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Seagrass ecology: environmental conditions and processes that affect the establishment and fate of seedlings

Laura Pereda Briones

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Seagrass ecology: environmental conditions and processes that affect the establishment and fate of seedlings

Dissertation submitted by

Laura Pereda Briones

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Supervisors:

Fiona Tomàs Nash & Jorge Terrados Muñoz

Tutor:

Guillem Mateu Vicens

“Lo que sabemos es una gota de agua,
lo que ignoramos es el océano”
(Isaac Newton)

Posidonia oceanica seedling
(Bendinat, March 2015)
By Laura Pereda Briones

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Contents

ABSTRACT

Recruitment of new individuals into a reproductive plant population is a multistage process, from seed production, propagule dispersal and seed germination, to seedling establishment and further survival. This multistage process is decisive in plant life story, since it will determine the trajectory of plant community assembly. Importantly, the transition between seed germination and seedling establishment and further seedling survivorship represents a "bottleneck" for plant populations, limiting their recruitment success. To successfully overcome this transition, seedlings must surmount a series of biotic and abiotic environmental filters, and simultaneously be capable of resisting the progressively more abundant and frequent anthropogenic threats.

Seagrasses form extensive meadows in coastal waters, carrying out important ecosystem functions that provide essential services to society. Nonetheless, seagrasses are suffering a strong decline worldwide due to multiple anthropogenic stressors. In this context, early life stages are an essential step to guarantee population survivorship since propagules provide an essential genetic diversity source, necessary to adapt to environmental changes, and their dispersal allow seagrasses to colonize new areas. The present thesis examines how some abiotic and biotic factors, including anthropogenic disturbances, influences propagule dispersal and successful seedling establishment.

Throughout this thesis I have determined that seagrass recruitment is considerably affected by the hydrodynamic conditions imposed by waves and currents, which have a key role in determining propagule dispersal, as well as establishment and survivorship of the three species analysed (*Cymodocea nodosa, Posidonia oceanica* and *Zostera marina,*). Additionally, substratum type and complexity affect seedling establishment success, since more complex substrata reduce flow velocity and increase the bottom boundary layer thickness, enhancing seed and seedling retention. Once established, seedlings increase their survival in locations sheltered from waves, where the energy flux is lower. The hydrodynamic energy also varies with depth, and in shallow waters (i.e. 0.5-6 m), higher depths are associated to higher survival. Furthermore, certain biotic factors, such as the macroalgal community present at the settlement location, also affect seedling anchorage to the substrate and subsequent seedling survivorship.

In addition to the above-mentioned factors, threats derived from global change narrow even more this population bottleneck, since they directly influence seedling development and survival. Seawater warming (29ºC) negatively affects the development of *P. oceanica* seedlings and damages their photosynthetic apparatus. When the increase in temperature occurs in terms of marine heatwaves, different populations of the same species (*C. nodosa*) exhibit different responses. Although seedlings from all regions suffer a reduction in development and survival, being the effects much more severe under a marine heatwave of 32ºC than under the one of 29ºC, there are important regional differences in seedling responses. Seedlings from regions with higher thermal variability are more resistant than those seedlings from more stable regions, which highlights the importance of the intraspecific variability, basic for species evolution and distribution. On the other hand, the studied invasive algae (*Caulerpa cylindracea* and *Lophocladia lallemandii*) do not have negative effects on *P. oceanica* seedlings, and thus, additive effects to those caused by a simultaneously increase in temperature were not detected when seedlings where growing under both disturbances.

The present research takes a step forward towards the understanding of those environmental factors and processes that affect the establishment and fate of seedlings, thus contributing to seagrass meadow conservation, ecosystems of a paramount ecological and socio-economic importance.

RESUMEN

El reclutamiento de nuevos individuos a una población de plantas adultas es un proceso crítico que determina la trayectoria de la comunidad. Este proceso consta de varias etapas, desde la dispersión de los propágulos y la germinación de las semillas, hasta el establecimiento de las plántulas y su posterior supervivencia. La transición entre la germinación de las semillas y el establecimiento y posterior supervivencia de las plántulas supone un "cuello de botella" para la población. Para superar con éxito esta transición en su ciclo vital, las plántulas deben atravesar una serie de filtros bióticos y abióticos, y ser capaces de resistir las amenazas de origen antrópico.

Las fanerógamas marinas forman extensas praderas en aguas costeras, desarrollando funciones ecológicas que suministran importantes servicios a la sociedad. Sin embargo, están sufriendo una fuerte regresión a nivel mundial como resultado de muchas perturbaciones antrópicas. En este contexto, los estadios juveniles suponen un eslabón esencial para asegurar la supervivencia de las poblaciones, pues contribuyen a mantener la diversidad genética de la población, fundamental para su adaptación a los cambios, y su dispersión permite la colonización de nuevas áreas. La presente tesis analiza cómo actúan algunos factores abióticos y bióticos, incluyendo perturbaciones humanas, sobre la dispersión de propágulos y el establecimiento exitoso de las plántulas.

En esta tesis he comprobado que el reclutamiento de fanerógamas marinas está considerablemente afectado por las condiciones hidrodinámicas establecidas por olas y corrientes, las cuales tienen un papel clave en determinar la dispersión, establecimiento y supervivencia de los propágulos de *Cymodocea nodosa, Posidonia oceanica* y *Zostera marina.* Asimismo, el tipo de sustrato y su complejidad afectan al éxito del establecimiento, pues sustratos más complejos disminuyen la velocidad de la corriente y aumentan el espesor de la capa límite del fondo, facilitando la retención de semillas y plántulas. Una vez establecidas, las plántulas aumentan su supervivencia en zonas más protegidas de la influencia de las olas, donde el flujo de energía asociado a éstas es menor. La energía hidrodinámica también cambia con la profundidad, y en aguas someras (i.e. 0.5-6 m), mayores profundidades están asociadas a mayor supervivencia. Además, ciertos factores bióticos, como la comunidad de macroalgas presente en el lugar de asentamiento, también influyen en el anclaje al sustrato y la supervivencia de las plántulas.

Asimismo, las amenazas derivadas del “cambio global” estrechan todavía más este cuello de botella poblacional, al influir directamente en el desarrollo y supervivencia de las plántulas. El calentamiento del agua (29ºC) afecta negativamente el desarrollo de plántulas de *P. oceanica* y daña su aparato fotosintético. Cuando este calentamiento se produce en forma de olas de calor, las poblaciones de una misma especie (*C. nodosa*) responden de manera diferente. Si bien todas las regiones ven afectado su desarrollo y supervivencia, siendo los efectos más dañinos en la ola de calor de 32ºC que en la de 29ºC, la respuesta a nivel regional difiere. Aquellas plántulas que proceden de regiones con mayor amplitud térmica son más resistentes a este aumento de temperatura que regiones con un régimen térmico más estable, lo cual resalta la importancia de las variaciones intraespecíficas, básicas en la evolución y distribución de las especies. Por otro lado, las algas invasoras *Caulerpa cylindracea* y *Lophocladia lallemandii* no tienen efectos negativos en *P. oceanica* cuando esta se ve sometida a calentamiento.

La presente investigación da un paso hacia delante en la comprensión de los factores ambientales y los procesos que afectan el asentamiento y destino de las plántulas, contribuyendo así a la conservación de las praderas de fanerógamas marinas, ecosistemas de gran importancia ecológica y socio-económica.

RESUM

El reclutament de nous individus a una població de plantes adultes és un procés crític que determina la trajectòria de la comunitat. Aquest procés es composa de diferents etapes, des de la dispersió de propàguls i la germinació de llavors, fins a l'establiment de les plàntules i la seva posterior supervivència. La transició entre la germinació de les llavors i l'establiment i posterior supervivència de les plàntules suposa un coll d'ampolla per a la població. Per a poder superar amb èxit aquesta transició en el seu cicle vital, les plàntules han de travessar un seguit de filtres biòtics i abiòtics i, alhora, tenir la capacitat de resistir les amenaces d'origen antròpic.

Les fanerògames marines formen praderies en aigües costeres, desenvolupant funcions ecològiques que subministren serveis importants per a la societat. Tot i això, aquestes estan patint una forta regressió a nivell mundial com a resultat de moltes pertorbacions antròpiques. En aquest context, els estadis juvenils suposen una etapa essencial per a assegurar-ne la supervivència, contribuint en el manteniment de la diversitat genètica de les poblacions, la qual és fonamental per a la seva adaptació als canvis, essent la seva dispersió la que permet la colonització de noves àrees. Aquesta tesi analitza com actuen alguns factors abiòtics i biòtics, incloent-hi pertorbacions humanes, sobre la dispersió de propàguls i l'establiment exitós de les plàntules.

En aquesta tesi he comprovat que el reclutament de plàntules de fanerògames marines està fortament afectat per les condicions hidrodinàmiques establertes per l'onatge i les corrents, les quals tenen un paper clau en la determinació de la dispersió, l'establiment i la supervivència dels propàguls de *Cymodocea nodosa, Posidonia oceanica* i *Zostera marina*. No obstant, el tipus de substrat i la seva complexitat afecten l'èxit de l'establiment; els substrats més complexes disminueixen la velocitat de la corrent i augmenten el gruix de la capa límit de fons, facilitant la retenció de llavors i plàntules. Un cop establertes, les plàntules augmenten la seva supervivència en zones més protegides de la influència de l'onatge, on el flux d'energia associat a aquestes és menor. L'energia hidrodinàmica canvia amb la profunditat i, en aigües poc fondes (i.e. 0.5-6 m) les majors profunditats estan associades a una major supervivència. A més a més, alguns factors biòtics, com per exemple la comunitat de macroalgues existent en el lloc de

l'assentament, també influeixen en l'ancoratge al substrat i la supervivència de les plàntules.

A més a més, les amenaces derivades del «canvi global» estrenyen encara més el coll d'ampolla poblacional, exercint una influència directa en el desenvolupament i la supervivència de les plàntules. L'escalfament de l'aigua (29ºC) afecta negativament el desenvolupament de plàntules de *P. oceanica* i malmet el seu aparell fotosintètic. Quan aquest escalfament es produeix en forma d'onades de calor, les poblacions d'una mateixa espècie (*C. nodosa*) responen de manera diferent. Si bé les plàntules de totes les regions es veuen afectades negativament tant en desenvolupament com supervivència, essent els efectes de la onada de calor més nocius a 32ºC que a 29ºC, la resposta regional canvia. Aquelles plàntules que provenen de regions amb una major amplitud tèrmica són més resistents a aquest augment de temperatura en comparació amb les que provenen de regions amb règims tèrmics més estables, resultat que ressalta la importància de les variacions interespecífiques, bàsiques en l'evolució i la distribució d'espècies. D'altra banda, les algues invasores *Caulerpa cylindracea* i *Lophocladia lallemandii* no tenen efectes negatius en *P. oceanica* quan aquesta està sotmesa a l'escalfament.

Aquesta investigació dóna un pas endavant en la comprensió dels factors ambientals i els processos que afecten l'assentament i el destí de les plàntules, contribuint així a la conservació de les praderies de fanerògames marines, ecosistemes de gran importància ecològica i sòcio-econòmica.

LIST OF ABBREVIATIONS

α	Photosynthetic quantum efficiency
ADV	Accoustic Doppler Velocimeter
AF	Absorbance factor
A_F	Frontal area exposed to the flow
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BN	Bendinat
δ	Boundary layer thickness
C_g	Celerity of the waves group
DW	Dry weight
E	Total wave energy per surface unit
E_k	Saturating irradiance
ETR	Electron transport rate
ETR_{max}	Maximum electron transport rate
F	Wave energy flux
F_m	Maximal fluorescence
g	Acceleration of gravity
GLM	General Lineal Model
h	Depth
H_s	Significant wave height
IMEDEA	Mediterranean Institute for Advanced Studies
IPPC	Intergovernmental Panel on Climate Change
k	Wave number
L	Wave lenght
MDA	Maximum dissimilarity algorithm
MHW	Marine heatwave
PAM	Pulse Amplitude Modulated
PAR	Photosynthetically active radiation
PN	Punta Negra
PPF	Photosynthetic photon flux
ρ	Density of seawater

ρ_s	Density of the propagule
RLC	Rapid Light Curves
RBF	Radial Basis Function
Re	Reynolds number
SC	Son Caliu
SOCIB	Balearic Island Observing and Forecasting System
SST	Sea Surface Temperature
SWAN	Simulating WAves Nearshore
θ	Mean wave direction
TLA	Total Leaf Area
T_p	Wave period
V_s	Volumen of the propagule
W_s	Settling velocity
WW	Wet weight
Y	Optimum quantum yield

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1. INTRODUCTION

1. INTRODUCTION

Posidonia oceanica flower
(Son Caliu, December 2014)
By Laura Pereda Briones

1.1. Seagrass ecosystems

Seagrasses are angiosperms adapted to growing in marine environments, where they can form extensive meadows in shallow coastal waters (Den Hartog, 1970; Hemminga and Duarte, 2000). Seagrass meadows are present on all continents, except Antarctica, occupying about 177.000 km^2 (Green and Short, 2003), often being the dominant primary producers in coastal areas, and they are one of the most productive ecosystems on earth (Duarte and Chiscano, 1999).

Seagrasses are recognized as ecosystem engineers and habitat-forming species, since they considerably modify the abiotic environment of their biome (van der Heide et al., 2007). This fact contributes to providing important ecosystem services to humans (Hemminga and Duarte, 2000; Campagne et al., 2015; Cullen-Unsworth, 2014; Nordlund et al., 2016). To begin with, seagrass meadows support high biodiversity by offering a suitable habitat for colonization by many organisms. Seagrass canopy and in some cases the dense matrix of roots and rhizomes serve as a refuge to hide from predators (Jackson et al., 2001; Heck and Orth, 2006, Farina et al., 2009). Furthermore, seagrass canopy attenuates the water flow, guaranteeing a safe refuge for recruitment. Certainly, numerous larvae or juvenile stages use seagrass meadows as nursery areas (Hemminga and Duarte, 2000; Cullen-Unsworth, 2014). In addition, seagrass leaf surfaces can harbour numerous families of epiphytes (Ballesteros, 1984), that simultaneously provide an important habitat and food source for other marine organisms (e.g. mesograzers, sea urchins; Reynolds et al., 2014, Tomas et al., 2005). This complex structure offered by seagrasses allows considering them as habitat-forming species that create biodiversity hot spots.

The abovementioned attenuation of water flow and waves by seagrass canopies (Koch et al., 2009; Infantes et al., 2012) also causes momentum loss of the suspended particles in the water, leading to increased sediment deposition and decreased resuspension (Koch, 1999; Terrados and Duarte, 2000; Gacia and Duarte, 2001; Hendriks et al., 2008). This capacity contributes to coastal protection (Koch et al., 2009) as well as to decreasing water turbidity and consequently promoting seagrass photosynthesis and growth (van der Heide et al., 2007).

Seagrass meadows also export litter that accumulates in adjacent ecosystems, providing organic matter towards deep waters and beaches (Cebrian et al., 1997; see review by Boudouresque et al., 2017) and protecting the seashore from erosion (Coupland et al., 2007; see review by Ondiviela et al., 2014). This litter creates structure, which promotes sand accumulation and facilitates dune formation (Hemminga and Nieuwenhuize, 1990). Seagrass meadows accumulate a big amount of their production in their sediments, which represent approximately 15 % of the carbon reservoir in the ocean (Duarte, 2000; Lavery et al., 2013; Marbà et al., 2014a). On the other hand, seagrasses also export carbon to higher trophic levels (Tomas et al., 2005; Prado et al., 2007; Lal et al., 2010; Kollars et al., 2017). All these functions and associated ecosystem services imply that seagrasses are one of the most valued ecosystems on earth (Constanza et al., 1997; Barbier et al., 2011). Nonetheless, it is necessary to consider that not all seagrasses provide the same ecosystem services, since it seems to vary through genera and bioregions (Nordlund et al., 2016).

1.2. Main threats to seagrass ecosystems

Seagrasses form extensive meadows in coastal environments, areas with high natural disturbance regimes (Nicholls et al., 2007) as well as with concentrated human populations (Short et al., 2015), thus consequently being exposed to multiple natural and anthropogenic threats (Orth et al., 2006a). Certainly, seagrasses are facing the most quickly decline among earth ecosystems (7% yr-1, Waycott et al., 2009; Boudouresque et al., 2009). Seagrass decline and its degradation influences the ecosystem services they provide, disturbing the coastal environment, and generating much larger and long-term impacts than the loss of the meadows themselves (Hughes et al., 2009).

A wide variety of causes, acting from local to regional and global scales, are responsible for seagrass loss and degradation (Orth et al., 2006a; Waycott et al., 2009). Globally, seagrasses are exposed to all five of the most severe threats to marine biodiversity (Norse, 1993): overexploitation, physical modification, nutrient and sediment pollution, introduction of non-native species, and climate change. Specifically, reduction in water clarity due to increased turbidity and nutrient loading from wastewater outfalls, and sediment runoff in human altered watersheds, has been identified as the primary cause of seagrass loss worldwide (Waycott et al., 2009; Short et al., 2011). In addition, other disturbances are threatening seagrass communities, further magnifying potential overall impacts. Boating-related damage, attributed to propellers (Dawes et al., 1997), anchors (Creed and Filho, 1999), moorings, (Glasby and West, 2018), and destructive fishing practices (Orth et al., 2006a) scour the sea bottom and remove leaves, shoots and rhizomes of seagrasses and other benthic organisms. In addition, construction of harbours causes seagrass burial due to changes in sedimentary regimes (Ruiz and Romero, 2003). Overfishing indirectly affects seagrasses owing to the role of top-down control. Certainly, the loss of predators can have cascading effects on trophic dynamics, leading to the loss of the herbivores that clean seagrasses of fouling algae (Orth et al., 2006a, Hughes et al., 2013).

In addition, seagrasses are subjected to the proliferation of non-native marine species worldwide (see review by Williams and Smith, 2007). At least 56 non-native species have been introduced in seagrass beds, of which the majority have been associated with losses of biodiversity and other negative effects (Williams, 2007). Furthermore, since invasion pressure is accelerating as a result of increasing trade (Galil, 2009; Ruiz and Fofonoff, 2000), seagrasses will likely simultaneously experience multiple species introductions, which could have additive and even synergistic effects on seagrass populations and the ecosystems they sustain (Simberloff and von Holle, 1990).

Finally, global climate change has been recognised as one of the main threats to marine and coastal ecosystems (Brouns, 1994). Seagrasses are highly vulnerable to the impacts derived from climate change (Orth et al., 2006a; Waycott et al., 2009), such as increases in sea surface temperature, sea level rise, and frequency and intensity of extreme climatic events (e.g. storm surges, marine heatwaves). Temperature is a major factor influencing seagrass fitness, growth and metabolic rates (Koch et al., 2007). The current and predicted increase in sea surface temperatures (IPCC, 2013; 2014), threatens seagrasses in different ways. Long-lasting exposure to elevated temperatures affect physiological processes (decrease in photosynthesis and increase in respiration rates, altering the carbon budget and growth, Bulthuis, 1987; Ralph, 1998; Collier et al., 2011), and influences life history events (e.g. reproduction, Díaz-Almela et al., 2009). Short-term exposure to more frequent and intense extreme climatic events can often exceed temperature thresholds of species, leading to a decrease in shoot density (Smale et al. 2019) and even to shoot mortality (e.g. Marbà and Duarte, 2010). Furthermore, exposure of seagrass to these extreme climatic events could significantly affect their nutritional value due to the alteration of the fatty acid metabolism, producing important concerns in the health state of next trophic levels (Beca-Carretero et al., 2018). Therefore, increased sea surface temperature linked to global warming will be important in determining the present and future distribution and condition of seagrasses (Bulthuis, 1983; Ralph, 1998;

Short et al., 2000) and will negatively affect the provision of ecological goods and services in the near future (Smale et al., 2019).

Together with anthropogenic disturbances, natural stressors also challenge seagrass performance. Several physical, chemical and biological factors influence survival, growth and reproduction of seagrasses (Nejrup and Pedersen, 2008). When these factors are beyond their normal range of variation, having negative consequences on individual physiology or population performance, they are considered stressors (Vinebrooke et al., 2004). In particular, physical disturbances associated to hydrodynamic conditions (e.g. wave exposure, current velocity) and geological characteristics (e.g. substratum type, bottom roughness) are very important in determining survival of adult and juvenile stages (Orth et al., 1994; Terrados, 1997; Cabaço et al., 2008; Piazzi et al., 1999; Koch et al., 2010; Infantes et al., 2011a) and thus determining the distribution and maintenance of seagrass meadows (Koch, 2001; Frederiksen et al., 2004; Infantes et al., 2009).

Importantly, seagrasses are often exposed to multiple threats and stressors at different temporal and spatial scales (Orth et al., 2006a), and generally interactive effects are more severe than cumulative ones (Crain et al., 2008). The combination of multiple perturbations possibly will reduce seagrasses' resilience to future environmental changes (Connell and Ghedini, 2015; Moreno et al., 2018). Therefore, it is necessary to study the effects of multiple threats and stressors to improve our understanding of these complex processes.

1.3. Reproduction in seagrasses

Seagrasses reproduce both vegetatively through rhizome elongation and sexually by seeds (Hemminga and Duarte, 2000; Kendrick et al., 2012). However, seagrass meadows maintenance and expansion are typically mainly attributed to clonal growth (Phillips et al., 1981; Williams, 1990). In fact, sexual reproduction in seagrasses was largely understudied until the last decades, when several studies have pointed out its importance in contributing to the persistence and maintenance of meadows (Orth et al., 2006b; Kendrick et al., 2012; Jarvis et al., 2014). Indeed, seagrass bed formation usually involves both reproductive strategies (Kendrick et al., 2012), since each of them have specific advantages. Asexual reproduction propagates healthy genotypes through growth and dispersal via fragmentation (e.g. Campbell, 2003) and avoids the existing costs of reproduction (Obeso, 2002). Sexual reproduction contributes to the genetic variation pool (which is essential for natural selection), provides the possibility to escape from disturbances through dormant seeds, and facilitates dispersal (e.g. Williams, 1995).

All seagrass species share a common architecture, due to their rhizomatous existence, but sexual reproductive strategies differ between species (Rasheed, 2004). For some species (e.g. *Zostera marina*) plants are annual or perennial and flowering is common and often abundant (Silberhorn et al., 1983), while for others (e.g. *Posidonia oceanica*) it is a rare event, very variable in frequency and intensity (Buia and Mazella, 1991). Most seagrasses produce negatively buoyant seeds which could move along the sediment surface depending on hydrodynamic conditions of the bottom boundary layer and on the physical properties of seeds (Ruiz-Montoya et al., 2012; Pereda-Briones et al., 2018a), while seeds of other species are produced at the base of the plant and will not move unless a disturbance re-suspends the upper layer of the sediment and transports them to other places (*e.g. Cymodocea nodosa*, Terrados, 1993). Some of them, such as *Z. marina* or *Halophila ovalis* are able to remain dormant, creating a "seed bank" from which populations could recover after a disturbance occurs (Jarvis and Moore, 2010; Kuo and Kirkman, 1992). Instead, other species, such as *P. oceanica*, *P. australis*, *Enhalus acoroides* or *Thalassia hemprichii*, produce non-dormant seeds that germinate immediately after release (Den Hartog, 1970). There are also species that have positively buoyant propagules that are transported on the water surface depending on currents generated from tides, wind, waves, seas and swell (Micheli et al., 2010). These propagules can be fruits (e.g. *Thalassia testudiunm*), seeds (e.g. *E. acoroides*) or reproductive shoots (e.g. *Z. marina* or *Z. noltii*), although some reproductive shoots have neutral buoyancy and move within the water column (Orth et al., 2006b).

Beyond interspecies differences, sexual reproductive strategies also differ within populations of the same species, mainly depending on environmental conditions, such as light, temperature, nutrients or salinity, but also on genetic factors (Smith and Walker, 2002; Barret et al., 1993). Flowering typically occurs only within a narrow range of one or more of these factors (McMillan, 1980; Terrados, 1993; Diaz-Almela et al., 2007). For example, *Z. marina* perennial populations invest strongly in sexual reproduction when growing under high resource availability (Johnson et al., 2017) or in response to environmental disturbance (Cabaço and Santos, 2012), whereas annual populations invest predominantly in sexual reproduction (Kim et al., 2014).

In summary, seagrass reproductive propagules are able to colonize new and distant habitats and also recolonize disturbed areas, enabling the resilience and recovery of existing meadows, and thus contributing to maintaining seagrass populations (Harwell and Orth, 2002; Källström et al., 2008; Johnson et al., 2018).

1.4. Importance of early life stages

In the current global scenario of increasing threats to marine coastal ecosystems (Orth et al., 2006a; Waycott et al., 2009; Short et al., 2011), the ability of seagrasses to deal with such disturbances depends on their capacity to colonize new areas and to acclimate to environmental changes (Orth et al., 2006b; Kendrick et al., 2012; McMahon et al., 2014). Over the last decades, a central goal of marine ecology has been to elucidate how anthropogenic and environmental stressors affect seagrasses and their recovery (Valdemarsen et al., 2011; Grech et al., 2012).

Seagrass reproductive propagules are a crucial life stage that contributes to the maintenance of existing meadows (Kendall et al., 2004; Zipperle et al., 2009; Jarvis et al., 2014) and to the formation of new ones via dispersal (Orth et al., 1994; 2006b). Seedling recruitment provides an essential genetic diversity source, necessary to adapt to anthropogenic and natural environmental changes and to guarantee the long-term persistence of species and the ecosystems they form (Hughes et al., 2008; Kendrick et al., 2012; 2017). In addition, due to the limited number of species present in seagrass communities, within-species diversity may replace the functional role of species diversity (Duffy, 2006; Hughes et al., 2008). Therefore, in order to maximize seagrass ecosystem potential to withstand and adapt to current and future changes, the conservation of genetic diversity has become a main interest for ecologists and coastal managers (e.g. Reynolds et al., 2012; van Katwijk, et al., 2016). In addition to these paramount functions, seeds and seedlings can also be used to recover endangered areas in seagrass restoration projects (Renton et al., 2011; Orth et al., 2012; van Katjwik et al., 2016). Seed-based restoration, in contrast to the traditional seagrass restoration made by transplantation of adult plant cuttings from donor beds to degraded areas (Fonseca and Bell, 1998; Duarte, 2002; van Katwijk et al., 2009; Pailing et al., 2009; Balestri et al., 2011), in general has minor damage to donor beds (Orth et al., 2000). Furthermore, this technique provides genetic diversity that facilitates the recovery of populations (Orth et al., 2000; Kendrick et al., 2012; Reynolds et al., 2012). However, since this technique has the limitation of seed availability and successful seedling recruitment, a careful selection of the recovery site and a cautious selection of the proper planting technique is essential (van Katwijk et al., 2016) and can't be used generally.

Overall, early life stages are of outstanding ecological importance, given the current scenario of seagrass threats and losses (Waycott et al., 2009), being crucial to simultaneously evaluate the multiple anthropogenic threats and environmental stressors affecting seagrasses.

1.5. Seagrass recruitment patterns

Recruitment of new individuals is a multistage process, from seed production, dispersion and germination, to seedling establishment and further incorporation into the adult reproductive population (Gómez-Aparicio, 2008). This process involves successive life history stages (from seed, germinant to emerged seedling) which represent the interface between a dispersal phase and a sessile phase (Ericksson and Ehrlén, 1992). Therefore, recruitment is considered a decisive stage in plant life story, since it will determine the trajectory of plant community assembly (Ericksson and Ehrlén, 1992; Cramer et al., 2008). Despite the recognition of the critical role of recruitment for plant populations (James et al., 2011), seagrass ecology studies have only started investigating its importance in relatively recent years (Kendrick et al., 2012; 2017; Statton et al., 2017; Guerrero-Meseguer et al., 2018). Each stage that takes part on the recruitment process can create a "bottleneck" for the plant life cycle if it is associated with low probability of success, and thus limit population success. The transition between seed germination and seedling establishment represents the most exposed stage for recruitment success of plant populations (Harper, 1977). This evident importance of post-germination phases is also confirmed in seagrass ecosystems (Piazzi et al., 1999; Olesen et al., 2004; Orth et al., 2006b; Statton et al., 2017; Johnson et al., 2018).

Recruitment patterns are driven by biotic and abiotic filters, which determine survival of new individuals (Harper, 1977). These filters are very heterogeneous in space and time (Stewart et al., 2000) and influence plant species differently. Some species are restricted to living under very specific environmental conditions (microsite-limited species), while others have no specific habitat requirements, being mostly influenced by seed availability (seed-limited species; Münzbergová and Herben, 2005). Once reproductive propagules are released, either in smaller (e.g. *P. oceanica*) or greater (e.g.

Z. marina) quantities, they must overcome the hazards of the environment they encounter. Therefore, it is vital to understand which factors the propagules are exposed to through the different stages of their recruitment process, and to identify "safe" (sensu Harper et al., 1961) recruitment microsites [(i.e. small-scale sites where seedlings have a larger probability of survival) Eriksson and Ehrlén, 1992].

A wide array of factors can influence seagrass early life history stages. Among them, hydrodynamic conditions are identified as one of the most decisive ones (e.g. Frederiksen et al., 2004; Infantes et al., 2009). Physical forces (mainly waves and currents, but also wind) influence the dispersal of reproductive propagules (e.g. Källström et al., 2008; Ruiz-Montoya, et al., 2012). Near-bottom orbital velocities produced by waves and current flow velocity can disturb the sediments, and may remove seeds (e.g. Marion and Orth, 2012) and cause seedling burial or dislodgement (e.g. Infantes et al., 2011a). Small-scale topography affects dispersion and seed germination (Orth et al., 1994, 2003; Inglis, 2000a; Balestri and Lardicci, 2008) and substratum type is determinant for seedling establishment and survival (Piazzi et al., 1999; Olesen et al., 2004). A successful recruitment also could depend on predation, herbivory and bioturbation rates. Seed predation could remove seeds (e.g. Orth et al., 2006b); herbivory could affect plant development by reducing its photosynthetic area (e.g. Kirkman, 1999) and bioturbators could dislodge seeds and seedlings from the recruitment pool (e.g. Statton et al., 2017). In addition, microsite characteristics, such as depth (Piazzi et al., 1999), light (Dominguez et al., 2012), composition of algal canopy (Alagna et al., 2013) or nutrient availability (Chapin et al., 1987) are major drivers of seagrass recruitment patterns.

Since the characteristics of early life stages differ amongst species, the biotic and abiotic filters that are more important for seagrass early life stages will also vary. Furthermore, the relative importance of those factors can also change within a species depending on the geographic contexts of the different populations. For instance, seagrass ability to withstand physical forces largely depends on species size and morphology (e.g. Cabaço et al., 2008; Infantes et al., 2011a). Larger species are exposed to higher drag coefficient than smaller ones, being more vulnerable to establish on high energy exposed sites. For example, *P. oceanica* seedlings, due to their higher drag coefficient in comparison to *Cymodocea nodosa*, are dislodged earlier from the substrate when undergoing the same flow velocity (Infantes et al., 2011a). Another important attribute are the anchoring systems of seedlings, which are essential for anchoring to the substrate and minimize hydrodynamic disturbance. These also vary among species. For instance, the genus *Phillospadix* and the species *Amphibolis antartica* have a specialized appendage with a comb-like structure that enhances seed attachment to the substrate (Blanchette et al., 1999; Rivers et al., 2011). Seedlings of *P. oceanica*, on the other hand, produce adhesive root hairs and have the capacity to adjust root traits (such as length, direction of growth) according to substratum type (Badalamenti et al., 2015; Balestri et al., 2015, Guerrero-Meseguer et al., 2018).

Importantly, due to the paramount functions and associated ecosystem services provided by seagrasses, and considering that they are often exposed to multiple disturbances at different temporal and spatial scales (Orth et al., 2006a), it is essential to study in detail the processes affecting seagrass early life stages. Overall, given the heterogeneity of the marine environment, the multiple biotic and abiotic factors, and multiple threats and stressors controlling recruitment patterns, and the different mechanisms of seagrass species and populations to cope recruitment “bottlenecks”, it is essential to improve our understanding of all these processes in order to incorporate them in future research.

1. INTRODUCTION

2. OBJECTIVES

2. OBJECTIVES

Posidonia oceanica fruit
(Cala Xinxell, May 2015)
By Laura Pereda Briones

The present thesis aims to contribute to the progress in the understanding of seagrass recruitment patterns, examining factors that influence propagule dispersal, seedling establishment and further survival. This multistage process represents a series of “bottlenecks” of seagrass survival and development and is influenced by multiple biotic and abiotic environmental factors and anthropogenic disturbances. Given the many threats posed by many human activities on seagrass ecosystems, this thesis also aims to contribute to understanding how two of the main menaces associated with global change (i.e. warming and biological invasions) affect seedling survival and recruitment. To achieve these goals, three different seagrass species have been used: *Posidonia oceanica*, *Cymodocea nodosa* and *Zostera marina* (see the General Methodology section for more details of these species).

The above-mentioned main targets were accomplished through four specific objectives, included in the results section. Each of the fourth chapters includes one of the main specific objectives, and constitutes a separate article in preparation or published in a peer reviewed journal (Q1).

To examine the interplay among hydrodynamic conditions, substratum characteristics and early life stages features on propagules dispersal and trapping. This specific objective was assess for the three species (*P. oceanica*, *C. nodosa* and *Z. marina*) and it was reached by (1) estimating transport velocities of floating propagules at different current velocities, (2) determining the minimum flow velocities at which seeds and seedlings start moving over the substratum (threshold velocities) and (3) analysing the capacity of different substrata to retain propagules (“trapping”) and how this process is influenced by the propagules physical characteristics. (**Chapter 1**: *“Dispersal of seagrass propagules: interaction between hydrodynamics and substratum type”*). This chapter has been published in the journal Marine Ecology Progress Series[1].

To identify microsites suitable for seedling survival and evaluate the importance of the different biotic and abiotic factors regulating early seedling development and survival. To evaluate this specific objective, we selected the species *P. oceanica*, and it was reached

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[1] Pereda-Briones L, Infantes E, Orfila A, Tomas F, Terrados J. 2018. Dispersal of seagrass propagules: interaction between hydrodynamics and substratum type. *Marine Ecology Progress Series* **593**, 47-59.

by: (1) analysing the influence of wave energy flux on seedling survival, (2) evaluating the influence of substratum type on seedling survival and development, and (3) examining if the association with certain macroalgae favours seedling development and survival. (**Chapter 2**: *"Influence of biotic and abiotic drivers of seagrass (Posidonia oceanica) seedling recruitment: identifying suitable microsites"*). This chapter is being prepared for submission in a peer-reviewed journal.

To evaluate the potential independent and cumulative impacts of warming and the presence of invasive algae on the biological status (development, physiology and survivorship) of seagrass seedlings. To analyse this specific objective, we selected the invasive species *Caulerpa cylindracea* and *Lophocladia lallemandii* and the seagrass *P. oceanica*, and it was reached by: (1) assessing the effects of increase in temperature on seedling development and survival (2) evaluating the effects of the presence of the invasive algae on seedlings vegetative development, and (3) examining the interaction of seedlings with invasive algae under warming. (**Chapter 3**: *"Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae"*). This chapter has been published in the journal Marine Pollution Bulletin[2].

To assess the vulnerability of seagrass seedlings to marine heatwaves. To assess this specific objective, we selected the species *C. nodosa,* and we have evaluated it response to MHWs at three levels: survivorship, morphology and physiology, along its distribution range, to connect seedling responses to the thermal variability of their local environments. This specific objective was reached by: (1) cultivating under a common garden experiment *C. nodosa* seedlings of the different regions prior to the heatwave experiment performance, (2) measuring seedling morphology and photophysiology parameters during the MHW and after the recovery period, and (3) comparing seedling responses with the climatic contexts of the different regions. (**Chapter 4**: *"Resilience of seagrass seedlings to marine heatwaves is modulated by local thermal variation"*). This chapter is being prepared for submission in a peer-reviewed journal.

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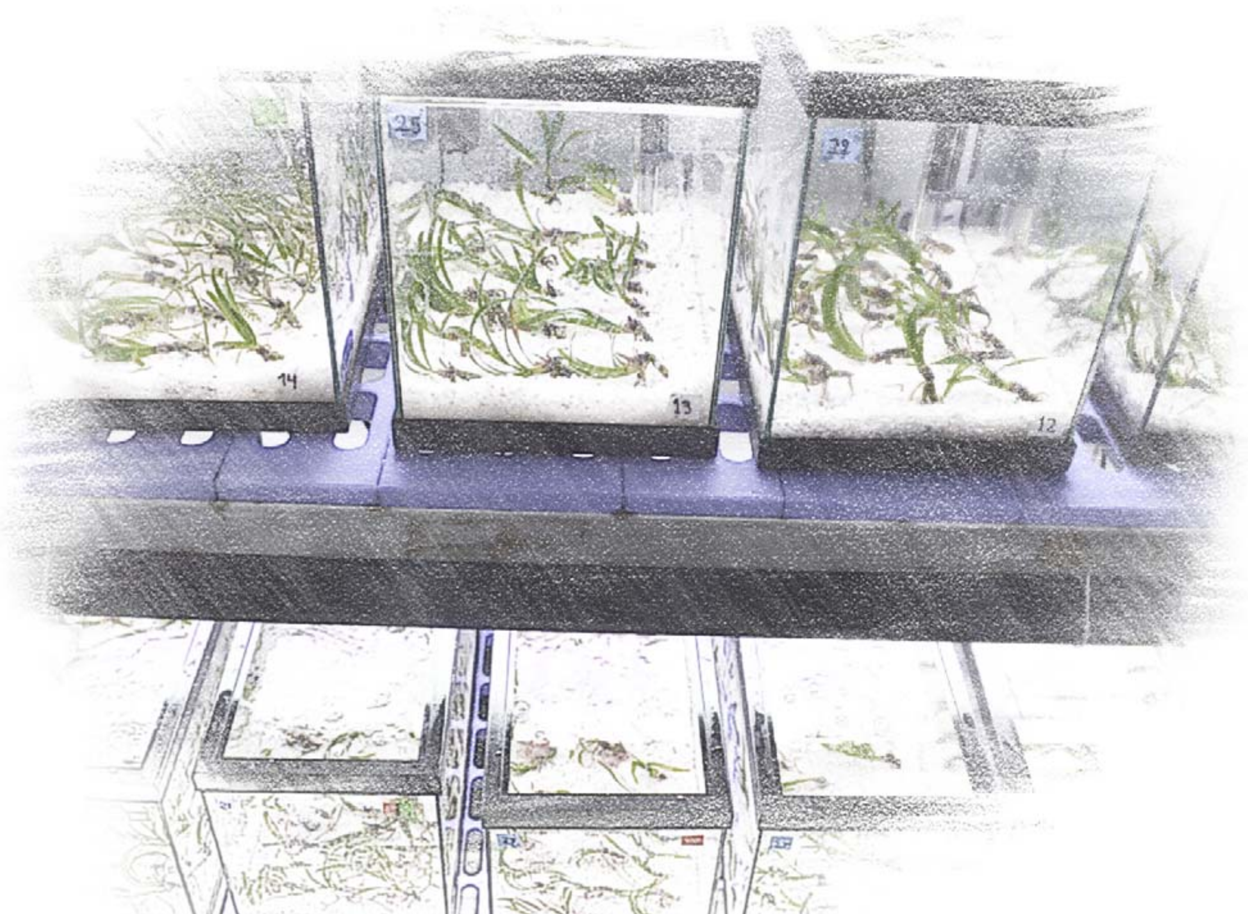
[2] Pereda-Briones L, Terrados J, Tomàs F. 2019. Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae. *Marine Pollution Bulletin* **141**, 36-45.



3. GENERAL METHODOLOGY

3. GENERAL METHODOLOGY

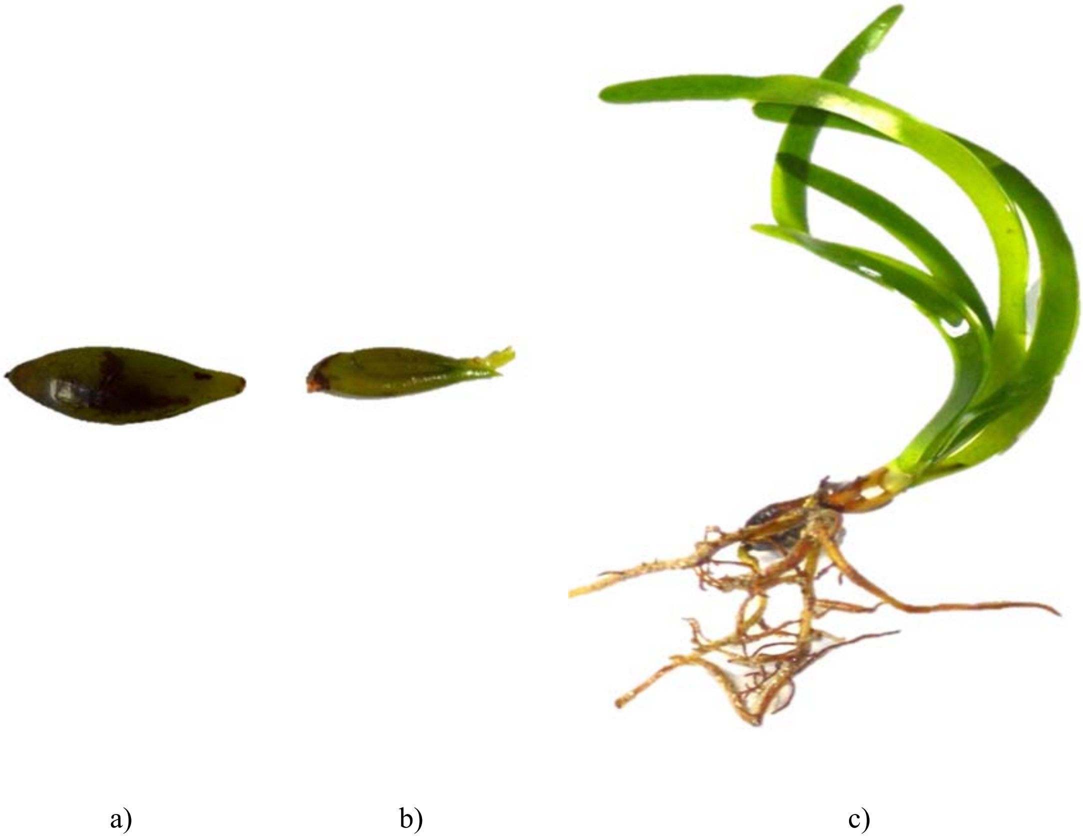
Experiment at IMEDEA
(Esporles, March 2016)
By Laura Pereda Briones

In order to study how seagrass recruitment is affected by multiple biotic and abiotic factors, several methodological approaches were performed: from observational field studies, flume experiments, to mesocosm experiments as well as a physical oceanography approach. The particularities of each method, as well as the description of the species analysed and the areas of study for each chapter are described in detail in the third section (i.e., in the material and methods section of each chapter).

3.1. Study species

3.1.1. *Posidonia oceanica*

P. oceanica is a seagrass endemic to the Mediterranean Sea. This species undergoes very slow growth (Marbà and Duarte, 1998) and very variable sexual reproduction, both in frequency and intensity (e.g. Buia and Mazzella, 1991; Díaz-Almela et al., 2007). It produces floating fruits, which are transported by surface currents until they dehisce and the seeds are released (Fig. 1). Seeds are negatively buoyant and fall to the bottom, where they must settle and establish.



a) b) c)

Figure 1. *Posidonia oceanica* early life stages: fruit (a), seed (b) and seedling (c).

Fruits and seeds of *P. oceanica,* were collected on the shore in Mallorca (Balearic Islands, Spain). Once collected, some fruits were maintained intact for further analysis, while others were opened and their seeds removed and placed in aquaria with seawater at a constant temperature (Terrados et al., 2013).

3.1.2. *Zostera marina*

Z. marina is the most widely distributed seagrass species in northern latitudes (den Hartog 1970; Olesen et al., 2015). *Z. marina* populations are annual or perennial (Robertson and Mann, 1984; Kim et al., 2014) and flowering is common and often abundant (Silberhorn et al., 1983). This species produces reproductive shoots which contain negatively buoyant seeds that mostly dehisce within the meadow (Fig. 2), although some are transported inside the reproductive shoots by surface currents until they dehisce.

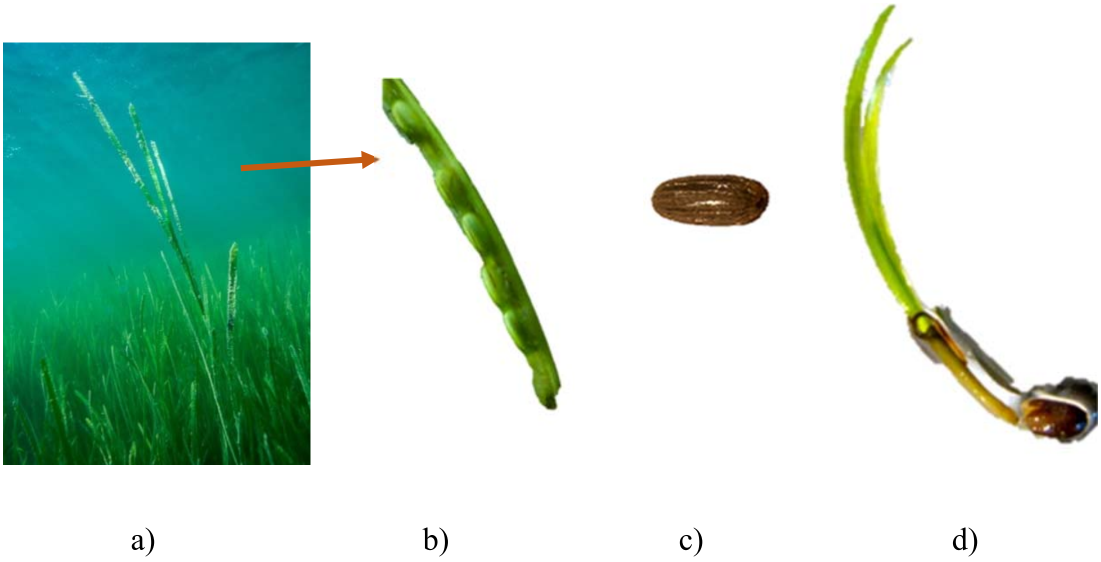


Figure 2. *Zostera marina* early life stages: reproductive shoot (a), fertilized inflorencence detail (b), (photographs by Eduardo Infantes), seed (c) and seedling (d).

Reproductive propagules of *Z. marina* were collected by harvesting reproductive shoots at 1-3 m depth in the Gullmars fjord, Gasö, Sweden. Some reproductive shoots were stored for a short time period for further analysis in outdoor tanks, while others were stored for a longer time period until seeds were released. Seeds were maintained at salinity 34 and 5ºC to prevent germination (Infantes and Mocksnes, 2018).

3.1.3. *Cymodocea nodosa*

C. nodosa is representative of the Mediterranean Sea and the Atlantic coast of North Africa. Flowering and fruiting occurs annually, being abundant in dense meadows (e.g. Caye and Meinesz, 1986; Terrados, 1991). It produces negatively buoyant fruits, which develop buried at the shoot base. Once the fruit pericarp decays, its negatively buoyant seed is released within the meadow (Fig. 3), although it may be exported during sediment erosion events, such as storms.

Figure 3. *Cymodocea nodosa* early life stages: fruit (a), seed (b) and seedling (c).

Seeds of *C. nodosa* were collected from shallow meadows of 7 different regions along the Mediterranean Sea and at the Canary Islands Archipielago (Las Palmas de Gran Canaria, Spain). Then, seeds were placed in darkness inside aquariums to prevent germination.

3.1.4. Invasive algae

In the Mediterranean Sea, *Caulerpa cylindracea* and *Lophocladia lallemandii* (Fig. 4, 5) are two of the most invasive macroalgae threatening *P. oceanica* meadows (Verlaque, 1994; Streftaris and Zenetos, 2006; Ballesteros et al., 2007; Klein and Verlaque, 2008). *C. cylindracea* is widely distributed in tropical and warm temperate seas

(Verlaque et al., 2003), has rapidly colonized wide areas of the Mediterranean, forming permanent populations, interfering with the native assemblages and having an invasive behaviour (Streftaris and Zenetos, 2006; Klein and Verlaque, 2008). *L. lallemandii* has an Indo-Pacific distribution, was probably introduced into the Mediterranean Sea via the Suez Canal (Verlaque, 1994), and it can behave as an invasive species, being able to cover most types of substrates (Boudouresque and Verlaque, 2002; Ballesteros et al., 2007).

Figure 4. The invasive algae *Caulerpa cylindracea* over *Posidonia oceanica* matte.

Figure 5. The invasive algae *Lophocladia lallemandii*, (photograph by Enric Ballesteros).

The invasive algae, *C. cylindracea* and *L. lallemandii* were collected in Mallorca and maintained in separate tanks in the same conditions as *P. oceanica* seedlings.

3.2. Flume experiments

Hydrodynamic conditions affecting early life stages dispersal and trapping of *C. nodosa*, *P. oceanica* and *Z. marina* were simulated in a hydraulic flume (Fig. 6) located at Kristineberg Station, Sweden. In order to identify the threshold velocities of the propagules to initiate movement and to be retained by the substrata, one variable (substrata) was modified (Fig. 7), while leaving others constant (e.g. flow). Flow velocities and vertical velocity profiles were measured with an acoustic Doppler velocimeter (Nortek, Vectrino).

Figure 6. Hydrodynamic flume, (photograph by Eduardo Infantes).

Transport of floating propagules

The surface flow velocity was measured at 1-2 cm below water surface with the ADV positioned upwards. Fruits and reproductive shoots of each species were placed independently on the water surface, and their velocity was assessed at different flow velocities.

Threshold velocities for transport and trapping measurements

Seeds and seedlings were placed over a flat sand bed prepared in the flume, while increasing the water flow at 1 cm s^{-1} intervals until they started to move, and threshold velocity values were calculated. The substratum capability to retain propagules (i.e.

trapping), was calculated by measuring the distance travelled by seeds and seedlings over a specific substratum type under different flow velocities. We measured the distance travelled and the velocity of the propagules from the beginning of the test section until the propagule was trapped or until it passed over the entire test section without being trapped. In order to evaluate the effect of the substratum type on the bottom boundary layer, we estimated the boundary layer thickness, and the percentage of flow reduction (see chapter 1 in the result section for more details).

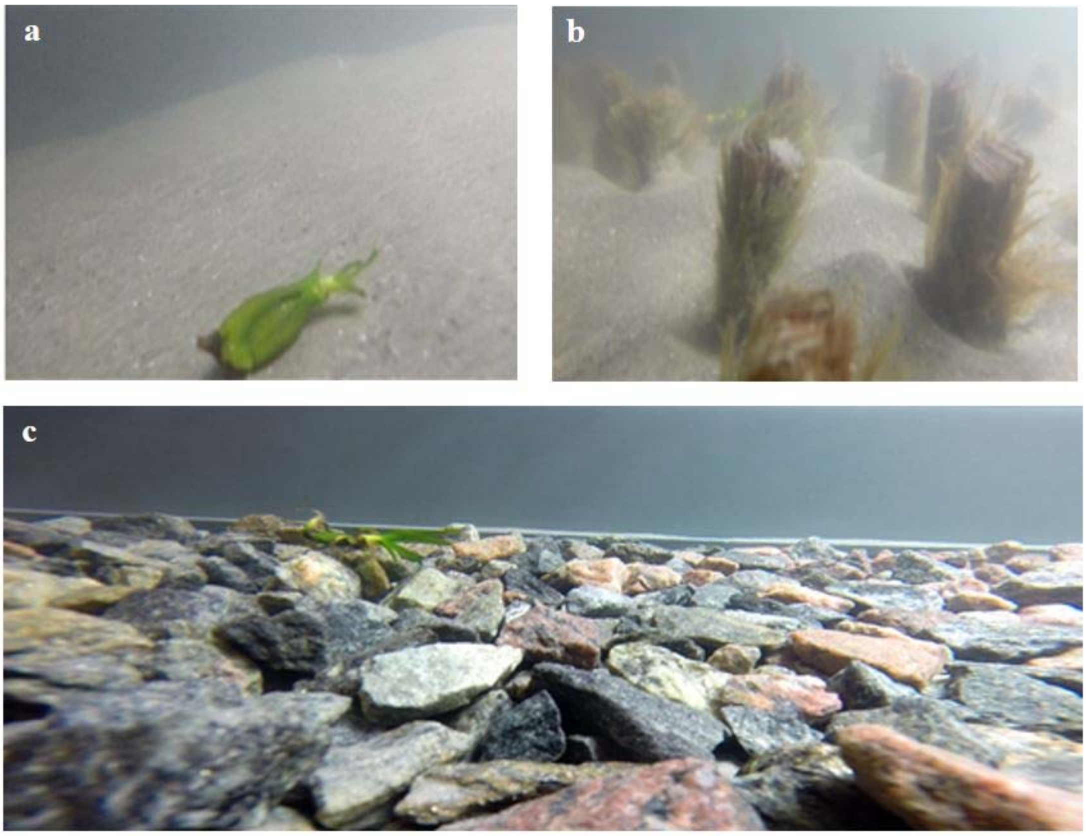


Figure 7. Substratum types analysed in the flume: (a) sand, (b) model of *Posidonia oceanica* matte and (c) coarse gravel, (photograph by Eduardo Infantes).

3.3. Field experiments

To identify microsites suitable for survival of *P. oceanica* seedlings, an observational field study was conducted. We marked groups of naturally-settled seedlings at different microsites (Fig. 8) around Palma Bay (Balearic Islands, Western Mediterranean), and followed their population dynamics during two years, while simultaneously characterizing the environmental and biological factors associated with the safe recruitment microsites.

At the first sampling date we recorded depth, substratum type and bottom roughness of each seedling microsite. Then, trough periodical samplings carried out on SCUBA, we measured seedling morphometry and survival, and percentage cover of the different macroalgae species at each microsite by photographical analysis. In addition, the hydrodynamic conditions were evaluated and compared (see the *"Physical oceanography"* section for more details).

Figure 8. *Posidonia oceanica* seedlings on different microsites (sand, a; matte, b; gravel, c; rock, d) at Palma Bay, Balearic Islands (Spain).

3.4. Mesocosm experiments

We have conducted manipulative experiments in mesocosms to assess how global warming and biological invasions affect seagrass seedling survival and development. The in situ manipulation of water temperature is unfortunately not a viable approach, thus in order to analyse the effects of warming on seagrasses, most studies use aquaria (e.g. Nejrup and Pedersen, 2008; Hernán et al., 2017), chambers (e.g. Kaldy, 2014), or mesocosms with closed systems (e.g., Koch et al., 2007; Olesen et al., 2012). Our experiments were conducted in aquaria equipped with white fluorescent lamps, an air pump and a water filter, in temperature-controlled chambers at IMEDEA (Fig. 9). We manipulated seawater temperature to reach the desired temperature treatments, at a constant temperature and at increasing temperatures to simulate marine heatwaves, in both cases after an acclimation period. To evaluate the effect of invasive algae, both species of invasive macroalgae were added to the different aquaria with the seedlings under the different temperature regimes.

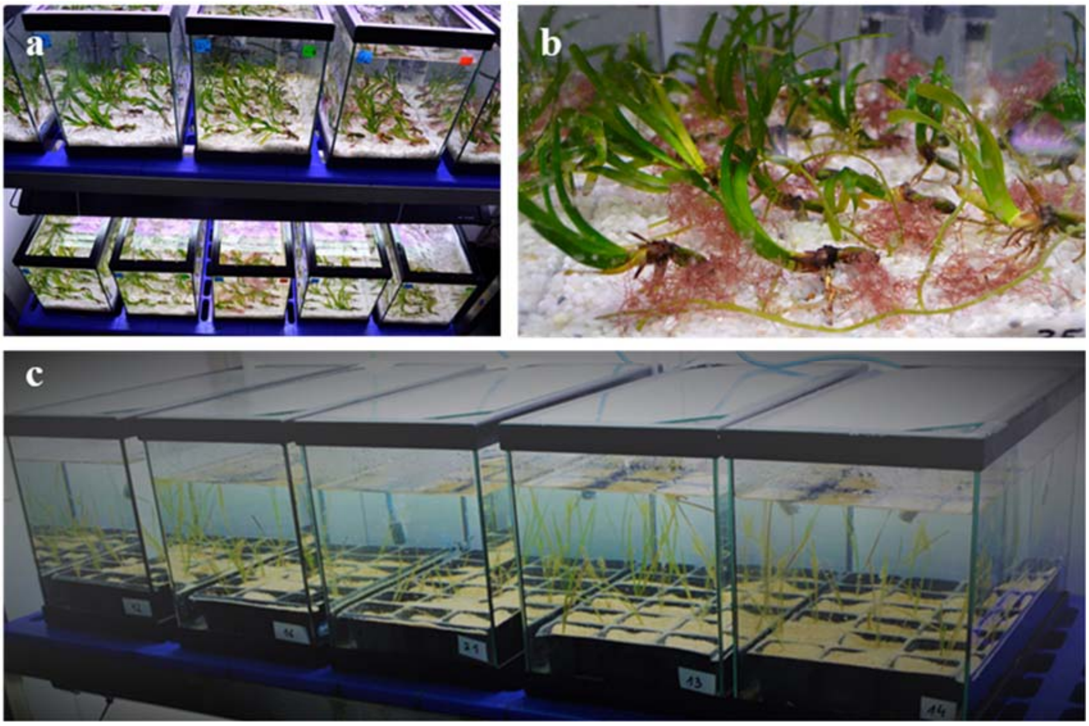


Figure 9. Aquaria with (a and b) *Posidonia oceanica* seedlings, *Caulerpa cylindracea* and *Lophocladia lallemandii*, and (c) *Cymodocea nodosa* seedlings, within the temperature-controlled rooms at IMEDEA.

Common garden

To evaluate the effect of extreme climatic events (i.e. marine heatwaves) within *C. nodosa* seedling populations, a common garden was set prior to the abovementioned mesocosm experiments. Firstly, seed germination was induced by a hyposaline shock (Zarranz et al., 2010). Germinated seedlings were transplanted into seedbeds filled with siliceous sediment, inside 300L plastic boxes with continuous seawater flow and temperature controlled by light and temperature HOBOS (Fig. 10). This common garden infrastructure was located outdoors, at the Visitors Centre of Cabrera National Park

(Mallorca), and seedlings were growing during 15 months under the same conditions, prior to the conduction of the experiment.

Figure 10. *Cymodocea nodosa* seedlings growing in outdoors common gardens at the Visitors Centre of Cabrera National Park, Colonia de Sant Jordi, Balearic Islands (Spain).

3.5. Plant analysis

In order to assess the characteristics of the different early life stages and species, physical and morphological properties of the plants were measured and compared. As traits that may influence transport, propagule length, width, height, volume and mass were measured, and density values and frontal leaf area were calculated. A set of seedling morphological variables were measured in response to warming and the presence of invasive algae: number of leaves, leaf width of the second youngest leaf, maximum leaf length, total leaf area, percentage of necrotic surface on each leaf, total root number, total root length and number of new roots. In the observational field study, number of bites in leaves was also measured.

In addition, plant biochemical and photochemical traits were measured to examine responses to warming and invasive species. Carbon and nitrogen content of plant tissues were analysed using a carlo-Erba CNH elemental analyser, and carbohydrates (starch and sucrose) in seeds were quantified using the methodology described by Invers et al. (2004). Rapid light curves were used to examine seedling photophysiology by pulse amplitude modulated (PAM) fluorometry (e.g. Campbell et al., 2003).

3.6. Physical oceanography

Hydrodynamic conditions

With the purpose of relating the variation of seedling abundance over time at each microsite in our observational field study, we evaluated the degree of wave exposure. To analyse the hydrodynamic forces to which the seedlings were exposed, it was necessary to assess the significant wave height (H_s), and its associated period (T_p) (Holthuisjen, 2007). H_s, T_p as well as mean direction of the wave (θ), were obtained from a climate database. We compared this near-shore database with respect to the real wave conditions in the study area, registered by an oceanographic buoy. Additionally, to further ensure the reliance of the database, we placed accelerometers (HOBO Pendant® G Data Logger) in situ. Then, we propagated the maritime climate from deep to shallow water using a numerical model (SWAN-*Simulating WAves Nearshore*). From this near-shore maritime climate, the characteristic storm surges were identified from the Wave Energy Flux (F) parameter, which represents the energy that arrives to the coast due to the incident waves (Holthuijsen, 2007). Finally, we identified and quantified extreme events along the time period analysed and related them with seedling persistence.

Thermal context characterization

To describe marine heatwaves (MHWs) along the Mediterranean Sea and the Canary Islands, we used the daily sea surface temperatures (SSTs) from satellite data, following the methodology of Hobday et al., 2016. Then, in order to relate thermal and oceanographic variability with seedling responses to extreme climatic events (i.e. MHWs), we analysed and compared several physical parameters among regions, such as mean number of warm days, seasonal cycle amplitude or the standard deviation of the whole SST time series.

4. RESULTS

4. RESULTS

Posidonia oceanica seed
(Son Caliu, May 2015)
By Laura Pereda Briones

4. RESULTS

This section contains the results of the thesis and is organized into four chapters. The chapters are linked by a temporal thread, following the stages along the plant recruitment process. It starts by analysing the drivers of (*Cymodocea nodosa, Posidonia oceanica* and *Zostera marina*,) propagule dispersal and retention, especially the effects of hydrodynamic conditions and substratum type (**chapter 1**). Then, attention is focused on propagule establishment and further survival, which determine the successful recruitment of new individuals. Firstly, some abiotic and biotic factors present at seedlings (*P. oceanica*) settlement microsite, which affect their development and survival are evaluated (**chapter 2**). Secondly, the effects of invasive algae and seawater warming on seagrass seedlings (*P. oceanica*) are assessed (**chapter 3**). Finally, how extreme climatic events, specifically marine heatwaves, influence the resilience of (*C. nodosa*) seedling populations along its distribution range is evaluated (**chapter 4**).

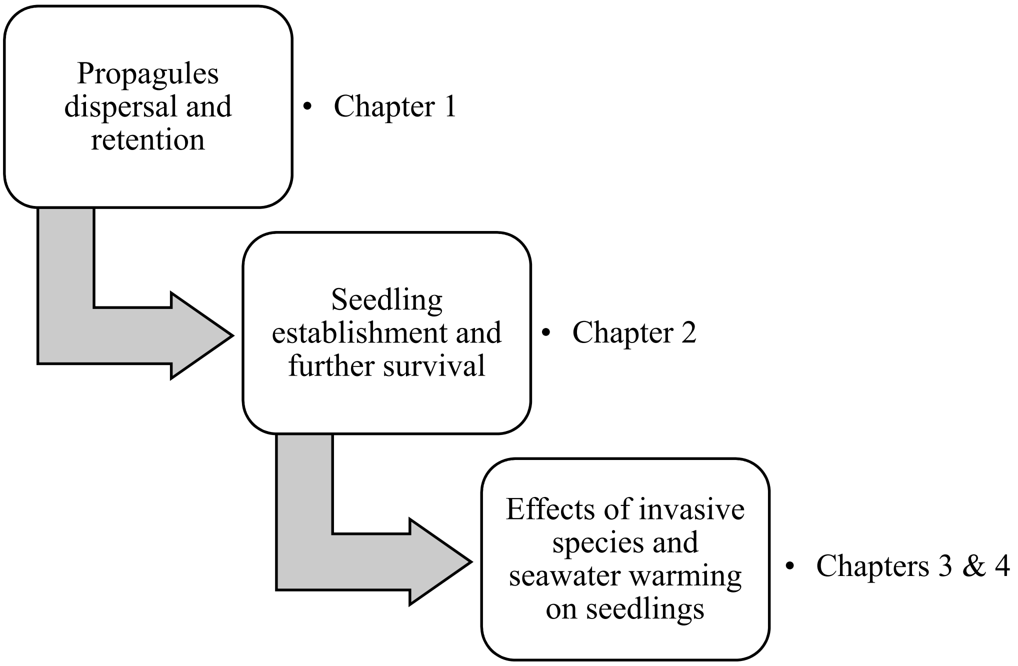


Figure 11. Conceptual organization of thesis chapters.

4. RESULTS

Dispersal of seagrass propagules: interaction between hydrodynamics and substratum type

4. RESULTS

Zostera marina seed
(Kristineberg, 2016)
By Eduardo Infantes

4.1. Dispersal of seagrass propagules: interaction between hydrodynamics and substratum type

4.1.1. Abstract

Waves and currents influence not only the spatial distribution of seagrass meadows but also the transport, establishment and survivorship of seagrass propagules and hence the success of seagrass recruitment from sexual reproduction. We quantified the dispersal of propagules of three seagrass species (*Posidonia oceanica, Cymodocea nodosa* and *Zostera marina*) over substrata of different complexity (sand, coarse gravel, and *P. oceanica* matte of different shoot abundances) under unidirectional flow in a hydraulic flume. Threshold velocities indicate that *Z. marina* seeds start to move earlier over a flat sandy bottom (14 cm s^{-1}) than *P. oceanica* (20 cm s^{-1}) and *C. nodosa* (21 cm s^{-1}) seeds. Propagule trapping increased with bottom complexity, which was related to the flow reduction that each substratum generated and their boundary layer thickness. Trapping rates were higher in coarse gravel and in high dead shoot abundance matte, where flow was reduced more than 50 %. Over sand, flow reduction was minimal and propagules were not trapped. Furthermore, notable differences between *P. oceanica* early life stages were observed, being seeds trapped first, followed by seedlings of increasing ages, which appears to be related with the more hydrodynamic shape of the younger stages. Together these results provide important insights into the drivers of seagrass recruitment, which are of interest for restoration purposes and numerical modelling.

4.1.2. Introduction

Seagrasses are a dominant feature of coastal environments and provide a suite of important ecosystem services such as coastal protection, carbon sequestration, and nursery areas for many commercially and ecologically valuable marine species, among others (Hemminga and Duarte, 2000). As a result of different human stressors, seagrasses have suffered important declines worldwide (Waycott et al., 2009). Sexual reproduction is an essential part of the life cycle of seagrasses, for its role in the consolidation of existing meadows and for the colonization of new ones. The products of sexual reproduction such as fruits, seeds and seedlings are critical for dispersal and for the genetic variation for seagrasses (Kendrick et al., 2012; 2017). In fact, studies on early life stages of other aquatic plants indicate that they are decisive in limiting species distribution and abundance (Les et al., 2003). Furthermore, they could be used as a source of transplanting material in seagrass restoration projects (Ganassin and Gibbs, 2008; Renton et al., 2011).

Hydrodynamics (waves and currents) influence the spatial distribution of seagrass meadows (Frederiksen et al., 2004; Infantes et al., 2009), being also crucial for seedling survival (Infantes et al., 2011a). In fact, hydrodynamic conditions strongly determine the success of seagrass restoration projects (Bos and van Katwijk, 2007). Substratum type may also contribute to seagrass propagule trapping and establishment (Piazzi et al., 1999; Infantes et al., 2011b), and substratum roughness determines the height of the bottom boundary layer which influences the transport of seeds and seedlings (Koch et al., 2010; Alagna et al., 2015). Indeed, identification of adequate substratum type is critical for the survival and growth of transplanted seedlings in restoration activities (Balestri et al., 1998). Furthermore, bottom shear stress from the combined effect of waves and currents can mobilize unconsolidated sediment, producing erosion or accretion that can affect seagrasses negatively through uprooting and burial (Orth et al., 1994; Terrados, 1997; Cabaço et al., 2008).

Beyond hydrodynamics per se, the physical properties of seagrass propagules, such as morphology, density, settling velocity and drag coefficient are highly determinant of their dispersal potential (Infantes et al., 2011a; Ruiz-Montoya et al., 2012; 2015). Once dispersed, seedling establishment, survival and development depend on the environmental characteristics of the settlement area, such as depth, substratum type, hydrodynamic conditions or erosion events (Piazzi et al., 1999, Orth et al., 2006b; Infantes et al., 2011a; Alagna et al., 2013). In fact, the period between seed germination and seedling establishment is one of the most vulnerable phases for plant development, undergoing the highest mortality rates (Orth et al., 2006b; Alagna et al., 2013). Thus, elucidating the interactions between hydrodynamics and substratum type affecting the dispersal of seagrass propagules is necessary to understand natural colonisation processes and, in particular, secondary dispersal (*sensu* Chambers and McMahon, 1994) of seagrasses species which have floating propagules stages. In order to identify safe-recruitment microhabitats and the establishment potential of seagrass seedlings in different substrata, it is essential to consider these processes from a fluid dynamics perspective.

The present study attempts to understand how hydrodynamics and substratum types affect the transport and establishment of propagules of different seagrass species. We focus on three temperate species that have different morphological and physical traits that will likely affect their interaction with hydrodynamics and substratum: *Posidonia oceanica* (endemic to the Mediterranean Sea), *Cymodocea nodosa* (representative for the Mediterranean Sea and the Atlantic coast of North Africa) and *Zostera marina* (widespread in northern latitudes; Green and Short, 2003). *P. oceanica* and *Z. marina* produce floating propagules (fruits and reproductive shoots, respectively), which are transported by the surface currents until they dehisce and the seeds are released. In contrast, the fruits of *C. nodosa*, which are negatively buoyant, develop buried at the shoot base and are released within the meadow, although they may be exported during sediment erosion events, such as storms. As the seeds of the three species are negatively buoyant and fall to the bottom, their dispersal will depend on hydrodynamic processes occurring within the bottom boundary layer (Orth et al., 1994; 2006b). Hereafter, their secondary dispersal depends on their propagule physical characteristics and the interaction between hydrodynamic conditions and substratum type (Infantes et al., 2011a, b; Ruiz-Montoya et al., 2012; 2015; Alagna et al., 2015). In this study we specifically aimed to: 1) estimate transport velocities of floating-propagules of *P. oceanica* and *Z. marina* at different current velocities, 2) determine the minimum flow velocities at which seeds and seedlings of the three species start moving over the substratum (threshold velocities) and 3) estimate the capacity of different substrata to retain propagules (trapping) and how this process is influenced by the propagules' physical characteristics.

4.1.3. **Materials and methods**

4.1.3.1. **Seed collection and physical properties**

Seeds of *Posidonia oceanica* (Fig.12a) were obtained from fruits collected on the shore during June 2015 in Mallorca (Balearic Islands, Western Mediterranean Sea). Fruits were opened and their seed removed and placed in 20L aquariums with seawater (salinity, 37), at a temperature of 20ºC and illuminated by white fluorescent lamps that provided 76 ± 2 µmol. m^{-2} s^{-1} of photosynthetically active radiation in a 14/10 h light/dark photoperiod. Seeds successfully germinate under these conditions and first leaves and roots are produced within two weeks (Terrados et al., 2013). In addition, fruits collected in June were also conserved without opening in seawater under the same conditions.

Seeds of *Cymodocea nodosa* (Fig.12b), which are typically buried in the sediment, were collected during February 2015 in Mallorca from shallow meadows by SCUBA.

They were placed in darkness inside 20L aquariums under the same above-mentioned conditions. All seeds were transported to the Lovén Centre for Marine Science, Kristineberg station, Sweden, and were kept in seawater aquariums under the same conditions as explained above. Seeds of *Zostera marina* (Fig. 12c) were collected by harvesting reproductive shoots at 1-3 m depth in the Gullmars fjord, Gåsö in Sweden, in July 2015. Reproductive shoots were stored in outdoor tanks at Kristineberg until the seeds were released. *Z. marina* seeds were stored at salinity 34 and temperature of 5°C to prevent germination (Infantes et al., 2016).

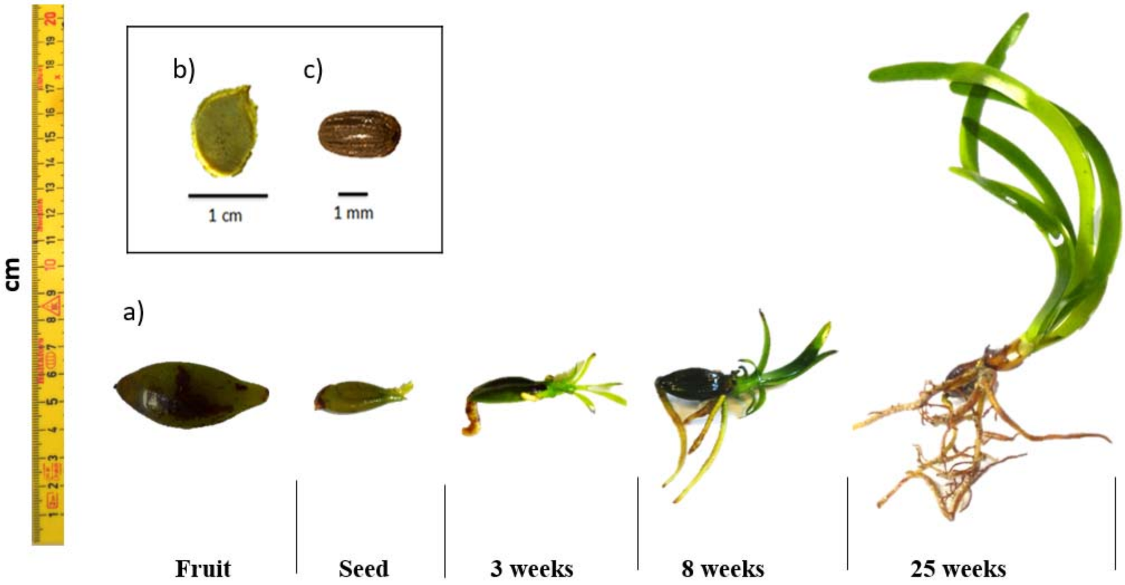


Figure 12. *Posidonia oceanica* (a) early life stages: fruit and seeds (0 weeks old), and seedling at 3 weeks, 8 weeks and 25 weeks of age, seeds of (b) *Cymodocea nodosa* and (c) *Zostera marina.*

Physical characteristics of fruits, reproductive shoots, seeds and seedlings were measured and compared, since they influence propagule transport and trapping (Koch et al., 2010; Infantes et al., 2011a; Ruiz-Montoya et al., 2012). Propagule length, width and height were measured using an electronic precision caliper (mm) and the volume (ml) was determined by fluid displacement in a 12-mL graduated cylinder. Propagule mass was measured as wet weight (g). The density of each fruit, reproductive shoot, seed or seedling, was calculated from mass and volume measurements. The number of spathes was measured for *Z. marina* reproductive shoots. The number and length of leaves, width of the second youngest leaf and root number and length were measured for all *P. oceanica* seedlings.

Settling velocities (w_s) of seagrass propagules (n=10) were estimated as the time needed to sink to the bottom in a vertically placed glass tube (70 cm long and 20 cm diameter) filled with seawater. Each measurement was repeated 3 times for each individual propagule and the mean value was calculated. Propagule drag coefficient (C_d) was calculated following Dean and Dalrymple (2004) as:

$$C_d = 2\left(\frac{\rho_s - \rho}{\rho}\right)\frac{gV_s}{w_s^2 A_F}$$

where ρ_s is the density of the propagule, ρ is the density of seawater, g is the acceleration of gravity, V_s is the volume of the propagule and A_F is the estimated exposed propagule frontal area exposed to the flow.

Seed frontal area (A_F) for *Z. marina* and *P. oceanica* was determined assuming an elliptical shape with semi axes a and b, (i.e. $A_{F\,SEED} = \pi \cdot a \cdot b$). In contrast, the frontal area of *C. nodosa* seeds was estimated multiplying its maximum length by its width since their shape resembles a flat disc (Fig. 12b). The frontal area of *P. oceanica* seedlings was determined by adding the foliar surface (estimated as a rectangular shape) to the area of the seed (approximated to an ellipsoid) and the area of roots (obtained by multiplying the root diameter by the total root length), i.e., $A_{F\,SEEDLING} = A_{F\,SEED} + (\sum_{i=1}^{n} l_i * w_i) + (d_r \cdot tl_r)$, where l and w are the length and width of each leaf, n is the number of leaves, d_r the root diameter and tl_r the total root length. Seawater density used for the calculations was taken as a constant 1025 kg m^{-3} (equivalent to the value at 20ºC).

In order to evaluate the effect of the substratum type on the boundary layer, we estimated the boundary layer thickness assuming the flow turbulent as:

$$\delta = \frac{0.37x}{Re^{\frac{1}{5}}}$$

where Re is the Reynolds number and x is the distance downstream from the start of the boundary layer.

4.1.3.2. Flume description and flow-substrate characterization

Hydrodynamic conditions were simulated in a flume to identify the threshold velocities of the propagules to initiate movement and to be retained by the substrata. A unidirectional (current) flume was used to simulate the horizontal transport of propagules such as dispersal and trapping. A flume was used to identify threshold velocities and propagule trapping since it allows modifying one variable (substratum) while leaving other ones constant (e.g. flow). Current velocities from 5 to 30 cm s^{-1}, typical for the locations where the propagules were collected (Balbín et al., 2012; Anthony and Svane, 1994), were applied in a hydraulic flume at Kristineberg Station. The flume was 4 m long, 0.5 m wide and 0.5 m depth (Fig. 13). The test section was 2 m long and the water level was maintained at 0.15 m. Flow velocities were generated by a motor-run propeller at the far end of the flume controlled by an adjustable speed drive (Dayton Electronic, model 6K119). The measurements were conducted during two periods, July and October 2015 in order to examine the properties of different plant developmental stages.

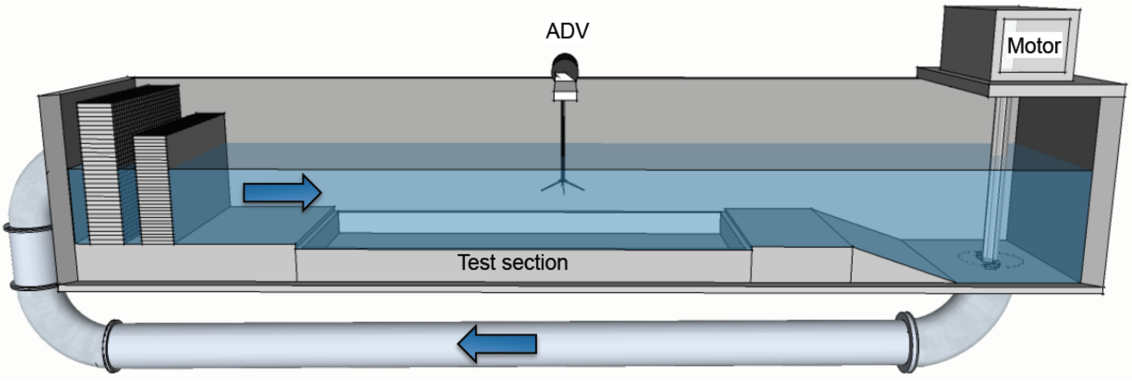


Figure 13. Diagram of the hydraulic flume (by Selwyn Hoeks).

Flow velocities were measured with an Acoustic Doppler Velocimeter, ADV (Nortek, Vectrino) at a sampling rate of 25 Hz. Vertical velocity profiles were measured over different substratum at 6 positions (0.5, 1, 2, 3, 5 and 7 cm above the bottom) before and after the test section. Percentage of flow reduction due to substratum type was calculated from the flow before and after the test section applying a current velocity of 20 cm s^{-1} in order to ensure that all propagules had initiated their movement (see Results).

4.1.3.3. Transport of fruits and reproductive shoots

Transport velocities of floating propagules, i.e. *P. oceanica* fruits and *Z. marina* reproductive shoots, were measured in the unidirectional flume. The surface flow velocity was measured at 1-2 cm below the water surface with the ADV positioned upwards. Floating-propagules of each species (n=10) were placed independently on the water surface and their velocity was assessed by recording the time needed to travel along one meter at different flow velocities (from 5 to 30 cm s^{-1}).

4.1.3.4. Threshold velocities for transport and trapping measurements

Threshold velocities, defined as the flow velocity at which a propagule initiates and maintains a continuous movement along the whole test section, were measured for each of the three seed species and for four different developmental stages of *P. oceanica* propagules (i.e., seeds, 3 week-, 8 week- and 25 week-old seedlings). Propagules were separately placed over a flat sand bed prepared in the flume. The water flow was increased at 1 cm s^{-1} intervals until they started to move. The velocity of the propagule was estimated measuring the time needed to cross 1 m distance once it had initiated the movement at different flow velocities. Trials were repeated ten times (using different propagules each time) for each propagule type and the mean threshold velocity values were calculated.

Trapping was defined as the substratum capability to retain *P. oceanica* seeds and seedlings, and it was calculated by measuring the traveled distance of the propagules over a specific substratum type under different flow velocities. Three common substratum types were evaluated: sand (0.25-0.50 mm in diameter), coarse gravel (in the range of 5 x 3 x 2 cm), and matte (i.e. a network of rhizomes from dead plants) of *P. oceanica*. We constructed a model of matte by burying in the sand different numbers of orthotrophic (i.e. vertical growth) fragments of *P. oceanica* rhizomes collected from beach-cast plants after a storm. Six different matte types were built combining three dead shoot abundances (75, 150 and 300 shoots per m^2) and two heights above sand (1 and 4 cm). These shoot abundances represent the range of shoot density values found in highly degraded meadows (Pergent et al., 1995).

In order to recreate the propagule transport over the substratum types at several flow velocities, we used a 2 m length plastic box fixed at the bottom of the flume containing the substrate analyzed (Fig. 13). Seeds and seedlings were placed over a 10 cm thick, flat sand bed of 1 m longitude that preceded the box with the specific substratum. This enabled the propagules to develop an initial movement and continue over the 100 cm of the corresponding substrata. We measured the distance traveled and the velocity of the propagules from the beginning of the test section (containing a specific substrate) until the propagule was trapped or until it passed over the entire test section without being trapped.

Trapping results are presented depending on the difference between the length of the test section and the travelled distance by the propagules. If the travelled distance was equal to the total length of the test section, the trapping was considered zero, while trapping was maximum (i.e. 100) if the propagule was retained at the beginning of the test section. Substrata were ranked according to their complexity, from the lowest complexity (i.e. sand), along the different matte substrata of increasing rhizome abundance and height, to the highest (i.e. coarse gravel). The complexity of matte was calculated as the number of rhizomes m^{-2} x rhizome height (cm). For coarse gravel, complexity was calculated as the number of rocks m^{-2} x rock height. Complexity assigned to sand was zero. Therefore, substratum complexity increased from sand (0) to matte with rhizomes of 1 cm high (75*1 = 75, 150*1 = 150, 300*1 = 300), 4 cm high (75*4 = 300, 150*4 = 600, 300*4 = 1200) and coarse gravel (1500).

One-way analysis of variance (ANOVA) was used to compare physical characteristics and threshold velocities among seeds of the different species and among *P. oceanica* early life stages. An analysis of covariance (ANCOVA) was used to compare the slopes of the two regression lines obtained from the data on floating propagule velocity (dependent variable) of the two species analysed (categorical factor) in relation to the surface flow velocities (continuous co-variable). There were done two regression lines to evaluate the relation between *Z. marina* morphological characteristics (length and number of shoots) and their velocity. A factorial ANOVA was used to evaluate the effects on trapping of *P. oceanica* propagules regarding two factors: substratum type (with eight levels: sand, six different *P. oceanica* matte treatments, and coarse gravel) and life stage (with four levels: seeds and seedlings of 3, 8 or 25 weeks of age). For a better comparison between the treatments, this analysis was conducted using the results obtained with a flow velocity of 20 cm s^{-1}, since at this velocity all the propagules moved. To examine differences between levels of each factor, a post-hoc Tukey test was conducted. Data were analysed after ensuring normality and homogeneity of variances using Kolmogorov-Smirnov and Cochran's tests.

4.1.4. **Results**

A linear relationship between floating-propagule velocity and the surface flow velocity was found for *Posidonia oceanica* fruits and *Zostera marina* reproductive shoots, which was different between the two species (Fig. 14; ANCOVA: $F_{(1,7)} = 7.6054$; $p < 0.05$).

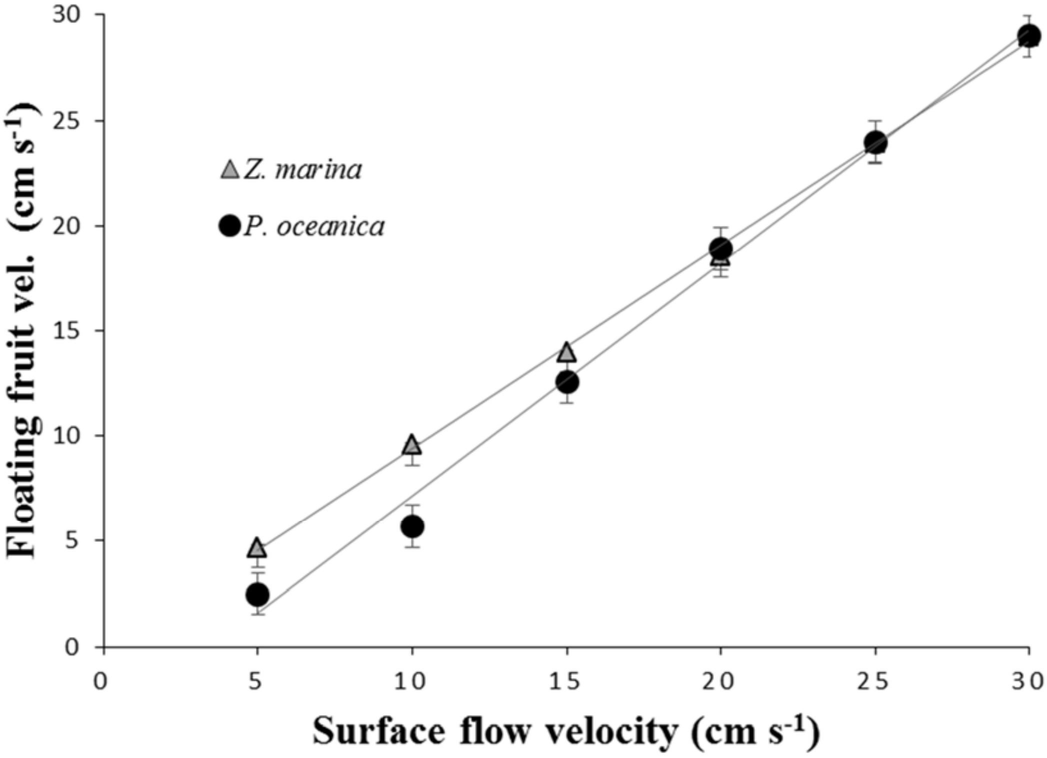


Figure 14. Velocity of *Zostera marina* reproductive shoots ($y = 0.92x + 0.24; R^2 = 0.99$) and *Posidonia oceanica* fruits ($y = 1.12x - 4.11; R^2 = 0.98$), transported by different flow velocities.

At low flow velocities (5 and 10 cm s^{-1}) *P. oceanica* fruits, whose density is lower than water (Table 1), emerge and move at half the flow velocity of the water (2.5 and 5.7 cm s^{-1}, respectively). In contrast, at higher flow velocities (15 and 20 cm s^{-1}) the fruit velocity resembles the flow velocities of the water (12.6 and 18.9 cm s^{-1}, respectively). In contrast, *Z. marina* reproductive shoots move almost at the same velocity as the water flow throughout all the range of measured velocities (Fig. 14). The length of *Z. marina* reproductive shoots varied from 20 to 135 cm and the number of spathes between 4 and 35 per shoot, and their mean weight, volume and density were 7.4 ± 1.4 g, 9.9 ± 1.4 cm^3 and 0.74 ± 0.05 g cm^{-3}, respectively. We did not find a significant relationship between the length of *Z. marina* reproductive shoots and their velocity ($R^2 = 0.33$); nor between the number of shoots and their velocity ($R^2 = 0.43$).

Properties	***P. oceanica*** (Po)	***C. nodosa*** (Cy)	***Z. marina*** (Zo)	**F (significance)**	**Tukey test**
Length (cm)	2.08 ± 0.67	1.02 ± 0.34	0.33 ± 0.07	2033.53**	Po>Cy>Zo
Width (cm)	0.83 ± 0.10	0.64 ± 0.16	0.152 ± 0.04	6930.53**	Po>Cy>Zo
Height (cm)	0.49 ± 0.07	0.14 ± 0.04	0.152 ± 0.04	6599.40**	Po>Cy>Zo
Weight (g)	0.65 ± 0.02	0.078 ± 0.01	0.0073 ± 0.001	674.17**	Po>Cy>Zo
Volume (cm^3)	0.58 ± 0.05	0.075 ± 0.001	0.007 ± 0.001	275.44**	Po>Cy>Zo
Density (g cm^{-3})	1.12 ± 0.04	1.035 ± 0.001	1.065 ± 0.001	2617352**	Po>Zo>Cy
Settling velocity (cm s^{-1})	9.73 ± 0.51	6.78 ± 0.124	7.44 ± 0.18	1865.28**	Po>Zo>Cy
Frontal area (cm^2)	5.42 ± 0.06	0.147 ± 0.003	0.039 ± 0.001	10023886**	Po>Cy>Zo
Drag coefficient	0.21 ± 0.03	0.18 ± 0.002	0.24 ± 0.005	474348.7**	Zo>Po>Cy

Table 1. *Posidonia oceanica*, *Cymodocea nodosa* and *Zostera marina* physical characteristics (n=10). The 2nd, 3rd and 4th column represent the mean and SE values of the measured properties. The 5th column is the resultant F value of a one-way ANOVA analysis comparing properties across seeds of the different species. P-values < 0.001 are denoted as **.

Seed size strongly differed amongst seagrass species, with *P. oceanica* exhibiting the largest frontal area, density, settling velocity and volume, while *Cymodocea nodosa* had higher frontal area, weight and volume than *Z. marina* (Table 1, Tukey). Density and settling velocity of *Z. marina* seeds were higher than those of *C. nodosa*, and drag coefficient of *Z. marina* was highest, followed by *P. oceanica* and *C. nodosa* (Table 1, Tukey). Regarding *P. oceanica* early life stages, as propagules grew, their frontal area, weight, volume and drag coefficient increased while their settling velocities decreased (Table 2, Tukey).

				Seedling			
Properties		**Fruit**	**Seed**	**3 weeks**	**8 weeks**	**25 weeks**	**F (Sig.)**
Seed or Fruit (cm)	Length	3.32 ± 0.73	2.08 ± 0.07	1.97 ± 0.18	1.88 ± 0.17	1.89 ± 0.12	6448.40**
	Width	1.24 ± 0.30	0.83 ± 0.05	0.83 ± 0.01	0.92 ± 0.05	0.97 ± 0.08	6381.21**
	Height	1.33 ± 0.32	0.49 ± 0.02	0.52 ± 0.04	0.61 ± 0.03	0.58 ± 0.01	2383.02**
Leaves	Area (cm^2)			1.04 ± 0.04	7.52 ± 0.72	25.8 ± 2.73	322.76**
	Max. length (cm)			1.24 ± 0.04	3.57 ± 0.29	13.0 ± 1.31	578.96**
Roots (cm)	Number			5.21 ± 0.13	8.21 ± 0.29	7.80 ± 0.24	2733.08**
	Total length			1.64 ± 0.12	5.60 ± 0.18	40.0 ± 2.78	574303**
Whole propagule	Weight (g)	2.34 ± 0.15	0.65 ± 0.02	0.86 ± 0.03	1.89 ± 0.05	4.70 ± 0.16	1532.79**
	Volume (cm^3)	2.66 ± 0.14	0.58 ± 0.05	0.80 ± 0.02	1.81 ± 0.01	4.54 ± 0.23	2509.94**
	Density (g/cm^3)	0.87 ± 0.01	1.12 ± 0.04	1.11 ± 0.03	1.09 ± 0.01	1.08 ± 0.03	1498.41**
	Settling vel. (cm/s)		9.73 ± 0.06	9.56 ± 0.09	6.16 ± 0.12	5.28 ± 0.32	1478.65**
	Frontal area (cm^2)		5.42 ± 0.51	5.76 ± 0.42	6.90 ± 0.38	15.2 ± 0.21	267793.7**
	Drag coefficient		0.21 ± 0.03	0.24 ± 0.02	0.86 ± 0.05	1.17 ± 0.05	546.11**

Table 2. *Posidonia oceanica* early life stages physical characteristics (n=10). The statistics column contains the resultant F value of a one-way ANOVA analysis comparing properties across early life stages. P-values < 0.001 are denoted as **.

The minimum current velocity needed to move seeds or seedlings at the bottom (threshold velocities) was lower for *Z. marina* seeds (14 cm s^{-1} ± 0.01 SE) followed by *P. oceanica* seeds (which started at 20 cm s^{-1} ± 0.03 SE) and *C. nodosa* seeds (21 cm s^{-1} ± 0.01 SE; Fig. 15a. $F_{(2,27)} = 2019.6$; $p < 0.001$).

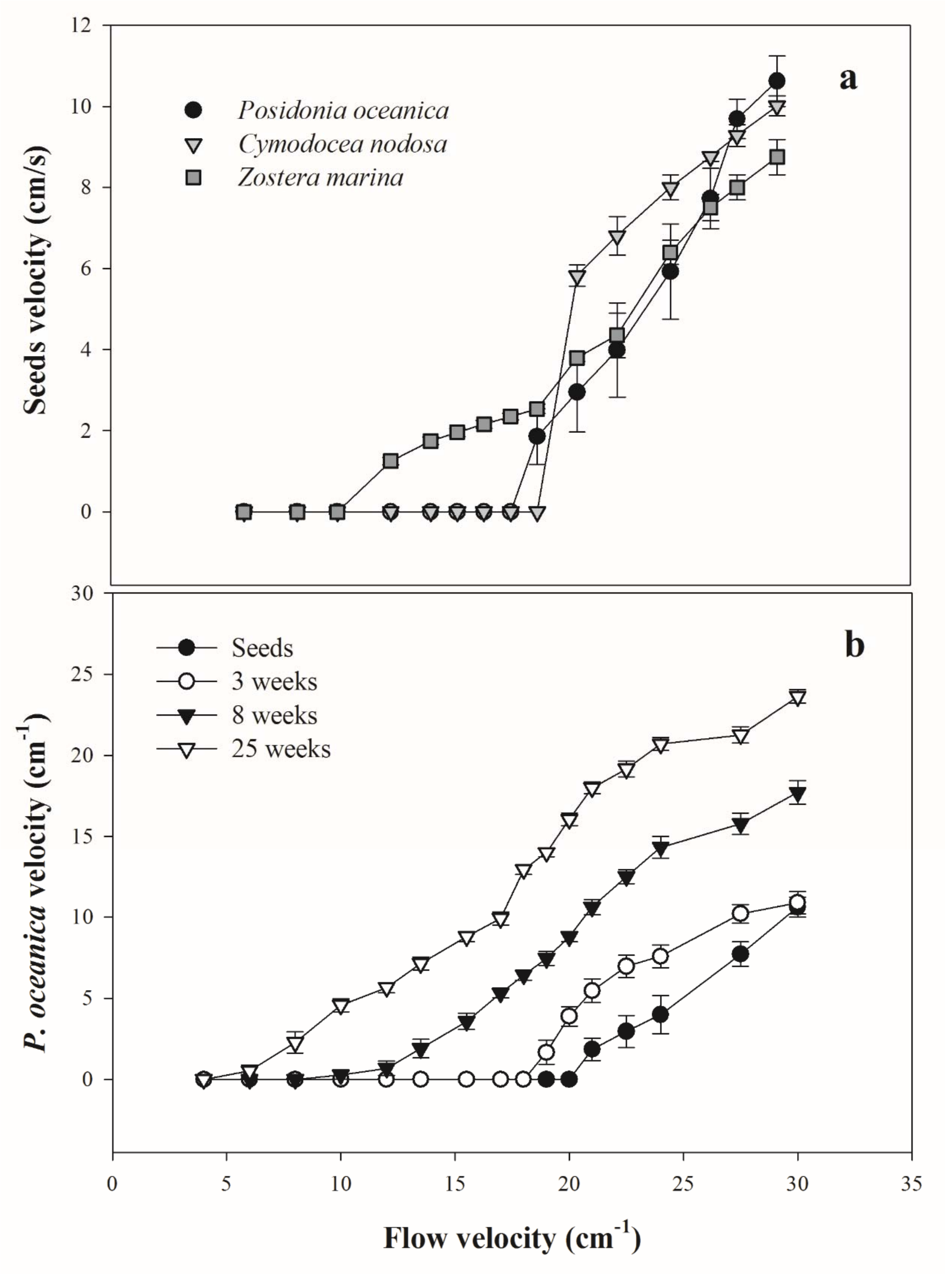


Figure 15. Threshold velocities of a) *Posidonia oceanica* (black circles), *Cymodocea nodosa* (grey triangles) and *Zostera marina* (black squares) seeds and b) *P. oceanica* early life stages. Mean ± SE.

Regarding *P. oceanica* early life stages, 25-week-old seedlings were the first to move (4 cm s^{-1} ± 0.3 SE), followed by 8-week-old seedlings (7 cm s^{-1} ± 0.2 SE), 3-week-old seedlings (17.5 cm s^{-1} ± 0.04 SE) and seeds (20 cm s^{-1} ± 0.06SE; Fig. 15b. $F_{(3,36)}$ = 1893.6; $p < 0.001$). Threshold velocities decreased linearly with drag coefficient for every seed species and developmental stage of *P. oceanica* (Fig. 16a).

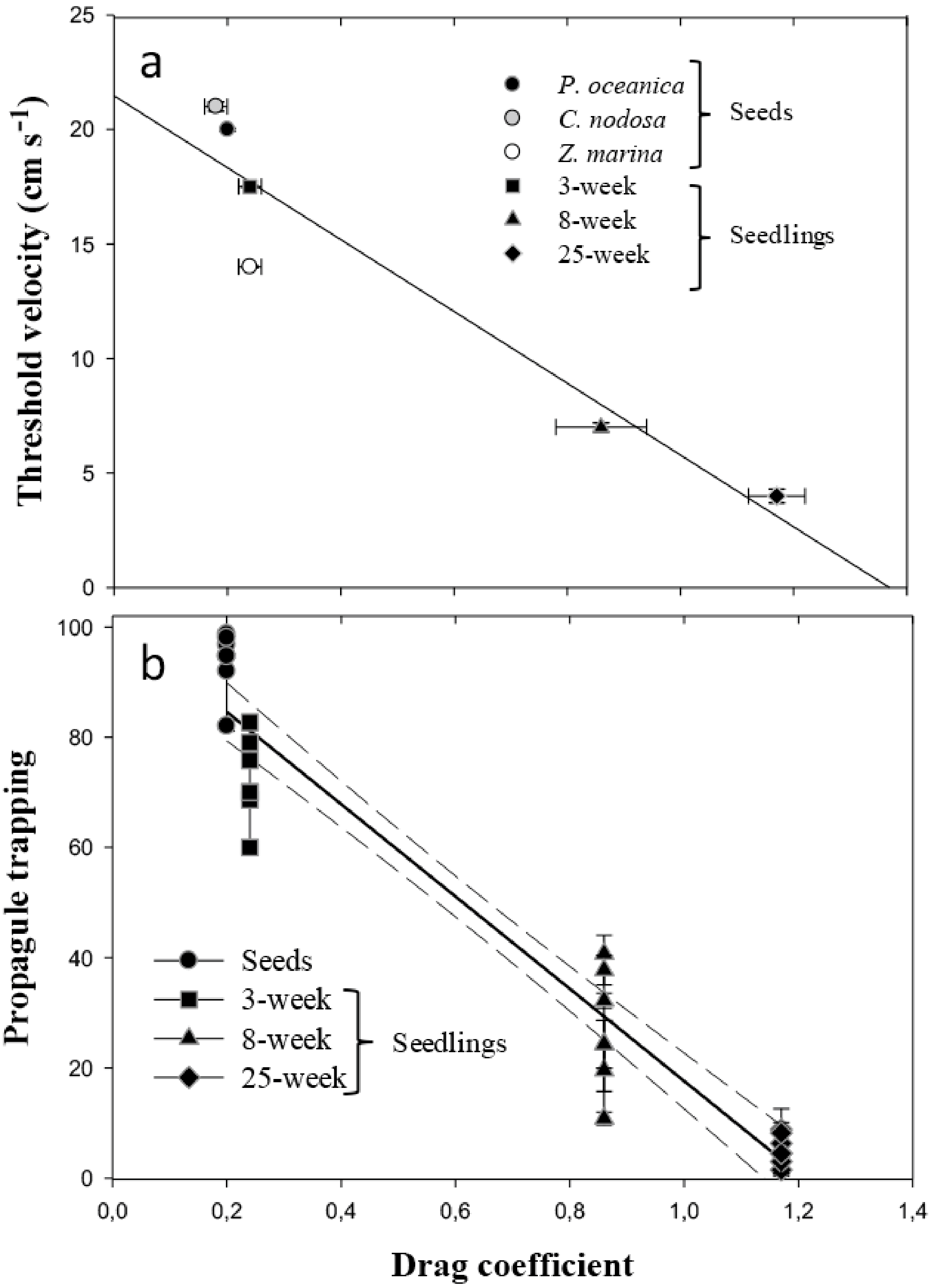


Figure 16. Drag coefficient related to a) threshold velocities over a flat sandy bottom for *Posidonia oceanica* (black circles), *Cymodocea nodosa* (grey circles) and *Zostera marina* (black circles) seeds ($y = 21.47x - 15.68; R^2 = 0.90$); and b) trapping for the four stages of *P. oceanica* by each substrata at 20 cm s^{-1}, ($y = 101.31x - 83.71; R^2 = 0.93$). Mean ± SE.

Trapping differed across substratum (S) and propagule (P) types (significant S x P interaction; Table 3). All *P. oceanica* seeds and seedlings went through the sand substratum without being trapped, whereas trapping decreased with flow velocity for all other substrata analysed, being maximum at flow velocities below 7 cm s^{-1} for all the propagules.

Factor	df	MS	F	Tukey test
Early life stage (A)	3	32359.4	2467.67**	25W<8W<3W<Seeds
Substratum type (B)	7	35230.2	2686.59**	S<M1<M2=M5<M3<M4<M6
A*B	21	1810.7	138.08**	

Table 3. Results of the factorial ANOVA analysis for evaluate early life stages and substratum type effects on trapping and the post-hoc Tukey test for evaluate differences between means. 25W, 8W, 3W represents 25-8-3 weeks old seedlings of *Posidonia oceanica* respectively, and S=Sand, M1-M2-M3-M4-M5-M6 represents the 6 combinations of matte substrata = 75 (1) - 75 (4) – 150 (1) - 150 (4) - 300 (1) - 300 (4) being: rhizome abundances (rhizomes height) respectively.

Seeds always underwent higher trapping than seedlings, and seedling trapping decreased with increasing age (Table 3). Trapping of propagules by matte and coarse gravel decreased linearly $(y = 101.31x - 83.71; R^2 = 0.93)$ with higher drag coefficients (Fig. 16). Trapping increased with rhizome abundance and height (Fig. 17). Over coarse gravel, all seeds and seedlings were trapped independently of flow velocity, except for the 25 week old seedlings which were not trapped at velocities higher than 16 cm s^{-1} (Fig. 18).

Flow reduction was higher over substrata of higher complexity such as 4-cm-height matte and coarse gravel. The flow reduction over sand was minimal, while over coarse gravel it was reduced between 20 - 90 % in the first 3 cm above the bottom (Fig. 19a). Flow reduction over 1-cm-matte varied between 10 - 40 % in the first 2 cm above the bottom (Fig. 19b), while over 4-cm-matte it varied between 10 - 60 % in the first 5 cm (Fig. 19c). The boundary layer thickness (δ) increased with substratum complexity, reaching a value nearly two times higher over coarse gravel than over the other substrates (Table 4).

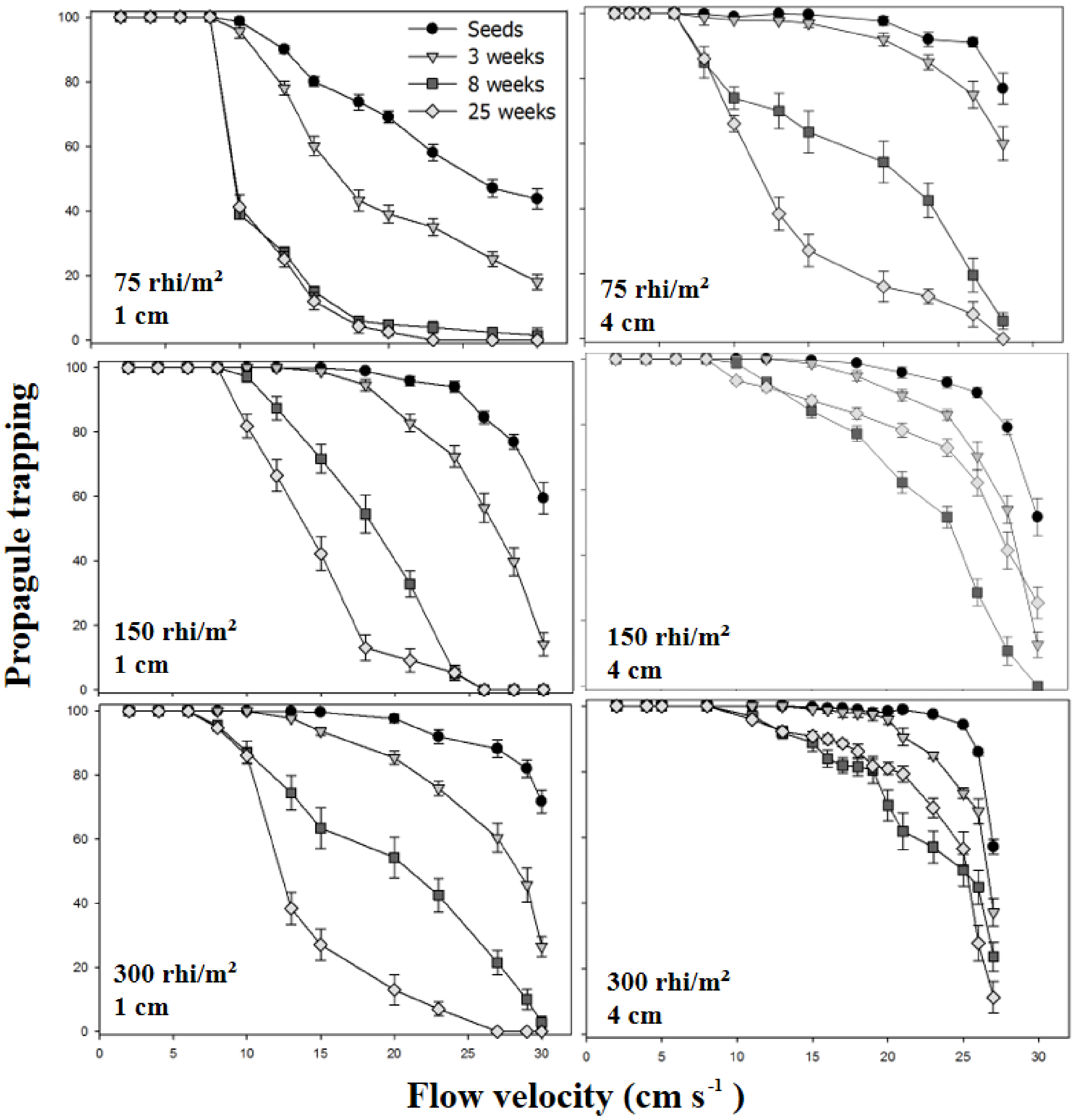


Figure 17. Trapping for the four stages of *Posidonia oceanica* over matte substrata of different dead shoot abundances and heights. Mean ± SE.

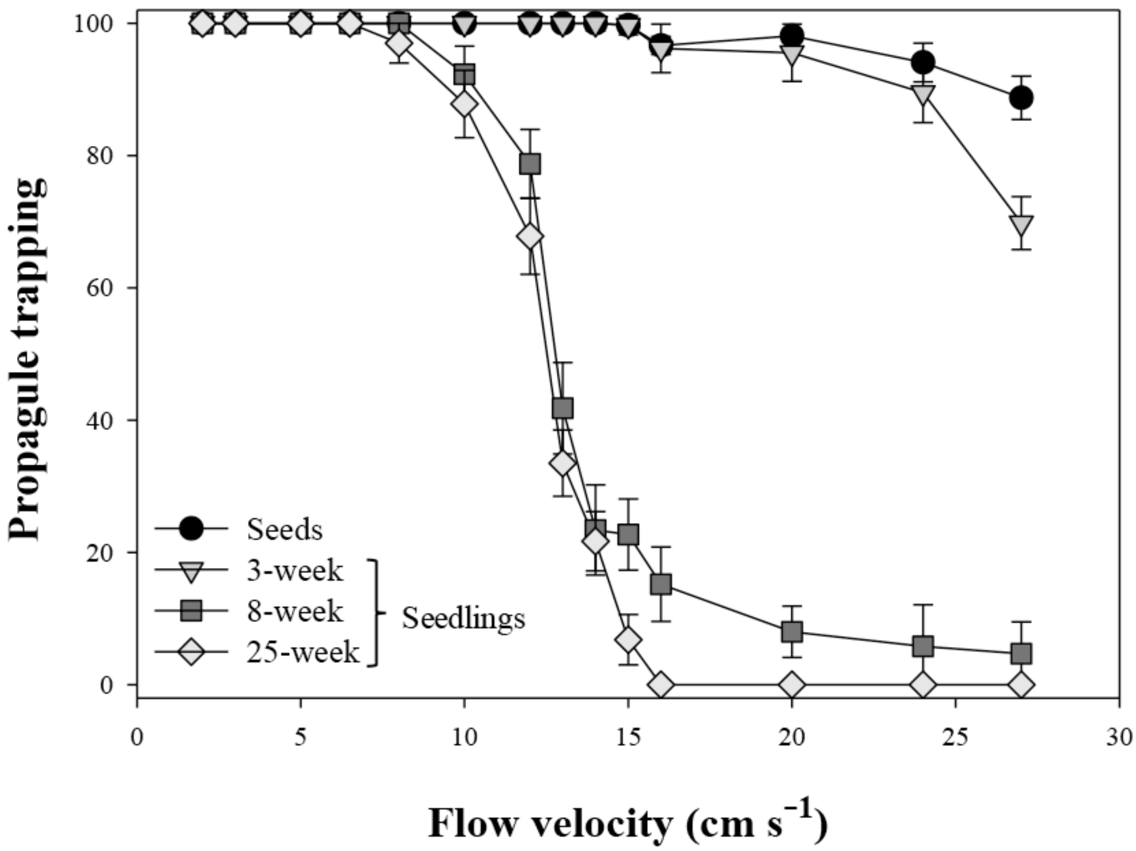


Figure 18. Trapping for the four stages of *Posidonia oceanica* over coarse gravel substrata. Mean ± SE.

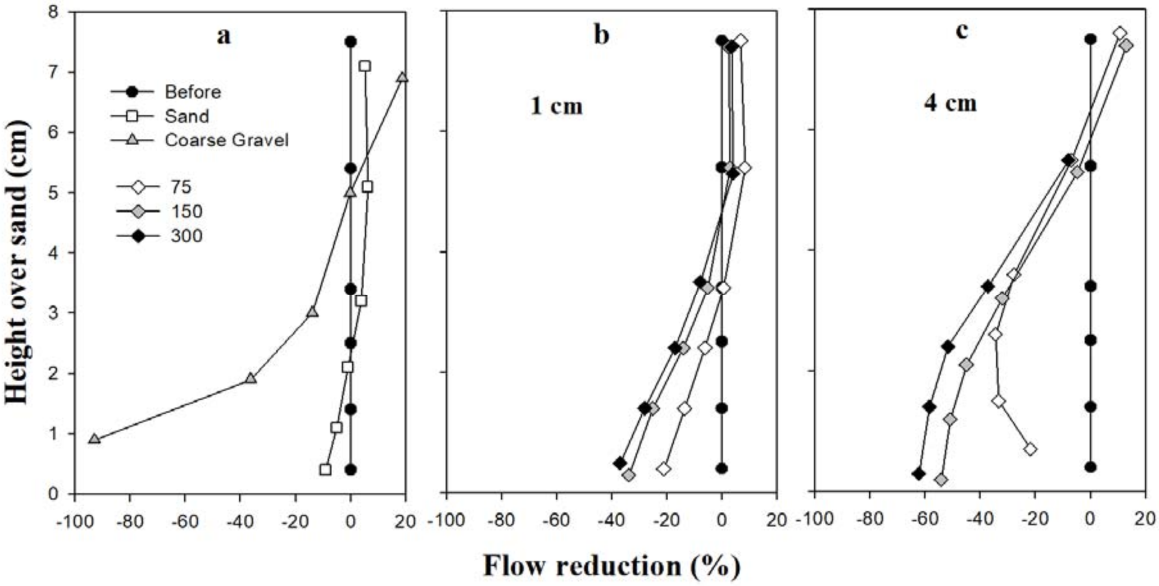
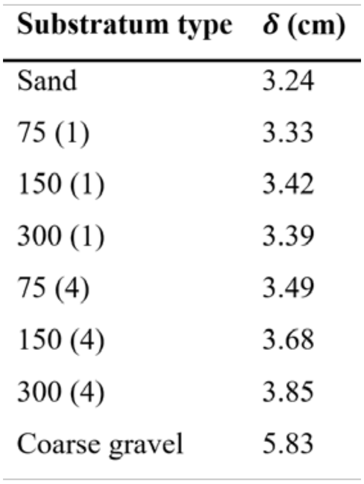


Figure 19. Percentage of flow reduction in a 20 cm s^{-1} flow compared between sand and coarse gravel (a), rhizomes of 1 cm height (b) and rhizomes of 4 cm height (c). Three rhizomes abundances were used (Rhi = 75, 150 and 300 dead shoots m^{-2}). “Before” represents the flow over sand at the beginning of the flume.



Substratum type	δ (cm)
Sand	3.24
75 (1)	3.33
150 (1)	3.42
300 (1)	3.39
75 (4)	3.49
150 (4)	3.68
300 (4)	3.85
Coarse gravel	5.83

Table 4. Boundary layer thickness at 1 m downstream for the different substratum types: sand, the 6 combinations of matte substrata = 75 (1) - 75 (4) – 150 (1) - 150 (4) - 300 (1) - 300 (4) being: rhizome abundances (rhizomes height) respectively, and coarse gravel. Velocity flow was for all experiments 20 cm s^{-1}.

4.1.5. Discussion

Our experiments highlight the existence of important inter- and intraspecific differences on how hydrodynamics and substratum type determine the movement of seagrass propagules. Such differences are strongly influenced by the different physical characteristics of the three species examined. Overall, trapping of seagrass propagules increases with bottom complexity and boundary layer thickness as the different substrata generate different flow reduction. The threshold velocities needed to start propagule movement decrease with developmental stage and their drag coefficient. Furthermore, notable differences were observed between *Posidonia oceanica* seeds and seedlings, as drag coefficient increased with developmental stage, hampering trapping and indicating that younger stages have a hydrodynamic shape which enhances their establishment in comparison to older ones. Therefore, if *P. oceanica* seedlings cannot successfully colonize a new substrate in the first few days of development, their capacity of being trapped and thus their establishment ability might become limited by the modification of their physical characteristics as they grow.

The velocity of floating-propagules (i.e. fruits and shoots) varies in proportion to the surface current velocity and differs between species. While reproductive shoots of *Zostera marina* move nearly at the same velocity than the surface current, *P. oceanica* fruits move slower than the surface current when flow velocities are low (< 10 cm s^{-1}). Such reduction for the fruits may be because part of the fruit emerges from the sea surface while part of it is submerged, therefore interacting with the sea-air boundary layer and slowing the overall speed. Velocities achieved by *Z. marina* and *P. oceanica* propagules might be related to their different interaction with the surface boundary layer, being *Z. marina* less emerged than *P. oceanica*, having therefore higher velocities. We did not manipulate wind conditions, but in addition to the current velocity, wind also contributes to propagule dispersal, allowing fruits to move faster than the surface currents, which allows fruits to be transported at rates ca 2 - 4 % of the wind velocity (Källström et al., 2008; Ruiz-Montoya et al., 2012). Further research is necessary to assess the combined effects of wind, waves and currents on floating-propagules in order to further assess dispersal of floating propagules.

Threshold velocity for seeds of *Z. marina* have been estimated as 8 cm s^{-1} over a flat sandy bottom (Orth et al., 1994), which is nearly half the velocity that we measured (14 cm s^{-1}). Such differences may be explained by the fact that during the measurements by Orth et al. (1994) only some of the *Z. marina* seeds tested started to move, and it was not a continuous movement. Moreover, the substratum that they used was slightly different to ours, a false bottom composed of sandpaper and sand (0.4 - 0.425 mm in diameter). Interestingly, *P. oceanica* and *Cymodocea nodosa* seeds required about 50 % higher threshold velocities than *Z. marina*, and this pattern is likely explained by the difference in seed physical characteristics (e.g. frontal area exposed to the flow) of the three species, providing *Z. marina* with a higher drag coefficient than *P. oceanica* or *C. nodosa*. The higher floating velocities of reproductive spathes and the lower threshold velocities of seeds could contribute to explain the wide geographical range that *Z. marina* colonizes in comparison to the other two species (Green and Short, 2003). Furthermore, under current and future scenarios of local and global human stressors (e.g. eutrophication, global change), these physical traits may provide an advantage in future scenarios of environmental impacts as *Z. marina* may be able to colonize more places and thus have a larger potential for escaping to other areas with better environmental conditions.

Seeds of *P. oceanica* required higher threshold velocities than older seedlings, likely as a result of their lower drag coefficient. The threshold velocity decrease observed from seeds to seedlings has also been reported for other aquatic macrophytes such as *Ruppia maritima, Potamogeton perfoliatus* and *Stuckenia pectinate* (Koch et al., 2010), and they are in the same order of magnitude as the ones we have determined for *P.*

oceanica. These species increase their drag after germination and consequent organ development, and some seedlings actually acquire buoyancy, hampering their establishment (Koch et al., 2010). Drag coefficient is a good proxy for propagule movement, since a higher value results in decreasing propagules' threshold velocities and trapping success, yet estimations of drag coefficient still remain scarce for most species of marine vascular plants (Backhaus and Verduin, 2008; Infantes et al., 2011a; Ruiz-Montoya et al., 2012).

The positive correlation between substratum complexity and trapping indicates that complex substrata, such as 4-cm-height matte and coarse gravel, are more capable of retaining propagules. Indeed, sand (which we had given a value of 0 complexity) was not able to retain propagules at any of the tested velocities, while matte and coarse gravel, with higher complexity, were able to trap some of them depending on current velocity and propagule characteristics (Fig. 20).

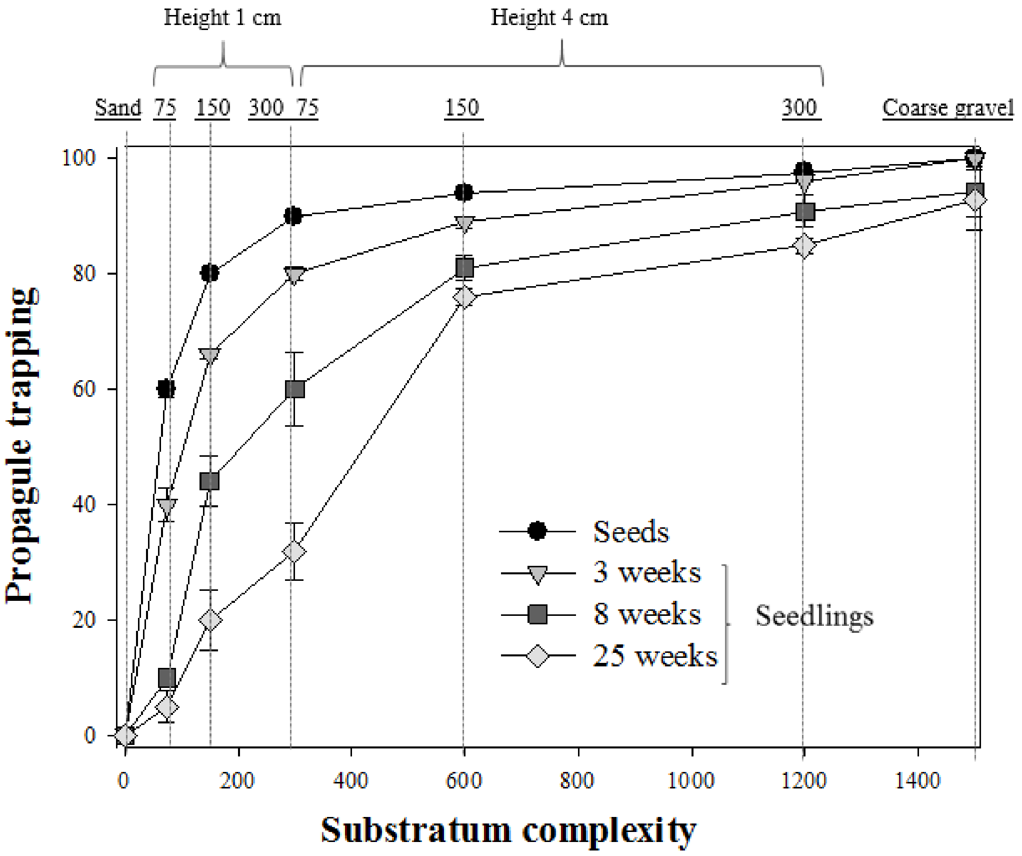


Figure 20. Relation between trapping of *Posidonia oceanica* seeds and seedlings and substratum complexity in a 20 cm s^{-1} flow.

Our first trapping measurements over matte were conducted at shoot abundances of 300 m^{-2}, which is equivalent to a highly degraded meadow (Pergent et al., 1995) and we observed seed and seedling trapping of almost 100 %. On account of these results, we decreased rhizome abundance and modified rhizome height to evaluate the trapping by matte at further stages of deterioration. Here we provide the first experimental evidence of the trapping variability of *P. oceanica* propagules by matte depending on its complexity, and our results highlight that rhizome height contributes more strongly to trapping than the actual shoot numbers, suggesting that even highly degraded meadows could allow for recovery if enough complexity is provided by matte. Indeed, matte is considered a favourable substratum for the establishment of *P. oceanica* seedlings, both naturally and for transplanting (Balestri et al., 1998; Piazzi et al., 1999; Terrados et al., 2013). In comparison to sand, coarse gravel was the substratum with the highest complexity and almost all propagules were trapped independently of the flow velocity. While *P. oceanica* seedlings are able to establish in gravel, they do not appear to survive on this substrate in the long term, probably because of damage and abrasive action caused by the gravel particles (Piazzi et al., 1999). Similarly, seedlings are also able to establish in sand (Balestri and Lardicci, 2008), but their survival in the long term is unclear (Infantes et al., 2011a). In consolidated substrata such as matte or rocks, higher survivorship of *P. oceanica* seedlings is often observed in nature (Balestri et al., 1998; Piazzi et al., 1999; Alagna et al., 2013; Terrados et al., 2013), which is in accordance with our results of higher trapping.

In addition to substratum type and its associated complexity, we also detected that other factors will be important in influencing seagrass propagules transport and trapping. A relevant finding was the positive correlation between flow reduction over complex substrata and propagule trapping. At high substratum complexity, propagule trapping is highly independent of its physical characteristics. Conversely, at lower substratum complexity, propagule trapping depends on the propagules physical characteristics, because the effect of substratum on flow is weaker. Therefore, the drag coefficient is a strong predictor of trapping, particularly for substrata of low, but some, complexity (i.e. not sand).

After release by floating-fruits, *P. oceanica* seeds sink and, depending on the hydrodynamic conditions and substratum type, are trapped or transported along the bottom until a microhabitat which facilitates their trapping is encountered. Given that seeds lack dormancy, if they cannot establish in the first few days after fruit dehiscence, trapping may be hampered because their development modifies their physical characteristics. Once retained in a safe recruitment microhabitat, the presence of adhesive root hairs (Baladamenti et al., 2015) reinforces establishment and facilitates persistence over consolidated substrates, explaining the prevalence in rocky substrata a few years after establishment (Alagna et al., 2013). In addition to the adhesive properties of root hairs, the complexity of the substratum facilitates seedling anchoring (Alagna et al., 2015). Additionally, our observations of seedling movement in the flume suggest that roots, which are somewhat rigid and show an apparent negative phototropism, might become anchoring structures that could facilitate propagule trapping and establishment. The primary root (first root produced at the distal end of the seed) grows frequently curved resembling a hook (Fig. 12a) that could possibly enhance trapping. Indeed, anchor-like structures that are suggested to contribute to trapping have been described in propagules of *Phyllospadix spp.* and *Amphibolis spp* (Kuo and den Hartog, 2006).

Our study provides important insights into the drivers of seagrass dispersal recruitment, which could be applicable for numerical hydrodynamic dispersal models and restoration efforts. Nowadays, the structure and function of seagrasses in many ecosystems is affected by anthropogenic and natural disturbances that have caused their strong decline worldwide (Waycott et al., 2009), as well as a renovated interest on seagrass restoration (Orth et al., 2006b; Ganassin and Gibbs, 2008; Renton et al., 2011). Hydrodynamic exposure (Orth et al., 2006b; Bos and van Katwijk, 2007; van Katwijk et al., 2009) and the availability of a suitable substratum (Rivers et al., 2011; Infantes et al., 2011b) frequently determine restoration success. More complex substrata such as rocky areas or dense mattes of *Posidonia oceanica* may facilitate the retention and establishment of seagrass propagules and therefore substratum complexity could be an additional criterion in the site selection process during restoration.

Influence of biotic and abiotic drivers of seagrass (*Posidonia oceanica*) seedling recruitment: identifying suitable microsites

4. RESULTS

Field Study
(Bendinat, January 2016)
By Eduardo Infantes

4.2. Influence of biotic and abiotic drivers of seagrass (*Posidonia oceanica)* seedling recruitment: identifying suitable microsites

4.2.1. Abstract

The period between seedling germination and successful establishment is considered the most vulnerable phase for plant development. To better understand recruitment patterns within populations, it is essential to identify the abiotic constrains and biotic interactions that allow the successful colonization of species. We evaluate which combination of factors are associated with successful survival and development of *Posidonia oceanica* seedlings, in order to identify which are the most important microsite features acting together on the recruitment success. Our results show that *P. oceanica* seedlings are rather specific in their environmental requirements during their first 18 months of life, because their survival and development are favored in microsites of consolidated substratum (rock, *P. oceanica* matte) covered by macroalgae (mainly crustose algae) and located in sheltered locations (with energy flux values not exceeding $7x10^5$ kg s^{-2} m s^{-1}). After this phase, their probability of surviving becomes more independent from microsite characteristics. In addition, we detect a positive correlation between seed volume and seedling survivorship, i.e. above a certain volume (0.7 cm^3), all seedlings survive.

4.2.2. Introduction

The identification of factors influencing recruitment rates is a main objective of plant population biology (Harper, 1977; Crawley, 1990). To establish a new individual, a seed must be placed at a suitable microsite, germinate, and the developing plant must overcome the threats to plant survival at that site (Eriksson and Ehrlén, 1992). Thus, identifying the processes affecting the early phases of the life cycle of a species, from those limiting seed dispersal to those affecting seed germination and seedling establishment, is a prerequisite for understanding the factors controlling recruitment (Seddon, 2004; Irving et al., 2010). Within this multistage process, the period between seedling germination and successful establishment is considered the most vulnerable phase for plant development (Harper, 1977), and represents a bottleneck in the plant life-cycle, compromising species abundance and distribution (Gomez-Aparicio, 2008). Recruitment patterns result from processes operating at multiple scales that determine the survival and growth of new individuals (Harper, 1977), being crucial to identify the environmental conditions that characterize a site as "safe" (sensu Jarèr et al., 1961). These small-scale locations suitable for seedling establishment are known as "microsites" (Eriksson and Ehrlén, 1992), and the abiotic constraints and biotic interactions within them determine the colonization success of the species (Gotelli et al., 2010; Soliveres et al., 2011).

Engineering organisms (i.e., seagrasses, mangroves, or salt marsh plants) carry out important ecosystem functions, such as modulating sedimentary processes, attenuating wave action and creating and regulating the habitat for other organisms (Gutiérrez et al., 2012). In this study we focus on seagrasses, which develop key coastal ecosystems (Hemminga and Duarte, 2000) and have been suffering worldwide declines during the last century associated to human activities (Boudouresque et al., 2009; Waycott et al., 2009). Seagrass meadow expansion occurs mainly through clonal growth (Lewis and Phillips, 1980; Phillips et al., 1981), even though the products of sexual reproduction are essential for the consolidation of existing meadows and for the colonization of new ones (Olesen et al., 2004). Furthermore, seedlings provide genetic variation, necessary for adaptation (Kendrick et al., 2012; 2017) that contributes to the recovery of degraded meadows and long distance dispersal. However, as mentioned before, the period between seed germination and seedling successful establishment represents a demographic "bottleneck" limiting recruitment within populations (James et al., 2011; Statton et al., 2017). Consequently, a better understanding of the biotic and abiotic filters regulating seagrass recruitment represents a main goal to predict meadow formation and recovery.

Here we focus our attention on the dominant seagrass of the Mediterranean Sea, *Posidonia oceanica.* This species has a very slow growth rate (Marbà and Duarte, 1998) and sparse sexual reproduction, which varies unpredictably in both frequency and intensity (e.g. Buia and Mazzella, 1991; Balestri, 2004; Diaz Almela et al., 2006; 2007). Indeed, in situ observations of seedlings are few (Buia and Piraino, 1989; Piazzi et al., 1999; Gambi and Guidetti, 1998; Alagna et al., 2013), since one-year survival represents a major bottleneck in their life cycle (Balestri et al., 1998; Piazzi et al., 1999). Due to the strong decline suffered by *P. oceanica* meadows in the last decades (Boudouresque et al., 2009), and given the increased rates of environmental change predicted for the coming years (IPPC, 2014), it is of special interest to evaluate the main factors controlling seedling recruitment of this species. Here, we evaluate the association of microsite features with successful seedling settlement and development, in order to identify the most important variables acting together on recruitment success. In addition to intrinsic traits of seedlings, such as their seed reserves (Balestri et al., 2009) or their morphology (Pereda-Briones et al., 2018a) in determining seedling settlement and survivorship, the type and number of variables comprising the dimensions of an environmental niche vary from one species to another and according to the abiotic and biotic contexts (Krebs, 2001; Gray et al., 2015). Seeds are transported by wave energy and currents until settlement occurs. Later, physical dislodgement of seedlings by wave and current forces is a primary factor impeding successful recruitment (Statton et al., 2017; Marion and Orth, 2012; Rivers et al., 2011; Infantes et al., 2011a; Balestri et al., 1998), hence the characterization of the hydrodynamic conditions is crucial to evaluate seedlings recruitment success. Small-scale topography is important in determining dispersion and germination patterns (Orth et al., 1994; 2006b; Inglis, 2000b; Balestri and Lardicci, 2008, Pereda-Briones et al., 2018a) and substratum type can strongly influence seedling survival (Piazzi et al., 1999; Olesen et al., 2004; Pereda-Briones et al., 2018a). Indeed, *P. oceanica* seedlings are firmly established when they occur in consolidated substrates (Balestri et al., 1998; Piazzi et al., 1999; Alagna et al., 2013; Baladamenti et al., 2015), as these provide strong resistance when plants are pulled. In addition, biological factors also influence the success of seedling recruitment. For instance, the presence and composition of the macroalgal community associated to the settlement area can also affect anchoring success, nutrient uptake, vegetative development and herbivory pressure, among others (Blanchette et al., 1999; Alagna et al., 2013). The aim of the present study was to identify microsites suitable for survival of *P. oceanica* seedlings and evaluate the importance of different factors affecting early seedling development and survival. We examined the following questions: 1) hydrodynamic conditions limit successful recruitment, 2) substratum type determines seedling survival, and 3) the association with certain macroalgae favours seedling development and survival. To this end, we marked groups of naturally-settled seedlings at different microsites around Palma Bay (Balearic Islands, Western Mediterranean) and followed their development and survival during almost two years while simultaneously characterizing the environmental and biological factors associated with the safe recruitment microsites.

4.2.3. Materials and methods

4.2.3.1. Study area

Seedlings of *Posidonia oceanica* were detected in May 2015 at three sites along Palma Bay: Son Caliu (Long. 2.553ºE – 39.525ºN), Punta Negra (2.548ºE – 39.525ºN) and Bendinat (Long. 2.579ºE – 39.532ºN), south-west of Mallorca, in the Balearic Islands archipelago (Western Mediterranean) (Fig. 21).

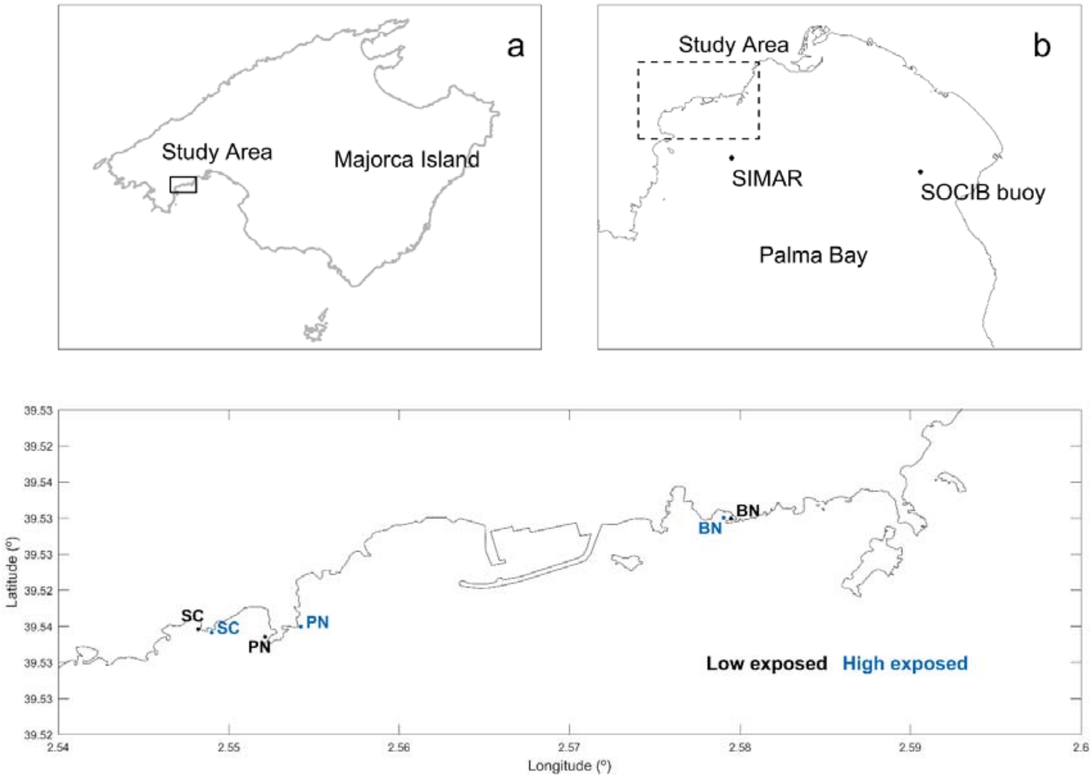


Figure 21. Location of study area in Mallorca Island (a), location of SIMAR reanalysis point and SOCIB oceanographic buoy in Palma bay (b), location of the experimental sites: Son Caliu (SC), Punta Negra (PN) and Bendinat (BN) (c). The high exposed zones are marked in blue.

The three sites are located in shallow embayments, between 0.5 and 6 m deep, and are southward orientated. The sea floor in these embayment's consists of rocky substrate interspersed with matte of dead *P. oceanica* (Boudouresque et al., 1980), and some sand and coarse gravel throughout. Rocky substrates and *P. oceanica* matte were mostly covered by a macroalgal assembly, although there some bare rock was also present, while sand and coarse gravel were almost devoid of vegetation, with occasional exceptions of patches covered with macroalgae or seagrass. At each of the three sites we marked 56 seedlings haphazardly distributed in two zones which were separated from each other about 50 m and were differentiated by their degree of wave exposure (see Results). Seedlings were estimated to have germinated and attached during late spring or early summer 2014, since their vegetative development (see next section, Sampling procedures) was similar to those measured for one-year-old seedlings (Gambi and Guidetti, 1998; Balestri et al., 2009).

4.2.3.2. **Sampling procedures**

Each individual seedling was tagged with a number and its location was additionally marked with a peg labelled with the seedling number next to the plant. At the time of marking, all seedlings were still bearing the seed at the base of the rhizome, which had a volume of 0.59 cm^3 ± 0.016 (mean ± SE, N= 168), and had 5 to 6 leaves amounting a total leaf surface of 36.64 ± 0.3 (mean ± SE, N= 168).

To evaluate the importance of the biotic and abiotic factors controlling *P. oceanica* seedling recruitment, we examined a set of features at each microsite while measuring the seedlings' population dynamics. Samplings were carried out on SCUBA every four months from May 2015 until January 2017. During each survey we measured seedling morphometry: number of leaves, length of all leaves, the width of the second youngest leaf, number of bites in leaves and we estimated seed volume (derived from length and width measurements of the seed). Survival was expressed as the percentage of seedlings present with respect to the initial number present in each zone at the three sites. We also estimated the dominance of the different macroalgae species at each microsite by photographical analysis, using a 20 x 20 cm frame centered on each seedling. In order to quantify the main species covering the microsite we used the software CPCe-Program "Coral Point with Excel extensions" (Kohler and Gill, 2005) to distribute 30 random points over each 20 x 20 cm site image and identified the species below them. The macroalgal cover was then calculated by adding all the points that were in contact with the identified species. In addition, to estimate the percentage cover of macroalgae in the area directly in contact with seedling roots, we randomly overlapped 10 random points over a 5 x 5 cm plot cut out from the original 20 x 20 cm image of each microsite. Subsequently, macroalgae species were grouped in functional groups, following the classification proposed by Steneck and Dethier (1994), except for *Caulerpa cylindracea, Caulerpa prolifera* and *Dasycladus vermiculais*, which did not fit in at any of these functional groups, and therefore we grouped them as "others". Additionally, at the first sampling date we recorded depth, substratum type and bottom roughness of each seedling microsite. Microsite roughness was estimated by laying a metal chain over the two diagonals of the 20 x 20 cm quadrats centered on each seedling, and measuring the length required by the chain to follow the substratum surface along the diagonals (Luckhurst and Luckhurst, 1978). Once this length was measured, it was compared with the same distance on a flat surface, by subtracting this distance, which would be representative of a roughness value of zero.

4.2.3.3. **Hydrodynamic conditions**

Significant wave height (H_s), peak period (T_p), and mean direction (θ) were obtained from the climate database of Puertos del Estado (Martínez-Asensio et al., 2013; SIMAR node – 2.583ºE - 39.50ºN), about 3.6 Km from the study area, from January 2015 until July 2018. The validation of this near-shore database (see Supplementary material, Text S 1; Fig. S 1 and Fig. S 2) confirmed its reliability with respect to the real wave conditions in the study area. At that point we propagated the maritime climate from deep to shallow water by using a numerical model (SWAN-*Simulating WAves Nearshore*), in order to obtain the historical wave series at our study sites (for further details of the propagation methodology see Supplementary material, Text S 2, Fig. S 3). Then, we identified the extreme events of every zone of each site from the Wave Energy Flux (F). This parameter accurately represents the energy that arrives to the coast due to the incident waves, taking into account the significant wave height (H_s) and its associated period (T_p) for each sea state. In order to analyze the hydrodynamic forces to which the seedlings are exposed, it is necessary to measure the H_s (m) and its associated T_p (s), since the energy transfer from surface to bottom depends jointly on both parameters (Holthuijsen, 2007). First, the total wave energy per surface unit (E) was calculated, where ρ is the seawater density (1.025 g cm^{-3}), g is the gravity constant, and H_s the significant wave height.

$$E = \frac{1}{8} * \rho * g * H_S{}^2$$

Second, we calculated the celerity of the waves group, where k is the wave number ($k = 2\pi/L$), L is wave length, and h is the depth.

$$C_g = \frac{1}{2} * \left(1 + \left(\frac{2 * k * h}{\sinh(k * h)}\right)\right)$$

Third, we multiplied $E * C_g$ to obtain the Wave Energy Flux (F) parameter:

$$F = E * C_g$$

Then, the time series was divided into six periods, each of 4 months, from January 2015 until January 2017, and those mean energy flows which overcame the 95 % percentile in every period were selected as they represent the amount of energy related to extreme events (Galanis et al., 2011) that potentially would affect seedling persistence. To take into account not only storm intensity, but also its persistence, all F values at each period were integrated (see Supplementary material Fig. S 4). Finally, we evaluated the wave exposure degree (in terms of wave flux energy) of each zone in the three study sites and related the variation in seedling abundance over time at each microsite with the wave energy flux characteristic of each period.

4.2.3.4. **Data analysis**

To analyse the influence of the environmental factors (wave energy flux, bottom roughness, depth and substratum type) on *P. oceanica* seedling survivorship, a Generalized Linear Model (GLM) was performed. A binomial family of errors with a "logit" link function was selected to meet the assumptions of linearity and normality of errors, which were checked by a visual inspection of residuals. Similarly, another GLM was performed to evaluate the importance of the same environmental factors on seedling total leaf area (TLA). In this case we selected a Gaussian family of errors with an "identity" link function. Furthermore, to ensure that the flux energy values (F), were significantly different between the high and the low exposed zones at every site, a pairwise t-test was performed.

In order to identify differences in survivorship along time depending only on substratum type, a homogeneity of slopes model was conducted. Survival was the dependent variable, substratum the categorical predictor [with 7 levels: rocks, matte, gravel and sand (all covered with macroalgae), and rocks, gravel and sand (all devoid of vegetation)], and time was the continuous predictor. A post-hoc Tukey test was performed to detected differences in survival between levels of the categorical predictors. In addition, we assessed if the variation of seed volume among seedlings influenced seedling survival and foliar development (TLA) by conducting One-way ANOVAs (fixed factor seed volume). Since seed volume measurements did not vary through time, we have taken the initial values for the current analysis. Seed volume was categorized into nine levels of 0.1cm^3 increments (from 0.4 cm^3 to 1.2 cm^3). Furthermore, we tested if different substrata retained seedlings with different seed volumes by another One-way ANOVA (fixed factor substrata).

4.2.4. Results

The reconstruction of the maritime climate in the three study sites by the SWAN propagation model did not identify strong storm surges at our study sites (H_s q95 = 0.49 m ± 0.14). Nonetheless, at the three sites, we confirmed different wave exposure regimes between the two zones, being one of the zones always more exposed than the other one according to the F values of the extreme events (pairwise t-test p-value < 0.05 for the three sites; Fig. 22a). Accordingly, hereafter, factor zone will represent the degree of exposure, with a low-exposed and a high-exposed zones at each site. Seedlings established in the high exposed zone had higher mortality rates than those established in the less exposed ones (Fig. 22b), except for in Son Caliu.

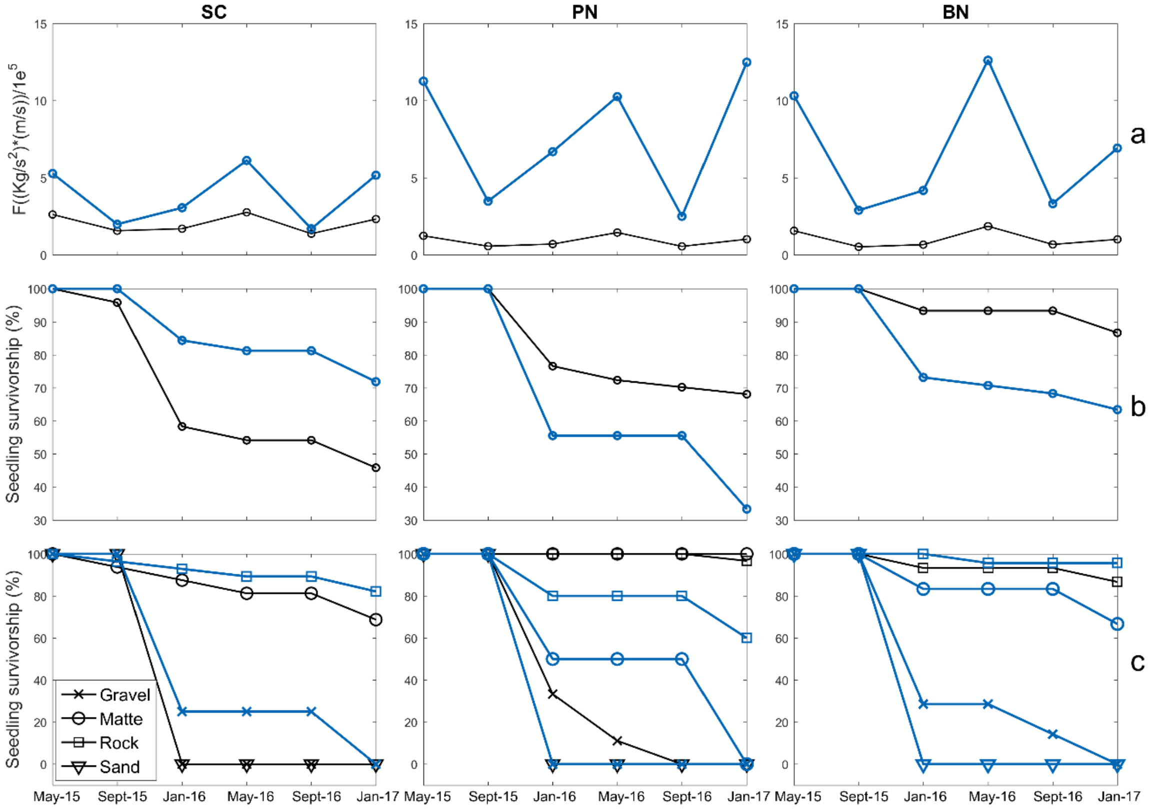


Figure 22. Wave Energy Flux (F) (a) and percentage of seedling survivorship (b) across time at the low exposure (black line) and high exposure (blue line) zones for each site: Son Caliu (SC), Punta Negra (PN) and Bendinat (BN). Percentage of seedling survivorship along the different substrates (c): gravel (crosses), matte (circles), rocks (squares) and sand (triangles).

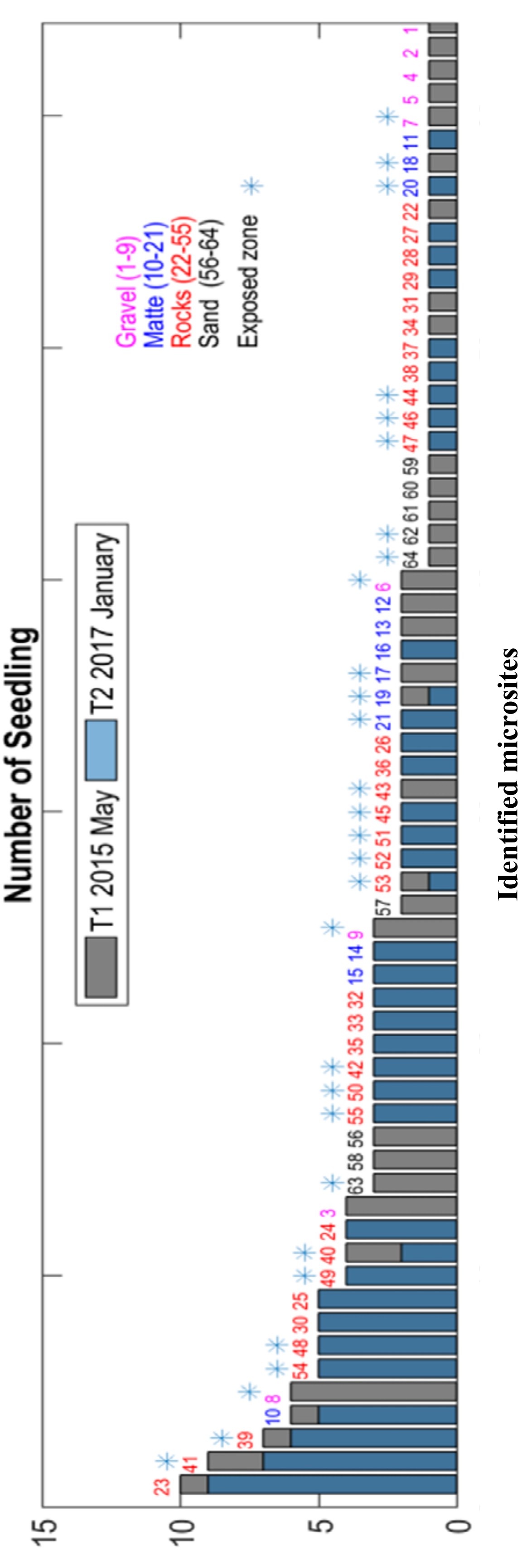
By analysing at each of the initial 168 microsites the main biotic and abiotic factors together (degree of exposure, depth, substratum type and dominant macroalgal species associated), 64 microsite types were identified (Fig. 23). These microsite types represent the combination of the biotic and abiotic factors associated to successful seedling establishment, since all seedlings initially found were between 9 to 11 months old. However, we observed that these microsites differed in time in terms of successful recruitment of the established seedlings. After almost two years, certain microsites had higher survivorship and plant development, while others underwent high seedling mortality (Fig. 23).

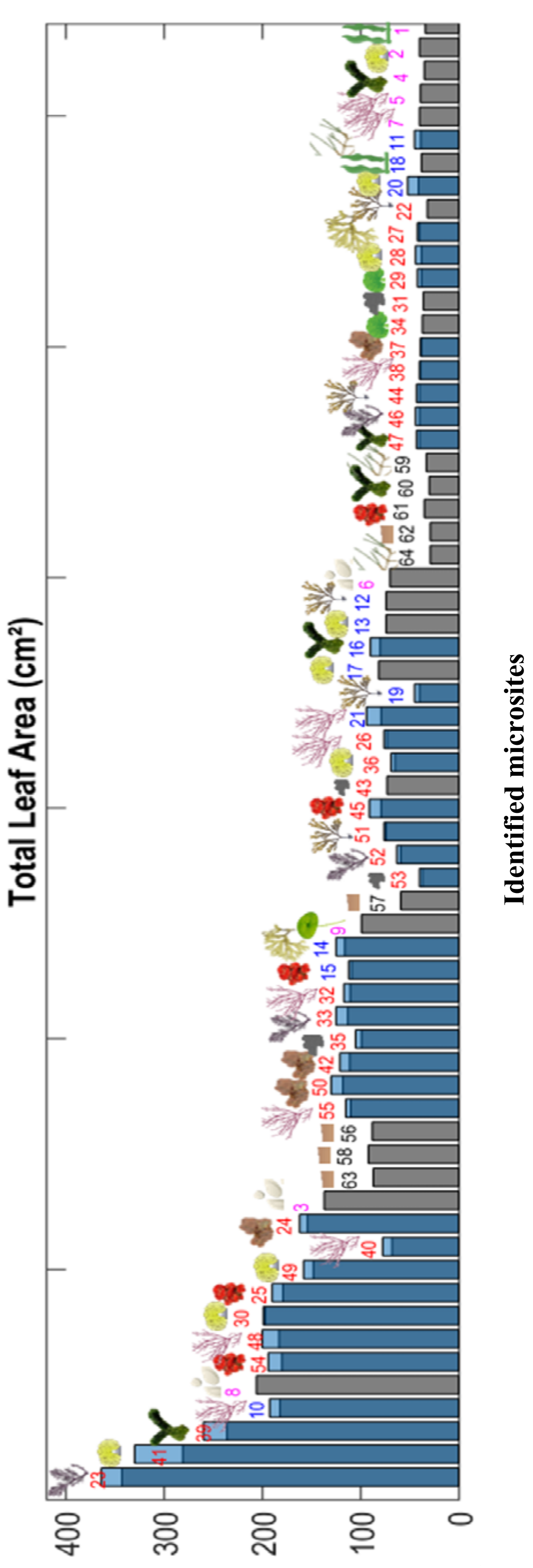
Survivorship ~ depth + energy flux + roughness + substratum)			
Family	binomial (logit)		
AIC	101.22		
Coefficients:		Estimate	P-value
	Depth	1.167	**0.01**
	Energy flux	-0.369	**0.001**
	Roughness	0.754	0.08
	Subs.(Matte)	20.281	0.99
	Subs.(Rocks)	22.729	0.99
	Subs.(Sand)	-0.423	0.99

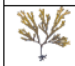
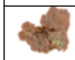
Table 5. Results of the Generalized Linear Model (GLM) analysing the influence of the environmental factors energy flux, roughness, depth and substratum type on *Posidonia oceanica* seedling survivorship.

Overall, seedling survivorship decreased significantly with higher energy flux (GLM coefficient = -0.37; Table 5), and increased at higher depths (coefficient = + 1.17). Regarding substratum type, the vast majority of seedlings were established on consolidated substrates, i.e. rocks (60 %) or dead matte (16 %) rather than sand or gravel (Fig. 22c; Fig. 23).

4. RESULTS


Number of Seedling
T1 2015 May
T2 2017 January
Gravel (1-9)
Matte (10-21)
Rocks (22-55)
Sand (56-64)
Exposed zone
15
10
5
0
23 41 39 10 8 54 48 30 25 49 40 24 3 63 58 56 55 50 42 35 33 32 15 14 9 57 53 52 51 45 43 36 26 21 19 17 16 13 12 6 64 62 61 60 59 47 46 44 38 37 34 31 29 28 27 22 20 18 11 7 5 4 2 1
Identified microsites


Total Leaf Area (cm²)
400
300
200
100
0
23 41 39 10 8 54 48 30 25 49 40 24 3 63 58 56 55 50 42 35 33 32 15 14 9 57 53 52 51 45 43 36 26 21 19 17 16 13 12 6 64 62 61 60 59 47 46 44 38 37 34 31 29 28 27 22 20 18 11 7 5 4 2 1
Identified microsites

Symbol	Legend
	*Acetabularia acetabulum*
	*Caulerpa prolifera*
	*Cymodocea nodosa*
	*Dasycladus vermicularis*
	*Dictyopteris polipodioides*
	*Dictyota dichotoma*
	*Ellisolandia elongata*
	*Flabellia petiolata*
	Coarse gravel
	*Jania adhaerens*
	*Litophyllum incrustans*
	*Padina pavonica*
	*Peyssonelia rubra*
	Rocks
	Sand

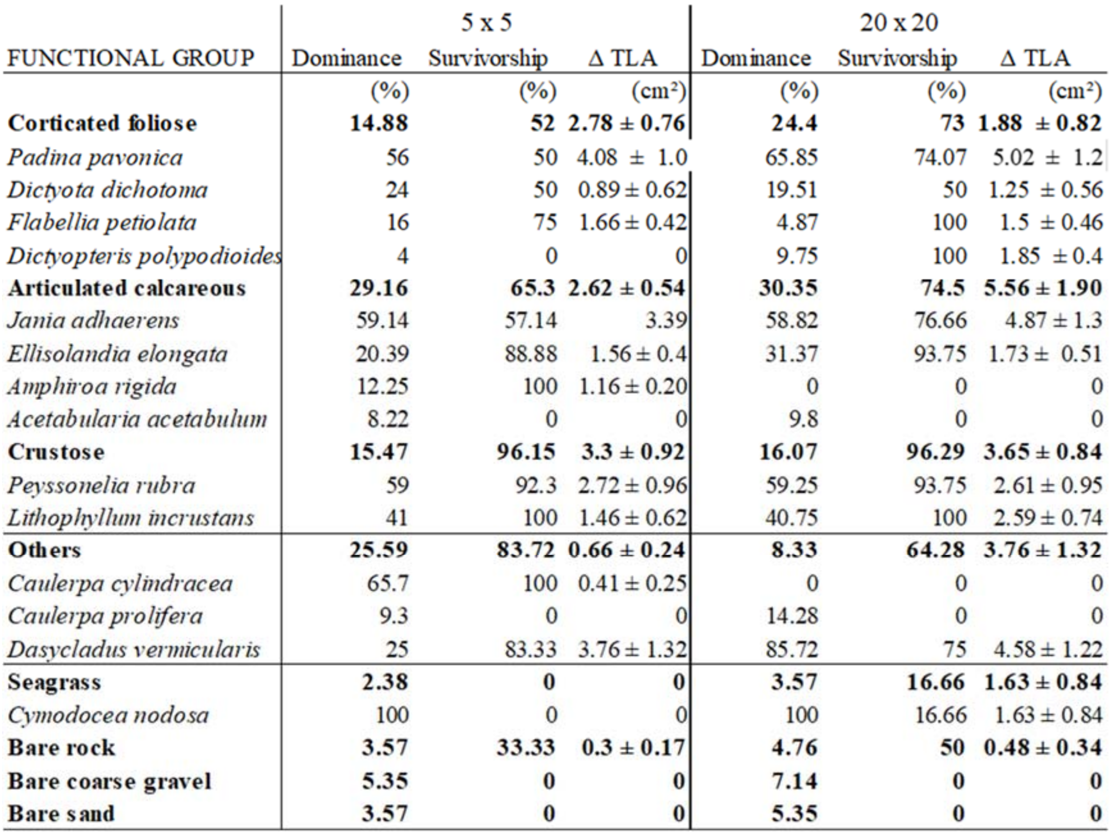
Depth interval (m)	Microsite number
0-1.5	22…29
1.5-3	6, 30…34, 39…46
3-4.5	1…5, 7…18, 35…38, 47…54, 56, 57, 62
4.5-5.5	19, 20, 21, 55, 58…61, 63,64

Figure 23. Number of seedling (upper panel) and seedling total leaf area (TLA) (lower panel) for the different microsite types, categorized by degree of exposure to waves, depth, substratum type and dominant macroalgal species associated, at the beginning (May 2015) and at the end (January 2017) of the studied period. The microsites are ordered from highest to lowest seedling abundance in the first panel. The number of seedlings at the beginning of the study are indicated with grey colour, while the seedlings present at the end of the study are in light blue (the superposition of both colours is transformed into a dark blue, representing the number of seedling present at the end of the experiment), i.e., a vertical bar totally grey indicates that no seedlings survived at this microsite, while a totally dark blue indicates 100 % survival. The degree of exposure is identified with a blue asterisk (*) over the high exposed microsites, while depth is indicated with a number over each vertical bar (each number is associated to a specific depth interval showed in the adjunct table). Substratum types are identified with different combinations of colours and numbers, and the macroalgal species or bare substrates are showed in the lower panel with a symbol (see the legend above for its meaning). The lower panel shows the variation of TLA along the study period at each microsite. All the vertical bars where the light blue overlays the dark blue indicate that these microsites underwent TLA increments through time.

4. RESULTS

Due to the unequal distribution of settled seedlings between consolidated and unconsolidated substrates from the beginning of the monitoring phase, the substratum variable is not balanced, and so the GLM analysis was not able to detect significant differences amongst substratum types. However, we did observe (non-significant) trends of the effect of substratum type on seedling survivorship (see coefficients in Table 5). The GLM analysis compared seedling survivorship over rocks, matte and sand in relation to coarse gravel substrate, and through the coefficients (although not significant) we see that seedling survivorship over rocks and matte is around 20 times higher than over coarse gravel substrate; while it is 0.42 times lower over sand than over coarse gravel. Furthermore, survival rates, taking into account all microsites together, were significantly different between substrates across the time period analysed (homogeneity of slopes model; Substratum * Time < 0.05). Rocks and matte covered with macroalgae were the most successful substrates for seedling survivorship, followed by bare rock, coarse gravel covered with macroalgae, and lastly by bare coarse gravel and sand, both covered by macroalgae or bare (Tukey). Rocks and matte substrate were also those where development was higher (Fig. 23, see also Supplementary material Fig. S 5 and Fig. S 6). In contrast, all seedlings settled on sand were lost during the first autumn-early winter; and only 25 % of the seedlings remained in the coarse gravel habitat at that time (i.e. January 2016). Bottom roughness was strongly related with substratum type, having each substratum a specific roughness interval, increasing from sand to rocks. As with the case with unbalanced data between substrates, the GLM was not able to detect significant differences between degrees of roughness.

We detected a negative relationship between seedling survival and bare substrates (homogeneity of slopes model, Fig. 23, see also Supplementary material Fig. S 5 and Fig. S 6), being seedling survivorship always below 34 %, and seedling TLA $\Delta < 0.5$ cm^2 on these substrates (Table 6). On the other hand, we detected a positive relationship with survivorship and TLA when microsites were dominated by macroalgae. Certain species were associated with higher survival and plant development, whereas others contributed less to these responses.



FUNCTIONAL GROUP	5 x 5 Dominance	5 x 5 Survivorship	5 x 5 Δ TLA	20 x 20 Dominance	20 x 20 Survivorship	20 x 20 Δ TLA
	(%)	(%)	(cm²)	(%)	(%)	(cm²)
Corticated foliose	**14.88**	**52**	**2.78 ± 0.76**	**24.4**	**73**	**1.88 ± 0.82**
Padina pavonica	56	50	4.08 ± 1.0	65.85	74.07	5.02 ± 1.2
Dictyota dichotoma	24	50	0.89 ± 0.62	19.51	50	1.25 ± 0.56
Flabellia petiolata	16	75	1.66 ± 0.42	4.87	100	1.5 ± 0.46
Dictyopteris polypodioides	4	0	0	9.75	100	1.85 ± 0.4
Articulated calcareous	**29.16**	**65.3**	**2.62 ± 0.54**	**30.35**	**74.5**	**5.56 ± 1.90**
Jania adhaerens	59.14	57.14	3.39	58.82	76.66	4.87 ± 1.3
Ellisolandia elongata	20.39	88.88	1.56 ± 0.4	31.37	93.75	1.73 ± 0.51
Amphiroa rigida	12.25	100	1.16 ± 0.20	0	0	0
Acetabularia acetabulum	8.22	0	0	9.8	0	0
Crustose	**15.47**	**96.15**	**3.3 ± 0.92**	**16.07**	**96.29**	**3.65 ± 0.84**
Peyssonelia rubra	59	92.3	2.72 ± 0.96	59.25	93.75	2.61 ± 0.95
Lithophyllum incrustans	41	100	1.46 ± 0.62	40.75	100	2.59 ± 0.74
Others	**25.59**	**83.72**	**0.66 ± 0.24**	**8.33**	**64.28**	**3.76 ± 1.32**
Caulerpa cylindracea	65.7	100	0.41 ± 0.25	0	0	0
Caulerpa prolifera	9.3	0	0	14.28	0	0
Dasycladus vermicularis	25	83.33	3.76 ± 1.32	85.72	75	4.58 ± 1.22
Seagrass	**2.38**	**0**	**0**	**3.57**	**16.66**	**1.63 ± 0.84**
Cymodocea nodosa	100	0	0	100	16.66	1.63 ± 0.84
Bare rock	**3.57**	**33.33**	**0.3 ± 0.17**	**4.76**	**50**	**0.48 ± 0.34**
Bare coarse gravel	**5.35**	**0**	**0**	**7.14**	**0**	**0**
Bare sand	**3.57**	**0**	**0**	**5.35**	**0**	**0**

Table 6. Percentages of dominance of the different algal functional groups (and the contribution of each macroalgal species), and seagrass, bare rock, bare coarse gravel and bare sand at 5 x 5 and 20 x 20 plots, in relation to seedling survivorship and foliar development [increment in total leaf area (Δ TLA)].

When grouping macroalgae species in functional groups, seedlings survived mostly in the presence of crustose algae (more than 96 %, Table 6), with *Litophyllum incrustans* and *Peyssonnelia rubra* as the dominant species. Articulated calcareous algae were the most abundant functional group, with *Jania adhaerens* being the dominant species and being associated to the higher TLA increments (Table 6, Fig. 23). *Ellisolandia elongata* contributed less to the TLA increment, but it was associated with almost 94 % of seedling survivorship. *Acetabularia acetabulum* was associated to total seedling mortality whereas seedlings associated with *Amphiroa rigida*, even though it was only dominant in 10 % of the microsites, exhibited 100 % survivorship. Corticated foliose was the second most abundant functional group. *Padina pavonica* was the dominant species, also having the highest TLA increment associated with it. *Dictyota dichotoma* was the second species in terms of abundance, but only half of the seedlings settled over it survived. The other species of this group, although more scarce, *Flabellia Petiolata* and *Dictyopteris polypodioides*, contributed to more than 75 % of seedling survivorship. Lastly, the species grouped as “others” were mostly dominant at the base of the seedling radicular system (5 x 5 microsites) mainly represented by *Caulerpa cylindracea*, which was associated with 100 % survivorship. In contrast, seedlings growing next to *Caulerpa prolifera* underwent total seedling mortality, whereas *Dasycladus vermicularis* was associated with the higher TLA increment and more than 75 % survivorship (Table 6, Fig. 23).

At the beginning of the sampling period (May 2014), seedlings were 9 to 11 months old and were still bearing their seed attached to the base of the rhizome. However, around the age of 24 months old (i.e. in September 2016), all survivors were lacking their seed. Seed measurements revealed that seedling survival was positively related to seed volume (F$_{(8,100)}$ = 1.95; P-value < 0.05; One-Way ANOVA), and above a certain volume (0.7 cm^3), all seedlings survived (Tukey). Furthermore, bigger seeds were found on harder substrates (rocks and matte; F$_{(3,100)}$ = 38.6; P-value < 0.01; One-Way ANOVA, Tukey). On the other hand, seedlings with bigger seeds (higher seed volume) did not exhibit higher TLA (F$_{(8,100)}$ = 0.72; P-value > 0.05). Seedling TLA slightly increased with energy flux (GLM, Table 7), being seedlings established at the more exposed site bigger than those in the less exposed ones.

ΔTLA ~ depth + energy flux + roughness + substratum)			
Family	Gaussian (identity)		
AIC	658.57		
Coefficients:		Estimate	P-value
	Depth	-0.734	0.214
	Energy flux	0.509	**0.01**
	Roughness	-0.925	0.187
	Substratum	0.853	0.655

Table 7. Results of the Generalized Linear Model (GLM) analysing the influence of the environmental factors energy flux, roughness, depth and substratum type on *Posidonia oceanica* seedling total leaf area (TLA).

4.2.5. Discussion

In this study we have been able to identify certain abiotic and biotic factors that provide the most suitable microsites for successful recruitment of *P. oceanica* seedlings. These microsites are locations which are sheltered from waves, generally between 1.5 and 4.5 m depth (and even less than 1.5 m in very sheltered areas), over consolidated substrates, and dominated by macroalgal assemblies, particularly those associated to crustose algae.

To begin with, seedling establishment is clearly biased towards sheltered locations, with their survival decreasing significantly with higher energy flux. Preference for calmer areas has also been previously reported for *P. oceanica* seedlings (Balestri et al., 1998; Infantes et al., 2011a) and for other seagrass species. In fact, physical removal by wave energy is the primary factor hampering successful recruitment of *Amphibolis antartica* and *Posidonia australis,* especially by winter storms (Rivers et al., 2011; Statton et al., 2017), and is also the major cause of transplant failure in restoration efforts for many species (van Katwijk and Hermus, 2000; Irving et al., 2010). The energy supported by the seedlings is caused by the accelerations generated in the bottom, both by wave height and period in the entire water column (Holthuijsen, 2007). At the three studied sites we identified two different zones in terms of degree of exposure to waves. In Punta Negra and Bendinat, maximum F values at the exposed area during winter months were between $10x10^5$ and $13x10^5$ Kg s^{-2} m s^{-1}, decreasing to around $7x10^5$ Kg s^{-2} m s^{-1} during the summer months, when storms are less frequent and of lesser magnitude. Instead, in Son Caliu, maximum F values were much lower, not exceeding $7x10^5$ Kg s^{-2} m s^{-1} at the exposed area during the winter months. This lower wave energy flux (due to the fact that the most energetic waves come from SSW directions, see Supplementary material Fig. S 3,) indicates that Son Caliu is overall the more protected site. The minor differences in degree of exposure between the two zones in Son Caliu, (and the fact that the more exposed site is largely dominated by rocky substrate) likely contribute to explaining the different responses of survival to the degree of exposure observed at this site in comparison to the other two sites. Importantly, at the three sites, the number of seedlings scarcely changes during summer of 2015, i.e. when F values do not exceed $3x10^5$ Kg s^{-2} m s^{-1}. The greatest decrease in the number of seedlings occurred between September 2015 and January 2016, (with 30 - 50 % of seedlings lost in the exposed area of Punta Negra and Bendinat, and almost 50 % in the less exposed area of Son Caliu), when these areas underwent F values of 7 $x10^5$ Kg s^{-2} m s^{-1}. Conversely, from January 2016 onwards, practically all seedlings survived until January 2017 (with moderate decreases of between 5 and 20 % for all the sites; Fig. 22a, b). Indeed, by analysing the raw data, total seedling survivorship from 9 - 11 months to 18 months was around 65 %, while from 18 until 30 months it was around 90 %. This suggests that seedlings are more vulnerable to hydrodynamic conditions during their first year and a half of life. After reaching about 18 months of age, the surviving seedlings are more resistant, despite withstanding higher magnitude extreme events (~10^5 Kg s^{-2} m s^{-1}). In addition, most successful establishment of seedlings took place on consolidated substrates, whereas unconsolidated substrates appear to hamper seedling retention and survival. Indeed, seedlings between fourteen and eighteen months old experienced increased mortality on unconsolidated substrates, such as sand and gravel, while their populations were more stable over consolidated ones.

Another aspect which suggests that consolidated substrates contribute to higher seedling survival is that seedlings with bigger seeds were found on them (rocks and matte), and our results show that seed volume is positively correlated with seedling survival. This correlation was also observed by Balestri et al. (2009), who suggested that during the first year of life, seeds supply N and P to the plant, guaranteeing rapid growth of leaves and roots. Bigger seeds, which contain more reserves, likely provide seedlings with a higher capacity to face environmental stressors than the smaller ones. In addition, smaller seeds are associated with lower drag coefficient values (Pereda-Briones et al., 2018a), being higher flow velocities necessary to move them over the bottom. Perhaps, this contributed to the higher amount of smaller seeds over sand and coarse gravel substrates.

Furthermore, the positive effect of macroalgae cover on seedling survival was also identified as an important factor, which may indirectly favour seedlings by ameliorating environmental conditions. Environmental facilitation by co-habiting plants is a common phenomenon in other systems (e.g. Mediterranean forests; Gómez-Aparicio, 2008, salt marshes; Hacker and Bertness, 1999), and has also been suggested for *P. oceanica* (Alagna et al., 2013). In their study, Alagna et al., (2013) observed higher seedling persistence on rock substratum covered by *Cystoseira spp*., which was attributed to the macroalgae decreasing water flow and providing anchoring substrate for seedling roots. Yet, not all functional groups appear to influence seedling recruitment and development in the same way. Crustose algae, with *Litophyllum incrustans* and *Peyssonelia rubra* as the dominant species, appear to be the most favourable functional group regarding seedling survival. Crustose algae are characterized by a stony thallus, and probably this solid structure has allowed the adequate anchorage of the seedling's radicular system to the hard substrates. Indeed, *P. oceanica* seedlings can regulate root traits during their early development in relation to substrate typology (Balestri et al., 2015), and the presence of sticky hairs covering *P. oceanica* seedling roots (Baladamenti et al., 2015) allows them to successfully anchor and persist over hard substrates, improving their resistance against physical disturbances and ensuring nutrient uptake. Articulated calcareous algae, with *Jania adhaerens* as the most representative species, is the predominant functional group in terms of seedling abundance. *J.adhaerens* is fixed to the substrate by small discs, and it is relatively common in well illuminated environments that are slightly exposed to waves (Litter and Litter, 2000). Perhaps, the higher abundance of seedlings over *J. adhaerens* is owing to the predominant presence of this algae in the study area, whose fixation structure may facilitate seedlings to properly attach to hard substrates. *Ellisolandia elongata* was the second most important species of articulated calcareous and it is considered a "stress-tolerant species" (Arévalo et al., 2007). Its calcified structure gives it resistance to abrasion and predation, and due to having an algal body composed of both prostrate filaments and protruding upright filaments, it is able to recover rapidly after disturbances (Littler and Kauker, 1984). Perhaps, under a wide range of stress factors, the presence of *E. elongata* provides a stability that allows *P. oceanica* seedlings to persist longer.

The corticated foliose functional group was also highly abundant, and it was dominated by *Padina pavonica*. Seedlings growing on microsites dominated by *P. pavonica* had the maximum increment in TLA. This alga is a common and characteristic species in well-illuminated communities of the infralittoral (Rodriguez-Prieto et al., 2013). Lastly, *Caulerpa cylindracea* was mostly dominant at the base of the seedling radicular system (5 x 5 microsites), which may play an important role regarding initial attachment of the roots. The presence of *C. cylindracea* may facilitate seedling retention and further survival on rocky and matte substrates. In fact, this invasive species promotes *P. oceanica* seedling root development, facilitating the anchorage to the matte substrate (Pereda-Briones et al., 2018b).

To conclude, it is important to highlight that once seedlings have overcome the second winter, at an age of approximately 18 months old, they have a high probability of persistence in the population. Their risk of mortality decreases as they become older, independently of the environmental conditions. Thus, we suggest that the recruitment success of *P. oceanica* seedling is limited largely by the availability of suitable microsites. *P. oceanica* seedlings appear to be very specific in their environmental requirements (microsite-limited species) during their first two years of life, when they are favoured by microsites such as those characterized by rocky or matte substrates, covered by macroalgae, and located at sheltered locations. After this phase, their probability of surviving becomes more independent from microsite characteristics.

4. RESULTS

Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae

4. RESULTS

Posidonia oceanica seedlings, Caulerpa cylindracea and Lophocladia lallemandii
(IMEDEA, Esporles, March 2016)
By Laura Pereda Briones

4.3. Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae

4.3.1. Abstract

The observed and projected rise in sea surface temperature challenges marine biodiversity worldwide, and particularly in temperate ecosystems dealing with the arrival of novel species of tropical provenance. When the impacted biota are early life stages of ecosystem engineers, the effects of those impacts are of major concern for ecologists and coastal managers. We experimentally examined the individual and potential additive effects of seawater warming and the presence of the invasive algae on the development of seedlings of the seagrass *Posidonia oceanica* in a three-month mesocosm experiment. Whereas the presence of the invasive algae (*Caulerpa cylindracea* and *Lophocladia lallemandii*) did not result in detrimental effects on seedlings, warming negatively affected seedling development. Interestingly, the presence of both invasive algae may ameliorate the negative effects of warming.

4.3.2. Introduction

The gradual increase of global sea surface temperature (SST) observed since the 1950s represents a threat to marine ecosystems worldwide (IPPC, 2014). Global responses of marine biota already indicate that climate change can have a strong impact on marine life (Diaz-Almela et al., 2009; Lejeusne, 2010; Harley et al., 2012; Poloczanska et al., 2013). The rise in SST is predicted to increase between 0.6 and 1.5ºC in the next 40 years (IPCC, 2014). This can be particularly relevant for temperate ecosystems dealing with novel species of tropical provenance, such as introduced species or species that have undergone a range expansion (Raitsos et al., 2010, Vergés et al., 2014). Furthermore, when the species impacted by warming are ecosystem engineers, such as corals, kelps or seagrasses, the consequences of those impacts may cascade throughout the ecosystems they form, significantly magnifying overall impacts (e.g. Hoegh-Guldberg et al., 2007; Wernberg et al., 2013). Certainly, the introduction of invasive species and global warming are emerging as main threats to seagrass ecosystems (e.g. Orth et al., 2006a; Williams, 2007; Koch et al., 2013; Collier and Waycott, 2014).

Sexual reproduction is crucial for the persistence of seagrass beds, allowing for the consolidation of existing meadows and also for the formation of new ones via dispersal

(Jarvis et al., 2014; Olesen et al., 2004; Orth et al., 1994; 2006b). Moreover, seedling production represents a critical genetic diversity source that provides adaptive capacity to future environmental changes, essential for the long term persistence of the species (Jump et al., 2009; Kendrick et al., 2012; 2017). These early stages are considered the most critical phases in the life cycle of seagrasses, when plants experience the highest mortality rates (Orth et al., 2006b; Alagna et al., 2013). Indeed, the establishment rates of newly emerged seedlings are generally very low (Orth et al., 2006b), and are recognized as a major population bottleneck (Gomez-Aparicio, 2008). Given the ecological importance of seagrass seedlings, it is pressing to understand the environmental and biological factors which contribute to their successful establishment, survival and development.

The Mediterranean Sea is a hotspot of biodiversity (Myers et al., 2000). However, it exhibits rates of seawater warming that exceed threefold those of the global ocean (IPCC, 2007; Burrows et al., 2011). The increase in SST also stimulates the successful proliferation of alien species, particularly those from tropical and subtropical origin entering through the Suez Canal (Raitsos et al., 2010, Vergés et al., 2014). In fact, the Mediterranean Sea is one of the areas with more introductions of non-native species (e.g. Galil et al., 2014; Streftaris and Zenetos, 2006), some of which are considered as invasive and are affecting the integrity of natural communities, including loss of seagrass meadows (Vitousek et al., 1997; Williams and Smith, 2007; Thomsen et al., 2010).

The endemic seagrass *Posidonia oceanica* (L.) Delile is one of the most representative foundation species to inhabit littoral Mediterranean ecosystems due to its importance as a habitat former, and supporter of biodiversity (Duarte, 2000). It is a slow-growing species which forms extensive meadows, characterized by a very variable sexual reproduction in space and time (Green and Short, 2003; Diaz-Almela et al., 2009). These features contribute to a low capacity of resilience and adaptation to the rapid warming projected for the Mediterranean Sea (Raitsos et al., 2010; Unsworth et al., 2015). Negative effects (e.g. increased mortality) in response to increasing temperatures have been observed in *P. oceanica* adult plants (e.g. Díaz-Almela et al., 2009, Marbà and Duarte, 2010) and there are indications that seedlings of this species are also sensitive to warming (Guerrero-Meseguer et al., 2017; Hernán et al., 2017). Presumably, the presence of invasive algae could further enhance the negative effects of warming on *P. oceanica* seedlings, as this species can suffer mortality, biomass loss, or deterioration of its sediment quality deterioration when colonized by some invasive algae (Marbà et al.

2014b; Ballesteros et al., 2007; Holmer et al., 2009). On the other hand, invasive seaweed could also have facilitative interactions under elevated temperatures (Harley et al., 2012). For example, shading by the invaders could soften the effects of temperature rise by reducing photochemical stress (Wernberg et al., 2010).

In the Mediterranean Sea, *Caulerpa cylindracea* and *Lophocladia lallemandii* are two of the most invasive macroalgae threatening *P. oceanica* meadows (Verlaque, 1994*;* Streftaris and Zenetos, 2006; Ballesteros et al., 2007; Klein and Verlaque, 2008*)*. Both species are able to colonize *P. oceanica* meadows, and can be particularly abundant at the edge of the meadows, over matte, as well as in low density seagrass patches (Ballesteros et al., 2007; Marin-Guirao et al., 2015), which are often the same habitats where *P. oceanica* seedlings successfully settle (Balestri et al., 1998; Piazzi et al., 1999; Balestri and Lardicci, 2008; Alagna et al., 2013). Therefore, these invasive and native macrophytes are likely to interact during the seedling establishment phase. In addition, the proliferation of both invasive species may be enhanced by increases in growth rates under warming (Samperio-Ramos et al., 2015), possibly accelerating the invasion of native habitats. Due to the important ecosystem services offered by seagrasses, including sediment and nutrient trapping, carbon fixation, nursery areas, and high primary production (Costanza et al., 1997; Pergent et al., 2014), examination of the potential interactive impacts of warming and biological invasions on seagrasses is of main relevance for conservation and management strategies. The aim of the present work was to experimentally evaluate the potential independent and cumulative impacts of warming and the presence of invasive algae (*C. cylindracea* or *L. lallemandii*) on the biological status (development, physiology and survivorship) of seedlings of the Mediterranean seagrass *P. oceanica*. We conducted a 3-month laboratory experiment where *P. oceanica* seedlings where exposed to different seawater temperatures and the presence of the invasive algae, in order to test the following hypotheses: (1) the increase in temperature will negatively affect *P. oceanica* development, promoting seedling death (2) the presence of the invasive algae reduces the vegetative development of *P. oceanica*, and (3) the interaction of seedlings with the invasive algae alters the effects of warming on *P. oceanica* seedlings.

4.3.3. Materials and methods

4.3.3.1. Seed and algae collection and maintenance

Seeds of *Posidonia oceanica* were obtained from fruits collected on the shore during June 2015 in Mallorca (Balearic Islands, Western Mediterranean). Fruits were opened and their seed removed and placed in 20L aquariums with seawater (salinity, 37), at a temperature of 20ºC and illuminated by white fluorescent lamps that provided 76 ± 2 μmol. m^{-2} s^{-1} of photosynthetically active radiation in a 14/10 h light/dark photoperiod. Seeds successfully germinate under these conditions and first leaves and roots are produced within two weeks (Terrados et al., 2013). These seedlings were maintained on a gravel substrate for the following four months under the described conditions with monthly replacement of seawater and weekly manual elimination of epiphytic algae growing on seedling leaves. Seedlings were examined for proper vegetative development (presence of seed, leaves and roots) and 800 seedlings with homogeneous morphological characteristics (in terms of seed area, number and size of leaves and roots; Table S1 and S2) were chosen for the experiment. *Caulerpa cylindracea* and *Lophocladia lallemandii* were collected by SCUBA during October 2015 in Mallorca and were maintained during 15 days, in separate tanks, in the same conditions as the seagrass seedlings. *C. cylindracea* rhizoids were buried in the gravel, allowing for the usual establishment pattern of this species, attaching to the substrate. *L. lallemandii*, which typically grows as an epiphyte on other macrophytes, was maintained freely on the bottom. Both seaweeds remained in perfect condition during the acclimation period.

4.3.3.2. Experimental design

A factorial experiment was designed considering two factors: temperature (with three levesl: 25ºC, 27ºC and 29ºC) and presence of invasive algae (with three levels: absence, presence of *C. cylindracea,* and presence of *L. lallemandii*). We also analyzed the effects of both invasive species occurring concurrently, but we were only able to examine this effect at 27ºC due to logistical constraints.

According to Sureda et al. (2008), the ratio of *P. oceanica* biomass (including leaves, sheaths and rhizomes) versus *L. lallemandii* biomass in an invaded area was 10.7 (in dry weight, DW). We used this ratio to estimate the biomass of *L. lallemandii* needed in each aquarium (which contained 20 seedlings; i.e. 5.62 g *P. oceanica* DW), which was equivalent to ca. 5 g of wet weight (WW). For *C. cylindracea*, we used the biomass quantified by Holmer et al. (2009), and Pereda-Briones et al. (2018b) in the field (i.e. 8 g *C. cylindracea* DW m^{-2}), which amounted to 7.5 g *C. cylindracea* WW in each aquarium. The biomass of both invasive algae was replaced by fresh material on a monthly basis to ensure the optimal condition of the invasive algae material.

We manipulated seawater temperature to reach three values: 25ºC as the control treatment [the summer mean SST in the Mediterranean Sea measured over the last 20 years (1982–2012); Shaltout and Omstedt, 2014]; 27°C (temperature in the range of the mean summer SST forecasted for the end of this century; Shaltout and Omstedt, 2014); and 29°C (temperature in the range of the mean summer SST forecasted for next century in the Mediterranean Sea; Shaltout and Omstedt, 2014).

Treatment temperatures were reached after an acclimation period in which temperature was increased at a rate of 1ºC per week, using the same methodology as Hernán et al. (2017). Seedlings and invasive algae were then exposed to the treatment temperatures for three months. Each treatment had 4 replicate 20L aquaria each containing 20 randomly selected seedlings, commercial aquarium gravel as substratum, a heater, and a recirculating pump. Aquaria were filled with filtered seawater (10 µm plus UV filter), which was replaced monthly in concordance with the addition of new invasive algae biomass. Epiphytic algae different from *L. lallemandii* growing on seedling leaves were manually eliminated every week. Salinity and temperature were measured in alternating days, and maintained at 37 PSU and at 25, 27 and 29ºC respectively. Water samples of each aquarium were taken at the beginning, middle and end (coinciding with the water replacement events) of the experiment for nutrient content analysis.

4.3.3.3. **Seedling survival and development**

Survival of *P. oceanica* seedlings was calculated as the percentage of alive seedlings at the end of the experiment relative to the initial number of seedlings contained in each replicate aquarium. Seedling development was assessed by measuring the following morphological characteristics on ten seedlings per aquarium at the beginning and at the end of the experiment: leaf width of the second youngest leaf, maximum leaf length, total leaf area, number of leaves, percentage of necrotic surface on each leaf, total root number, total root length and number of new roots (which are characterized by white coloration and being flexible).

4.3.3.4. Seedling biomass and physiology

Biomass of *P. oceanica* seedlings was measured by drying the samples in an oven at 60ºC to constant weight for approximately 48 h. Then, three seedlings of each aquarium were randomly selected to analyze carbon and nitrogen contents in leaves, seeds, rhizomes, and roots, using a Carlo-Erba CNH elemental analyzer. Additionally, carbohydrates in seeds (sucrose and starch) were measured using the methodology described by Invers et al. (2004). Sucrose and starch content were obtained after three sequential extractions with 95 % (v/v) ethanol at 80ºC for 15 min. The remaining pellet of seeds was dissolved in 0.1N NaOH for 24h at room temperature for starch extraction. Sugars and starch content of extracts were determined by spectrophotometry using an anthrone assay with sucrose as standard.

Rapid light curves (RLCs) were used to examine the effects of increased temperature and presence of invasive algae on seedling photophysiology. Fluorescence yield was measured in ten seedlings of each aquarium by pulse amplitude modulated (PAM) fluorometry, using an underwater device (Diving-PAM Walz GmbH, Germany). RLCs were generated automatically using an incremental sequence of actinic illumination periods, with light intensities increasing in 8 steps from 0 to 260 $\mu mol\ quanta\ m^{-2}s^{-1}$ PAR at intervals of 15 seconds between measurements. The fraction of photosynthetic photon flux (PPF) absorbed by the leaf, the absorptance factor (AF), was derived from measuring the incident irradiance from a halogen lamp before and after the Diving-PAM's sensor was covered with 1 to 4 layers of the seedling leaves, then AF was calculated according to the method described by Beer et al. (1998).

Fluorescence measurements were done by placing the tip of the instrument's main optical fiber ca. 1 cm away from the base of the leaf. Then, a saturating-light with a pre-set PPF was irradiated. This allows to obtain the effective quantum yield (Y) of photosynthetic electron transport through PSII ($Y = \left(F_{m'} - F\right)/F_{m'}$) , were $F_{m'}$ is the maximal fluorescence of a light-adapted plant when all reaction centers are reduced, or closed, and F is the fluorescence in the light when part of the reaction centers are open. At that point, rates of electron transport (ETR) were calculated as: $ETR = Y \times incident\ PAR\ \times AF\ \times 0.5$ (assuming that half of the photons absorbed were absorbed by photosystem II; Schreiber et al., 1994). Photosynthetic quantum efficiency (α) was quantified as the initial slope of the linear portion of the RL curve. Saturating irradiance (E_k) was obtained by dividing the $\mathrm{ETR}_{\max}$ by α.

4.3.3.5. Data analysis

Factorial ANOVAs (with two fixed and crossed factors; temperature and invasive algae) were used to test for differences in the initial morphological features of all the seedlings, and to compare final values of seedlings' morphological characteristics, carbon, nitrogen and carbohydrate content, as well as photophysiological variables. In addition, one-way ANOVAs were used to examine effects on seedling morphological traits between the four different levels of invasive algae presence (i.e. absence, *C. cylindracea*, *L. lallemandii*, and *C. cylindracea* + *L. lallemandii*) at 27ºC. Repeated measures ANOVAs were used to compare the concentration of dissolved inorganic nutrients present in the seawater of each aquarium at the beginning, in the middle and at the end of the experiment.

Prior to statistical analyses, data were examined for normality and homogeneity of variances using Kolmogorov-Smirnov and Cochran's tests, respectively. Post-hoc Tukey test were conducted to examine differences between levels of each factor.

4.3.4. Results

All *Posidonia oceanica* seedlings survived for the entire duration of the experiment, but their development differed among treatments (Table 8). The total length of new roots decreased significantly with temperature, being significantly longer at 25ºC than the other temperatures (Fig. 24, Tukey). Similarly, the number of leaves and total leaf area decreased about 30 % and 50 % at 27ºC when seedlings were growing alone or with *Caulerpa cylindracea*, respectively. At 29ºC, the number of leaves continued to decrease. In addition, total leaf area decreased at 29ºC but only when *P. oceanica* seedlings were growing alone, whereas seedlings co-ocurring with *Lophocladia lallemandi* did not exhibit such decreases in either number of leaves or leaf area at 29ºC (Fig. 24, Tukey). On the other hand, the percentage of necrotic leaf surface tended to increase with temperature, being significantly higher at 29ºC for seedlings growing alone or with *L. lallemandii* (Fig. 24, Tukey).

Carbon content in seedling tissues was generally not affected by temperature nor the presence of invasive algae (Table 9), except for in the presence of *L. lallemandii*, whereby carbon in new roots was significantly lower than for seedlings growing alone or in the presence of *C. cylindracea*. Nitrogen content of leaves and new roots did not differ amongst treatments, whereas nitrogen content of old roots, rhizomes and seeds was generally higher at 29ºC than the other temperatures (Table 9; Fig. 25; Tukey). Regarding soluble carbohydrates in seeds, sucrose content was higher in the presence of *L. lallemandii* than in the other invasive treatments for all temperatures, and starch content was not affected by either treatment or their interaction (Table 10; Fig. 26, Tukey).

Whereas saturating irradiance did not differ amongst treatments (Table 11), seedling maximum electron transport rate (ETR_{max}) and photosynthetic efficiency (α) increased significantly at 27ºC, and decreased again at 29ºC (29 = 25 < 27; Table 11; Fig. 27, Tukey).

The presence of the two invasive species (tested only at 27ºC) had very few effects on the development of *P. oceanica* seedlings; with only total leaf area changing, which was lower in seedlings interacting with invasive species, due to the presence of *C. cylindracea*, in comparison to seedlings growing alone (see Supplementary material Table S 3; Tukey).

Water nutrient content changed through time (i.e. with each water change) but did not differ between treatments (see Supplementary material Table S 4). Ammonium concentrations decreased through time from 7.1 ± 0.41 to 2.9 ± 0.32 μM, whereas nitrate increased from 0.5 ± 0.02 to 3.5 ± 0.29 μM.

Variable	Source	df	MS	F	p
Nª of leaves	Invasion	2	0.164	0.327	0.724
	Temperature	2	11.184	22.322	**< 0.01**
	Inv x T	4	1.580	3.154	**0.03**
	Error	27	0.501		
Width (cm)	Invasion	2	0.001	0.428	0.656
	Temperature	2	0.002	1.257	0.301
	Inv x T	4	0.001	0.362	0.833
	Error	27	0.002		
Maximum leaf lenght (cm)	Invasion	2	6.041	3.848	**0.03**
	Temperature	2	4.221	2.688	0.086
	Inv x T	4	2.483	1.582	0.208
	Error	27	1.570		
Total leaf area (cm^2)	Invasion	2	68.21	4.788	**0.017**
	Temperature	2	468.83	32.912	**< 0.01**
	Inv x T	4	109.93	7.717	**< 0.01**
	Error	27	14.25		
Necrotic leaf surface (cm^2)	Invasion	2	4.415	1.566	0.227
	Temperature	2	23.482	8.873	**< 0.01**
	Inv x T	4	3.452	1.304	0.293
	Error	27	26.464		
Seed area	Invasion	2	0.013	0.309	0.737
	Temperature	2	0.034	0.773	0.472
	Inv x T	4	0.022	0.494	0.740
	Error	27	0.044		
Nº of roots	Invasion	2	31.001	0.971	0.391
	Temperature	2	29.659	0.929	0.407
	invasion x ten	4	29.911	0.937	0.457
	Error	27	31.912		
Total root length (cm)	Invasion	2	35.940	2.004	0.154
	Temperature	2	7.990	0.446	0.645
	Inv x T	4	23.590	1.315	0.289
	Error	27	17.930		
Maximum root length (cm)	Invasion	2	1.192	0.9464	0.401
	Temperature	2	1.922	15.263	0.236
	Inv x T	4	0.73	0.5798	0.680
	Error	27	1.259		
Nª new roots	Invasion	2	74.104	1.226	0.309
	Temperature	2	51.780	0.856	0.436
	Inv x T	4	55.269	0.914	0.470
	Error	27	60.460		
Total new roots length	Invasion	2	9.723	3.696	**0.038**
	Temperature	2	26.298	10.235	**< 0.01**
	Inv x T	4	0.435	0.165	0.954
	Error	27	2.631		
Maximum new root length (cm)	Invasion	2	3.864	3.480	**0.045**
	Temperature	2	8.761	7.890	**< 0.01**
	Inv x T	4	0.150	0.135	0.968
	Error	27	1.110		

Table 8. Results of the Factorial ANOVAs analysing the morphological features of *Posidonia oceanica* seedlings in response to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects are highlighted in bold.

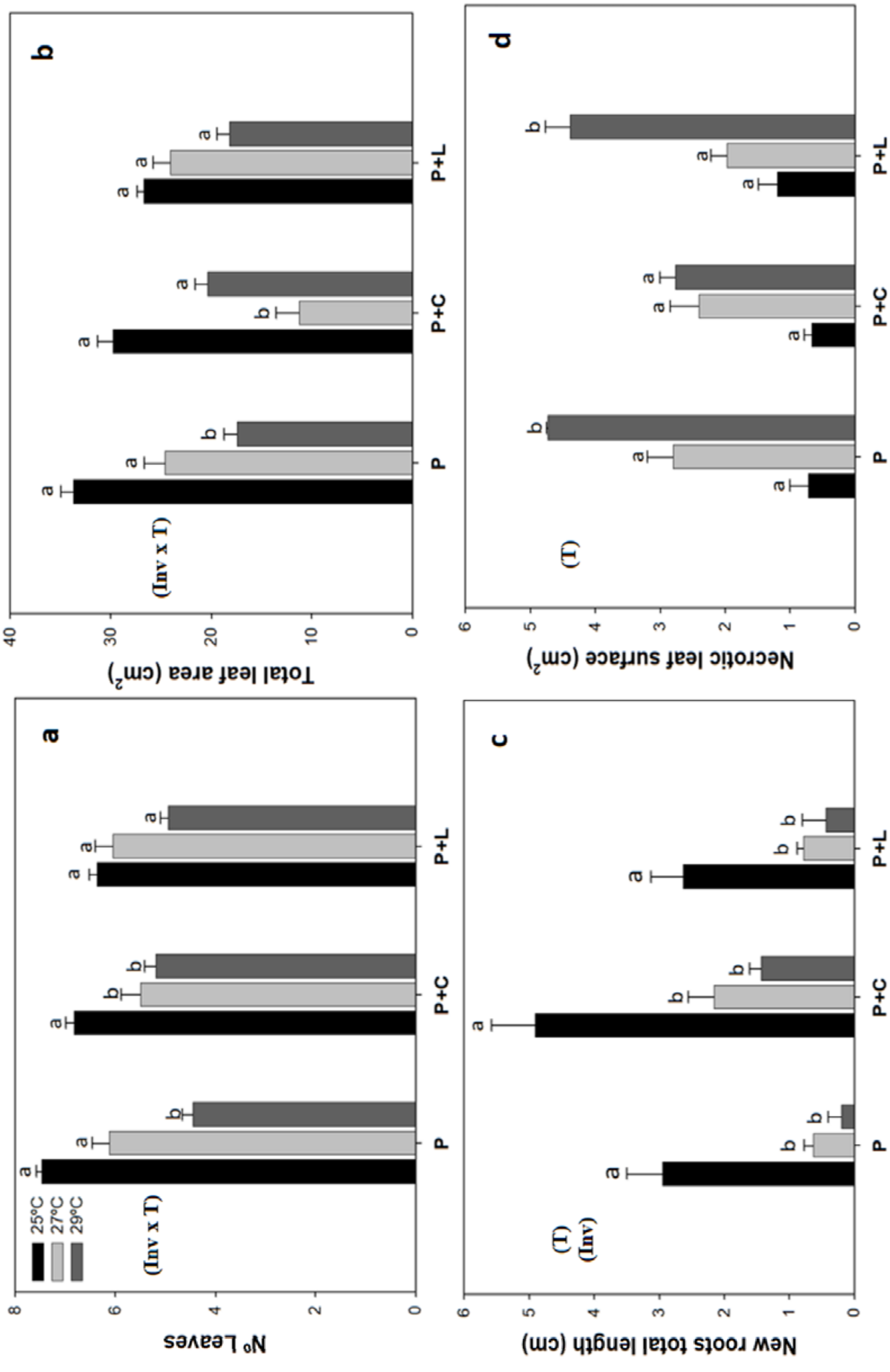


Figure 24. *Posidonia oceanica* seedling's number of leaves (a), total leaf area (b), total length of new roots (c) and necrotic leaf surface (d) in the different experimental treatments: P (only *P. oceanica* seedlings), P+C (*P. oceanica* seedlings and *Caulerpa cylindracea*), P+L (*P. oceanica* seedlings + *Lophocladia lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

Nutrient	Variable	Source	df	MS	F	p
Carbon	Seeds	Invasion	2	17.3	2.908	0.071
		Temperature	2	0.79	0.134	0.875
		I x T	4	5.38	0.904	0.475
		Error	27	5.95		
	Old roots	Invasion	2	12.07	1.647	0.211
		Temperature	2	18.88	2.578	0.094
		I x T	4	12.88	1.758	0.166
		Error	27	7.33		
	New roots	Invasion	2	43.95	4.557	**0.020**
		Temperature	2	1.17	0.122	0.886
		I x T	4	3.67	0.381	0.820
		Error	27	9.64		
	Leaves	Invasion	2	1.29	2.44	0.106
		Temperature	2	0.54	1.02	0.373
		I x T	4	0.23	0.43	0.784
		Error	27	0.53		
	Rhizomes	Invasion	2	0.79	0.570	0.570
		Temperature	2	3.62	2.640	0.091
		I x T	4	1.43	1.051	0.404
		Error	27	1.37		
Nitrogen	Seeds	Invasion	2	0.200	3.123	0.060
		Temperature	2	1.089	17.002	< **0.01**
		I x T	4	0.135	2.121	0.105
		Error	27	0.064		
	Old roots	Invasion	2	0.006	0.475	0.626
		Temperature	2	0.069	5.515	< **0.01**
		I x T	4	0.005	0.421	0.792
		Error	27	0.012		
	New roots	Invasion	2	0.0319	0.035	0.066
		Temperature	2	0.006	0.600	0.556
		I x T	4	0.012	1.162	0.350
		Error	27	0.01		
	Leaves	Invasion	2	0.003	0.151	0.860
		Temperature	2	0.012	0.663	0.523
		I x T	4	0.015	0.813	0.527
		Error	27	0.018		
	Rhizomes	Invasion	2	0.102	0.650	0.531
		Temperature	2	1.086	6.894	< **0.01**
		I x T	4	0.141	0.898	0.480
		Error	27	0.157		

Table 9. Factorial ANOVAs analysing the differences in percentage of nitrogen in the *Posidonia oceanica* seedlings tissue components: seeds, old roots, new roots, rhizomes and leaves, according to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects are highlighted in bold.

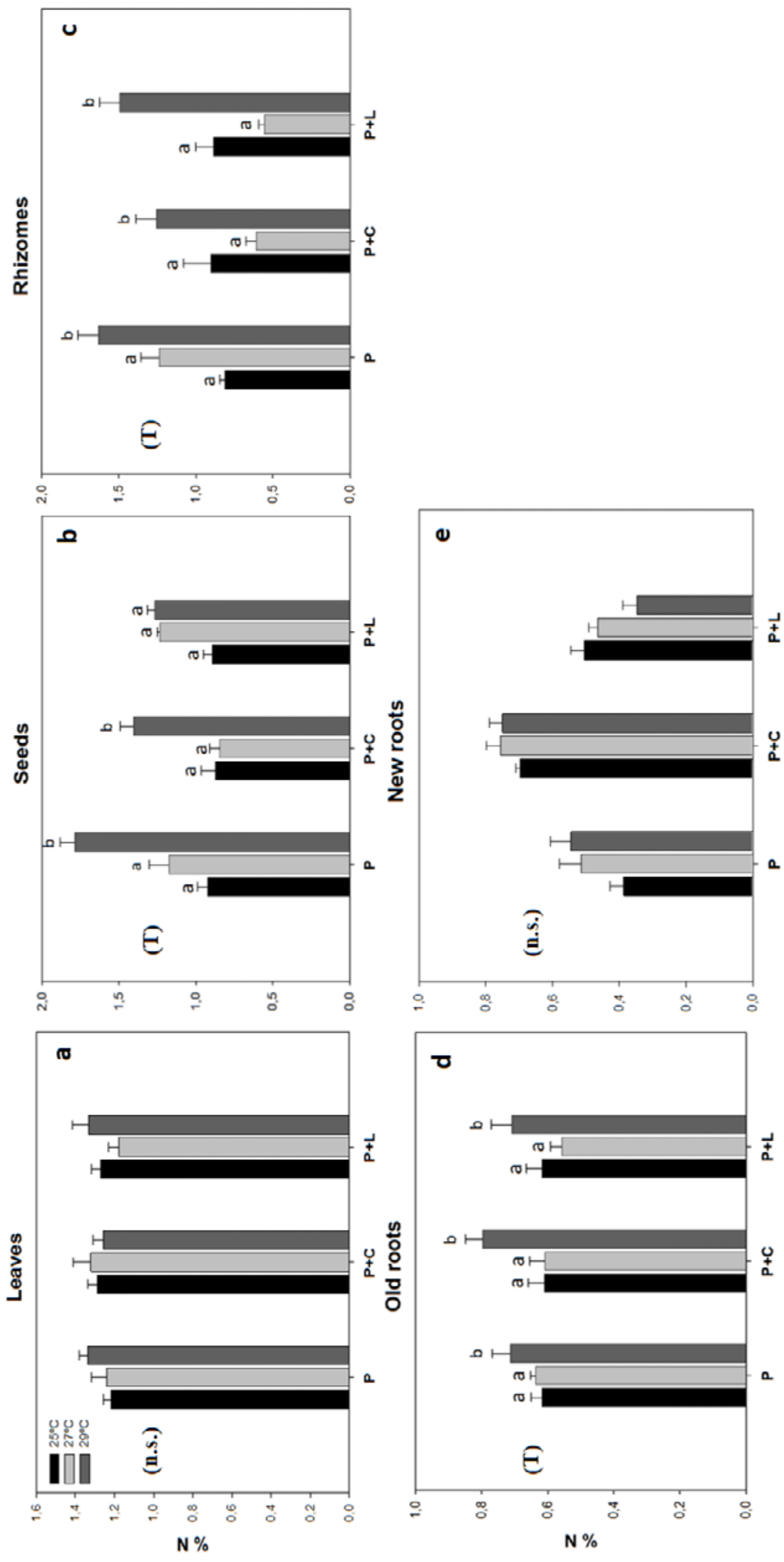


Figure 25. *Posidonia oceanica* mean nitrogen concentration (% of dry weight) of seedling tissues: leaves (a), seeds (b), rhizomes (c), old roots (d) and new roots (e), across experimental treatments: P (only *P. oceanica* seedlings), P+C (*P. oceanica* seedlings and *Caulerpa cylindracea*), P+L (*P. oceanica* seedlings + *Lophocladia lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

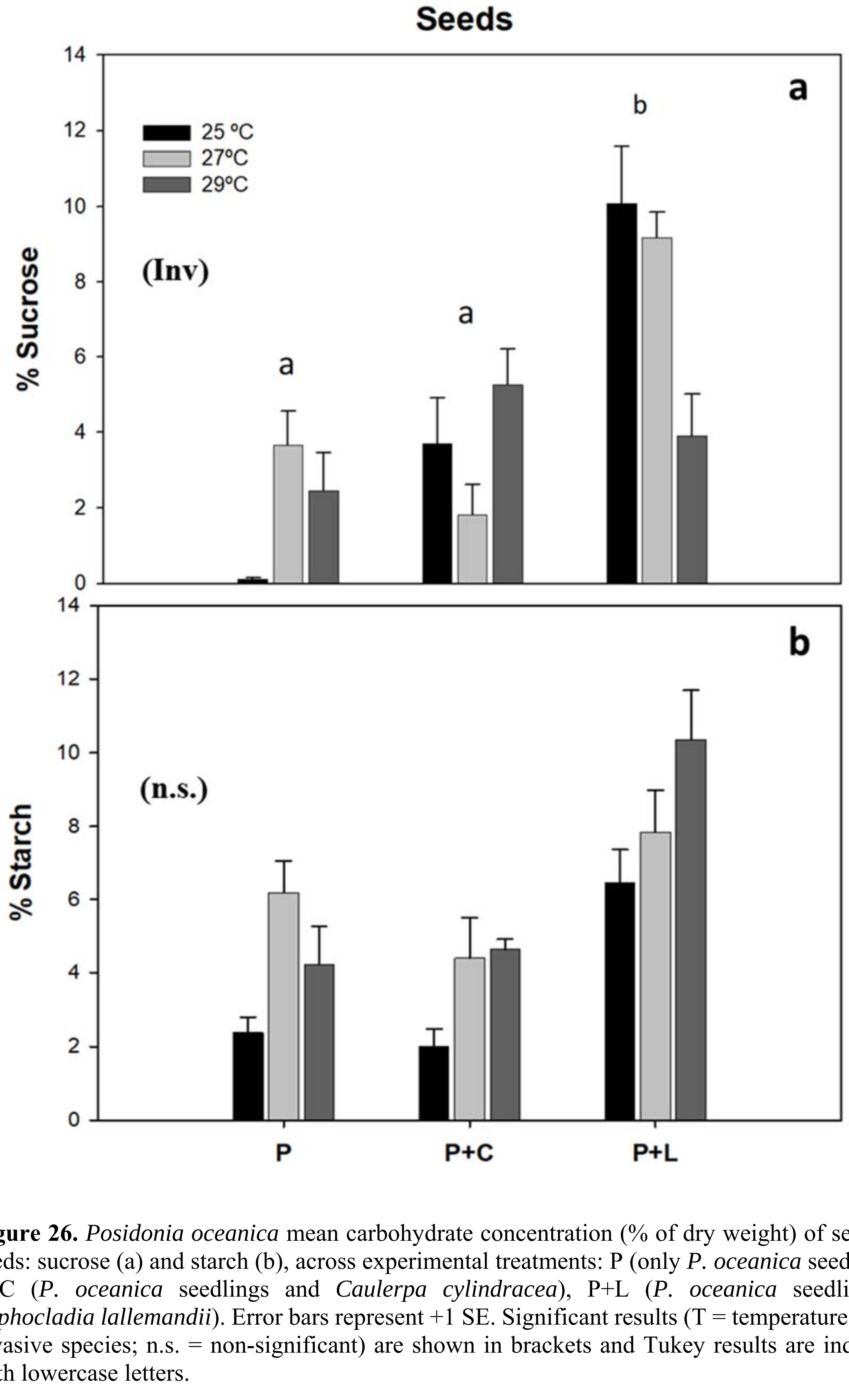


Figure 26. *Posidonia oceanica* mean carbohydrate concentration (% of dry weight) of seedling seeds: sucrose (a) and starch (b), across experimental treatments: P (only *P. oceanica* seedlings), P+C (*P. oceanica* seedlings and *Caulerpa cylindracea*), P+L (*P. oceanica* seedlings + *Lophocladia lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

Variable	Source	df	MS	F	p
Sucrose	Invasion	2	72.92	6.852	**< 0.01**
	Temperature	2	2.437	0.229	0.797
	Inv x T	4	23.38	2.197	0.112
	Error	17	10.64		
Starch	Invasion	2	2.37E+09	1.829	0.191
	Temperature	2	5.61E+08	0.431	0.656
	Inv x T	4	5.94E+08	0.457	0.765
	Error	17	1.29E+09		

Table 10. Factorial ANOVAs analysing the percentage of sucrose and starch in the seeds of *Posidonia oceanica* seedlings according to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects highlighted in bold.

Variable	Source	df	MS	F	p
ETR max	Invasion	2	5.197	0.268	0.765
	Temperature	2	169.055	8.717	**< 0.01**
	Inv x T	4	9.032	0.466	0.761
	Error	99	19.395		
Alpha	Invasion	2	0.003	0.469	0.627
	Temperature	2	0.076	13.928	**< 0.01**
	Inv x T	4	0.000	0.061	0.993
	Error	99	0.005		
Ek	Invasion	2	767.1	0.677	0.511
	Temperature	2	1009.9	0.891	0.414
	Inv x T	4	1002.9	0.885	0.476

Table 11. Factorial ANOVAs analysing the photophysiology parameters: ETRmax, alpha and Ek of *Posidonia oceanica* seedlings according to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects are highlighted in bold.

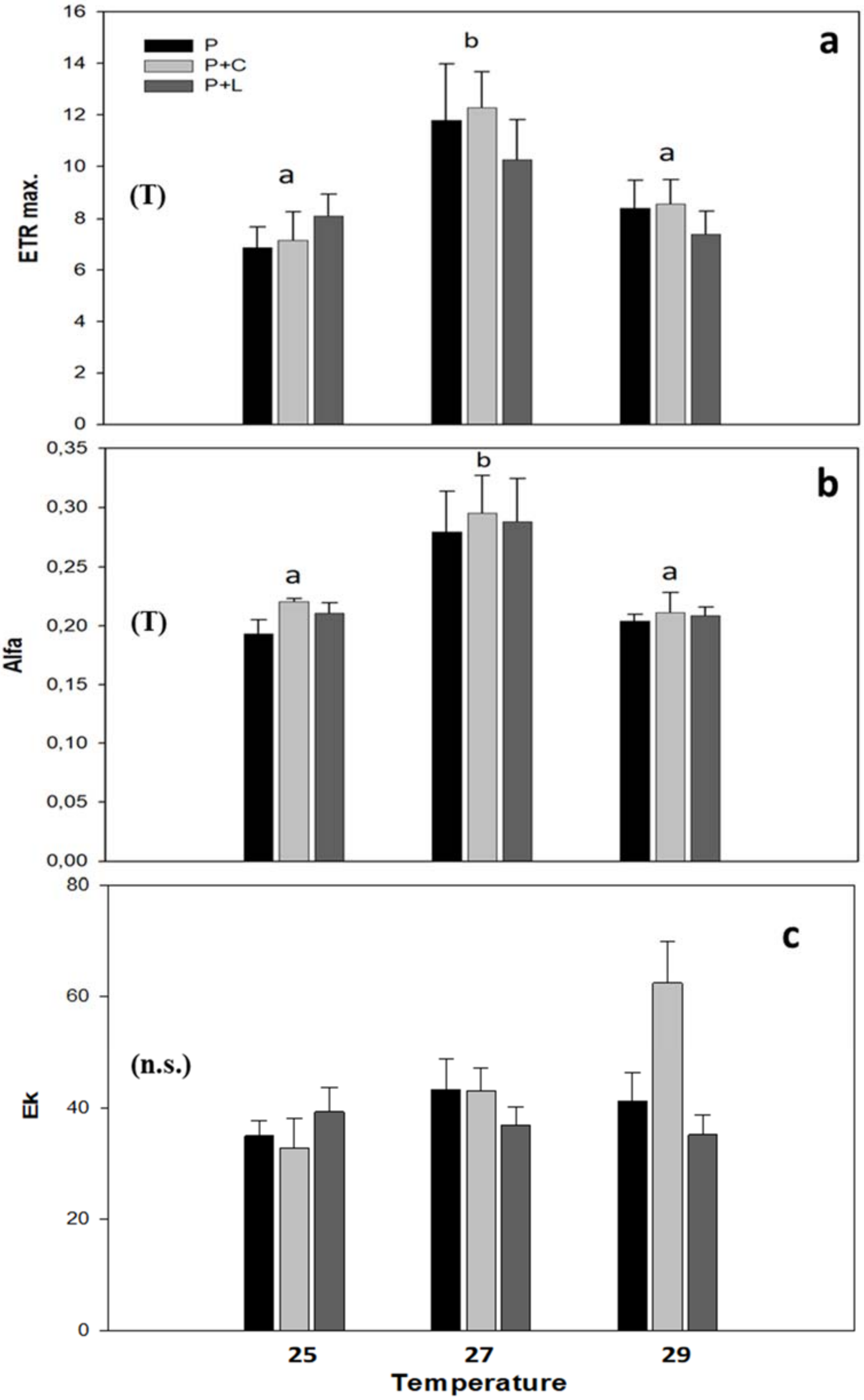


Figure 27. *Posidonia oceanica* photophysiology parameters: ETRmax (a), alpha (b) and Ek (c) across experimental treatments: P (only *P. oceanica* seedlings), P+C (*P. oceanica* seedlings and *Caulerpa cylindracea*), P+L (*P. oceanica* seedlings + *Lophocladia lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

4.3.5. Discussion

Our results indicate that warming can negatively affect the seedlings of *Posidonia oceanica*, and that the presence of invasive seaweed can potentially alter some of these responses to warming. Increasing temperatures have affected not only the photophysiology of the plants, but also leaf development and the production of new roots. Interestingly, the presence of invasive algae does not necessarily result in negative effects on seedlings.

Our observations supported our first hypothesis (i.e. the increase in temperature will negatively affect *P. oceanica* development, promoting seedling death), as we detected seedling deterioration with increasing temperatures. Optimal growth temperatures for temperate seagrasses are reported to be between 11.5 and 26ºC (Lee et al., 2007), and once the threshold temperature is exceeded, seagrass performance typically declines (Nejrup and Pedersen, 2008). Critical temperature threshold for adult *P. oceanica* plants are considered to be between 29ºC and 30ºC (Marbà and Duarte, 2010; Olsen et al., 2012), and similar to those of seedlings (Guerrero-Meseguer et al., 2017; Hernán et al., 2017). Our four-month-old seedlings survived along the 90 days of the experiment in all the treatments, including those maintained at 29ºC, whereas previous studies with six-month old seedlings observed an increase of ca. 13 % in mortality rates at this temperature (Hernán et al., 2017). Such contrasting responses may be related to carbohydrate reserves (i.e. starch and sucrose) of seeds, which were not affected by temperature in our experiment. Carbohydrates are crucial storage compounds of plants (Tetlow et al., 2004), which reflect the recent energetic balance of the plant, and their reduction can strongly influence plant survival (Genot et al., 1994) and make seagrass meadows more vulnerable to additional stress (Eklof et al., 2009). Presumably, our younger seedlings should have higher carbon reserves than older ones, which may have increased their resistance to warming. Consequently, we suggest that the lethal temperature threshold of young seedlings of *P. oceanica* is age-dependent based on carbon reserves available in the seed and would be above 29 ºC for younger seedlings. The results of Guerrero-Meseguer et al. (2017), who worked with five-month old *P. oceanica* seedlings, support this notion since they did not observe mortality at 27 nor at 29ºC, whereas mortality peaked to 33% when temperatures went over 29ºC.

Even though our seedlings did not suffer mortality under warming, we did observe sublethal responses which may affect seedling survival success in the longer term. For example, during seedling development, *P. oceanica* seedlings typically undergo a decrease in the number of leaves formed, but an increase in total leaf area as these new leaves are larger (Terrados et al., 2013). In our experiment, seedlings experiencing warming underwent a stronger reduction in leaf formation and an overall decrease in total leaf surface. This response indicates that increasing temperatures reduce leaf formation rates, which has also been reported for adults (Mayot et al., 2005) and seedlings (Olsen et al., 2012; Guerrero-Meseguer et al., 2017; Hernán et al., 2017) of *P. oceanica* as well as for adult plants of other temperate (Nejrup and Pedersen, 2008) and tropical (Edwards 1995) seagrasses. In addition, the proportion of necrotic leaf surface increased, indicating that warming creates stressful conditions for temperate seagrass seedlings (Niu et al., 2012, Guerrero-Meseguer et al., 2017; Hernán et al., 2017). Increased necrotic surface may be stimulated by a deterioration of the photosynthetic apparatus when temperature overpasses certain values. In fact, warming can lead to improved fitness and an increase in photosynthesis rate (with an estimated optimum temperature range for net photosynthesis of 16ºC to 35 ºC in temperate seagrasses; Lee et al., 2007) until the thermal threshold of a species is exceeded and damage occurs (Pörtner and Farrel, 2008; Pedersen et al., 2016). We observed that maximum electron transport rate (ETR_{max}) and photosynthetic efficiency (α) of seedlings increased significantly at 27ºC, but decreased again at 29ºC, suggesting that the photosynthesis process suffers when temperatures are above 27ºC (see also Guerrero-Meseguer et al., 2017).

Regarding seedlings' nutrient content, we detected higher nitrogen at higher temperatures in seeds, old roots and rhizomes, but no differences in leaves or new roots. Presumably, as the number of leaves and leaf surface decrease with temperature, seedlings don't need to mobilize a larger fraction of their nitrogen reserves (stored in seeds, old roots and rhizomes) to produce leaf biomass, resulting in higher final nitrogen concentrations in these tissues. In contrast, new roots may have higher nitrogen requirements since they are an active growth tissue where the available resources are mobilized and used, and their development may be prioritized regardless of the environmental conditions.

Interestingly, concerning our second hypothesis (i.e. the presence of the invasive algae reduces seedlings' vegetative development), our results suggest the opposite. While it has been demonstrated that *Caulerpa cylindracea* and *Lophocladia lallemandii* can negatively affect adult plants of *P. oceanica*, decreasing shoot size and increasing leaf necrosis and shoot mortality (Holmer et al., 2009; Ballesteros et al., 2007; Marbà et al., 2014b), effects on seedlings remain practically unknown. In fact, *C. cylindracea* can favor the production of new roots of *P. oceanica* seedlings, facilitating their early establishment (Pereda-Briones et al., 2018b), and effects of *L. lallemandi* on *P. oceanica* seedlings have not been examined thus far. The lack of negative effects of invasive algae on the seedlings observed in our experiment and detected in the field (Pereda-Briones et al., 2018b), in addition to the enhancement of root development in the presence of *C. cylindracea*, and the tendency of the presence of *L. lallemandii* to improve the negative effects of warming on seedling leaf development, all suggest that there may be positive rather than negative influences of invasive algae on seedlings during their recruitment phase under the tested conditions. Yet, and considering the cautionary principle, it is worth highlighting that, albeit not statistically significant, we did observe a trend towards lower leaf area in the presence of *L. lallemandii*. Perhaps other experimental conditions (e.g. duration of the experiment or biomass of *L. lallemandii*), would have yield more obvious negative effects.

Warming also led to a decrease of total new root length, which is likely to negatively affect the establishment capacity of seedlings if they do not reach the adequate root length for successful anchoring (Infantes et al., 2011a). In addition to the length of the root system, the ability of the seedlings to remain anchored to the substratum is strongly dependent on the hydrodynamic conditions (Frederiksen et al., 2004; Infantes et al., 2009; Rivers et al., 2011, Pereda-Briones et al., 2018a) and the substratum type (Balestri and Lardicci, 2008; Alagna et al., 2013; Pereda-Briones et al., 2018a). In this sense, the presence of *C. cylindracea,* by favoring the production of new roots, could facilitate the establishment of seedlings, and thus, maximize the possibilities of remaining in the substratum facing waves and currents. Indeed, the survival and growth of one-year old *P. oceanica* seedlings transplanted to dead matte substrate are enhanced by the presence of *C. cylindracea* (Pereda-Briones et al., 2018b). Furthermore, this facilitative interaction was not diminished under warming conditions, thus potentially counteracting negative warming effects by enabling the anchoring of the seedlings and the consequent nutrient uptake in adverse thermal scenarios.

Elevated temperatures can increase or decrease competition, and even transform competitive interactions into facilitative ones (Harley et al., 2012). In our case study, the effects of rising temperatures on *P. oceanica* seedlings have not been modified in the presence of invasive algae, rejecting our third hypothesis (i.e. the interaction of seedlings with the invasive algae alters the effects of the temperature on *P. oceanica* seedlings). Although the presence of *C. cylindracea* attenuated the decrease in the number of leaves and total leaf area of *P. oceanica* seedlings at 29ºC, and in the presence of *L. lallemandii* there were no temperature effects in these two variables, the general trend of decreasing foliar development with higher temperatures is maintained independently of the presence of invasive algae.

Warming is expected to continue in the coming years (IPPC, 2014), and this may also modify the response of both invasive algae, with further consequences for their interactions with *P. oceanica* seedlings. For instance, *L. lallemandii* may be more vulnerable to warming as it does not tolerate well temperatures above 29ºC, while *C. cylindracea* maintains high growth rates from 23 to 31ºC (Samperio-Ramos et al., 2015). On the other hand, global warming on marine ecosystems is accelerating the settlement of new alien species (Raitsos et al., 2010), and there are numerous examples of invasive species being more tolerant to higher temperatures than native ones (e.g. Smith et al., 2004; Sorte et al., 2010). This is particularly relevant for the Mediterranean Sea, where the predicted warming exceeds threefold that of the global ocean (Burrows et al., 2011), further encouraging the proliferation of invasive species from tropical and subtropical origin entering through the Suez Canal (Bianchi, 2007). Therefore, even though *P. oceanica* seedlings appear to be resistant in their interaction with *C. cylindracea* and *L. lallemandii* (at least in the conditions of our study), since they are sensitive to warming (Guerrero-Meseguer et al., 2017; Hernán et al., 2017; this study), they may not cope well with these or other invasive species if global warming trends continue.

4. RESULTS

Resilience of seagrass seedlings to marine heatwaves is modulated by local thermal variation

4. RESULTS

Cymodocea nodosa seedling
(IMEDEA, Esporles, July 2017)
By Laura Pereda Briones

4.4. Resilience of seagrass seedlings to marine heatwaves is modulated by local thermal variation

4.4.1. Abstract

The increase of extreme climatic events, such as marine heatwaves (MHWs), may push marine organisms to exceed their resilience limits. In this scenario, early life stages are critical for adaptation to global warming. Habitat-forming species, which are already stressed by multiple perturbations, are also vulnerable to these abrupt events. Using a common garden mesocosm experiment, we evaluated the response of seedlings of the seagrass *Cymodocea nodosa* from eight regions covering its distribution range in the Mediterranean and Atlantic to two MHWs of different intensity. Specifically, we aimed to understand how these extreme events affected the survival, photophysiology and morphometry of seedlings. We expected that a larger local environmental (particularly, thermal) variability would be connected with increased resilience to MHWs. Our findings show that seedlings suffer a decrease in survival, leaf development and photosynthesis efficiency under both MHWs, being stronger for the severe one (32ºC), but seedling responses are modulated by local thermal variation, with larger local (population) thermal variability promoting higher seedling resistance to MHWs. In addition, we suggest that these responses might not only be due to phenotypic plasticity, but also be the result of adaptation to local thermal regimes. This study also suggested that early life stages of seagrasses may be more vulnerable to heat stress than their adult counterparts. This outcome has implications for the future distribution patterns of marine species in response to global warming.

4.4.2. Introduction

Global warming is affecting marine ecosystem structure and function worldwide (Hoegh-Guldberg and Bruno, 2010), and might considerably modify the provision of ecosystem services (Nordlund et al., 2016). The actual trend of increasing sea surface temperatures [(SST) IPCC, 2014], in conjunction with the progressively frequent occurrence of extreme climatic events (IPCC, 2012), is causing abrupt ecological changes in the oceans. In particular, MHWs, which are episodes of extreme warm seawater temperatures that persist for days to months (Hobday et al., 2016), are increasing in frequency and duration (Hobday et al., 2018; Oliver et al., 2018). MHWs have important impacts on marine ecosystems due to the lower capacity of species to respond to unexpected events rather than to gradual changes (Garrabou et al., 2009; Wernberg et al., 2012; Smale and Wernberg, 2013; Wernberg et al., 2016; Hughes et al., 2017; Jones et al., 2018) and may push marine organisms to exceed their resilience limits (Winters et al., 2011; Frölicher and Laufköter, 2018). Furthermore, the ecological impacts of MHWs can be particularly dramatic when they involve habitat-forming species (e.g. corals, kelps, seagrasses), as negative impacts on their abundance and distribution can cascade and magnify throughout the ecosystems they form (e.g Wernberg et al., 2010; 2011), and will disrupt the provision of ecological goods and services associated to these paramount ecosystems (Smale et al., 2019).

Importantly, when predicting species responses to warming (e.g. extinctions, range shifts) species are often considered as physiologically homogeneous (e.g. Burrows et al., 2014), ignoring the fact that populations of the same species may differ in their thermal tolerance across their distribution range (King et al., 2018). For instance, the climatic variability hypothesis, which predicts that climatic tolerances are broader for species living in more variable climates (e.g. Khaliq et al., 2014), could also be applied at the intraspecific level. Indeed, regional thermal tolerance of the coral *Acropora tenuis* is driven by the local adaptations of the symbiont populations (Howells et al., 2011). These local differences in thermal tolerances would lead to the prediction that, within a species, populations experiencing lower variations in temperature will be more vulnerable to warming and extreme events than populations undergoing wider thermal variations.

Since many marine habitat-forming species are sessile organisms with limited dispersal capacity (Buia and Mazzela, 1991; Kendrick et al., 1995; Markey et al., 2016), short and intense climatic events, such as MHWs, provide little time for these sessile species to modify their distribution ranges (reviewed by Reusch and Wood, 2007). Therefore, in the context of these rapid changes, phenotypic plasticity (i.e. the ability of a given genotype to adjust the phenotype acclimating to different environmental conditions; *sensu* Angilleta, 2009) plays a critical role in determining population resilience (*sensu* Gunderson et al., 2010), i.e. resistance to- and recovery from- climate change. Together with acclimation, which can arise over reasonably rapid timescales (Munday et al., 2013), local adaptation of populations through genetic variation is essential for longer-term population resilience under this changing environment (Donelson et al., 2019). The breadth of the thermal niche of different populations of a species will be determined both by phenotypic plasticity and adaptation, and identifying their contributions has strong implications for understanding and predicting the resilience capacity of species to warming.

The ecological impacts of MHWs can be especially harmful in species that are already stressed by multiple perturbations, such as seagrasses (Scavia et al., 2002; Waycott et al., 2009). Despite the goods and services provided by seagrass meadows (Heminga and Duarte, 2000), these key ecosystems are one of the most threatened on earth (Waycott et al., 2009), and are experiencing widespread regression as a consequence of cumulative impacts, such as physical modification, nutrient and sediment pollution, introduction of non-native species, and climate change (Orth et al., 2006a). For seagrasses, there is evidence of several physiological and demographic processes that are affected by seawater warming. In temperate seagrasses, photosynthetic performance and survival of adult plants are negatively correlated with temperature (Massa et al., 2009 and Marbà and Duarte, 2010, respectively) and shoot mortality increases after the occurrence of MHWs (Diaz-Almela et al., 2009; Oviatt, 2004; Reusch et al., 2005).

In this changing environment, the products of seagrass sexual reproduction (i.e. early life stages) provide a new genetic variation pool necessary to adapt to anthropogenic and natural environmental changes and to guarantee the long-term persistence of species and the ecosystems they form (Hughes et al., 2008; Kendrick et al., 2012, 2017). Moreover, early life stages contribute to the maintenance of existing meadows (e.g. Jarvis et al., 2014) and the formation of new ones via dispersal (Orth et al., 1994; 2006b). Furthermore, seagrasses form a genetic mosaic, derived from the combination of clonal and sexual reproduction (Hemminga and Duarte, 2000; Kendrick et al., 2012), and higher intra-specific genetic diversity favours resilience after disturbances (Hughes and Stachowicz, 2004), including heatwaves (Ehlers et al., 2008; Reusch et al., 2005). Therefore, understanding resilience of early life stages to MHWs becomes critical in order to predict and manage the effects of extreme climatic events on crucial marine ecosystem such as seagrass meadows, thus contributing to seagrass population conservation.

In this study, we use the seagrass *Cymodocea nodosa* (Ucria) Ascherson, as the model species to examine how extreme climatic events affect seedling functioning and resilience in relation to their local thermal context, since this species covers a wide geographical range (the Mediterranean Sea and the contiguous eastern Atlantic coasts; Green and Short, 2003, Fig. 28), encompassing different thermal regimes. We collected seeds from eight regions and cultivated them into a common garden mesocosm facility during 15 months under the same conditions, prior to simulating two MHW of varying intensity. By doing this, although maternal effects may still be at play, we expected that the observed seedling responses would not only be due to acclimation (phenotypic plasticity), but would also be the result of adaptation to the local thermal regimes of their region of origin. Specifically, the objective of our work was to assess the resilience of this species to MHWs, connecting seedling responses to the thermal variability of their local (population) environments of provenance. We expected that a larger local environmental (particularly, thermal) plasticity would be connected with an increased resilience to MHWs.

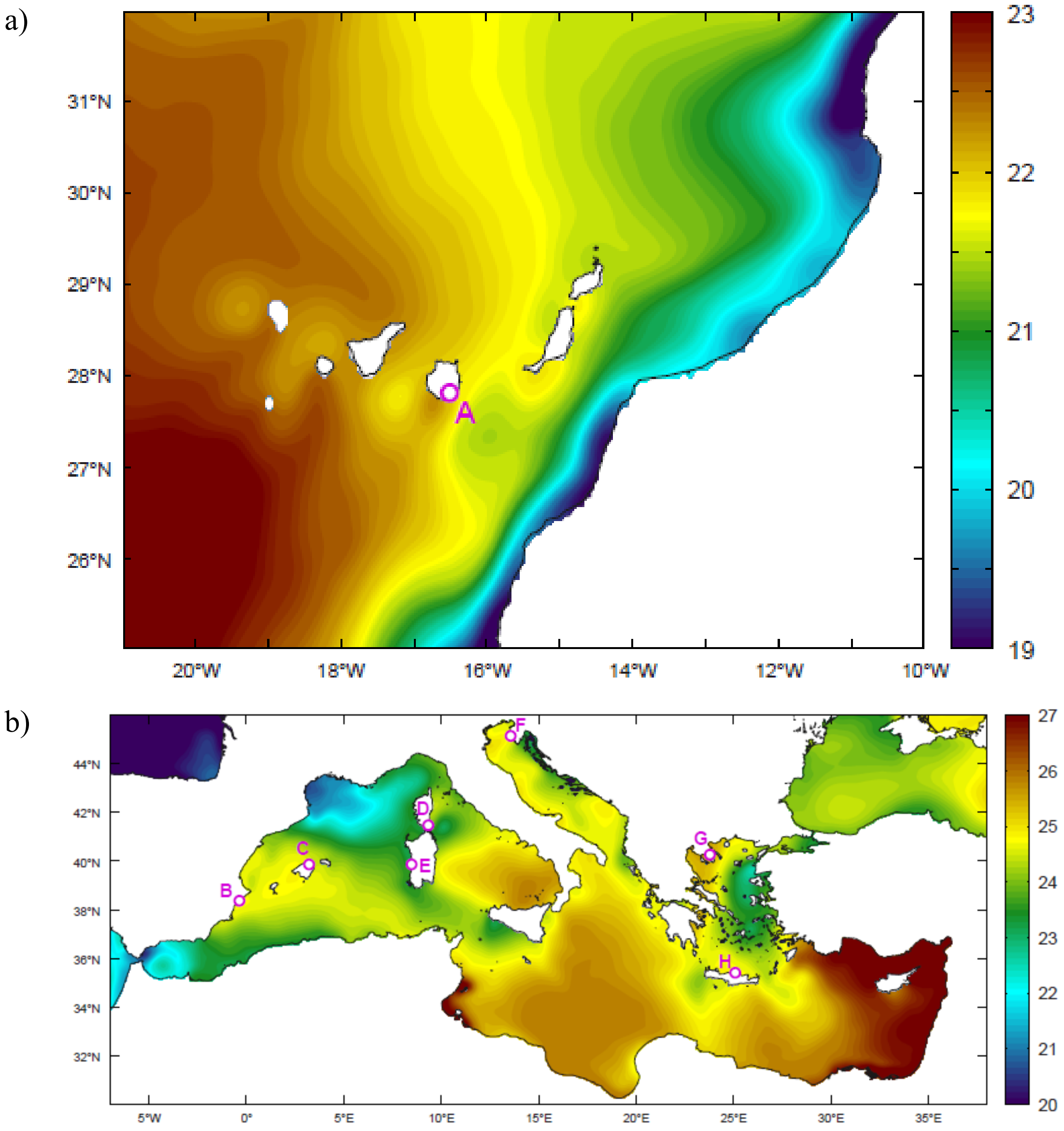


Figure 28: Mean summer (June-July-August) sea surface temperature (SST) values (ºC) of the 2006 - 2016 period along the distribution range of the seagrass *Cymodocea nodosa* in the Atlantic Ocean (a) and the Mediterranean Sea (b). Sampling regions are labeled with a letter: A (Gran Canaria), B (Alicante), C (Mallorca), D (Corsica), E (Sardinia), F (Istria), G (Halkidiki) and H (Crete).

4.4.3. Materials and methods

4.4.3.1. Study regions: thermal and productivity context, and MHWs definition

This study encompassed eight regions across the distribution range of *Cymodocea nodosa* in the Mediterranean Sea and the adjacent Atlantic Ocean. The thermal and productivity contexts of the eight regions were described by means of satellite data (GHRSST Level 4 OSTIA). For each region, daily SST values were obtained from 2006 to 2016 (Fig. 28). Then, for each of the eight regions, the following metrics were obtained (Table 12, see Supplementary material, Fig. S 7): number of warm days (i.e. sum of the total number of days for all the MHWs observed throughout the 10 years), summer mean maximum and mean minimum SST (ºC), seasonal cycle SST amplitude (mean maximum SST – mean minimum SST; ºC), SST standard deviation of the whole ten-year time series (ºC), seasonal standard anomalous SST, in ºC (i.e. standard deviation of the anomalous SST, where the anomaly is defined as the SST after removing its seasonal cycle, representing the inter-annual variability), standard anomalous lowpass, in ºC (i.e. Standard deviation of the anomalous SST, where the anomaly is defined as SST after removing its low frequency variability; the lowpass filter uses a 30-days symmetric blackman window, *https://www.mathworks.com/help/signal/ref/blackman.html,* it represents the high frequency variability), Chl-Max seasonal cycle (i.e. maximum value of the chlorophyll concentration; mg m^{-3}), and Chl-Amplitude seasonal cycle, in mg m^{-3} (i.e. amplitude of the chlorophyll concentration measured for the seasonal cycle (i.e. maximum – minimum chlorophyll concentration).

Environmental variables	Gran Canaria	Alicante	Mallorca	Sardinia	Corsica	Istria	Halkidiki	Crete
Number of warm days	177	96	120	181	184	135	106	137
Mean max. seasonal cycle (°C)	23.61	26.76	26.54	24.86	25.10	26.12	27.06	26.21
Mean min. seasonal cycle (°C)	21.56	24.05	24.14	22.82	22.37	21.44	22.94	24.25
Amplitude seasonal cycle (°C)	4.96	12.83	12.55	11.23	11.74	16.21	14.18	10.49
Std SST	1.79	4.59	4.56	4.14	4.24	5.67	5.11	3.83
Std anomalous (seasonal) SST	0.61	0.72	0.72	0.86	0.86	1.03	0.83	0.66
Std anomalous (lowpass) SST	0.18	0.24	0.25	0.28	0.29	0.39	0.31	0.24
Chl - Max Seasonal Cycle (mg m^{-3})	0.23	0.77	0.62	1.51	0.52	1.33	0.58	0.23
Chl - Amplitude Seasonal (mg m^{-3})	0.11	0.64	0.48	1.36	0.39	0.94	0.46	0.13

Table 12. Environmental variables for the eight studied regions ordered from West to East.

MHWs have been defined in different ways depending on the metrics used to assess this extreme thermal event (e.g. Meehl and Tebaldi, 2004; Selig et al., 2010; Marbà et al., 2015; Hobday et al., 2016). In the present study we define a MHW as a period of time when the SST is 0.2ºC above its maximum seasonal cycle (in our case for the period 2006

- 2016; See Supplementary material Fig. S 8 for details on MHW definition and Supplementary material Table S 5 for a description of the main characteristics of the different MHWs detected for each region). To describe the MHWs, we used the daily SSTs from the level 4 High Resolution Sea Surface Temperature satellite data (GHRSST Level 4 OSTIA), which is gridded on a regular 0.054º grid and available for the period 2006 - 2016. We also used the chlorophyll concentration from the MODIS-Terra, mapped at 4 km resolution, as a proxy of regional productivity in order to incorporate other possible variables (besides the thermal ones) that may affect seedling traits. Considering the satellite data obtained, and taking into account that water temperatures in these extreme events will soon surpass 29ºC (Hobday et al., 2018), we selected 29ºC as our moderate MHW, and 32ºC as the severe one.

4.4.3.2. Seed collection, germination, and seedling maintenance

Seeds of *C. nodosa*, which are typically buried in the sediment on shallow soft substrates (Terrados, 1993; Barberá et al., 2005), were collected during February and March 2016 (Dominguez et al., 2010) in Gran Canaria (the Canary Islands) and at 7 Mediterranean regions (Alicante, Mallorca, Sardinia, Corsica, Istria, Halkidiki and Crete; Fig. 28). At each region, seeds were collected from two sites. All seeds were collected from shallow-water meadows (Table 13) by gently removing the sediment by hand while scuba diving.

Region	Country	Latitude	Longitude	Depth (m)	S (‰)	Sediment type	# seeds collected
Gran Canaria	Spain	N 27°47'22.45"	W 015°29'33.63"	9	37.0	Medium sand	400
		N 27º52'12.42"	W 015º23'02.80"	14	37.0		400
Alicante	Spain	N 38°21'34.83"	W 000°26'17.66"	2.5	37.2	Fine sand and mud	440
		N 38°11'08.58"	W 000°32'24.09"	3.5	37.3		400
Mallorca	Spain	N 39°51'36.68"	E 003° 6'37.22"	2.5	38.0	Fine sand	670
		N 39°50'13.38"	E 003°10'4.58"	3	38.0		410
Sardinia	Italy	N 39° 53'6.00"	E 008° 26'52.2 0"	2.0	37.0	Fine sand	320
		N 39° 51'34.80"	E 008° 26'16.20"	1.5	37		413
Corsica	France	N 41°28'11.97"	E 009° 4'16.34"	0.5	37.0	Fine gravel and mud	750
		N 41°28'31.16"	E 041°28'31.16"	0.5	37.0		556
Istria	Croatia	N 45° 5'51.85"	E 013°37'53.03"	2.5	39.5	Medium sand with mud	770
		N 45° 7'2.68"	E 013°36'58.73"	4	39.5		510
Halkidiki	Greece	N 40°13'52.60"	E 023°44'29.38"	0.5	39.0	Mud and coarse sand	411
		N 40°12'42.99"	E 023°46'29.09"	0.5	39.0		613
Crete	Greece	N 35°24'0.96"	E 025° 1'34.04"	5	38.0	Medium sand	571
		N 35°19'17.21"	E 025°23'31.59"	1.5	38.0		145

Table 13. Location and environmental characteristics of the *Cymodocea nodosa* meadows sampled at each region.

Seeds were stored in darkness in water bottles with seawater, including aeration and water replacement if necessary. The same day of collection, or the following day, seeds were transported by plane to the Mediterranean Institute for Advance Studies (IMEDEA), in Mallorca (Western Mediterranean). Once at IMEDEA, seeds were placed under constant darkness inside 60L aquariums with seawater at a temperature of 19ºC. The aquariums were equipped with aeration and a water filter, providing a flow rate of 200L per hour.

Seeds germination was induced in April 2016 through a hyposaline shock (Zarranz et al., 2010). Initially, all seeds were placed in recipients where salinity was reduced until 11. Once seeds germinated, they were transferred into other recipients with higher salinities at one week intervals (24 first and subsequently to 31), in order to favor their acclimation to final salinities of 37. At the beginning of May 2016, all surviving seedlings were transplanted to outdoor mesocosms (300L). Each seedling was planted into a 4 x 4 cm seedbed filled with medium-fine siliceous sediment, inside the mesocosms, which were supplied with a continuous seawater flow and natural solar illumination. Seedlings were randomly distributed across seedbeds and mesocosms. Available light and temperature of the mesocosms were monitored using light Hobo Pendant loggers. This common garden infrastructure was located at the Visitors Centre of Cabrera National Park, at La Colonia de Sant Jordi (Mallorca). Hence, seedlings from all populations grew during 15 months under the same environmental conditions. During this period, a weekly manual elimination of epiphytic algae growing on seedling leaves and tanks was carried out. In August 2017 (i.e. when seedlings were ca. one and a half years old), 60 seedlings from each region were selected for the heatwave experiment.

4.4.3.3. **Heatwaves simulation: experimental design**

Seedlings were distributed into 30 (20L) aquaria filled with UV-filtered seawater at 24ºC, under a salinity of 37 and illuminated by white fluorescent lamps that provided 200 µmol m^{-2} s^{-1} of photosynthetically active radiation in a 14/10 h light/dark photoperiod; all aquaria had aeration and a filter. In each aquarium, two seedlings from each region were placed, for a total of 16 seedlings per aquarium. After an acclimation period of 10 days, seawater temperature was increased in 20 aquaria, at a rate of 0.5ºC per day. After 10 days, these 20 aquaria reached 29ºC; ten aquaria were maintained at this temperature, while the temperature was further increased until 32ºC in the remaining 10 aquaria. In summary, there were 10 control (24ºC) aquaria, 10 aquaria at 29ºC ('moderate MHW') and 10 aquaria at 32ºC ('severe MHW'). Both heatwaves (i.e. “disturbance phase”) had a duration of 30 days, after which seawater temperature was decreased 0.5ºC per day, until all the aquaria reached 24ºC, when the “recovery phase” period started (which also lasted 30 days).

4.4.3.4. **Seedling responses**

Survival of *C. nodosa* seedlings was calculated as number of living seedlings at the end of both experimental MHWs and the recovery phase, relative to the initial number of seedlings contained in each treatment. Seedling development was assessed at the start of the experiment, in the middle of both MHWs, as well as at the end of the recovery phase, by measuring the length and width of each leaf and the number of leaves, which allowed us to calculate total leaf area (TLA).

Effects of increasing temperatures on seedling photophysiology were analyzed in the middle of both MHWs, and at the end of the recovery phase, using an Aquation® Classic Submersible Fluorometer. Fluorescence measurements were performed by placing the tip of the instrument’s main optical fiber at ca. 2 cm away from the base of the leaf and irradiating a saturating-light with a pre-set photosynthetic photon flux. We then obtained the optimum quantum yield (Y) of the photosynthetic electron transport through the PSII ($Y = (F_m - F_o)/F_m$), were F_m is the maximal fluorescence of a dark-adapted plants when all PSII reaction centers are “open”, and F_o is the basal fluorescence. The absorptance factor (AF), i.e. the fraction of absorbed light by the leaf, was calculated using the Beer et al. (1998) method, by measuring the incident irradiance from a halogen lamp before and after the sensor was covered with 1 to 3 layers of seedling leaves. Finally, we calculated the absolute electron transport rate (ETR) as: $ETR = Y \times incident\, PAR \times AF \times 0.5$ (Schreiber et al., 1994). We chose ETR as the photophysiological parameter used for the statistical analysis since it can be related to carbon balance changes experienced by plants undergoing warming (Greve et al., 2003; Winters et al., 2011).

4.4.3.5. Statistical analysis

The interdependence (collinearity) between each pair of predictor environmental variables (Table 12) was examined through a Pearson-product moment correlation analysis (see Supplementary material, Table S 6) and was performed in order to select the environmental variables used to examine seedling responses.

Generalized Lineal Models (GLMs) were used to analyse the effects of region and temperature on the three types of seedling responses analysed (i.e. survival, morphometry and photophysiology). Survival at the end of both phases was analyzed through a GLM, using a “binomial” family error structure and a “logit” link function in the R statistical package. Two models were applied, with and without an interaction term between the predictive fixed factors “Treatment” (moderate MHW and severe MHW) and “Region”. Similarly, morphometry (in terms of TLA) and ETR values were analyzed through GLMs at the end of each phase using a “Gaussian” family error structure, with an “identity” link function and also considering two models (with and without an interaction term between temperature treatment and region). For each response variable, the AIC criterion was used to select the model of largest parsimony and the odds ratios were calculated for each region, as a measure of the 'effect size' between regions. Differences in TLA and ETR between treatments for each region were tested using a post-hoc Tukey test.

We examined the relationships between seedling response regional differences, i.e. regional 'effect sizes', of survival, morphometry (TLA) and ETR, with the regional variations in their thermal environments (environmental variables, Table 12) through lineal regression analyses. We selected the environmental variable which was more strongly related with our responses (i.e. higher coefficient of determination). All modelling and testing was implemented in the R.3.4.3 statistical package.

4.4.4. Results

4.4.4.1. Regional thermal regimes

Strong differences in thermal variability among regions were found (Table 12, see Supplementary material, Fig. S 7); in terms of standard deviation of the SST temperature and amplitude of the seasonal cycle, Gran Canaria was the most stable region, while Halkidiki and Istria were the most variable regions. Halkidiki and Istria exhibited the highest summer temperatures, reaching 28ºC. Some variability amongst regions was also detected in terms of productivity contexts (Table 12, Supplementary material Fig. S 7i, S 7j).

4.4.4.2. Seedling responses to experimental MHWs

At the end of the experimental warming, seedling survival was higher in the controls than in the warming treatments (Fig. 29, Table 14). Under control conditions, more than 70 % of the seedlings from the different regions remained alive; except for the seedlings of Gran Canaria, for which only 37 % survived (Fig. 29a). Under the moderate MHW, survival particularly decreased for seedlings from Gran Canaria and Alicante (5 % and 40 %, respectively; Fig. 29b). Under the severe MHW, all seedlings from Gran Canaria died, and survival strongly decreased for the rest of regions, particularly for Crete (36.7 %) and Alicante (24 %).

Leaf development measurements at the end of both phases revealed a decrease of TLA under both MHWs with respect to the control treatment (Fig. 30a, Tukey, Table 14). For all the regions (except Halkidiki) the severe MHW caused a significantly higher reduction in TLA than the moderate one.

Seedlings from different regions differed in their photophysiological (ETR) response to warming (Fig. 30b, Tukey, Table 14). For instance, while ETR of seedlings from Mallorca, Crete and Halkidiki decreased with increasing temperatures, seedlings from Sardinia, Corsica and Istria exhibited an increase in their ETR at the moderate MHW, but a decrease under the severe one. Seedlings from Alicante, on the other hand, underwent a decrease in ETR under the moderate MHW but an increase under the severe one (Fig. 30b, Tukey).

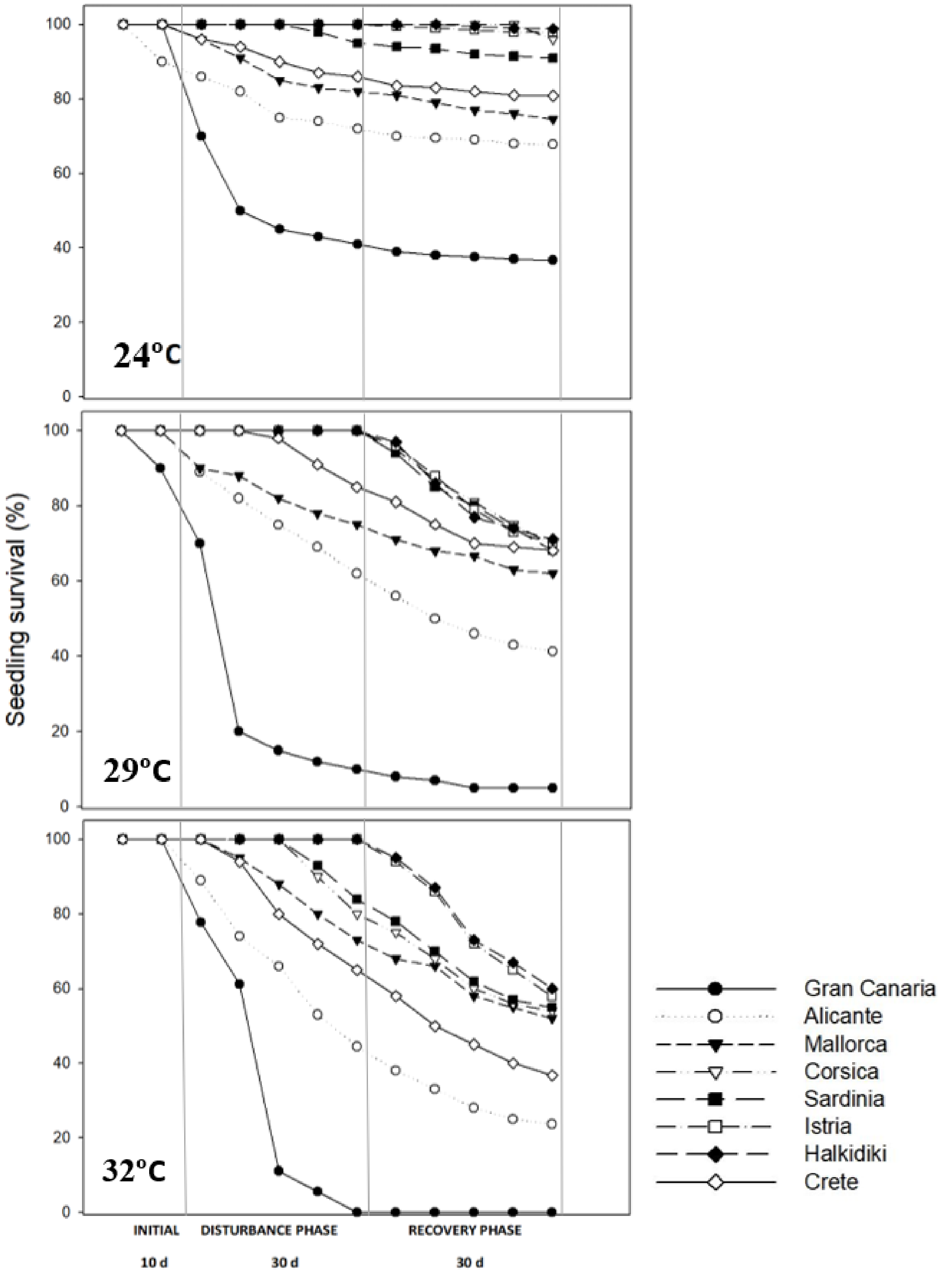
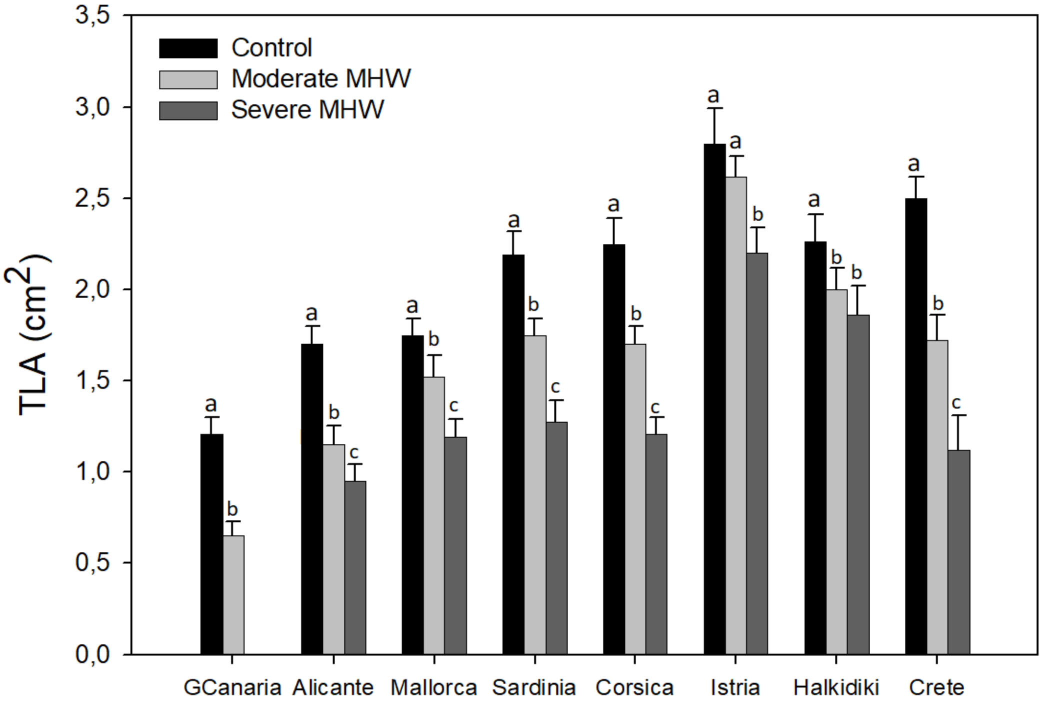


Figure 29. Percentage of *Cymodocea nodosa* seedling survival at the end of experimental warming, including (a) control (24ºC), (b) moderate MHW (29Cº) and (c) severe MHW (32ºC), for each region.

a)



b)

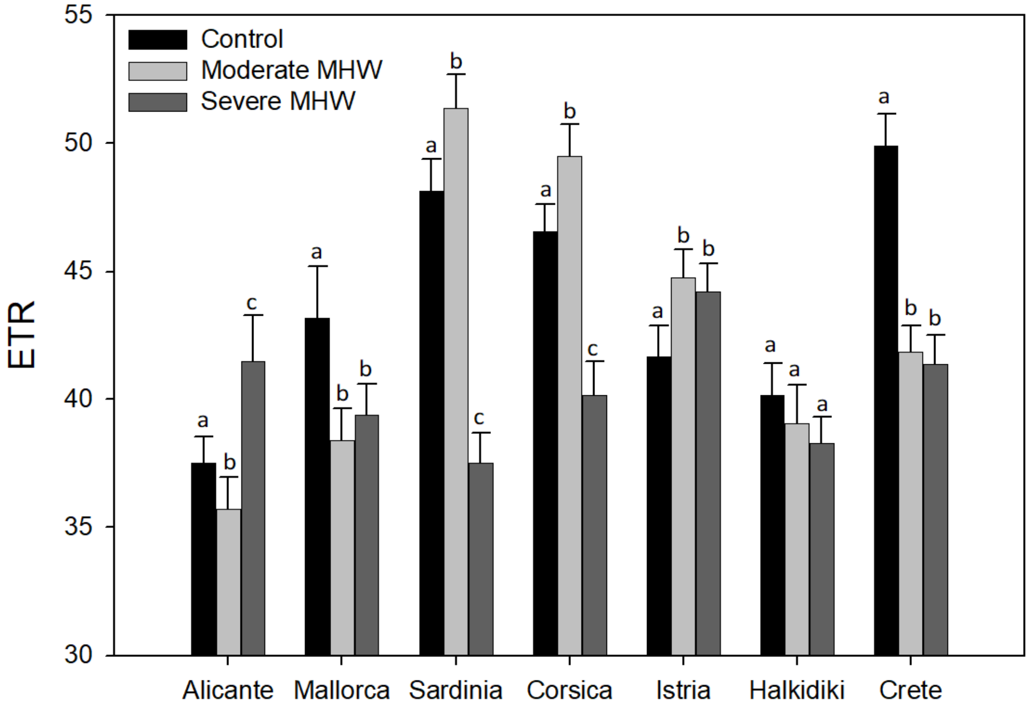


Figure 30. (a) Mean (+SE) total leaf area (TLA) and (b) electron transport rate (ETR) of *Cymodocea nodosa* seedlings at the end of experimental warming, including control (24ºC), moderate MHW (29Cº) and severe MHW (32ºC) treatments, for all Mediterranean regions. For panel (a) no data are available for the severe MHW for Gran Canaria since all seedlings died, whereas for panel (b) no data are available for Gran Canaria since the leaves of the survivors at the moderate MHW were degraded and none survived at the severe MHW. Letters indicate significant differences among temperature treatments (for each region separately). Regions are ordered from West to East.

Response variable	**Survival**		**TLA**		**ETR**	
Family	binomial (logit)		gaussian (identity)		gaussian (identity)	
	Estimate	P-value	Estimate	P-value	Estimate	P-value
Region (G. Canaria)	-1.91	< **0.01**	-1.83	< **0.01**		
Region (Alicante)	1.00	< **0.01**	1	< **0.01**	1	**0.03**
Region (Mallorca)	0.80	**0.04**	1.21	**0.03**	4.19	0.1
Region (Sardinia)	1.14	< **0.01**	1.34	< **0.01**	13.16	< **0.01**
Region (Corsica)	1.23	< **0.01**	1.48	< **0.01**	11.42	< **0.01**
Region (Istria)	1.15	< **0.01**	1.36	< **0.01**	6.61	**0.04**
Region (Halkidiki)	1.31	< **0.01**	1.78	< **0.01**	3.01	0.12
Region (Crete)	0.58	0.09	1.11	**0,05**	9.28	< **0.01**
Treatment (moderate)	-0.62	**0.01**	-0.96	**0.01**	-0.92	0.09
Treatment (severe)	-0.94	< **0.01**	-1.38	< **0.01**	-3.53	**0.04**

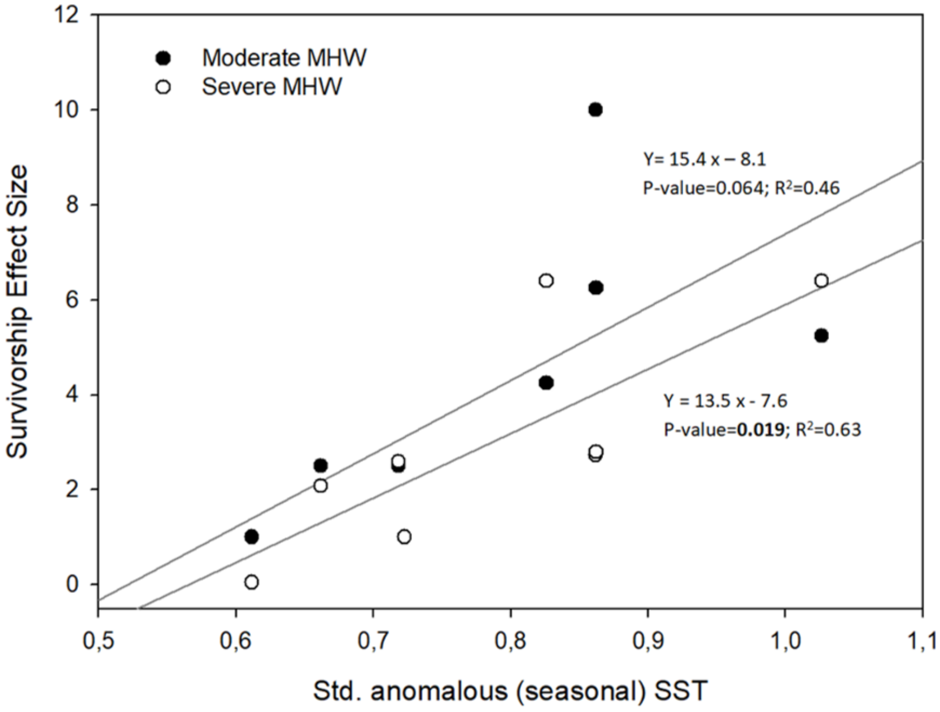
Table 14. Results of the General Lineal Model (GLM) analysis for the three response variables: survival, total leaf area (TLA) and electron transport rate (ETR). No ETR data is available for Gran Canaria since the leaves of the survivors at the moderate MHW were degraded and none survived at the severe MHW. Significant effects are highlighted in bold.

4.4.4.3. Regional differences in seedling resilience according to their thermal regimes of origin

Significant relationships were obtained between regional differences in the effect sizes of response variables and regional variations in their thermal contexts (Fig. 31, 32, 33, Supplementary material, Table S 7), whereas no significant relationships were detected between seedling responses and variability in productivity (i.e. variations in chlorophyll concentration).

In general, seedlings from regions with higher thermal variability (Table 12) were always more resistant (i.e. higher effect sizes) to increasing temperatures with respect to controls. For example, regions with a higher thermal variability had higher survival (Fig. 31), higher TLA (Fig. 32) and higher ETR (Fig. 33) effect sizes when undergoing warming, which correspond to the proportion that a region is more capable to survive, to increment their TLA or ETR values than a region of reference. However, seedling responses differed between MHW intensity, being more harmful the effects of the severe MHW than those of the moderate one. Furthermore, we detected that effect sizes of the recovery phase were more strongly related with regional thermal variability than those of the disturbance phase (see P-values and R^2 of Fig. 31, 32 and 33). For instance, while the relationship between survival and thermal variability was similar between MHWs for the disturbance phase (Fig. 31a), it differed considerably during the recovery phase, with the seedlings exposed to the moderate MHW exhibiting higher effect sizes than those exposed to the severe MHW (Fig. 31b). TLA responses followed a similar pattern than that of survival (Fig. 32). On the other hand, the relationship between ETR and thermal variability differed between the two MHWs at the disturbance phase (Fig. 33a); whereas under the severe MHW, the ETR effect size exhibited a positive relationship with thermal variability, it underwent the opposite pattern under the moderate MHW, i.e. regions with higher thermal variability had lower ETR effect sizes. This pattern shifted at the recovery phase, when ETR response was positively related with thermal variation for the moderate MHW whereas there was no significant relationship with thermal variability for the severe MHW.

a) DISTURBANCE PHASE



b) RECOVERY PHASE

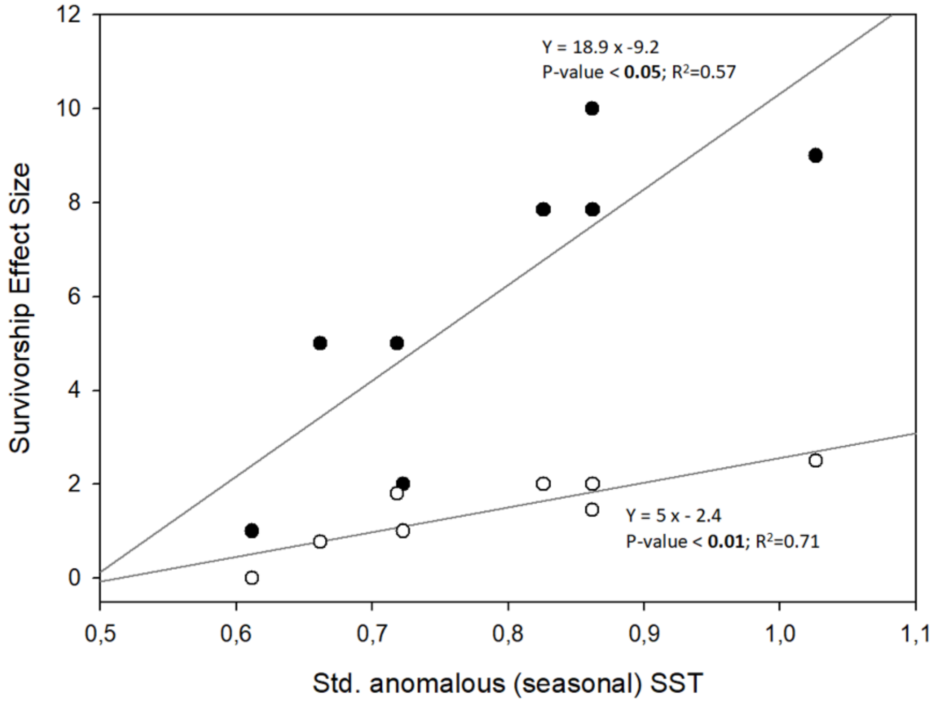
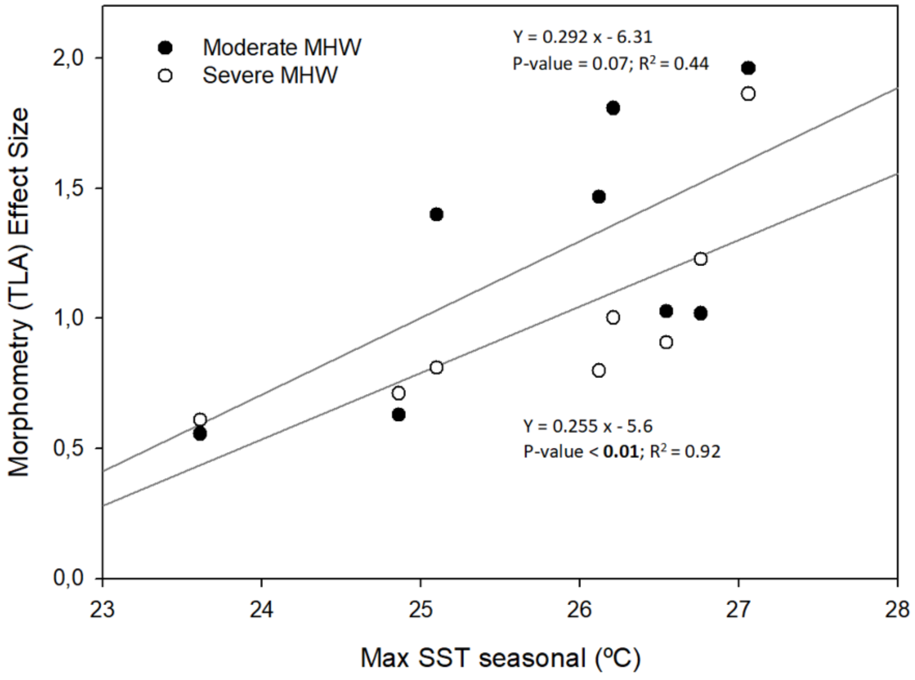


Figure 31. Regressions between the 'effect sizes' of *Cymodocea nodosa* seedling survivorship from different regions and their environmental variability during (a) the disturbance phase and (b) the recovery phase. The environmental variability was typified according to the Std. anomalous (seasonal) sea surface temperature (SST).

a) DISTURBANCE PHASE



b) RECOVERY PHASE

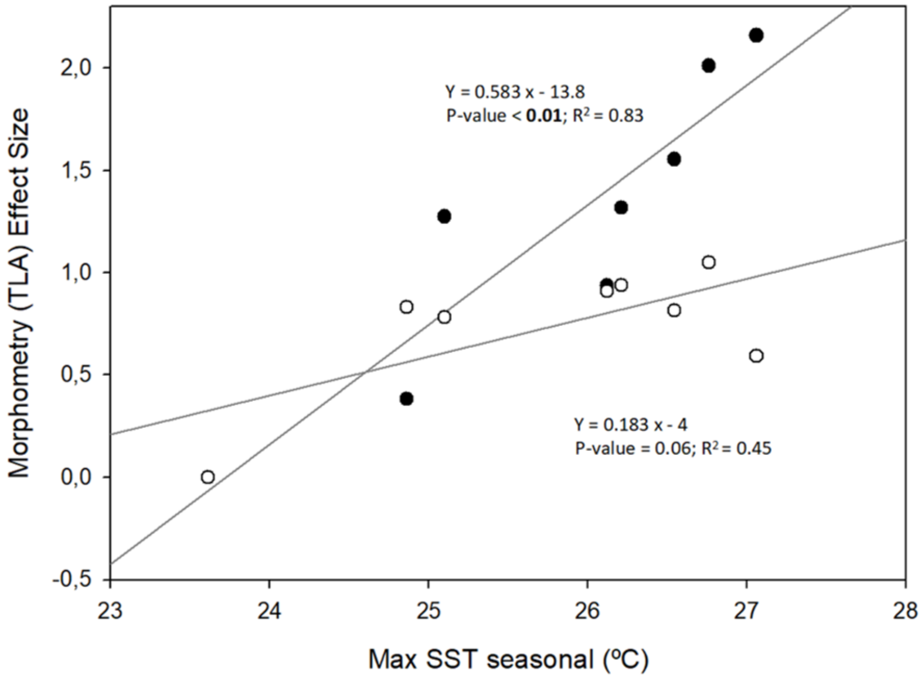
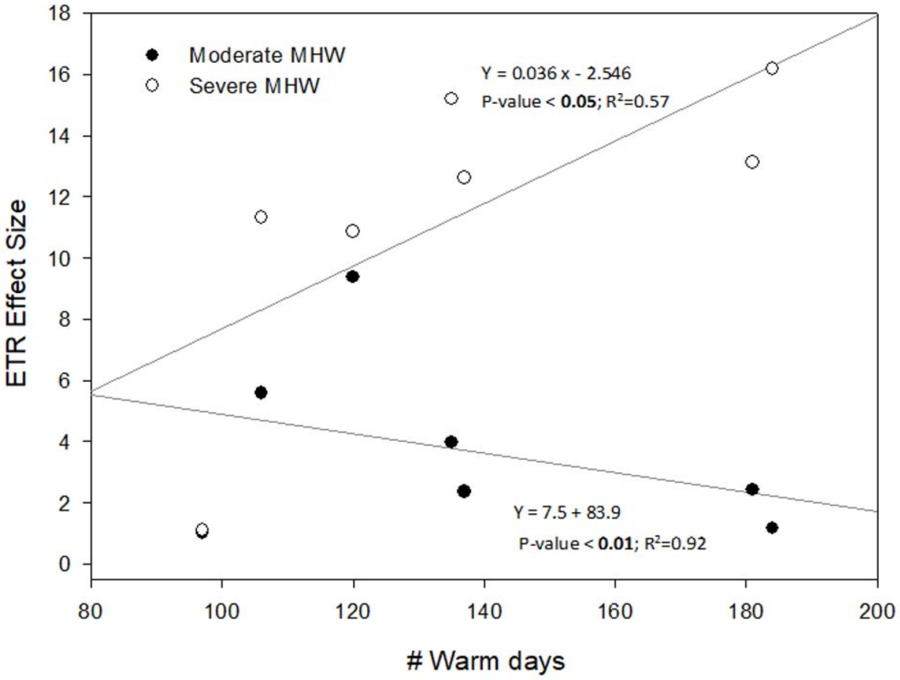


Figure 32. Regressions between the total leaf area (TLA) 'effect sizes' of *Cymodocea nodosa* seedlings from different regions and their environmental variability [in terms of maximum seasonal sea surface temperature (SST)] during (a) the disturbance phase and (b) the recovery phase. Note that the scale of the Y axis is different between the two phases.

a) DISTURBANCE PHASE



b) RECOVERY PHASE

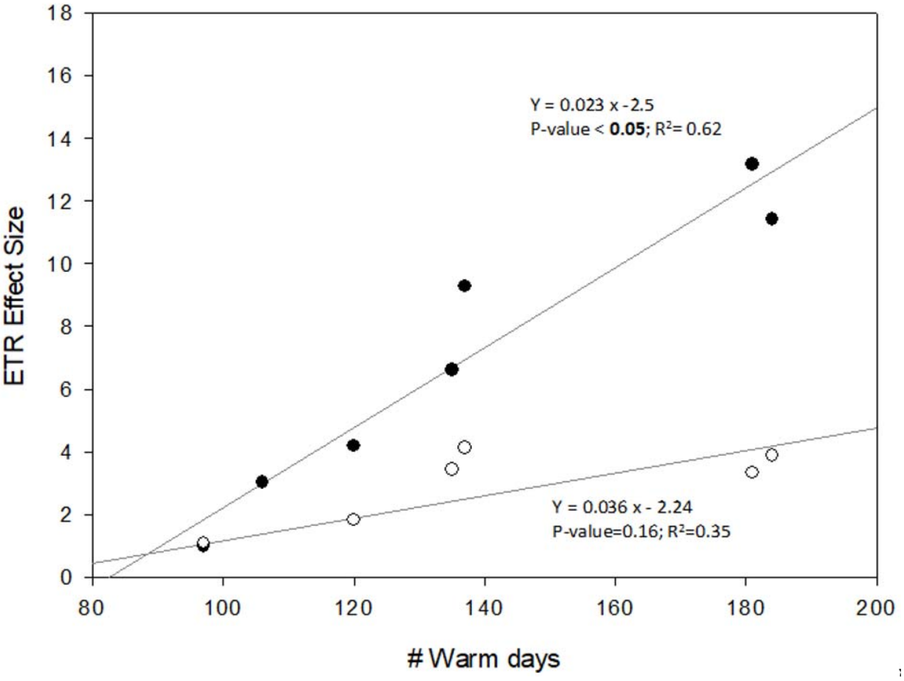


Figure 33. Regressions between the electron transport rate (ETR) 'effect sizes' of *Cymodocea nodosa* seedlings from different regions and their environmental variability (in terms of number of warm days) during (a) the disturbance phase and (b) the recovery phase.

4.4.5. Discussion

Here we have experimentally demonstrated that seagrass seedling responses to MHWs differed according to their location of origin along their distribution range; and that such responses are highly related with their thermal contexts. Furthermore, our results indicate that populations from the different regions are adapted to their local thermal contexts, and that seedlings from regions with rapid and frequent temperature changes, including cold winters and warm summers, have higher capacity to respond to unexpected changes (in this case MHWs) than more thermally stable populations. The detailed analysis of regional thermal contexts along the species distribution range, and its comparison with seedling responses at different levels (survival, morphometry and photobiology) to MHWs, provide solid evidence for the existence of regional acclimation patterns within species. Furthermore, because seeds and seedlings of the different regions were grown under a common garden during a long period of time, the detected pattern could be attributed not only to acclimation, but also to evolutionary adaptation to the local thermal conditions, even though transgenerational plasticity from maternal effects cannot be ruled out (Schmitz and Ecker, 2012).

Under our experimental MHW simulation, survival rates and leaf development decreased with warming for all populations under both MHWs, and particularly under the severe MHW conditions, i.e., exposure to this rapid and maintained temperature rise caused mortality and a reduction in TLA in all regions, suggesting that MHWs of 29ºC or above might have serious consequences for *C. nodosa* seedlings throughout most of its distribution range (although some populations from shallow enclosed lagoons may be more tolerant; e.g. Terrados and Ros, 1995). Importantly, in our study, seedlings from certain regions exhibited a particularly reduced resistance to induced thermal stress. For instance, seedlings from Gran Canaria (Canary Islands), a region which undergoes high seasonal thermal stability and lower summer temperatures, suffered an abrupt drop in survival and in TLA. This region is also close to the western and southern distributional limit of the species (Cunha and Araujo, 2009), and is more genetically isolated (Alberto et al., 2006), which may further hamper its resilience capacity (e.g. Tuya et al., 2018). Certainly, when seagrass populations are next to the peripheral distribution range of the species, they can be more susceptible to stress events, since they are already suffering other disturbances associated to their environmental contexts, and thus extreme events may have a longer-lasting impact (Fraser et al., 2014). Alicante was the second region in terms of suffering high mortality of seedlings. Interestingly, Alicante is located at the distribution limit between two ecoregions (Masucci et al., 2012; Spalding et al., 2007): the Western Mediterranean and the Alboran Sea, the latter being under strong Atlantic (i.e. and thus colder water) influence.

TLA responses are probably explaining, at least in part, the observed survival patterns. For instance, seedlings with lower TLA will have less area available for photosynthesis, decreasing their capacity to incorporate and accumulate carbon reserves. Furthermore, respiratory demands likely increase with increasing metabolic demands associated with warming (Pérez and Romero, 1992; Lee et al., 2007; Marin-Guirao et al., 2018), leading to a negative carbon balance (i.e. photosynthesis < respiration). Moreover, given the age of the seedlings (more than 1.5 years old) carbohydrate reserves in the seed, which could help compensate for higher carbon demands, would have already been depleted, likely ultimately leading to seedling collapse and death.

Regarding photobiological parameters, ETR did not exhibit the same consistent pattern that we observed for survivorship and TLA. For instance, while TLA was higher for the controls than for the MHWs regardless of the region, this was not the case for ETR. In fact, seedlings from some regions (e.g. Sardinia and Corsica) exhibited an increase in ETR under the moderate MHW but a strong decrease for the severe, while others (e.g. Crete and Mallorca) were negatively affected under both MHWs, and some regions were either positively or not affected by warming. Furthermore, ETR responses regarding regional thermal variations were also strikingly different from TLA or survivorship. In fact, during the disturbance phase, we observed a negative relationship between ETR effect size and the number of warm days for the moderate heat wave, indicating that populations with a higher number of warm days have lower ETR effect sizes (i.e. minor ETR values in relation to regions with a lower number of warm days). ETR is a parameter derived from an instantaneous measurement, and therefore does not provide an integrative measure of photobiology, which may be blurring the responses to altered thermal regimes that we do see with TLA and ETR. On the other hand, we did find a positive relation between ETR effect size and the number of warm days for the severe MHW. Perhaps the temperature experienced during the moderate MHW (i.e. 29ºC) was not a stressful condition for the more thermally variable regions, and only the most stable ones needed to increment their ETR values to compensate for a presumable increment in respiration rates (Greve et al., 2003). Interestingly, this negative relationship observed for the moderate MHW switched to a positive one in the recovery phase. Perhaps, the more thermally variable populations took advantage of the return to normal conditions (i.e. 24ºC) to increment their energetic reserves (Marin-Guirao et al., 2016). In contrast, adult plants of *Zostera marina* suffered a reduction in ETR under a simulated MHW (Winters et al., 2011), and low-latitude populations were able to recover after water temperatures returned to control levels, suggesting that adult plants may have higher resilience capacity than seedlings.

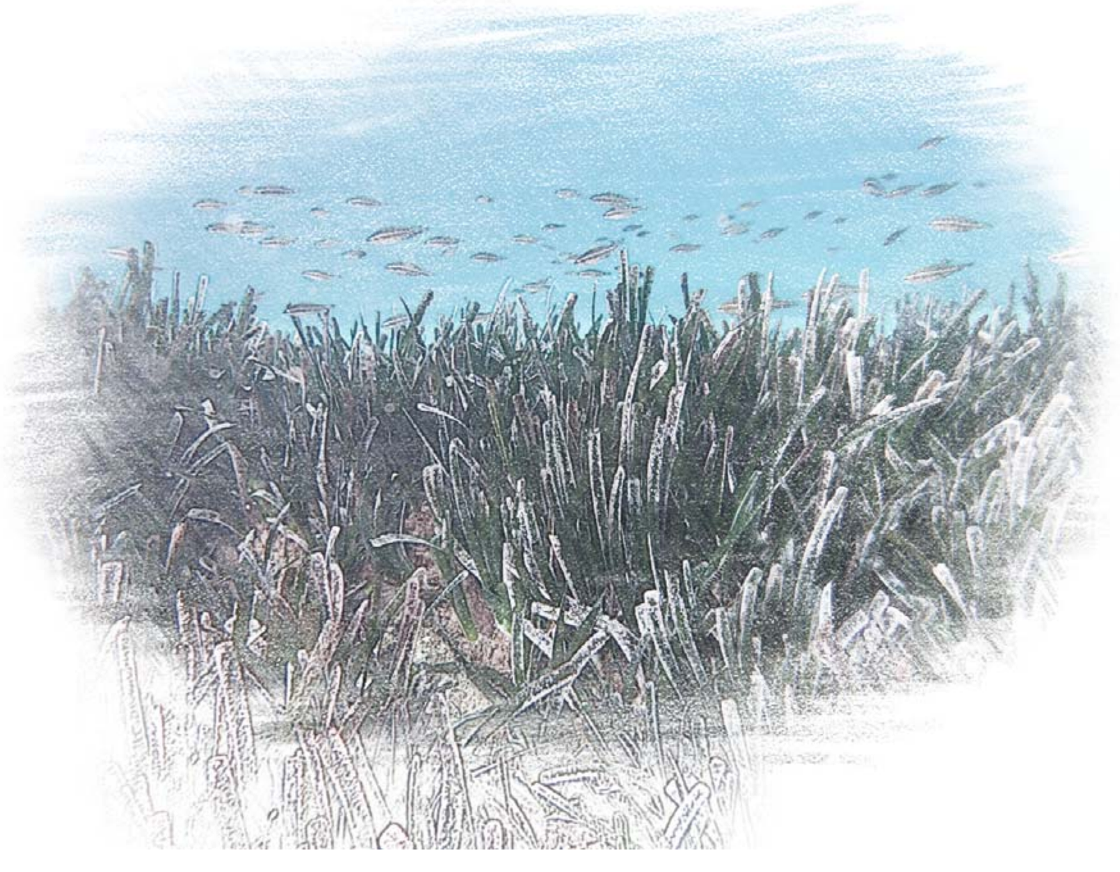
An important point to highlight is that recovery from either of the two MHWs was not detected for any of the regions. Indeed, after the disturbance stopped, survival, photo-physiology and leaf development did not converge to those values of the control treatments. In fact, 30 days after returning seawater temperature to control levels, delayed effects of warming became even more evident than the ones examined immediately after the thermal stress, all of which suggests that rather than an actual "recovery" phase, we were observing a "post-disturbance" phase. A time lag in the response to increased temperatures was also identified for the seagrass *Zostera marina* (Reynolds et al., 2016), and highlights the importance of following species responses after the disturbance has finished, since in experimental works only immediate responses to stressors are typically examined (Wernberg et al., 2012). It seems that under our experimental conditions, *C. nodosa* seedlings were negatively affected beyond a threshold from which they were not able to recover properly, leading to the maintenance of further degradations of the negative responses exhibited during the disturbance phase throughtout the recovery phase. Presumably, exposure to extreme temperatures exceeded the acclimation capacity of seedlings, suggesting that early life stages, at least for this species, are much more vulnerable to heat stress than adult plants (Pérez and Romero, 1992; Savva et al., 2018). In addition, the duration of the recovery phase may not have been long enough to allow for recovery, particularly for the least resistant populations.

Numerous studies analysing the effects of warming on seagrasses have identified *C. nodosa* as one of the more resistant species to increasing seawater temperatures (e.g. Pérez and Romero, 1992; Savva et al., 2018), being considered as an eurobiotic species (i.e. which could live under a wide range of environmental conditions, Marin-Guirao et al., 2016). There are even recent studies (Egea et al., 2018) suggesting that the interaction of an increase in temperature with acidification and, to some extent, nutrient enrichment may benefit *C. nodosa* productivity and resistance in future climate change scenarios.

However, this work indicates that *C. nodosa* seedlings are expected to suffer serious impacts due to the projected warming trends for the coming decades (IPCC, 2014), and points out to the fact that early life stages may be much more sensitive to thermal stress than their adult counterparts. In fact, the transition between seed germination and seedling establishment represents the most exposed stage for recruitment success of plant populations, and this "bottleneck" is largely attributed to the vulnerability of early life stages to biotic and abiotic environmental factors (Harper, 1977). For instance, kelp recruitment success requires the concurrence of critical levels of several conditions (e.g. nutrients, light, temperature, and water motion), and is often limited by environmental stressors, such as sedimentation or grazing (Schiel and Foster, 2006; Brawley and Johnson, 1991). So, considering that early life stages already are highly vulnerable to "natural" environmental stressors, adding disturbances derived from global change may have dramatic consequences for the recovery capacity of adult populations.

In conclusion, this study supports the climatic variability hypothesis, as it demonstrates the positive relationship between resilience to heatwaves and local thermal variability. Furthermore, our results highlight the critical importance of incorporating intra-specific variability when making predictions about species responses to climate change.

4. RESULTS



5. GENERAL DISCUSSION

5. GENERAL DISCUSSION

Posidonia oceanica meadow
(Punta Negra, April 2016)
By Laura Pereda Briones

Since the results obtained throughout the present thesis have been discussed in detail in each of the respective chapters, the purpose of this section is to provide a more synthetic and general overview in order to connect the general outcomes. Here I aim to integrate the different results related to the environmental conditions and processes that affect the dispersal, establishment and fate of seagrass seedlings. As a final corollary, possible future directions for further research are presented.

Seagrasses form extensive meadows in coastal environments worldwide, areas with high natural disturbance regimes (Nicholls et al., 2007), and of concentrated human population, being exposed to multiple natural and anthropogenic threats (Orth et al., 2006a; Waycott et al., 2009; Short et al., 2011). Under this changing environment, the capacity of seagrasses to colonize new areas and to acclimate to the current global changes is crucial to guarantee the long-term persistence of species and the ecosystems they form. Although seagrass expansion is largely driven by clonal growth, dispersal of propagules that result from sexual reproduction (e.g. seeds, seedlings) can be important for both meadow maintenance and colonization of new areas (Hemminga and Duarte, 2000; Orth et al., 2006b). The latter reproductive strategy also ensures population genetic diversity, increasing the potential to adapt to anthropogenic and natural environmental changes (Kendrick et al., 2012; McMahon et al., 2014). However, the effective transfer of genes from a source population into a new area is only effective when propagules containing those new genes reach a suitable microsite, establish and survive.

Dispersal patterns and the modification of propagule physical characteristics to facilitate their anchorage capacity differ across species, and also depend on the propagule's developmental stage. The results of this thesis highlight the importance of propagule size and shape, [in terms of drag coefficient (C_d)] for propagule movement of the three species examined (*Cymodocea nodosa, Posidonia oceanica* and *Zostera marina*). Higher drag values result in decreasing the propagules' threshold velocities to start moving and therefore hamper propagule "trapping" by the substratum. Drag coefficient increases with developmental stage (e.g. *P. oceanica* seed C_d = 0.21; 3-week seedling = 0.24; 8-week seedling = 0.86 and 25-week seedling = 1.17), and thus older (i.e. and more developed) stages are at a disadvantage when establishing. Therefore, for seedling survival it is essential to reach a suitable microsite during their first days-weeks of life, suggesting that there could be a threshold age for each species beyond which propagules can no longer be retained by the substratum. Additionally, seed and seedling retention (i.e. trapping) increases with bottom complexity and boundary layer thickness as the different substrata generate different flow reductions. Complex substrata, such as coarse gravel or dead matte of *P. oceanica*, facilitate propagule trapping due to the higher reduction of flow and higher thickness of the bottom boundary layer. Furthermore, over complex substrata, propagule trapping is highly independent of the propagule's physical characteristics. Conversely, for substrata with lower complexity, such as sand, propagule trapping strongly depends on the propagules' physical characteristics, because the effect of substratum on flow is weaker. This result explains to a large extent why we have found much more seedlings over complex substrata (i.e. rocks, *P. oceanica* dead matte and coarse gravel) than over sand substrate in the field observational study. Nonetheless, seedlings established over coarse gravel did not survive long. Probably, gravel particles, due to their abrasive action when moved by waves, cause damage to the seedling radicular system (Piazzi et al., 1999). Similarly, we have found that *P. oceanica* seedlings are able to establish on sand, but they are not able to survive, so sand habitats become, in practice, an uncolonizable substrate, which begs the question: how did *P. oceanica* initially colonize sandy bottoms? From clonal growth from neighbouring rocky habitats? By establishing in areas highly protected from hydrodynamic disturbances (e.g. lagoons, deep bottoms), even though in those areas sediments may have been very anoxic? These questions lead me to think that, even though the patterns of recruitment in seagrasses are becoming better understood, a lot of information is still lacking. For example, if the rocky substrate is so appropriate for successful seedling establishment and recruitment, why is *P. oceanica* dominant in soft bottoms? Perhaps, if seedlings are more visually apparent on rocky substrates than on sandy ones, they suffer more herbivory pressure and thus decrease their survival success; or perhaps, beyond a certain seedling size, seedling drag coefficient is too high to withstand winter storms, while seedlings might have the ability to penetrate deeper into the sandy substrates, favouring their persistence.

Beyond these speculations, our observations of seedling movement in the flume suggest that roots, which are somewhat rigid and show an apparent negative phototropism, are important anchoring structures that facilitate propagule trapping and establishment. In this sense, the positive effect of macroalgae cover on seedling survival could be a result of macroalgae facilitating the anchorage of the radicular system into the hard substrates, which may also enhance seedling resistance against physical disturbances. Indeed, hydrodynamic conditions not only influence the dispersal process, but also significantly affect the survival of the established seedlings. Seedling establishment is clearly biased towards sheltered locations, with low energy flux (a physical parameter dependent on the height and period of the waves; Holthuijsen, 2007). Once established, seedling survivorship decreases significantly with higher energy flux. Therefore, any biotic (i.e. macroalgal assembly) or abiotic (substratum type) factor that enhances the permanence of the seedlings in the substrate is essential to guarantee their survival. In this sense, we have identified certain abiotic and biotic factors that provide the most suitable microsites for successful (*P. oceanica*) seedling recruitment. These microsites are locations which are sheltered from waves, generally between 1.5 m and 4.5 m depth, over hard substrates, and dominated by macroalgal assemblies, particularly those associated to crustose algae.

Interestingly, the invasive algae *Caulerpa cylindracea*, may play an important role in initial attachment of seedling roots. Certainly, all seedlings established at microsites where this macroalga was the dominant species in the 25 cm^2 radius around the radicular system survived throughout our observational field study, suggesting that *C. cylindracea* may facilitate seedling retention and further survival on rocky and matte substrates. In fact, an in situ transplant experiment also suggests that this invasive species promotes *P. oceanica* seedling root development, facilitating anchorage to the matte substrate (Pereda-Briones et al., 2018b). Moreover, under laboratory conditions, we detected significantly higher root production by *P. oceanica* seedlings in the presence of *C. cylindracea* in our ambient temperature treatment (Pereda-Briones et al., 2019). In addition to this positive interaction, we did not observe any negative effects of *C. cylindracea* on *P. oceanica* seedlings, suggesting that this species could facilitate successful establishment and recruitment of *P. oceanica* seedlings. Similarly, no negative effects were found for the other invasive macroalgae studied (*Lophocladia lallemandii*) either. This lack of negative effects of invasive algae on seedlings under the tested conditions and the positive effect of *C. cylindracea* presence on root development, suggest that there might be a facilitative effect of some invasive algae on seedlings during their recruitment phase.

However, I have not assessed the indirect effects of increased temperature on invasive species, and how this can influence seedling responses. For instance, if invasive algae are favoured by an increase in temperature (Samperio-Ramos et al., 2015), they could considerably increment their abundance and could have negative effects on seedlings. Warming is expected to continue in the coming years (IPCC, 2014), and this could affect the response of both invasive algae. Indeed, there are numerous examples of invasive species being more tolerant to higher temperatures than native ones (e.g. Smith et al., 2004; Sorte et al., 2010) and global warming on marine ecosystems is accelerating the settlement of new alien species (Raitsos et al., 2010). Additionally, it is necessary to consider that climate-driven plastic responses may enhance biological invasions, since invasive species appear to have greater plasticity than non-invasive species (Davidson et al., 2011). Then, under this scenario of progressively increasing sea surface temperature and extreme climatic events, invasive species may have an opportunity to establish and spread, posing a major barrier to future seedling recruitment. Therefore, even though *P. oceanica* seedlings appear to be resistant in their interaction with *C. cylindracea* and *L. lallemandii* (at the abundance levels that we tested), they may not cope well with these or other invasive species if global warming trends continue.

Moreover, warming directly affects *P. oceanica* seedling performance. Under our experimental conditions, a maintained increase in temperature has led to a decrease of total new root length, leaf formation and total leaf surface. Furthermore, we also observe an increase in necrosis and a deterioration of seedling photosynthesis processes when temperatures reach 29ºC. These sublethal responses may affect seedling survival success in the longer term, and could influence their resistance to other disturbances, such as biological invasions. The other species analysed in this regard, *C. nodosa*, also appears to suffer under warming. In this case, we have assessed how extreme climatic events (in particular, marine heatwaves MHWs) would affect seedling resilience and functioning. Importantly, climate change is not only increasing sea surface temperatures gradually, but it is also increasing the occurrence of extreme climatic events (IPCC, 2012). In particular, MHW (i.e. periods of extreme warm seawater temperature that persist for days to months, Hobday et al., 2016), are increasing in frequency and duration (Hobday et al., 2018; Oliver et al., 2018). Seagrasses, which are already stressed by multiple perturbations (Waycott et al., 2009), can be especially vulnerable to such extreme events (Diaz-Almela et al., 2009; Oviatt, 2004; Reusch et al., 2005). In this changing environment, the ability of a given genotype to produce a plastic response, i.e. to acclimate under different environmental conditions, is essential for resisting to- and recovering from those climatic events (O'Brien et al., 2017). Therefore, it is necessary to analyse not only the inter-specific, but also the intra-specific variability of seagrasses when facing thermal stress (see review by Violle et al., 2012). We have experimentally demonstrated that *C. nodosa* seedling responses to MHWs differ along its distribution range, and that such responses are highly related to their local thermal contexts. The exposure to this rapid and maintained temperature rise caused mortality and reduction in leaf development in all regions analysed, suggesting that MHWs of 29ºC of above have serious consequences for *C. nodosa* seedlings throughout most of its distribution range. However, seedlings from certain regions exhibited particularly reduced resistance to induced thermal stress, while others were able to cope quite well with such disturbance. Seedlings from populations which are adapted to varying thermal contexts throughout their annual cycles had higher capacity to acclimate to unexpected changes than populations from more thermally stable locations. Moreover, those populations that are at the periphery of the distribution range of the species or in the middle between two ecoregions (i.e. an area defined in terms of its natural features and environment, e.g. Western Mediterranean or Alboran Sea), were more susceptible to thermal stress, thus, populations cannot exist anywhere within its distribution range. Therefore, identifying which populations are more resistant to global warming is essential to improve our knowledge of seagrass recruitment patterns. Furthermore, our results suggest that early life stages of *C. nodosa* are much more sensitive to thermal stress than their adult counterparts, since adults of this species have been identified as highly resistant to increasing seawater temperatures (e.g. Pérez and Romero, 1992; Savva et al., 2018), being considered as an eurobiotic species (i.e. which could live under a wide range of environmental conditions; Marin-Guirao et al., 2016). On the other hand, the fact that early life stages of both species analysed (*P. oceanica* and *C. nodosa*), which cohabit in the Mediterranean Sea, are negatively affected by global warming, leads to the prediction that Mediterranean seagrass seedlings are likely to suffer irreversible damage in the near future if the warming trends are maintained. This dramatic scenario endangers the recruitment success of these species and threatens their genetic diversity. It is also feasible that the most resistant species (*C. nodosa*) starts to displace the most vulnerable one (*P. oceanica*), although this may be particularly true through clonal growth, since differences in resistance to increased temperatures between both species are especially marked in adult plants (e.g. Savva et al., 2018). Yet, recruitment of both species, as demonstrated here, is much more sensitive to warming, endangering the acclimation and adaptive potential of both species to the more frequent and intense environmental changes. Certainly, due to the limited number of species present in seagrass communities, within-species diversity may replace the functional role of species diversity (Duffy, 2006), and particularly regarding resilience to disturbances (e.g. Hughes and Stachowicz, 2004, Reusch et al., 2005), which has important implications for the future distribution patterns of these species in response to global warming.

Since global change is rising at a time when natural environments are becoming progressively more fragmented through habitat destruction (review by Hoffmann and Sgrò, 2011), and more degraded through multiple perturbations (Waycott et al., 2009), conservation strategies to cope with and improve this situation are indispensable. Natural recovery of degraded habitats may be very slow (Walker et al., 2006; Uhrin et al., 2011), so the first target should be to stop those anthropogenic threats that are attainable with current management and policy strategies (e.g. reduce the dumping of waste into the sea or improve boat anchoring systems). In order to facilitate the recovery process, restoration plans could be also implemented. In this context, seed-based restoration emerges as a technique which provides genetic diversity, thus enhancing the recovery of populations (Orth et al., 2000; Kendrick et al., 2012; Reynolds et al., 2012). In this sense, the results presented in this thesis could be applicable to improve restoration techniques. For instance, degree of exposure to waves, substratum complexity or macroalgal cover, could be additional criteria in the site selection process during restoration. Additionally, the appropriate selection of genotypes when performing restoration plans is essential to contribute to the resistance to environmental stressors and anthropogenic disturbances.

Overall, the outcomes of this thesis have identified key elements of some environmental conditions and processes (e.g. hydrodynamic conditions, microsites, species interactions, temperature thresholds) affecting reproductive propagule dispersal, retention, establishment and further survival. In this multistage process, it is essential for seagrass seedlings to reach a suitable microsite which facilitates their survival, a step which is strongly determined by hydrodynamic conditions and the presence of adequate hard substrates. However, once seedlings are able to establish, threats derived from global change, such as those analysed here also exert a strong influence on this process.

FUTURE DIRECTIONS

Throughout the elaboration of this thesis, some exciting questions have risen that could not be explored during the development of my PhD thesis, but constitute thrilling new research directions. Some of them delve into the chapters of the thesis, being their natural progression, while others correspond to identified knowledge gaps on the research topic.

Deepening the understanding of how propagule physical characteristics determine their dispersal

Important inter- and intraspecific differences on how hydrodynamics and substratum type determine the movement of seagrass propagules have been identified in this thesis. Such differences were strongly influenced by the different physical characteristics between species and developmental stages (i.e. seed and seedlings of increasing ages). Among these features, drag coefficient was identified as good indicator of propagule movement. In order to improve our predictive capacity of the minimum threshold velocities that initiate propagule movement and determine how size and shape of individuals determine their trapping success, it would be necessary to measure propagule physical characteristics of different species, especially their drag coefficient. Then, the information obtained in the present thesis could be extended to other species and developmental stages, and could be applicable for numerical hydrodynamic dispersal models around the world as well as to make predictions of adequate establishment sites.

Disentangling the facilitative effects of macroalgal assemblies on seedling establishment

A positive relationship was identified between macroalgae cover and seedling survival. However, to be able to infer causality, manipulative experiments where seedlings are transplanted at microsites dominated by certain macroalgae could be done to improve our understanding of how this biotic interaction affects seedling recruitment success. In addition, controlled experimental approaches could be useful to assess in more detail how the radicular systems of seedlings anchor to the different macroalgae. Both field and laboratory studies could elucidate the specific mechanisms driving the facilitative interaction that we have observed, and which appears to play an important role in seedling resistance against physical disturbance.

Elucidating the mechanisms whereby Caulerpa cylindracea promotes root development of Posidonia oceanica seedlings.

The increase in production of new roots by *P. oceanica* seedlings observed in our mesocosm experiment, and the positive influence of this algae on seedling survival detected in the field, suggest a facilitative interaction between both species. Root development may have been promoted by the physical structure provided by this algae, whose stolons and rhizoids could increase substratum complexity, enhancing root attachment to hard substrates. However, it is also possible that certain chemical cues released by *C. cylindracea* or symbiotic/associated microorganisms to it may also facilitate seedling root development. To assess this second possibility, controlled experimental conditions where *P. oceanica* seedlings and *C. cylindracea* are growing together but without physical contact, would be required. Under this experimental conditions, allopathic (i.e. biochemical products that influence the germination, growth, survival or reproduction of other organisms) effects between both species could be identified, clarifying the role of this interaction in seedling recruitment success.

Unravelling the importance of plastic and adaptive potential of seagrass seedling to resist the currently changing climate

The actual trend of increasing sea surface temperatures, in conjunction with the progressively frequent occurrence of extreme climatic events, may push marine organisms to exceed their resilience limits. In this scenario, early life stages are critical for adaptation to global warming. We have demonstrated that *Cymodocea nodosa* seedling responses to marine heatwaves differ along its distribution range, and that such responses are modulated by their local thermal contexts. To further analyse how biogeographic distribution influences seedling responses and adaptive potential, it is noteworthy to integrate genomic and ecological techniques in inter- and intraspecific studies. For instance, analysing which genotype-specific traits will confer resistance to global warming could be a useful tool in restoration projects. Such interdisciplinary experiments will give a more holistic understanding on adaptation and plasticity patterns.

6. CONCLUSIONS

6. CONCLUSIONS

Posidonia oceanica seedling
(Bendinat, January 2016)
By Eduardo Infantes

1. Dispersal and trapping of seagrass propagules exhibit important inter- and intraspecific differences. How hydrodynamics and substratum type determine the movement of seagrass propagules is strongly influenced by the different physical characteristics of the three species examined (*Cymodocea nodosa, Posidonia oceanica* and *Zostera marina*). Threshold flow velocities (i.e. the flow velocity necessary for seagrass propagules to start a continuous movement over the substratum) depend on the drag coefficient of the propagules. Moreover, during the dispersal process, trapping of propagules by the substratum increases with higher drag coefficients, and drag coefficient increases with propagule developmental stage, and thus older (i.e. more developed) stages are at a disadvantage.

2. Seeds and seedling trapping increases with bottom complexity and boundary layer thickness as the different substrata generate different flow reduction. Complex substrata, such as coarse gravel or matte, facilitate propagule trapping due to the higher reduction of flow and higher thickness of the bottom boundary layer.

3. *P. oceanica* seedling establishment is clearly biased towards sheltered locations, with their survivorship decreasing significantly with higher energy flux. In addition, most successful establishment occurs on consolidated substrates (i.e. rocks and matte), whereas unconsolidated substrates (i.e. sand and gravel) appear to hamper seedling establishment and survival. Furthermore, a positive effect of macroalgae cover on seedling survival has been identified, whereby crustose algae appear to be the most favourable functional group regarding seedling survival, and the invasive algae *Caulerpa cylindracea* may play an important role in initial attachment of seedling roots on rocky and matte substrates. Additionally, a positive correlation has been detected between seed volume and seedling survivorship, i.e. above a certain volume (0.7 cm^3), all seedlings survive.

4. Once *P. oceanica* seedlings overcome the second winter after establishment, at an age of approximately 18 months old, they have a high probability of persistence in the population. Thus, *P. oceanica* seedlings appear to be very specific in their environmental requirements (microsite-limited species) during their first one year and a half of life, when they are favoured by microsites such as those characterized by rocky or matte substrates, covered by macroalgae, and located at sheltered locations. After this phase, their probability of surviving becomes more independent from microsite characteristics.

5. High seawater temperatures have negative effects on seedlings of the two species examined (*P. oceanica* and *C. nodosa*). An increase of 3ºC and 5ºC with respect to the control temperature during three months have led to sublethal effects on *P. oceanica* seedlings. These seedlings undergo a decrease of total new root length, leaf formation and total leaf surface, an increase in necrosis and a deterioration of seedling photosynthesis processes when temperatures reach 29ºC. On the other hand, under an abrupt and maintained increase in temperature during one month [i.e marine heatwave (MHW)] *C. nodosa* seedlings have suffered a reduction in leaf development, photosynthesis efficiency and survivorship, especially when temperatures reach 32ºC.

6. *C. nodosa* seedling responses to marine heatwaves differ along its distribution range, and such responses are modulated by their thermal contexts. Populations which are adapted to varying thermal conditions throughout their annual cycles, with rapid and frequent temperature changes, including cold winters and warm summers, have higher capacity to adapt to unexpected changes (in this case MHWs), than more thermally stable populations. Moreover, those populations that are at the periphery of the distribution range of the species or in the middle between two ecoregions are more susceptible to thermal stress.

7. The invasive algae *Caulerpa cylindracea* and *Lophocladia lallemandii* do not have detrimental effects on the development of *P. oceanica* seedlings. This lack of negative effects, in addition to the enhancement of seedling root development in the presence of *C. cylindracea*, and the tendency of the presence of *L. lallemandii* to improve the negative effects of warming on seedling leaf development, all suggest that there may be positive rather than negative influences of invasive algae on seedlings during their recruitment phase under the tested conditions.

Together these conclusions provide important insights into the drivers of seagrass recruitment. The results of this thesis have identified key elements of some environmental conditions and processes (e.g. hydrodynamic conditions, microsites, species interactions, temperature thresholds) affecting reproductive propagule dispersal, trapping, establishment and further survival. In this multistage process, it is essential for seagrass seedlings to reach a suitable microsite which facilitates their survival. Yet, once seedlings are able to establish, threats derived from global change, such as those analysed here have also strong influence on this process. In addition, the outcomes of this thesis highlight the importance of considering inter- and intraspecific variability when assessing recruitment patterns and effects of environmental changes on plant populations.

6. CONCLUSIONS

7. REFERENCES

7. REFERENCES

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(Port des Canonge, December 2015)
By Laura Pereda Briones

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Cymodocea nodosa meadow
(Cabrera, April 2016)
By Laura Pereda Briones

8.1. Supplementary Material Chapter 2: Influence of biotic and abiotic drivers of seagrass (*Posidonia oceanica*) seedling recruitment: identifying suitable microsites

Text S 1. Validation of the Puertos del Estado climate database

To validate the reliability of Puertos del Estado nearshore database with respect to the real wave conditions in the study area, we compared its oceanographic parameter values [significant wave height (H_s), wave period (T_p) and wave direction (θ)], with those registered by an oceanographic buoy located at 2.70ºE – 39.493ºN (Fig. 21), 10 km from the SIMAR node. Despite being at different locations, since both are at the same depth (30 m) and enclosed in the same large Bay (i.e. Palma Bay), we can reasonably assume that the wave conditions coming from the South should be analogous, not being affected by the boundary conditions of the coastline (Holthuijsen, 2007). Therefore, we selected those wave conditions originating at a direction range between 140 and 220 degrees (based on the information offered by the corresponding wave rose), and then plotted the variation of the significant wave height between both databases across time and calculated their correlation, with a satisfactory result (Fig. S 1). To further validate our data, we placed accelerometers (HOBO Pendant® G Data Logger) in two different zones of one of the three study sites (Son Caliu). Both zones were at the same depth (i.e. 2.8 m), but undergoing different wave exposure according to our results, and therefore we were able to compare if this assumed H_s variation is also registered by the accelerometers. Acceleration data were registered every second during 3 hours along 3 consecutive days included in the study period analyzed (Fig. S 2). Although the climate database registered H_s (m) values and the accelerometers register acceleration (m s^{-2}), they are proportionally related ($a_x = \frac{H}{2}\omega^2 \frac{ch(k(h+z))}{shkh}$; Holthuijsen, 2007; i.e. the greater the H_s, the greater the acceleration), and thus, we can relate both measurements. After its comparison, we detected equivalent values to those observed by the SIMAR node, further ensuring the reliance of the Puertos del Estado database.

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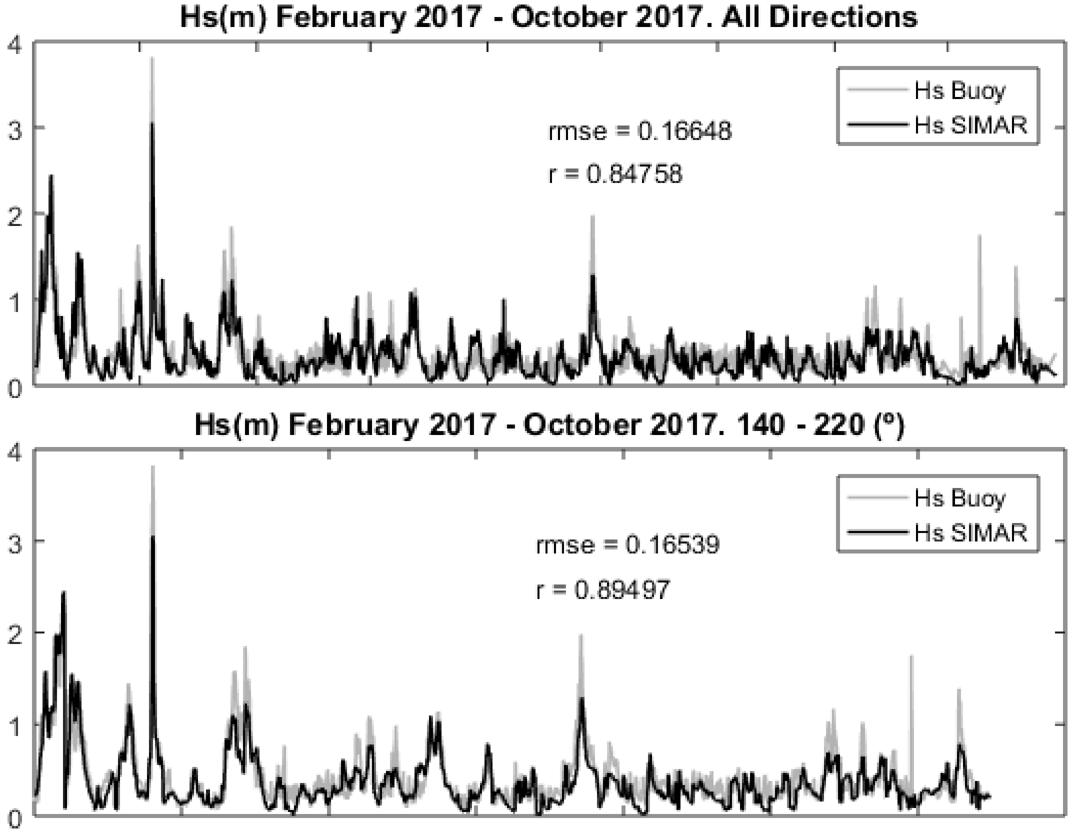
Text S 2. Wave propagation methodology

Taking into account the totality of the sea states included in the SIMAR reanalysis point, we selected 200 cases representative of all the range of casuistic wave conditions using Maximum Dissimilarity Algorithm (MDA; Camus et al., 2011). Then, we propagated these 200 selected sea states from deep sea to shallow water using a numerical model (SWAN-*Simulating WAves Nearshore*). Once the sea states were propagated, we reconstructed the maritime climate at the three study sites by Radial Basis Function (RBF, Camus et al., 2011), to identify the hydrodynamic conditions to which the seedlings were exposed during our sampling period (i.e. from May 2015 to January 2017). Fig. S 3 shows two characteristic wave condition cases propagated with SWAN model. Case 1 represents those sea states coming from the Southwest (180 - 270 degrees) which are diffracted by the East coast and a little islet. For these wave conditions, Son Caliu remains protected, whereas Bendinat is the most exposed site. Case 2 represents sea states coming from the Southeast (90 - 180 degrees), which affect the sites of Son Caliu and Punta Negra, being Bendinat more protected.


Hs(m) February 2017 - October 2017. All Directions

Hs(m) February 2017 - October 2017. 140 - 220 (°)

Figure S 1. Validation of the SIMAR off-shore database (black line) in relation to SOCIB oceanographic buoy (grey line). The upper panel shows the correlation for all direction wave conditions, and the lower panel shows only those wave conditions coming from the South (140 - 220 degrees).

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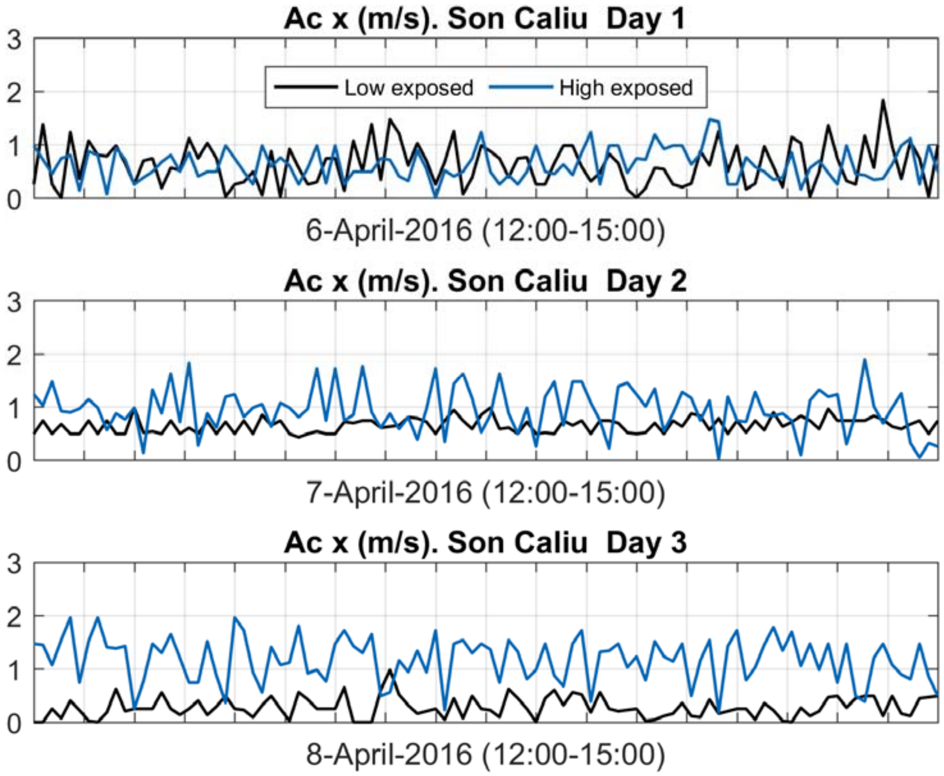


Figure S 2. Acceleration data recorded from accelerometers located at Son Caliu. The duration of the recording data was three days. Black lines indicate the low exposure zone and blue lines the high exposed one.

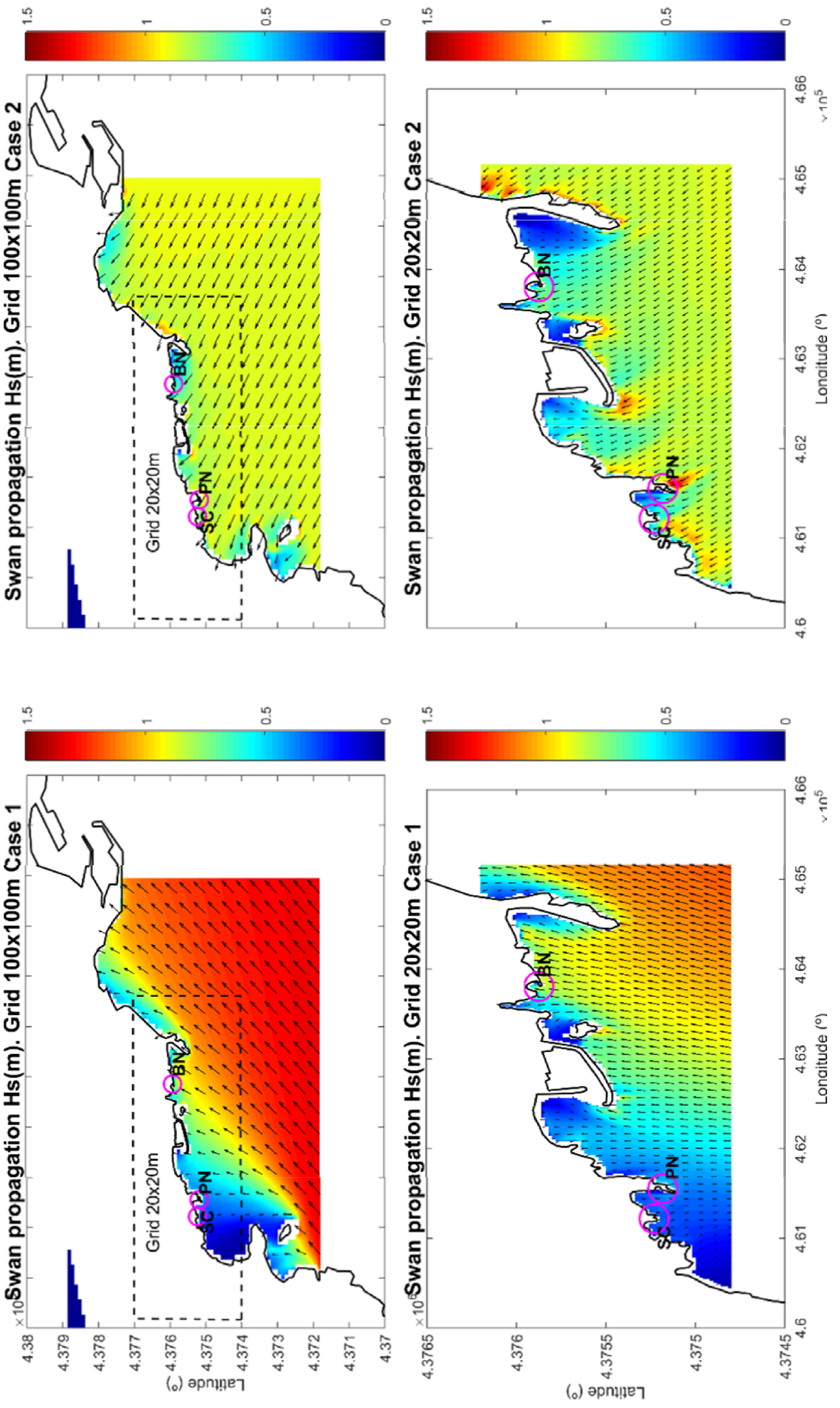


Figure S 3. SWAN propagation model. Example of two characteristic cases: case 1: Hs = 1.3 m; Tp = 6 s; θ = 220º and case 2: Hs = 1.2 m; Tp = 7.5 s; θ = 122º.

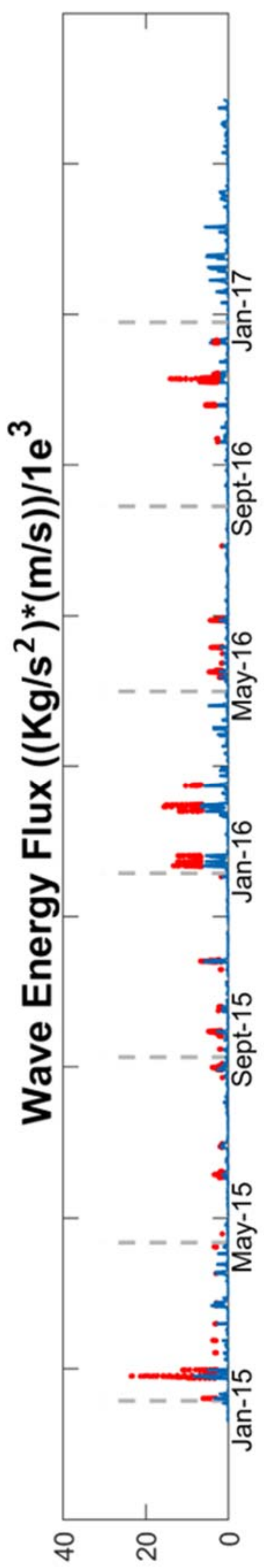
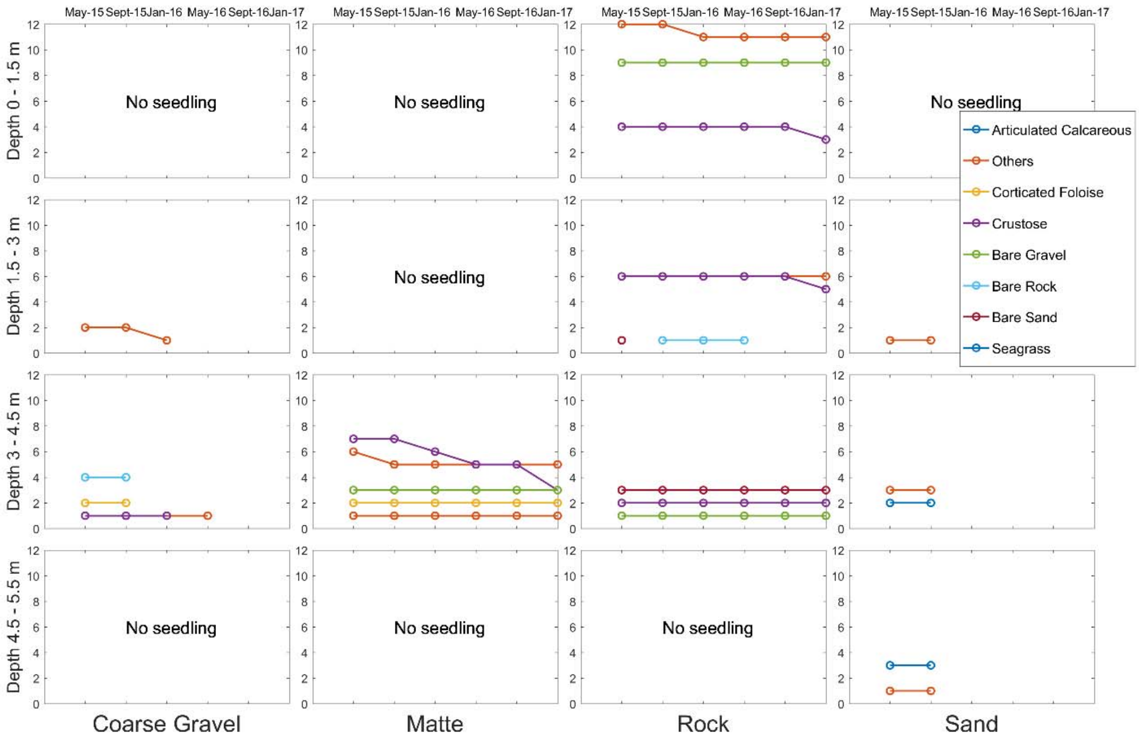


Figure S 4. Example of the identification of characteristic extreme events for every period. The Wave Energy Flux (F) values which overcome the 95 % percentile are marked in red.

Nº of Seedlings - 1



Nº of Seedlings - 2

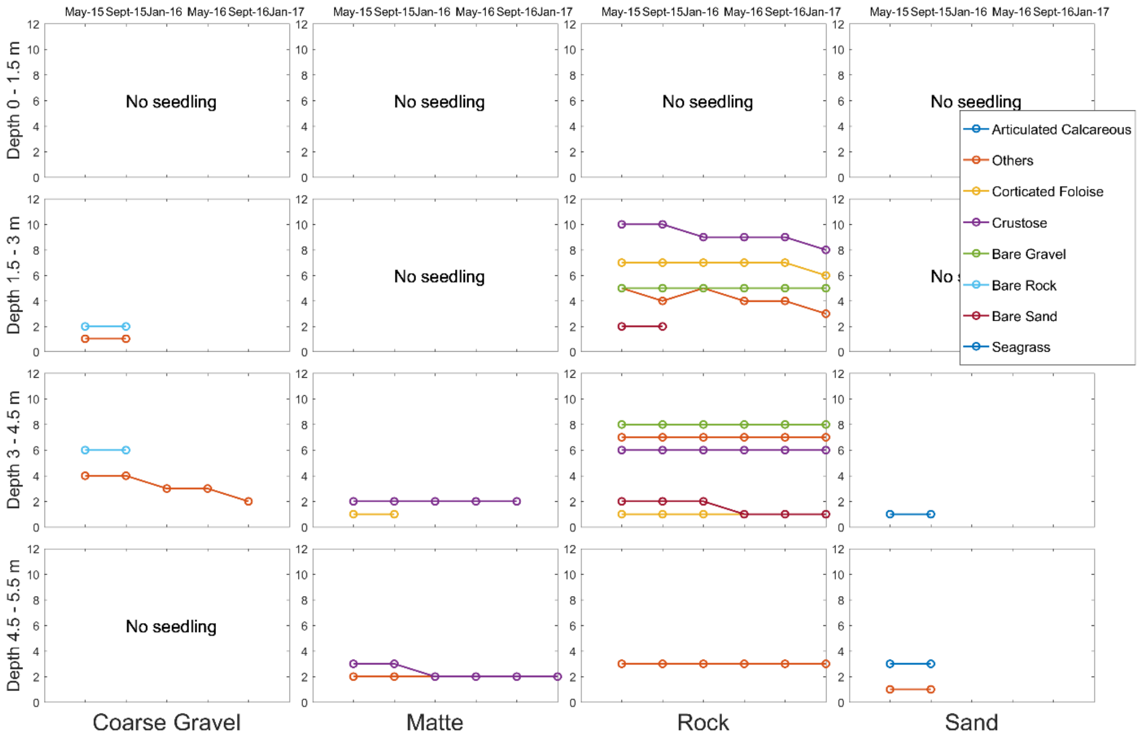


Figure S 5. Number of *Posidonia oceanica* seedlings present at each sampling period depending on substratum type, depth and dominant macroalgae functional group or bare substrate at 20 x 20 cm plots in low exposed zone (1: upper panel) and high exposed (2: lower panel).

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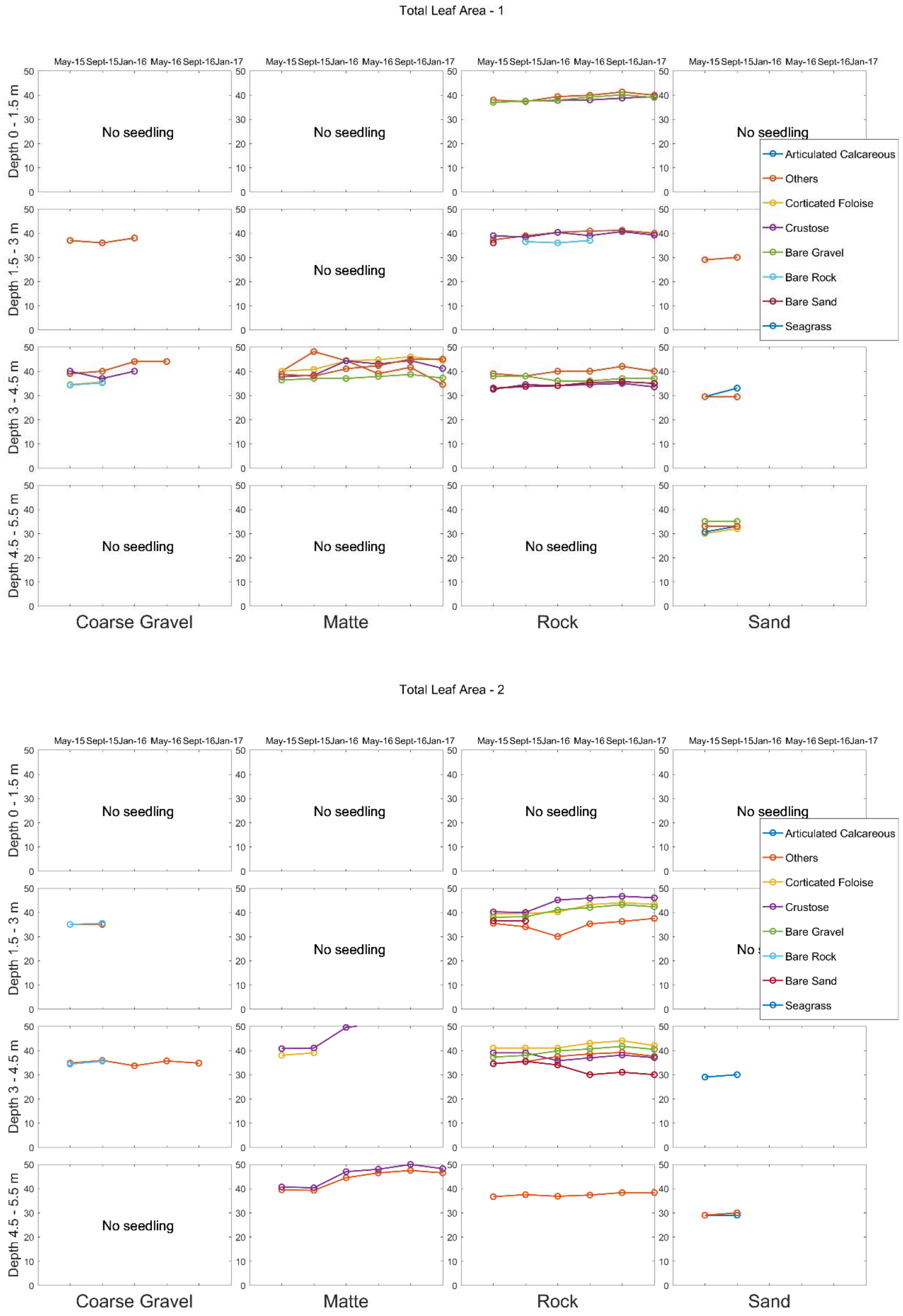


Figure S 6. Evolution of the mean total leaf area (cm^2) of *Posidonia oceanica* seedlings across time depending on substratum type, depth and dominant functional group or bare substrate at 20 x 20 plots in low exposed zone (1: upper panel) and high exposed zone (2: lower panel).

8.2. Supplementary Material Chapter 3: Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae

Variables	Mean ± SE
Nº of leaves	6.89 ± 0.07
Leaf width (cm)	0.53 ± 0.01
Maximum leaf length (cm)	6.99 ± 0.23
Total leaf area (cm²)	16.05 ± 0.62
Seed area	1.03 ± 0.01
Nº of roots	4.41 ± 0.08
Total root length (cm)	17.70 ± 0.61
Maximum root length (cm)	5.92 ± 0.21

Table S 1. Morphological features (mean ± SE) of *Posidonia oceanica* seedlings at the beginning of the experiment.

Variable	**Source**	**df**	**MS**	**F**	**p**
Nª of leaves	Invasion	2	0.013	0.09	0.915
	Temperature	2	0.231	1.53	0.234
	Inv x T	4	0.404	2.68	0.072
	Error	27	0.151		
Width (cm)	Invasion	2	0.362	2.025	0.151
	Temperature	2	0.113	0.632	0.539
	Inv x T	4	0.177	0.987	0.431
	Error	27	0.179		
Maximum leaf lenght (cm)	Invasion	2	2.301	0.947	0.4
	Temperature	2	1.817	0.748	0.482
	Inv x T	4	0.323	0.133	0.968
	Error	27	2.428		
Total leaf area (cm^2)	Invasion	2	13.513	0.718	0.496
	Temperature	2	6.922	0.367	0.695
	Inv x T	4	2.745	0.145	0.963
	Error	27	18.819		
Seed area	Invasion	2	13.100	0.130	0.878
	Temperature	2	145.700	1.451	0.251
	Inv x T	4	79.100	0.788	0.543
	Error	27	100.400		
Nº of roots	Invasion	2	0.003	0.018	0.982
	Temperature	2	0.151	0.760	0.477
	Inv x T	4	0.645	3.231	0.074
	Error	27	0.199		
Total root length (cm)	Invasion	2	1612	0.734	0.489
	Temperature	2	1233	0.561	0.576
	Inv x T	4	3872	1.763	0.165
	Error	27	2196		
Maximum root length (cm)	Invasion	2	32.200	0.217	0.805
	Temperature	2	38.900	0.263	0.770
	Inv x T	4	293.400	1.985	0.125
	Error	27	147.800		

Table S 2. Results of the Factorial ANOVAs examining the morphological features of *Posidonia oceanica* seedlings at the beginning of the experiment according to the presence of invasive algae (Inv), temperature (T) and their interaction.

Variable	**Source**	**df**	**MS**	**F**	**p**
Nª of leaves	Invasion	3	0.825	0.487	0.698
	Error	12	1.695		
Width (cm)	Invasion	3	0.001	0.325	0.807
	Error	12	0.003		
Maximum leaf lenght (cm)	Invasion	3	6.363	1.912	0.182
	Error	12	3.329		
Leaf surface (cm^2)	Invasion	3	153.172	3.845	**0.039**
	Error	12	39.836		
Necrotic leaf surface (cm^2)	Invasion	3	2.146	0.352	0.788
	Error	12	6.091		
Seed area	Invasion	3	0.019	0.569	0.646
	Error	12	0.034		
Nº of roots	Invasion	3	69.015	0.963	0.442
	Error	12	71.655		
Total root length (cm)	Invasion	3	34.220	0.968	0.440
	Error	12	35.351		
Maximum root length (cm)	Invasion	3	0.952	0.441	0.728
	Error	12	2.161		
Nª new roots	Invasion	3	136.152	1.004	0.425
	Error	12	135.666		
Total new roots length	Invasion	3	1.904	0.936	0.454
	Error	12	2.034		
Maximum new root length (cm)	Invasion	3	1.511	0.825	0.505
	Error	12	1.831		

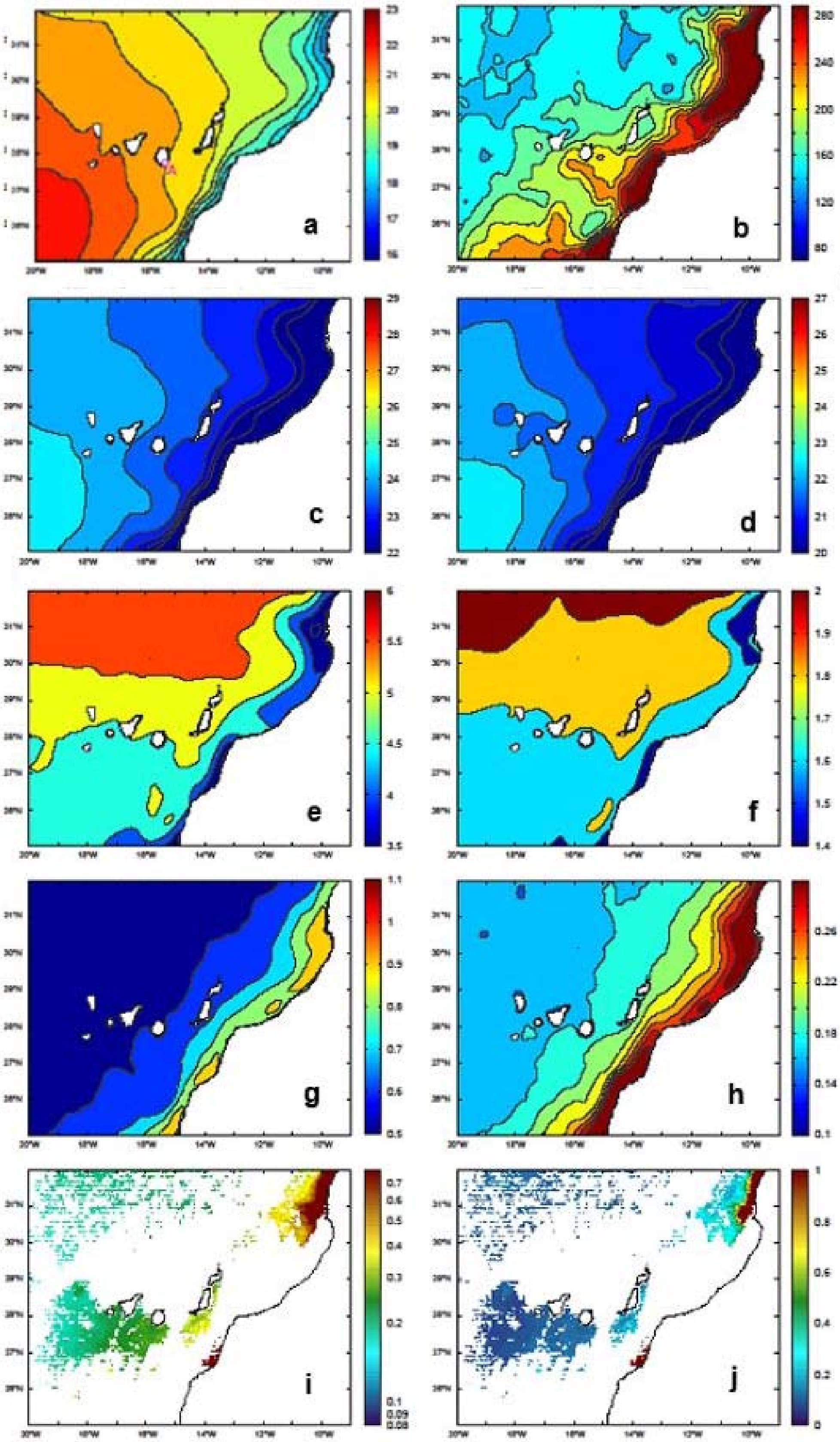
Table S 3. Results of the One way ANOVAs examining the morphological features of *Posidonia oceanica* seedlings in response to invasive algae at 27ºC. Significant effects are highlighted in bold.

Variable	**Source**	**df**	**df error**	**MS**	**MS error**	**F**	**p**	**Tukey**
Nitrite+Nitrate	Invasion	2	27	0.325	1.309	0.248	0.782	
	Temperature	2	27	1.499	1.309	1.145	0.333	
	Inv x T	4	27	1.053	1.309	0.804	0.533	
	TIME	2	54	105.511	1.683	62.694	**< 0.01**	$t_0 < t_1 = t_2$
	Time x Inv	4	54	1.084	1.683	0.644	0.634	
	Time x T	4	54	0.853	1.683	0.507	0.731	
	Time x Inv x T	8	54	0.722	1.683	0.429	0.899	
Ammonium	Invasion	2	27	9.622	33.414	0.288	0.752	
	Temperature	2	27	1.396	33.414	0.042	0.959	
	Inv x T	4	27	12.641	33.414	0.378	0.822	
	TIME	2	54	371.898	34.472	10.788	**< 0.01**	$t_0 > t_1 = t_2$
	Time x Inv	4	54	8.014	34.472	0.232	0.919	
	Time x T	4	54	2.076	34.472	0.060	0.993	
	Time x Inv x T	8	54	9.222	34.472	0.268	0.974	
Nitrite	Invasion	2	27	0.001	0.004	0.255	0.777	
	Temperature	2	27	0.002	0.004	0.207	0.815	
	Inv x T	4	27	0.004	0.004	0.907	0.474	
	TIME	2	54	0.114	0.004	26.463	**< 0.01**	$t_0 < t_1 > t_2 = t$
	Time x Inv	4	54	0.010	0.004	2.285	0.072	
	Time x T	4	54	0.002	0.004	0.402	0.806	
	Time x Inv x T	8	54	0.004	0.004	1.013	0.438	
Phosphate	Invasion	2	27	0.000	0.016	0.002	0.998	
	Temperature	2	27	0.000	0.016	0.007	0.993	
	Inv x T	4	27	0.004	0.016	0.274	0.892	
	TIME	2	54	0.076	0.012	6.463	**< 0.01**	$t_0 < t_1 \neq t_2 = t_0$
	Time x Inv	4	54	0.004	0.012	0.317	0.865	
	Time x T	4	54	0.008	0.012	0.661	0.622	
	Time x Inv x T	8	54	0.007	0.012	0.628	0.751	
Nitrate	Species	2	27	0.363	1.250	0.290	0.751	
	Temperature	2	27	1.570	1.250	1.256	0.301	
	Inv x T	4	27	0.951	1.250	0.761	0.560	
	TIME	2	54	103.195	1.706	60.485	**< 0.01**	$t_0 < t_1 = t_2$
	Time x Inv	4	54	1.160	1.706	0.680	0.609	
	Time x T	4	54	0.835	1.706	0.490	0.743	
	Time x Inv x T	8	54	0.732	1.706	0.429	0.898	

Table S 4. Repeated measures ANOVAs analysing the evolution of the water nutrient content through time according to the presence of invasive algae (Inv), temperature (T) and their interactions. Significant effects are highlighted in bold.

8.3. Supplementary Material Chapter 4: Resilience of seagrass seedlings to marine heatwaves is modulated by local thermal variation

1)



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2)

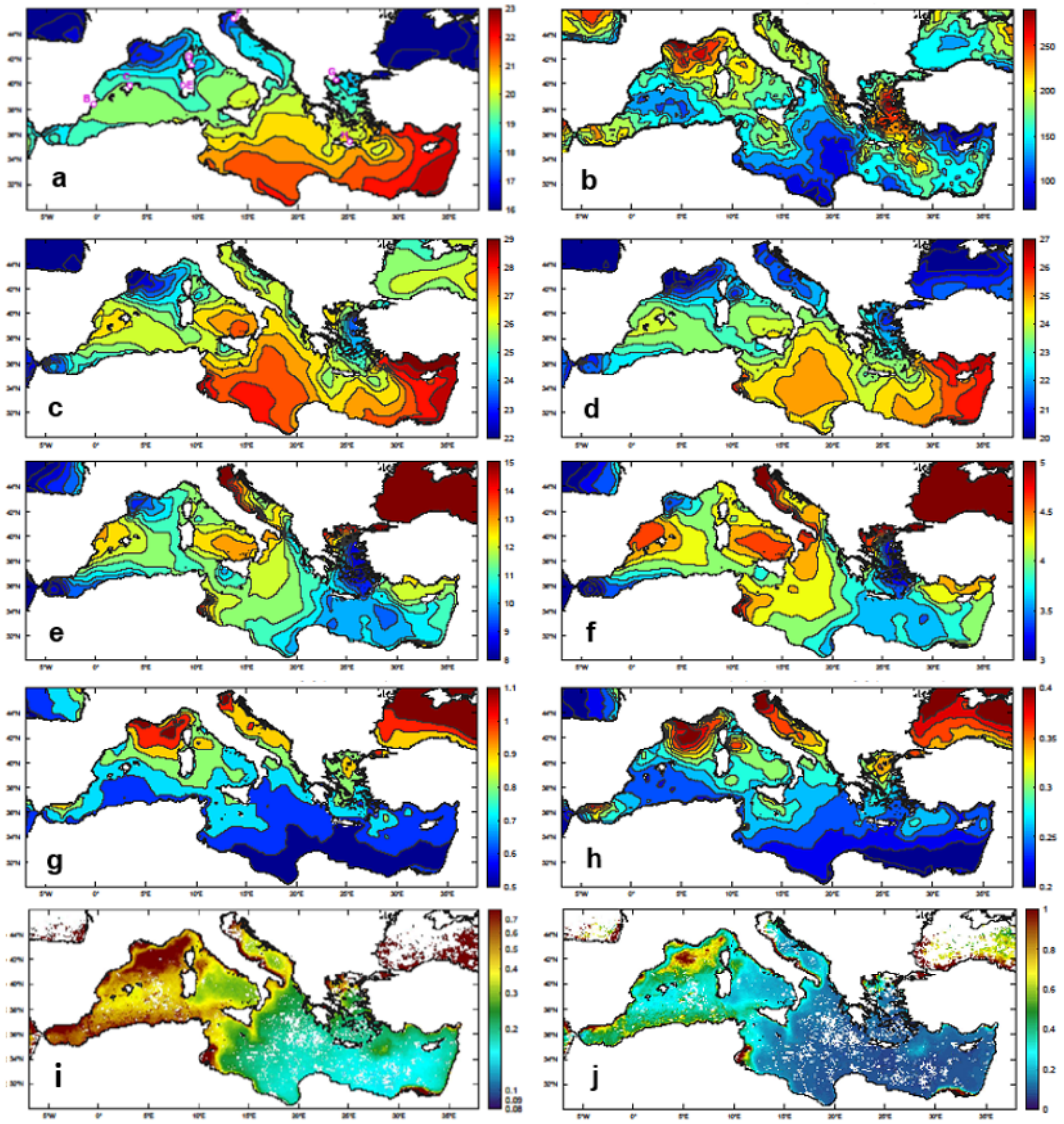

Figure S 7. Representation of the environmental variable metrics for (1) the Canary Islands archipelago and (2) the entire Mediterranean Sea: (a) SST-Mean (ºC) contours 0.5ºC, (b) Number of warm days, contours 20 days, (c) SST-Max seasonal cycle (ºC) contours 0.5ºC, (d) SST-Min summer (ºC), contours 0.5ºC, (e) SST Amplitude seasonal cycle (ºC) contours 0.5ºC, (f) SST-Standard deviation (ºC) contours 0.2ºC, (g) Anomalous-SST Standard deviation (ºC) contours 0.1ºC, (h) Anomalous-SST (lowpass) Standard deviation (ºC) contours 0.02ºC, (i) Chlorophyll maximum seasonal cycle (mg m^{-3}) and (j) Chlorophyll amplitude seasonal cycle (mg m^{-3}). All the regions analysed are labelled with a letter in the first subplot of each panel: A (Gran Canaria), B (Alicante), C (Mallorca), D (Corsica), E (Sardinia), F (Istria), G (Halkidiki) and H (Crete).

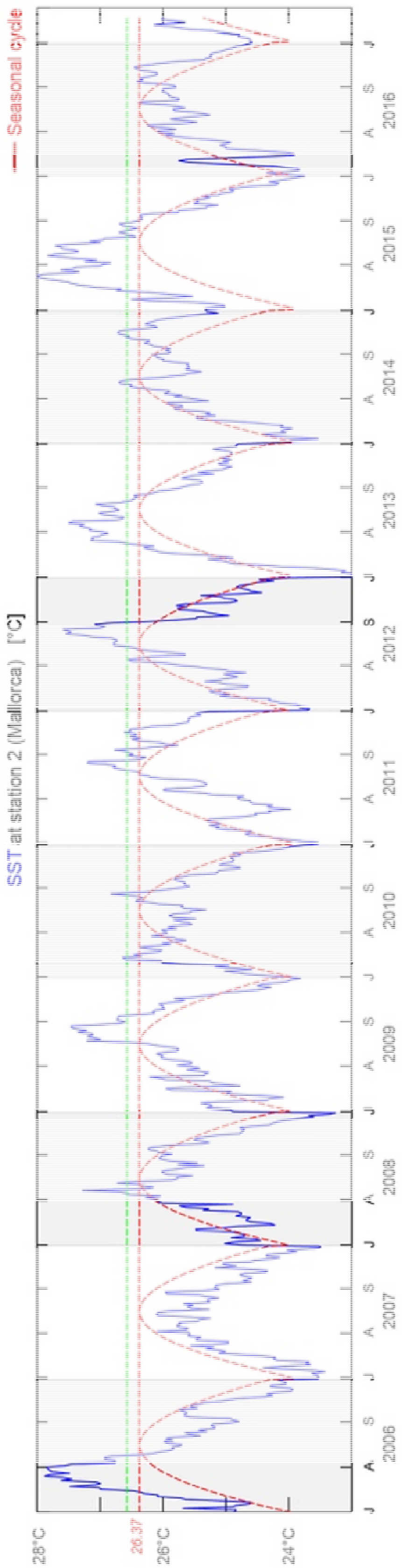

Figure S 8. Heatwave characterization. The seasonal cycle (dashed red line) is defined by a harmonic analyses using sinusoids with frequencies of 1, 2 and 3 cycles per year. This figure illustrates the identification method of MHWs, which are defined as the time where the sea surface temperature (SST, blue time series) is greater than the dashed green line (26.57ºC).

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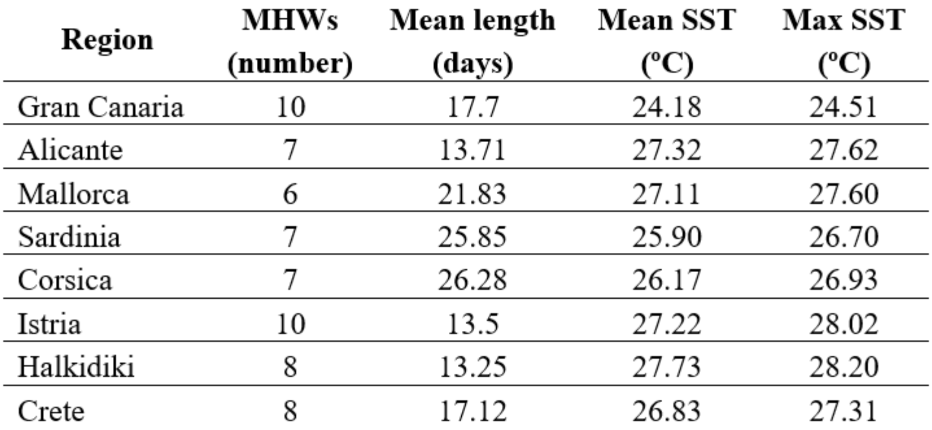
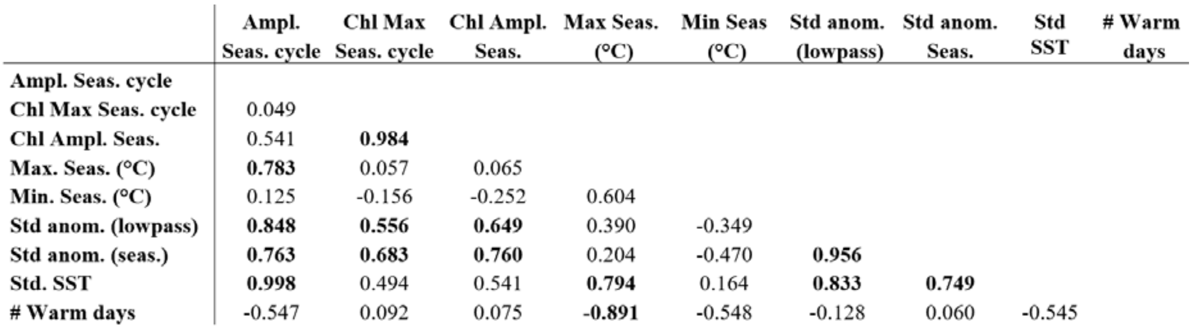


Region	MHWs (number)	Mean length (days)	Mean SST (ºC)	Max SST (ºC)
Gran Canaria	10	17.7	24.18	24.51
Alicante	7	13.71	27.32	27.62
Mallorca	6	21.83	27.11	27.60
Sardinia	7	25.85	25.90	26.70
Corsica	7	26.28	26.17	26.93
Istria	10	13.5	27.22	28.02
Halkidiki	8	13.25	27.73	28.20
Crete	8	17.12	26.83	27.31

Table S 5. Characteristics of the marine heatwaves (MHW) experienced at the eight studied regions between 2006 and 2016.



	Ampl. Seas. cycle	Chl Max Seas. cycle	Chl Ampl. Seas.	Max Seas. (°C)	Min Seas (°C)	Std anom. (lowpass)	Std anom. Seas.	Std SST	# Warm days
Ampl. Seas. cycle									
Chl Max Seas. cycle	0.049								
Chl Ampl. Seas.	0.541	**0.984**							
Max. Seas. (°C)	**0.783**	0.057	0.065						
Min. Seas. (°C)	0.125	-0.156	-0.252	0.604					
Std anom. (lowpass)	**0.848**	**0.556**	**0.649**	0.390	-0.349				
Std anom. (seas.)	**0.763**	**0.683**	**0.760**	0.204	-0.470	**0.956**			
Std. SST	**0.998**	0.494	0.541	**0.794**	0.164	**0.833**	**0.749**		
# Warm days	-0.547	0.092	0.075	**-0.891**	-0.548	-0.128	0.060	-0.545	

Table S 6. Pearson's correlation coefficients of the environmental variables. Significant correlations are highlighted in bold.

		Effect Size			
		Disturbance phase		Recovery phase	
Response variable	**Region**	**Moderate MHW**	**Severe MHW**	**Moderate MHW**	**Severe MHW**
Survival	Gran Canaria	0.90	0.05	0.75	0.01
	Alicante	1.00	1.00	1.00	1.00
	Mallorca	2.50	2.60	5.00	2.25
	Corsica	10.00	2.73	10.00	1.45
	Sardinia	6.25	2.80	7.86	2.00
	Istria	5.25	6.40	8.00	2.50
	Halkidiki	4.25	6.40	7.86	2.00
	Crete	2.50	2.08	5.00	0.77
TLA	Gran Canaria	0.56	0.61	0.00	0.00
	Alicante	1.00	1.00	1.00	1.00
	Majorca	1.03	0.91	1.55	0.81
	Corsica	1.40	0.81	1.27	0.78
	Sardinia	0.63	0.71	0.38	0.83
	Istria	1.47	0.80	0.94	0.91
	Halkidiki	1.96	1.86	2.16	0.593
	Crete	1.81	1.00	1.31	0.94
ETR	Alicante	1.00	1.00	1.00	1.00
	Corsica	1.17	16.18	11.43	3.88
	Crete	2.35	12.61	9.29	6.14
	Istria	3.98	15.18	6.62	3.44
	Halkidiki	5.58	11.33	3.02	0.26
	Majorca	9.38	10.86	4.19	1.83
	Sardinia	2.43	13.11	13.16	3.33

Table S 7. Effect sizes of survival, total leaf area (TLA) and electron transport rate (ETR) for the different regions for both marine heatwaves (MHWs) and both experimental phases. The effect size number represents how many times every region has more probability of survival in comparison with a region of reference, in this case Alicante, with an effect size of 1.

8. APPENDIX

8.4. Pereda-Briones L, Infantes E, Orfila A, Tomas F, Terrados J. 2018. Dispersal of seagrass propagules: interaction between hydrodynamics and substratum type. *Marine Ecology Progress Series*, 593, 47-59.

8. APPENDIX

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Dispersal of seagrass propagules: interaction between hydrodynamics and substratum type

L. Pereda-Briones[1,*], E. Infantes[2], A. Orfila[1], F. Tomas[1,3], J. Terrados[1]

[1]Instituto Mediterráneo de Estudios Avanzados, IMEDEA (CSIC-UIB), Miquel Marques 21, 07190 Esporles, Illes Balears, Spain
[2]Department of Marine Sciences, University of Gothenburg, Kristineberg, Fiskebäckskil, 45178, Sweden
[3]Department of Fisheries and Wildlife, Oregon State University, 104 Nash Hall, Corvallis, OR 97331, USA

ABSTRACT: Waves and currents influence not only the spatial distribution of seagrass meadows but also the transport, establishment and survivorship of seagrass propagules and hence the success of seagrass recruitment from sexual reproduction. We quantified the dispersal of propagules of 3 seagrass species (*Posidonia oceanica, Cymodocea nodosa* and *Zostera marina*) over substrata of different complexities (sand, coarse gravel and *P. oceanica* mattes of different shoot abundances) under unidirectional flow in a hydraulic flume. Threshold velocities indicate that *Z. marina* seeds start to move earlier over a flat sandy bottom (14 cm s^{-1}) than seeds of *P. oceanica* (20 cm s^{-1}) and *C. nodosa* (21 cm s^{-1}). Propagule trapping increased with bottom complexity, which was related to the flow reduction that each substratum generated and the boundary layer thickness. Trapping rates were higher in coarse gravel and in mattes with higher abundances of dead shoots, where flow was reduced more than 50%. Over sand, flow reduction was minimal and propagules were not trapped. Furthermore, notable differences between *P. oceanica* early life stages were observed, with seeds trapped first, followed by seedlings of increasing ages. This result may be related to the smaller frontal area (area exposed to water flow) and higher settling velocity of the younger life stages. Together, our results provide important insights into the drivers of seagrass recruitment, which are of interest for restoration purposes and numerical modelling.

KEY WORDS: Establishment · Seed transport · Recruitment · Drag coefficient · Morphology · Seedling · *Posidonia oceanica* · *Cymodocea nodosa* · *Zostera marina*

INTRODUCTION

Seagrasses are a dominant feature of coastal environments and provide a suite of important ecosystem services, including coastal protection, carbon sequestration and nursery areas for many commercially and ecologically valuable marine species, among others (Hemminga & Duarte 2000). As a result of different human stressors, seagrasses have suffered important declines worldwide (Waycott et al. 2009). Sexual reproduction is an essential part of the life cycle of seagrasses, for its role in the consolidation of existing meadows and for the colonization of new ones, and thus may be an important process for seagrass recovery following disturbance (Preen et al. 1995, Olesen et al. 2004). Furthermore, the products of sexual reproduction such as fruits, seeds and seedlings are critical not only for dispersal but also for providing genetic variation, which is crucial for adaptation (Kendrick et al. 2012, 2017). In fact, early life stages of other aquatic plants are decisive in limiting species distribution and abundance (Les et al. 2003). In addition to these ecological functions, early life stages can also be used as a source of transplanting material in seagrass restoration projects (Ganassin & Gibbs 2008, Renton et al. 2011, Orth et al. 2012), which is an important issue to consider given the dramatic rates of seagrass loss worldwide (Waycott et al. 2009).

Hydrodynamics (waves and currents) influence the spatial distribution of seagrass meadows (Koch 2001,

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[*] Corresponding author: lpereda@imedea.uib-csic.es

Frederiksen et al. 2004, Infantes et al. 2009), and are also crucial for seedling survival (Infantes et al. 2011a). Substratum type may also contribute to seagrass propagule trapping and establishment (Piazzi et al. 1999, Infantes et al. 2011b), and substratum roughness determines the height of the bottom boundary layer which influences the transport of seeds and seedlings (Koch et al. 2010, Alagna et al. 2015). Similarly, hydrodynamics are also an important factor in determining the success of seagrass restoration projects (Bos & van Katwijk 2007), and identification of adequate substratum type is critical for the survival and growth of transplanted seedlings in restoration activities (Balestri et al. 1998). Furthermore, bottom shear stress from the combined effect of waves and currents can mobilize unconsolidated sediment, producing erosion or accretion that can affect seagrasses negatively through uprooting and burial (Orth et al. 1994, Terrados 1997, Cabaço et al. 2008).

Beyond hydrodynamics, the physical properties of seagrass propagules, such as morphology, density, settling velocity and drag coefficient, are highly determinant of their dispersal potential (Infantes et al. 2011a, Ruiz-Montoya et al. 2012, 2015). Once dispersed, seedling establishment, survival and development depend on the environmental characteristics of the settlement area, such as depth, substratum type, hydrodynamic conditions or erosion events (Piazzi et al. 1999, Orth et al. 2006a, Infantes et al. 2011a, Alagna et al. 2013). In fact, the period between seed germination and seedling establishment is one of the most vulnerable phases for plant development, when plants experience the highest mortality rates (Orth et al. 2006a, Alagna et al. 2013). Thus, elucidating the interactions between hydrodynamics and substratum type affecting the dispersal of seagrass propagules is necessary to understand natural colonization processes and, in particular, secondary dispersal (sensu Chambers & MacMahon 1994) of seagrass species that have floating propagule stages. In order to identify safe recruitment microhabitats and the establishment potential of seagrass seedlings in different substrata, it is essential to consider these processes from a fluid dynamics perspective.

Here we investigated how hydrodynamics and substratum types affect the transport and establishment of propagules of different seagrass species. We focussed on 3 temperate species with different morphological and physical traits that likely affect their interaction with hydrodynamics and substratum: *Posidonia oceanica* (endemic to the Mediterranean Sea), *Cymodocea nodosa* (representative of the Mediterranean Sea and the Atlantic coast of North Africa) and *Zostera marina* (widespread in northern latitudes; Green & Short 2003). *P. oceanica* produces floating fruits, which are transported by surface currents until they dehisce and the seeds are released, whereas *Z. marina* produces reproductive shoots which contain seeds that mostly dehisce within the meadow, although some are transported inside the reproductive shoots by surface currents until they dehisce. In contrast, the fruits of *C. nodosa*, which are negatively buoyant, develop buried at the shoot base and are released within the meadow, although they may be exported during sediment erosion events, such as storms. As the seeds of the 3 species are negatively buoyant and fall to the bottom, their dispersal at the bottom will depend on hydrodynamic processes occurring within the bottom boundary layer (Orth et al. 1994, 2006a). Hereafter, their secondary dispersal depends on the physical characteristics of the propagules and on the interaction between hydrodynamic conditions and substratum type (Infantes et al. 2011a,b, Ruiz-Montoya et al. 2012, 2015, Alagna et al. 2015). In this study, we specifically aimed to (1) estimate transport velocities of floating propagules of *P. oceanica* and *Z. marina* at different current velocities, (2) determine the minimum flow velocities at which seeds and seedlings of the 3 species start moving over the substratum (threshold velocities) and (3) estimate the capacity of different substrata to retain propagules ('trapping') and how this process is influenced by the propagules' physical characteristics.

MATERIALS AND METHODS

Seed collection and physical properties

Seeds of *Posidonia oceanica* (Fig. 1a) were obtained from fruits collected on the shore during June 2015 in Mallorca (Balearic Islands, Western Mediterranean Sea). Fruits were opened and their seeds removed and placed in 20 l aquaria with seawater (salinity 37), at a temperature of 20°C and illuminated by white fluorescent lamps that provided 76 ± 2 μmol m^{-2} s^{-1} of photosynthetically active radiation in a 14:10 h light:dark photoperiod. Seeds successfully germinate under these conditions and first leaves and roots are produced within 2 wk (Terrados et al. 2013). In addition, fruits collected in June were also conserved unopened in seawater under the same conditions.

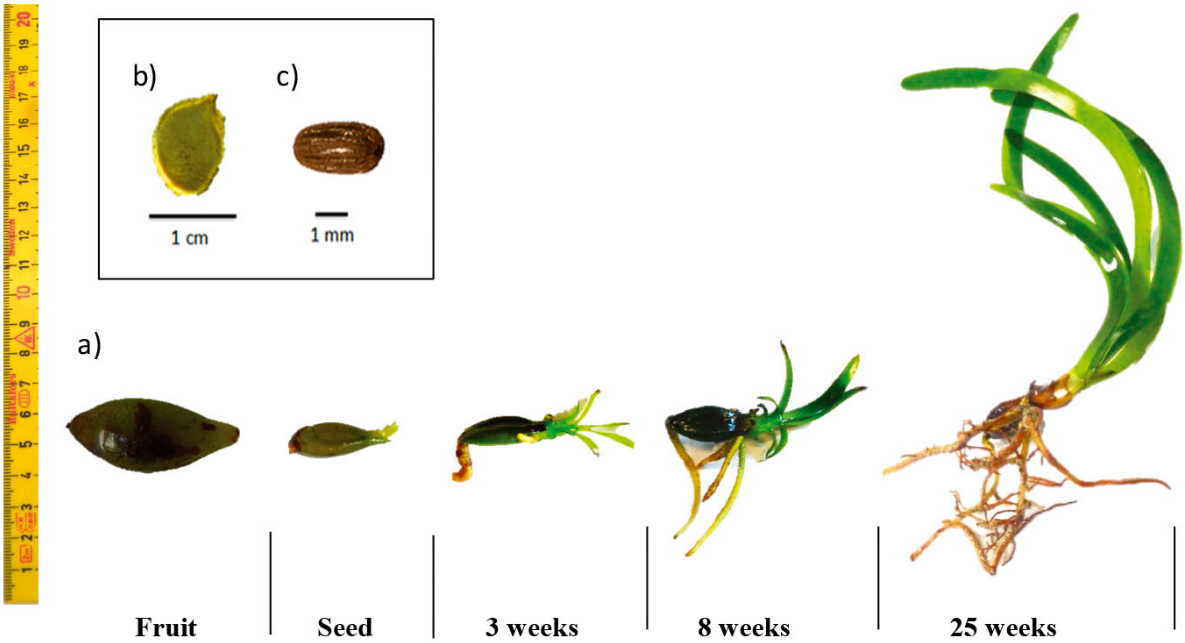

Fig. 1. *Posidonia oceanica* early life stages: (a) fruit and seed (0 wk old), and seedlings at 3, 8 and 25 wk of age. Also shown are seeds of (b) *Cymodocea nodosa* and (c) *Zostera marina*

Seeds of *Cymodocea nodosa* (Fig. 1b), which are typically buried in the sediment, were collected during February 2015 in Mallorca from shallow meadows by scuba diving. They were placed in darkness inside 20 l aquaria under the same conditions as described above. All seeds were transported to the Lovén Centre for Marine Science, Kristineberg Station, Sweden, and were kept in seawater aquaria under the same conditions as described above.

Seeds of *Zostera marina* (Fig. 1c) were collected by harvesting reproductive shoots at 1–3 m depth in the Gullmars fjord, Gåsö, Sweden, in July 2015. These reproductive shoots were stored in outdoor tanks at Kristineberg until the seeds were released (Infantes & Moksnes 2018). *Z. marina* seeds were stored at salinity 34 and temperature of 5°C to prevent germination (Infantes et al. 2016).

Physical characteristics of fruits, reproductive shoots, seeds and seedlings were measured and compared, since they influence propagule transport and trapping (Koch et al. 2010, Infantes et al. 2011a, Ruiz-Montoya et al. 2012). Propagule length, width and height were measured using an electronic precision calliper (mm), and the volume (ml) was determined by fluid displacement in a 12 ml graduated cylinder. Propagule mass was measured as wet weight (g). The density of each fruit, reproductive shoot, seed or seedling was calculated from mass and volume measurements. The number of spathes was measured for *Z. marina* reproductive shoots. The number and length of leaves, width of the second youngest leaf and root number and length were measured for all *P. oceanica* seedlings.

Settling velocities (w_s) of seagrass propagules (n = 10) were estimated as the time needed to sink to the bottom in a vertically placed glass tube (70 cm long and 20 cm diameter) filled with seawater. Each measurement was repeated 3 times for each individual propagule, and the mean value was calculated. Propagule drag coefficient (C_d) was calculated following Dean & Dalrymple (2004) as:

$$C_d = 2\left(\frac{\rho_s - \rho}{\rho}\right)\frac{gV_s}{w_s^2 A_F} \qquad (1)$$

where ρ_s is the density of the propagule, ρ is the density of seawater, g is the acceleration of gravity, V_s is the volume of the propagule, and A_F is the estimated propagule frontal area exposed to the flow.

Seed frontal area (A_F) for *Z. marina* and *P. oceanica* was determined assuming an elliptical shape with semi axes a and b, (i.e. $A_{F\ SEED} = \pi \times a \times b$). In contrast, the frontal area of each *C. nodosa* seed was estimated by multiplying its maximum length by its width since the seed shape resembles a flat disc (Fig. 1b). The frontal area of *P. oceanica* seedlings was determined by adding the foliar surface (estimated as a rectangular shape) to the area of the seed (approximated to an ellipsoid) and the area of roots (obtained by multiplying the root diameter by the total root length), i.e. $A_{\mathrm{F\,SEEDLING}} = A_{\mathrm{F\,SEED}} + \left(\sum_{i=1}^{n} l_i * w_i\right) + (d_{\mathrm{r}} \cdot l_{\mathrm{rT}})$, where l and w are the length and width of each leaf, n is the number of leaves, d_{r} is the root diameter, and l_{rT} the total root length. Seawater density used for the calculations was taken as a constant 1025 kg m^{-3} (equivalent to the value at 20°C).

In order to evaluate the effect of the substratum type on the boundary layer, we estimated the boundary layer thickness assuming the flow turbulence as:

$$\delta = \frac{0.37x}{\mathrm{Re}^{\frac{1}{5}}} \tag{2}$$

where Re is the Reynolds number and x is the distance downstream from the start of the boundary layer.

Flume description and flow-substrate characterization

A unidirectional (current) flume located at Kristineberg Station was used to (1) identify the threshold velocities of the propagules to initiate movement and to be retained by the substrata, and (2) to simulate the horizontal transport of propagules, including dispersal and trapping. A flume was used to identify threshold velocities and propagule trapping since it allows modifying 1 variable (substratum) while leaving others constant (e.g. flow). Current velocities from 5 to 30 cm s^{-1}, typical for the locations where the propagules were collected (Anthony & Svane 1994, Balbín et al. 2012), were applied in the flume. The flume was 4 m long, 0.5 m wide and 0.5 m deep (Fig. 2). The test section was 2 m long, and the water level was maintained at 0.15 m. Flow velocities were generated by a motor-run propeller at the far end of the flume controlled by an adjustable speed drive (Dayton Electronic, model 6K119). The measurements were conducted during 2 periods, July and October 2015, in order to examine the properties of different plant developmental stages.

Flow velocities were measured with an acoustic Doppler velocimeter (ADV, Nortek, Vectrino) at a sampling rate of 25 Hz. Vertical velocity profiles were measured over different substrata at 6 positions (0.5, 1, 2, 3, 5 and 7 cm above the bottom) before and after the test section. Percentage of flow reduction due to substratum type was calculated from the flow before and after the test section at a current velocity of 20 cm s^{-1} in order to ensure that all propagules had initiated their movement (see 'Results').

Transport of fruits and reproductive shoots

Transport velocities of floating propagules, i.e. *P. oceanica* fruits and *Z. marina* reproductive shoots, were measured in the unidirectional flume. The surface flow velocity was measured at 1–2 cm below the water surface with the ADV positioned upwards. Floating propagules of each species (n = 10) were placed independently on the water surface, and their velocity was assessed by recording the time needed to travel along 1 m at different flow velocities (from 5 to 30 cm s^{-1}).

Threshold velocities for transport and trapping measurements

Threshold velocity, defined as the flow velocity at which a propagule initiates and maintains a continuous movement along the whole test section, was measured for each of the 3 seed species and for 4 different developmental stages of *P. oceanica* propagules

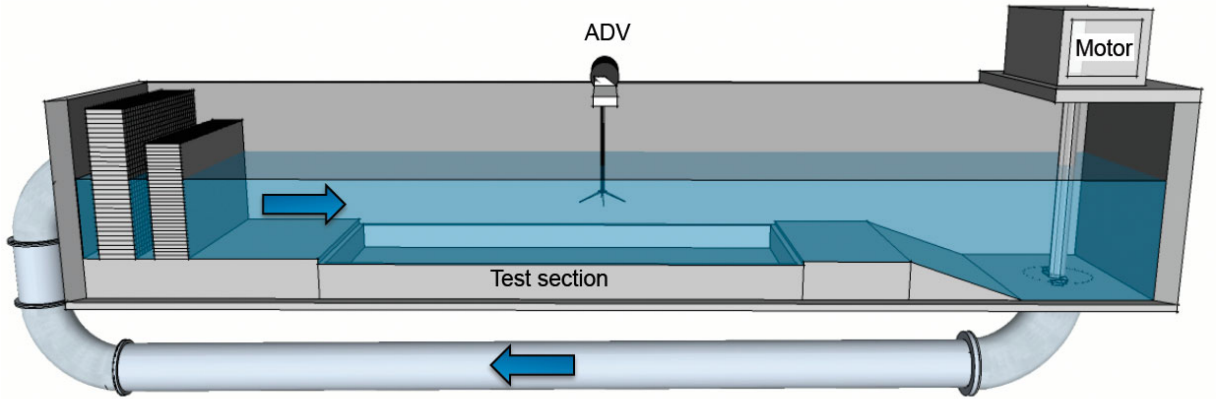

Fig. 2. The hydraulic flume used in the laboratory to simulate hydrodynamic conditions. ADV: acoustic Doppler velocimeter

(i.e. seeds and 3, 8 and 25 wk old seedlings). Propagules were separately placed over a flat sand bed prepared in the flume. The water flow was increased at 1 cm s^{-1} intervals until they started to move. The velocity of the propagule was estimated by measuring the time needed to cross 1 m distance once it had initiated the movement at different flow velocities. Trials were repeated 10 times (using different propagules each time) for each propagule type, and the mean threshold velocity values were calculated.

Trapping was defined as the substratum capability to retain *P. oceanica* seeds and seedlings, and it was calculated by measuring the distance travelled by the propagules over a specific substratum type under different flow velocities. Three common substratum types were evaluated: sand (0.25–0.50 mm in diameter), coarse–very coarse gravel (in the range of 5 × 3 × 2 cm) and *P. oceanica* matte (i.e. a network of rhizomes from dead plants). We constructed a model of matte by burying in the sand different numbers of orthotrophic (i.e. vertical growth) fragments of *P. oceanica* rhizomes collected from beach-cast plants after a storm. Six different matte types were built combining 3 dead shoot abundances of 75, 150 and 300 shoots m^{-2} (75, 150 and 300 shoots placed in the flume test section, respectively) and 2 heights above sand (1 and 4 cm) by haphazardly distributing the shoots along the box. These shoot abundances represent the range of shoot density values found in highly degraded meadows, but can also be contextualized in terms of depth variations, since shoot density typically decreases with increasing depth (Pergent et al. 1995).

In order to recreate the propagule transport over the substratum types at several flow velocities, the flume had a 2 m long test section containing the substrate analysed (Fig. 2). Seeds and seedlings were placed over a 10 cm thick, flat sand bed of 1 m longitude that preceded the box with the specific substratum. This enabled the propagules to develop an initial movement and continue over the 100 cm of the corresponding substrata. We measured the distance travelled and the velocity of the propagules from the beginning of the test section (containing a specific substrate) until the propagule was trapped or until it passed over the entire test section without being trapped.

Trapping results are presented depending on the difference between the length of the test section and the distance travelled by the propagules. If the distance travelled was equal to the total length of the test section, then trapping was considered 0, while trapping was maximum (i.e. 100) if the propagule was retained at the beginning of the test section. Substrata were ranked according to their complexity, from the lowest complexity (i.e. sand), along the different matte substrata of increasing rhizome abundance and height, to the highest (i.e. coarse gravel). The complexity of matte was calculated as the number of rhizomes m^{-2} × rhizome height (cm). For coarse gravel, complexity was calculated as the number of gravel pieces m^{-2} × gravel height. Complexity assigned to sand was 0. Therefore, substratum complexity increased from sand (0) to matte with rhizomes of 1 cm height (75 × 1 = 75, 150 × 1 = 150, 300 × 1 = 300), 4 cm high (75 × 4 = 300, 150 × 4 = 600, 300 × 4 = 1200) and coarse gravel (1500).

Data analysis

One-way analysis of variance (ANOVA) was used to compare physical characteristics and threshold velocities among seeds of the different species and among *P. oceanica* early life stages. An analysis of covariance (ANCOVA) was used to compare the slopes of the 2 regression lines obtained from the data on floating propagule velocity (dependent variable) of the 2 species analysed (categorical factor) in relation to the surface flow velocities (continuous covariable). Two different regressions were conducted to evaluate the relation between *Z. marina* morphological characteristics (length and number of shoots) and their velocity. A factorial ANOVA was used to evaluate the effects on trapping of *P. oceanica* propagules regarding 2 factors: substratum type (with 8 levels: sand, 6 different *P. oceanica* matte treatments and coarse gravel) and life stage (with 4 levels: seeds, and seedlings of 3, 8 or 25 wk of age). For a better comparison between the treatments, this analysis was conducted using the results obtained with a flow velocity of 20 cm s^{-1}, since at this velocity all of the propagules moved. To examine differences between levels of each factor and between factors, a post hoc Tukey test was conducted. Data were analysed after ensuring normality and homogeneity of variances using Kolmogorov-Smirnov and Cochran's tests.

RESULTS

A linear relationship between floating-propagule velocity and the surface flow velocity was found for *Posidonia oceanica* fruits and *Zostera marina* reproductive shoots, which was different between the 2 species (Fig. 3; ANCOVA: $F_{1,7} = 7.6054$, $p < 0.05$). At

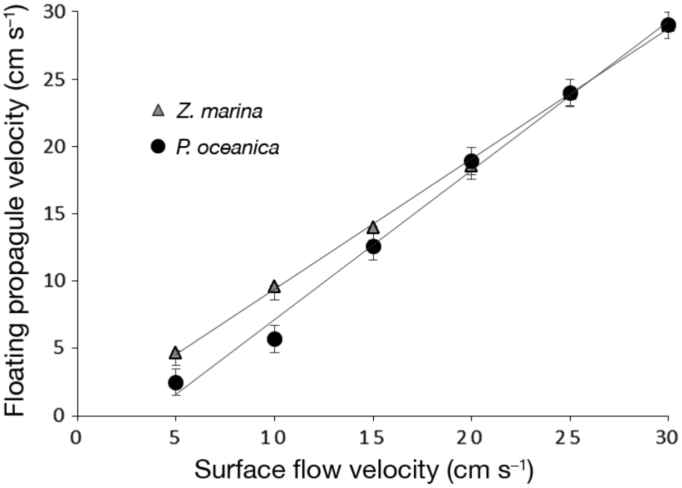

Fig. 3. Velocity of *Zostera marina* reproductive shoots ($y = 0.92x + 0.24$, $R^2 = 0.99$) and *Posidonia oceanica* fruits ($y = 1.12x - 4.11$, $R^2 = 0.98$), transported by different flow velocities

low flow velocities (5 and 10 cm s^{-1}) *P. oceanica* fruits, whose density is lower than water (Table 1), emerge and move at half the flow velocity of the water (2.5 and 5.7 cm s^{-1}, respectively). In contrast, at higher flow velocities (15 and 20 cm s^{-1}) the fruit velocity resembles the flow velocities of the water (12.6 and 18.9 cm s^{-1}, respectively). In contrast, *Z. marina* reproductive shoots move almost at the same velocity as the water flow throughout the range of measured velocities (Fig. 3). The length of *Z. marina* reproductive shoots varied from 20 to 135 cm, and the number of spathes between 4 and 35 shoot^{-1}, and their mean weight, volume and density were 7.4 ± 1.4 g, 9.9 ± 1.4 cm^3 and 0.74 ± 0.05 g cm^{-3}. We did not find a significant relationship between the length of *Z. marina* reproductive shoots and their velocity ($R^2 = 0.33$), nor between the number of shoots and their velocity ($R^2 = 0.43$).

Seed size differed amongst seagrass species, with *P. oceanica* exhibiting the largest frontal area, density, settling velocity and volume, while *Cymodocea nodosa* had higher frontal area, weight and volume than *Z. marina* (Table 1, Tukey tests). Density and settling velocity of *Z. marina* seeds were higher than those of *C. nodosa*, and drag coefficient of *Z. marina* was highest, followed by *P. oceanica* and *C. nodosa* (Table 1, Tukey tests). Regarding *P. oceanica* early life stages, as propagules grew, their frontal area, weight, volume and drag coefficient increased while their settling velocities decreased (Table 2, Tukey tests).

The minimum current velocity needed to move seeds or seedlings at the bottom (threshold velocities) was lower for *Z. marina* seeds (mean ± SE, 14 ± 0.01 cm s^{-1}) followed by *P. oceanica* seeds (which started at 20 ± 0.03 cm s^{-1}) and *C. nodosa* seeds (21 ± 0.01 cm s^{-1}; $F_{2,27} = 2019.6$, $p < 0.001$; Fig. 4a). Regarding *P. oceanica* early life stages, 25 wk old seedlings were the first to move (4 ± 0.3 cm s^{-1}), followed by 8 wk old seedlings (7 ± 0.2 cm s^{-1}), 3 wk old seedlings (17.5 ± 0.04 cm s^{-1}) and seeds (20 ± 0.06 cm s^{-1}; $F_{3,36} = 1893.6$, $p < 0.001$; Fig. 4b). Threshold velocities decreased linearly with drag coefficient for seeds of all species and for all developmental stages of *P. oceanica* (Fig. 5a).

Trapping differed across propagule types and substratum (significant interaction; Table 3). There was no trapping over sand for any of the life stages, and trapping differed across the other substratum types for every early life stage, except for the mattes with 75 and 150 rhizomes of 4 cm height, where trapping results were equal. Overall, while all *P. oceanica* seeds and seedlings went through the sand substratum without being trapped, trapping tended to decrease with flow velocity for all other substrata analysed, being maximum at flow velocities below 7 cm s^{-1} for all propagules. Seeds underwent higher trap-

Table 1. Mean ± SE physical characteristics of *Posidonia oceanica*, *Cymodocea nodosa* and *Zostera marina* (n = 10). Tukey tests of a 1-way ANOVA comparing properties across seeds of the different species are shown. In all cases, results were significant at $p < 0.01$

Properties	*P. oceanica* (Po)	*C. nodosa* (Cy)	*Z. marina* (Zo)	$F_{2,27}$	Tukey test
Length (cm)	2.08 ± 0.67	1.02 ± 0.34	0.33 ± 0.07	2033.53	Po > Cy > Zo
Width (cm)	0.83 ± 0.10	0.64 ± 0.16	0.152 ± 0.04	6930.53	Po > Cy > Zo
Height (cm)	0.49 ± 0.07	0.14 ± 0.04	0.152 ± 0.04	6599.40	Po > Cy > Zo
Weight (g)	0.65 ± 0.02	0.078 ± 0.01	0.0073 ± 0.001	674.17	Po > Cy > Zo
Volume (cm^3)	0.58 ± 0.05	0.075 ± 0.001	0.007 ± 0.001	275.44	Po > Cy > Zo
Density (g cm^{-3})	1.12 ± 0.04	1.035 ± 0.001	1.065 ± 0.001	2 617 352	Po > Zo > Cy
Settling velocity (cm s^{-1})	9.73 ± 0.51	6.78 ± 0.124	7.44 ± 0.18	1865.28	Po > Zo > Cy
Frontal area (cm^2)	5.42 ± 0.06	0.147 ± 0.003	0.039 ± 0.001	10 023 886	Po > Cy > Zo
Drag coefficient	0.21 ± 0.03	0.18 ± 0.002	0.24 ± 0.005	474348.7	Zo > Po > Cy

Table 2. Mean ± SE physical characteristics of *Posidonia oceanica* early life stages (n = 10). Tukey tests of a 1-way ANOVA comparing properties across early life stages are shown. In all cases, results were significant at $p < 0.01$

	Properties	Fruit (Fr)	Seed (Se)	Seedlings			$F_{3,36}$	Tukey
				3 wk	8 wk	25 wk		
Seed or fruit (cm)	Length	3.32 ± 0.73	2.08 ± 0.07	1.97 ± 0.18	1.88 ± 0.17	1.89 ± 0.12	6448.40	Fr > Se > 3 > 8 = 25
	Width	1.24 ± 0.30	0.83 ± 0.05	0.83 ± 0.01	0.92 ± 0.05	0.97 ± 0.08	6381.21	Fr > 25 > 8 > 3 = Se
	Height	1.33 ± 0.32	0.49 ± 0.02	0.52 ± 0.04	0.61 ± 0.03	0.58 ± 0.01	2383.02	Fr > 8 > 25 > 3 = Se
Leaves	Area (cm^2)			1.04 ± 0.04	7.52 ± 0.72	25.8 ± 2.73	322.76	25 > 8 > 3
	Max. length (cm)			1.24 ± 0.04	3.57 ± 0.29	13.0 ± 1.31	578.96	25 > 8 > 3
Roots	Number			5.21 ± 0.13	8.21 ± 0.29	7.80 ± 0.24	2733.08	8 > 25 > 3
	Total length (cm)			1.64 ± 0.12	5.60 ± 0.18	40.0 ± 2.78	574 303	25 > 8 > 3
Whole propagule	Weight (g)	2.34 ± 0.15	0.65 ± 0.02	0.86 ± 0.03	1.89 ± 0.05	4.70 ± 0.16	1532.79	25 > Fr > 8 > 3 > Se
	Volume (cm^3)	2.66 ± 0.14	0.58 ± 0.05	0.80 ± 0.02	1.81 ± 0.01	4.54 ± 0.23	2509.94	25 > Fr > 8 > 3 > Se
	Density (g cm^{-3})	0.87 ± 0.01	1.12 ± 0.04	1.11 ± 0.03	1.09 ± 0.01	1.08 ± 0.03	1498.41	Se > 3 > 8 > 25 > Fr
	Settling velocity (cm s^{-1})		9.73 ± 0.06	9.56 ± 0.09	6.16 ± 0.12	5.28 ± 0.32	1478.65	Se > 3 > 8 > 25
	Frontal area (cm^2)		5.42 ± 0.51	5.76 ± 0.42	6.90 ± 0.38	15.2 ± 0.21	267 793.7	25 > 8 > 3 > Se
	Drag coefficient		0.21 ± 0.03	0.24 ± 0.02	0.86 ± 0.05	1.17 ± 0.05	546.11	258 > 3 > Se

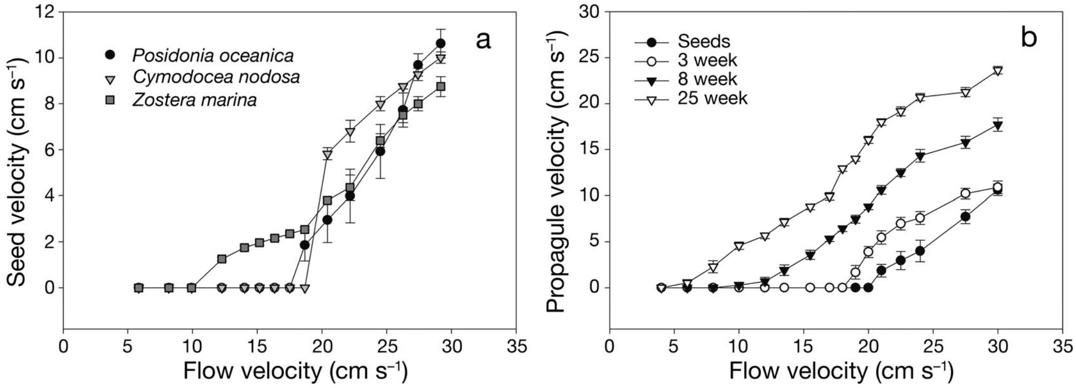

Fig. 4. Mean ± SE threshold velocities of seeds of (a) *Posidonia oceanica*, *Cymodocea nodosa* and *Zostera marina*, and of (b) *P. oceanica* early life stages (seeds, and seedlings at 3, 8 and 25 wk of age)

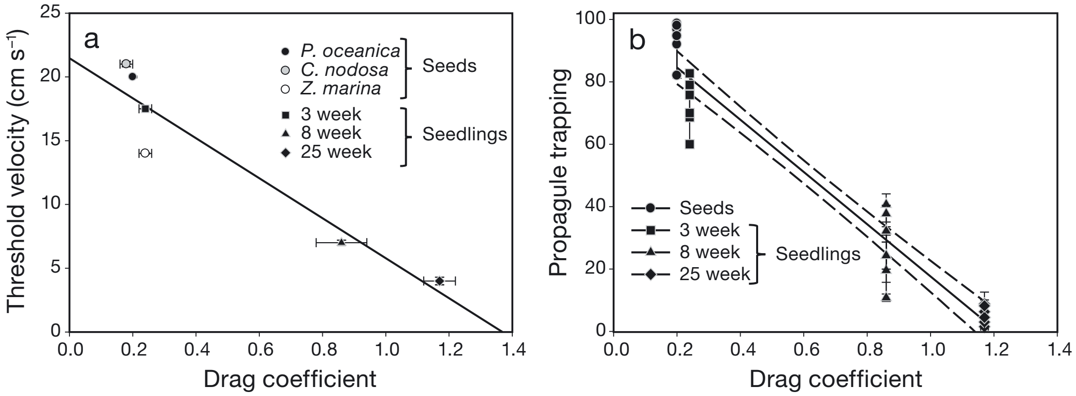

Fig. 5. (a) Mean ± SE drag coefficient related to threshold velocities over a flat sandy bottom for seeds and seedlings of *Posidonia oceanica*, seeds of *Cymodocea nodosa* and seeds of *Zostera marina* ($y = 21.47x - 15.68$, $R^2 = 0.90$). (b) Trapping of the 4 stages of *P. oceanica* by each substratum at 20 cm s^{-1} ($y = 101.312x - 83.71$, $R^2 = 0.93$). Trapping was calculated by measuring the distance travelled by the propagules over a specific substratum type under different flow velocities. If the distance travelled was equal to the total length of the test section, the trapping was considered 0, while trapping was maximum (i.e. 100) if the propagule was retained at the beginning of the test section

Table 3. Factorial ANOVA evaluating early life stages (ELS) and substratum type (ST) effects on trapping. All results were significant at $p < 0.01$

Factor	df	MS	*F*
ELS	3	32 359.4	2467.67
ST	7	35 230.2	2686.59
ELS × ST	21	1810.7	138.08
Error	288	13.1	

ping than seedlings, and seedling trapping decreased with increasing age. Trapping of propagules by matte and coarse gravel decreased linearly ($y = 101.31x - 83.71$, $R^2 = 0.93$) with higher drag coefficients (Fig. 5b). Trapping increased with rhizome abundance and height (Fig. 6) and, over coarse gravel, all seeds and seedlings were trapped independently of flow velocity, except for the 25 wk old seedlings, which were not trapped at velocities above 16 cm s^{-1} (Fig. 7).

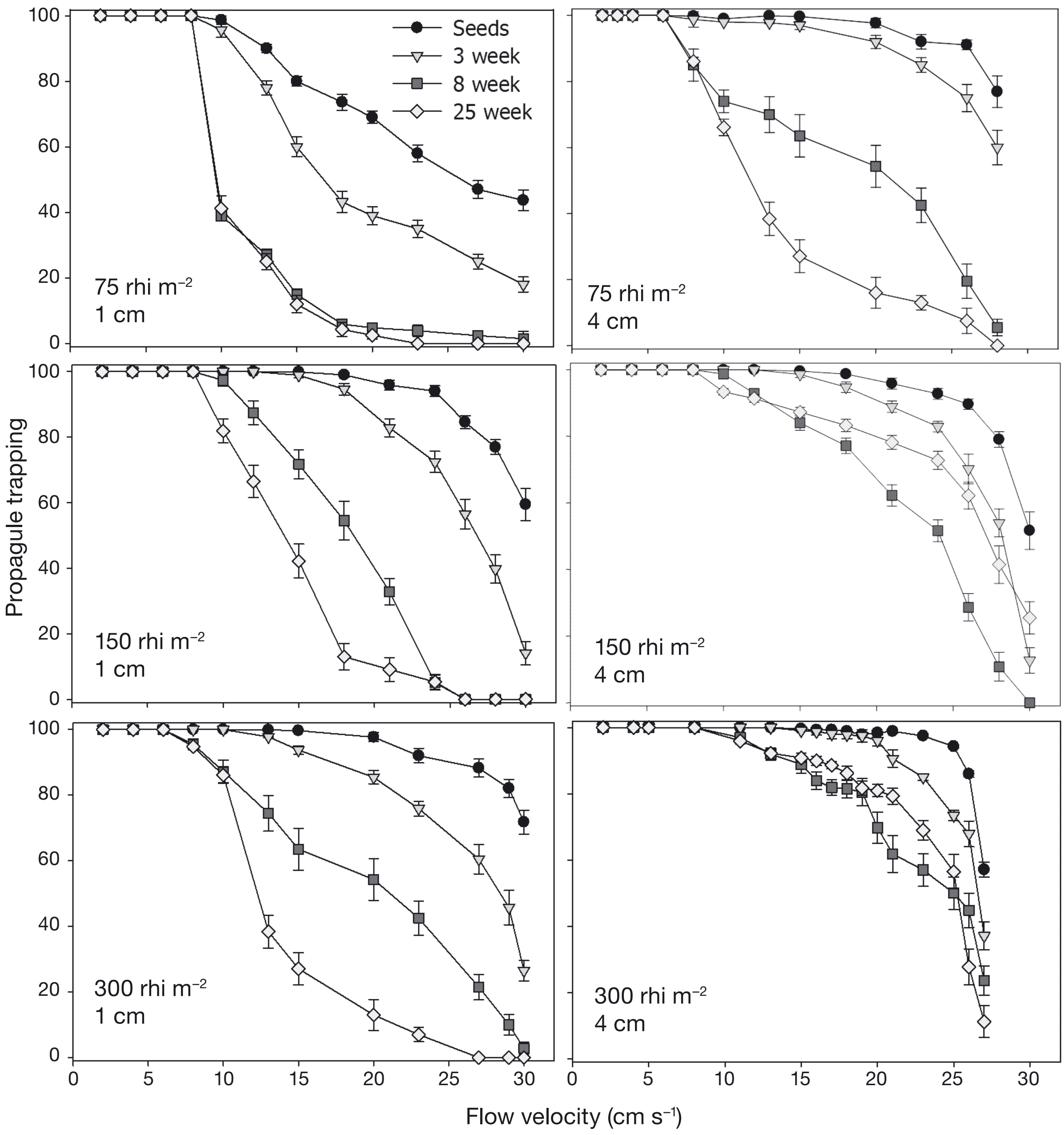

Fig. 6. Mean ± SE trapping rates for the 4 stages of *Posidonia oceanica* over matte substrata of different dead shoot abundances (rhizomes m^{-2}) and heights (cm). See Fig. 5 for a brief explanation of how trapping was calculated

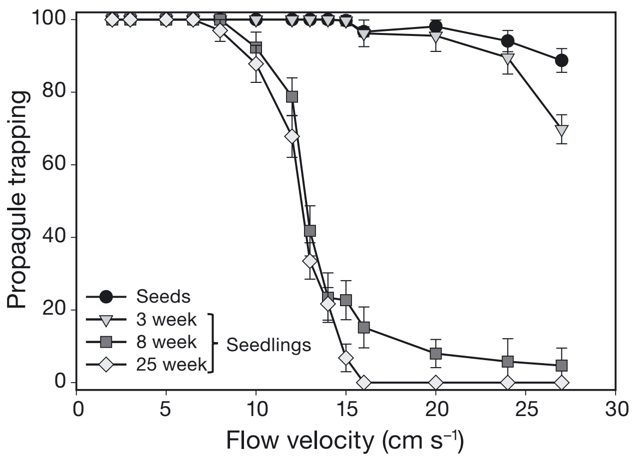

Fig. 7. Mean ± SE trapping rates for the 4 stages of *Posidonia oceanica* over coarse gravel substrata. See Fig. 5 for a brief explanation of how trapping was calculated

Flow reduction was higher over substrata of higher complexity, such as 4 cm matte and coarse gravel. The flow reduction over sand was minimal, while over coarse gravel it was reduced between 20 and 90% in the first 3 cm above the bottom (Fig. 8a). Flow reduction over 1 cm matte varied between 10 and 40% in the first 2 cm above the bottom (Fig. 8b), while over 4 cm matte it varied between 10 and 60% in the first 5 cm (Fig. 8c). The boundary layer thickness (δ) increased with substratum complexity, reaching a value nearly 2 times higher over coarse gravel than over the other substrates (Table 4).

DISCUSSION

Our experiments highlight important inter- and intraspecific differences in how hydrodynamics and substratum type determine the movement of seagrass propagules. Such differences are strongly influenced by the different physical characteristics of the 3 species examined. Overall, trapping of seagrass propagules increases with bottom complexity and boundary layer thickness as the different substrata generate different flow reduction. The threshold velocities needed to start propagule movement decreased with developmental stage and drag coefficient. Furthermore, notable differences were observed between *Posidonia oceanica* seeds and seedlings, as drag coefficient increased with developmental stage, hampering trapping and indicating that younger stages have a lower frontal area and higher settling velocity in comparison to older stages (Table 2). Therefore, if *P. oceanica* seedlings cannot successfully colonize a new substrate in the first few days of development, their capacity for being trapped and thus their ability to become established might become limited by the modification of their physical characteristics as they grow.

The velocity of floating propagules (i.e. fruits and shoots) varies in proportion to the surface current velocity and differs between species. While reproductive shoots of *Zostera marina* move at nearly the same velocity as the surface current, *P. oceanica* fruits move slower than the surface current when

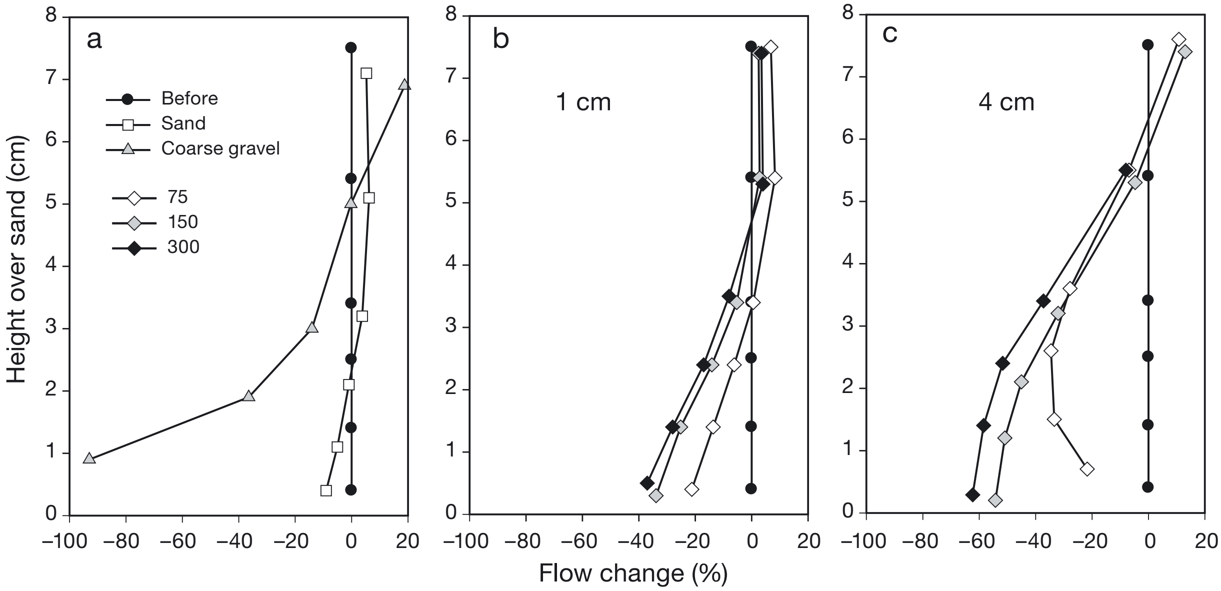

Fig. 8. Percentage of flow reduction in a 20 cm s⁻¹ flow compared between (a) sand and coarse gravel, (b) rhizomes of 1 cm height and (c) rhizomes of 4 cm height. Three rhizome abundances were used (75, 150 and 300 dead shoots m⁻²). 'Before' represents the flow over sand at the beginning of the flume

Table 4. Boundary layer thickness (δ) at 1 m downstream for the different substratum types: sand, the 6 combinations of matte substrata (given as rhizome abundance in shoots m^{-2} and [rhizome height in cm]) and coarse gravel. Velocity flow in all experiments was 20 cm s^{-1}

Substratum type	δ (cm)
Sand	3.24
75 [1]	3.33
150 [1]	3.42
300 [1]	3.39
75 [4]	3.49
150 [4]	3.68
300 [4]	3.85
Coarse gravel	5.83

flow velocities are low (<10 cm s^{-1}). Such a reduction may occur because part of the fruit emerges from the sea surface while part of it is submerged, therefore interacting with the sea–air boundary layer and decreasing the overall speed. Velocities achieved by *Z. marina* and *P. oceanica* propagules might be related to their different interaction with the surface boundary layer, with the fruits of *P. oceanica* more emerged than *Z. marina* shoots, and therefore having lower velocities. We did not manipulate wind conditions, but in addition to the current velocity, wind also contributes to propagule dispersal, allowing fruits to move faster than the surface currents, which allows fruits to be transported at rates ca. 2–4% of the wind velocity (Harwell & Orth 2002, Källström et al. 2008, Ruiz-Montoya et al. 2012). Further research is necessary to assess the combined effects of wind, waves and currents on floating propagules in order to further assess dispersal of floating propagules.

The threshold velocity for seeds of *Z. marina* has been estimated to be 8 cm s^{-1} over a flat sandy bottom (Orth et al. 1994), which is nearly half the velocity that we measured (14 cm s^{-1}). Such differences may be explained by the fact that during the measurements by Orth et al. (1994), only some of the *Z. marina* seeds tested started to move, and it was not a continuous movement. Moreover, the substratum that they used was slightly different from ours, i.e. a false bottom composed of sandpaper and sand (0.4–0.425 mm in diameter). We used bare sand in order to compare different levels of substratum complexity. Nevertheless, if we had simulated the interface created by benthic organisms in soft sediments such as sand, it would probably have modified the bottom roughness, and our results may therefore have been different (Graf & Rosenberg 1997).

Interestingly, *P. oceanica* and *Cymodocea nodosa* seeds required about 50% higher threshold velocities than *Z. marina*, and this pattern is likely explained by the difference in seed physical characteristics (e.g. frontal area exposed to the flow) of the 3 species, providing *Z. marina* with a higher drag coefficient than *P. oceanica* or *C. nodosa*. The higher floating velocities of reproductive spathes and the lower threshold velocities of seeds could contribute to explain the wide geographical range that *Z. marina* colonizes in comparison to the other 2 species (Green & Short 2003). Furthermore, under current and future scenarios of local and global human stressors (e.g. eutrophication, climate change), these physical traits may provide an advantage, as this species may be able to colonize more places and may thus have a larger potential for escaping to other areas with better environmental conditions. Nevertheless, Orth et al. (1994) observed that when mature seeds of *Z. marina* are released from reproductive shoots in the field, they fall to the bottom while transported by water currents, often dispersing only up to a few metres.

Regarding *C. nodosa* seeds, they are formed at the base of the shoots buried in the sediment and normally are retained there, contributing mainly to the maintenance of already established meadows (Buia & Mazzella 1991). However, if seeds are over soft sediments, such as sand, they could be resuspended and moved tens of metres, contributing to the formation of new meadows. Under these circumstances, seeds could be accumulated in bottom depressions like subaqueous sand dunes (Marbà & Duarte 1995). In addition, Terrados (1993) found relatively few seeds of *C. nodosa* inside shallow meadows, but did note some low-density patches originating from seeds in the surroundings, apparently formed by seeds which were re-suspended, transported and accumulated there.

Seeds of *P. oceanica* required higher threshold velocities than older seedlings, likely as a result of their lower drag coefficient. The threshold velocity decrease observed from seeds to seedlings has also been reported for other aquatic macrophytes such as *Ruppia maritima*, *Potamogeton perfoliatus* and *Stuckenia pectinata* (Koch et al. 2010), and the values are of the same order of magnitude as the ones we have determined for *P. oceanica.* These species increase their drag after germination and consequent organ development, and some seedlings actually acquire buoyancy, hampering their establishment (Koch et al. 2010).

Drag coefficient is a good proxy for propagule movement, since a higher value results in decreasing the propagules' threshold velocities and trapping success, yet estimations of drag coefficient still remain scarce for most species of marine vascular plants (Backhaus & Verduin 2008, Infantes et al. 2011a, Ruiz-Montoya et al. 2012).

The positive correlation between substratum of different complexities and trapping indicates that complex substrata, such as 4 cm height matte and coarse gravel, are more capable of retaining propagules. Indeed, sand (which we had given a value of 0 complexity) was not able to retain propagules at any of the tested velocities, while matte and coarse gravel, with higher complexity, were able to trap some of them depending on current velocity and propagule characteristics (Fig. 9).

Our first trapping measurements over matte were conducted at shoot abundances of 300 m^{-2}, which is equivalent to a highly degraded and/or deeper meadow (Pergent et al. 1995), and we observed seed and seedling trapping of almost 100 %. On account of these results, we decreased rhizome abundance and modified rhizome height to evaluate the trapping by matte at further stages of deterioration. Here we provide the first experimental evidence of the trapping variability of *P. oceanica* propagules by matte depending on its complexity, and our results highlight that rhizome height contributes more strongly to trapping than the actual shoot numbers, suggesting that even highly degraded meadows could allow for recovery if enough complexity is provided by matte. Indeed, matte is considered a favourable substratum for the establishment of *P. oceanica* seedlings, both naturally and for transplanting (Balestri et al. 1998, Piazzi et al. 1999, Terrados et al. 2013). In comparison to sand, coarse gravel was the substratum with the highest complexity, and almost all propagules were trapped independently of the flow velocity. While *P. oceanica* seedlings are able to establish in gravel, they do not appear to survive on this substrate in the long term, probably because of damage and abrasive action caused by the gravel particles (Piazzi et al. 1999). Similarly, *P. oceanica* seedlings are also able to establish in sand (Balestri & Lardicci 2008), but their survival over the long term is unclear (Infantes et al. 2011a). In consolidated substrata such as matte or rocks, higher survivorship of *P. oceanica* seedlings is often observed in nature (Balestri et al. 1998, Piazzi et al. 1999, Alagna et al. 2013, Terrados et al. 2013), which is in accordance with our results of higher trapping.

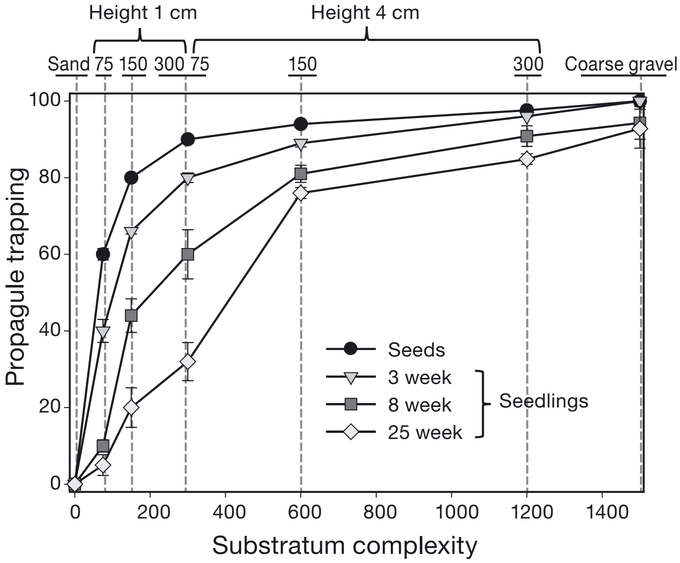

Fig. 9. Relation between trapping of *Posidonia oceanica* seeds and seedlings and substratum complexity in a 20 cm s^{-1} flow. Three rhizome abundances were used (75, 150 and 300 dead shoots m^{-2}, at heights of 1 and 4 cm). Matte complexity was calculated as the number of rhizomes m^{-2} × rhizome height (cm). For coarse gravel, complexity was calculated as the number of gravel pieces m^{-2} × gravel height. Complexity assigned to sand was 0

In addition to substratum type and its associated complexity, we also found that other factors are important in influencing the transport and trapping of seagrass propagules. A relevant finding was the positive correlation between flow reduction over complex substrata and propagule trapping. At high substratum complexity, propagule trapping is highly independent of its physical characteristics. Conversely, at lower substratum complexity, propagule trapping depends on the propagules' physical characteristics, because the effect of substratum on flow is weaker. Therefore, the drag coefficient is a strong predictor of trapping, particularly for substrata of low, but some, complexity (i.e. not sand).

After release by floating fruits, *P. oceanica* seeds sink and, depending on the hydrodynamic conditions and substratum type, are trapped or transported along the bottom until a microhabitat which facilitates their trapping is encountered. Given that seeds lack dormancy, if they cannot establish in the first few days after fruit dehiscence, trapping may be hampered because their development modifies their physical characteristics. Once retained in a safe recruitment microhabitat, the presence of adhesive root hairs (Badalamenti et al. 2015) reinforces establishment and facilitates persistence over consolidated substrates, explaining the prevalence in rocky substrata a few years after establishment (Alagna et al. 2013). In addition to the adhesive properties of root hairs, the complexity of the substratum facilitates seedling anchoring (Alagna et al. 2015). Additionally, our observations of seedling movement in the flume suggest that roots, which are somewhat rigid and show an apparent negative phototropism, might become anchoring structures that could facilitate propagule trapping and establishment. The primary root (first root produced at the distal end of the seed) frequently grows curved, resembling a hook (Fig. 1a) that could possibly enhance trapping. Indeed, anchor-like structures that are suggested to contribute to trapping have been described in propagules of *Phyllospadix* and *Amphibolis* spp. (Kuo & den Hartog 2006).

Our study provides important insights into the drivers of seagrass dispersal recruitment, which could be applicable for numerical hydrodynamic dispersal models and restoration efforts. The structure and function of seagrasses in many ecosystems is affected by anthropogenic and natural disturbances that have caused their strong decline worldwide (Waycott et al. 2009), prompting a renewed interest in seagrass restoration (Orth et al. 2006b, Ganassin & Gibbs 2008, Renton et al. 2011). Hydrodynamic exposure (Orth et al. 2006a, Bos & van Katwijk 2007, van Katwijk et al. 2009) and the availability of a suitable substratum (Infantes et al. 2011b, Rivers et al. 2011) frequently determine restoration success. More complex substrata such as coarse gravel areas or dense and high mattes of *Posidonia oceanica* may facilitate the retention and establishment of seagrass propagules, and therefore substratum complexity could be an additional criterion in the site selection process during restoration. Further data are needed to better understand the role played by rocky substrata.

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8. APPENDIX

1.1. Pereda-Briones L, Terrados J, Tomas F. 2019. Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae. *Marine Pollution Bulletin*, 141, 36-45.

8. APPENDIX

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Negative effects of warming on seagrass seedlings are not exacerbated by invasive algae

L. Pereda-Briones[a,*], J. Terrados[a], F. Tomas[a,b]

[a] Instituto Mediterráneo de Estudios Avanzados, IMEDEA (CSIC-UIB), Miquel Marques 21, 07190 Esporles, Illes Balears, Spain
[b] Department of Fisheries and Wildlife, Oregon State University, 104 Nash Hall, Corvallis, OR 97331, USA

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ABSTRACT

The observed and projected rise in sea surface temperature challenges marine biodiversity worldwide, and particularly in temperate ecosystems dealing with the arrival of novel species of tropical provenance. When the impacted biota are early life stages of ecosystem engineers, the effects of those impacts are of major concern for ecologists and coastal managers. We experimentally examined the individual and potential additive effects of seawater warming and the presence of the invasive algae on the development of seedlings of the seagrass *Posidonia oceanica* in a three-month mesocosm experiment. Whereas the presence of the invasive algae (*Caulerpa cylindracea* and *Lophocladia lallemandii*) did not result in detrimental effects on seedlings, warming negatively affected seedling development. Interestingly, the presence of both invasive algae may ameliorate the negative effects of warming.

1. Introduction

The gradual increase of global sea surface temperature (SST) observed since the 1950s represents a threat to marine ecosystems worldwide (IPPC, 2014). Global responses of marine biota already indicate that climate change can have a strong impact on marine life (Díaz-Almela et al., 2009; Lejeusne et al., 2010; Harley et al., 2012; Polozanska et al., 2013). The rise in SST is predicted to increase between 0.6 and 1.5 °C in the next 40 years (IPCC, 2014). This can be particularly relevant for temperate ecosystems dealing with novel species of tropical provenance, such as introduced species or species that have undergone a range expansion (Raitsos et al., 2010; Vergés et al., 2014). Furthermore, when the species impacted by warming are ecosystem engineers, such as corals, kelps or seagrasses, the consequences of those impacts may cascade throughout the ecosystems they form, significantly magnifying overall impacts (e.g. Hoegh-Guldberg et al., 2007; Wernberg et al., 2012). Certainly, the introduction of invasive species and global warming are emerging as main threats to seagrass ecosystems (e.g. Orth et al., 2006b; Williams, 2007; Koch et al., 2013; Collier and Waycott, 2014).

Sexual reproduction is crucial for the persistence of seagrass beds, allowing for the consolidation of existing meadows and also for the formation of new ones via dispersal (Jarvis et al., 2014; Olesen et al., 2004; Orth et al., 1994, 2006a). Moreover, seedling production represents a critical genetic diversity source that provides adaptive capacity to future environmental changes, essential for the long term persistence of the species (Jump et al., 2009; Kendrick et al., 2012, 2017). These early stages are considered the most critical phases in the life cycle of seagrasses, when plants experience the highest mortality rates (Orth et al., 2006a; Alagna et al., 2013). Indeed, the establishment rates of newly emerged seedlings are generally very low (Orth et al., 2006a), and are recognized as a major population bottleneck (Gómez-Aparicio, 2008). Given the ecological importance of seagrass seedlings, it is pressing to understand the environmental and biological factors which contribute to their successful establishment, survival and development.

The Mediterranean Sea is a hotspot of biodiversity (Myers et al., 2000). However, it exhibits rates of seawater warming that exceed threefold those of the global ocean (IPCC, 2007; Burrows et al., 2011). The increase in SST also stimulates the successful proliferation of alien species, particularly those from tropical and subtropical origin entering through the Suez Canal (Raitsos et al., 2010; Vergés et al., 2014). In fact, the Mediterranean Sea is one of the areas with more introductions of non-native species (e.g. Galil et al., 2014; Streftaris and Zenetos, 2006), some of which are considered as invasive and are affecting the integrity of natural communities, including loss of seagrass meadows (Vitousek et al., 1997; Williams and Smith, 2007; Thomsen et al., 2010).

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[*] Corresponding author.
E-mail address: lpereda@imedea.uib-csic.es (L. Pereda-Briones).

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The endemic seagrass *Posidonia oceanica* (L.) Delile is one of the most representative foundation species to inhabit littoral Mediterranean ecosystems due to its importance as a habitat former, and supporter of biodiversity (Duarte, 2000). It is a slow-growing species which forms extensive meadows, characterized by a very variable sexual reproduction in space and time (Green and Short, 2003; Díaz-Almela et al., 2009). These features contribute to a low capacity of resilience and adaptation to the rapid warming projected for the Mediterranean Sea (Raitsos et al., 2010; Unsworth et al., 2015). Negative effects (e.g. increased mortality) in response to increasing temperatures have been observed in *P. oceanica* adult plants (e.g. Díaz-Almela et al., 2009; Marbà and Duarte, 2010) and there are indications that seedlings of this species are also sensitive to warming (Guerrero-Meseguer et al., 2017; Hernan et al., 2017). Presumably, the presence of invasive algae could further enhance the negative effects of warming on *P. oceanica* seedlings, as this species can suffer mortality, biomass loss, or deterioration of its sediment quality deterioration when colonized by some invasive algae (Marbà et al., 2014; Ballesteros et al., 2007; Holmer et al., 2009). On the other hand, invasive seaweed could also have facilitative interactions under elevated temperatures (Harley et al., 2012). For example, shading by the invaders could soften the effects of temperature rise by reducing photochemical stress (Wernberg et al., 2010).

In the Mediterranean Sea, *Caulerpa cylindracea* and *Lophocladia lallemandii* are two of the most invasive macroalgae threatening *P. oceanica* meadows (Verlaque, 1994; Streftaris and Zenetos, 2006; Ballesteros et al., 2007; Klein and Verlaque, 2008). Both species are able to colonize *P. oceanica* meadows, and can be particularly abundant at the edge of the meadows, over matte, as well as in low density seagrass patches (Ballesteros et al., 2007; Marín-Guirao et al., 2015), which are often the same habitats where *P. oceanica* seedlings successfully settle (Balestri et al., 1998; Piazzi et al., 1999; Balestri and Lardicci, 2008; Alagna et al., 2013). Therefore, these invasive and native macrophytes are likely to interact during the seedling establishment phase. In addition, the proliferation of both invasive species may be enhanced by increases in growth rates under warming (Samperio-Ramos et al., 2015), possibly accelerating the invasion of native habitats. Due to the important ecosystem services offered by seagrasses, including sediment and nutrient trapping, carbon fixation, nursery areas, and high primary production (Costanza et al., 1997; Orth et al., 2006b; Pergent et al., 2014), examination of the potential interactive impacts of warming and biological invasions on seagrasses is of main relevance for conservation and management strategies. The aim of the present work was to experimentally evaluate the potential independent and cumulative impacts of warming and the presence of invasive algae (*C. cylindracea* or *L. lallemandii*) on the biological status (development, physiology and survivorship) of seedlings of the Mediterranean seagrass *P. oceanica*. We conducted a 3-month laboratory experiment where *P. oceanica* seedlings where exposed to different seawater temperatures and the presence of the invasive algae, in order to test the following hypotheses: (1) the increase in temperature will negatively affect *P. oceanica* development, promoting seedling death (2) the presence of the invasive algae reduces the vegetative development of *P. oceanica*, and (3) the interaction of seedlings with the invasive algae alters the effects of warming on *P. oceanica* seedlings.

2. Materials and methods

2.1. Seed and algae collection and maintenance

Seeds of *Posidonia oceanica* were obtained from fruits collected on the shore during June 2015 in Mallorca (Balearic Islands, Western Mediterranean Sea). Fruits were opened and their seed removed and placed in 20 L aquariums with seawater (salinity, 37), at a temperature of 20 °C and illuminated by white fluorescent lamps that provided 76 ± 2 $\mu mol \cdot m^{-2} s^{-1}$ of photosynthetically active radiation in a 14/10 h light/dark photoperiod. Seeds successfully germinate under these conditions and first leaves and roots are produced within two weeks (Terrados et al., 2013). These seedlings were maintained on a gravel substrate for the following four months under the described conditions with monthly replacement of seawater and weekly manual elimination of epiphytic algae growing on seedling leaves. Seedlings were examined for proper vegetative development (presence of seed, leaves and roots) and 800 seedlings with homogeneous morphological characteristics (in terms of seed area, number and size of leaves and roots; Tables S1 and S2) were chosen for the experiment. *C. cylindracea* and *L. lallemandii* were collected by scuba-diving during October 2015 in Mallorca and were maintained during 15 days, in separate tanks, in the same conditions as the seagrass seedlings. *C. cylindracea* rhizoids were buried in the gravel, allowing for the usual establishment pattern of this species, attaching to the substrate. *L. lallemandii*, which typically grows as an epiphyte on other macrophytes, was maintained freely on the bottom. Both seaweeds remained in perfect condition during the acclimation period.

2.2. Experimental design

A factorial experiment was designed considering two factors: temperature (see details below) and presence of invasive algae (with three levels: absence, presence of *C. cylindracea*, and presence of *L. lallemandii*). We also analyzed the effects of both invasive species occurring concurrently, but we were only able to examine this effect at 27 °C due to logistical constraints.

According to Sureda et al. (2008), the ratio of *P. oceanica* biomass (including leaves, sheaths and rhizomes) versus *L. lallemandii* biomass in an invaded area was 10.7 (in dry weight). We used this ratio to estimate the biomass of *L. lallemandii* needed in each aquarium (which contained 20 seedlings; i.e. 5.62 g *P. oceanica* DW), which was equivalent to ca. 5 g of wet weight (WW). For *C. cylindracea*, we used the biomass quantified by Holmer et al. (2009), and Pereda-Briones et al. (2017) in the field (i.e. 8 g *C. cylindracea* DW/m^2), which amounted to 7.5 g *C. cylindracea* WW in each aquarium. The biomass of both invasive algae was replaced by fresh material on a monthly basis to ensure the optimal condition of the invasive algae material.

We manipulated seawater temperature to reach three values: 25 °C as the control treatment [the summer mean SST in the Mediterranean Sea measured over the last 20 years (1982–2012); Shaltout and Omstedt, 2014]; 27 °C (temperature in the range of the mean summer SST forecasted for the end of this century; Shaltout and Omstedt, 2014); and 29 °C (temperature in the range of the mean summer SST forecasted for next century in the Mediterranean Sea; Shaltout and Omstedt, 2014).

Treatment temperatures were reached after an acclimation period in which temperature was increased at a rate of 1 °C per week, using the same methodology as Hernan et al. (2017). Seedlings and invasive algae were then exposed to the treatment temperatures for three months. Each treatment had 4 replicate 20 L aquaria each containing 20 randomly selected seedlings, commercial aquarium gravel as substratum, a heater, and a recirculating pump. Aquaria were filled with filtered seawater (10 μm plus UV filter), which was replaced monthly in concordance with the addition of new invasive algae biomass. Epiphytic algae different from *L. lallemandii* growing on seedling leaves were manually eliminated every week. Salinity and temperature were measured in alternating days, and maintained at 37 PSU and at 25, 27 and 29 °C respectively. Water samples of each aquarium were taken at the beginning, middle and end (coinciding with the water replacement events) of the experiment for nutrient content analysis.

2.3. Seedling survival and development

Survival of *P. oceanica* seedlings was calculated as the percentage of alive seedlings at the end of the experiment relative to the initial number of seedlings contained in each replicate aquarium. Seedling development was assessed by measuring the following morphological characteristics on ten seedlings per aquarium at the beginning and at the end of the experiment: leaf width of the second youngest leaf, maximum leaf length, total leaf area, number of leaves, percentage of necrotic surface on each leaf, total root number, total root length and number of new roots (which are characterized by white coloration and being flexible).

2.4. Seedling biomass and physiology

Biomass of *P. oceanica* seedlings was measured by drying the samples in an oven at 60 °C to constant weight for approximately 48 h. Then, three seedlings of each aquarium were randomly selected to analyze carbon and nitrogen contents in leaves, seeds, rhizomes, and roots, using a Carlo-Erba CNH elemental analyzer. Additionally, carbohydrates in seeds (sucrose and starch) were measured using the methodology described by Invers et al. (2004). Sucrose and starch content were obtained after three sequential extractions with 95% (v/v) ethanol at 80 °C for 15 min. The remaining pellet of seeds was dissolved in 0,1 N NaOH for 24 h at room temperature for starch extraction. Sugars and starch content of extracts were determined by spectrophotometry using an anthrone assay with sucrose as standard.

Rapid light curves (RLCs) were used to examine the effects of increased temperature and presence of invasive algae on seedling photophysiology. Fluorescence yield was measured in ten seedlings of each aquarium by pulse amplitude modulated (PAM) fluorometry, using an underwater device (Diving-PAM Walz GmbH, Germany). RLCs were generated automatically using an incremental sequence of actinic illumination periods, with light intensities increasing in 8 steps from 0 to 260 $\mu mol\ quanta\ m^{-2}s^{-1}$ PAR at intervals of 15 s between measurements. The fraction of photosynthetic photon flux (PPF) absorbed by the leaf, the absorptance factor (AF), was derived from measuring the incident irradiance from a halogen lamp before and after the Diving-PAM's sensor was covered with 1 to 4 layers of the seedling leaves, then AF was calculated according to the method described by Beer et al. (1998).

Fluorescence measurements were done by placing the tip of the instrument's main optical fiber ca. 1 cm away from the base of the leaf. Then, a saturating-light with a pre-set PPF was irradiated. This allows to obtain the effective quantum yield (Y) of photosynthetic electron transport through PSII ($Y = (F_{m'} - F)/F_{m'}$), were $F_{m'}$ is the maximal fluorescence of a light-adapted plant when all reaction centers are reduced, or closed, and F is the fluorescence in the light when part of the reaction centers are open. At that point, rates of electron transport (ETR) were calculated as: $ETR = Y \times incident\ PAR \times AF \times 0.5$ (assuming that half of the photons absorbed were absorbed by photosystem II; Schreiber et al., 1994). Photosynthetic quantum efficiency (α) was quantified as the initial slope of the linear portion of the RL curve. Saturating irradiance (E_k) was obtained by dividing the $\text{ETR}_{\max}$ by α.

2.5. Data analysis

Factorial ANOVAs (with two fixed and crossed factors; temperature and invasive algae) were used to test for differences in the initial morphological features of all the seedlings, and to compare final values of seedlings' morphological characteristics, carbon, nitrogen and carbohydrate content, as well as photophysiological variables. In addition, one-way ANOVAs were used to examine effects on seedling morphological traits between the four different levels of invasive algae presence (i.e. absence, *C. cylindracea*, *L. lallemandii*, and *C. cylindracea* + *L. lallemandii*) at 27 °C. Repeated measures ANOVAs were used to compare the concentration of dissolved inorganic nutrients present in the seawater of each aquarium at the beginning, in the middle and at the end of the experiment.

Table 1
Results of the Factorial ANOVAs analysing the morphological features of *Posidonia oceanica* seedlings in response to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects highlighted in bold.

Variable	Source	df	MS	F	p
Nª of leaves	Invasion	2	0,164	0,327	0,724
	Temperature	2	11,184	22,322	**< 0,01**
	Inv × T	4	1580	3154	**0,03**
	Error	27	0,501		
Width (cm)	Invasion	2	0,001	0,428	0,656
	Temperature	2	0,002	1257	0,301
	Inv × T	4	0,001	0,362	0,833
	Error	27	0,002		
Maximum leaf length (cm)	Invasion	2	6041	3848	**0,03**
	Temperature	2	4221	2688	0,086
	Inv × T	4	2483	1582	0,208
	Error	27	1570		
Total leaf area (cm^2)	Invasion	2	68,210	4788	**0,017**
	Temperature	2	468,830	32,912	**< 0,01**
	Inv × T	4	109,930	7717	**< 0,01**
	Error	27	14,250		
Necrotic leaf surface (cm^2)	Invasion	2	4145	1566	0,227
	Temperature	2	23,482	8873	**< 0,01**
	Inv × T	4	3452	1304	0,293
	Error	27	2646		
Seed area	Invasion	2	0,013	0,309	0,737
	Temperature	2	0,034	0,773	0,472
	Inv × T	4	0,022	0,494	0,740
	Error	27	0,044		
N° of roots	Invasion	2	31,001	0,971	0,391
	Temperature	2	29,659	0,929	0,407
	invasion × temperature	4	29,911	0,937	0,457
	Error	27	31,912		
Total root length (cm)	Invasion	2	35,940	2004	0,154
	Temperature	2	7990	0,446	0,645
	Inv × T	4	23,590	1315	0,289
	Error	27	17,930		
Maximum root length (cm)	Invasion	2	1192	0,946	0,401
	Temperature	2	1922	1526	0,236
	Inv × T	4	0,730	0,580	0,680
	Error	27	1259		
Nª new roots	Invasion	2	74,104	1226	0,309
	Temperature	2	51,780	0,856	0,436
	Inv × T	4	55,269	0,914	0,470
	Error	27	60,460		
Total new roots length	Invasion	2	9723	3696	**0,038**
	Temperature	2	26,928	10,235	**< 0,01**
	Inv × T	4	0,435	0,165	0,954
	Error	27	2631		
Maximum new root length (cm)	Invasion	2	3864	3480	**0,045**
	Temperature	2	8761	7890	**< 0,01**
	Inv × T	4	0,150	0,135	0,968
	Error	27	1110		

The superscripted letter correspond to "squared" (square centimeters)

Prior to statistical analyses, data were examined for normality and homogeneity of variances using Kolmogorov-Smirnov and Cochran's tests, respectively. Post-hoc Tukey test were conducted to examine differences between levels of each factor.

3. Results

All *Posidonia oceanica* seedlings survived for the entire duration of the experiment, but their development differed amongst treatments (Table 1). The total length of new roots decreased significantly with temperature, being significantly longer at 25 °C than the other temperatures (Fig. 1, Tukey). Similarly, the number of leaves and total leaf area decreased about 30% and 50% at 27 °C when seedlings were growing alone or with *C. cylindracea*, respectively. At 29 °C, the number

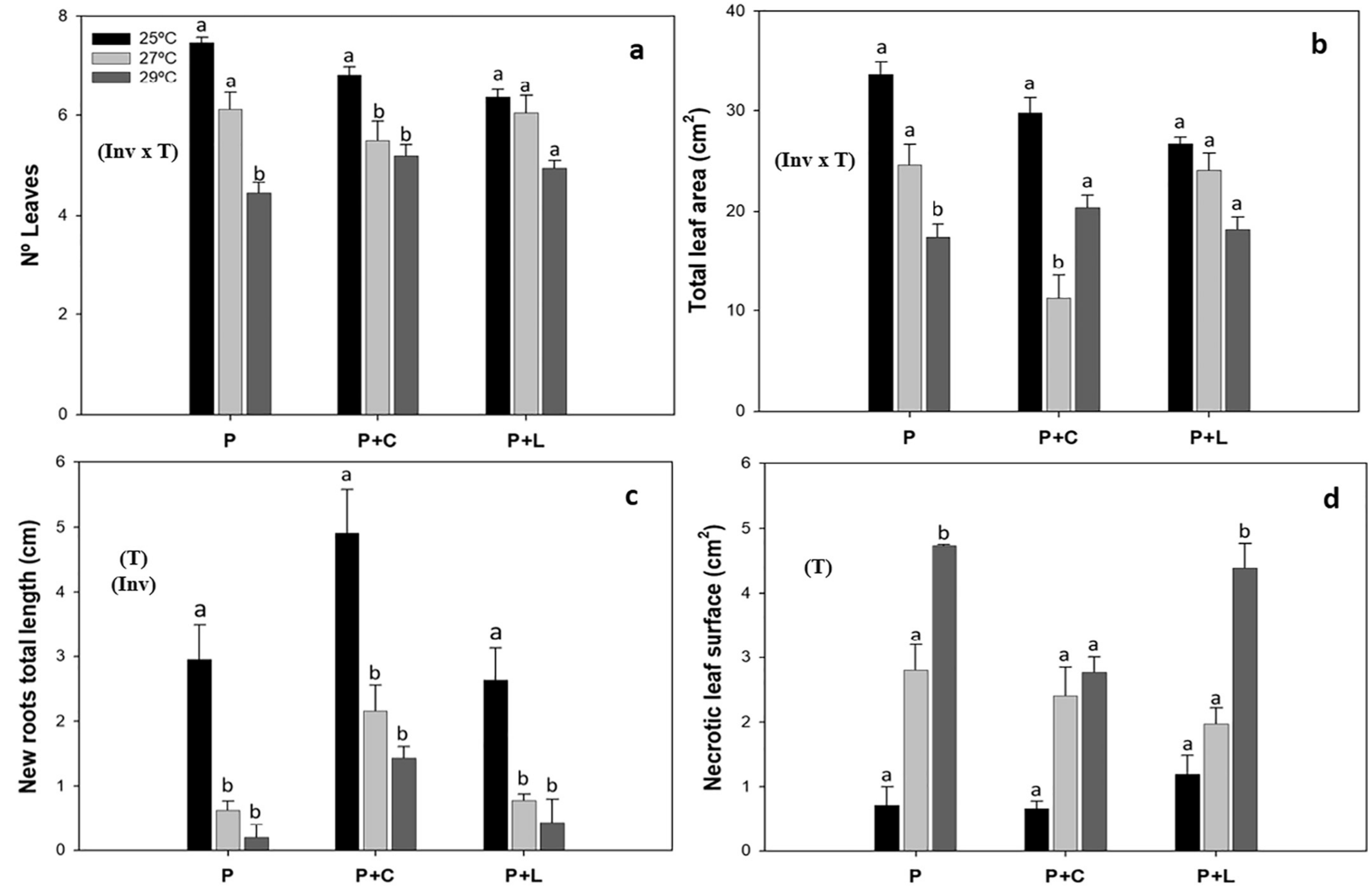

Fig. 1. *P. oceanica* seedling's number of leaves (a), total leaf surface (b), total length of new roots (c) and necrotic leaf surface (d) in the different experimental treatments: P (only *P. oceanica* seedlings), P + C (*P. oceanica* seedlings and *C. cylindracea*), P + L (*P. oceanica* seedlings + *L. lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

of leaves continued to decrease. In addition, total leaf area decreased at 29 °C but only when *P. oceanica* seedlings were growing alone, whereas seedlings co-occurring with *L. lallemandi* did not exhibit such decreases in either number of leaves or leaf area at 29 °C (Fig. 1, Tukey). On the other hand, the percentage of necrotic leaf surface tended to increase with temperature, being significantly higher at 29 °C for seedlings growing alone or with *L. lallemandii* (Fig. 1, Tukey).

Carbon content in seedling tissues was generally not affected by temperature nor the presence of invasive algae (Table 2), except for in the presence of *L. lallemandii*, whereby carbon in new roots was significantly lower than for seedlings growing alone or in the presence of *C. cylindracea*. Nitrogen content of leaves and new roots did not differ amongst treatments, whereas nitrogen content of old roots, rhizomes and seeds was generally higher at 29 °C than the other temperatures (Table 2; Fig. 2; Tukey). Regarding soluble carbohydrates in seeds, sucrose content was higher in the presence of *L. lallemandii* than in the other invasive treatments for all temperatures, and starch content was not affected by either treatment or their interaction (Table 3; Fig. 3, Tukey).

Whereas saturating irradiance did not differ amongst treatments (Table 4), seedling maximum electron transport rate (ETR_{max}) and photosynthetic efficiency (α) increased significantly at 27 °C, and decreased again at 29 °C ($29 = 25 < 27$; Table 4; Fig. 4, Tukey).

The presence of the two invasive species (tested only at 27 °C) had very few effects on the development of *P. oceanica* seedlings; with only total leaf area changing, which was lower in seedlings interacting with invasive species, due to the presence of *C. cylindracea*, in comparison to seedlings growing alone (Table S3; Tukey).

Water nutrient content changed through time (i.e. with each water change) but did not differ between treatments (Table S4). Ammonium concentrations decreased through time from 7.1 ± 0.41 to 2.9 ± 0.32 μM, whereas nitrate increased from 0.5 ± 0.02 to 3.5 ± 0.29 μM.

4. Discussion

Our results indicate that warming can negatively affect the seedlings of *Posidonia oceanica*, and that the presence of invasive seaweed can potentially alter some of these responses to warming. Increasing temperatures have affected not only the photophysiology of the plants, but also leaf development and the production of new roots. Interestingly, the presence of invasive algae does not necessarily result in negative effects on seedlings.

Our observations supported our first hypothesis (i.e. the increase in temperature will negatively affect *P. oceanica* development, promoting seedling death), as we detected seedling deterioration with increasing temperatures. Optimal growth temperatures for temperate seagrasses are reported to be between 11.5 and 26 °C (Lee et al., 2007), and once the threshold temperature is exceeded, seagrass performance typically declines (Nejrup and Pedersen, 2008). Critical temperature threshold for adult *P. oceanica* plants are considered to be between 29 °C and 30 °C (Marbà and Duarte, 2010; Olsen et al., 2012), and similar to those of seedlings (Guerrero-Meseguer et al., 2017; Hernan et al., 2017). Our four-month-old seedlings survived along the 90 days of the experiment in all the treatments, including those maintained at 29 °C, whereas previous studies with six-month old seedlings observed an increase of ca. 13% in mortality rates at this temperature (Hernan et al., 2017). Such contrasting responses may be related to carbohydrate reserves (i.e.

Table 2
Factorial ANOVAs analysing the differences in percentage of nitrogen in the *P. oceanica* seedlings tissue components: seeds, old roots, new roots, rhizome and leaves, according to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects are highlighted in bold.

Nutrient	Variable	Source	df	MS	F	p
Carbon	Seeds	Invasion	2	17,300	2908	0,071
		Temperature	2	0,790	0,134	0,875
		Inv × T	4	5380	0,904	0,475
		Error	27	5950		
	Old roots	Invasion	2	12,070	1647	0,211
		Temperature	2	18,880	2578	0,094
		Inv × T	4	12,880	1758	0,166
		Error	27	7330		
	New roots	Invasion	2	43,950	4557	**0,020**
		Temperature	2	1170	0,122	0,886
		Inv × T	4	3670	0,381	0,820
		Error	27	9640		
	Leaves	Invasion	2	1,29	2,44	0,106
		Temperature	2	0,54	1,02	0,373
		Inv × T	4	0,23	0,43	0,784
		Error	27	0,53		
	Rhizomes	Invasion	2	0,790	0,570	0,570
		Temperature	2	3620	2640	0,091
		Inv × T	4	1430	1051	0,404
		Error	27	1,37		
Nitrogen	Seeds	Invasion	2	0,200	3123	0,060
		Temperature	2	1089	17,002	< **0,01**
		Inv × T	4	0,135	2121	0,105
		Error	27	0,064		
	Old roots	Invasion	2	0,006	0,475	0,626
		Temperature	2	0,069	5515	< **0,01**
		Inv × T	4	0,005	0,421	0,792
		Error	27	0,012		
	New roots	Invasion	2	0,032	3035	0,066
		Temperature	2	0,006	0,600	0,556
		Inv × T	4	0,012	1162	0,350
		Error	27	0,010		
	Leaves	Invasion	2	0,003	0,151	0,860
		Temperature	2	0,012	0,663	0,523
		Inv × T	4	0,015	0,813	0,527
		Error	27	0,018		
	Rhizomes	Invasion	2	0,102	0,650	0,531
		Temperature	2	1086	6894	< **0,01**
		Inv × T	4	0,141	0,898	0,480
		Error	27	0,157		

starch and sucrose) of seeds, which were not affected by temperature in our experiment. Carbohydrates are crucial storage compounds of plants (Tetlow et al., 2004), which reflect the recent energetic balance of the plant, and their reduction can strongly influence plant survival (Genot et al., 1994) and make seagrass meadows more vulnerable to additional stress (Eklof et al., 2009). Presumably, our younger seedlings should have higher carbon reserves than older ones, which may have increased their resistance to warming. Consequently, we suggest that the lethal temperature threshold of young seedlings of *P. oceanica* is age-dependent based on carbon reserves available in the seed and would be above 29 °C for younger seedlings. The results of Guerrero-Meseguer et al. (2017), who worked with five-month old *P. oceanica* seedlings, support this notion since they did not observe mortality at 27 nor at 29 °C, whereas mortality peaked to 33% when temperatures went over 29 °C.

Even though our seedlings did not suffer mortality under warming, we did observe sublethal responses which may affect seedling survival success in the longer term. For example, during seedling development, *P. oceanica* seedlings typically undergo a decrease in the number of leaves formed, but an increase in total leaf area as these new leaves are larger (Terrados et al., 2013). In our experiment, seedlings experiencing warming underwent a stronger reduction in leaf formation and an overall decrease in total leaf surface. This response indicates that increasing temperatures reduce leaf formation rates, which has also been reported for adults (Mayot et al., 2005) and seedlings (Olsen et al., 2012; Guerrero-Meseguer et al., 2017; Hernan et al., 2017) of *P. oceanica* as well as for adult plants of other temperate (Nejrup and Pedersen, 2008) and tropical (Edwards, 1995) seagrasses. In addition, the proportion of necrotic leaf surface increased, indicating that warming creates stressful conditions for temperate seagrass seedlings (Niu et al., 2012; Guerrero-Meseguer et al., 2017; Hernan et al., 2017). Increased necrotic surface may be stimulated by a deterioration of the photosynthetic apparatus when temperature overpasses certain values. In fact, warming can lead to improved fitness and an increase in photosynthesis rate (with an estimated optimum temperature range for net photosynthesis of 16° to 35 °C in temperate seagrasses; Lee et al., 2007) until the thermal threshold of a species is exceeded and damage occurs (Pörtner and Farrel, 2008; Pedersen et al., 2016). We observed that maximum electron transport rate (ETR_{max}) and photosynthetic efficiency (α) of seedlings increased significantly at 27 °C, but decreased again at 29 °C, suggesting that the photosynthesis process suffers when temperatures are above 27 °C (see also Guerrero-Meseguer et al., 2017).

Regarding seedlings' nutrient content, we detected higher nitrogen at higher temperatures in seeds, old roots and rhizomes, but no differences in leaves or new roots. Presumably, as the number of leaves and leaf surface decrease with temperature, seedlings don't need to mobilize a larger fraction of their nitrogen reserves (stored in seeds, old roots and rhizomes) to produce leaf biomass, resulting in higher final nitrogen concentrations in these tissues. In contrast, new roots may have higher nitrogen requirements since they are an active growth tissue where the available resources are mobilized and used, and their development may be prioritized regardless of the environmental conditions.

Interestingly, concerning our second hypothesis (i.e. the presence of the invasive algae reduces seedlings' vegetative development), our results suggest the opposite. While it has been demonstrated that *C. cylindracea* and *L. lallemandii* can negatively affect adult plants of *P. oceanica*, decreasing shoot size and increasing leaf necrosis and shoot mortality (Holmer et al., 2009; Ballesteros et al., 2007; Marbà et al., 2014), effects on seedlings remain practically unknown. In fact, *C. cylindracea* can favor the production of new roots of *P. oceanica* seedlings, facilitating their early establishment (Pereda-Briones et al., 2017), and effects of *L. lallemandi* on *P. oceanica* seedlings have not been examined thus far. The lack of negative effects of invasive algae on the seedlings observed in our experiment and detected in the field (Pereda-Briones et al., 2017), in addition to the enhancement of root development in the presence of *C. cylindracea*, and the tendency of the presence of *L. lallemandii* to improve the negative effects of warming on seedling leaf development, all suggest that there may be positive rather than negative influences of invasive algae on seedlings during their recruitment phase under the tested conditions. Yet, and considering the cautionary principle, it is worth highlighting that, albeit not statistically significant, we did observe a trend towards lower leaf area in the presence of *L. lallemandii*. Perhaps other experimental conditions (e.g. duration of the experiment or biomass of *L. lallemandii*), would have yield more obvious negative effects.

Warming also led to a decrease of total new root length, which is likely to negatively affect the establishment capacity of seedlings if they do not reach the adequate root length for successful anchoring (Infantes et al., 2011). In addition to the length of the root system, the ability of the seedlings to remain anchored to the substratum is strongly dependent on the hydrodynamic conditions (Frederiksen et al., 2004; Infantes et al., 2009; Rivers et al., 2011, Pereda-Briones et al., 2018) and the substratum type (Balestri and Lardicci, 2008; Alagna et al., 2013; Pereda-Briones et al., 2018). In this sense, the presence of *C. cylindracea*, by favoring the production of new roots, could facilitate the establishment of seedlings, and thus, maximize the possibilities of remaining in the substratum facing waves and currents. Indeed, the survival and growth of one-year old *P. oceanica* seedlings transplanted to dead matte substrate are enhanced by the presence of *C. cylindracea* (Pereda-Briones et al., 2017). Furthermore, this facilitative interaction

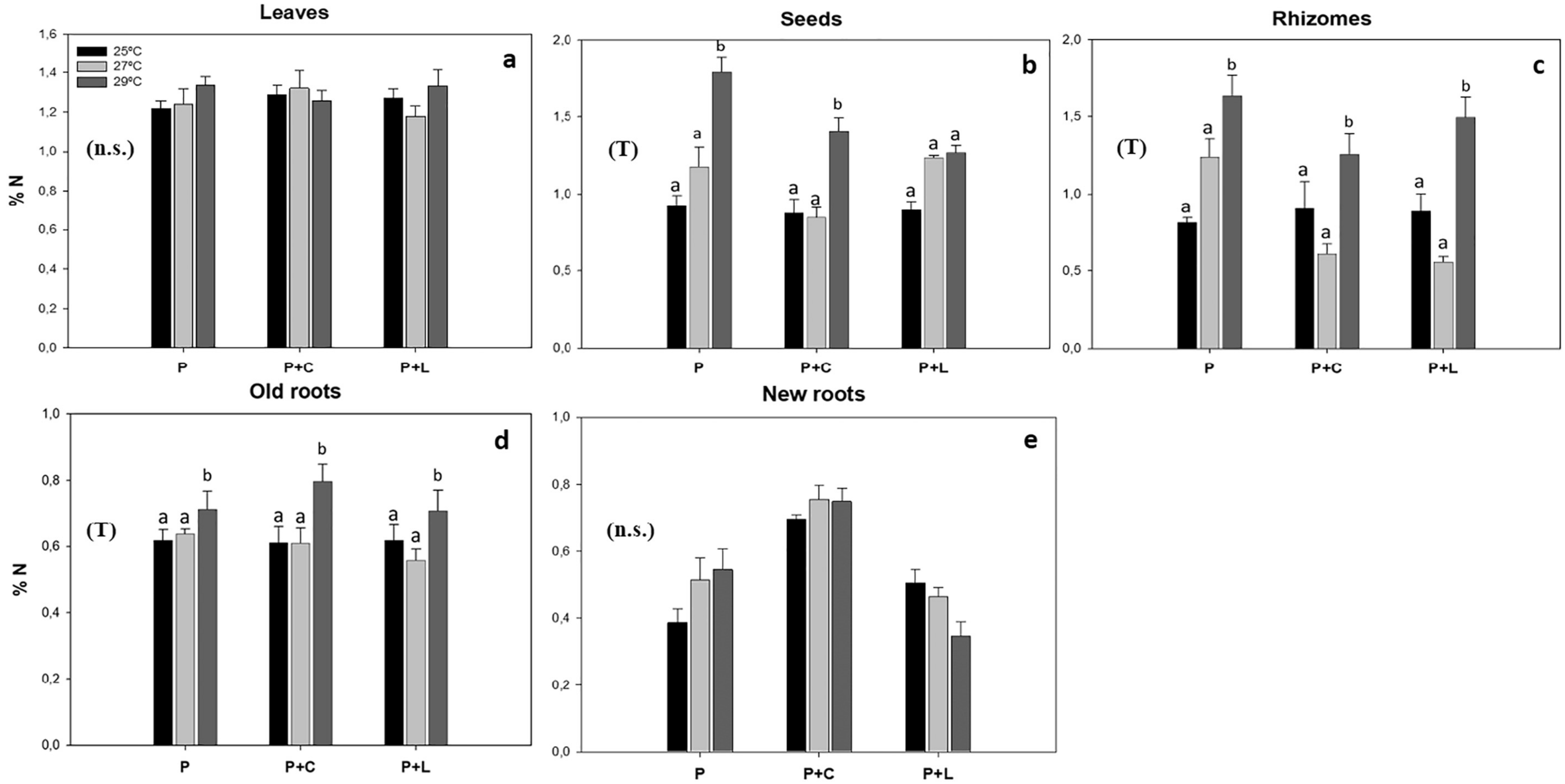

Fig. 2. *Posidonia oceanica* mean nitrogen concentration (% of DW) of seedling tissues: leaves (a), seeds (b), rhizomes (c), old roots (d) and new roots (e), across experimental treatments: P (only *P. oceanica* seedlings), P + C (*P. oceanica* seedlings and *C. cylindracea*), P + L (*P. oceanica* seedlings + *L. lallemandii*). Error bars represent +1 SE.
Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

Table 3
Factorial ANOVAs analysing the percentage of sucrose and starch in the seeds of *P. oceanica* seedlings according to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects highlighted in bold.

Variable	Source	df	MS	F	p
Sucrose	Invasion	2	72,920	6852	**< 0,01**
	Temperature	2	2437	0,229	0,797
	Inv × T	4	23,380	2197	0,112
	Error	17	10,640		
Starch	Invasion	2	2,37E + 09	1829	0,191
	Temperature	2	5,61E + 08	0,431	0,656
	Inv × T	4	5,94E + 08	0,457	0,765
	Error	17	1,29E + 09		

was not diminished under warming conditions, thus potentially counteracting negative warming effects by enabling the anchoring of the seedlings and the consequent nutrient uptake in adverse thermal scenarios.

Elevated temperatures can increase or decrease competition, and

Table 4
Factorial ANOVAs analysing the photophysiology parameters: ETR_{max}, alpha and E_k of *Posidonia oceanica* seedlings according to the presence of invasive algae (Inv), temperature (T) and their interaction. Significant effects highlighted in bold.

Variable	Source	df	MS	F	p
ETR max	Invasion	2	5197	0,268	0,765
	Temperature	2	169,055	8717	**< 0,01**
	Inv × T	4	9032	0,466	0,761
	Error	99	19,395		
Alpha	Invasion	2	0,003	0,469	0,627
	Temperature	2	0,076	13,928	**< 0,01**
	Inv × T	4	0,000	0,061	0,993
	Error	99	0,005		
Ek	Invasion	2	767,100	0,677	0,511
	Temperature	2	1009,900	0,891	0,414
	Inv × T	4	1002,900	0,885	0,476

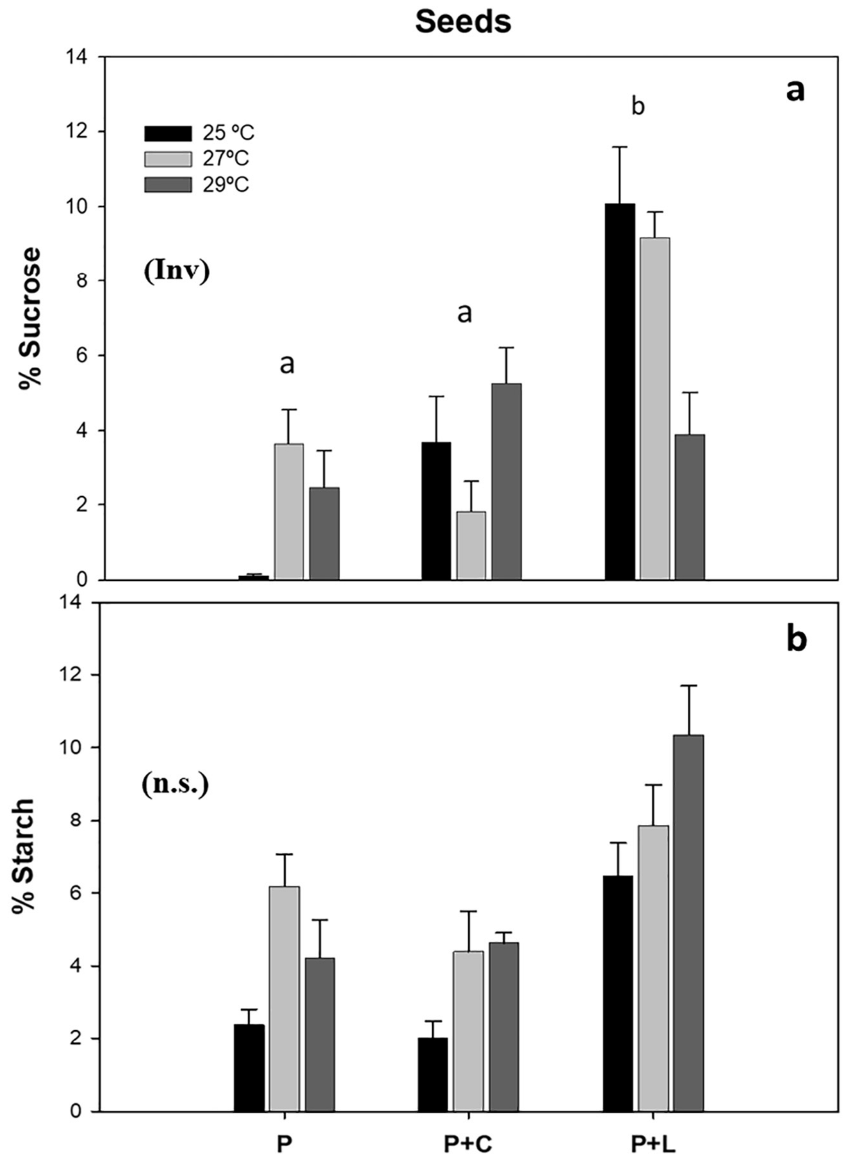

Fig. 3. *Posidonia oceanica* mean carbohydrate concentration (% of DW) of seedling seeds: sucrose (a) and starch (b), across experimental treatments: P (only *P. oceanica* seedlings), P + C (*P. oceanica* seedlings and *C. cylindracea*), P + L (*P. oceanica* seedlings + *L. lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

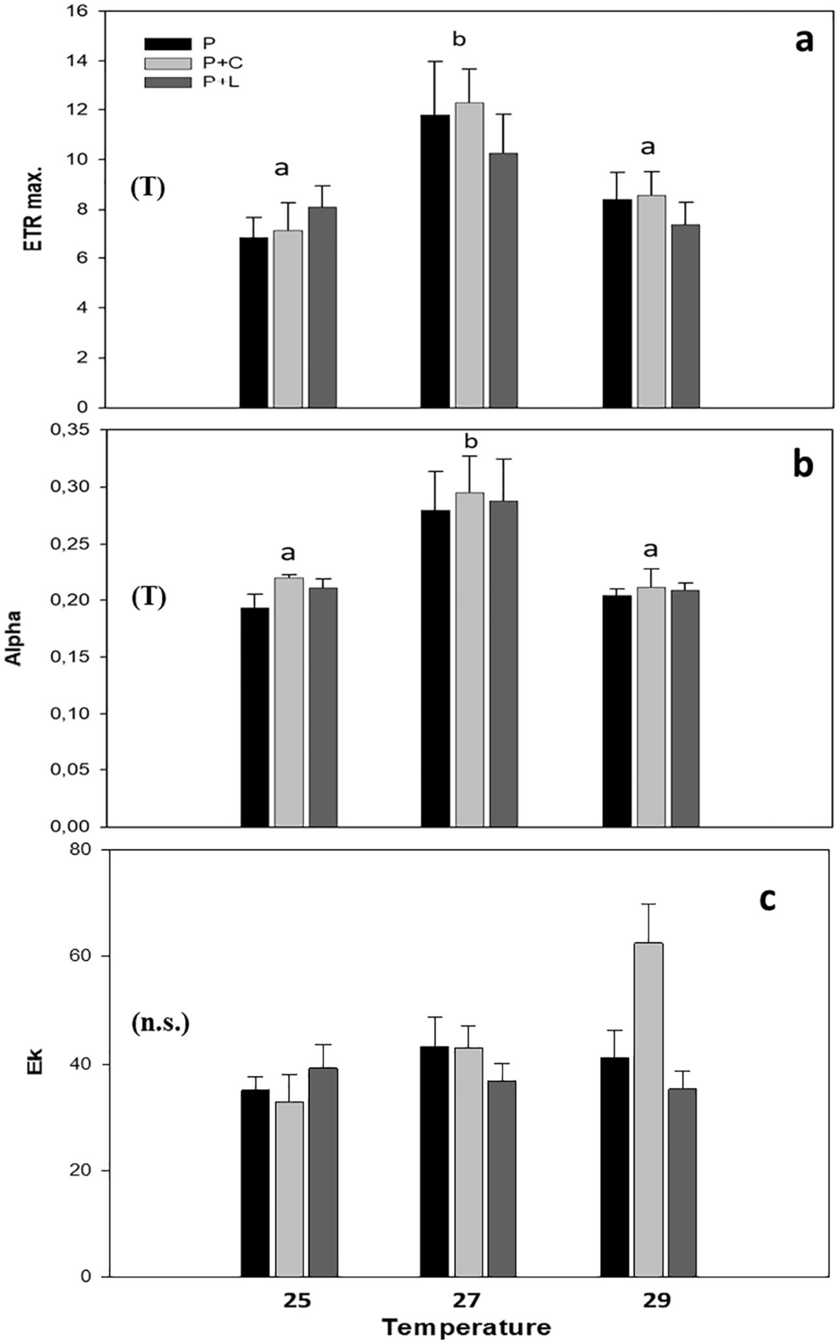

Fig. 4. *Posidonia oceanica* photophysiology parameters: ETR_{max} (a), alpha (b) and E_k (c) across experimental treatments: P (only *P. oceanica* seedlings), P + C (*P. oceanica* seedlings and *C. cylindracea*), P + L (*P. oceanica* seedlings + *L. lallemandii*). Error bars represent +1 SE. Significant results (T = temperature; Inv = invasive species; n.s. = non-significant) are shown in brackets and Tukey results are indicated with lowercase letters.

even transform competitive interactions into facilitative ones (Harley et al., 2012). In our case study, the effects of rising temperatures on *P. oceanica* seedlings have not been modified in the presence of invasive algae, rejecting our third hypothesis (i.e. the interaction of seedlings with the invasive algae alters the effects of the temperature on *P. oceanica* seedlings). Although the presence of *C. cylindracea* attenuated the decrease in the number of leaves and total leaf area of *P. oceanica* seedlings at 29 °C, and in the presence of *L. lallemandii* there were no temperature effects in these two variables, the general trend of decreasing foliar development with higher temperatures is maintained independently of the presence of invasive algae.

Warming is expected to continue in the coming years (IPPC, 2014), and this may also modify the response of both invasive algae, with further consequences for their interactions with *P. oceanica* seedlings. For instance, *L. lallemandii* may be more vulnerable to warming as it does not tolerate well temperatures above 29 °C, while *C. cylindracea* maintains high growth rates from 23 to 31 °C (Samperio-Ramos et al., 2015). On the other hand, global warming on marine ecosystems is accelerating the settlement of new alien species (Raitsos et al., 2010), and there are numerous examples of invasive species being more tolerant to higher temperatures than native ones (e.g. Smith et al., 2004; Sorte et al., 2010). This is particularly relevant for the Mediterranean Sea, where the predicted warming exceeds threefold that of the global ocean (Burrows et al., 2011), further encouraging the proliferation of invasive species from tropical and subtropical origin entering through the Suez Canal (Bianchi, 2007). Therefore, even though *P. oceanica* seedlings appear to be resistant in their interaction with *C. cylindracea* and *L. lallemandii* (at least in the conditions of our study), since they are sensitive to warming (Guerrero-Meseguer et al., 2017; Hernan et al., 2017; this study), they may not cope well with these or other invasive species if global warming trends continue.

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Contributors

L.P., F.T. and J.T. conceived and conducted the experiment. L.P. conducted plant analyses. F.T., and J.T. contributed with reagents/material/laboratory equipment. L.P., F.T. and J.T prepared and reviewed the manuscript and have approved the final article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.01.049.

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8.6. Declaration according to the University of the Balearic Islands

Universitat de les
Illes Balears

Dr Fiona Tomàs Nash, and Dr. Jorge Terrados Muñoz of the Mediterranean Institute for advanced studies (IMEDEA-CSIC-UIB)

WE DECLARE:

That the thesis titles *Seagrass ecology: environmental conditions and processes that affect the establishment and fate of seedlings,* presented by Laura Pereda Briones to obtain a doctoral degree, has been completed under our supervision and meets the requirements to opt for an International Doctorate.

For all intents and purposes, we hereby sign this document.

Signature

Dr. Fiona Tomàs Nash

Dr. Jorge Terrados Muñoz

Esporles, 8th April, 2019

8. APPENDIX

Muchas gracias a todas las personas
que me han ayudado a llegar hasta aquí.