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Effects of disturbance and stress on the reproductive modes of marine plants

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"We make our world significant by the courage of our questions and the depth of our answers."

Carl Sagan in Cosmos

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Abstract

Seagrass populations are declining worldwide due to human activities and rapid climate changes. Marine plants achieve population growth by a combination of sexual and clonal reproduction, and their relative contribution to population life history is poorly studied. Seagrasses are limited by light at the lower depth limit of distribution and by hydrodynamic action at the upper limit. Literature suggests a differentiated demography and genetic structure along the depth gradient, from higher contribution of sexual reproduction at shallower depths to a progressively larger contribution of clonal propagation deeper. In the first chapter we found that along a depth gradient more *Cymodocea nodosa* seedlings where lost at shallow depths than at deeper levels. The hypothesis of a differentiated reproductive balance along the depth gradient was tested in the second chapter, by analysing the demography and genetic structure of seagrass meadows along depth gradients. The results showed a heterogeneous pattern of genotypic richness and clonal structure, likely influenced by local acclimation and stochasticity. In the third chapter, the re-colonization strategies after perturbation along a *C. nodosa* meadow were investigated. Initially recolonization was mainly clonal, but after winter the study areas had loss biomass and only few shoots remained. Though the areas did not fully recover, genotypic richness increased along time in the perturbed areas, demonstrating the relevance of sexual reproduction at any depth level. In the fourth chapter, a *Zostera marina* meadow that disappeared after extreme perturbation was studied. Previous to its disappearance, the meadow was known to be genetically rich. Sexual reproduction, via seed bank, played a crucial role in its recovery. In a later stage, clonal growth was essential for colonization and spread. In the fifth chapter, seagrass transplants initiated via clonal propagation were tested in an area where 10 ha of seagrass were loss due to continued impacts. The result was the selection of the most successful species, donor population and time of the year to attempt future transplants. Furthermore, it is likely that larger initial transplant areas are able to surpass the required threshold to achieve vegetated stability, allowing for long term persistence. The challenges of seagrass restoration highlight the urgent need to protect seagrass meadows worldwide.

Key words: Seagrass; Recruit survival; Depth gradient; Life-history traits; Recover from disturbance; Marine Habitat Restoration.

Resumo

As plantas marinhas são importantes ecossistemas costeiros com elevados índices de biodiversidade, são a base de muitas atividades económicas como a pesca e o turismo, contribuindo para a proteção da erosão costeira e no sequestro de carbono atmosférico. No entanto este ecossistema está ameaçado em todo o mundo devido a atividades humanas e rápidas alteração climáticas. As populações de plantas marinhas colonizam o espaço através da combinação entre reprodução sexual e clonal. A importância relativa da reprodução sexual e clonal na história de vida das populações de plantas marinhas foi pouco estudada, no entanto sabe-se que existem muitos fatores que têm um papel importante na alocação sexual ou clonal das populações como o stress induzido pela poluição, eventos climatéricos extremos que reduzem a biomassa das pradarias, impactos regulares como a herbívora e o hidrodinamismo; e stress como a limitação de luz ou falta de nutrientes. É importante compreender a importância dos modos de reprodução das plantas marinhas na resposta a eventos de perturbação e stress para planear medidas de conservação e proteção futuras. A distribuição vertical das plantas marinhas é limitada pela falta de luz em profundidade, impossibilitando a fotossíntese, e no limite superior pelo hidrodinamismo que remove mecanicamente as plantas. Assim, pradarias que colonizem perfis verticais são excelentes casos de estudo para investigar o equilíbrio entre os dois modos reprodutivos. Existem evidências na literatura que sugerem a existência de diferenciação entre a estrutura genética e demográfica ao longo de perfis ambientais, como a profundidade e poluição. No caso de perfis de profundidade a teoria aponta para uma maior alocação sexual perto do limite superior e menos alocação sexual no limite inferior. No primeiro capitulo desta tese foi estudada a sobrevivência de recrutas da planta marinha *Cymodocea nodosa* ao longo da distribuição vertical da pradaria. Foi descoberto que no limite superior de distribuição os recrutas perdem-se devido ao forte hidrodinamismo, enquanto que no limite inferior há menos percas. Sugerindo que ainda que exista uma maior alocação sexual no limite superior, como indicado na literatura, o contributo para a diversidade genética pode não ser tão elevado como o esperado no limite superior; e que em profundidade é provável que ainda que com menor alocação sexual o contributo para a diversidade genética seja superior ao que era até aqui sugerido. No segundo capitulo foi feito um estudo pormenorizado da demografia e genética ao longo do perfil de distribuição vertical para a mesma espécie. Os

resultados mostraram um padrão demográfico e genético heterogéneo, suportando a hipótese levantada no capítulo anterior. Este estudo revelou que esta espécie tem uma grande capacidade de adaptação a condições locais e o padrão demográfico e genético encontrado é o reflexo da adaptação local e de fatores estocásticos. Numa perspetiva de conservação é interessante saber se as plantas marinhas respondem de forma sistemática a perturbações capazes de remover áreas significativas de biomassa. Assim no terceiro capítulo foi efetuada uma experiência manipulativa em que foi seguido o processo de recuperação natural das áreas removidas ao longo de um perfil de profundidade. Numa primeira fase a recuperação foi feita através de crescimento clonal, sem existir um padrão de profundidade. No entanto, devido a impactos sucessivos durante períodos de agitação marítima intensa o processo de recolonização foi revertido ficando limitado a poucas plantas. Nenhuma área recuperou completamente, no entanto a diversidade genotípica aumentou ao longo do tempo, o que quer dizer que a lenta recolonização está a ser feita em grande parte através de reprodução sexual. No quarto capítulo segui a recuperação natural de uma pradaria de *Zostera marina* que foi extremamente impactada por eventos climatéricos extremos, levando ao seu desaparecimento repentino. Antes do seu desaparecimento a pradaria foi alvo de um estudo genético que revelou uma diversidade genética elevada e inesperada para uma população no limite da distribuição. A elevada diversidade genética é a função de reprodução sexual frequente na população. Numa primeira fase a reprodução sexual foi fundamental para a recuperação desta pradaria, uma vez que a área foi repovoada por recrutas provenientes de um banco de sementes. Numa segunda fase a propagação clonal foi essencial para a colonização da restante área disponível. Este estudo demostrou que populações com elevada diversidade genética têm hipótese de sobrevivência quando afetadas por fenómenos extremos. Os habitats subtidais podem ser afetados de forma significativa por eventos de perturbação naturais ou artificiais de forma irreversíveis, resultando na perca da estabilidade do ecossistema. Caso as populações não sejam suficientemente resilientes ou se os impactos forem continuados a recuperação de habitat não é possível. Caso os fatores que levaram ao desaparecimento das populações sejam eliminados é possível iniciar um processo de recuperação de habitat. No caso das plantas marinhas esse processo pode ser feito através de transplantes onde a propagação clonal é favorecida e o processo de ocupação de espaço acelerado, numa tentativa de reverter a estabilidade do sistema sem vegetação para um sistema com

vegetação. No capitulo cinco foram testadas várias combinações de transplante numa zona que outrora albergava 10 ha de plantas marinhas. Como resultado deste estudo sabemos qual a espécie, de que população dadora e a ser transplantada em que altura do ano tem mais probabilidades de sucesso. Esta informação foi fundamental para fazer um teste piloto com o tamanho das áreas iniciais de transplante. Este estudo não foi conclusivo por falta de replicados, mas deu origem à hipótese de que quanto maior for o transplante inicial, mais provável será o seu sucesso e resiliência. Esta tese provou a relevância da reprodução sexual e clonal nas plantas marinhas, em pradarias naturais o balanço entre os dois modos de reprodução permite que as plantas sobrevivam e prosperem em condições locais distintas, originando um padrão heterogéneo que é o reflexo das diferentes pressões ao longo da história da pradaria. Ficou mais uma vez provado que a diversidade genética e a reprodução sexual são fundamentais para a recuperação natural das populações afetadas por fenómenos de perturbação extremos. Por fim, é importante referir que existe um ponto de estabilidade do ecossistema, que assim que é ultrapassado o sistema deixa de estar estável e a sua recuperação é muito pouco provável. Para recuperar a estabilidade de um ecossistema é fundamental que as ameaças que levaram ao seu desaparecimento sejam eliminadas. É urgente que as pradarias marinhas sejam alvo de proteção uma vez que depois de atravessado um determinado ponto de instabilidade o processo de recuperação de resiliência é extremamente difícil.

Palavras-chave: Plantas marinhas; Sobrevivência dos recrutas; Gradiente de profundidade; Estratégias de vida; Recuperação de impacto; Restauração de habitats marinhos.

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Introduction

Seagrasses comprise all the marine flowering plants, the small number (only 13 genera) of plants that colonized completely marine environments, living on shallow shores and estuarine environments of every sea in the world except Antarctica (Green and Short 2003). Seagrasses evolved from freshwater plants 100 million years ago (den Hartog 1970, Les et al. 1997), in at least 3 independent events, resulting into six families *Zosteraceae, Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, Ruppiaceae* and *Zannichelliaceae* (Short et al. 2011). However, the definition of seagrasses (as fully marine) does not always include the latter two families, resulting in only four families in some literature (Les et al. 1997, Larkum et al. 2007, Nguyen et al. 2015) (Fig.1). The processes involved in adaptation from terrestrial plants to living in the sea include loss of genes for stomata, volatiles and defence, and increase in genes involved in salt tolerance, light harvesting and cell wall gas exchange (Olsen et al. 2016) (Fig. 2).

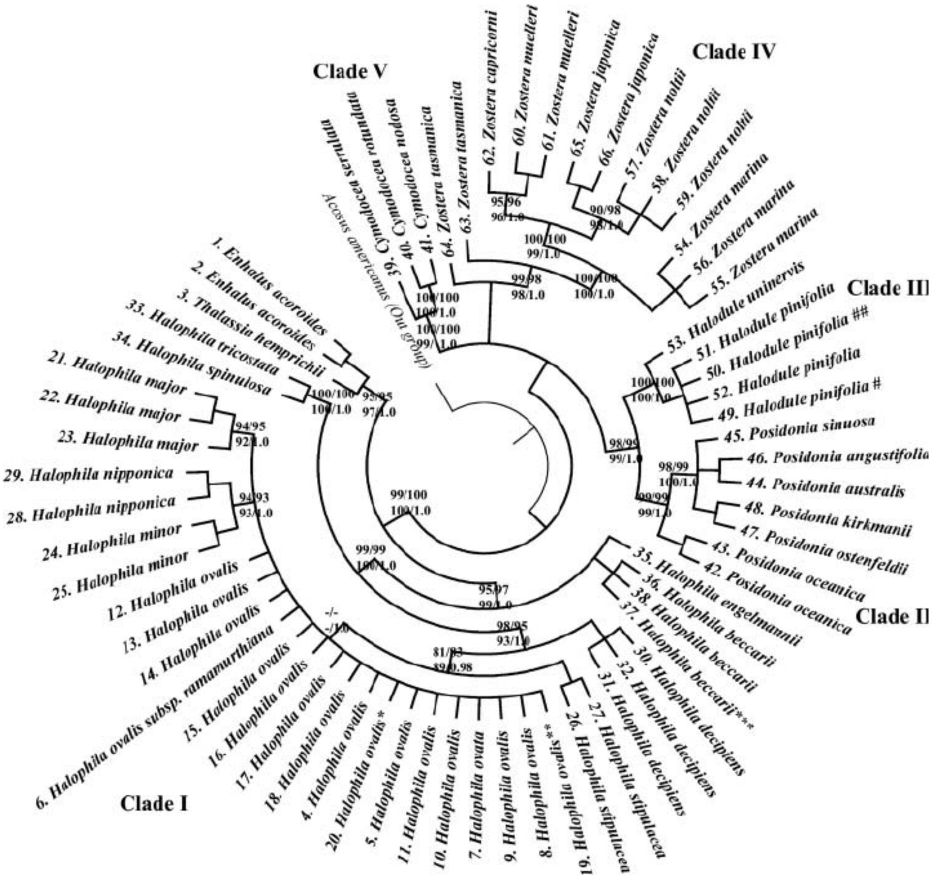

Fig 1: Seagrass phylogeny inferred by Nguyen et al. 2015.

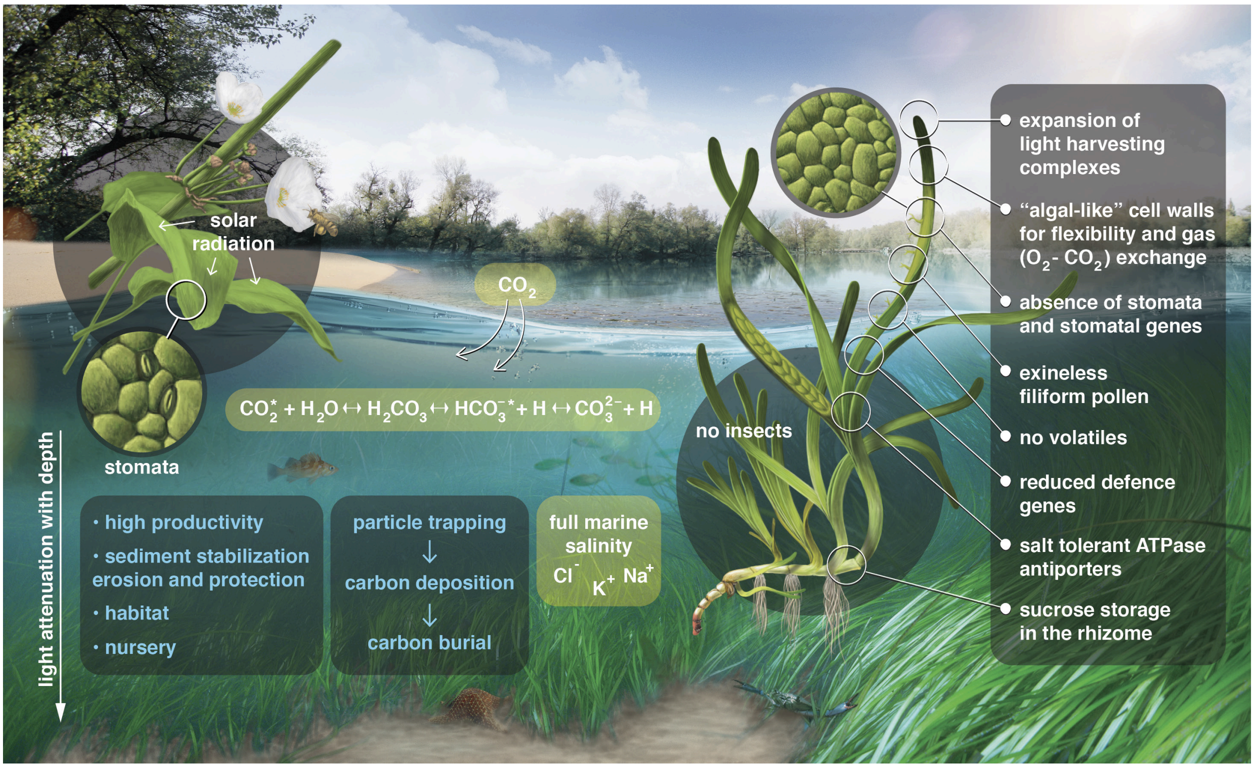

Figure 2: Structural and physiological adaptations to life in the sea by seagrasses (Olsen et al. 2016). In this example *Zostera marina*. In blue: Ecosystem services; In the green boxes: Physical processes related to CO2, light and salinity; In the dark green boxes: Physiological and morphological process which lead to gene losses and gains.

Seagrass meadows form important coastal ecosystems due to their high productivity and biodiversity, sediment stabilization capacity and water flow reduction (Duffy 2006, Larkum et al. 2007). Seagrasses have great socio-economic importance providing shoreline protection, carbon sequestration, fishing grounds and tourism opportunities (Orth et al. 2006, Barbier et al. 2011, Fourqurean et al. 2012, Christianen et al. 2013, Maxwell et al. 2017). These properties make seagrass meadows one of the most valuable ecosystems in the world (Costanza et al. 1997). The important ecosystem functions that seagrasses provide are however under threat, as seagrass populations are declining worldwide due to multiple causes related to human activities. These range from global effects like climate change, to more local effects such as eutrophication reducing water quality; and physical impacts such as dredging, boat propellers and storm events (Hughes et al. 2009, Waycott et al. 2009, Carey et al. 2002, Duarte et al. 2004).

Seagrasses occur mainly in tropical waters, where their species diversity is highest, but some species can occur in temperate and even polar waters (Fig 3). Seagrass depth distribution is limited by light, ranging from 1 m in murky waters such as coastal lagoons to 50 m in clear water areas such as some Mediterranean locations (Duarte 1991). The upper limit of the distribution is controlled by desiccation stress for intertidal species, and hydrodynamic disturbance for subtidal species (Bjork et al. 1999), although in high latitudes sea ice can also limit their vertical distribution.

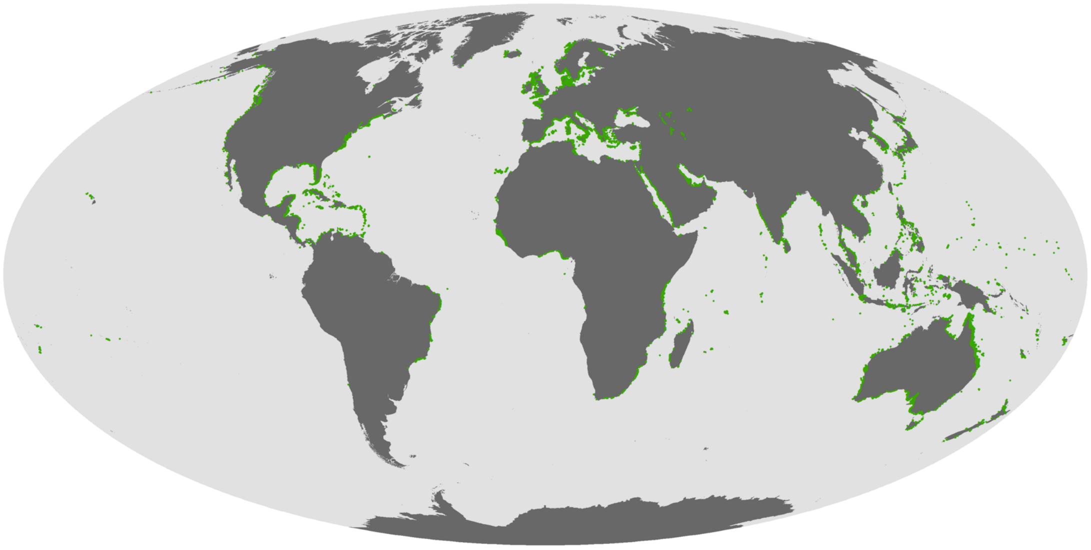

Figure 3: World map of seagrass distributions represented in green (from 2016 UNEP-WCMC).

Seagrass reproduction mechanisms

All seagrass species achieve population growth by a combination of sexual reproduction and clonal growth (Ackerman et al. 2006). They spread from established areas by means of horizontal elongation of rhizomes and by dispersion of seeds (i.e., Duarte & Sand-Jensen 1990) (Fig. 4). Vegetative proliferation, clonal growth, is the critical reproductive mode for seagrass meadows to spread and persist (Marbà et al. 2004). Rhizomes grow under the sediment and connect shoots. Due to horizontal growth many shoots in the same meadow are part of a clone (Hemminga et al. 2000).

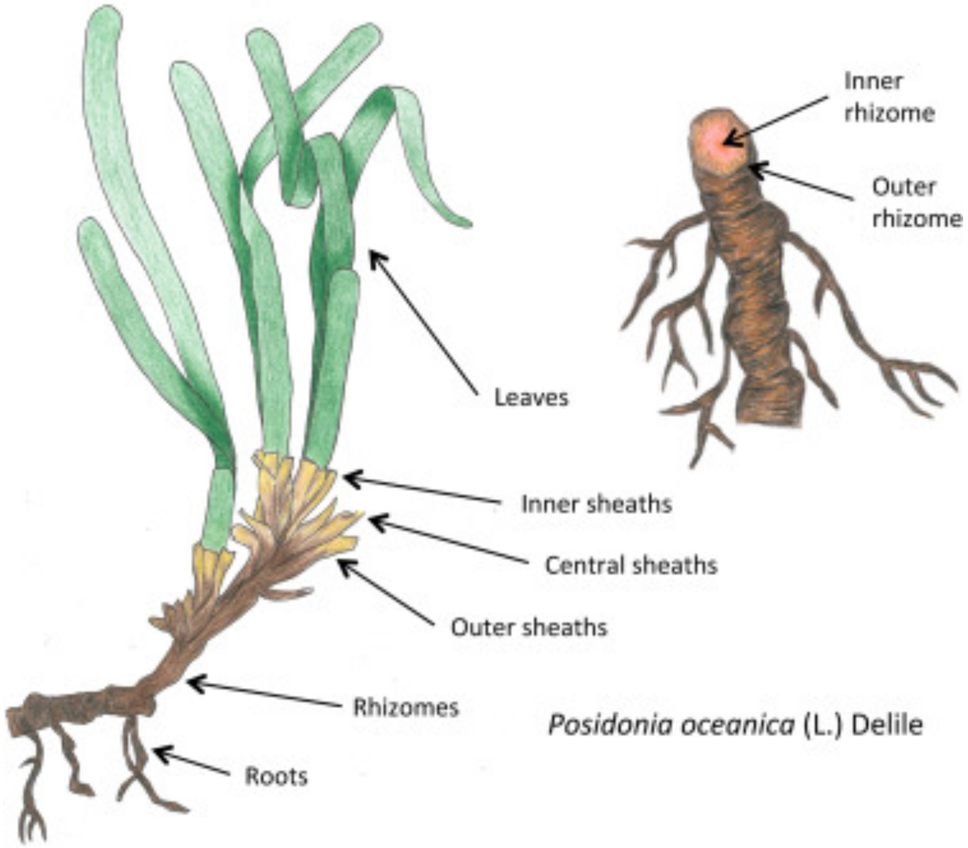

Figure 4: General structure of seagrasses, depicting the ramet unit containing roots, rhizome and leaves (Kaal et al. 2016).

Marine plant clones can grow for many years, even millennia (Arnaud-Haond et al. 2012). Seagrass sexual reproduction is usually water mediated, hydrophily (Faegri & van der Pijl 2013), in all species but one, *Enhalus accoroides* (Ackerman 2007), and fertilization occurs when the male flower releases pollen from the stamens into the water and it reaches the stigma of a female flower. Recently it was described that *Thalasssia testudium*, a common Caribbean species, can be pollinized not only by water but also by small invertebrates, zoophilous pollination (Van Tussenbroek et al. 2016), suggesting that maybe mixed pollination (abiotic-biotic) occurs in other seagrass species.

Most seagrasses are dioecious, with separated male and female plants (Waycott & Les 1996). Some species are monoecious, with male and female flowers in the same plant, and are self-compatible, allowing self-pollination which can reduce genetic variation. To minimize the chances of self-pollination most monoecious plants (i.e., *Zostera marina*) produce male and female flowers at different times (Hammerli & Reusch 2003).

Both reproduction modes have important contribution on seagrasses establishment and colonization. While clonal growth reduces the cost of sexual reproduction (Price & Marshall 1999), sexual reproduction increases population genetic diversity, but dilutes female genetic contribution to the next generation by half (Maynard 1978, Crowley & McLetchie 2002). High levels of genetic diversity enhance population resilience and increase species ability to adapt to unfavourable conditions (Balloux et al. 2003, Reusch 2006). The balance between the two reproductive modes has effects in demography (Eriksson 1989), dispersal (Winkler & Fischer 2001) and population structure (McLellan et al. 1997).

Environmental conditions may affect the related contribution of each of the reproductive modes (Eckert 2002), which have been found to be reflected in genotypic richness gradients (Gonzales et al. 2008). Genotypic richness measures the proportion of different genetic individuals in a clonal population and it integrates the recent generations history balance between sexual and asexual allocation. There are few studies on the allocation to both modes of reproduction (Piquot et al. 1998, Prati & Schmid 2000, Pluess & Stocklin 2005), and the response strategies to disturbance are poorly studied (Waycott et al. 2007).

Studied species

In this thesis, I studied the reproductive modes of two seagrass species and their relative contribution to population growth after disturbance and along environmental gradients.

Cymodocea nodosa (Ucria) Ascherson is a dioecious species with seed basicarpy (Bornet 1864). This species occurs in the Atlantic Ocean from central Portugal to Mauritania, including Madeira and Canary Archipelagos and spreads throughout the Mediterranean Sea (Mascaro et al. 2009, Oliva et al. 2012). The species includes very divergent genetic groups (Alberto et al. 2006, 2008) in the easternmost Mediterranean and the southernmost Atlantic (i.e., Canary Islands and adjacent African coastline) ranges, which correspond to ancient long-term persistence zones (Chefaoui & Serrao 2017), and a contact zone in the Atlantic-Mediterranean transition zone (Alberto et al. 2008, Masucci et al. 2012). This plant is of great importance acting as “habitat engineer” (Reyes et al. 1995, Tuya et al. 2014a, b) forming extensive and fragmented meadows from 1 m to 35 m depth depending on water clarity (Reyes et al. 1995, Barberà et al. 2005, Tuya et al. 2013). Sand movement can stimulate *C. nodosa* flowering rate, producing seed banks which can germinate and recolonize the impacted areas when conditions are more suitable (Marbà & Duarte 1995). In the Mediterranean and Canary Islands, it has a seasonal pattern of high biomass and shoot density during summer (Terrados & Ros 1993, Rismondo et al. 1997, Reyes et al. 1995, Tuya et al. 2006, Tuya et al. 2013). Although its worldwide status is non-threatened (Short et al. 2011) in some locations such as in the Canary Islands it is declining due to increasing human pressures (Zarranz et al. 2010, Fabbri et al. 2015).

Zostera marina is a monoecious species. It is the most widely distributed seagrass in the North hemisphere, occurring all around the globe (den Hartog 1970), with several distinct phylogenetic lineages (Olsen et al. 2004). This species forms perennial and annual populations (Bos et al. 2007). In annual populations, at some time during the year, all vegetative shoots turn into flowering shoots, originating seed banks that can germinate when conditions become favourable (Keddy & Patriquin 1978, van Lent and Verschuure 1994, Meling-López & Ibarra-Obando 1999, Santamaría-Gallegos et al. 2000, Jarvis et al. 2014). Perennial population colonize areas by a balance of clonal growth and sexual reproduction. It is thus expected that perennial populations exhibit lower genotypic diversity then the annual ones (Olesen & Sand-Jensen 1994, Olesen 1999, Kim et al. 2008). Along its southernmost European range in Iberia, populations are very genetically distinct, locally adapted (Billingham 2003, 2007) and highly clonal. Although, as a whole this region contains similar total genetic diversity as the central European region (Diekmann & Serrao 2012).

Seagrass reproductive strategies as a response to disturbance and stress

In population ecology, disturbance is an interference to the community defined as the total or partial loss of biomass. It deviates populations from a steady state. It will open space which can be recolonized either by the same or different species (Grime 1979). Disturbance can be regular and predictable or irregular and unpredictable. When disturbance is predictable, for example grazing fish over seagrass meadows, it is considered to be an impact. When disturbance is unpredictable, such as a hurricane, it is considered to be a perturbation. Stress is an interference to the community that does not deviate populations from a steady state. It limits or decreases growth rate, as it can happen in reduced light conditions due to water quality reduction or pollution.

Seagrass meadows are present in highly variable environments (Green & Short 2003). When light conditions are adequate, some seagrass species, such as *C. nodosa*, can colonize and grow from 1 m to 35 m depth forming continuous meadows (Reyes et al. 1995). Along such depth gradient, environmental conditions change drastically in a small spatial scale. While shallow subtidal *C. nodosa* is exposed to high light intensity and hydrodynamics, deeper meadows experience light limitation but are less impacted by hydrodynamic disturbance.

Depth gradients constitute a perfect setting to study the balance between sexual and asexual reproduction in clonal organisms such as seagrasses. Few studies have characterized reproduction mode variation in seagrasses along depth gradients (Olesen et al. 2004). Demographic pattern observed at different depths are expected to co-vary with environmental variability along the depth gradient (Olesen et al. 2002, Duarte 1991).

According to Grime (1977) plant life strategies are expected to change due to different levels of stress and disturbance. When in high stress and high disturbance environments plant persistence and survival is unlikely. In conditions of low stress and high disturbance sexual allocation is favoured; in low stress and low disturbance environments a balance between sexual modes is reached; in low disturbance and high stress clonal growth is favoured.

Accordingly, in the upper depth limit of subtidal seagrass meadows where hydrodynamic disturbance opens space periodically, higher sexual allocation and dispersal rates are expected. Here the genotypic richness pattern should be characterized by many small clones. While at the deeper colonization limits, due to light limitation, shoot longevity decreases presenting a risk for patch mortality (Olesen et al. 2002). As sexual reproduction requires more energy, it is expected that space colonization will depend more on clonal growth spread with depth. This should result in a genotypic richness composed of few large clones. Finally, at intermediate depths a balance between sexual modes and the presence of fewer and larger clones is expected.

Impact and perturbation effects in seagrass life-history

Perennial seagrass populations are expected to have low sexual allocation when compared to annual populations. But contrarily to perennial populations, annual population long term persistence depends on sexual reproduction to produce seed banks. Seed banks are necessary for meadows recovery when conditions become favourable. Nevertheless, multiple studies have reported high levels of sexual allocation in perennial *Z. marina* populations (van Lent & Verschuure 1994, Meling-López and Ibarra-Obando 1999, Olesen 1999, Santamaría-Gallegos et al. 2000). Those unexpected situations are likely linked to disturbance events (Cabaço & Santos 2012, Qin et al. 2014), which may not be sufficient to qualify the environment as unsuitable but are responsible for significant biomass losses. Therefore, different levels of disturbance can result in different proportions of clonal and sexual reproduction contribution to the recovery of physically impacted meadows.

It is crucial to unveil the importance of each reproductive mode used by seagrasses in the recovery process from physical impact. Researching the importance of sexual and clonal reproduction along environmental disturbance gradients, such as depth, can provide precious information on seagrass meadows recovery time from impact at various depths.

Shifting stable states

Seagrass ecosystems exist in given locations due to a combination of physical, chemical and biological factors (Maxwell et al. 2015). When all conditions are stable, seagrass meadows can persist for long periods of time (*sensu* Holling 1973). When key components are disturbed beyond a threshold the ecosystem stable state is disrupted, resulting in a shift from vegetated to un-vegetated seafloor (Fig. 5).

Seagrass restoration represents an attempt to shift an ecological state, from un-vegetated to vegetated. Although seagrass restoration has been conducted for nearly 70 years (e.g. Addy 1947), the majority of the projects have been conducted in sheltered waters. Restoration attempts in wave exposed coasts are few (Bull et al. 2004, van Katwijk et al. 2009, Fonseca et al. 1998), as it poses great logistical and environmental challenges (Paling et al. 2003). Furthermore, high wave energy, whether stochastic or periodic, can result in meadow erosion and/or plant burial which can quickly shift the ecosystem state back to un-vegetated (Fonseca & Bell 1998, Turner et al. 1999, Bryars 2008). Factors governing seagrass stable states are poorly understood as few studies have linked disturbance regimes with seagrass meadow response on a landscape scale (Duarte & Sand-Jensen 1990, Kendrick et al. 1999, Kirkman & Kuo 1990, Fonseca and Bell 1998, Turner et al. 1999, Maxwell et al. 2015).

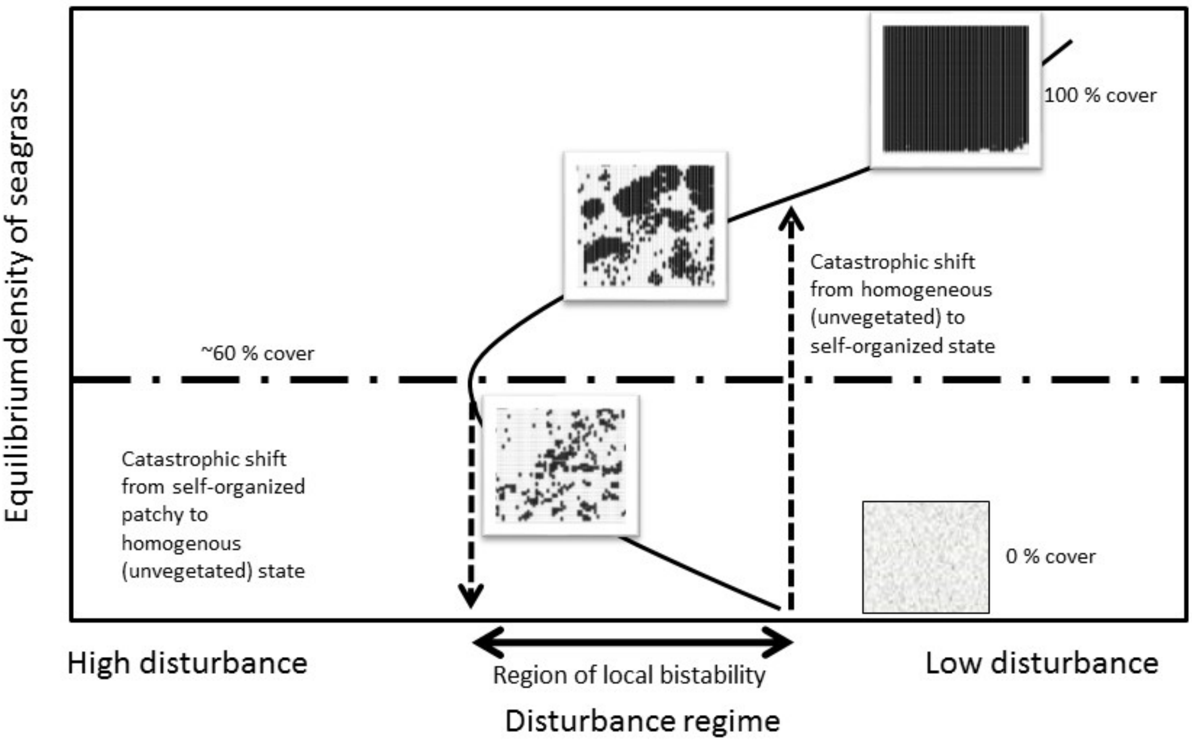

Figure 5: Schematic showing the relationship of a seagrass restoration project to ecosystem stability. Adapted from Rietkerk et al. (2004) by Dr. Mark Fonseca. The horizontal dashed line refers to the level where a theorized threshold in coverage may occur (Fonseca and Bell 1998).

Thesis goals

The main objectives of this thesis are to bring knowledge on how does depth, as an environmental gradient, affects the sexual and clonal reproduction balance of *C. nodosa* meadows; to determine the contribution of clonal and sexual reproduction in *Z. marina* and *C. nodosa* meadows recovery from disturbance; and to determine the factors that allow to shift ecosystem stable state thru clonal propagation in open ocean seagrass restoration actions.

Specifically, this thesis aimed to study:

- Seedling survival of *C. nodosa* along a depth gradient (Chapter I)

- Genotypic richness and demography patterns of *C. nodosa* along a depth gradient (Chapter II)

- Recolonization strategies of *C. nodosa* after disturbance along a depth gradient (Chapter III)

- The importance of clonal and sexual reproduction to the recovery of a *Z. marina* meadow after extreme disturbance (Chapter IV)

- Infer the most successful seagrass transplants conditions in an exposed costal area, initiated with clonal growth propagation (Chapter V).

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Chapter I: Recruit survival of *Cymodocea nodosa* along a depth gradient

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Abstract

Clonal plants can reproduce sexually and clonally, expressing different proportions of these different life histories in distinctive environments. In seagrass meadows, light attenuation hinders marine plant colonization in deeper areas of its vertical distribution. Therefore it is expected that the fluorescence maximum quantum yield of seedlings will be higher at deep limits of the meadow relative to shallower depths. We hypothesized that seagrass seedlings experience higher mortality in deeper areas of the meadows than at shallower levels. Our objective was to test survival of seagrass seedlings along a depth gradient in meadows of *Cymodocea nodosa* in the Canary Islands. Seedlings germinated in laboratory conditions, were transplanted to a natural meadow at three water depths: shallow (5 m), medium (8 m) and deep (12 m). Transplanted seedlings were monitored by measuring maximum quantum yield, leaf length, number of leaves per shoot and survival. Our data suggest that light does not hinder survival of seedlings along the depth gradient of the meadow. Before establishing clonal growth, seedlings in deeper areas had higher survival rates than those in shallow areas. Even though seeds are present at shallow depths and sexual allocation is high, sexual contribution can be lower than expected due to seedling loss.

Key words

Recruit survival; seagrasses; *Cymodocea nodosa*; depth gradient

Introduction

Clonal plants have the capacity to reproduce both sexually and clonally, expressing different proportions of these life histories in distinctive environments. Clonal angiosperms are able to occur in areas subject to frequent disturbance such as seashores, rivers and wastelands, where non-clonal plants are less common (Fahrig et al. 1994). If clonal propagation and sexual reproduction contribute differently to population recovery from disturbance, then environmental gradients of disturbance are expected to result in different proportions of the contribution of sexual versus clonal propagation. In the marine realm, sharp disturbance gradients occur across the photic zone, from high irradiance and wave exposure near the surface, to low irradiance and less hydrodynamic disturbance at the depth limit. However, few studies have focused on the role of clonal versus sexual contribution of sessile marine benthos (Olesen et al. 2004) along vertical distribution patterns.

Cymodocea nodosa (Ucria) Ascher, a dioecious marine angiosperm, can propagate both clonally and sexually. Male and female flowers have a clumped spatial distribution and reduced seed dispersal (Alberto et al. 2005). This species occurs in the Mediterranean and in the eastern Atlantic from Portugal to Senegal (Masucci et al. 2012). In the Canary Islands *C. nodosa* has been declining dramatically in the past two decades (Fabbri et al. 2015), due to several threats such as competition with the green algae *Caulerpa prolifera*, urban wastewater outfalls, sediment run-off and coastal infrastructures construction (Tuya et al. 2014).

Clonal growth of *C. nodosa* lateral rhizomes from established patches maintains the existing population and increases dispersal at a local scale (Duarte & Sand-Jensen 1990, Marbà & Duarte 1995). The vertical distribution of *C. nodosa* occurs along an environmental gradient of hydrodynamic disturbance in the upper limits and light limitation at the deeper limits (Duarte 1991b). In the Canary Islands, extensive monospecific meadows of *C. nodosa* can be found ranging from the intertidal to 35 m depth, with flowers and seeds occurring from 3 to 10 m (Reyes et al. 1995), producing seed banks in some localities throughout the year (Caye & Meinesz 1985, Caye et al*.* 1992, Reyes et al*.* 1995). However, seed recruitment in *C. nodosa* may be low (Caye & Meinesz 1986, Buia & Mazzella 1991, Reyes et al.1995).

In the Canary Archipelago, authors have described seeds and flowers only at the upper limit of distribution (Zarranz et al*.* 2010, Reyes et al*.* 1995), suggesting that sexual contribution at depth might be lower. Nonetheless, we have observed seedlings present along the entire depth gradient of meadows, indicating that *C. nodosa* meadows benefit from sexual contribution along its entire depth extent.

Yet, survival of recruits along the vertical profile of the meadow remains unknown. To test the recruit survival of *C. nodosa* along a depth gradient we transplanted seedlings germinated in laboratory conditions to different depths in a *C. nodosa* meadow. We monitored seedling survival, leaf length, number of leaves per shoot and maximum quantum yield as an indicator of post-transplant physiological condition.

Methods

Sampling area and seed collection

The study was conducted at Juan Grande bay, off the southeastern coast of Gran Canaria (27845945.480 N; 15833904.010 W, Spain), where a monospecific *C. nodosa* meadow extends from 3 to 15 m of depth. A search for seeds was done by SCUBA diving along the entire meadow to start our laboratory seed germination and seedling cultures. However, because very few seeds were found at depths greater than 5 m, we collected 1000 seeds from the shallow margin of the meadow, at an average depth of 4 m.

Seed germination

We followed seed germination and cultivation in laboratory conditions as described in Zarranz et al. (2010). Seeds were cleaned from epiphytes with a toothbrush and washed for 10 min with 10% commercial bleach diluted in autoclaved seawater. After this step, seeds were washed three times with autoclaved seawater and were individually placed in prepared culture vessels with PES solution (Provasoli 1968), substituting ammonium for nitrate and mono-potassium phosphate for glycerol phosphate (García-Jiménez et al. 2006, Zarranz et al. 2010). Autoclaved sand was used as a substrate. Culture vessels (MagentaR – G7 300 ml Sigma Co., Chicago, IL, USA) were prepared with 200 ml of PES culture medium and 40 cc of substrate. The culture medium salinity was 18 psu in order to induce germination (Caye & Meinesz 1986, Caye et al. 1992). Culture vessels were maintained in a growth chamber at Las Palmas University, under 30 µmol photons m^{-2} s^{-1} irradiance, 20 ºC and a photoperiod of 16 h light: 8 h dark. After thirty days, the culture medium was renewed and salinity was increased to 36 psu. After thirty days at the new salinity conditions, 180 seedlings developed new leaves and sufficient roots to be transferred to the field.

Seedlings were transplanted to the Juan Grande meadow (Fig. 1) at three depth levels: Shallow (5 m), Medium (8 m) and Deep (12 m), covering the depth gradient present in the meadow. At each depth level, three stations were created with 20 transplanted seedlings each, distant five meters from each other.

Field measurements

Survival of the seedlings, measured as the proportion of surviving seedlings, was monitored at all stations four times, at day 3, 41, 90 and 339 after the transplant. The longest leaf per shoot was measured at the same time as the survival of seedlings with the exception of day 3.

Photosynthetic efficiency, assessed by chlorophyll fluorescence parameters, measures plant instant physiological performance and sensibility to stress (Hanelt 1992, Beer & Björk 2000. Although photosynthetic efficiency alone does not ensure long term plant survival, it can be considered an indication of plant recovery from adverse conditions (Malta et al. 2006). To measure the fluorescence of chlorophyll *a* and to calculate the maximum quantum yield of photosynthesis, an underwater pulse amplitude modulate fluorometer (Diving PAM, Walz) was used. Photosynthetically active radiation (PAR) was measured at mid day, with the Diving PAM sensor over the seedlings transplant sites. Dark leaf clips were attached at 0.5 cm above the base of the second youngest leaf of all plants, to maintain a constant distance of the PAM sensor to the plant and to be able to dark acclimate the tissue. To oxidate the electron transport chain a far-red weak pulse was applied for five seconds (Hanelt 1998) after which the shutters of the dark leaf clips were closed and plants acclimated for 10 min. Basal fluorescence (F0) was obtained by turning on the PAM measuring light and opening the shutter. To measure maximum fluorescence (Fm), a saturating light pulse (≈ 5000 µmol photons m^{-2} s^{-1}) was applied for 0.6 seconds. After this step, variable fluorescence (Fv = Fm – F0) and maximum quantum yield (Fv/Fm) were calculated.

For all seedlings the maximum quantum yield was measured in the laboratory right before the transplant. In all stations, Fv/Fm was measured on a random sample of 10 seedlings and of 10 adult plants immediately after the transplant, 2 days and 40 days after the transplant.

Statistics

Differences in seedling survival between depth levels along time were tested with a General Linear Mixed Model (GLMM) using the function glmmPQL in package MASS from R (R core team 2014). This model used binomial error terms and depths analysed within time levels. We set two planned orthogonal contrasts to test differences between 1) the shallow level against the deeper levels and 2) between medium and deep levels.

Differences in leaf length and number of leaves per shoot, between depth levels and measurement dates were tested using a linear mixed model using function lme in package nlme from R (R core team 2014), with error terms set to depth levels measurements within dates. We set two planned orthogonal contrasts to test differences between 1) the shallow level against the deeper levels and 2) between medium and deep levels. For this analysis we excluded the last date, because there were no seedlings present in the shallow level.

The maximum quantum yield (Fv/Fm) was compared using a factorial design with plant stage (adult or seedling) and depth (shallow, medium, deep) nested with time (three measurement dates: transplant day, 2 and 40 days after transplant). The model was analysed with the lme function in package nlme from R (R core team 2014). We set two planned orthogonal contrasts to test differences between 1) the shallow level against the deeper levels and 2) between medium and deep levels.

A one-way ANOVA was used to test for differences in PAR light along the depth gradient (shallow, medium, deep). A Tukey's honest significant test was performed, as a post-hoc analysis, to identify differences between depth levels shallow and medium, shallow and deep, medium and deep.

Results

Depth affected seedling survival with the shallow level having higher mortality rate than deep and medium levels pooled together (contrast 1: $t = -4.06$; $df = 30$; $P < 0.001$). There were no fixed effect differences in survival between medium and deep levels (contrast 2 : $t = 1.59$; $df = 30$; $P = 0.122$) (Fig. 2). The transplanted seedlings were subject to increased mortality in all depth levels through time. The effect time, modelled as a random intercept, is expressed by the range of the 95 % confidence interval around the predicted survival probability for each depth level. The lower, mean and upper 95 % confidence interval for seedling survival probability were 0.011 (lower), 0.199 (mean) and 0.852 (upper), for the shallow level; 0.119 (lower), 0.756 (mean) and 0.986 (upper), for the medium level; and 0.057 (lower), 0.581 (mean) and 0.970 (upper), for the deep level. These confidence intervals are very wide demonstrating a strong effect of time on seedling survival.

Leaf length did not change between the shallow and the medium and deep levels pooled together (contrast 1 : $t = 1.55$; $df = 4$; $P = 0.196$) nor between the medium and deep levels (contrast 2 : $t = 0.23$; $df = 4$; $P = 0.833$). All depth levels followed the same trend, with decreasing leaf length in the first 41 days, increasing at 90 days and maintaining size until the last monitoring (339 days) (Fig. 3). The mean leaf lengths across all times were 10.64, 10.76 and 9.92 cm for shallow, medium and deep levels, respectively. The confidence intervals of these means were 9.33 cm wide, revealing an expected effect of time since transplant on leaf length (random intercept in the model). At the end of the monitoring time (339 days), the leaf length of the transplanted plants was 11.6 ± 4.3 cm (mean ± SE), less than half of the height of the native meadow, which averaged 27.4 ± 0.8 cm (mean ± SE).

The number of leaves per shoot did not change between all depth levels (contrast 1 : t = 0.27; df = 4; P = 0.802; contrast 2 : t = 0.27; df = 4; P = 0.797). All depth levels followed the same trend, maintaining an average of 3.9 ± 0.1 leaves per shoot along the experiment (Fig. 4).

There was a significant difference in PAR between depth levels (F (2, 51) = 364.9; P < 0.001) (Fig. 5). Tukey's multiple comparison post-hoc test showed significant differences between shallow and deep level (P < 0.001), shallow and medium level (P < 0.001) and medium and deep level (P < 0.001).

There were no differences in maximum quantum yield (Fv/Fm) between the shallow and the pooled medium and deep depth levels (contrast 1: t = -074; df = 4; P = 0.502) nor between medium and deep level (contrast 2 : t = -0.005; P = 0.996). Adult and seedling Fv/Fm was significantly different (t = -6.19; df = 8; P < 0.001). The interaction between depth and plant stage was not significant and was removed from the final model (full model AIC : 2073.6 and reduced model AIC : 2063.5, analysis of deviance between the two models P = 0.922). The Fv/Fm of seedlings did not change with depth but changed along time. From the transplant until day 2 the Fv/Fm of seedlings was lower when compared to native plants and after 40 days it increased, reaching the values of the native adult plants at all depth levels (Fig. 6).

Discussion

Our results showed that survival of seagrass seedlings was not reduced with increasing depth in meadows of *C. nodosa*. Seedlings were capable of surviving at all depth levels within a meadow and had rapid light adaptation at all depth levels (40 days). Light attenuation has been shown to hinder plant colonization in the deeper areas of the vertical distribution (Duarte 1991). It was thus expected that seedlings in deeper areas experience higher photosynthetic efficiency when compared to seedlings in shallower depths, as described by Silva et al. (2013) in a light deprivation study and by Olesen et al. (2002) along a depth gradient. Our results in this case study show that light attenuation at increasing depths was not a limiting factor for seedling survival or for growth, since the number of leaves per shoot was constant along the time. At the deepest station (15 m) PAR was higher than 150 µmol photons m-2 s-1, which is above *C. nodosa* compensation irradiance and saturation irradiance (Olesen et al. 2002, Olivé et al. 2013, Silva et al. 2013). The green algae *Caulerpa prolifera* was found at our study site at depths deeper than 15 m forming extensive meadows, in an identical pattern to El Medano meadows in Tenerife Island (Reyes et al. 1995) were *C. nodosa* meadow limit was at 20 m, being thereafter substituted by *C. prolifera*. It as been indicated that the regression of *C. nodosa* and the expansion of *Caulerpa* can be based on competition for nutrients in the sediment (Ceccherelli & Cinelli, 1997). Therefore the deeper limits are likely to be limited by other factors than light.

The fluorescence maximum quantum yield of seedlings equalled that of local control plants at all depth levels, suggesting that light conditions are suitable for the survival of transplanted seedlings. In contrast, the steady decrease of transplanted plant numbers at all depths and the lack of increase in leaf length, suggests that despite suitable light conditions, other factors influence plant survival and shoot length to the size of local control plants. Physical disturbance after transplant can be limiting for recruit survival, as supported by Marbà et al. (2004), which reported that 50 to 70 % of seedlings die before clonal growth begins.

Clonal growth and patch integration were described to be important factors for plant survival (McRoy & Lloyd 1981, Olesen & Sand-Jensen 1994). Zarranz et al. (2010) described vegetative clonal growth in *C. nodosa* after 30 days of transplant with the development of plagiotrophic rhizomes. Therefore the first 30 days after transplant are critical for long-term survival of transplanted seedlings. Our results point in this direction, since the biggest losses in seedling numbers at all depth levels occurred in the first 50 days after transplant. The results obtained are congruent with other studies (Lau & Young 1988, Duarte & Sand-Jensen 1996), which also concluded that young seedlings, prior to the establishment of clonal growth and plant integration, are more susceptible to natural environmental stressors such as hydrodynamic disturbance, burial by sediments, light and nutrient availability.

At our study site, Juan Grande, seeds were concentrated at shallow depths, as also described for other localities (Reyes & Sansón 1994). However, our results showed that at shallow depths seedlings disappeared completely, while on the contrary, at deeper levels, seedlings persisted. These data need to be carefully interpreted. Although no seedling persisted in the shallow level at the end of the experiment, the sample size was probably too low to detect rare events of survival. We have no data to distinguish between the hypotheses of local mortality in place or loss due to physical disturbance dislodging the seedlings. Nevertheless, the low transplant survival at shallow areas, suggests a low contribution of sexual propagation to the local population growth. In the future we plan to analyse genotypic richness to test this idea. It is possible that seeds, although rarer, occur at deeper depths below densities that are easily sampled given SCUBA diving constraints. This is supported by the observation of seedlings in deeper areas that are likely more successful than seedlings observed at shallow depths. Our results open the possibility for an attempt to restore *C. nodosa* meadows in deeper areas using seedlings to form initial patches, promoting patch growth and survivability, which are considered critical bottlenecks for seagrass colonization (Hemminga & Duarte 2000).

This study has shown that survival of *C. nodosa* recruits (seedlings) does not decrease with depth, and can be very low at the shallower levels. The presence of more flowering shoots and of seed banks at shallow depths suggests a larger allocation to sexual reproduction there, but we show here that its contribution to population increase at shallow depths may be low. Instead, at medium and deep areas, where sexual allocation is lower, its contribution to population growth can be higher. These conclusions imply that the meadow dynamics and persistence is dependent on a tight integration of population demography across the vertical environmental gradient.

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Supporting information

Figure 1. Researcher transplants seedling at Juan Grande bay. The rhizome part of the seedlings was carefully hand buried into the sediment at three depth levels: Shallow (5 m), Medium (8 m) and Deep (12 m), covering the depth gradient present in the meadow. At each depth level, three stations were created with 20 transplanted seedlings each, distant five meters from each other.

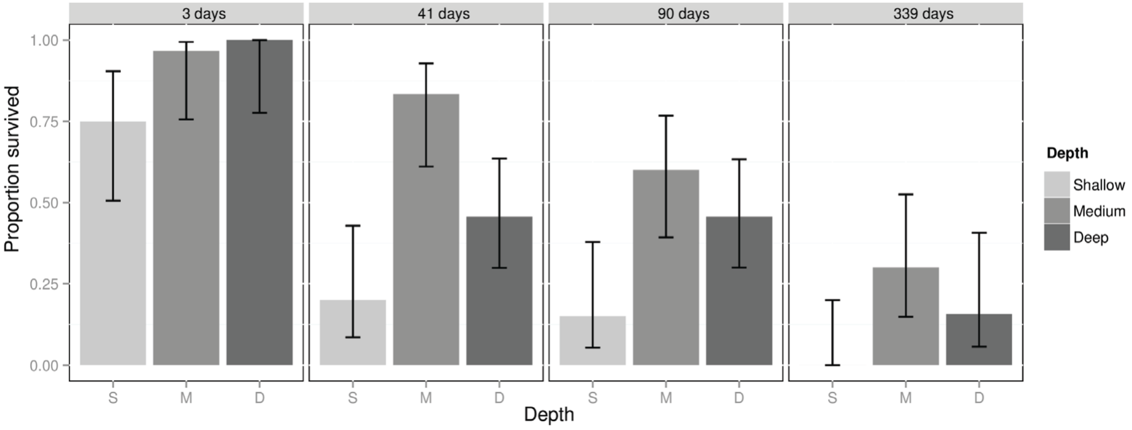

Figure 2. Mean proportion of transplanted *Cymodocea nodosa* seedlings that survived (bar) and 95% confidence interval (error bar) per depth level (S: shallow; M: medium; and D: deep). Seedling survival was monitored four times during one year (3, 41, 90 and 339 days after the transplant).

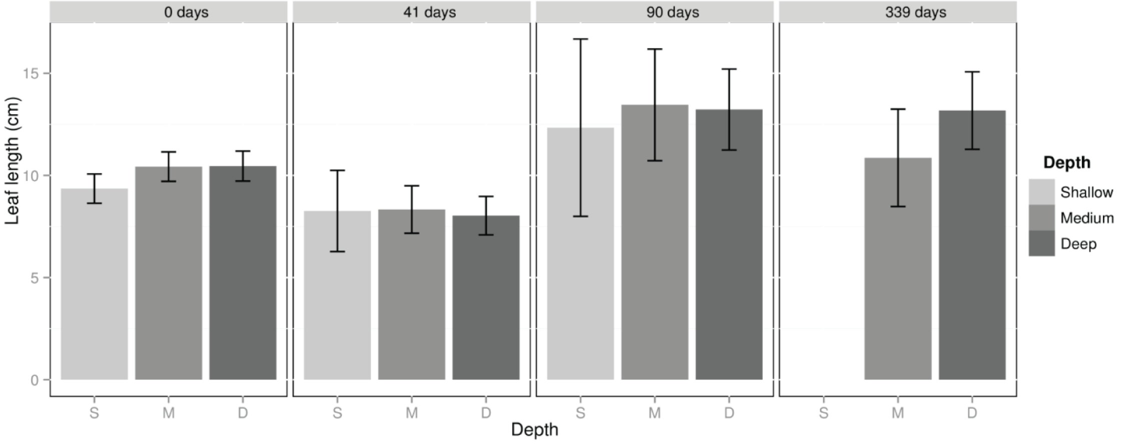

Figure 3. Mean leaf length (bar) and 95% confidence interval (error bar) of *Cymodocea nodosa* transplanted seedlings at three depth levels (S: shallow; M: medium; and D: deep). Leaf size was measured at the start of the experiment (time zero) and three times throughout one year (41, 90 and 339 days after the transplant date).

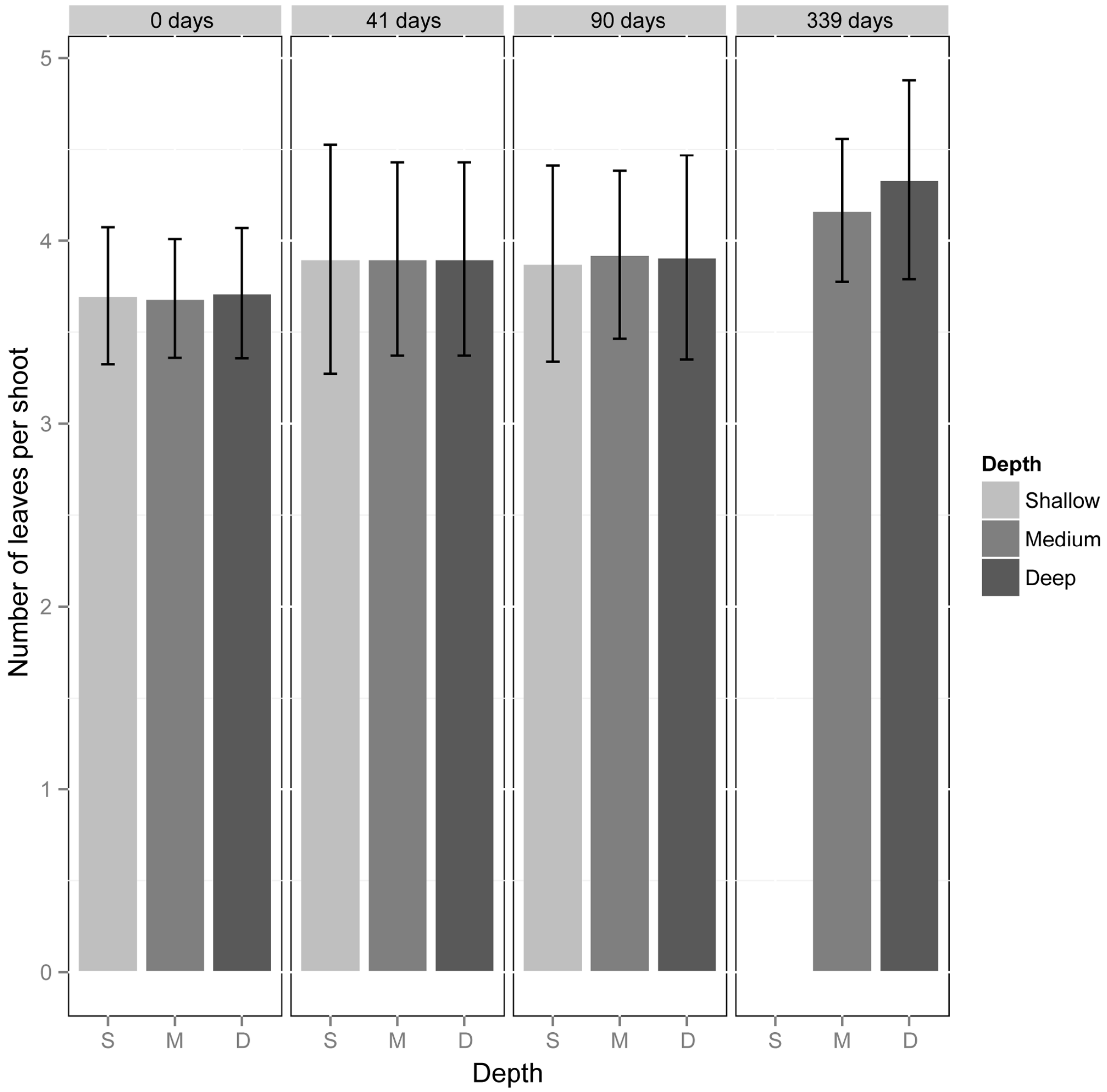

Figure 4. Number of leaves per shoot and 95% confidence interval (error bar) of *Cymodocea nodosa* transplanted seedlings at three depth levels (S: shallow; M: medium; and D: deep). Leaves per shoot were counted at the start of the experiment (time zero) and three times throughout one year (41, 90 and 339 days after the transplant date).

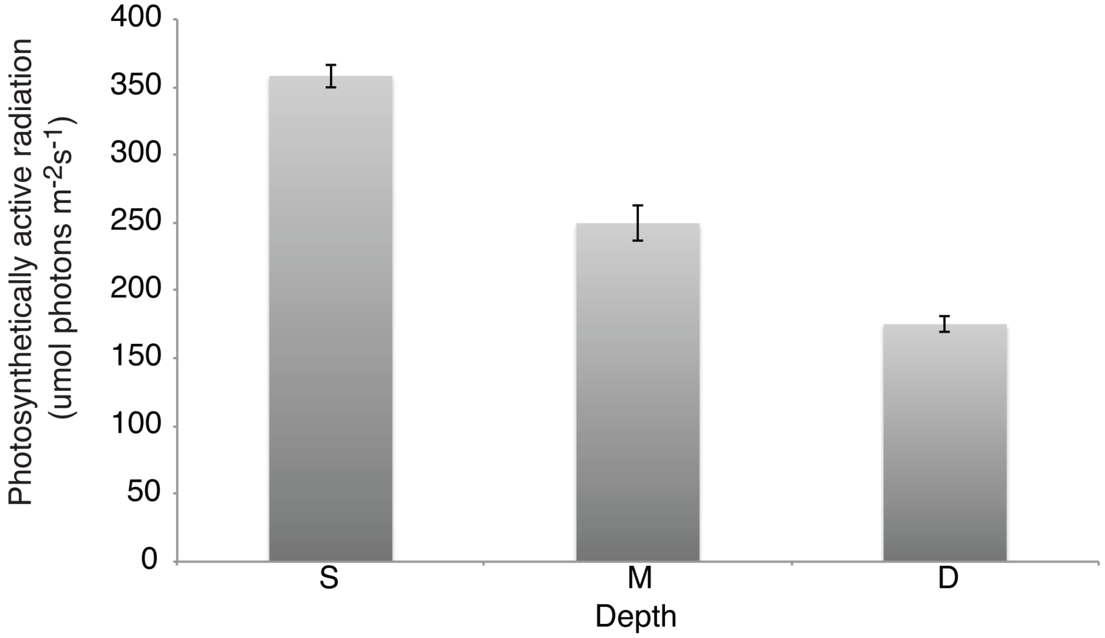

Figure 5. Photosynthetically active radiation (PAR) µmol photons $m^{-2}s^{-1}$ and 95% confidence interval (error bar) measured at three different depths (S: shallow, M: medium and D: deep) over the seedlings transplant sites. PAR measured with an underwater pulse amplitude modulate fluorometer (Diving PAM, Walz).

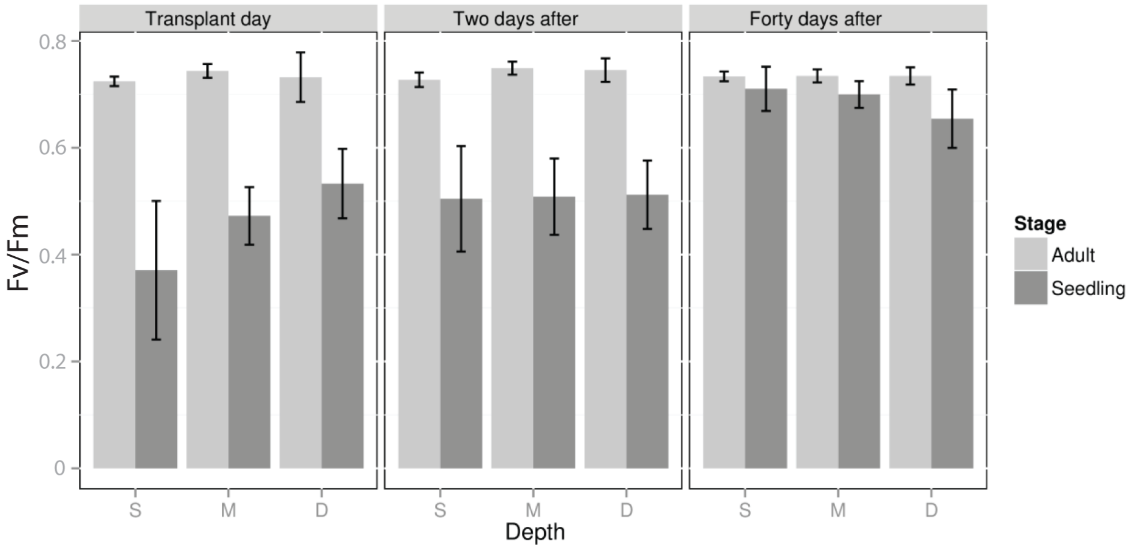

Figure 6. Maximum quantum yield (bar) and 95% confidence interval (error bar) measured for *Cymodocea nodosa* transplanted seedlings (dark grey) and native adult plants (light grey) at three different depths (S: shallow, M: medium and D: deep). The maximum quantum yield, Fv/Fm, was measured during the transplant day, 3 and 38 days after seedling transplant.

Chapter II: Sexual and asexual reproductive contributions along a depth gradient in *Cymodocea nodosa* (Ucria) meadows.

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Abstract

Environmental conditions can affect the relative contribution of sexual and clonal reproduction in clonal organisms. As a result, variation in genotypic diversity (the proportion of different clones in the population) along environmental gradients is expected. Gradients of disturbance and resource limitation are often associated with seagrass depth distribution, including wave action disturbance at the surface and light limitation at depth. Our goal was to determine if the reproduction mode (sexual versus clonal) in the clonal seagrass *Cymodocea nodosa* varied along its natural depth distribution. The meadows of this marine clonal plant in the Canary Islands are spread along a vertical gradient, providing an ideal model system to study the association between environment (depth) and reproductive strategies in clonal organisms. Here we combined demographic (seed density, flower density, rhizome elongation rate) and genetic analyses (genotypic richness, clonal structure and spatial genetic structure) to study reproductive variation along a depth gradient in four sites in the Canary Islands. Our results indicate that *C. nodosa* meadows in the Canary Islands experience repeated seedling recruitment events at all depth levels (shallow, medium and deep). We found that one meadow mirrored the expected pattern along the depth gradient, showing evidence of a ruderal life strategy at the shallow limit, a competitive strategy in medium depths and a tolerant strategy at the deep limit. In the other meadows the life strategy identified most frequently was competitive. No general demographic or genetic pattern was found along the depth gradient of *C. nodosa* meadows in the Canary Islands. Our results highlight the importance of both reproductive strategies along depth for species maintenance and that reproductive balance of clonal plants along environmental gradients might be more stochastic than simply predicted by theory.

Key words

Life traits, depth gradient, clonal organism

Introduction

Partially clonal organisms are able to propagate by clonal or sexual means. Clonality allows for plants to grow and spread horizontally (Silvertown 2008) translocating resources between ramets, and reducing the costs of sexual reproduction in heterogeneous environment (Price and Marshall 1999). Although sexual reproduction dilutes female genetic contribution to the next generation by half, when compared with clonal reproduction (Maynard 1978, Crowley and McLetchie 2002), it increases population genetic diversity. Genetic recombination enhances genotypic diversity, increasing resilience and the ability to adapt to disturbances in natural populations (Balloux et al 2003; Reusch 2006). The relative contribution of each reproductive mode can be affected by environmental conditions (Eckert 2002) as shown in other plants (Gonzales et al 2008) where fewer genotypes were found stressful environments, when compared with more suitable habitat. Current knowledge leads to the prediction that in clonal organisms genotypic diversity (the proportion of distinct genotypes in a population) might vary along environmental gradients.

Spatial genetic structure (SPG), which is the non-random spatial distribution of alleles and genotypes, is the result of the balance between genetic drift, migration and local selection (Epperson 2003, Vekemans and Hardy 2004). Population genetic differentiation increases as a factor of limited dispersal over time, having an effect on genetic variation and neighbourhood size of genetically related individuals (Epperson 2003). In plants and sessile organisms, spatial genetic structure is important for species persistence and distribution (Epperson and Chung 2001, Escudero et al 2003). Research on the relationship between SGS and habitat heterogeneity, such as depth, can contribute for a better understanding on how species adapt to within population environmental variability. For example, if dispersal of sexual propagules exceeds the scale of spatial heterogeneity that putatively affects the sexual vs. clonal reproductive balance, the effect of the environment might go undetected. Therefore, when studying the reproductive balance of clonal organisms it is important to study dispersal to disentangle the local and immigrant contributions to sexual reproduction.

Seagrasses are marine flowering plants living in shallow shores and estuarine environments of every sea in the world except Antarctica. All seagrasses achieve population growth through a balance of clonal growth and sexual reproduction, spreading from established patches by means of seeds and horizontal elongation of lateral rhizomes (e.g. Duarte and Sand-Jensen 1990). Seagrasses can present a more or less continuous depth distribution, forming a habitat characterized by extreme environmental variability within a small spatial scale (Green and Short 2003). The upper and lower ends of this distribution are characterized by hydrodynamic disturbance and light-limitation respectively, allowing for the study of clonal life history trade-offs.

In this study, we investigate how the reproductive strategy of the marine plant *Cymodocea nodosa* changes along a depth gradient. We combine genetic and demographic approaches to estimate genotypic richness, sexual and vegetative proxies for population growth.

At the upper limit of the species depth range, where physical disturbance opens space periodically, we hypothesize that higher sexual allocation and higher dispersal rates will be favoured, as a strategy for rapid colonization (ruderal strategy, Grime 1977). At intermediate depths low stress and low disturbance are expected, thus, clonal structure should be dominated by fewer larger and older clones (competitive strategy). Finally, near the depth limit we expect that energy demand due to light limitation should hinder sexual reproduction and space colonization will depend mostly on clonal spread (tolerant strategy). We also characterized genetic structure along the depth range to clarify the role of migration in generating the observed genotypic richness at different depths.

Methods

Model species

Cymodocea nodosa is a dioecious seagrass that exhibits fast clonal growth (Duarte and Sand-Jensen 1990) and seed basicarpy (Buia and Mazzella 1991) with reduced seed dispersal (Alberto et al. 2005). Its distribution encompasses the Mediterranean basin and the North Atlantic, from Central Portugal to Senegal, as well as Madeira Island and the Canaries Archipelago (Masucci et al 2012).

Study site and sampling design

Mono-specific seagrass meadows of *C. nodosa* can be found in the Canary Islands, ranging from 5 m to 35 m depth (Reyes et al 1995). Two islands in the Canary archipelago, Tenerife and Fuerteventura separated by 240 km, were used to conduct our study (Figure 1). Two populations in Tenerife Island, Los Cristianos and El Puertito meadows, separated by 7 km; and two populations in Fuerteventura Island, Punta Jandía and Morro Jable meadows, separated by 19 km.

In each meadow three depth levels were defined: 1) Shallow (upper limit of the meadow); 2) Medium (intermediate depth between deeper and shallower limits of the meadow); and 3) Deep (deeper limit of the meadow). A total of 40 ramets were collected inside rectangular plots of 4.5 m per 10 m at regular distances (1 m) along four parallel transects (10 ramets per transect). In each meadow nine rectangular plots were sampled, three at each depth, covering horizontal and vertical axes along the slope. All sampling was performed by SCUBA diving.

Demography

Demographic data was recorded at each depth level in all meadows by collecting three 20 cm cores to estimate shoot density, seed density and flowering density. Additionally, rhizomes were collected in the same sites to calculate shoot and horizontal rhizome apices densities, shoot maximum age and age structure, horizontal and vertical rhizome extension rates as traits relevant for clonal propagation. Demographic parameters from recent years in *C. nodosa* meadows were reconstructed using techniques based on the information recorded in vertical growth internodes as described in Duarte et al (1994).

Rhizome elongation rate (RER) was used to estimate *C. nodosa* clonal contribution to population growth. We estimated RER using a method based on leaf Plastochrone Intervals (PI). PI represents the time interval between the production of consecutive leafs on a shoot, allowing to estimate the difference in age between two consecutive shoots. We first measured the difference in leaf scars between two consecutive shoots along a rhizome. This difference can be easily converted to a time by multiplying it by the PI. The number and length of rhizome internodes between consecutive shoots was also measured. Using linear regression, we then estimated the rate of rhizome internodes produced per PI and multiplied it by the mean rhizome internode length to get our RER estimate in cm/PI.

To test the null hypotheses of mean equality for seed density, flowering rate and rhizome elongation rates, among depth levels in each population, we used one-way ANOVAs, followed by Tukey-HSD post hoc test in R software (R Core Team 2017).

Microsatellite genotyping

Genomic DNA was extracted from silica gel-dried plant material following Doyle and Doyle (1988). Thirteen microsatellite loci (Cy-1; Cy-4; Cy-16; Cy-18; Cn2-16; Cn2-18; Cn4-29; Cn2-45; Cn2-14; Cn2-38; Cn2-24; Cn4-19; Cn4-6) were PCR amplified using fluorescently labeled primers as described in Alberto et al. (2005). Amplified fragments were analysed on an ABI PRISM 3130 XL Genetic Analyser (Applied Biosystems) using the GeneScan-350 ROX standard. A total of 1440 samples were successfully genotyped and a minimum of thirty-four samples per plot was amplified for all microsatellite loci.

Raw allele sizes were scored with STRAND (http://www.vgl.ucdavis.edu/informatics/STRand/), binned using the R package MsatAllele (Alberto 2009) in R software (R Core Team 2017), and manually reviewed for ambiguities.

Genotypic richness

Equal multilocus genotypes (MLGs) can either result from finding by chance the same alleles in two ramets from sexual reproduction, or from asexual reproduction replicating the same genet in different ramets sampled. Thus, to unambiguously determine genetic individuality, the number of distinct genets was estimated for all loci in all data subsets by testing the null hypothesis that identical MLG might have been produced by sexual recombination (*Psex*). Rejecting the null hypothesis provides confidence that identical MLGs are caused by asexual reproduction. *Psex* was estimated using GenClone 2.0 (Arnaud-Haond and Belkhir 2007). Genotypic richness, the proportion of different genets G in the sample was then estimated as

$$R = (G-1) / (N-1),$$

where G the number of genets and N the number of ramets sampled (Arnaud- Haond et al. 2007). Genotypic richness ranges from R = 0 (all samples belong to the same genetic individual or clone) to R = 1 (all samples are genetically unique individuals) and it is a proxy of the multi-generation contribution of sexual reproduction to population growth. One-way ANOVAs were performed to test for differences in genotypic richness between the three depth levels in each population, followed when necessary by Tukey HSD to test for multiple pairwise comparisons between depth levels.

Clonal structure

At each sampling station, the maximum length between sampled ramets belonging to the same clone was calculated. The average clone length (m) per depth level at each population was obtained. Differences between depth levels, at each sampled population, was assessed with ANOVAS and Tukey HSD when necessary.

Population genetics

Allelic richness was calculated using software GenePop (Raymond and Rousset 1995). Expected (*He*) and observed (*Ho*) heterozygosity, and the *Gis* estimator of inbreeding according to Nei (1987) were estimated with software GenoDive (Meirmans and Van Tienderen 2004). For all statistics 95% confidence intervals (CI) were obtained through bootstrapping over loci. Deviation from Hardy–Weinberg equilibrium was tested using the least squares method (Amova).

Spatial genetic structure

Spatial genetic structure within plots was characterized by determining the association between a kinship coefficient F_{ij} (Loiselle et al 1995), which estimates the pairwise probability of identity by descent, and pairwise spatial distance. Average pairwise kinship coefficient was calculated for eight class distances within a plot. Distance class bin limits were defined in a way that the number of pairs of individuals within each class was constant. The slope of the regression (*blog*) between pairwise kinship coefficients for each distance as a function of the logarithm of the spatial distance was calculated. The 95% CI was obtained by a permutation test available in software Spagedi (Hardy and Vekemans 2002). Spatial locations were permuted 1000 for each population to test the null hypothesis of no spatial genetic structure (i.e., H_0: *blog* = 0). Analyses were run at the genet level in software Spagedi, by including a single copy of each MLG in the sample or all MLG in the sample, respectively

(Hardy and Vekemans 2002). Variables *Fij* and *blog* were compared between depth levels within populations using ANOVA and compared within and among depths using T-Student test in R software (R Core Team 2017).

Results

Demography

No differences in seed density were found between depth levels in all meadows with the exception of Los Cristianos where more seeds were found in shallow (50.52 seeds m^{-2}) than in deep (9.18 seeds m^{-2}) plots (Tukey HSD p = 0.041). Seed density ranged from 9.18 seeds m^{-2} in Los Cristianos deep level to 76.54 seeds m^{-2} in El Puertito shallow level, both in Tenerife. Likewise, differences in flowering density were only found in a single meadow and depth comparison, in El Puertito meadow between Shallow (16.89 flowers m^{-2}) and Deep (1.53 flowers m^{-2}) levels (Tukey HSD p = 0.007). Flower density ranged from 19.37 flowers m^{-2} to 1.53 flowers m^{-2} in Los Cristianos Medium and El Puertito Deep level respectively.

Inconsistent patterns were found in different meadows for differences in rhizome elongation rate (clonal growth) between depths. Los Cristianos meadow had significant differences in RER between all depth levels (Tukey HSD p < 0.001 between all comparisons). The highest mean RER value was found in medium plots (4.78 cm/PI), followed by shallow (2.91 cm/PI) and deep level (1.57 cm/PI), with all Tukey HSD p < 0.01. For Morro Jable differences were found between shallow (6.60 cm/PI) and deep (2.40 cm/PI), and shallow and medium (2.68 cm/PI) depth levels (Tukey HSD p value < 0.001 in both cases), indicating a gradient from shallow to deep. However, in Punta Jandía RER was lower at the shallow level compared to medium and deep (2.54, 3.65, 3.69 cm/PI; respectively) (Tukey HSD p < 0.001 on both comparisons). RER pattern along the depth gradient was similar in El Puertito, Tenerife and Morro Jable, Fuerteventura and opposite to Punta Jandía, Fuerteventura. Los Cristianos showed a unique pattern with a higher RER at Medium.

Genotypic richness

Genotypic richness in Los Cristianos was higher in shallow than in medium and deep levels (Tukey HSD p = 0.023; p = 0.018 respectively). In El Puertito meadow we found the opposite pattern, genotypic richness in deep was higher than in shallow and medium depth levels (Tukey HSD P = 0.039; P = 0.015 respectively). The average *R* per depth level ranged from 0.37 and 0.87 in Morro Jable and El Puertito deep levels, respectively. No differences between depth levels were found in Morro Jable and Punta Jandía (Figures 2 to 5).

Clonal structure

Overall, the clonal structure of the sampled sites at all the depth levels were characterized by few large clones. Clone size did not differ between depth levels in Punta Jandía, Morro Jable and el Puertito meadows. Yet significant differences were found for Los Cristianos, Shallow depth level clones were smaller when compared with Deep and Medium levels (Tukey HSD P = 0.00; P = 0.00; respectively).

Genetic statistics

The allelic richness ranged from 3.5 in Los Cristianos Medium level and El Puertito Shallow level and 6.0 in El Puertito Deep level. The inbreeding estimator *Gis* was significant (95% CI) in all populations, showing heterozygosity deficit. None of the analysed populations were at Hardy Weinberg equilibrium (Table 1).

Spatial Genetic Structure

We did not find a consistent within plot pattern for the association between genetic co-ancestry (F_{ij}) and spatial distance across all depth levels and sites. A total 22 plots had no significant association while 14 had a negative association. When plots were used as replicates to compare genetic structure across depths, there were no significant differences in the mean kinship coefficient at the first distance class and in *blog* values at any of the studied populations (ANOVA $p > 0.05$ for all comparisons). When pairwise comparisons included individuals from different plots in the same site, but restricted to either individuals in same or different depths, there were no significant differences at the fist distance class F_{ij} and *blog* between the vertical and horizontal axes (*t*-test $p > 0.05$, with sites as replicates). This suggests that genetic differentiation along horizontal (same depth) and vertical (different depth) axes is similar.

Discussion

The depth gradient colonized by *Cymodocea nodosa* meadows in the Canary Islands was not associated with a particular pattern of sexual vs asexual reproductive balance. Using both direct demographic measurements and indirect genetic estimates, accounting for multiple generation contributions, we found inconsistent variation that did not match our predictions of environmental based effects on reproductive system. Additionally, across the different demographic and genetic metrics used, only in one site out of four there were congruent patterns between metrics and only for a single pairwise combination of metrics. This was found in Los Cristianos, Tenerife, where average clone sizes in shallow levels were smaller and more clones per sample were found when compared to medium and deep levels where fewer, larger clones were found.

Different factors can explain our results. First, the spatial scale of *C. nodosa* distribution in these open ocean Canary slopes and that of hydrodynamic disturbance might not create the heterogenous effects hypothesized along the depth gradient. High-energy swells, particularly during winter, have been pointed as an impact factor un-rooting *C. nodosa* in the Canary Islands (Tuya et al 2013). This seasonal winter disturbance creates fragmented meadows (Tuya et al 2006). In summer when conditions are favourable re-colonization of the available areas takes place, as well as an increase of shoot density and biomass on the persistent patches (Reyes et al 1995, Tuya et al 2006). These could keep the meadow in a permanent state of patch extinction and colonization where the required equilibrium to observe our predictions would be rarely achieved. To conduct this study, we searched for continuous meadows across and along the depth profile. Although our sites had high cover (> 80% across the meadow) there was a patchy distribution of seagrass clearings that supports the idea that disturbance might be more evenly distributed across depth than assumed by our predictions.

In contrast with our results, variation in the sexual-asexual reproductive balance was observed in *C. nodosa* along an eutrophic gradient in the Mediterranean coastal lagoon of Alfacs bay (Oliva et al 2014). The environmental heterogeneity was mostly anthropogenic and eutrophic populations had lower sexual allocation and genotypic richness, when compared with non-eutrophic populations.

The uniqueness of our results is that contradicting to expectations we have found evidence of high genotypic richness at all depths. Sexual reproduction was site dependent rather than depth dependent. It has been shown that seedlings at deeper areas have higher survival rates than those at shallow levels, probably because of hydrodynamic disturbance and subsequent seedling lost (Paulo et al 2016). In some cases, such as in El Puertito, we have observed simultaneously the lowest sexual proxies (flower and seed density) and the highest genotypic richness at the deep area. Our results emphasize the value of combining demographic and genetic methods to understand clonal plant reproductive biology. Because genotypic richness is the composite of realised multi-generation sexual reproduction, including migration, it does not necessarily match demographic estimates collected in a single season.

The observed SGS at the plot level revealed site specific variability. There were no differences in SGS between depths, suggesting that at the tested scale, the dispersal, genetic drift and population history is identical along the depth gradient. Genetic differentiation decayed with distance similarly within and between depths. The genetic richness analyses showed heterozygosity deficit at all depth levels which is likely due to inbreeding. This suggests that sexual reproduction occurs repeatedly over time at all depths. Our results suggest that although the sampled meadows were not in a colonization phase, sexual reproduction is still of significant importance for population maintenance (Alberto et al 2006).

Previous studies at the Canary Islands concluded that changes over the last decade have been spatially heterogeneous, with 60% declining versus 40% stable seagrass meadows (Tuya et al 2013). A combination of natural factors, like strong winter swell, and negative human actions, such as land runoffs, aquacultures and human constructions (Dalton 2004, Halpern et al 2008, Shahidul and Tanaka 2004), can further increase the disturbance experienced by *C. nodosa* in the Canary Islands. The capacity of this clonal organism to colonize such a dynamic environment is reflected in its heterogeneous life strategies, which are responsible for species maintenance and long-term persistence.

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Supporting information

Table 1: *A* allelic richness; *He* expected heterozygoty; *Ho* observed heterozygoty; *Gis* estimator of inbreeding, in bold significant values within a 95% CI. Negative values indicate

an excess of heterozygotes, and positive values a deficit of heterozygotes; mean kinship coefficient slope *blog.*

Location	Depth	*A*	*He*	*Ho*	*Gis*	*blog*
Morro Jable	Shallow	4.23	0.43	0.29	**0.32**	-0.027
Morro Jable	Medium	4.54	0.39	0.33	**0.16**	-0.016
Morro Jable	Deep	3.77	0.46	0.38	**0.17**	-0.023
Punta Jandía	Shallow	3.77	0.40	0.32	**0.20**	-0.011
Punta Jandía	Medium	3.85	0.38	0.27	**0.31**	-0.024
Punta Jandía	Deep	4.54	0.43	0.23	**0.46**	-0.026
Los Cristianos	Shallow	5.77	0.47	0.34	**0.28**	-0.022
Los Cristianos	Medium	3.54	0.35	0.32	**0.07**	-0.014
Los Cristianos	Deep	4.31	0.46	0.43	**0.08**	-0.02
El Portito	Shallow	3.54	0.35	0.31	**0.12**	-0.027
El Portito	Medium	4.15	0.38	0.31	**0.18**	-0.014
El Portito	Deep	6.00	0.46	0.23	**0.51**	-0.028

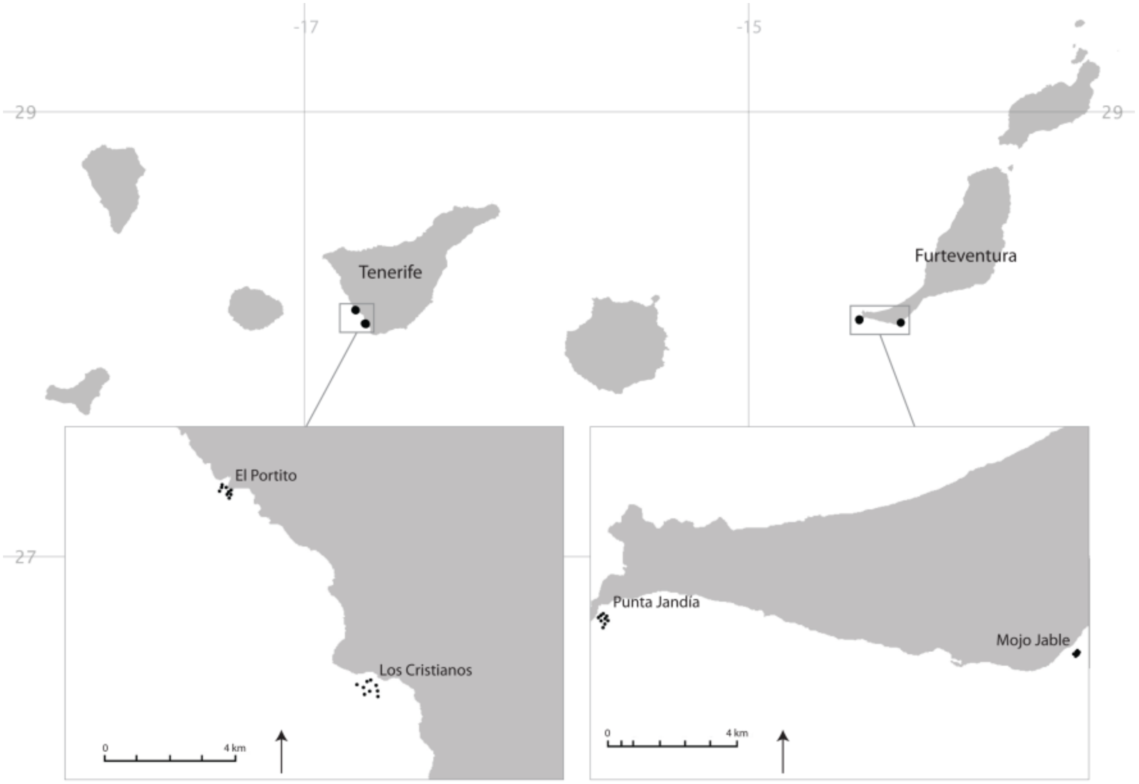

Figure 1: Map of the Canary archipelago, where Tenerife and Fuerteventura Islands are represented. In each Island two meadows were sampled, El Puertito and Los Cristianos in Tenerife; and Punta Jandía and Morro Jable in Fuerteventura. At each population three replicates were done at each depth level (Shallow level, Medium level and Deep level). Depth levels were set according to meadow limits.

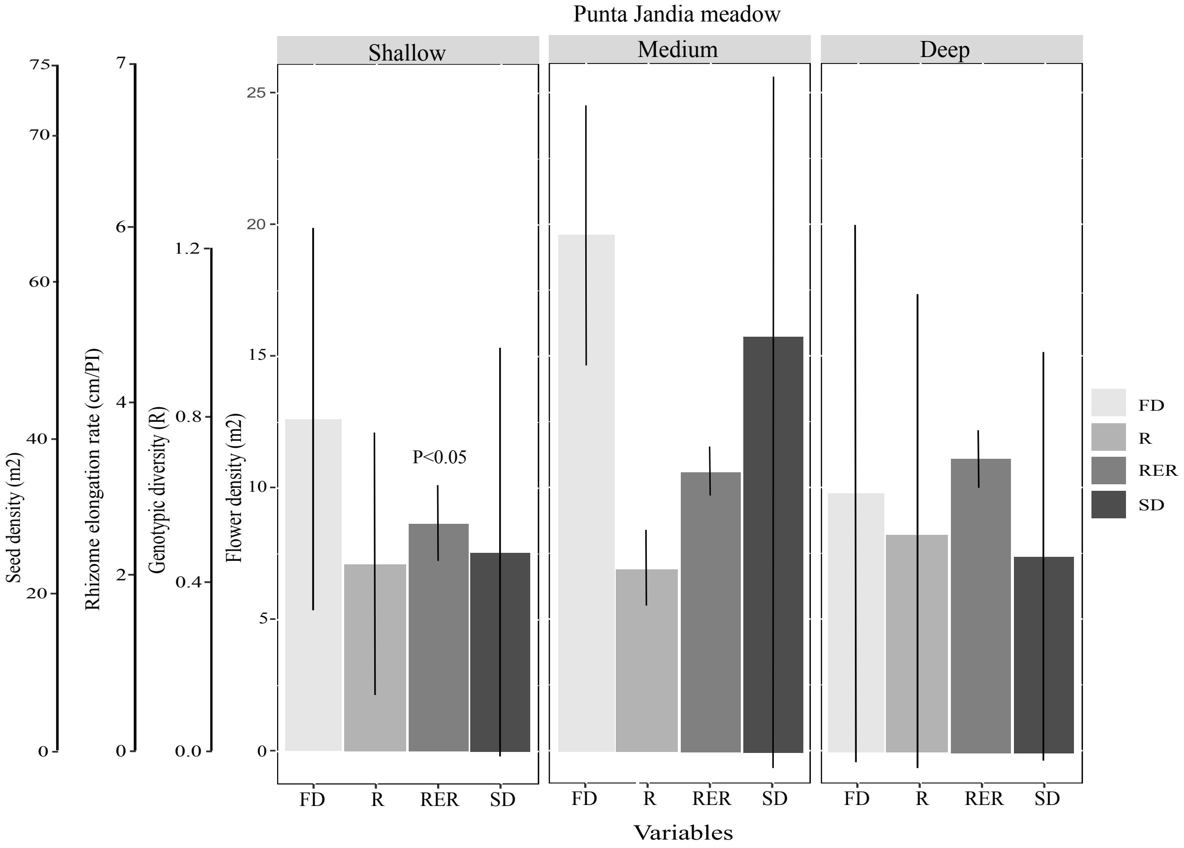

Figure 2: Punta Jandia seagrass meadow demographic (FD - Flower density (m^2); SD - Seed density (m^2); RER - Rhizome elongation rate (cm/PI)) and genetic (R - Genotypic richness) variables at three depth levels (Shallow, Medium, Deep).

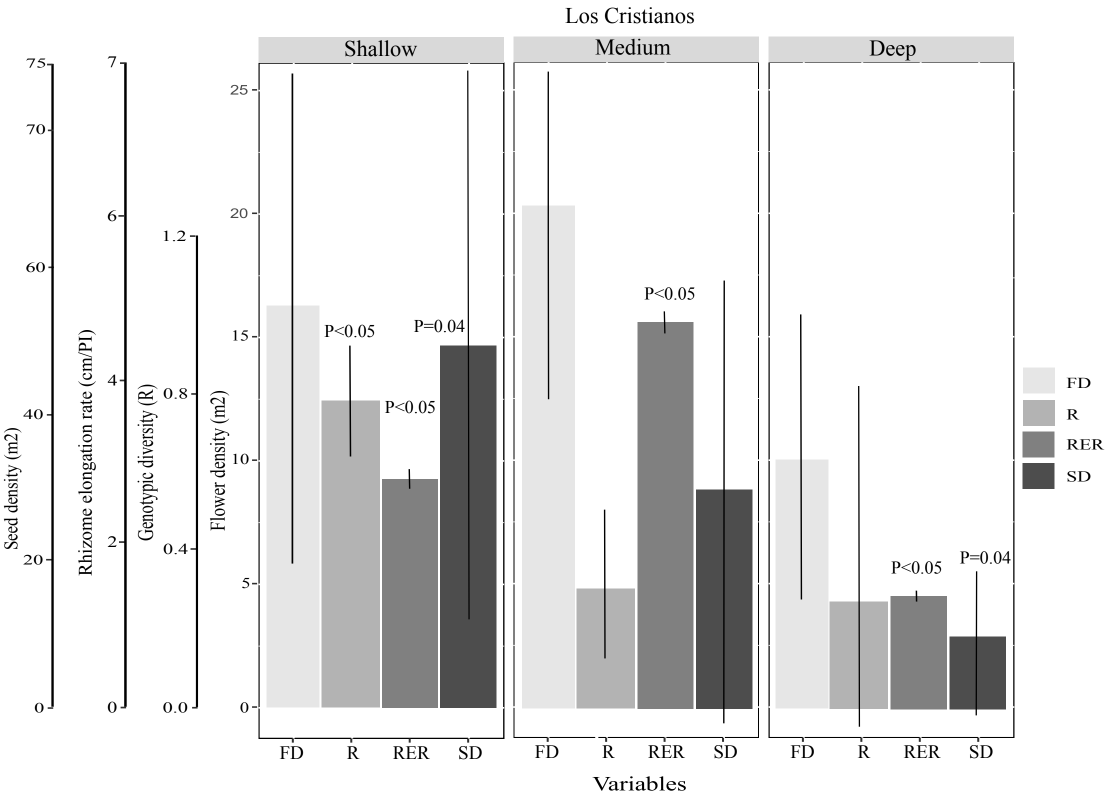

Figure 3: Los Cristianos seagrass meadow demographic (FD - Flower density (m^2); SD - Seed density (m^2); RER - Rhizome elongation rate (cm/PI)) and genetic (R - Genotypic richness) variables at three depth levels (Shallow, Medium, Deep).

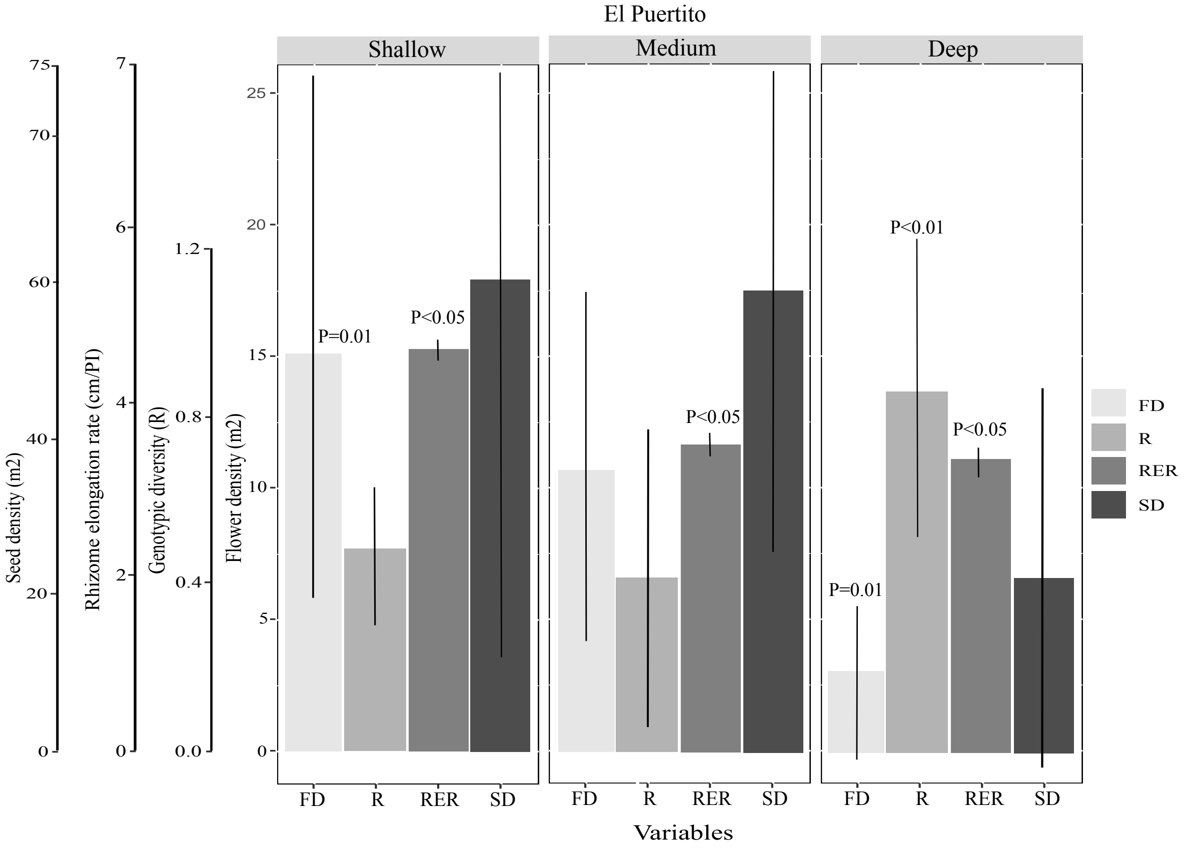

Figure 4: El Puertito seagrass meadow demographic (FD - Flower density (m^2); SD - Seed density (m^2); RER - Rhizome elongation rate (cm/PI)) and genetic (R - Genotypic richness) variables at three depth levels (Shallow, Medium, Deep).

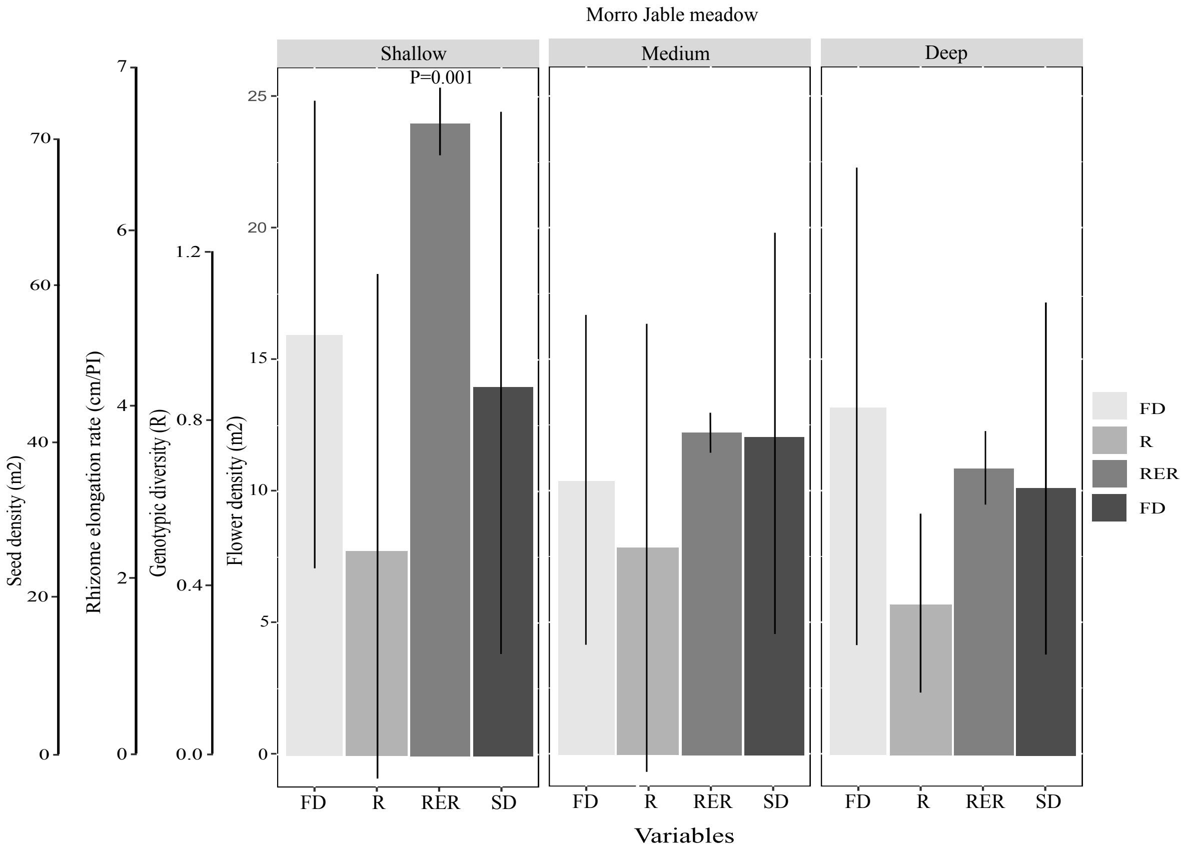

Figure 5: Morro Jable seagrass meadow demographic (FD - Flower density (m^2); SD - Seed density (m^2); RER - Rhizome elongation rate (cm/PI)) and genetic (R - Genotypic richness) variables at three depth levels (Shallow, Medium, Deep).

Chapter III: Re-colonization following impact in *Cymodocea nodosa* (Ucria) meadows.

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Abstract

Seagrasses are declining world-wide due to several threats, which often results from the removal of plant biomass and loss of habitat. As clonal organisms, seagrasses can reproduce both sexually and clonally, yet the relative importance of these life traits in the recolonization of impacted areas is poorly known. *Cymodocea nodosa*, a marine angiosperm, forms large meadows in the Canary Islands covering a depth gradient between 5 m to 35 m, making the region a perfect scenario to test the importance of sexual and clonal reproduction in the response to physical impact. This study aims to test experimentally the relative roles of sexual and clonal reproduction in recovery from disturbance. Two Islands were selected for this experiment, Tenerife and Fuerteventura, and two meadows per Island were chosen to create artificial disturbance plots. In total, twelve artificial plots were created, each with 0.25 m^2. Three depth levels according with meadow limits were selected: Deep, Medium and Shallow; at each depth four plots were created. The removal plots were monitored five times over a total period of 788 days in which: plant cover, seedling density and rhizome elongation rate were measured. The genotypic richness of the removal plots was calculated prior to removal and at the end of the experiment. There were no differences in the recolonization dynamics at different depth levels. Rhizome elongation rates and plant cover increased from spring to autumn, but after the first winter most of the plots lost all its biomass. The genotypic richness increased from an average 0.5 prior removal to 0.7 at the end of the experiment, indicating an important role of sexual reproduction in the recovery of impacted areas regardless of the depth level. Recovery from impact in *C. nodosa* meadows in the Canary Islands proved to be much slower than observed by studies with the same species in the Mediterranean, which raises the concern for its protection.

Key words

Cymodocea nodosa, Impact recovery, Depth gradient

Introduction

Seagrass populations are declining worldwide due to several threats, from climate change to direct and indirect human activities (Waycott et al. 2009). Physical impact such as plant removal are at the top of the most severe impacts marine plants can suffer. Leisure boat moorings, dredging, propellers and strong weather events are among the origins of such impact (Duarte et al. 2004). Seagrasses are marine flowering plants with the possibility to reproduce sexually or clonally, colonizing unvegetated areas by means of seeds and horizontal elongation of lateral rhizomes (e.g. Duarte & Sand-Jensen 1990). Because of the decrease in photosynthetic available radiation with depth, light has also been pointed as a limitation for sexual reproduction in some seagrass species (i.e. the Indo-Pacific seagrass *Enhalus acoroides*; Rollón et al. 2003). The combination of frequent hydrodynamic impacts in with high light intensity in shallow areas may allow for energy allocation into sexual reproduction, creating a heterogeneous genotypic richness pattern, decreasing from shallow to deep areas. Variation in seagrass life-traits has been observed along different types of environmental gradients such as from eutrophic areas to non-eutrophic areas (e.g., Oliva et al. 2014). In the Canary Islands, *Cymodocea nodosa* (Ucria) Aschers, a marine angiosperm, forms extensive meadows from 5 m to 35 m of depth (Reyes et al. 1995), and seeds have been observed at the deeper limit of its distribution. Paulo et al. (2016) observed that *C. nodosa* seedlings can successfully thrive at the deeper limits of the meadow, whereas at shallow depths there was a major loss of new recruits, suggesting an overall balance of effective clonal and sexual contributions to population growth along the depth gradient. Clonal strategies of plants are known to be variable; three different strategies have been suggested as response of species to different combinations of disturbance and stress factors: low disturbance and low stress, competitive strategy; low disturbance high stress, tolerant strategy; and low stress high disturbance ruderal strategy (Grime 1977). The above strategies can be hypothesized to dominate at different depths in a seagrass meadow, were the upper limit is frequently disturbed by hydrodynamics and the deeper end limited by light.

To test this pattern, we used the meadow depth gradient as a factor prone to trigger different life strategies. Understanding population growth strategies (the balance between sexual and clonal growth) used by marine angiosperms recovering from physical impact along an environmental gradient not only provides a test of life history trait variation theory but also provides important information on recovery time. We hypothesize that re-colonization will reflect the life strategies of the marine plant *C. nodosa* where we expect: high sexual contribution at a shallow level; a balanced input between sexual and clonal at intermediate depths; and less sexual allocation at greater depths.

Methods

To study *C. nodosa* population growth strategy during recovery from removal impacts we simulated impact by clearing a 0.25 m^2 area from meadows in two islands of the Canary archipelago: (1) Tenerife and (2) Fuerteventura. In each Island we have worked in two different meadows, in Tenerife: Los Cristianos and El Puertito; and in Fuerteventura: Morro Jable and Punta Jandía. All the removed plant material was used for a demography study (Paulo et al. in prep.). The plots were set in different meadows to isolate for site-specific differences, assuring for independent replicates. All fieldwork was done using SCUBA.

We cleared three plots per meadow, one at each depth level: shallow, medium and deep, adding a total of four plots at each depth. The depth levels were selected based on the depth limits founding each meadow. The shallowest plots were cleared between 8 and 10 m depth, in the medium level between 11 and 13 m and in the deepest plots between 14 and 19 m. After plant removal, the bare sediment remained levelled with the surrounding meadow. In order to find the removal plots over time, we installed four metal staples (one per corner) and attached a small buoy with a 1.5 m cable to their upper end (Fig. 1).

Plots were monitored five times: right after clearing (May 2010), 100 days (August 2010), 167 days (November 2010), 377 days (March 2011) and 788 days (May 2012) after plant removal. Plant cover was estimated visually in situ over the removal plots using a 0.25 m^2 grid (Fig. 2). As a control we haphazardly placed the grid over the surrounding meadow and measured plant cover over time. Seedling density (seedlings 0.25 m^{-2}) was estimated in every monitoring using the same sampling grid over the removal plot. Rhizome elongation rate (RER) was estimated by marking the new rhizomes with small plastic tie wraps. The sampling grid was used to identify the new individuals and rhizome horizontal growth was measured in every monitoring, allowing for the estimation of the RER in cm.day^{-1}.

To infer the genotypic richness at each depth level, at the beginning and end of the study, ten plants per removal area were randomly colleced. At the end of the study all shoots that had grown inside the removal plot were collected. Genomic DNA was extracted from silica dried plant material by a CTAB (2% beta-mercaptoethanol) method (Coyer et al. 2004). Fluorescently labeled primers were used to PCR-amplify nine microsatellite loci (Cn2-16; Cn2-18; Cn4-29; Cn2-45; Cn2-14; Cn2-38; Cn2-24; Cn4-19; Cn4-6). Fragments were analyzed on an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems) using the GeneScan-350 ROX standard. Raw allele sizes were scored and manually reviewed for ambiguities using STRAND (http://www.vgl.ucdavis.edu/informatics/STRand/), and MsatAllele (Alberto 2009) in R software (R Development Core Team 2009). Genotypic richness was estimated as by Dorken & Eckert (2001): $R = (G-1)/(N-1)$.

To test effects on response variables (plant cover, seedling presence, rhizome elongation rate and genotypic richness) of differences between depth level along the time, repeated measures ANOVAs were performed using package “ez” in R software (R core team 2014).

Results

Plant cover

There were no significant differences in the plant cover of the removal plots between depths (ANOVA P = 0.07). Plant cover was higher at 167 days than at 788 days (ANOVA P = 0.02). In the last monitoring only two plots in the shallow level, one at medium level and two at deep level had plant cover (Fig. 3).

There were no significant differences in plant cover in the control areas between any of the tested depths (ANOVA P = 0.50). Although not significant, in the control meadows there was a 20% drop in the percent cover between the beginning and end of the experiment (Fig. 4). This drop reflects the extreme reduction of plant cover in one of the studied meadows (Morro Jable). This reduction was likely due to overgrowth of epiphytes over the entire meadow, which started to be noticed in March 2011 (Fig. 5).

Seedling presence

There were no differences for seedling density in the removal plots between depth levels at any sampled time (ANOVA P = 0.54) and within any depths along time (ANOVA P = 0.13). The average number of seedlings present in the removal plots was always low (under 0.25 seedlings (0.25 m^{-2}) during all the monitoring times, with the exception of the last one (788 days). At the shallow and deep levels, seedling density was above 1.5 seedlings.0.25 m^{-2} in the last monitoring time (Fig. 6).

Rhizome Elongation Rate

Differences in RER between depth levels were not found (ANOVA P = 0.10). Rhizome elongation rates ranged from a maximum of 0.35 $cm.day^{-1}$ (+- 0.06 SE) at 167 days at the shallow level and minimum of 0.03 $cm.day^{-1}$ (+- 0.01 SE) at 377 days at the medium depth level. Along time RER was different (ANOVA P = 0.019), in the third monitoring time after 2010 winter (377 days) RER decreased to less than half (Fig. 7). In the last monitoring (788 days) all ramets under monitoring were absent.

Genotypic richness

Genotypic richness in the removal plots was different between the beginning and the end of the experiment (ANOVA P = 0.01). The genotypic richness changed from an average *R* = 0.5 before removal to values over 0.7 at the end of the monitoring period. No differences in *R* were found between depth levels at any sampled time (ANOVA P = 0.09) (Fig. 8).

Discussion

In this research, we have tested the different growth strategies of *C. nodosa* along a vertical gradient, by creating physical disturbance plots and following its recolonization. Depth was not a contributing factor for the tested response variables. Both plant cover and rhizome elongation rate increased from spring (May 2010) to autumn (November 2010), followed by a drastic drop to near zero after winter 2010. These results indicated that periodic disturbance over impacted areas can reset the recolonization process. Sexual reproduction was identified as an important contribution for plant recovery as genotypic diversity increased with time. Our results are in line with other seagrass removal experiments in which disturbance increased genotypic richness (Reusch 2006). Our results show that sexual reproduction is important along the depth gradient of *C. nodosa* meadows in the Canary Islands, as suggested in previous research (Paulo et al. 2016) in which seedlings were found to be successful at considerable depth. We suggest that sexual reproduction plays an important role in recovery from impact in *C. nodosa* meadows at the Canary Islands.

This species has been described in the Mediterranean as the most plastic of all European seagrass species, able to colonize areas with intense sediment dynamics including submerged sand dunes (Marbà 2004). In the Mediterranean, *C. nodosa* was reported to recolonize an area of 300 m^2 in 7 years (Marbà et al. 2004), demonstrating that recovery in sheltered areas is possible. The rhizome elongation rates found in our study are within the observed range for the Mediterranean, where Terrados & Ros (1992) reported a range from 0.005 cm day^{-1} and 0.465 mm day^{-1}. Leading to the expectation that recovery in the Canary Islands would be possible in few months, given the small sized plots. *C. nodosa* recovery from disturbance in the Canary Islands showed to be extremely slow, from the twelve plots only five showed signs of recovery after more than two years of monitoring.

A key finding of this research is that when the impacted areas are greatly disturbed during recolonization process, clonal growth is not sufficient to resist and the ongoing recovery of impacted areas are reset to bare sediment. This phenomenon was observed after the 2010 winter with a decrease of plant cover in the impacted plots, though the natural surrounding meadows persisted. It has been described for the region that winter swells can uproot *C. nodosa* plants creating meadow fragmentation (Tuya et al. 2013, Tuya et al. 2006). Furthermore, it is known that 60% of seagrass meadows in the Canary Islands have declined over the last ten years (Tuya et al. 2013), likely due to natural weather events and human actions (Halpern et al. 2008). Eutrophication, a factor known to negatively affect seagrass meadows (Hughes et al. 2009) was observed in Morro Jable (Pers. Obs.). Most likely this factor played an important role in the drastic biomass decrease across the meadow, in which the impacted plots were no exception.

In the North Atlantic and Mediterranean, *C. nodosa* was characterized according to IUCN Red List of Threatened Species as “least concern” and its population trend as “stable” (Short et al. 2011). In 2010, the Canarian Autonomous Government reduced *C. nodosa* protection status (BOC n112, Law 4/2010 of the Canarian Catalogue of Protected Species). A thorough study on the condition of *C. nodosa* meadows in the Canary Islands was conducted by Tuya el al. 2013, in where it was concluded that there is scarce data to support its conservation status, and given the observed decadal changes, the conservation status of *C. nodosa* should be re-evaluated. Our findings raise the importance for the protection of *C. nodosa* meadows in the Canary Islands.

Acknowledgements

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Supporting information

Figure 1: Removal plot in Los Cristianos meadow - Tenerife Island. To mark the plots, four staples (one per corner) were installed and a small buoy was attached to each.

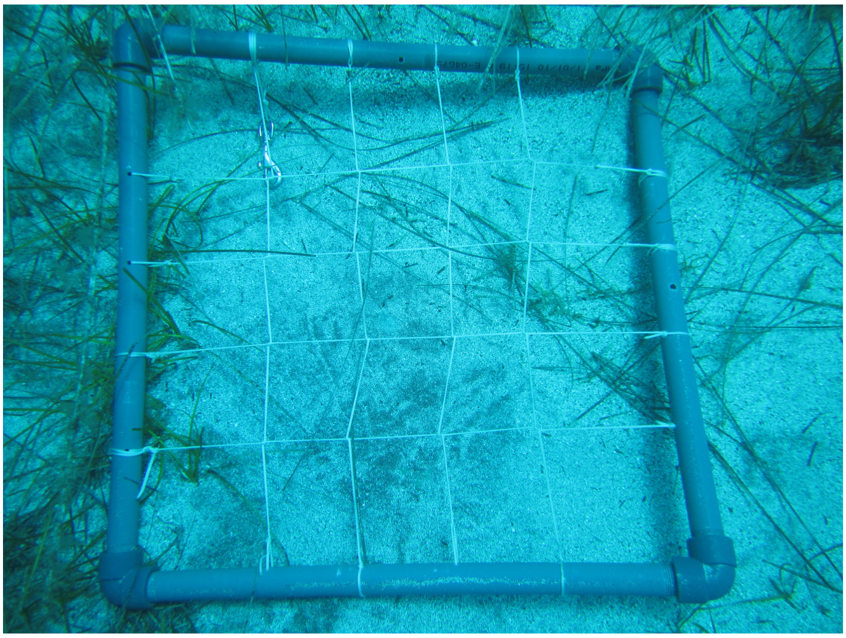

Figure 2: Sampling grid design to follow clonal growth and seedling presence inside the removed 0.25 m^2 *C. nodosa* areas.

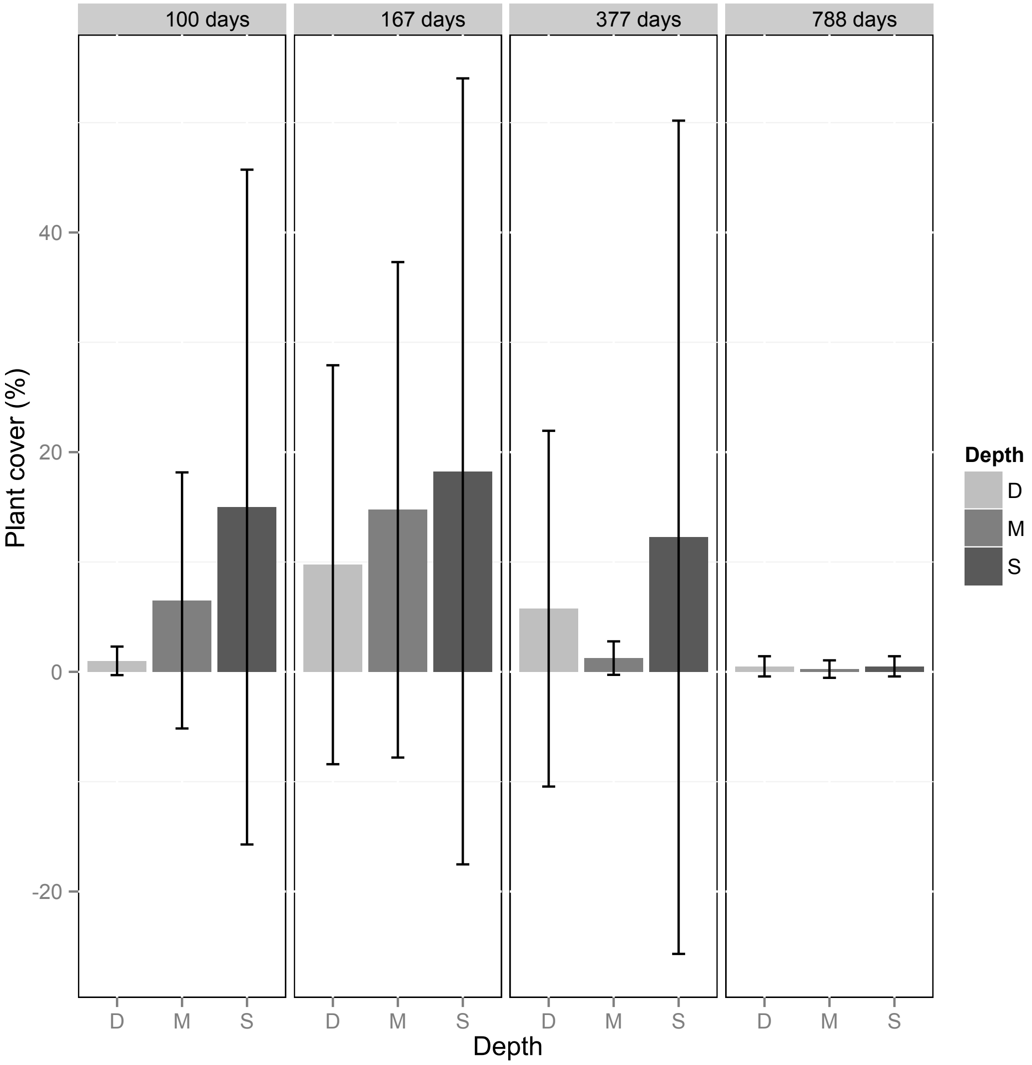

Figure 3: Average percent plant cover in the removal plots after 100 days (August 2010), 167 days (November 2010), 377 days (March 2011) and 788 days (May 2012) after physical disturbance. Depth levels represented by: D – Deep; M – Medium and S – Shallow.

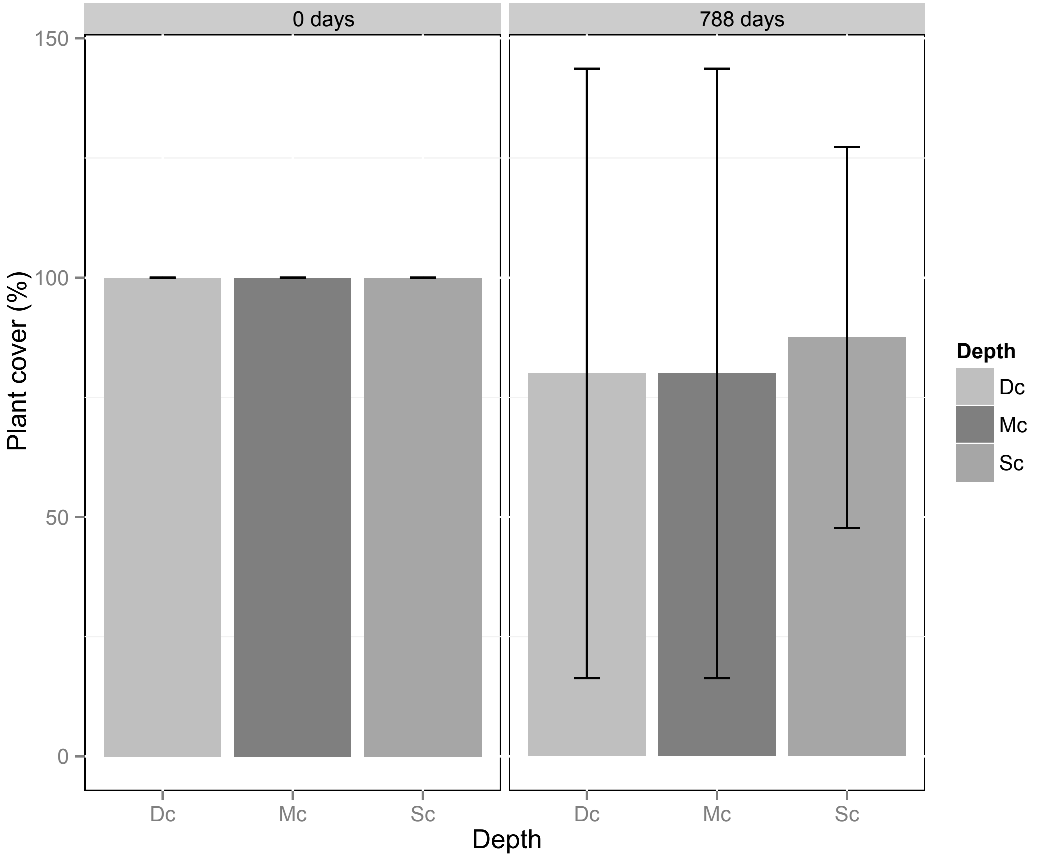

Figure 4: Average percent plant cover in the control meadow at the beginning of the experiment (May 2010) and at the end after 788 days (May 2012). Depth levels represented by: D – Deep; M – Medium and S – Shallow.

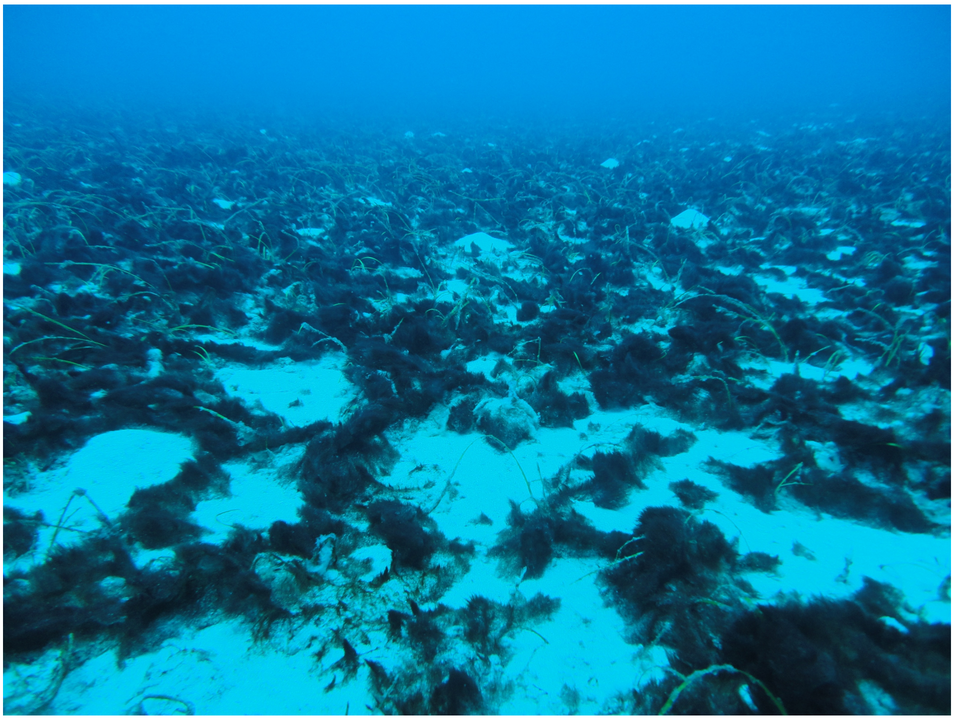

Figure 5: Epiphyte overgrowth at Morro Jable *C. nodosa* meadow at Fuerteventura Island, March 2011.

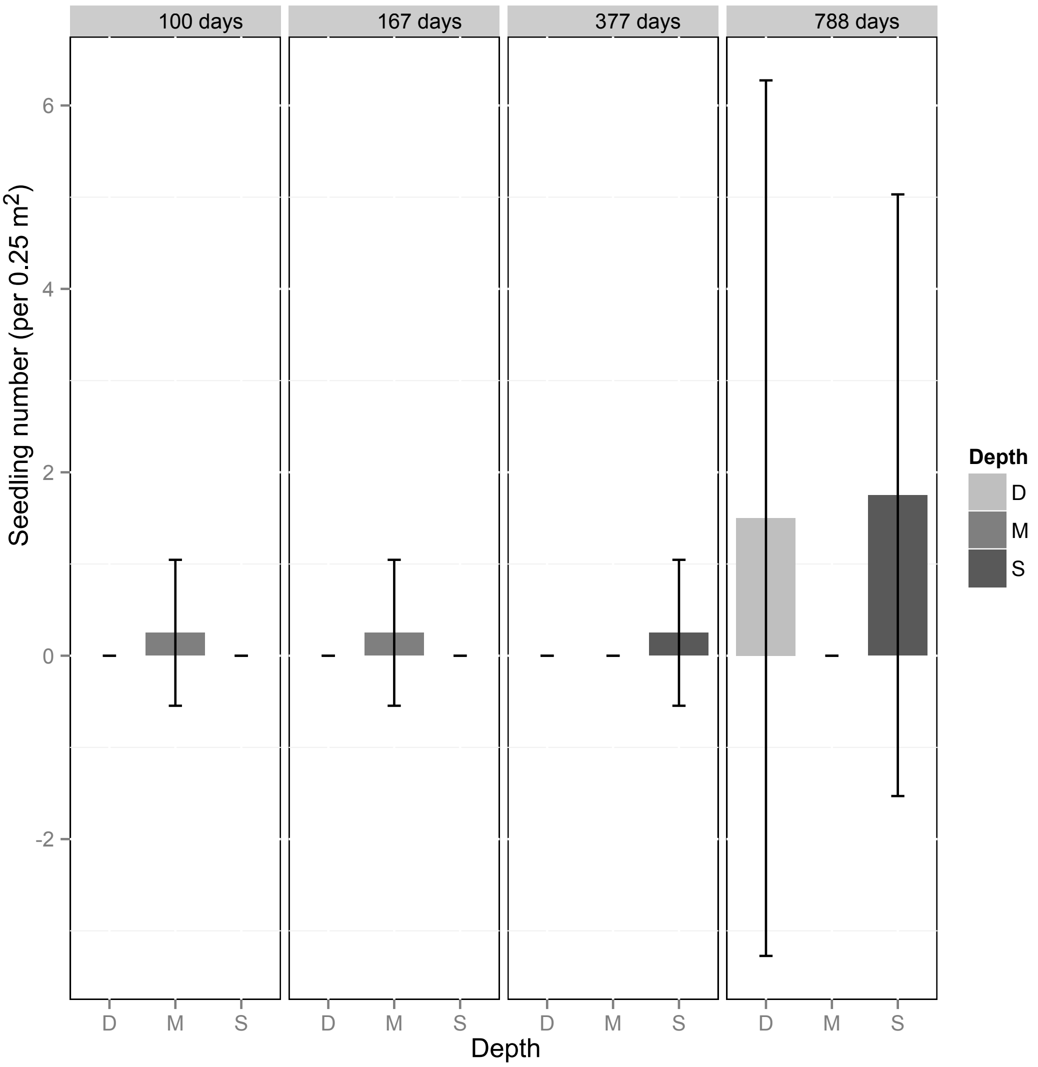

Figure 6: Average seedling numbers inside the removal plots (0.25 m^2) after 100 days (August 2010), 167 days (November 2010), 377 days (March 2011) and 788 days (May 2012) after physical disturbance. Depth levels represented by: D – Deep; M – Medium and S – Shallow.

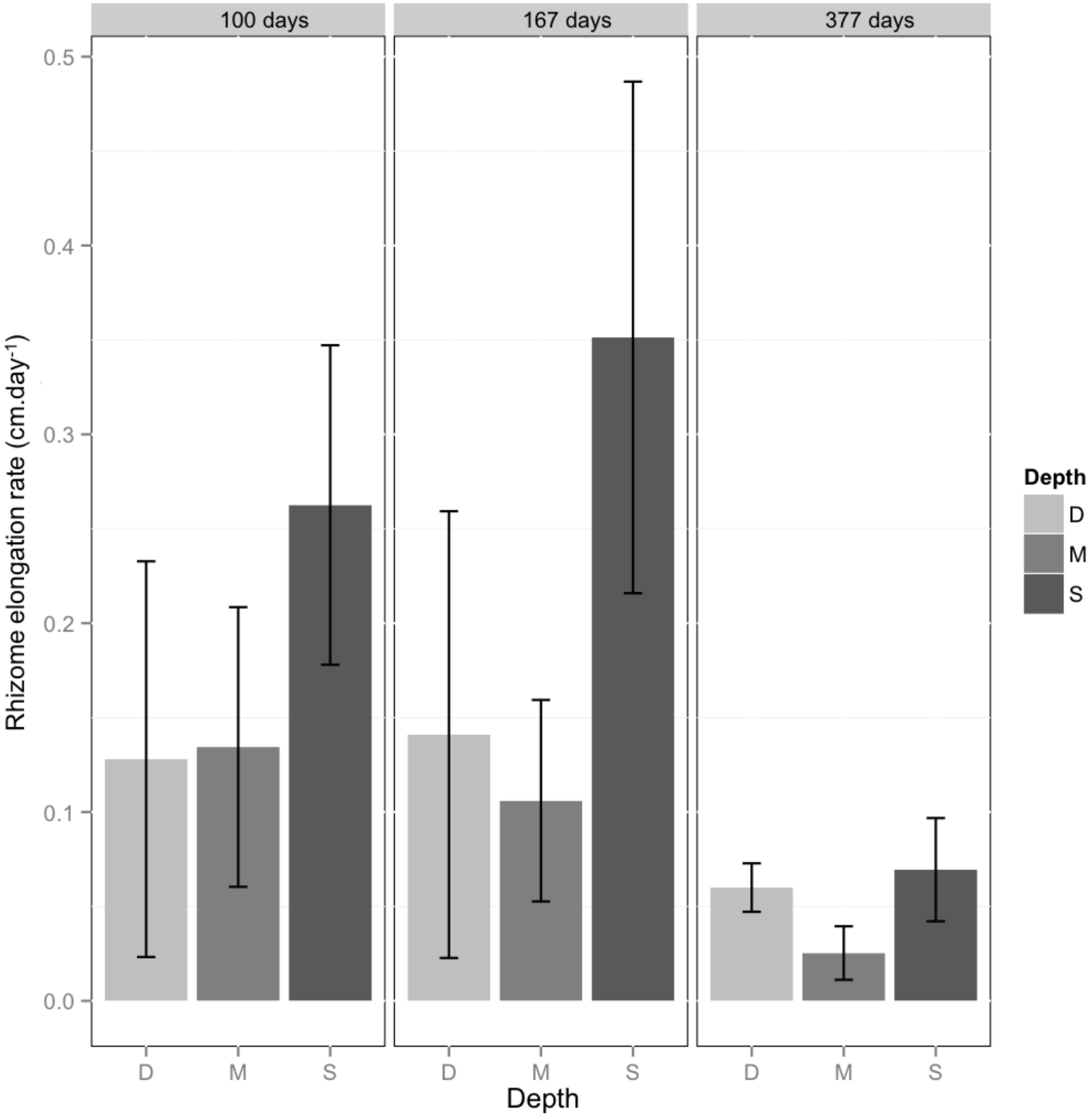

Figure 7: Average Rhizome Elongation Rate (cm.day^{-1}) in the removal plots after 100 days (August 2010), 167 days (November 2010) and 377 days (March 2011) after physical disturbance. Depth levels represented by: D – Deep; M – Medium and S – Shallow.

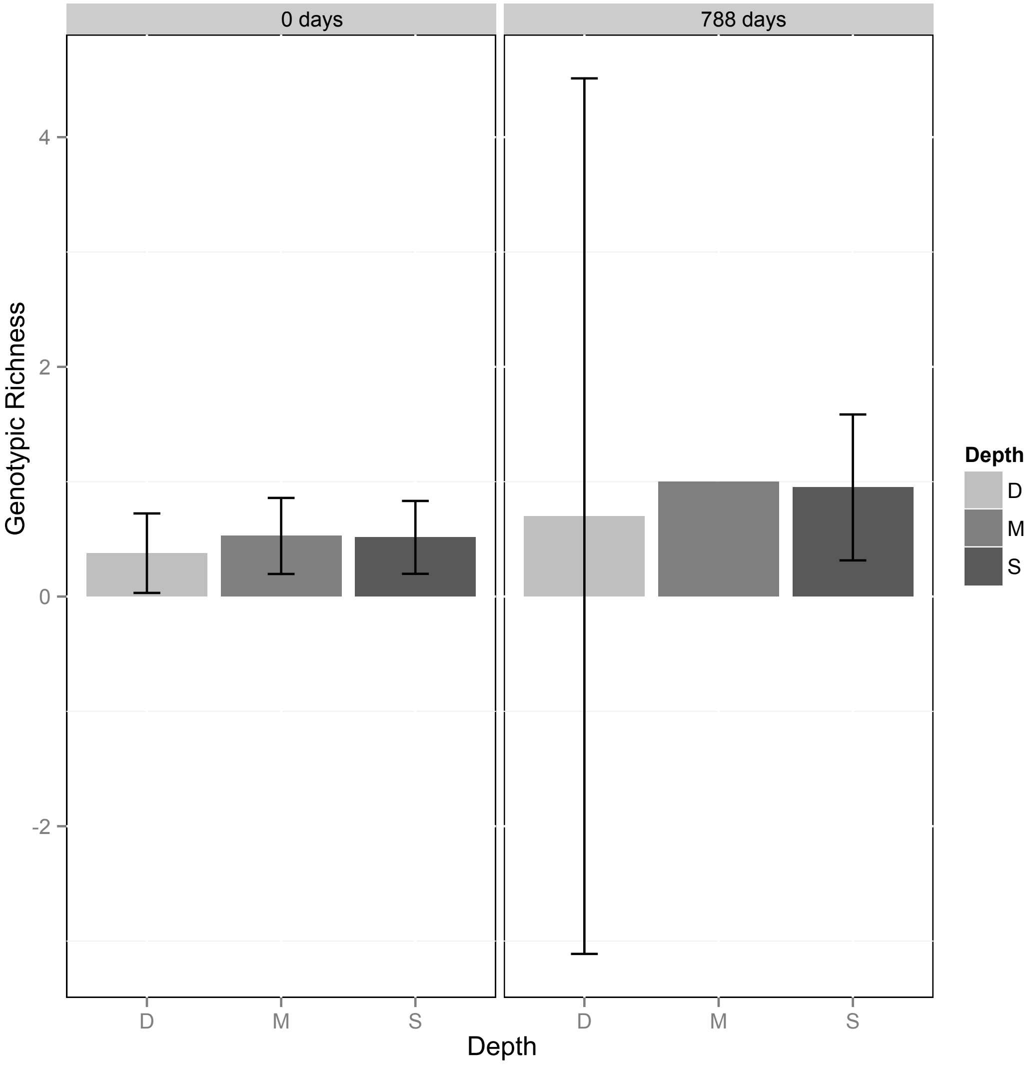

Figure 8: Average Genotypic Richness (*R*) in the removal plots in the beginning of the experiment (May 2010) and at the end after 788 days (March 2012). Depth levels represented by: D – Deep; M – Medium and S – Shallow.

Chapter IV: Sexual versus clonal propagation during recovery of a seagrass (*Zostera marina*) meadow from an extreme weather event.

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Abstract

Marine flowering plants can reproduce sexually and asexually, and the relative contribution of these two modes can be dependent on the environmental conditions. *Zostera marina*, a seagrass widely distributed in the northern hemisphere can form annual and perennial meadows with distinct proportions of sexual versus clonal propagation depending on the environmental disturbance regime. We study the hypothesis that the contribution of sexual propagation varies during the recovery of a seagrass meadow. In this case study, we compare the proportion of sexual versus clonal propagation of a perennial *Z. marina* meadow, before its disappearance due to winter storms and after recovery. Before disturbance, genotypic diversity was high, indicating frequent sexual reproduction events, likely to create an abundant seed bank. Seedling germination allowed the population to recover after the extreme disturbance. As months passed, seedlings became rare and finally absent, giving place to adult shoots. On an advanced stage of colonization, the shoots colonized the area by vegetative growth, which lowered the genotypic diversity. Despite this reduction over time, genotypic diversity of the new meadow is still high, demonstrating the importance of sexual reproduction in meadow recovery and persistence.

Key words

Recover from disturbance, *Zostera marina*, genotypic diversity

Introduction

Seagrasses are declining worldwide due to human and natural causes (Waycott et al. 2009), leading to loss of their important ecosystem functions (Hemminga & Duarte 2000). Disturbance and stress are well known environmental factors affecting successful plant establishment (Craine 2009). Catastrophic events in particular, can change seagrass cover area drastically (den Hartog 1987, Williams 1988, Larkum & den Hartog 1989), which may trigger energy allocation into sexual reproduction (e.g., Reusch 2006, Alexandre et al. 2005, Hammerstrom et al. 2006). Although clonal propagation is the major mode of space occupation for many seagrasses (Marbà & Duarte 1998), under some environmental conditions sexual reproduction can be very important in population persistence and in particular for recovery from disturbances (Deslous-Paoli & Dagault 2003, Greve et al. 2005, Bell et al. 2008, Park et al. 2011).

Several studies have focused on the relationship between genotypic diversity and resilience and resistance to perturbations and extreme stress events in seagrasses (e.g., Hughes & Stachowicz 2004, 2011, Reusch et al. 2005, Massa et al. 2013; Evans et al. 2017). These studies report that higher genotypic diversity can have several positive effects in the response to perturbations, including increased resistance to loss and faster recovery. A better understanding of how seagrass meadows recover from extreme events and of the relative role of sexual versus clonal propagation (i.e., genotypic diversity) may thus be an important concern in conservation and management decisions (Reusch et al. 2005, Qin et al. 2014). However, most of the evidence is based on short term experiments or at most a year of recovery. Very little is known about the relationship between genotypic diversity and disturbances in the long term, analysed at scales encompassing several years, therefore this paper aims to focus on understanding the relationship between sexual versus clonal propagation and disturbance along a multi-year time scale.

It is very rare to have the opportunity to follow seagrass meadow recovery before and after a disturbance due to multiple factors, particularly lack of previous knowledge on the meadow condition or when the meadow never recovers. In this study, we used as model a seagrass meadow that we had sampled prior to its disappearance, and we followed it after it recovered from disturbance. The meadow had high genotypic diversity (Diekmann & Serrão 2012); thus we raised the hypothesis of meadow recovery being mostly driven by seed banks.

Zostera marina (L.), a monoecious seagrass species, is the most widely distributed seagrass species in the northern hemisphere (den Hartog 1970), forming perennial and annual populations (Bos et al. 2007). Annual populations are characterized by high sexual allocation, where all vegetative shoots turn into flowering shoots, creating seed banks which can germinate when conditions are favourable (Keddy & Patriquin 1978, van Lent & Verschuure 1994, Meling-López & Ibarra-Obando 1999, Santamaría-Gallegos et al. 2000, Jarvis et al. 2014). The genotypic (i.e., clonal) diversity of annual meadows is therefore expected to be very high. Perennial populations are characterized by lower sexual allocation, and therefore lower genotypic diversity, with clonal growth being the favoured strategy for area occupation (Olesen & Sand-Jensen 1994, Olesen 1999, Kim et al. 2008). Despite these general predictions, high sexual allocation has been observed in some perennial populations (van Lent & Verschuure 1994, Meling-López & Ibarra-Obando 1999, Olesen 1999, Santamaría-Gallegos et al. 2000). Such unpredicted high sexual allocation in persistent populations may be linked to disturbance events (Cabaço & Santos 2012, Qin et al. 2014), such as storms.

The aim of this study is to assess how the importance of sexual and clonal reproduction varies before, during and after recovery from natural storm disturbance in a natural seagrass population followed over a 5 year period. We propose to test two alternative hypotheses that result in contrasting genotypic diversity in the recovered meadow; we hypothesize that in the long term, seagrass recovery may lead to either 1) clone dominance due to vegetative propagation being more effective than sexual reproduction, resulting in low genotypic diversity; or 2) high genotypic diversity due to recurrent seedling recruitment.

Methods

Location

The studied meadow is located at the tip of the Troia Peninsula (Ponta do Adoche) at the mouth of the Sado estuary, Portugal (38º29.525’ N 08º54.507’W, Fig. 1). The area is a sand point characterized by very strong tidal currents. The perennial *Z. marina* bed is located at the beach and starts at 1.5 m depth extending to 3 m depth.

The meadow had been monitored and sampled for population genetic analysis before its disappearance in 2008 (Diekmann & Serrão 2012). In March 2010, after intense winter storms, we observed that all 1.12 ha were lost (Cunha et al. 2013). *Z. marina* recovery was monitored during the spring and summer of 2010. By 2013 complete recovery of the total original area was observed (continuous seagrass cover of 0.57 ha) and was resampled for genetics.

Field work

The total area of the meadow was estimated in the spring of 2009 using GPS in tracking mode while following its contour from the surface in a good visibility day. In 2007, 2013 and 2015, the area was estimated using Google Earth images.

Before the disturbance, shoot density had been recorded in 2009. Immediately after the winter disturbance, we recorded seedling and shoot densities in April, June, and September 2010. The meadow was resampled in July 2013 (3 years after the disturbance), when cover was visually estimated to be back to 100% as before its disappearance. Monitoring was done by SCUBA divers along two 50 m transects perpendicular to shore. Systematically every 2 m along the transects, divers counted seedling and shoot density using a 25 x 25 cm quadrat at the interception point, and measured leaf lengths using a ruler, in a total of 25 quadrats per transect.

We performed ANOVA and, when necessary, Tukey tests (R core team 2017) to test before and after disturbance mean differences for the following variables: seedling density (seedling m^{-2}), shoot density (shoots m^{-2}) and leaf length (cm).

Genetics

Plant leaf collection was done haphazardly by SCUBA diving, maintaining a distance of 1.5 m in between sampled shoots. The pre-disturbance samples were collected in May 2008 (n = 83) and the post-recovery samples were collected in May 2013 (n = 31). Samples were conserved in silica gel desiccant.

Plant DNA was extracted following the CTAB method (Doyle & Doyle 1988). After extraction, two chloroform ⁄ isoamyl alcohol (24:1) extractions and one ethanol (100%) precipitation were used to purify the DNA. Samples were genotyped for eight microsatellite loci (Stam et al. 1999, Reusch 2000), and followed protocols of Reusch et al. (2000) for multiplexing. The analyses of fluorescently labelled PCR fragments were made on an ABI PRISM 3130 Genetic Analyser (Applied Biosystems) at CCMAR. The software STRAND (http://www.vgl.ucdavis.edu/informatics/strand.php) was used to score raw allele sizes. The R package MsatAllele (Alberto 2009) was used to bin the allele sizes, and ambiguities were manually reviewed.

The genotype data were analysed to estimate the number of sampled genets (G) for comparison with the number of sampled ramets (N= sample size). The probability that identical multilocus genotypes (MLG) were produced by sexual recombination (*Psex*) rather than being clones was estimated using the GenClone software (Arnaud-Haond & Belkhir 2007). The proportion of genets per population was estimated following Dorken & Eckert (2001): $R = (G-1) / (N-1)$. We also analysed both before and after disturbance samples together in GenClone to infer if we could find the same clones in both populations from distinct years.

Population genetic parameters were calculated after the removal of clone replicates for pre-disturbance and post-disturbance samples. The observed and expected heterozygosity (H_O; H_E) and the inbreeding coefficient (F_{IS}) were estimated using the software GenoDive (Meirmans & van Tienderen 2004) and F_{IS} were tested for significant difference from zero using a permutation test (Goudet 1995). Pairwise population differentiation (F_{ST}) was estimated and significance (difference from zero) was tested by permutations (AMOVA, Excoffier 1992, Michalakis & Excoffier 1996) using the same software.

Using the allele frequencies in the two samples, we calculated the likelihood of an individual genotype found in 2013 to be found in each of the samples (Paetkau et al. 1995) allowing us to assign from which sample each post-recovery individual is most likely to come from. Individual population assignment was inferred in Genodive (Mermaids).

Results

Before and after meadow comparison

Total meadow area: The seagrass meadow increased its area from 0.79 ha in 2007 to 1.12 ha in 2009. After disturbance in the winter of 2009/2010, seagrass cover was completely absent. Meadow recovery occurred throughout the next five years; in 2013 it had 0.57 ha and in 2015 reached 1.47 ha.

Seedling recruitment: In April 2010, the number of seedlings was significantly higher than in any of the subsequent monitoring times (June 2010, September 2010 and July 2013) (Tukey HSD $P < 0.05$ between all comparisons; Table 1; Figure 2). There were no differences in seedling density between June 2010, September 2010 and July 2013 (Tukey HSD $P > 0.05$ between all comparisons). Seedling density was maximum in April 2010 (10.2 seedlings m^{-2} +- 2.71 SE), decreasing to 0.07 seedlings m^{-2}(+- 0.04 SE) in June 2010 and no seedlings were detected in September 2010 and July 2013 (Fig. 2).

Clonal shoot density: Shoot density also varied along time, with an opposite trend relative to that of seedling density. It changed from 288.48 shoots m^{-2} (+- 19.30 SE) in July 2009 (before the disturbance), to total loss of shoots in February 2010. In that same year, in April only seedlings were observed, none of which had initiated clonal growth. By June 2010, shoot density was 12.62 (+- 3.91 SE) and by September it had nearly duplicated to 22.19 (+- 4.05 SE) shoots m^{-2}. Finally, the population reached densities similar to the pre-disturbance values in July 2013 (314.80 shoots m^{-2} +- 9.35 SE), representing 100% plant cover (Fig. 3).

There were significant shoot density differences between all dates (Tukey HSD $P < 0.05$) with the exception of June and September 2010 (Tukey HSD $P = 0.73$). Leaf length also increased significantly from April 2010 to July 2013, as shown by significant differences between all dates along the monitoring period (Tukey HSD $P < 0.05$) (Fig. 4).

Population genetic structure

Different clones were identified in the samples collected before and after disturbance. The genotypic diversity estimated decreased from R = 0.89 in 2008 to R = 0.60 in 2013 (Table 1). Based on allelic frequencies, the individual population assignment test tagged only a single individual from the post-disturbance population as belonging to the pre-disturbance population.

Expected and observed heterozygosity were similar in both samples (Permutation test, P = 1.00). Although the inbreeding coefficient was also similar between populations (Permutation test P = 1.00) the significant positive F_{IS} of the pre-disturbance population revealed heterozygote deficiency, whereas the post-disturbance did not show a deviation from Hardy-Weinberg equilibrium. The pairwise population differentiation fixation index F_{ST} between samples was 0.091 and not significantly different from zero (Permutation test P = 0.001).

Discussion

Our results show that both sexual and clonal reproduction of *Z. marina* played an important role, but contributed differentially, in different stages of recovery of a meadow from a severe natural disturbance event that had caused its disappearance. At an early stage, sexual reproduction played a crucial role in the meadow recovery, shown by seedling recruitment. It has been hypothesised (Orth et al. 2006) that the natural recover of *Z. marina* banks is likely to be possible due to small remnant stands. We have made a considerable effort to cover the total area that the seagrass meadow used to cover after its decay and no evidences of remaining shoots were found. Therefore our results demonstrated the important role of the seed bank in recovering a population in which all plants were lost.

On the long term, clonal growth increased shoot density increasing the vegetated area. This perennial meadow behaved as an annual meadow during extreme climate conditions. This demonstrates the species capacity to recover from large scale losses, via seedling recruitment, as similarly observed in other locations (Deslous-Paoli & Dagault 2003, Greve et al. 2005, Jarvis & Moore 2010, Jarvis et al. 2012, Kim et al. 2014, Qin et al. 2016). Most of all, it shows that the contribution of sexual and clonal propagation is a population trait that is very variable and this plasticity plays a key role in population persistence despite catastrophic disturbance events.

The new meadow was exclusively originated by sexual recombination from the previous population and not from seed dispersal from a differentiated population, as shown by the absence of genetic differentiation between the pre- and post-disturbance samples. All other populations in the region are very differentiated and separated by >100 km (Diekmann & Serrao 2012). Closer to this study site there were small patches within a distance viable for seed dispersal but only one which survived the storm, is a very small patch inside the Sado estuary but it does not contain sufficient genotypic diversity to have originated this new population (Diekmann & Serrao 2012).

Our study site is located at an extremely dynamic area, mainly due to strong tidal currents and storms during winter months. In such dynamic environments, high sexual allocation by seagrasses is well documented (Keddy & Patriquin 1978, van Lent & Verschuure 1994, Meling-López & Ibarra-Obando 1999, Santamaría-Gallegos et al. 2000). The high genotypic diversity found in the pre-disturbance population demonstrates that this meadow allocated resources into sexual reproduction in the years previous to disturbance. As a result, a seed bank allowed the population to recover. The observed reduction in genotypic diversity in 2013 shows that shoot recruitment from sexual propagules, which started as 100% seedlings (R = 1), was diluted with clonal growth over time. Nevertheless, the post-disturbance population genetic diversity was still high.

The pre-disturbance population showed heterozygosity deficiency, which may be explained by inbreeding (Diekmann & Serrão 2012), suggesting that in the past, sexual reproduction occurred between closely related individuals or selfing. The post-disturbance population, sampled after 3 years of recovery, had no heterozygote deficiency, indicating random mating. This change in mating system might hypothetically be a consequence of the different clonal structure of the population. Although male and female flowers in the same inflorescence are not mature in synchrony, those in different flowering shoots from the same clone can reproduce with each other (Reusch 2001). In contrast, in a recent population formed entirely by smaller clones, each clone is not likely to have many more flowering shoots than any other one.

Southern edge populations, such as the one studied here, are particularly important for the persistence and evolution of *Z. marina* as a species. These populations are small and reproductively isolated from others (Diekmann & Serrão 2012). Although at the worldwide scale, eelgrass conservation status is of least concern, the current trend is for populations to decline (Short el al. 2010). The southern populations in particular are considered highly endangered and many have recently disappeared (Cunha et al. 2013). As an example of the species vulnerability in this region of the world, 5 km northwest from our study area, 10 ha of eelgrass meadow were recently lost and did not recover naturally (Cunha et al. 2014). Due to the opportunistic nature of this research, we haven't got the opportunity to study sexual and clonal allocation. As future recommendations, we suggest the study of the sexual investment in this populations to better understand the chances of recolonization after localized extinction. Seedbank size should be assessed yearly, together with the meadow flowering rate, this information in vital to understand the chances of survival in case of another mass mortality event. Such knowledge can be used to plan seed based restoration programs (Tanner & Parham 2010, Marion & Orth 2010). Because *Z. marina* seed banks have a transient nature, the window of opportunity for recolonization might be short. Seed banks highest germination success is limited to 6 months and it is reduced to less than 5% in 15 months (Jarvis et al. 2014). To predict for how many years a recovering population is fragile to new disturbances, it is important to be address in future research the age to sexual maturity. Therefore, it is important to highlight that once a seagrass bed is removed conservation efforts should continue to assure the success of the new cohort of seedlings.

This study demonstrated that the relative roles of sexual versus clonal reproduction in semi-clonal organisms, such as seagrasses, vary with the temporal scales of population disturbance and are best understood when analysed on multi-year scales, and revealed also that sexual reproduction is of extreme importance in population maintenance and resilience in the face of unpredicted future climate regimes.

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Supporting information

Table 1: Comparing two *Z. marina* samples from before (Pre) and after (Post) disturbance. Number of ramets (N), number of genets (G), genotypic diversity (R), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}). Pre-disturbance population (Pre) and Post-disturbance population (Post). P- values calculated by Permutation test.

	Pre	Post	P-value
N	83	31	
G	74	19	
R	0.89	0.60	
H_O	0.479	0.497	1.000
H_E	0.547	0.530	1.000
F_{IS}	0.125	0.062	1.000

Figure 1: Portugal map showing the region studied. Troia peninsula with a star indicating the study site in Ponta do Adoche, followed before and after disturbance of a *Zostera marina* meadow.

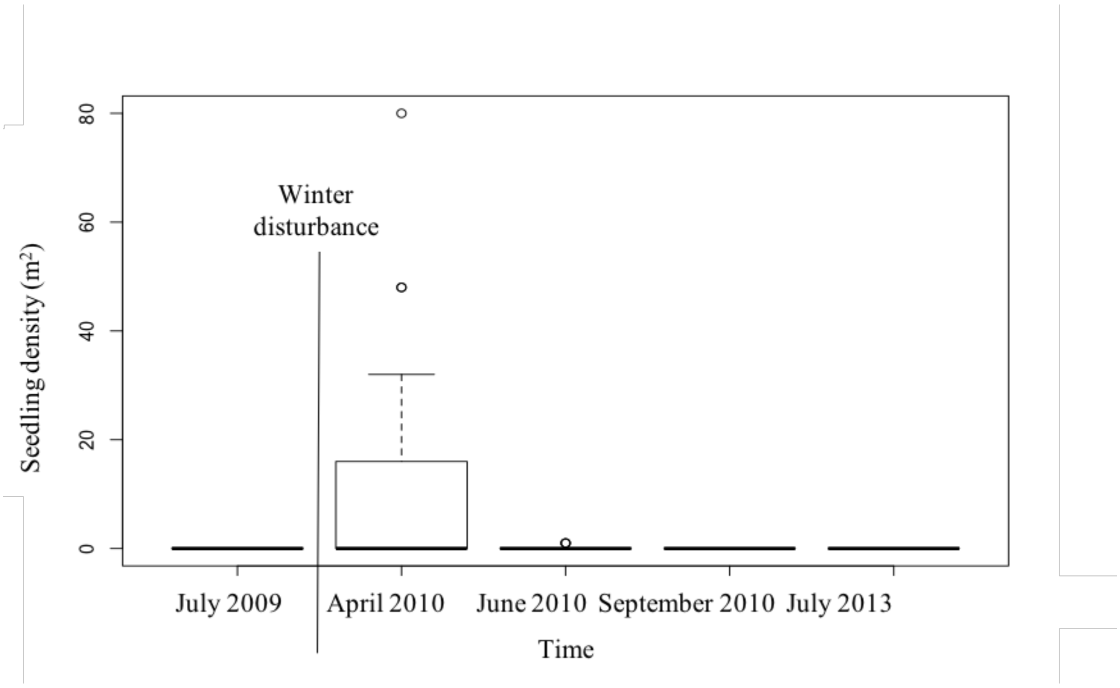

Figure 2: Seedling density (m^2) in a *Zostera marina* meadow at Ponta do Adoche sampled before disturbance, and after disturbance in four periods from April, 2010 to July 2013.

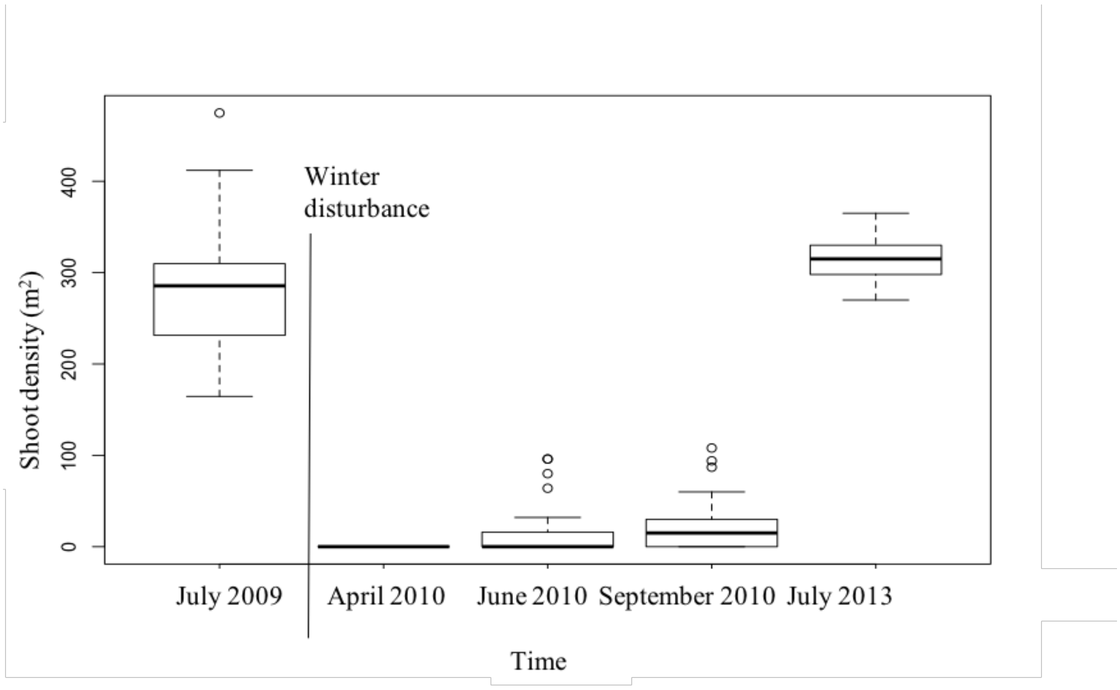

Figure 3: Shoot density (m^2) in a *Zostera marina* meadow at Ponta do Adoche sampled in July 2009, before disturbance, and after disturbance in four periods from April, 2010 to July 2013.

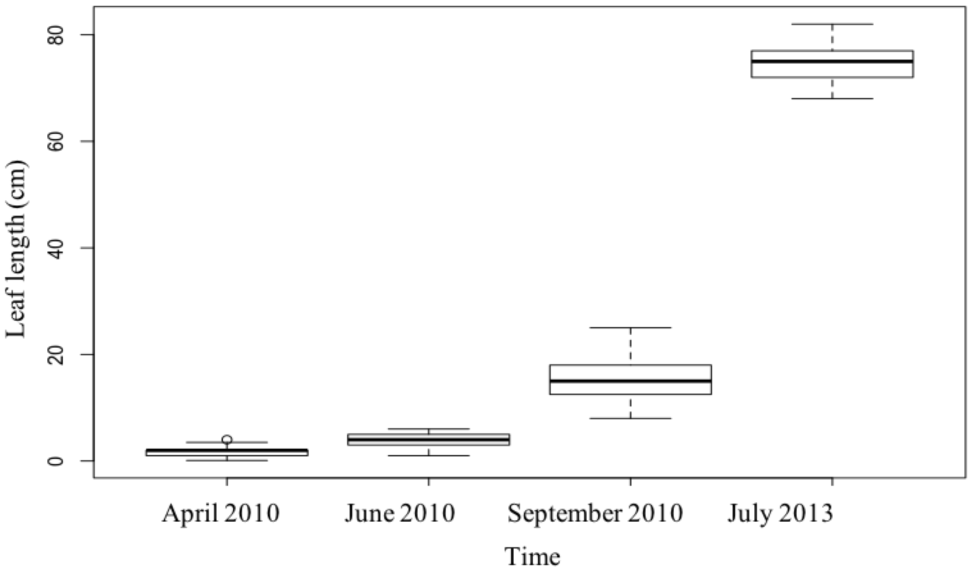

Figure 4: *Zostera marina* meadow leaf length (cm) at Ponta do Adoche measured after disturbance in four periods from April, 2010 to July 2013.

Chapter V: Open ocean seagrass transplants. Can we do it?

Authors

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Abstract

Some of the major challenges in open ocean seagrass restoration are the choice of transplant design that is optimal for coastlines periodically exposed to high water motion, and understanding the survival and dynamics of the transplanted areas on a long time scale over many years. To contribute to a better understanding of these challenges, we describe here part of a large-scale seagrass restoration program conducted in a Marine Park in Portugal, the results of which suggest hypotheses to be tested in future restoration studies. This open ocean site had lost all of its 30 ha of seagrass cover. A series of pilot studies were done to infer which transplant method would be more effective, revealing that blocks of plants in their sediment (sods) was the most suitable. The goal of this study was to recover the seagrass habitat that was lost, in order to continue to provide its ecosystem functions.

Factors assessed were donor seagrass species, transplant season and source location. The three species native to the region, *Zostera marina*, *Z. noltii* and *Cymodocea nodosa* were transplanted, with the objective to find which species would produce better long term persistence to recover the seagrass habitat. To test for the most suitable season, transplants were conducted in spring, summer and autumn. Two distinct source locations were tested: Ria Formosa coastal lagoon and Sado Estuary. Monitoring was done three times a year for 8 years, in which planting unit areas and densities were measured, to assess survival and growth. The best results were obtained with the species *Z. marina* transplanted from the location Sado Estuary during spring and summer. Long-term persistence of established (well rooted) transplants was mainly affected by extreme winter storms but there was evidence of fish grazing effects also. Our results indicate that persistence assessments should be done in the long term, as all transplants were successful (survived and grew) in the short term, but were not resistant in the long term after a winter with exceptionally strong storms. The interesting observation that only the largest (11 m^2) transplanted plot of *Z. marina* persisted over a long time, increasing to 103 m^2 in 8 years, overcoming storms and grazing, raise the hypothesis that for a successful shift to a vegetated state it might be necessary to overpass a minimum critical size or tipping point. This hypothesis was tested in 2017 with replicates from two donor populations and it was proven that bigger planting units from Ria Formosa persist and expand. Based on direct observation of a fast reduction of canopy height, grazing preference is pointed as an hypothesis to explain the fast decay of the transplants from the nearest donor population, Sado Estuary. It is suggested that in future habitat restoration efforts big planting units are considered and grazing preferences hypothesis is tested.

Keywords

Restoration; seagrass; transplanting.

Introduction

Seagrass restoration has been conducted for nearly 70 years, since the middle of the last century (e.g. Addy 1947). However, the vast majority of seagrass projects have been of limited extent (< 0.5 ha), often experimental and almost exclusively in sheltered estuarine waters. Restoration attempts in more wave exposed coasts are still few (Scott et al., 2004;

van Katwijk et al., 2009). To our knowledge, open-ocean large-scale, non-experimental restoration has only been attempted in Western Australia (Fonseca et al., 1998(b); Paling et al., 2003, 2007).

Open ocean restoration of seagrasses poses special challenges, both logistical and environmental (Paling et al., 2003), and the operation of divers and safety issues in open ocean settings raise costs significantly (Calumpong and Fonseca, 2001). In addition, high wave energy, whether stochastic or periodic, limits operations and can quickly erode planted areas or mobilize sediments that bury seagrasses, particularly for young meadows that have not yet reached sufficient abundance to reach an equilibrium with the disturbance regime (den Hartog, 1971; Patriquin, 1975; Fonseca et al., 1983; Marbá et al., 1994; Fonseca and Bell, 1998; Turner et al., 1999; Bryars, 2008).

Restoration of seagrasses represents an attempt to induce a change in ecological state, from a condition of low structural complexity (typically unvegetated seafloor) to a more complex form (vegetated). Theory on alternative stable states predicts that ecosystems can exist in a given location under multiple combinations of physical, chemical and biological conditions or “states” (Lewontin, 1969; Sutherland, 1974; May, 1977; Maxwell et al., 2015). These states are considered to be stable because they persist for relatively long periods of time and only shift from one state to another when key components are disturbed beyond a threshold characterizing their resistance to change (*sensu* Holling, 1973). Introduction of seagrass transplants qualifies as just such a perturbation. Once a state shift (persistent seagrass transplant) is achieved, the system is also characterized by resistance to revert back to the original state, even when the pressures that lead to the regime shift are released. However, factors governing stable states of seagrass beds are not well-known, as few studies of seagrass ecosystems quantitatively link disturbance regimes with seagrass responses on a landscape-scale (Duarte and Sand-Jensen, 1990; Kendrick et al., 1999; Kirkman and Kuo, 1990; Fonseca and Bell, 1998; Turner et al., 1999). Recent work by Maxwell et al. (2015) more clearly defines environmental thresholds that may drive seagrass coverage from one state to another. Therefore if seagrass restoration is viewed as an effort to catalyze a state shift (from an unvegetated to a vegetated state), the factors limiting that transition must be understood as a basis for setting realistic restoration goals and expectations. For restoration projects in physically dynamic environments, the challenge is to create resilient seagrass habitat before a perturbation could cross a critical environmental threshold and revert the system back to the bare sand condition; i.e., failure of the restoration effort.

Here we describe what became an adaptive process in an attempt to restore an open ocean (Atlantic) seagrass habitat in Portugal. What was once an extensive seagrass bed of approximately 30 ha, had disappeared completely by 2007, leaving unvegetated sandy seafloor (Cunha et al., 2012). Various scenarios were considered for how this transition came about. From historical photographic evidence (Fig. 1a) and anecdotal information obtained from local residents, it appeared that both mooring and anchor scarring from the seasonal influx of recreational vessels, and clam dredging prior to designation of Marine Protected Area status, likely combined to directly eliminate a significant part of the seagrass cover (Cunha et al., 2012). Simultaneously it drove the system to a fragmented state (as seen in aerial photographs) that may have been susceptible to collapse (*sensu* Scheffer et al., 2012). The vessel mooring impact scenario is a potential ‘threshold-like state response’ (Anderson et al., 2008) where the driver (mooring damage) continues to increase while the ecosystem state (seagrass cover) collapses below some critical threshold of cover needed to persist in this open ocean setting (*sensu* Fonseca and Bell, 1998). Alternative or additional scenarios included the possibility that local (bivalve) trawling practices would have contributed to bed patchiness and collapse if not outright full elimination of the seagrass cover. Such a scenario would fit Anderson et al.’s (2008) ‘driver-state hysteresis’ model because trawling pressure would likely drop as the resource was exhausted, but leaving the resource unable to recover to its previous state. These state change scenarios are rarely considered in seagrass restoration planning, largely because the drivers of previous state changes are, as seen here, often conjectural. However, consideration of these scenarios ultimately provided the basis upon which an adaptive decision was made to increase the initial size of the planting plot.

At the time the restoration project was planned an on-site sampling survey revealed one remaining patch of *Z. marina* covering approximately 60 m^2, in which only 4 distinct plants (i.e., genotypes) were found (Diekmann and Serrao, 2012). However, this last meadow did not survive the winter of 2006/2007 (Cunha et al., 2013), just before the start of the restoration project. The causes for this winter loss are unknown.

This paper reports on how various exploratory attempts shaped our understanding of the factors controlling the unvegetated state of the area. We describe the adaptive strategy employed for interpreting our results in an attempt to define and overcome unexpected stressors that limited seagrass recolonization and eventually led to persistent seagrass establishment.

The goal of the transplants was to bring seagrass habitat to the Marine Park again, to restore the ecosystem functions provided by seagrass habitat. Due to the exploratory nature of seagrass restoration in an exposed open costal area, we designed transplant areas to answer the following questions: 1) which seagrass species (among the three native species)? 2) which time of the year is best for transplanting? 3) which donor populations provide higher restoration success? and 4) with which initial transplant size?

Methods

The initial seagrass restoration goal followed traditional approaches which was to create as many patches as possible, spaced over the past seagrass area, to achieve patches over a large area that could eventually coalesce in the future. The initial approach consisted in spreading small, individual planting units with the intent that plant survival, rhizome spreading and meadow coalescence (with associated increasing stability) could occur to form a patch before a major storm or disturbance event. Monitoring occurred over an nine-year period, representing a very long time series as is rarely seen in such restoration efforts (van Katwijk, 2015).

Several seagrass species were used in the transplanting efforts because *Zostera marina*, the species that used to occupy the area (Palminha, 1958), was not initially (at the beginning of the project in 2007) very abundant in any of the possible donor populations, in contrast with the species *Zostera noltii* and *Cymodocea nodosa.* Therefore, to assess the optimal strategy to recolonize the area with seagrasses, all three species were used.

Site description

The Marine Park Professor Luiz Saldanha is part of the Arrábida Natural Park, which covers 52 km^2 and stretches over a 38 km coastline (Fig. 2). The coastline is rocky with high cliffs, punctuated by occasional sandy bays. These bays, with low sediment organic content (pers. obs.), include the previous seagrass area (Cunha et al., 2013) where the transplanting was performed (Portinho da Arrábida and Galapos Bay, Fig. 2)

In 2006, the restoration site was evaluated to meet seven key criteria (*sensu* Fonseca et al. 1998(a), Calumpong & Fonseca 2001): 1) Has the same water depths as nearby (Sado Estuary) natural seagrass meadows; 2) Had a history of having seagrasses; 3) Was not naturally recolonizing; 4) Had sufficient area to accommodate the desired transplant size; 5) Was not regularly disturbed; 6) Could support similar quality (seagrass) habitat; 7) Was no longer experiencing human impacts. The transplant site clearly met points 1-4 and presumably point 6. Points 5 and 7 are later revisited to evaluate their contribution to restoration performance in a qualitative retrospective analysis.

Donor populations

Two donor populations were selected for the transplant operation, due to their species composition, abundance and to a lesser degree, accessibility: (1) Sado Estuary and (2) Ria Formosa (Fig. 2). The Sado Estuary was the closest population, located only 5-10 km east of the transplant areas. At this site plants were harvested using a sod method, the sods were covered with wet cloth and transported by boat for ~30 min before being submersed on site at the Arrábida planting site until planted (within 24-36 h). In contrast, Ria Formosa is a coastal lagoon located 250 km south from the transplant area and was the only other location that had large amounts of seagrass coverage for all three species (Cunha et al., 2009). The sod method was used with plants collected at this site, which were similarly exposed to 30 min boat drive after harvest but were then loaded to trays with seawater covering the sods for a 3 h automobile drive until being submersed at the Arrábida site prior to planting (also within 24-36 h of arrival).

Harvesting method

Three different methods were tested in an initial optimization phase: sediment-free seagrass fixation with 1) staples or 2) mesh frames and a with-sediment method 3) sods containing seagrass in their natural sediment. These were utilized in small-scale test plots during 2007 and the sod method was selected as the best method for this environment. For the sod method, seagrasses were collected with their original sediment, in sections of approximately 20 x 20 x 5 (sediment depth) cm. Sods were harvested from water depths ranging from 1 to 5 m (depending on location and tide, Fig. 1c) by SCUBA divers using a small shovel and were placed in non-buoyant plastic trays (1 x 0.5 m). Harvest sites were spread across ~ 100 m of each donor area.

Transplant method

Because of the high failure rate of sediment-free methods (typically 100% within 1-3 months), here we report only on sod methods. Sods of seagrasses were planted as individual Planting Units (PU) at 1m from each other to create discrete test Plots. Fifty five sod transplants were done between 2008 and 2016, in spring, summer and autumn (including ten placement of sods conterminously in large patches; see below). Although, as this work was integrated in a habitat restoration project, many transplant areas were not replicated enough to create statistically testable data. Therefore, here we only report the planting efforts which were primarily used to test the key questions: what species, from which donor population, in which year season, would produce better long term results (Table 1).

At the transplant site, to minimize plant damage, sods were removed from the transport trays underwater by hand and placed into a depression in the sand created by the diver. The depth of the sod in the sediment was carefully aligned to match its natural vertical relationship to the sediment surface. After transplanting, the sediment removed to create the depression was gently redistributed amongst the sod and the adjacent seafloor to create a homogeneous elevation of the planting plot.

Transplant monitoring and statistics

Monitoring was done in winter, spring, summer and autumn over the period 2008-2016 or until no biomass was observed. The individual planting unit area (m^2) within a test plot was computed by measuring the two longest perpendicular axis of all individual planting units per plot. PU shoot density (shoots m^{-2}) was computed by counting shoots within a quadrat (25 x 25 cm) centred on the PU. An individual PU was considered a success if shoot density and/or area increased after planting.

To determine the most successful plot settings, three variables were analyzed and compared between transplant attempts: net change in PU area (m^2); net change in PU shoot density (shoots m^{-2}); and Growth, a binomial response based on the positive or negative output of area and or density.

To deal with the repeated measures nature of our sampling we used linear mixed models (LMM) in package nlme (Linear and nonlinear mixed effects models) from R (R core team 2014) to test differences in area (LMM 1) and density (LMM 2) as a function of the factors: species composition (3 levels), source population (2 levels) and time of the year (3 levels); including in the model a term for repeated measures for each Plot.

Growth was considered to be positive when either net area or net density had a positive result. However, by using a binomial response of Growth, the use of a LMM model was not adequate. To test differences in Growth a Generalized Linear Mixed Model (GLM) was built using function glm (generalized linear mixed model) in package glm2 from R (R core team 2015). This model differs from the previous used LMM in that it allows for the choice of a binomial data distribution.

Photosynthetic efficiency and effects of relocation

Because sources of seagrass for this project were obtained from two geographic areas at very different distances, the effect of relocation on the photosynthetic status of the plants was examined. Plants taken from the nearby Sado Estuary were less than 10 km distant and were quickly moved by boat. However, plants from Ria Formosa were approximately 250 km distant and had to be transferred to a surface vehicle and transported overland. If a substantial depression of photosynthetic efficiency was detected as a consequences of relocation, this could then affect the comparative analysis of source stock. For short distances, plants were placed in tubs and covered with wet towels. For the long-distance transport, plants are kept in plastic trays with seawater for periods of maximum 4 hours.

Maximum quantum yield of photosynthesis can be used as an indicator of plant recovery from adverse conditions (Hanelt 1992, Beer & Björk 2000, Malta et al. 2006), such as transplants. To calculate the maximum quantum yield of photosynthesis, an underwater pulse amplitude modulate fluorometer was used (Diving PAM, Walz). To maintain a constant distance between the PAM sensor and the plant in order to dark acclimate the tissue, leaf clips were attached at 0.5 cm above the base of the second youngest leaf of all plants. A far-red weak pulse was applied for five seconds (Hanelt 1998) to oxidate the electron transport chain, after which the shutters of the dark leaf clips were manually closed and plants acclimated for 10 min. Base fluorescence of chlorophyll *a* (F0) was obtained by turning on the PAM measuring light and opening the shutter. A saturating light pulse (≈ 5000 µmol photons m^{-2} s^{-1}) was applied for 0.6 seconds to measure maximum fluorescence (Fm), therefore variable fluorescence (Fv = Fm – F0) and maximum quantum yield (Fv/Fm) were calculated.

Maximum quantum yield was measured in 10 plants previously to harvest, after transport, immediately after transplant, 10, 15 and 30 days after the transplant in *Z. marina* and *Z. noltii* from Ria Formosa and Sado Estuary, and in *C. nodosa* from Ria Formosa monitoring finished at the end of 15 days. Previously to transplant Fv/Fm values were used as reference to be compared to the subsequent monitor timings. Differences between Fv/Fm in the harvest moment and along time were tested using One-way ANOVA test in R version 2.15.2 (The R Foundation for Statistical Computing, 2012).

Sampling design for Plots

To address the questions (1) which species in (2) what time of the year, from (3) which donor population, provides better restoration results we transplanted in autumn 2008 three seagrass species plots: *Z. marina* (PU = 10)*, Z. noltii* (PU = 10) and *C. nodosa* (PU = 20). Due to natural donor population areas and densities we have opted to do this test with plants form Ria Formosa Coastal Lagoon. After knowing which species had better transplant results, we chose to transplant *Z. marina* plots from Sado Estuary during autumn (PU = 27), spring (PU = 27) and summer (PU = 27), to test the best season. Donor population source was tested with *Z. marina* plots collected in Ria Formosa Coastal Lagoon (PU = 27) and Sado Estuary (PU = 27) during summer.

Mega Plots

In spring 2010 we have attempted to overcome the 2009 winter storms impacts by creating two mega plots with one planting unit each of 11 m^2 initial transplant area. Mega plots donor populations were Sado Estuary and Ria Formosa. Unfortunately, at this time the main funding source for transplant initiatives was coming to an end. Due to scarce funding, logistic complexity and the loss of one donor population (Sado Estuary) as a consequence of the same storms that have impacted the transplants, it was only possible to set a pilot test. Therefore, not enough replicates were secured to test a new theory of the effect of minimum patch size in long term persistence.

In spring 2017, with new funding, a new experiment was set to test minimum patch size with four *Zostera marina* plots: two plots with three planting units of 6 m^2 initial size; and two plots with three planting units of 0.04 m^2 initial size. Half of the plants (three big and three small planting units) came from Ria Formosa and the other half from Sado Estuary. The plots were all transplanted in the vicinity of the pilot test transplanted in 2010. The experiment was monitored periodically in spring, summer, autumn, winter 2017 and spring 2018. Monitoring included measuring the height (cm) of ten shoots per planting unit to determine canopy height. Density was determined by using three 25x25 cm quadrats in the PU with 6m^2 initial area. A non-parametric Wilcoxon test was performed to compare differences between the canopy height and density along the monitoring times of the surviving PU (R core team 2014).

Results

Testing species

From the three transplanted plots in autumn 2008, *Z. noltii* persisted 100 days not surpassing 2008 winter; *Z. marina* persisted 500 days; and *C. nodosa* 400 days. None of the transplants survived the 2009 winter storms. The species that had highest growth was *Z. marina* (GLM $P < 0.01$ between the two other species), followed by *C. nodosa* which had higher growth than *Z. noltii* (GLM $P < 0.03$). Net area variation was different between all species (LME $P < 0.01$ between *Z. marina* and the two other species; and LME $P < 0.01$ between *C. nodosa* and *Z. noltii*). The species *Z. marina* had the biggest net area increase (Fig. 3). The lowest net density was measured in *Z. noltii* (LME $P < 0.01$), there were no differences between *Z. marina* and *C. nodosa* net densities (LME $P > 0.90$) (Fig. 4).

Testing transplant season

Based on the 2008 transplant results (that compared species), the species chosen to test transplant season was *Z. marina*. Due to logistic considerations (less distante), a nearby (~ 10 km) Sado Estuary meadow was used as donor population for testing season. The spring transplant persisted 290 days, the summer one 260 days and the autumn transplant 170 days. Again none of the transplants resisted the 2009 winter. Before transplant abrupt senescence, there was positive growth in all. In the first 100 days after transplant there was no difference in growth between treatments. The autumn transplant had lower growth than the spring and summer ones (GLM $P < 0.01$). No growth differences were found between summer and spring transplants (GLM $P > 0.86$). Spring transplants generated bigger areas than summer and autumn ones (LME $P < 0.01$). No net area differences were found between summer and autumn (LME $P > 0.78$) (Fig. 5). Net density variation was similar for transplants done in summer and spring (LME $P > 0.80$). Autumn transplants had a lower net density increase compared with summer and spring (LME $P < 0.01$) (Fig. 6).

Testing donor population

Both transplants persisted 250 days, but similarly to the other tests, plants did not survive the 2009 winter storms. Growth was significantly higher in Sado *Z. marina* transplants than Ria Formosa ones (GLM $P < 0.01$). However, net area and density variations were not significantly different between different source populations (LME $P > 0.05$; LME $P > 0.062$) (Fig. 7 and 8).

Photosynthetic efficiency and effects of relocation

Plants maximum quantum yield was measured as a photosynthetic efficiency indicator at different stages of transplant operations. *Z. marina* plants collected in Ria Formosa showed a significant increase (ANOVA $P < 0.01$) in maximum quantum yield between the harvest and 30 days after transplant. The maximum quantum yield of *Z. marina* collected in Sado Estuary was significantly lower after transport (ANOVA $P < 0.01$), increasing until 15 days after transplant from which it stabilized in to values equal to the initial harvest moment. *Z. noltii* collected in Ria Formosa had no significant difference in maximum quantum yield during all the experiment (ANOVA $P > 0.05$). *Z. noltii* from Sado Estuary had a significant decrease in Fv/Fm between harvest and the end of the transport (ANOVA $P < 0.01$). After transplant

Fv/Fm increased to values not different from that observed at harvest time (ANOVA $P > 0.98$) until last monitoring 30 days after planted. *C. nodosa* maximum quantum yield increased significantly 1 day and 15 days after transplant (ANOVA $P < 0.01$) (Fig. 9)

Mega Plots

Based on hundreds of hours of *in-situ* observations by at least a dozen different scientists, several human and environmental factors, appear to have affected long term transplant persistence at the Arrábida MPA. However, one specific *Z. marina* plot harvested in Ria Formosa, set in spring 2010 as a pilot test with 11 m^2 initial PU size, appears to have mitigated many of the impacts that were eventually identified as limiting seagrass colonization in the bay (Fig. 1g). This plot increased almost ten times its initial area from 11 m^2 in 2010 to 103 m^2 in 2018 (Fig. 10).

The small plots (0.04 m^2) transplanted in spring 2017 from Ria Formosa and Sado Estuary were absent in summer 2017 time of the first monitoring. As expected the bigger plots persisted for a longer period but with different outcomes between the donor populations.

The transplants from the closest donor population (Sado Estuary) decreased area from spring 2017 to autumn 2017 and did not survived winter 2017. In autumn 2017 average canopy height was 3.4 cm (SE 1.9 cm) revealing intense grazing activity, which was also observed visually in the leaves. Canopy height and plant density decreased consistently along the monitoring time, following the same trend as the vegetated area.

The three big transplants form Ria Formosa donor population were more resilient than the ones from Sado Estuary. Although one planting unit did not survived 2017 winter, the remaining two planting units increased the initial vegetated area from 6 m^2 in spring 2017 to 8 and 9 m^2 in spring 2018. Canopy high and plant density changed along the monitoring time in the survival plots (fig. 11 and Fig. 12). Plant density was equal in between summer 2017 – winter 2017, summer 2017 - spring 2018, and winter 2017 - spring 2018 (Wilcox test P-value > 0.05). Plant density minimum values were detected in spring 2017 and maximum in autumn 2017. Canopy heights were equal in between spring 2017 - 2018 and spring 2017 – summer 2017 (Wilcox test P-value > 0.05). Minimum canopy height was detected in winter 2017 and maximum in autumn 2017.

Discussion

In our case study, all seagrass transplants presented growth, both in the form of area and or density increase. Seagrasses acclimated rapidly to local conditions as indicated by maximum quantum yield as a proxy for good physiological state. The results demonstrate the general suitability of the local conditions for the seagrasses transplanted since they all survived and grew. However, this initial success was not verified in the very long term (8 years, which encompassed strong winter storms), highlighting how persistence assessments can have distinct results depending on the time scale of the evaluation conducted. The results also showed that species, donor populations and transplant season influenced the possibility to colonize new unvegetated areas with the same level of performance. The most successful case coincided with using the seagrass species previously found at the site (*Z. marina*), from one particular donor population (Ria Formosa), transplanted during spring, which is the season that maximizes growth and minimizes impacts of water motion.

Our initial expectations of having higher transplant success with plants originating from the closest donor population (Sado Estuary located at approximately 5 km distance) were not confirmed. The lack of an impact to the photosynthetic capacity of the plants demonstrated not only that there was no apparent bias in our evaluation of transplant performance as the result of different handling but also revealed that seagrasses can be successfully transplanted over very long distances.

Almost all seagrass restoration projects face the problem of shifting the state of the seafloor from an unvegetated to a vegetated state (Fonseca, 2011; van Katwijk et al., 2015), achieving such changes under wave exposed conditions is particularly daunting given that extreme wave energy acts as an external driver holding the site in a persistent unvegetated state (*sensu* den Hartog, 1971; Calumpong and Fonseca, 2001). In this case however, the historical presence of seagrass in these open ocean settings and the success of seagrass transplanting in even higher wave energy regimes (Paling et al., 2003) suggested the possibility of successful restoration.

Numerous human impacts in the transplant sites were identified during the eight years of monitoring. As the study unfolded, we concluded that two key initial site selection criteria had not been fully met: was not regularly disturbed (criterion #5) and was no longer experiencing human impact (criterion #7). The site was observed to be disturbed by unpredictable large storms in winter and was still experiencing illegal human impacts such as boat moorings, despite existing regulations and our own project measures against those. Establishing a pilot study that could effectively exclude human impacts prior to the development of future seagrass restoration project may help to forecast such effects (Cunha et al., 2012).

There is no evidence that grazing had been a factor influencing the loss of the seagrass meadow but it might have contributed to prevent recovery. Through observation (Fig. 1e) and periodic underwater video deployments (not shown) heavy grazing impacts by the sparid fish *Sarpa salpa* were observed. Grazing by *S. salpa* has been identified to have a top down control on seagrass meadows modifying habitat structure (Pagès et al., 2012) and has been shown to limit seagrass in MPA settings in the Mediterranean (Ferrari et al., 2008). We presume that in large seagrass areas, such as the one previously existing at this site, grazing pressure would not be a key factor hindering seagrass persistence because grazing pressure would have been widely distributed. However, in a colonizing situation with seagrass as small patches as in this study, grazing by *S. salpa* was observed frequently, with both plant leaves and sheaths grazed down to within centimetres of the sediment surface, often within 24 h of planting. Additionally, heavy fouling of grazing exclusion cages (not part of the plot responses described here) by the invasive rhodophyta *Asparagopsis armata* (Bonnemaisoniaceae) (Fig. 1f) appeared to cause the cages to become dislodged which prevented their usefulness (data not shown).

Storms have been identified as a major threat to restoration of seagrass ecosystems (Calumpong and Fonseca, 2001). During winter months increased wave action, particularly those associated with a large storm in 2009 resulted in the relocation of sediments which in some cases covered plants completely, reverting vegetated to non-vegetated seafloor. Similar effects were observed in the source population, a naturally-occurring *Z. marina* meadow in the nearby Sado Estuary (Fig. 2) during the same 2009 storm event, resulting in the loss of approximately 3 ha of seagrass (pers. obs.).

The mega plot pilot test results suggest that under specific conditions (particularly with initial creation of large patch sizes), *Z. marina* transplanted form Ria Formosa may surpass a threshold that shifts unvegetated areas to stable states of vegetated seafloor. The minimum initial patch size hypothesis was later tested and confirmed. This findings bring important information for future efforts, suggesting that it may be better to concentrate efforts in achieving larger initial sizes rather than spreading the effort in scattered smaller planting units over a larger area. From direct observations we hypothesise that *Z. marina* from the nearby Sado Estuary were more grazed than the ones from the far donor site Ria Formosa. Both population PUs showed grazing evidences, as an alternative explanation to grazing preferences, plants from Ria Formosa may have a better capacity to recover from grazing in comparison to Sado Estuary plants. Future research should be focused on grazing preferences and recover from grazing pressure between the two donor populations.

This study reports a case in which we attempted to initiate a state shift from bare sediment to seagrass cover, which was successfully achieved but not in the majority of the attempts. We raise the hypothesis that these results could be case examples of regime shifts between different ecosystem states. In this location, seagrass previously formed homogenous and stable seafloor coverage that was resistant to the natural levels of grazing and storms. Past human-induced disturbance that fragmented the seagrass meadows may have pushed it past a tipping point that is now very difficult to reverse. Secondary factors such as storms and grazing, that used to be present also in the previous ecosystem state now might be acting against the possibility of seagrass recovery from very small patches. Alone, these may not have been able to influence the seagrass meadow in the previous stable state. Given that seagrass transplants survived well initially and were robust to transport, and because the successful restoration was achieved only in the case which used the largest planting unit size, it appears this open ocean area is receptive to seagrass recolonization and that the largest hurdle to overcome is post-planting disturbance from storms and grazing. We suggest that future restoration attempts consider the use of large initial transplant units (in this case over several square meters in size) increasing the likelihood to resist the current disturbance regime.

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Supporting information

Figure 1: a - Restoration site before plant disappearance, with clear evidence of mooring impact over the seagrass community (the bare patches around each mooring); b – same geographic area as previous image, in 2007 when restoration started, in which no seagrass cover is observed; c – diver transplanting seagrass in sods, from a tray to the restoration site; d – anchor found impacting directly over a recent transplant; e – evidence of herbivory by *Sarpa salpa*; f – invasive seaweed *Asparagopsis armata* covering part of a transplant, also evident the bite marks on the leaves; g – transplant with initial size bigger than 1 m^2; h - transplant with initial size smaller than 1 m^2. Images *a* and *b* are from unknown authors.

Figure 2: Coast of Portugal showing the geographical location of the project site. The continuous line identifies the limits of the marine protected area of the Arrábida Natural Park. Black star indicates the restoration area. Open stars indicate donor populations: Ria Formosa (250 km south) and Sado Estuary (ca. 5 km eastwards).

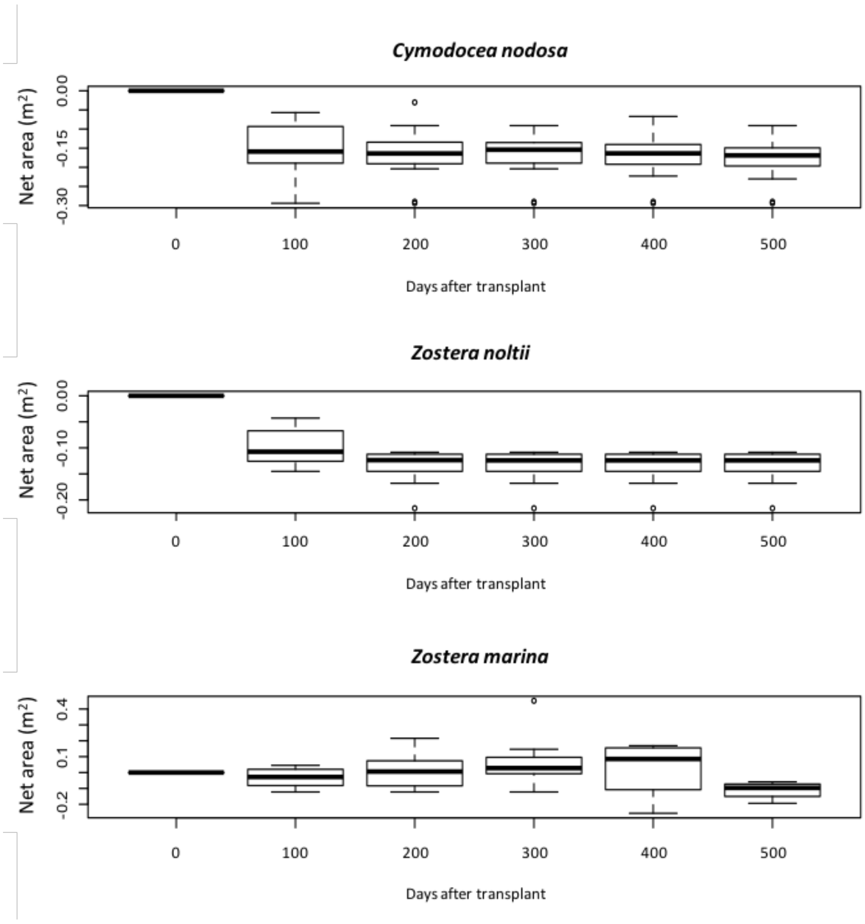

Figure 3: Net planting unit area in m^2 measured along time (0, 100, 200, 300, 400 and 500 days) for *Z. marina, Z. noltii* and *C. nodosa*, transplanted in autumn from Ria Formosa Coastal Lagoon.

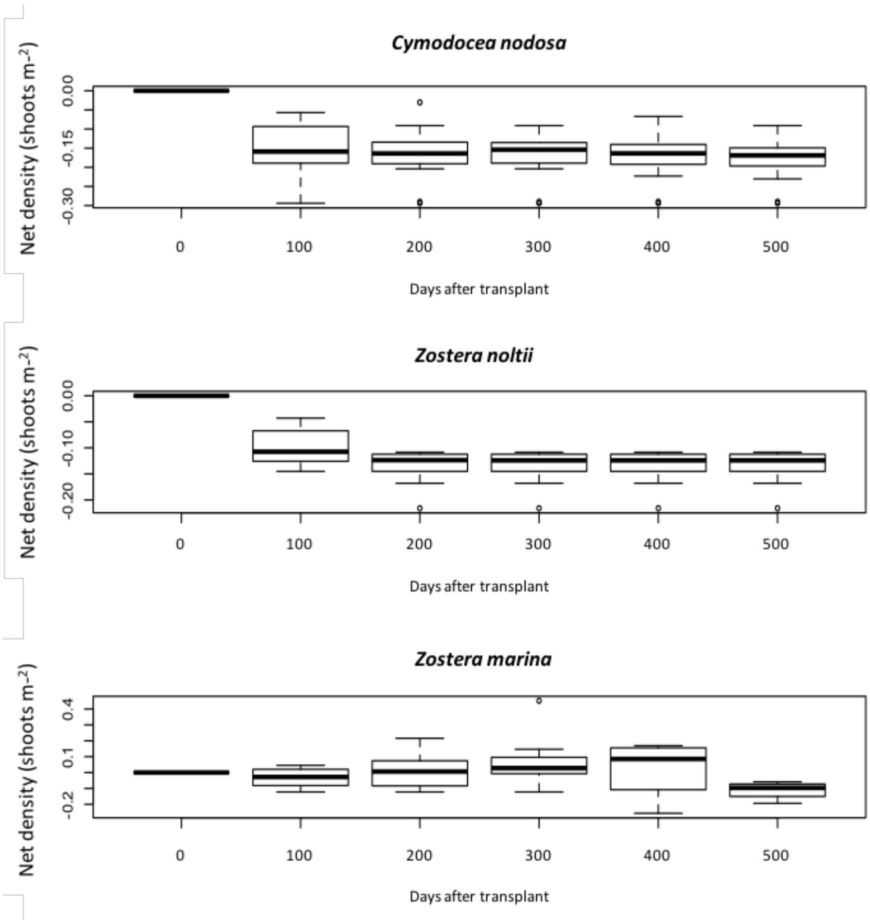

Figure 4: Net planting unit net density (shoots m-^{2}) measured along time (0, 100, 200, 300, 400 and 500 days) for *Z. marina, Z. noltii* and *C. nodosa*, transplanted in autumn from Ria Formosa Coastal Lagoon.

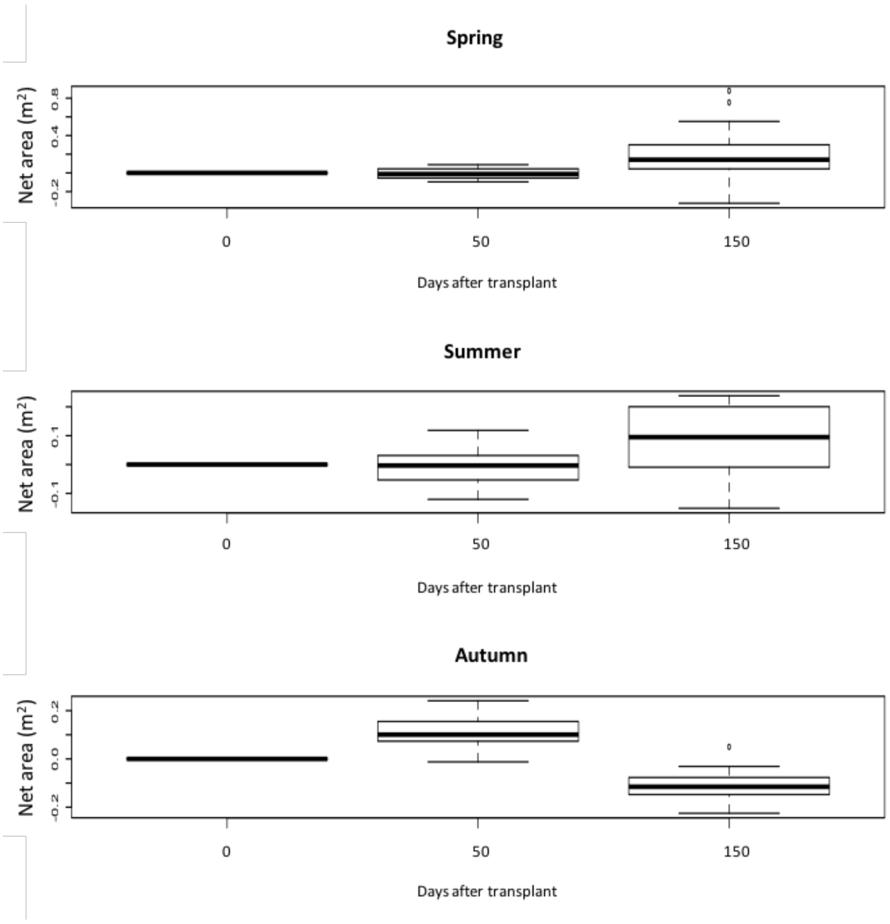

Figure 5: *Zostera marina* net area (m^2) variation along time (0, 50 and 150 days) transplanted in different year periods: autumn, spring, summer.

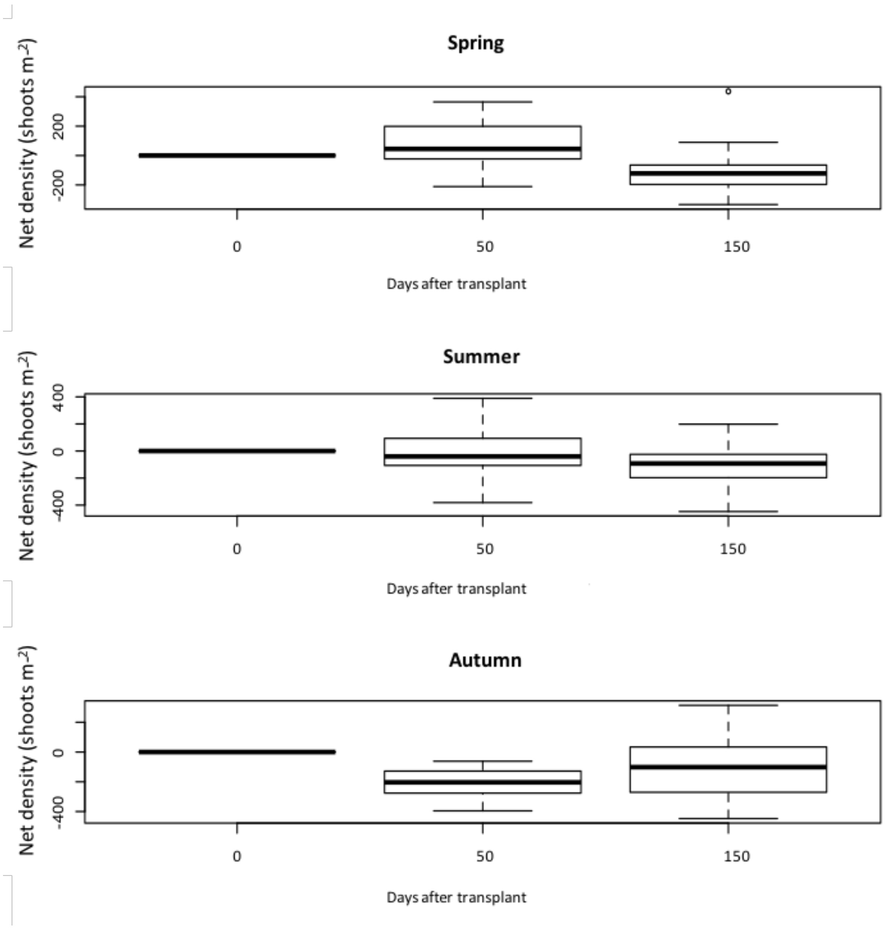

Figure 6: *Zostera marina* net density (shoots m^{-2}) variation along time (0, 50 and 150 days) transplanted in different year periods: autumn, spring, summer.

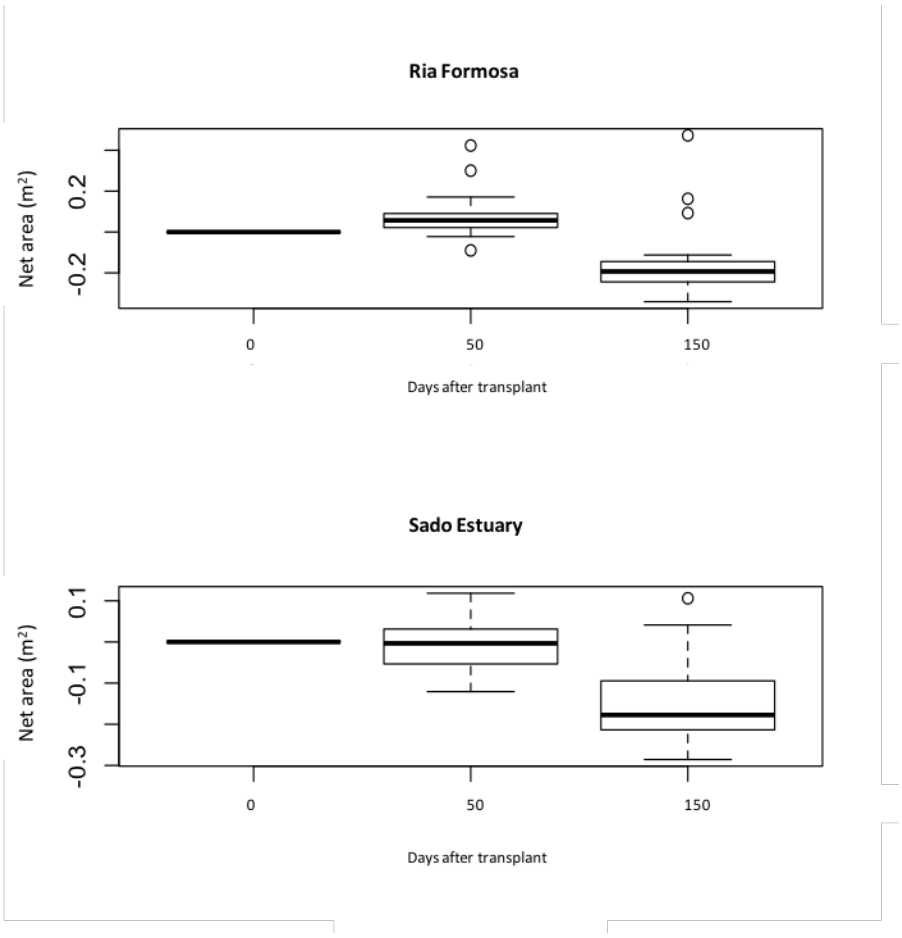

Figure 7. *Zostera marina* net area (m^2) variation along time (0, 50 and 150 days) when transplanted from different donor population sites: Ria Formosa Coastal Lagoon and Sado Estuary.

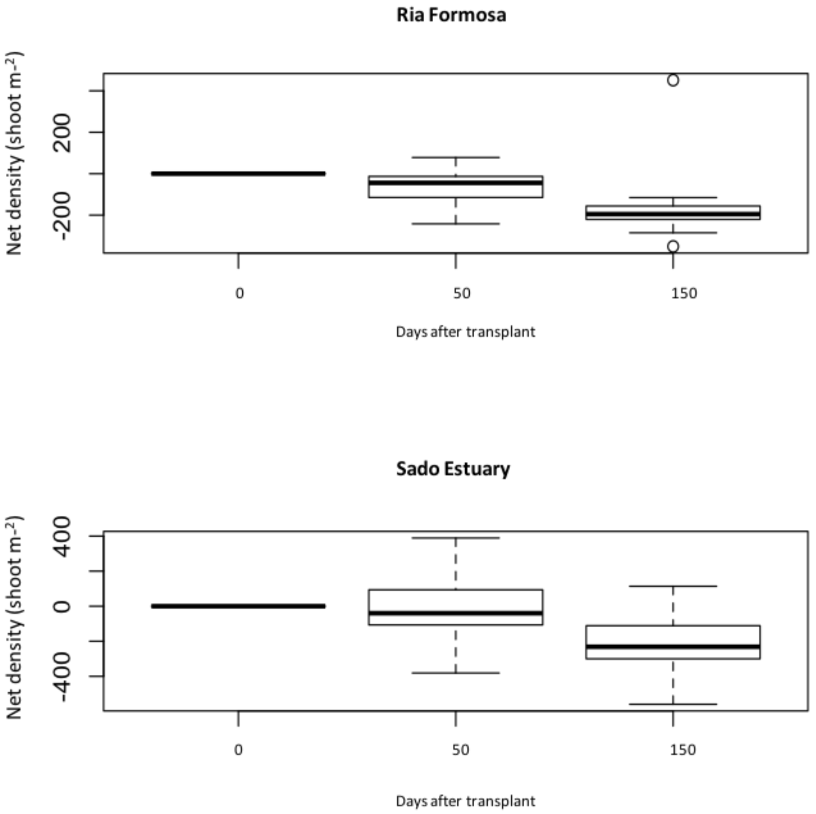

Figure 8. *Zostera marina* net density (shoots m-^{2}) variation along time (0, 50 and 150 days) when transplanted from too different donor population sites: Ria Formosa Coastal Lagoon and Sado Estuary.

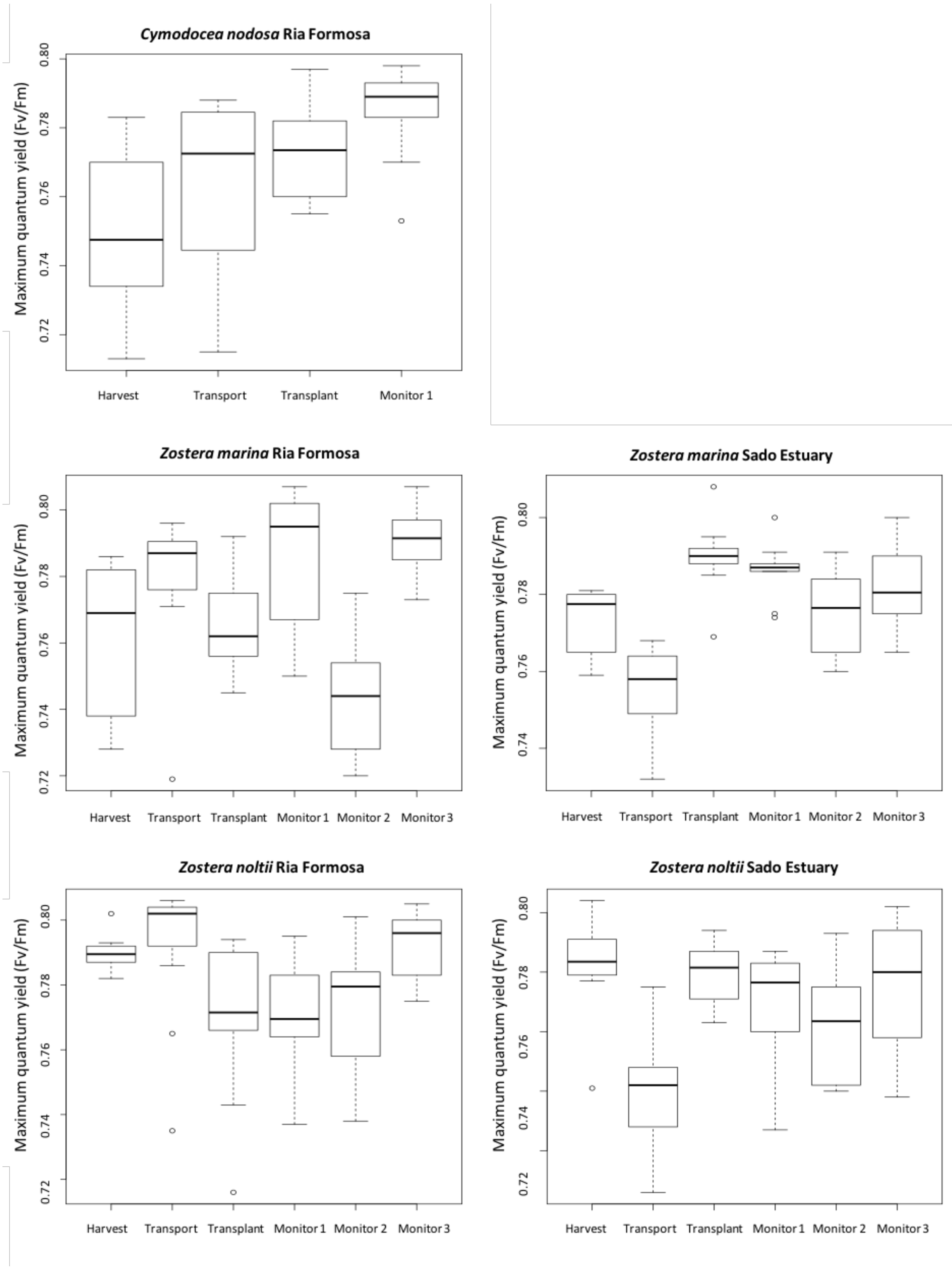

Figure 9: Maximum quantum yield (Fv/Fm) calculated for *Zostera marina* and *Z. noltii* harvested in Ria Formosa and Sado Estuary; and *Cymodocea nodosa* harvested in Sado Estuary. Fluorescence measures were taken during harvest, at the end of transport, after transplant, 10, 15 and 30 days after transplant for all treatments with the exception of *C. nodosa* in which the monitoring ended at the end of 15 days.

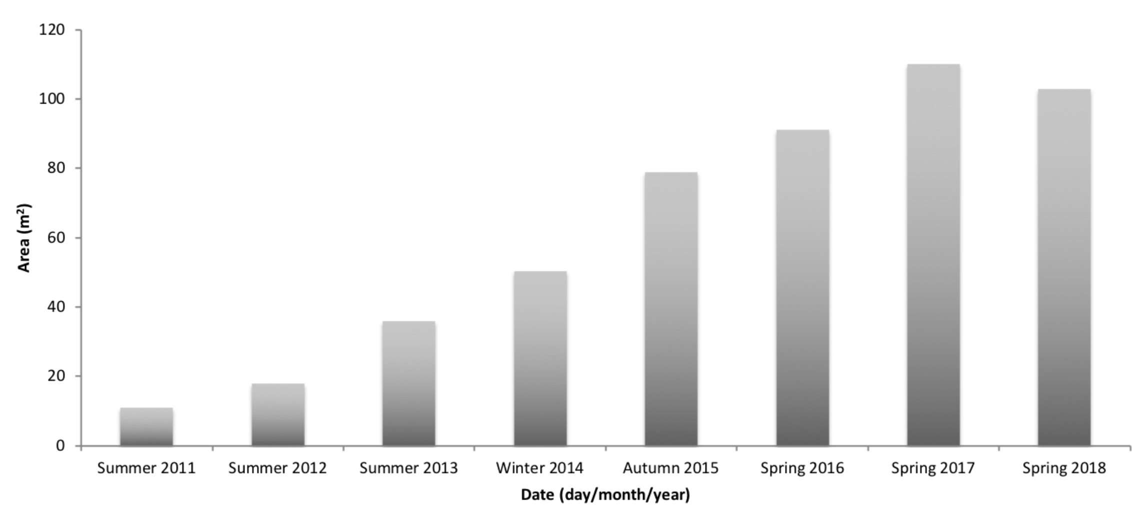

Figure 10: Area (m^2) increase along time of a *Zostera marina* plot, transplanted in spring 2010 from Ria Formosa donor population with an initial size of 11 m^2, which persists till this date (June 2018).

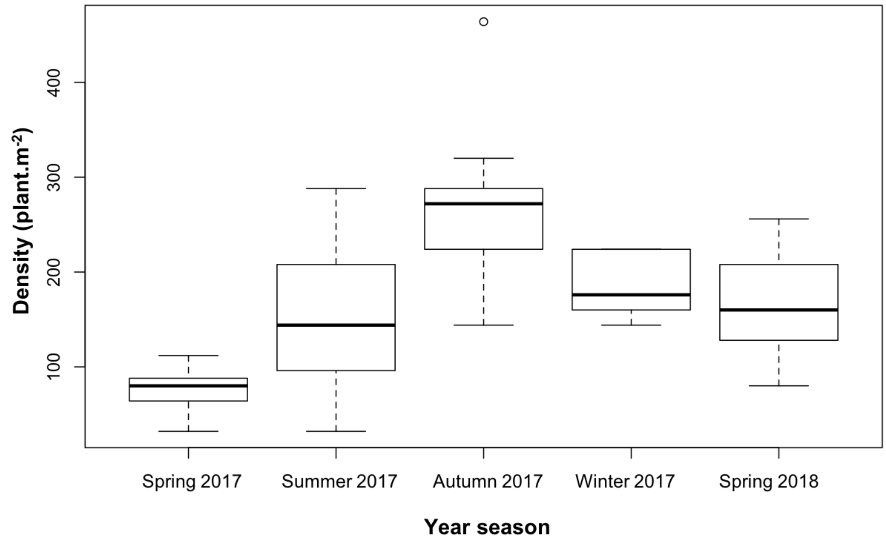

Figure 11: Density (plants.m^{-2}) variation along one year in a *Z. marina* plot from Ria Formosa transplanted in PMPLS in 2017, with three planting units of 6 m^2 initial transplant size.

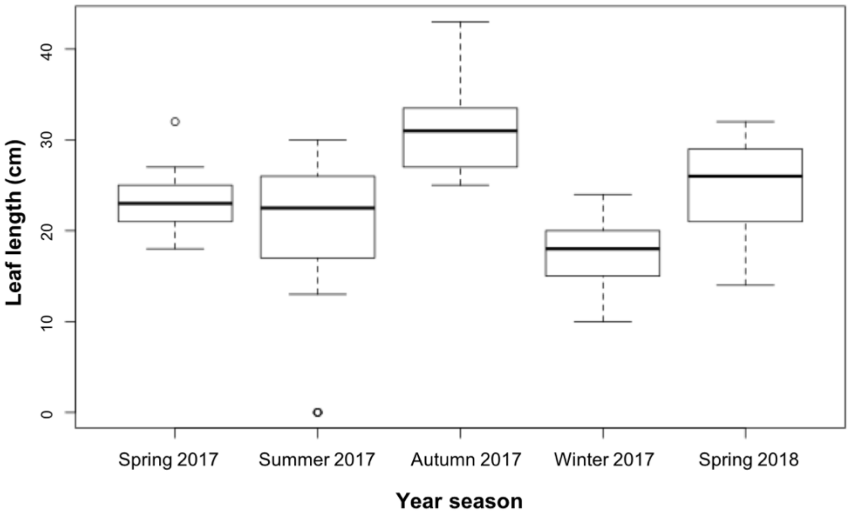

Figure 12: *Zostera marina* canopy height variation in the plot from Ria Formosa transplanted in the PMPLS in spring 2017 with three planting units of 6 m^2 initial transplant size. Canopy height monitoring over a year, once per season.

Table 1: Number of planting units transplanted per plot (Plot number in plot), of a given species (Species), of a given species (Species), in a year (Year) and year season (Season) which was collected from a source population (Population): Ria Formosa (Ria) or Sado estuary (Sado).

Plot number per plot	Year	Species	Season	Population
20	2008	*C. nodosa*	Autumn	Ria
10	2008	*Z. noltii*	Autumn	Ria
10	2008	*Z. marina*	Autumn	Ria
27	2009	*Z. marina*	Spring	Sado
27	2009	*Z. marina*	Summer	Sado
27	2009	*Z. marina*	Autumn	Sado
27	2009	*Z. marina*	Summer	Ria

Final Considerations

Sexual and clonal reproduction balance, along a depth gradient

The seagrass *C. nodosa* is well adapted to a range of environmental conditions, as proven by the heterogeneous genotypic richness and demographic pattern along depth gradients in the Canary Islands. Although there is evidence of higher sexual allocation at shallow depths (Reyes et al. 1995), I have found that hydrodynamic disturbance removes seedlings at that depth level. In this thesis, it is demonstrated that depth does not affect the balance between clonal and sexual reproduction in *C. nodosa* in the Canary Islands.

It was expected that as depth increases, plant physiological stress would also increase due to light attenuation. As depth decreases, physiological stress should also decrease, but simultaneously disturbance would increase due to hydrodynamic impact. At mid-range depths, a balance between disturbance and stress was expected, setting the optimal conditions for seagrass growth and establishment (Grime 1977). In the studied *C. nodosa* meadows, there was evidence of higher sexual allocation at shallow depths, where seed banks and flowering rates were reported to be higher (Reyes et al. 1995). It was thus hypothesized that sexual reproduction would decrease along the depth gradient due to high energy requirements (Silva et al. 2013, Olesen et al. 2002). My results have proven that seedling growth and survival was not affected by depth increase (Chapter 1), rejecting the hypothesis. At the shallow limits, were hydrodynamics creates periodical disturbance (impact), the persistency of early plant development stages is reduced due to seedling un-rooting. Hydrodynamic impact leads to limited *C. nodosa* seagrass recruitment success. Seed banks and higher flowering rates are likely to be an adaptation to frequent. In the studied meadow, higher sexual allocation did not represent an effective increase of genetically distinct individuals. Higher seedling survival rates were observed at medium and deeper depths, suggesting that sexual allocation can be more effective at this depth level when compared with shallower depths.

The previous findings lead to question if the demographic and genetic structure along the depth gradient was in fact stratified as suggested by other authors (Zarranz et al. 2010, Reyes et al. 1995). After sampling in four *C. nodosa* meadows in the Canary Islands (two in

Fuerteventura and two in Tenerife) it was obvious that meadows genetic diversity and demography was heterogeneous along the depth gradient (Chapter 2). Differences in between sites and depth levels are yet to be fully understood, as local adaptation and stochasticity are likely to play an important role in the observed pattern. With this study, it became clear that demographic and genetic heterogeneity is the reflect of how well this species is adapted to environmental changes. Seagrass meadow dynamics and persistency are dependent on the tight integration of population demography with the environment, which reflects the balance between plant reproductive modes.

Sexual and clonal reproduction roles in meadow recovery from perturbation

Sexual reproduction is crucial in the re-colonization process after extreme perturbation. When conditions are favourable, seeds can germinate and occupy available space, created due to unfavourable conditions (ex. Storm impact). During the initial re-colonization stage if a new impact occurs the meadow faces serious likelihood of failing to restore, as time greatly limits seed viability and germination success (Jarvis et al. 2014). Clonal growth is of extreme importance following seed germination, it increases density in the re-occupied areas by seedlings, contributing to meadow stability and resilience. When disturbance is not extreme and only parts of the meadow are affected, clonal growth allows for faster re-colonization. Nevertheless, successive impacts may limit successful colonization, in such situations re-colonization may be dependent on the integration of both sexual modes.

In a dynamic environment like the open ocean, seagrass meadows are subject to frequent disturbance events, which result in periodic biomass reduction. Also, un-expected events of disturbance (perturbation) can be created by big storms, land runoffs, aquacultures and other human actions, fragmenting the seagrass meadows into patches (Dalton 2004, Halpern et al. 2008, Tuya et al. 2014). When environmental conditions become favourable, the available space can potentially be colonized by the same or other species. In the case of seagrass meadows, the expectation would be that a balance between the two life strategies (sexual and clonal) would contribute to re-colonize the available areas. Unfortunately, in some cases the continuous impact over perturbed areas can reset the recolonization process back to bare sediment (Chapter 3). In the Canary Islands, *C. nodosa* did not recover from perturbation at any tested depths. In the first months after perturbation the disturbed areas were steadily recovering due to favourable plant growth conditions and absence of impact events. However, after the first winter storms the re-colonization process was re-set, transforming the perturbed areas back to bare sediment. Although full recovery did not happen during the monitoring time, genotypic diversity increased between the initial and final monitoring times in the perturbed areas, indicating that sexual reproduction is a key life trait in the maintenance and persistency of *C. nodosa* meadows.

Extreme disturbance events can seriously modify seagrass meadow landscape, to the point of complete biomass loss, as it happened to a 1.12 ha *Z. marina* meadow in the Sado Estuary after an intense storm period. The perturbation was so strong that it changed the seagrass habitat to bare sediment. Subsequent monitoring of the area revealed that the population started to recover from perturbation via seed bank, as described in other locations for annual *Z. marina* populations (i.e. Deslous-Paoli & Dagault 2003, Greve et al. 2005, Jarvis et al. 2012, Kim et al. 2014, Qin et al. 2016), and latter via clonal growth. Such an adaptation, from an annual to perennial life strategy, to our knowledge had not yet been documented (Chapter 4). Sexual reproduction played a crucial role in shifting the habitat from un-vegetated to vegetated state. In the long term, clonal growth contributed greatly for density and area increase, has confirmed by analysis of genotypic diversity.

This thesis proved that the balance between reproduction modes is of extreme importance for seagrass population maintenance and resilience, for both dioecious and monoecious species. While sexual reproduction was important to shift stable states (unvegetated to vegetated), clonal growth was crucial in the persistency and stability of the meadows. Given the importance of sexual reproduction in early stages of disturbance recovery, it is important to gather more knowledge on seagrass seed survival and germination success, targeting its application for seed based restoration programs (Tanner and Parham 2010, Marion and Orth 2010). When in a recovery process, seagrass impacted/perturbed areas are easily re-impacted, reducing its chances of a successful re-colonization. Therefore, it is suggested that conservation measures are taken in order to reduce all non-natural impacts during such periods. It is of extreme relevance that the survival and persistency of the new cohort is ensured, the formation of new seed banks is crucial to ensure that seagrass population have a chance to recover naturally from disturbance events.

When the ability to use seeds as an initial re-colonization resource is not possible, clonal growth will be the only way to attempt re-colonization. It is likely that for the re-colonization process to be successful via clonal growth, environmental conditions must to be stable until the impacted area is re-established. I have found that the most likely threshold conditioning the success of clonal re-colonization in *C. nodosa* in the Canary Islands is perturbation due to winter storms, which are likely to impact the recovering areas resetting it back to bare sediment. In the Canary Islands *C. nodosa* was subject of a research concluding that the meadows biomass suffered a reduction between 40% to 60% in ten years (Tuya el al. 2014). Therefore, in populations where sexual reproduction is limited (low genotypic diversity) or absent (i.e. as in Ria Formosa costal lagoon; Alberto et al. 2001) natural recolonization after perturbation may be unsuccessful.

The *Z. marina* meadow studied in Portugal in chapter 4 is at the species southern distribution, and unexpectedly this population is highly genotypically diverse (Diekmann et al. 2012), which has likely enabled for a successful natural recolonization after strong perturbation (Chapter 4). This provides support to the hypothesis that genotypic diversity (i.e., high sexual contribution) increases populations chances to survive to abrupt changes, enabling the persistence of the species. Therefore, the protection of such diverse populations is very important to ensure the survival of species and ecosystems.

As an example of the consequences of non-protecting seagrass meadows, in the nearby region of Portinho da Arrábida 10 ha of *Z. marina* meadow were lost due to continued human impacts. After the total loss of the biggest oceanic *Z. marina* meadow in Portugal, the area was the target of an ongoing restoration program (Cunha et al. 2014). This was a practical application of seagrass clonal propagation to recover lost seagrass habitat in an open coast area where natural seed recruitment is not likely due to high wave action shifting sediments in the absence of net of seagrass rhizomes to stabilize them (Chapter 5). This study showed that transplanting seagrasses in sods (promoting colonization thru clonal growth) can be successful in a short-term (Chapter 5). These efforts demonstrated, also as in chapter 4, that clonal growth alone may not be sufficient for population recovery when extreme disturbance events destroy the entire population, and in the absence of natural seed recruitment in an open coast site exposed to frequent disturbance it took very large transplanting efforts to recover a seagrass population that persisted over several years. Shifting stable states is not an easy task, specially in a wave exposed area due to many logistical and environmental factors (Paling et al. 2003). All tested species and donor populations were in a short time frame able to colonize the area after transplant, but as soon as it was disturbed (either by frequent impact or perturbation) the system would revert to its stable non-vegetated state. Transplant success should therefore be accessed through long-term monitoring programs which may last many years, as shown by this study over ten years. The most successful long-term case of seagrass transplant was a single *Z. marina* meadow which was transplanted with a bigger initial area than all others (11 m^2). The initial size is likely to have contributed to overcome a threshold which allowed the area to shift to a vegetated state. The minimum size hypothesis was proven by testing it with two donor populations. It is now known that the factors influencing seagrass long term survival in the studied area are the minimum size, the donor population source and transplant time of the year, as many factors remain unknown. Grazing preferences or the capacity to recover form grazing are pointed as likely factors to condition long term survival, though it remains as untested theory. Since it is likely that initial transplant size is a key factor for stable state shift, future efforts should be allocated in to this question.

It is important to state that seagrass restoration is used as a “Band-Aid” to try to revert an ongoing habitat degradation process, which in most cases is directly provoked by harmful human actions. Marine ecosystems are under threat due to repeated abuses and damages, such as overfishing, pollution, perturbation and climate change impacts (Richardson & Poloczanska 2008, Coll et al. 2012). The implementation of habitat restoration programs is important to attempt to stop the ongoing degradation and to try to reverse it, but is it very important to state that the thru solution is a drastic change of continued harmful human actions. It is urgent that mankind gains a holistic ecosystem conscience, as we Humans are part of it and It is our only planet. The implementation of effective conservation measures is crucial for the future existence of all Earth ecosystems in a rapidly changing world.

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