in areas where considerable human activities take place. In order to increase research into the influences of natural and anthropogenic drivers on the ecology, physiology and sexual reproduction of seagrasses, such as the information compiled in this thesis, determining which ecosystems are most susceptible to change is absolutely necessary. These approaches will allow us to take measures to manage and conserve seagrasses to prevent their meadows being lost in the future and to, therefore, improve the quality and biodiversity of marine ecosystems.

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4 General Conclusions

Chapter 1 – Understanding the sexual recruitment of *Posidonia oceanica*

1) The sexual reproduction of *P. oceanica* can be classified into three essential stages: fruit dispersion, seed adhesion and seedling anchorage.

2) In the fruit dispersion stage, the seed performs photosynthesis while being transported in the interior of fruits on the sea surface, which maintains the pericarp oxygenated.

3) In the seed adhesion stage, the seed develops microscopic adhesive hairs that will cover the primary and secondary roots and increase its range of light tolerance for photosynthesis, which favors adhesion to the substrate and seed development, respectively.

4) In the last seedling anchorage stage, roots attach the seedling to the substrate, and orientate it towards the direction of light to maximize photosynthesis and, thus, improve seedling development and establishment.

5) The adaptations observed in the sexual recruitment of *P. oceanica* are similar to other strategies developed in seagrasses with non dormant seed and fruit with a membranous pericarp, such as *Thalassia* sp. and *Enhalus* sp. This suggests a convergent evolution of this type of seagrasses as regards sexual recruitment.

Chapter 2 – Influence of substrata hardness and seed burial on the development of *Posidonia oceanica* seedlings

6) The culture of *P. oceanica* seedlings on sand promotes root system development, which compensates by slowing down leaf development, while hard substrata have the opposite effect.

7) The initial substrate has a legacy effect on the subsequent development of seedling transplants. The leaves of the seedlings that germinate on sand and glass slides are approximately the same length, but the roots from the seedlings that germinate on glass do not fully develop.

8) Shallow seed burial (0.5–1.5 cm) does not affect the development of the 5-month-old seedlings.

9) The culture of *P. oceanica* on sand prior to transplantation could enhance seedling survival in environmental restoration programs.

Chapter 3 – Responses of *Posidonia oceanica* seedlings to extreme temperature events

10) Heat waves of above 27 ºC limit the growth of the *P. oceanica* seedlings by inhibiting their photosynthetic system and limiting leaf growth. This causes a higher leaf senescence rate and, in some cases, even plant mortality.

11) The greater occurrence of extreme events, such as heat waves, due to future climate change could compromise the colonization ability and the genetic diversity of *P. oceanica* meadows.

Chapter 4 – The influence of extreme temperature events in *Posidonia oceanica* seedlings in combination with other environmental stressors

12) Heat waves can have major deleterious effects on the seagrass *P. oceanica* than other environmental stressors like grazing and burial.

13) Overgrazing seems to have a larger magnitude effect than seed burial and markedly lowers net leaf growth.

14) The combination of severe stressors produces synergic negative effects on *P. oceanica*: the net growth of *P. oceanica* leaves is much more impaired when sediment burial co-occurs with overgrazing.

Chapter 5 – Effects of ocean acidification on homospecific and heterospecific beds of *Halodule wrightii* and *Ruppia maritima*

15) In the estuarine systems of the Gulf of Mexico, increased CO_2 levels over several weeks may not stimulate the vegetative growth and community structure of the homospecific and heterospecific seagrass meadows of *H. wrightii* and *R. maritima*.

16) The conditions frequently encountered in mesohaline estuaries, such as oscillating salinity and reduced light availability, may produce antagonistic effects compared to those that occur by ocean acidification on seagrass development, which would limit the beneficial impacts of increased CO_2 availability**.**