Landscape genetics of a seagrass species in a tidal mudflat lagoon

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Declaração de autoria de trabalho

Tese: Landscape genetics of seagrass species in a tidal mudflat lagoon

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

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Abstract

In this thesis I looked at different components of dispersal in the seagrass *Zostera noltii*, from the dispersal potential of both sexual and asexual propagules to indirect genetic estimation and landscape genetics analysis.

Methodological approaches

The first part of the work focused on understanding the dispersal biology of the species, previously undescribed. Dispersal is defined by three phases: propagule separation, transport and settlement. Initial phases of dispersal were studied in the experimental study aiming to define the dispersal potential of the propagules. In the following study I evaluated success of post-dispersal settlement of the fragments – propagules shown to have the highest dispersal potential. To evaluate dispersal potential of sexual and asexual propagules of *Z. noltii* I quantified spatial and temporal scales of possible dispersal in still water and under different currents. I estimated sinking rates of seeds and changes over time in shoot buoyancy, shoot viability, fragment breakage, shoot growth rate and spathe release rate of floating fragments. To determine the fate of fragments after the dispersal, I quantified survival, rooting and development of vegetative fragments following dispersal and settlement. In case of successful establishment I analysed parameters which might depict the future of the established fragment, *i.e.* state of the apical shoot, branching and biomass. I tested the effect of two independent factors - the initial fragment size and the dispersal regime, *i.e.* different ratios of period of floating and settlement in sediment within 10 weeks of experiment.

The second part of this thesis aimed to estimate the spatial genetic structure across the whole population of *Z. noltii* in the Ria Formosa lagoon (about 84 km^2), using an individual-based sampling design where four ramets were sampled in each of 803 sampling plots randomly distributed over this seagrass habitat. Sampled ramets were processed utilizing nine polymorphic microsatellite markers to identify their multi locus genotypes (MLGs). Then I looked at the frequency of sampling particular MLGs (clonal frequency) and the spatial distribution of MLGs sampled multiple times. Due to the wide spatial distribution of clones observed, I applied a commonly used clonal age estimation method which excludes the possibility of long distance dispersal of vegetative fragments (LDD), but iteratively corrected the age estimates in each step by combining sampling locations with spatially

explicit sea-level data for the time the clone would have been initiated. Finally, I looked at the association between disturbance, *i.e.* sediment dynamics associated with the barrier islands, and clonal richness at the landscape scale.

The final chapter of this thesis presents the results of high resolution landscape genetics analyses carried out on the same data set as the clonal structure analysis. I used the digital terrain model to extract spatial variables (elevation, slope, aspect, curvature of the terrain), while the dynamic parameters (current velocity, direction and flow) were obtained from the hydrodynamic model EcoDynamo. Identification of clusters or groups was done using different clustering methods implemented in STRUCUTRE software, Geneland package and Discriminant Analysis of Principal Components. Spatial autocorrelation analysis was used to test the hypothesis of random spatial distribution of MLGs. Linear models served to test the association between landscape-derived pairwise distances and the pairwise genetic distances (Loiselle kinship coefficient). The dependent variables were Euclidian distance and four additional measures of connectivity across the landscape (cost distance, hydrographical distance and two tide-dependent resistance distances). I also modelled the per plot genotypic richness (R) as a function of the spatial and dynamic variables of the habitat; and looked into spatial organisation of plot-specific allelic richness (A). Hydrographical distance was derived from a propagule dispersal simulated within the lagoon using a transport routine implemented in EcoDynamo model. This simulation was also used to indicate important areas for connectivity of *Z. noltii* within the lagoon.

Main findings

Fast sinking rates of seeds suggest short spatial scale of dispersal, insufficient to connect disjunct meadows. Observed traits of floating fragments, *i.e.* shoot buoyancy and shoot survival (>55 d), suggest a potential for long distance dispersal (>2,300 km, assuming an unidirectional current) that could connect distant patches and allow colonization of new areas. These results indicate vegetative fragments have the potential to extend the dispersal achieved by detached seeds establishing asexual propagules as potential LDD vector in seagrasses. The subsequent experiments on post dispersal fragment establishment showed significantly higher survival for fragments which didn’t spend any time floating prior to the settlement (~60%) than for those subjected to floating periods (~30%). The state of the apical shoot showed a significant interaction between the initial fragment size and the experimental dispersal regime. Branching was only observed for settled fragments and was significantly higher for the initially larger fragments, decreasing with prolonged floating periods. Floating period did not have any effect on the average number of branches (overall mean 1.8 branches per branching fragment), but the initial size of the fragments did. The ratio of above:below ground biomass at the end of the experiment revealed that fragments are in the state typical for colonizing meadows. These results demonstrate survival and rooting of floating *Z. noltii* fragments is possible (average success of 34%), but in case of a prolonged floating period during dispersal their success of establishment will be lower, *i.e.* survival, rooting and branching tends to decrease. Implementing these results to the Ria Formosa lagoon system, I estimated, dependent on many assumptions, that successfully dispersed and established fragments could occupy over 68 ha every year, representing over 5% of the current *Z. noltii* coverage in the lagoon.

Genetic analysis provided 3,185 valid genetic samples (R=0.63, A=15.0). In this data set 1,999 unique multi-locus genotypes were identified, whose spatial distribution revealed many clones sampled multiple times and found across distances as large as the available habitat in the Ria Formosa lagoon (up to 26.4 km). If the distances between clonemates were based on clonal growth alone, then I estimated that the majority of large clones would be older than 40,000 years. This would be older than the Ria Formosa system itself, therefore LDD of asexual propagules is a more parsimonious explanation for the large spatial separation among clonemates of many different *Z. noltii* clones in Ria Formosa. The same analysis was performed for the sympatric seagrass *Cymodocea nodosa*, where I estimated an even older age, over 53,000 years, for a single dominant clone. Again this supported that also in *C. nodosa* asexual LDD is the likely cause for the large distribution of this clone. The probability of sampling a given multi-locus genotype 5, or more times, increased together with the distance from Ria Formosa's barrier islands, a putative source of disturbance by increased sedimentation. This association between disturbance regime and clonal structure of the population has been elusive to demonstrate previously with smaller sample sizes. Future research can determine whether demography or sexual allocation, or both, are the processes behind this pattern.

None of the clustering methods revealed clear spatially defined clusters. However, spatial autocorrelation analysis revealed a significant isolation by distance pattern where samples within 3 km were more genetically related than it would be expected under the null hypothesis of random mating, implying that natal dispersal is playing a role in shaping this small-scale structure. Regressions showed that the two resistance distances seem to be the best predictor of genetic distance (R2=0.80 and 0.85). The other distances were not good predictors of gene flow. Genotypic richness per sampling plot ranged from R=0 (all four samples sharing the same genotype) in 8% of the plots to R=1 (four different genotypes) in

37% of the plots. None of the modelled environmental variables indicated significant association with genotypic richness. I didn't observe a particular spatial organisation of plot-specific allelic richness. Simulations of propagule dispersal in the Ria Formosa indicated possible sink and source areas, potentially relevant for connectivity within the lagoon.

Conclusions

My studies show that the dispersal biology of *Z. noltii* is very dependent on commonly underestimated asexual dispersal. This asexual LDD is the most parsimonious explanation for the wide distribution of clones in the lagoon and dramatically changes our view of the species' life history. Furthermore, I propose that the observed lack of association between landscape features and spatial genetic structure is a consequence of this LDD of asexual propagules. The high dispersal capacity of *Z. noltii* combined with a tidal regime which homogenizes the otherwise complex lagoon habitat, are prevalent over any effects of landscape on gene flow caused by the lagoon complexity as shown by the landscape genetics analyses. Nevertheless, sexual reproduction also affects spatial genetic structure, as the positive kinship between clones within a 3 km range is a signature of restricted seed dispersal.

Keywords*:* long distance dispersal, landscape genetics, seagrass, asexual dispersal, clonal structure, *Zostera noltii*, microsatellites.

Sumário estendido

Nesta tese, investiguei as diferentes componentes de dispersão da erva marinha *Zostera noltii*, desde o potencial de dispersão de propágulos assexuados e sexuados até à sua estimação indirecta através de métodos genéticos e análise de genética paisagistica.

Abordagem Metodológica

A primeira parte deste trabalho focou-se na compreensão da biologia de dispersão desta espécie, a qual nunca tinha sido previamente descrita. A dispersão assexuada, tal como a definimos aqui, é definida por três fases: separação do propágulo (fragmento vegetativo), transporte e fixação. As fases iniciais da dispersão foram avaliadas num estudo experimental com vista a definir o potencial de dispersão de propágulos vegetativos de *Z. noltii*. Em seguida avaliei o sucesso de fixação deste fragmentos após a fase de transporte– As escalas espaciais e temporais de possível dispersão foram quantificadas sob o efeito de diferentes correntes, de forma a estimar o potencial de dispersão dos propágulos assexuados e sexuados de *Z. noltii*. Estimei as taxas de afundamento das sementes e alterações ao longo do tempo na flutuabilidade e viabilidade dos rebentos, ruptura dos fragmentos, taxa de crescimento dos rebentos e a taxa de libertação de espatas. A sobrevivência, enraizamento e desenvolvimento de fragmentos vegetativos após dispersão e fixação foram quantificados de modo a calcular o destino dos fragmentos após dispersão. Nos casos de successo de refixação, analisei parâmetros que me permitiram prever o futuro dos fragmentos refixados, tais como: estado do rebento apical, ramificação e biomassa. Testei o efeito de dois factores independentes – o comprimento do fragmento inicial e o regime de dispersão, *i.e.* diferentes rácios de períodos de flutuação e fixação no sedimento, num período de 10 semanas.

A segunda parte desta tese teve como objectivo estimar a estrutura genética espacial ao longo de toda a população de *Z. noltii* presente na Ria Formosa (cerca de 84 km^2). Usei uma amostragem onde quatro rebentos foram amostrados em cada um dos 803 pontos selecionados aleatoriamente ao longo do habitat desta espécie. Os rebentos amostrados foram posteriormente processados usando nove microssatélites polimórficos com o objectivo de identificar os diferentes genótipos multi locus (GML) presentes e caracterizar a diversidade genética da espécie. Calculei a frequência com que determinado GML foi amostrado (frequência clonal) e a distribuição espacial dos genótipos amostrados múltiplas vezes. Devido à ampla distribuição espacial dos clones observados, apliquei um método usado para a estimativa de idade clonal baseado na taxa de alongamento do rizoma da espécie e que exclui a possibilidade de dispersão a longa distância (DLD) de fragmentos vegetativos. As idades dos clones obtidas foram corrigidas iterativamente combinado a localização dos rebentos amostrados com o nível do mar para a ponto no passado durante o qual o clone teria sido iniciado. Finalmente, debrucei-me sobre a associação entre perturbação, *i.e.* dinâmica do sedimento associado às ilhas barreira, e probabilidade de amostrar clones de grande dimensão à escala paisagística.

O capítulo final desta tese apresenta os resultados da análise de genética paisagistica, baseada nos mesmos dados usados para a análise de estrutura clonal. Usei um modelo digital terreno, obtido através de uma análise de LIDAR, para extrair variáveis espaciais (elevação, inclinação, aspecto, curvatura do terreno), enquanto que os parâmetros de dinâmica (velocidade das correntes, direcção e fluxo) foram obtidos a partir do modelo hidrodinâmico EcoDynamo. A identificação de possíveis aglomerados, ou grupos genéticos, foi feita usando diferentes métodos de aglomeração implementados no software STRUCTURE, Geneland e análise discriminante de componentes principais. A hipótese de distribuição espacial aleatória dos diferentes genótipos foi testada através de análises de auto-correlação espacial. Modelos de regressão linear foram usados de modo a testar a associação entre distâncias entre pontos de amostragem baseadas na paisagem e distâncias genéticas (coeficiente de aparentamento de Loiselle). As variáveis independentes foram a distância Euclidiana e quatro medidas adicionais de conectividade ao longo da paisagem (distância baseada em custo de dispersão através da paisagem, distância hidrogeográfica e duas distâncias ponderadas baseadas em resistência dependente da maré). Modelei a riqueza genotípica (R) por ponto de amostragem como função das variáveis dinâmicas do habitat e investiguei a organização espacial da riqueza alélica específica (A) de cada ponto de amostragem. A dispersão de propágulos foi simulada na Ria usando uma rotina de transporte implementada no modelo EcoDynamo e foi usada para derivar a distância hidrogeográfica e como modo de detecção de áreas de conectividade importantes para *Z. noltii* dentro da Ria Formosa.

Resultados Principais

As taxas de afundamento rápido das sementes sugerem uma curta escala espacial de dispersão, insuficiente para conectar pradarias separadas. No que respeita aos fragmentos flutuantes, as características observadas, *i.e.* flutuabilidade e sobrevivência dos rebentos (>55 d), sugere potencial para dispersão a longas distâncias (>2,300 km, assumindo uma corrente unidireccional) possibilitando a conexão de pradarias distantes e permitindo a colonização de novas áreas. Estes resultados demonstram que os fragmentos vegetativos possuem potencial para estender a dispersão alcançada pelas sementes, estabelecendo os propágulos assexuados como potenciais vectores de DLD em ervas marinhas. As experiências posteriores, sobre a fixação dos fragmentos, mostraram uma sobrevivência significativamente mais elevada em fragmentos cuja fixação foi rápida (sem largos períodos de flutuação) (60%) comparativamente com aqueles que estiveram sujeitos a longos períodos de flutuação antes da fixação definitiva (30%). O estado do rebento apical demonstrou uma interacção significativa entre o tamanho inicial do fragmento e o regime de dispersão experimental. O processo de ramificação foi apenas observado em fragmentos fixados e foi significativamente mais elevada em fragmentes inicialmente maiores, decrescendo com o aumento do período de flutuação. Ao contrário do que se verificou para o tamanho inicial dos fragmentos, o período de flutuação não teve qualquer efeito no número médio de ramos (média geral de 1.8 ramificações por fragmento). O rácio da biomassa acima:abaixo do solo no final da experiência revelou que os fragmentos apresentavam um estado característicamente obervado na fase de colonização. Estes resultados demonstram que a sobrevivência e enraizamento de fragmentos flutuantes de *Z. noltii* é possível (com uma média de sucesso de 34%) sendo no entanto mais baixa com longos períodos de flutuação durante a dispersão, *i.e.* a sobrevivência, enraizamento e ramificação tende a decrescer. Ao implementar estes resultados ao sistema lagunar da Ria Formosa, estimei que, dependente de muitas suposições, fragmentos dispersos e fixados com sucesso poderiam ocupar mais de 68 ha todos os anos, representando mais de 5% da actual cobertura de *Z. noltii* na Ria Formosa.

As análises genéticas forneceram 3,185 amostras genéticas válidas (R=0.63, A=15.0). 1,999 GMLs únicos foram identificados neste conjunto de dados, cuja distribuição espacial revelou um elevado número de clones amostrados múltiplas vezes e encontrados ao longo de distâncias tão grandes quanto todo o habitat disponível na Ria Formosa (até 26.4 km). Se baseadas apenas no crescimento clonal, as distancias entre copias do mesmo clone revelaram que a maioria dos clones seriam mais velhos que 40,000 anos. Esta idade antecederia assim o próprio sistema lagunar da Ria Formosa por mais de duas dezenas de milhar de anos. Tendo em conta estas datações, e os resultados do meu trabalho experimental acima citados, a DLD é uma explicação mais parcimoniosa, do que uma explicação baseada em enlongamento do rizoma apenas, para a elevada separação observada entre cópias do mesmo clone. A mesma análise de datação foi efectuada para uma outra erva marinha que vive em simpatria com a *Z. noltii* - *Cymodocea nodosa.* Neste caso estimei uma idade ainda mais elevada, mais de 53,000 anos, para um único clone dominante. Mais uma vez, estes resultados suportam que, também para *C. nodosa*, DLD é a causa mais provável para a alargada distribuição espacial deste clone. A probabilidade de amostrar um determinado GMLs cinco ou mais vezes, aumentou juntamente com a distância para as ilhas barreira da Ria Formosa, uma possível fonte de perturbação através do aumento de sedimentação. Esta associação entre o regime de perturbação e a estrutura clonal da população tem sido difícil de demonstrar previamente com um menor número de amostras. Pesquisas futuras poderão determinar se a demografia ou distribuição sexual, ou ambos, são as causas deste padrão.

Nenhum dos métodos de aglomeração revelou uma clara separação de grupos espacialmente definidos. No entanto, as análises de autocorrelação espacial revelaram um isolamento significativo por padrão de distância, onde amostras no espaço de 3 km eram mais relacionadas genéticamente do que seria de esperar sob a hipótese nula de cruzamentos aleatórios. Isso sugere que a dispersão natural desempenha um papel na formação desta estrutura de pequena escala. As regressões demonstraram que as duas distâncias baseada em resistência parecem ser o melhor indicador de distâncias genéticas (R2=0.80 and 0.85). As restantes distâncias não mostraram ser bons indicadores do fluxo genético. A riqueza genética por local de amostragem variou entre R=0 (todas as quatro amostras partilhavam o mesmo genótipo) em 8% dos locais e R=1 (quatro genótipos diferentes) em 37% dos locais de amostrais. Nenhuma das variáveis ambientais utilizadas nos modelos indicou uma associação significativa com a riqueza genotípica. Não observei nenhuma organização espacial da riqueza alélica específica do local de amostragem em particular. As simulações de dispersão de propágulos na Ria Formosa sugeriram possíveis áreas de origem e fixação, potencialmente relevantes para a conectividade dentro deste sistema.

Conclusões

O meu estudo demonstra que a dispersão da *Z. noltii* possui um importante componente assexual, geralmente subestimada. A DLD assexual é a explicação mais parcimoniosa para a larga distribuição de clones na Ria Formosa e altera drasticamente a nossa visão do ciclo de vida da espécie. Para além disso, proponho que a falta de associação observada entre as características da paisagem e a estrutura genética espacial é uma consequência da DLD dos propágulos assexuados. Esta elevada capacidade de dispersão da *Z. noltii* combinada com o regime de marés que homogeneíza o de outra forma complexo habitat lagunar, predominam sobre quaisquer efeitos da paisagem no fluxo genético

causado pela complexidade lagunar. No entanto, há também um efeito da reprodução sexuada na estrutura genética espacial, dado que o grau positivo de parentesco entre clones dentro de uma área de 3 km representa um sinal de dispersão restrita de sementes.

Palavras chave: dispersão a longa distância, genética paisagistica, erva marinha, dispersão assexuada, estrutura clonal, *Zostera noltii*, microssatelites.

Structure of the thesis

The thesis is divided in five chapters with two main sections. **Chapter one** gives the introduction to the field of study and defines overall and particular aims of the study. Introduction is followed by three chapters organised in two sections. These are written in the format of scientific articles, and can be read independently. For each of them information about the co-authors and the status of the publication are given on the initial page of the chapter. First section brings together research done on the dispersal biology of *Zostera noltii* through two distinct chapters. **Chapter two** introduces the dispersal potential of *Z. noltii* presenting the results on its prolonged dispersal period and high survival rate of the transport phase of the dispersal. It emphasizes the before overlooked importance of asexual propagules in the species' life history. **Chapter three** then shows the results of an experimental study carried out to estimate the success of post-dispersal settlement of these propagules, showing they can play an important role in dispersal and maintenance of the population. This concludes the work done on species dispersal biology. Following section looks at the genetics of the population within the Ria Formosa lagoon, aiming to establish a relation between previously described asexual dispersal of the species and population and landscape genetics. This section includes **chapter four** on individual based genetic analysis carried out in the study area. It reveals long distance dispersal of asexual propagules indeed happens in this study system and links the disturbance, through the landscape features, with the clonal structure of the population. **Chapter five** is a report of landscape genetics analysis and interpretation of the results in the light of previous chapters. Finally, an overall discussion of the results presented in this thesis is given in **chapter six** which ends with final conclusions and remarks for the future work.

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Section I. – Dispersal biology

Section II. – Population and landscape genetics

List of abbreviations

A	Allelic Richness
ADV	Acoustic Doppler Velocimeter
DAPC	Discriminant Analysis of Principal Components
DTM	Digital Terrain Model
GLM	General Linear Model
Ho	Observed Heterozygosity
IBD	Isolation By Distance
LDD	Long Distance Dispersal
LiDAR	Light Detection and Ranging
LM	Linear model
MLG	Multi Locus Genotype
Pc	Clonal probability
Pc5	Probability that a clone will be sampled 5 or more times
Pc10	Probability that a clone will be sampled 10 or more times
Pc15	Probability that a clone will be sampled 15 or more times
PCR	Polymerase Chain Reaction
Psex	probability of a given MLG occurring n times as a consequence of different sexual reproduction events
R	Genotypic Richness
SGS	Spatial Genetic Structure
YBP	Years Before Present

Chapter 1.

Introduction

1.1. Gene flow through the landscape

This thesis focuses on the landscape genetics of seagrasses. In order to reliably evaluate the effect landscape has on organism and population, it is necessary to understand gene flow through the landscape. I studied an intertidal seagrass species, a sessile organism attached to the bottom of the intertidal lagoon. Movement of the organism itself through the landscape is therefore limited and this could affect the genetic differentiation throughout the landscape. But an important adaptive trait in the life history of a species is dispersal. This is a process in which either an individual or just a part of it (its propagule) spreads across space, away from the point of origin and/or parental organism (Croteau, 2010). Potentially, this may lead to gene flow (sensu Ronce, 2007). Dispersal is generally viewed as a process of “moving away”, even though in its strict sense it also includes the settlement of juveniles in the vicinity of the parental organism. Besides active dispersal, where an organism actively moves with the purpose of reaching a specific location, we recognize the passive dispersal in the case of organisms which are unable to move and use propagules to disperse (Croteau, 2010).

The process of dispersal can be divided in departure, vagrant phase and settlement (Ronce, 2007). Throughout this work I will refer to the vagrant phase as transport. The departure phase marks the initial movement of an organism from a given location, or the release/separation of the propagule from the parental organism. Transport is, as introduced above, either active or passive and ends with the organism or a propagule reaching a new location. Settlement refers to arrival and successful establishment of the organism or a propagule in the new location.

Looking at a sessile species’ dispersal in particular, it is important to clarify the process of formation of propagules. Some authors refer to propagules as disseminules (Croteau, 2010) or diaspores, in case of the plant propagules (Matlack, 1987). Propagule can be any part of the organism capable of independent establishment and survival. We can distinguish sexual and asexual propagules. Sexual propagules are spores, pollen, embryos, fruits, seeds or germlings, all reproductive units of the organism. Sexual dispersal can be either postmeiotic/prezygotic (*e.g.* broadcast spawned gametes, haploid algal spores, plant pollen) or postzygotic (*e.g.* plant seeds or planktonic larvae). These dispersal processes have been extensively studied and are the ones most frequently assumed in general considerations of dispersal.

Less often studied are the evolutionary and ecological consequences of asexual dispersal, also referred to as clonal dispersal, driving strong unbalance in our perception of dispersal. This is exceptionally important because clonality is common throughout many taxonomic levels. About 45% of vascular plant families exhibit some aspects of clonality (Tiffney and Niklas, 1985), while this proportion is over 70% in animal phyla (Hughes, 1989), but only 0.1% in vertebrates (Avise, 2008). Dispersal research thus needs to adjust its focus accordingly, to include the forms of dispersal occurring in clonal organisms. Clonal dispersal is defined by Lincoln (1998) as outward spreading of organisms or propagules from their point of origin or release, a definition which can also encompass the rhizome extension in clonal plants. It can take many forms and display a wide spectrum of processes (Sibly and Calow, 1982; Fischer and van Kleunen, 2002; Halkett *et al.*, 2005; Cornelissen *et al.*, 2014) resulting in dispersal distances from a few centimetres to thousands of kilometres. For clonal dispersal to occur, dispersal unit needs to get separated. Separation can be either the natural consequence of maturation (Oróstica *et al.*, 2012) or a consequence of disturbance producing the fragment (Riis and Sand-Jensen, 2006). The propagule needs to survive through the transport phase across space, independently or using a transport vector. The distance travelled by the propagule prior to the settlement is defined by its viability and dispersal vector (Jackson, 1986). Asexual dispersal is shown to be particularly advantageous under conditions in which sexual dispersal is not effective, such as in some species distributional ranges (Billingham *et al.*, 2003; Tatarenkov *et al.*, 2005).

As it was mentioned earlier, the terms clonal and asexual dispersal are used interchangeably in the literature. But while the term clonal dispersal is rather straightforward, the use of the term “asexual” in literature is somewhat ambiguous. When talking about asexual propagation of plants it often happens that only asexual production of reproductive units is looked at (*e.g.* Bengtsson and Ceplitis, 2000), clearly missing on propagule production as a consequence of environmental forcing and plant anatomy. It therefore seems that in case of clonal plants, ramets, *i.e.* modular units of a clonal plant, are considered relevant for dispersal once when viewed detached from the mother plant, *i.e.* fragmented. This is just one example of partial consideration of asexual propagation in clonal organisms. Similar bias in definition and study of asexual propagules and asexuality is observed in other studies of clonal or partially clonal organisms, especially in studies looking at evolutionary benefits of sexual vs asexual reproduction (Butlin, 2002; Engelstädter, 2008). One way to approach this issue is to define the terms as Judson and Normark (1996) did in their review. They proposed an inclusive definition of the term "asexual", taking into account apomixis, automixis and vegetative reproduction. Additional mechanism like pseudovivipary and fissiparity (Crump and Barker, 1985) should also be included here. In this thesis I use the term "asexual propagule" referring to fragments of the parental organism (*i.e.* one or more ramets linked together) which can establish in a new habitat. In sessile clonal organisms, a necessary condition for an asexual propagule to escape its population boundaries, and the classical modular replication pattern, is the production, separation or fragmentation of independent units.

The circumstances by which organisms benefit from sexual or asexual reproduction and how both systems are maintained have been the focus of much study and debate in evolutionary biology (Barton and Charlesworth, 1998; Butlin, 2002; Silvertown, 2008). Besides the evolutionary advantages of sexual reproduction that are relevant at the population level, sexual derived bodies (gametes or zygotes) are often thought of as the relevant dispersal propagules, adding a between population component to the list of important life history traits associated with sexual reproduction. On the contrary, the strategies advanced to justify asexual reproduction are generally constrained to the population spatial limits. For example, resource foraging through clonal extension, physical and physiological integration and population maintenance under mate limitation. Rhizome extension within the population is often the single component of asexual dispersal accounted for (Gliddon *et al.*, 1987; McMahon *et al.*, 2014).

While knowledge of dispersal can help explain observed population genetic structure, studying dispersal itself is a challenge. Dispersal can occur over large spatial and temporal scales, and is often carried out by very small propagules (Rius *et al.*, 2010). Direct tracking of dispersal is very difficult, due to small propagule size, and the temporal and spatial scales over which the process happens. Traditionally, dispersal was inferred by looking at occurrence data or assumed from expert knowledge, but recently modelling approaches are growing in popularity (Driscoll *et al.*, 2014). Expert knowledge was shown to be unreliable, particularly when it is used to expand the knowledge gained in one study on a seemingly similar study system. In the review of modes of dispersal in marine organisms, Winston (2012) mentioned several paradoxes where dispersal in one species differs greatly from the rules observed for other species with similar natural histories. Direct observation of the dispersal process is rare and is generally reported for the vertebrates which are mostly radio-collared (Byrom, 2002) or ear tagged (Drygala *et al.*, 2010). But even in those cases, Koenig *et al.* (1996) argue that the reported dispersal often falls short of the indirect estimates based on population genetic analysis of gene flow. Tracking dispersal in marine systems, especially following miniature dispersing propagules, can be a particularly complicated venture. Marking methods have been developed allowing to identify the source of the larvae of marine organisms once they are recaptured, but still with considerable effort necessary to tag sufficient numbers of propagules (Thorrold *et al.*, 2002). Often direct tracking is so challenging that researchers turn to modelling (Dytham, 2003; Leis, 2007; Travis *et al.*, 2012; Nicolle *et al.*, 2013). But even here, logistic constrains can lead to models which rely on data averaged over too long periods of time, leading to considerable inconsistencies with the field observations (Putman and He, 2013). Regardless of how our insights in dispersal process are obtained and theories made, the above considerations suggest these understandings should be tested in the real system. One way to do so, particularly in the case of complex and large systems, is employing population genetics tools (Gilg and Hiblish, 2003). Hypothesis about the type of the dispersal process can be tested by genetic analysis of patterns of differentiation, which can support or reject this hypothesis (Ouborg *et al.*, 1999). Ibrahim *et al.* (1996), using computer simulations, showed that different dispersal strategies produce different signatures in the population spatial genetic structure. But to thoroughly investigate the dispersal of a species, one needs to consider both the species dispersal traits and the environment in which dispersal is occurring.

Landscape genetics is a discipline (Manel *et al.*, 2003) that builds its working framework on the methodology and results of population genetics and landscape ecology. This was made possible by advances in molecular biology techniques on one side and increased computational power enabling complex geostatistical and statistical analyses on the other side (Guillot *et al.*, 2009). The main goal is to combine genetic, environmental, and spatial variation to understand how the environment shapes gene-flow and genetic divergence across space. This is done by linking spatially explicit ecological and environmental data with georeferenced genetics information (Riginos and Liggins, 2013). The typical questions addressed pertain to different time scales and hierarchical levels of organisation, from genes to populations (Riginos and Liggins, 2013).

Initially, landscape genetics was, as its name implies, mostly focused on terrestrial systems. Soon after the term was coined, the first studies applying this framework in marine systems were published (Gilg and Hiblish, 2003; Sköld *et al.*, 2003; Jørgensen *et al.*, 2005; Galindo *et al.*, 2006; Hansen and Hemmer-Hansen, 2007). While some authors applying this particular framework to marine environment use the term “landscape”, others define their work as “seascape” genetics (Selkoe *et al.*, 2008; Amaral *et al.*, 2012). Either way, it is important to account for distinct conditions and parameters that need to be taken into account when the study area is submerged in the ocean (see Riginos and Liggins, 2013). This was shown as the models developed for terrestrial systems do not function for the marine systems (see Galindo *et al.*, 2006).

There are several tools commonly used to study the spatial genetic structure of a population, Mantel tests or regression analysis of distances between the samples (Mantel, 1967), spatial autocorrelation (Griffith, 1992), Bayesian clustering (François and Durand, 2010), multivariate analyses (James and McCulloch, 1990; Jombart *et al.*, 2010), Monmonier algorithm and wombling (see Manel *et al.*, 2003). Researches aim to determine in which way the landscape influences the genetic identity of the organisms living in it. Does it impose barriers to gene flow, or its configuration shapes corridors for connectivity? Do local characteristics of the habitat determine which genotype can be found in it? Starting with different initial questions, most of these questions can still be answered with application of Mantel test, or one of its modifications (Manel *et al.*, 2003). These tests basically test correlations between matrices, either simply genetic and spatial distance ones, or including a third matrix referencing environmental distance, or some other putative driver of the observed genetic distances. Very recently though, Legendre *et al.* (2015) suggested Mantel test is being used wrong and its use should be restricted. Another basic goal in landscape genetic studies is to characterize spatially defined groups of individuals that share a common genetic background (*i.e.* Frantz *et al.*, 2009). Sampling without *a priori* defining “populations” provides a somewhat more objective approach to identify groups based on their similarity. Clustering methods can help identify the genetic groups, which can be useful in studies of biology, biogeography, evolution, dispersal, mating systems, management and conservation. Spatial autocorrelation analysis of genetic coancestry tests the correlation between spatial and kinship distances using individual pair-wise data, against a null hypothesis of random distribution of pairwise kinship. Hardy and Vekemans (1999) discussed the power of spatial analysis to infer evolutionary processes and suggested under certain conditions it can be applied to estimate the variance of the parent-offspring dispersal distribution.

1.2. Overview of Seagrass biology and ecology with reference to *Zostera noltii*

Seagrasses were mentioned for the first time rather early in the human history. In ancient Greece, Aristotle mentions *Posidonia oceanica* as early as the IV century BC (Larkum *et al.*, 2006). Still, it was only in the last 40 years that science turned its attention to seagrass research (Larkum *et al.*, 2006). To present day about 60 species have been described, distributed around the world in the shallow waters of all oceans and seas (Spalding *et al.*, 2003). They inhabit coastlines from intertidal mudflats to depths of 80-90m (Den Hartog, 1970; Gamulin-Brida *et al.*, 1973; Spalding *et al.*, 2003). Seagrasses are marine angiosperms, and not true grasses. They evolved from the terrestrial monocotyledons during the Cretaceous period as a polyphyletic group with common habitat and adaptations to marine life (Les *et al.*, 1997). Distinct lines of evolution led to five defining adaptations for this group: tolerance to submergence and salinity, developed anchoring roots system, submarine pollination and dispersal within the marine environment (Ackerman, 2006). They form meadows which are focal points of biodiversity, providing habitat, nursery and feeding grounds for many species of invertebrates (Cabanellas-Reboredo *et al.*, 2010), fish (Beck *et al.*, 2001), sea turtles (Bjorndal and Bolten, 2010), marine mammals (Heinsohn *et al.*, 1977) and algae. So far literature reported about 350 epiphytic macroalgae species in seagrass meadows, 170 epiphytic invertebrate species, and up to 50 species of fish in any given seagrass meadow (Duarte, 2000). Seagrasses are ecosystem engineers (Coleman and Williams, 2002), meadows protect the coast from erosion (Hendriks *et al.*, 2009), retain sediment particles (Bos *et al.*, 2007) and are important for nutrient cycling in coastal waters (*e.g.* van Engeland *et al.*, 2013), playing a crucial role in ocean carbon budget (Duarte and Chiscano, 1999; Duarte *et al.*, 2013 a) and climate regulation (Duarte *et al.*, 2013 b), which places them in the top spot of the economic evaluation of ecosystems (Costanza *et al.*, 1997).

Seagrass population genetics has been increasingly studied, particularly after the development of highly variable genetic markers (Procaccini and Waycott, 1998; Reusch, 2000; Alberto *et al.*, 2003 a; Alberto *et al.*, 2003 b; Coyer *et al.*, 2004 a; Ruggiero *et al.*, 2004; van Dijk *et al.*, 2007). As seagrasses are clonal organisms, balance between the clonality and genetic differentiation has been studied with special interest. Genetics were used to study historical processes shaping the studied population (Olsen *et al.*, 2013), clonality (Zipperle *et al.*, 2009; Becheler *et al.*, 2014) and connectivity (Campanella *et al.*, 2015), species resilience to disturbance (Hughes and Stachowicz, 2004; Massa *et al.*, 2013), taxonomic relations (Nguyen *et al.*, 2014), restoration efforts (Reynolds *et al.*, 2013; Olsen *et al.*, 2014) and conservation (see Procaccini *et al.*, 2007 and references therein). Some studies investigate the link between the environment and the genetics without strictly defining their field of studies as landscapes/seascape genetics (van Dijk and van Tussenbroek, 2010; Sinclair *et al.*, 2014) and it was done so even before the definition of landscape genetics (Schlueter and Guttman, 1998). The body of research on this topic is growing, though like in other seagrass research area, it mostly focuses on larger species and neglects the smaller ones.

Seagrass dispersal has often been studied in the light of restoration projects and mainly focusing on seeds, *i.e.* sexual propagation (Orth *et al.*, 1994; Marion and Orth, 2009; Balestri *et al.*, 2011; McMahon *et al.*, 2014). But during the last decade scientists started looking more into dispersal via plant fragments, uncovering valuable information on species biology and ecology (Hall *et al.*, 2006; Erftemeijer *et al.*, 2008; Källström *et al.*, 2008; McKenzie and Bellgrove, 2008; Kendrick *et al.*, 2012). We now know that seagrass fragments can disperse drifting over large spatial scales and occupy previously uninhabited areas (Ceccherelli and Piazzi, 2001; McKenzie and Bellgrove, 2008) and a similar strategy has been implied for seagrasses in particular (Hall *et al.*, 2006; Diaz-Almela *et al.*, 2008; Virnstein and Hall, 2009). Filling the gaps on our knowledge of seagrass dispersal can therefore improve the success of restoration practices (Balestri *et al.*, 2011) and help understand population connectivity and seagrass response to natural and human-induced disturbances (Thomson *et al.*, 2014).

When disturbance occurs in an ecosystem, two things matter for the survival of the population – resistance and resilience. Resistance determines how much change in the environment a species or a population can tolerate without changing its state (Downing *et al.*, 2012). Resilience describes the degree to which a species or a population is able to return to its previous state after a disturbance has taken place, and how fast it achieves it. In general, how an organism or a population will respond depends on the disturbance – its quality and quantity, but also on the species itself and the environment. Seagrasses are anchored to the bottom of the sea and in case of disturbance recovery is most difficult for slow growing species. Organismal recovery capacity shortcomings can be minimized by its resistance to disturbance. Huges and Stachowitz (2004) found in their experimental study that higher genetic diversity can increase the population resistance to physical disturbance, although authors didn't find it affects the resilience. Literature shows that when disturbance is moderate seagrasses show high levels of resistance (*e.g.* Longstaff and Dennison, 1999), and if changes persists over long periods of time they can adjust both at the individual and population levels (*e.g.* Collier, 2006; Ralph *et al.*, 2007). But in the case of a sudden and strong impact (such as sedimentation or shoot removal), seagrasses were not shown to respond equally fast, and in some cases do not recover from the impact (Duarte *et al.*, 1997; Rasheed, 2004). Nonetheless, seagrasses developed a set of mechanisms which may not necessarily ensure survival of the individual itself, but ultimately will work towards ensuring survival of the species. Cabaço and Santos (2012) report in their review that in 75% of studies seagrasses increased the reproductive effort in face of disturbance, this response being stronger in species with higher storage capacity (*i.e.* with bigger rhizome diameter). These authors suggested this type of response to be a mechanism to improve resilience. Specifically, increasing the reproductive effort increases genetic diversity which maximizes chances of adaptation to new conditions. Additionally, if the disturbance is strong enough to alter the habitat, it might become unsuitable for the species. In this scenario, the advantage of increased reproductive effort is the production of numerous propagules which are not attached to the bottom as parental plants, but are instead available for dispersal.

European waters are home to four seagrass species, from the large subtidal species *Posidonia oceanica*, *Cymodocea nodosa* and *Zostera marina*, to the small, often intertidal, *Zostera noltii* (Green and Short, 2003). In addition, the recently introduced Lessepsian species *Halophila stipulacea* is nowadays spread throughout the Mediterranean Sea and two widgeon grasses *Ruppia maritima* and *Ruppia chirrosa* (*Ruppia* is not a seagrass in strict sense but still often mentioned as such) found in European coastal waters (Green and Short, 2003).

The dwarf eelgrass, *Zostera noltii* Hornem is a small, perennial or annual plant (Figure 1.1), inhabiting coastal waters of Europe and Africa from Norway to Mauritania. It is found along the Atlantic Ocean, Kattegat, Mediterranean, Black, Azov, Caspian and Aral Seas (Green and Short, 2003). It is mostly intertidal, except in the areas of the Mediterranean and the Kattegat Sea where it can be found subtidally (Den Hartog, 1970). It can inhabit brackish waters and is often present in coastal lagoons (Hily *et al.*, 2003).

Figure 1.1. (a) *Zostera noltii* plant. (b) *Zostera noltii* meadow in the Ria Formosa (photo: Buga Berković)

Its wide geographic and niche distribution imply that *Z. noltii* is an adaptive species, inhabiting temperate waters of northern Atlantic and tropical Mauritanian coast equally (Fig.1.2), from dynamic and harsh intertidal mudflats, to calm and stable subtidal zones. *Z. noltii* is considered a pioneer species, among the first to inhabit an area, relying mostly on phalanx strategy (Ruggiero *et al.*, 2005; Brun *et al.*, 2007). In phalanx strategy connections between the ramets are shorter creating dense canopies, opposing to guerrilla strategy where spacing between the ramets is longer. Both strategies can be used by one species as a response to environmental condition (Ye *et al.*, 2006). *Z. noltii* spread is characterized both by fast rhizome growth (Marbà and Duarte, 1998) and frequent flowering as well as high seed production rates (Alexandre *et al.*, 2006). *Z. noltii* is a dominant seagrass species in the Ria Formosa lagoon, southern Portugal. Guimarães *et al.* (2012) reported that in 2002 it occupied about 45% of the intertidal area in the lagoon. Habitat occupied by *Z. noltii* is of great interest for humans as it is suitable for clam farming and harvesting, leading to frequent disturbances to local seagrass meadows (Cabaço *et al.*, 2005).

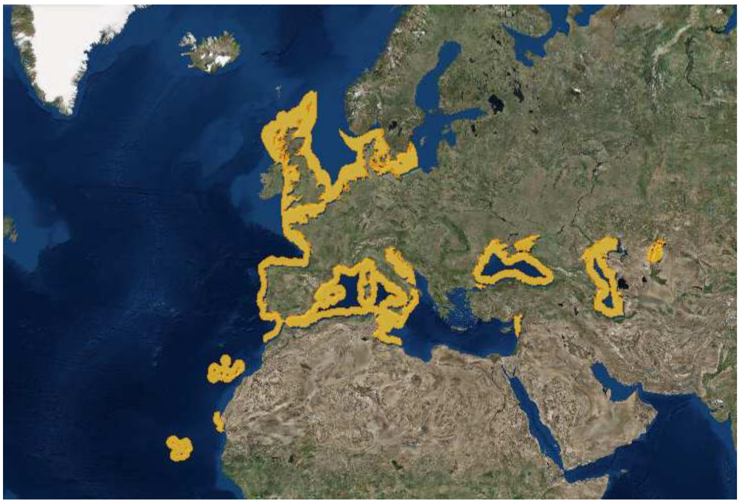

Figure 1.2. Distribution of *Zostera noltii* (from Short *et al.*, 2010)

Population genetics of *Z. noltii* have been studied since Coyer *et al.* (2004 a) developed nine microsatellite markers. Using this markers, Coyer *et al.* (2004 b) studied populations along the species' distribution range and found both genotypic diversity, *i.e.* number of distinct genotypes, (R=0.10-1.00) and standardized allelic richness (A=3.8-7.8) decrease from south to north. The fact that some populations exhibit annual and others semi-perennial reproduction mode has been offered as an explanation for high diversity in different populations, among other factors (Procaccini *et al.*, 2007). Researchers looking at isolation-by-distance (IBD) in *Z. noltii* found a limit of 100-150 km for a panmictic neighbourhood (Coyer at al., 2004 b), implying populations within this limits are susceptible to dispersal, possibly by rafting shoots (Procaccini *et al.*, 2007). While Procaccini *et al.* (2007) assumes that rafting shoots are only relevant as a vector for fruit/seeds dispersal, in this thesis I will argue that vegetative shoots dispersal plays a more important role in the dispersal process. The general IBD pattern observed by Coyer *et al.* (2004 b) was not consistent throughout the biogeographic area, *e.g.* in the Black and Azov Sea where genetic differentiation was rising much faster with increasing distance. Billingham *et al.* (2003) suggested this type of pattern can be explained with clonal reproduction dominating the margins of the range, while sexual reproduction is more relevant in the core areas. Coyer *et al.* (2004 b) found within 33 populations at 11 locations that clones were present in almost all the populations, and had about 3 m^2 in size. Clone size was varied geographically, as the populations in the Mauritania, Black and Azov Sea exhibited larger clones (Coyer *et al.*, 2004 b). Other population genetics parameters also varied greatly: expected heterozigosity H_{exp} between 0.279 and 0.675, observed heterozigosity H_o between 0.163 and 0.722 and percentage of unique alleles in population from 1.7 to 42.1% (Coyer *et al.*, 2004 b).

Seagrasses are clonal organisms, whose basic building unit is a ramet. A ramet is a plant segment comprised of one shoot, belonging rhizome and roots. Seagrass architecture (*i.e.* how ramets are organised in space) is shaped by apical dominance and clonal integration (Brun *et al.*, 2007). Apical dominance relates to the role of the apical shoot for plant growth, *e.g.* for the direction of the growth of the plant (Phillips, 1975). Clonal integration refers to the level of communication between the units of one plant, *e.g.* exchanging the nutrients between the distant parts of the clone but also defence signals and pathogens (Quereshi and Spanner, 1971; Stuefer *et al.*, 2004). In *Z. noltii*, both apical dominance and clonal integration strongly affect clonal architecture (Brun *et al.*, 2007). Brun *et al.* (2007) showed that apical dominance restricts branching and regulates the length of internodes, *i.e.* sections between the successive levels of leaf or branch insertion. Clonal integration is estimated to persist in *Z. noltii* for about 30 days, based on the maximum distance of resource translocation, and rhizome elongation rates (Marbà *et al.*, 2006). Marbà *et al.* (2002) estimated that the maximum distance of resource transport in *Z. noltii* was about 25 cm. In practice this means that even though the apical shoot is directing the growth of the rhizome and controlling the growth of branches (Brun *et al.*, 2007), a plant with damaged apical shoot will continue to grow via secondary ramets (*i.e.* branches on the main rhizome), facilitated by the transport of resources from the neighbouring parts of the plant. These mechanisms display plasticity of the plant, its ability to "bridge" the unsuitable habitat by translocating resources suitable ones (Cain, 1994) and occupy space in an optimal way.

Habitats in which *Z. noltii* is found are often highly dynamic and subject to both naturally occurring and anthropogenic disturbance. This can be observed in particular in the coastal lagoons and intertidal mud- and sand- flats along European shores, (*e.g.* Denmark, Flindt *et al.*, 1997; Netherlands, Govers *et al.*, 2014; Germany, Zipperle *et al.*, 2011; France, Plus *et al.*, 2010; Italy, Curiel *et al.*, 1996; Spain, Brun *et al.*, 2007; Valle *et al.*, 2011; Portugal, Cunha *et al.*, 2013). Intertidal populations need to adapt to prolonged periods of high irradiance and desiccation, high temperatures and freshwater inflow on occasion (*e.g.* rain), as well as hydrodynamics of incoming and leaving tides. Such environments often exhibit intense sediment dynamics, due to moving sand barriers or inlets (*e.g.* Cunha *et al.*, 2005). On the other hand, many habitats of *Z. noltii* coincide with areas of intense human activity. Multiple studies around the world evaluated impacts of human activities on coastal systems - either tourism related (Davenport and Davenport, 2006) different forms of agriculture (Lloret *et al.*, 2005), aquaculture (Ruiz *et al.*, 2001), fisheries (Blaber *et al.*, 2000) and clam harvesting (Cabaço *et al.*, 2005). Additionally, as they are often situated in shallow coastal lagoons, dredging for the purpose of maritime traffic is also an important stressor (Erftemeijer and Lewis, 2006). Dredging physically destroys the meadows but also leads to sediment loading, similarly to coastal construction, deforestation and sediment transport from inland via rivers. This was shown to have a strong negative impact on some seagrass species (Terrados *et al.*, 1998). Finally, eutrophication as a consequence of waste water discharge and nutrient transport, from agricultural or industrial zones through the watershed, is a major impact in developed and developing parts of the world (Björk *et al.*, 2008). All of these disturbances led to the decline of seagrasses around the world, placing them on the list of endangered species and habitats (Waycott *et al.*, 2009; Cabaço and Santos, 2014).

Studying the landscape genetics of an intertidal seagrass presents its own challenges. Habitat is changing with tidal cycle, from humid terrestrial one to the shallow coastal one. This means that for some individual's suitable habitat allowing uninterrupted gene flow is available twice a day, with periods of zero connectivity for the rest of the day. Thus, this temporal variation needs to be considered, as well as the spatial variation, in order to describe the habitat's dynamic character. No similar work has been done yet, regardless of the focus species. This study endeavours in unprecedented sampling effort focused on individuals, without a priori defined populations. Such design allows detailed insight into the structure of the population and landscape analysis with higher resolution than previously published body of work.

1.3. Thesis aims

The aim of this thesis is to understand the genetic spatial structure of the seagrass *Zostera noltii* in the mesotidal coastal lagoon Ria Formosa and link it to species' dispersal biology and landscape it inhabits. In particular, this work aimed to:

- give the insight in seagrass dispersal potential (Chapter 2),
- evaluate the success of dispersal via vegetative propagules (Chapter 3)
- providing description of species asexual dispersal biology (Chapters 2 and 3).

Furthermore, to

- use genetic analysis to support proposed dispersal pathways (Chapter 4) and
- discuss the importance of landscape complexity for population genetics (Chapter 4).

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Section I.

Dispersal biology

Chapter 2.

Extending the life history of a clonal aquatic plant: dispersal potential of sexual and asexual propagules of *Zostera noltii*

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Abstract

The dispersal potential of sexual and asexual propagules of *Zostera noltii* was experimentally quantified in still water and under different currents in microcosm and mesocosm facilities. We estimated sinking rates of seeds and changes over time in shoot buoyancy, shoot viability, fragment breakage, shoot growth rate and spathe release rate of floating fragments. The fast sinking rates of detached *Z. noltii* seeds suggest a small spatial scale of dispersal insufficient to connect fragmented populations, whereas the traits of floating fragments, particularly shoot buoyancy and shoot survival (>55 d), suggest a potential for long distance dispersal (>2,300 km) that could connect distant patches and allow colonization of new areas. We showed that the *Z. noltii* vegetative and reproductive fragments have the potential to extend the dispersal achieved by detached seeds alone.

Keywords: dispersal, aquatic plant, propagules, seagrass connectivity, *Zostera noltii*, seeds, fragments.

2.1 Introduction

Dispersal is essentially any movement of an organism, or any of its propagules, that potentially leads to gene flow (sensu Ronce, 2007). It is a life history trait, most typically associated with sexual reproduction, whether postmeiotic or prezygotic (*e.g.* broadcast spawned gametes, haploid algal spores, plant pollen) or postzygotic (*e.g.* plant seeds or planktonic larvae). Compared to sexual dispersal, much less attention has been given to the evolutionary and ecological consequences of clonal dispersal, although it is widespread across many organisms, particularly in plants. Clonal dispersal can be defined as outward spreading of organisms or propagules from their point of origin or release (Lincoln, 1998), which also includes the shoot multiplication through rhizome extension. A unit of clonal dispersal is often a fragment of an adult organism, although other means of asexual dispersal are possible (*e.g.*, apomixis). A dispersing fragment can be any unit capable of independent life. For clonal dispersal to occur, such a unit needs to get separated, either naturally as a consequence of maturation (Oróstica *et al.*, 2012), or fragmented by any disturbance (Riis and Sand-Jensen, 2006), and survive temporarily during transport in an appropriate dispersal vector. Dispersal distance is, hence, a function of fragment viability and dispersal vector (Jackson, 1986). Clonal dispersal is especially advantageous as a mean of propagation under conditions in which sexual dispersal is not effective, such as in some species distributional ranges (*e.g.*, Billingham, 2003; Tatarenkov *et al.*, 2005).

Fragment separation is a process of creation of asexual propagules that has been considered to be under selection (Highsmith, 1982). There is evidence that some aquatic plants may disperse only by vegetative fragments (*e.g. Ranunculus lingua*; Johansson and Nilsson, 1993), others propagate mainly via vegetative fragments (*e.g. Elodea canadiensis* and *Myriophyllum spicatum*; Nichols and Shawn, 1986), while few spread via more specific dormant apices *(e.g. Potamogeton crispus*; Nichols and Shawn, 1986). Boedeltje *et al.* (2003) found that out of all the propagules of 12 submerged plant species found in a stream almost 99% were vegetative ones. For different species, buoyancy properties of the fragments define their dispersal pattern and fate. While *e.g. E. canadiensis* floats under the surface of the water, and can therefore get entangled to other submerged vegetation, *Ranunculus peltatus* fragments float on the surface and more often strand on shallow sediments (Riis and Sand-Jensen, 2006). Still, relatively few studies have addressed the relevance of fragmentation in marine vegetation, which have the potential to disperse over much longer distances via drifting fragments (Ceccherelli and Piazzi, 2001; McKenzie and Bellgrove, 2008). Some seagrass genetic studies explicitly discuss populations' genetic structure in the light of possible fragment dispersal paths (Reusch, 2002), but again emphasizing fragments as vectors carrying sexual propagules.

Fragmentation and dispersal in aquatic clonal vegetation are concurrently thought to play a role in diminishing the negative effect of selfing, commonly observed in terrestrial clonal plants (Charpentier, 2002). Furthermore, for species with higher dispersal of fragments, clonal reproduction is more common (Silvertown, 2008). Dispersal is therefore, particularly in aquatic clonal plants, likely to be a crucial process in shaping populations' distribution and structure.

Seagrasses are marine clonal plants that disperse through both sexual and asexual propagules (Orth *et al.*, 1994). Both vegetative and reproductive fragments (*i.e.* fragments with flowering shoots composed of spathes containing flowers, fruits and seeds; Setchell, 1933) are important long distance dispersal vectors (Harwell and Orth, 2002; Hall *et al.*, 2006). In some seagrass species, floating seeds or fruits are as well a type of long distance dispersal (*e.g. Enhalus acoroides and Thalassia hemprichii* (Lacap *et al.*, 2002), *Posidonia oceanica* (Buia and Mazzella, 1991)), but seeds of most seagrass species are negatively buoyant (Pettit, 1984), and thus, unlikely to disperse far. Seagrass fragment formation, dispersal and establishment have rarely been studied and only for a few species, *e.g. Zostera marina* (Ewanchuk and Williams, 1996; Erftemeijer *et al.*, 2008; Källström *et al.*, 2008), *Thalassia testudinum* (Kaldy and Dunton, 1999) and *Posidonia oceanica* (Diaz-Almela *et al.*, 2008).

Understanding the dispersal and colonization potential of seagrasses is valuable to predict the natural potential for self-restoration and colonization (Rasheed, 2004; Boese *et al.*, 2009; Kendrick *et al.*, 2012) and in that way essential for the continuous efforts to conserve and restore the existing meadows (Harwell and Orth, 2002). Studies focused on restoration methods (*e.g.* Harwell and Orth, 1999; Marion and Orth, 2009) have revealed limited establishment success (Orth *et al.*, 2009; Paling *et al.*, 2009). Improving our knowledge on seagrass dispersal process can therefore improve the success of restoration practices (Balestri *et al.*, 2011). It can also help understand population connectivity, and their response to natural or human-induced disturbances. Most dispersal studies focused on dispersal of sexual propagules (Orth *et al.*, 1994; Harwell and Orth, 2002; Erftemeijer *et al.*, 2008; Källström *et al.*, 2008; Koch *et al.*, 2009), whereas less is known about the colonization potential of plant fragments (Barrat-Segretain and Bornette, 2000; Hall *et al.*, 2006).

Zostera noltii is the smallest seagrass among the native European species (Kuo and Hartog, 2001) exhibiting fast clonal growth rate (Peralta *et al.*, 2005), with common flowering events and high seed production rates (Alexandre *et al.*, 2006). The species inhabits mostly intertidal zones, even though some populations may develop in the subtidal, particularly where tides are very small. It is the dominant seagrass species in Ria Formosa lagoon, Southern Portugal, where it covers over 45% of the intertidal area (Guimarães *et al.*, 2012). In this lagoon, intensive clam culturing and harvesting occurs in intertidal areas along the *Z. noltii* meadows (Guimarães *et al.*, 2012). While the installation of clam beds removes local seagrass beds completely, clam digging within the remaining seagrass meadows decreases total biomass and shoot density (Alexandre *et al.*, 2005; Cabaço *et al.*, 2005). The latter activity leads to breakage and release of seagrass fragments, probably increasing the dispersal potential of the species. In the long term, such disturbed meadows invest more into reproductive shoots (Alexandre *et al.*, 2005), which can also become detached and dispersed with the vegetative ones.

The aim of this study was to investigate the dispersal potential of sexual and asexual propagules of *Zostera noltii*. Here, we focus on clonal dispersal as the transport of a genetically identical vegetative fragment after breakage from the sessile clone, with potential to contribute to gene flow (Ronce, 2007). Dispersal potential was experimentally quantified for both spatial and temporal scales by measuring seed sinking rates and different properties of floating fragments, with and without reproductive structures, *i.e.* shoot buoyancy, shoot viability, fragment break down, shoot growth rate and spathe release rate. We hypothesize that both the vegetative and reproductive fragments play a crucial role in *Z. noltii* population maintenance in the Ria Formosa lagoon, allowing higher dispersal over larger spatial scales than detached seeds alone.

2.2 Material and methods

Seeds, vegetative and reproductive fragments were collected in summer 2011 during low tide from a *Zostera noltii* meadow in Ria Formosa lagoon, South Portugal (37.01°N, 7.5°W). The dispersal potential of seeds, vegetative and reproductive fragments was evaluated in still water and under different currents in microcosm and mesocosm facilities.

2.2.1. *Dispersal potential of seeds*

Seeds of *Z. noltii* were obtained from flowering shoots that were collected, brought to the laboratory and placed in a seawater aquarium (20 L, 24°C, 33‰) with moderate aeration (Cabaço and Santos, 2010; Fig. 2.1 a). Seeds that naturally fell out from the spathes were collected every two days from the bottom of the aquarium. Forty-seven seeds were obtained over a period of four weeks.

The seed wet weight (precision of 0.1 mg) was determined after blotting each seed rapidly on a paper towel. The length and width of seeds (Fig. 2.1 b) were measured on photographs using the software ImageJ. Seed density was calculated by dividing the seed weight by their volume; seed volume was estimated using a formula for an ellipsoid body:

$$V= 4/3*\pi*a*b*c,$$

where a= half of width, b= half of thickness and c= half of length, assuming equality of width and thickness of the seed, independent of seed position along its longitudinal axis, as used by Koch *et al.* (2010) for seeds of similar shape. Seeds were kept separately in 1.5 mL tubes with autoclaved seawater until used in the still water and current dispersal experiments.

Sedimentation rate of seeds in still water was tested in a 40 cm tall seawater aquarium (24°C, 33‰). Seeds were released immediately under the surface of the water to avoid an effect of surface tension on their sinking behavior. The time required for a seed to reach the bottom was recorded three times for each seed. The average time was used to calculate the sedimentation rate. Linear regression of biometric variables on sedimentation rate of seeds was used to determine if seed characteristics explain sinking behavior.

The distance traveled by seeds was measured under three distinct current velocities (1.5, 4.0 and 6.5 cm/s) in a flume tank (1.5 m long, 7 cm water column depth, with a thin layer of sandy sediment on the bottom; Fig. 2.2 a) with re-circulating seawater (23°C, 33‰). The current velocity was measured by releasing a colored liquid in the flow. Experimental current velocities were chosen based on preliminary measurements used to determine the highest current velocity allowing tracking and retrieving seeds. This current velocity was within the range of currents measured 6 cm above *Z. noltii* canopy in Ria Formosa lagoon (ca. 3.5-8.0 cm/s; Urs Neumeier, pers. comm.). Seeds were placed immediately below water surface to avoid the surface tension effect and aligned with the longer axis in the direction of the current. The distance travelled by seeds until they reach the bottom was measured. The same procedure was repeated three times for each seed and the average distance was used.

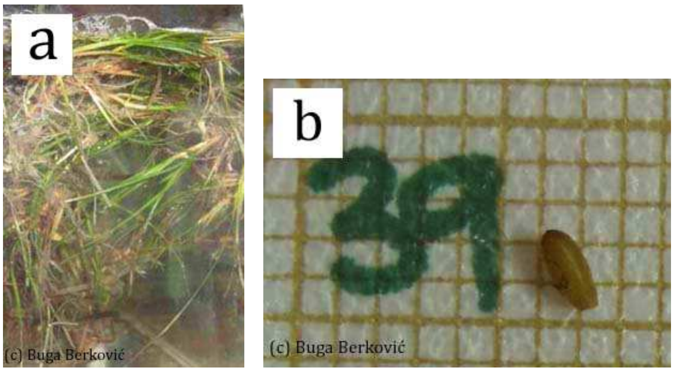

Figure 2.1. (a) Tank with flowering fragments for seed collection. (b) Seed of *Zostera noltii*. Squares in the back are 1x1 mm in size.

In order to determine the critical current velocity causing seed transport along the bottom, batches of eight seeds were placed in a line across the bottom of the flume tank, far from the tank walls and from each other (ca. 5 cm). Water current was slowly increased and when an individual seed moved more than 1 cm, the current velocity was recorded. To avoid flow disturbance, seeds were only removed from the flume after all of them moved from the starting line. This procedure was repeated three times for each batch of seeds, and the average current velocity recorded.

Seed viability was tested at the end of the experiments, by soaking them in a 0.5% tetrazolium solution at 25°C during 24 h (Conacher *et al.*, 1994). Seeds were considered viable if the hypocotyl stained red and the radicle either red or brown. A t-test was used to investigate the difference in seed biometric characteristics and dispersal behavior between the viable and non-viable seeds, after checking for normal distribution. When t-test assumptions were not verified, the non-parametric Mann-Whitney U test was used (Lehman

Figure 2.2. Flume tanks used to quantify (a) dispersal potential of *Zostera noltii* seeds and (b) damage of fragments occurring during exposure to currents, both in the biohydrodynamic laboratory of Sven Lovén Centre for Marine Sciences – Tjärnö, University of Gothenburg. (c) View of uncovered and covered outdoor tanks for still water experiments of the dispersal potential of vegetative fragments, in Ramalhete field station, University of Algarve.

and D'Abrera, 2006). For the analysis of dispersal in current and critical current velocity for bottom transportation only the viable seeds were considered, as dispersal of non-viable seeds has no biological meaning.

2.2.2. Dispersal potential of vegetative fragments

To estimate the time period during which floating vegetative fragments of *Z. noltii* remain viable, a mesocosm experiment (still water) was conducted using 34 vegetative fragments. The length of the rhizomes was measured and the number of shoots was counted for each fragment. Fragments were on average (± SE) 15.7±1.1 cm long and had 6.0±0.5 shoots. They were marked individually using colored, numbered, adhesive tape that was loosely fixed around the rhizome (1 cm away) to avoid any type of damage. Marked fragments were placed in outdoor tanks (550 L; Fig. 2.2 c) with low seawater renewal and covered with white plastic mesh to avoid overheating. Fragments were monitored after 5, 10, 15, 40 and 55 days for the number of shoots to estimate shoot production rate, as a proxy for growth. At the end of the experiment, the fragment production potential was evaluated by monitoring for tissue necrosis (brown-to-black colored leaves and rhizome with emphasis on meristems). Fragments were categorized as: growing (green leaves, without decaying tissue), stagnant (greenish–yellowish leaves with some decaying tissue) and dying (no green leaves, most of the tissue decaying).

To evaluate the damage occurring during exposure to currents, we mimicked the conditions observed in Ria Formosa lagoon where drifting patches of *Zostera noltii* fragments get entangled in the canopies of semi-submerged vegetation or physical structures set by humans; the flow of water current over these stationary fragments can cause breakage. To estimate the rate of fragment breakage, vegetative fragments were exposed to three distinct current velocities of 15, 30 and 40 cm/s. Batches of 30 fragments per treatment were placed in a flume tank (7 m long, re-circulating water, 23°C, 33‰; Fig. 2.2 b) with a plastic net (1 cm mesh) at the end of the working section to hold the fragments, exposing them to continuous water flow. The current velocity in the tank was monitored using an acoustic Doppler velocimeter (ADV). The current was measured 15 cm upstream of the working area and the probe was removed before fragment release. Fragments were released together, at the beginning of the tank and were exposed to the current during two hours. To synthesize our observations we categorized fragments in interval classes of number of shoots. All the fragments placed in the tank had more than three shoots, meaning that all fragments with one, two or three shoots at the end of experiment were a consequence of damage caused by the current. Therefore, we defined the first category to have three or less shoots and kept the range in shoot number for all other categories equal. Change rate of fragments in each shoot number class was calculated as: (N_a/ N_b) – 1, where N_a is the number of fragments in a given shoot number class after the exposure to the current and N_b is the number of fragments in the same class at the start. Positive change rate indicated an increase of fragments in a particular shoot number class, due to the damage of fragments from bigger size classes. Negative change indicated breakage of fragments in that shoot number class and their reclassification into a smaller shoot number class.

2.2.3. Dispersal potential of reproductive fragments

Twenty fragments with flowering shoots were kept in a seawater aquarium (20 L, 24°C, 33‰, as described in section 2.2.1.), and monitored for the natural release of the spathes and seeds in still water for 30 days. We assumed that reproductive fragments have the same dispersal potential as the vegetative ones, because there are no structural differences between them except for the presence of spathes in 1-2 shoots.

To test the damage caused by water current on the reproductive fragments, spathe release from the flowering shoots was quantified after two hours of current exposure (15, 30 and 40 cm/s) using the same experimental design as described for vegetative fragments (see section 2.2.2.). The change rate in the number of spathes per flowering shoot after exposure to current was used to quantify the damage imposed by water current. Change rate was calculated as before (see section 2.2.2.). Positive change rate was caused by the increase in number of fragments with target spathe – shoot ratio. As no new spathes could have been produced, this was indicative of spathe release for fragments with more spathes per shoot than the observed group. Negative change rate was considered to be indicative of spathe release in fragments of observed group.

2.3. Results

2.3.1. Dispersal potential of seeds

Sixty–six percent of *Z. noltii* seeds were viable, and viable seeds had higher density and weight than the non–viable ones (t-test, $p<0.05$, Tab. 2.1). Viable seeds showed a significant ($p<0.001$), three–fold faster sedimentation rate than the non–viable ones

(2.5±0.8 and 0.9±0.6 cm/s respectively). The seed weight had a significant effect on still water sedimentation rate ($R^2=0.35$, $p<0.001$); whereas the other biometric variables explained a smaller proportion of the variation ($R^2<0.15$, *e.g.* seed density, $p=0.01$). The critical resuspension current velocity for viable seeds was 8.2 cm/s.

Table 2.1. Biometric characteristics and dispersal behaviour of viable and non-viable seeds of *Zostera noltii* (mean ± SE).

Characteristic (units)	**Viable seeds**	**Non-viable seeds**
Length (mm)	2.3±0.2	2.3±0.2
Width (mm)	0.9±0.1	0.9±0.1
Weight (mg) **	1.4±0.5	1.0±0.4
Density (mg/mm) *	0.9±0.4	0.7±0.4
Sedimentation rate (cm/s) ***	2.5±0.8	0.9±0.6
Critical current for bottom transportation (cm/s)	8.2±1.2	N/A
Percentage (N)	66 (31)	34 (16)

The distance traveled by *Z. noltii* seeds increased with increasing currents (Fig. 2.3); seeds dispersed 2.3, 10.4 and 15.5 cm in current velocities of 1.5, 4.0 and 6.5 cm/s, respectively.

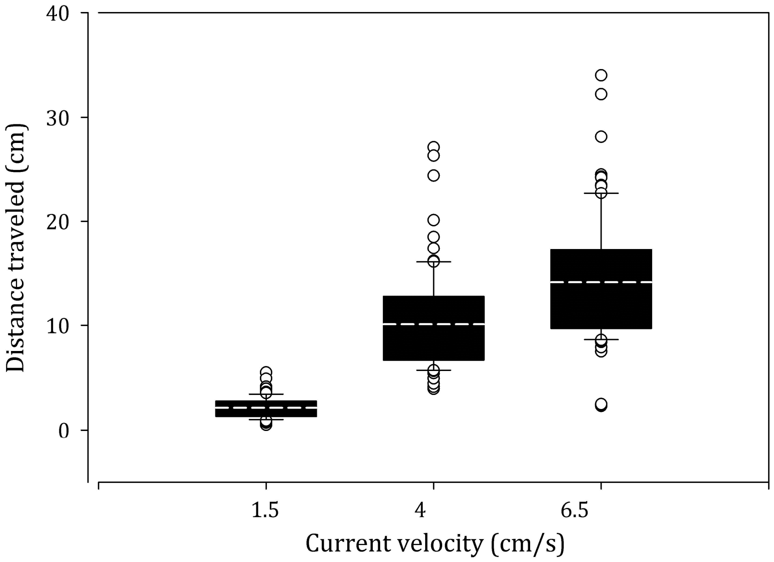

Figure 2.3. Observed *Zostera noltii* seed dispersal in experimental current velocities of 1.5, 4.0 and 6.5 cm/s. Dashed line inside the box shows the mean value, open circles represents outliers; the boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively.

Seed dispersal distance was significantly related to the seed weight in currents of 4.0 and 6.5 cm/s ($p<0.05$), but the association was poor ($R^2=0.26$ and 0.22, respectively).

2.3.2. *Dispersal potential of vegetative fragments*

Zostera noltii fragments floating in still water showed almost no change during the first five days of experiment, *i.e.* after the detachment from the sediment. Most of the fragments tested (68%) were viable and growing (0.40 shoots/d) after 15 days. After 40 days, half of the fragments were decaying although at a lower rate (-0.02 shoots/d) than the ones growing (0.11 shoots/d). At the end of the experiment, after 55 days, 15% of the fragments were still viable and growing (0.13 shoots/d; Fig. 2.4).

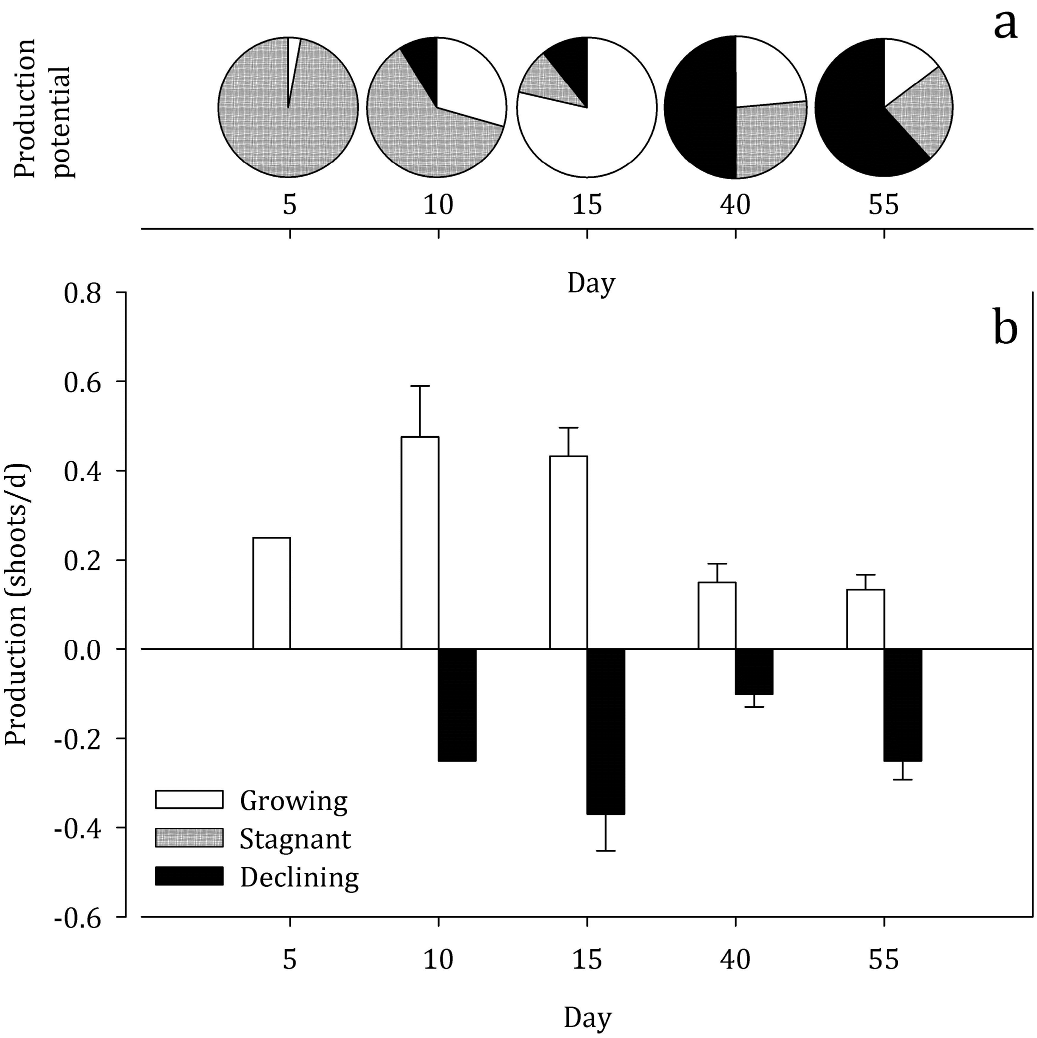

Figure 2.4. *Zostera noltii* capacity to produce new shoots while floating in still water (n=34). (a) Production potential shows proportion of fragments growing, stagnant or declining. (b) Production or decay of shoots per day; whiskers indicate standard error.

The proportion of damaged fragments increased with current velocity (Fig. 2.5). Ten percent of the fragments were damaged at 5 cm/s velocity, as indicated by the small positive change rate of the smallest shoot number class. A similar level of damage of 52 and 58% was observed for the current velocities of 15 and 30 cm/s, respectively, visible as higher positive change rates of the smallest shoot number class (Fig. 2.5). The damage caused by currents on *Z. noltii* vegetative fragments caused mostly the separation of single shoots, mainly in the older (terminal) part of the plant.

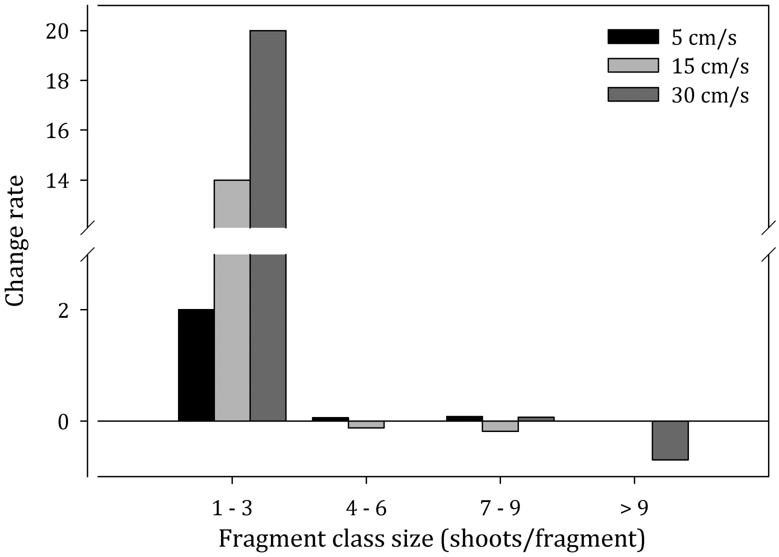

Figure 2.5. Change in size of *Zostera noltii* fragments after 2 h exposure in current velocities of 5, 15 and 30 cm/s. X-axis shows the fragments' size class.

2.3.3. Dispersal potential of reproductive fragments

Spathe release in still water occurred exclusively for the spathes carrying mature seeds, which had decaying tissues. The tissue decay precluded the precise quantification of the number of spathes released from the flowering shoots. None of the immature spathes was released from the flowering shoots during the whole experiment.

When the flowering shoots were exposed for 2 h to currents of 5 and 15 cm/s a similar low spathe release (3%) was observed. However, spathe release increased to 15% after 2 h exposure to 30 cm/s current. An increase in the number of flowering shoots with only one spathe per shoot (50% in 5 cm/s, 20% in 15 cm/s and 83% in 30 cm/s) was observed for the three tested currents, resulting from the transfer of flowering shoots from higher classes that lost all spathes except one. The release of spathes was strongest in fragments with more spathes per flowering shoot at the beginning of the experiment as shown by the negative change rate for the group of 5-7 spathes per flowering shoot (Fig. 2.6).

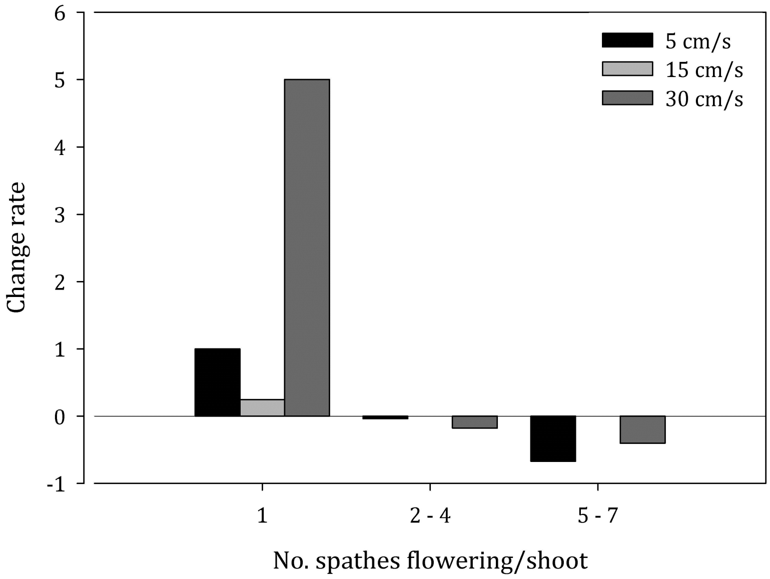

Figure 2.6. Change in number of spathes per flowering shoot of *Zostera noltii* reproductive fragments after 2 h exposure in current velocities of 5, 15 and 30 cm/s. The X-axis shows the number of flowering shoots class in relation to their number of spathes.

2.4. **Discussion**

Our results showed that floating asexual propagules of the seagrass *Zostera noltii* have a dispersal potential (in the order of thousands of km) that is not comparable to the dispersal of seeds (in the order of cm). Considering that fragments were still viable and growing after 55 days, that the average current velocity in Ria Formosa is about 50 cm/s (Duarte *et al.*, 2008) and assuming unidirectional and constant flow for the whole period and no wind effect in enhancing the transport of fragments, the potential dispersal of *Z. noltii* fragments would be 2,300 km. However, it is important to consider reestablishment success of floating fragments to assess effective dispersal. In any case, considering the *Z. noltii* fragments viability and the elevated number of fragments always floating in Ria Formosa it is expected that fragment dispersal is important for population connectivity. Other estimates obtained here, such as the fragment temporal viability, breakage and decay parameters, can provide valuable information to parameterize a hydrodynamic-based transport model that will allow a more accurate estimation of dispersal.

The observed long–term viability, survival and growth of *Z. noltii* vegetative fragments was similar to results found for *Zostera marina* with ca. 60% of fragments being viable six weeks after detachment (Ewanchuk and Williams, 1996). Prolonged viability of detached fragments is also frequently observed in macroalgae (*e.g.* Coleman and Brawley, 2005; Macaya *et al.*, 2005; McKenzie and Bellgrove, 2008), with reports of kelp viable propagules almost 18 weeks after detachment (Hernández-Carmona *et al.*, 2006). This prolonged viability indicates that floating fragments are a common dispersal vector in macrophytes (McKenzie and Bellgrove, 2008) and that fragmentation can be considered part of their life history, as is considered for corals (Highsmith, 1982). Survival capacity of *Z. noltii* fragments observed in this study was larger than that of other seagrasses, such as *Halophila johnsonii* and *Halodule wrightii* (Hall *et al.*, 2006). This higher survival may be related to the species adaptation to the dynamic intertidal habitat, where tidal currents and waves can easily brake off fragments, enhancing plant fragmentation and release of propagules. Seagrass fragmentation by hydrodynamic forces or human disturbances such as trampling or clam harvesting therefore appears to be an important factor mediating the species dispersal. To cope with habitat disturbances, *Z. noltii* may have been selected to promote fragmentation of the older parts of the clone, far from the apical shoot where clonal growth occurs. As such, the plant can still maintain its physiological integration (Marbà *et al.*, 2002) and also keep the meristem intact, increasing the chances of survival. Indeed, Cabaço *et al.* (2005) showed that damage had negative, significant effect on plant growth and survival only when the apical shoot was removed. Due to these mechanisms, even with high fragment damage (up to 58% in currents of 30 cm/s), the potential colonization capacity of *Z. noltii* fragments does not seem to be diminished. Even though in this study all the fragments were >10 cm long, which was described as the species' minimal functional length for clonal integration (Marbà *et al.*, 2002), variable success (growing vs. decaying) was observed.

The small dispersal capacity of individual *Z. noltii* seeds (<30 cm) was similar to what has been observed for other *Zostera* species (Orth *et al.*, 2000), suggesting that seeds falling from the spathes within a meadow are not likely to be exported to other areas. Other seagrass species generally have larger and heavier seeds, with faster sinking rates than *Z. noltii* (*e.g. Enhalus acoroides* and *Thalassia hemprichii*; Lacap *et al.*, 2002). Even in comparison to other submerged angiosperm species of similar seed size (*e.g. Ruppia maritima*, *Potamogeton perfoliatus* and *Stuckenia pectinata*; Koch *et al.*, 2010), viable *Z. noltii* seeds showed lower density, lower sinking rate and higher critical re-suspension velocities. The *Z. noltii* seed sedimentation rate in still water was partially explained by the seed weight (35%), as observed for some freshwater species by Koch *et al.* (2010), and both were significantly related to seed viability, suggesting that seed weight can be used as a quick, non-destructive proxy of seed viability. Seed weight explained 26% of variance in dispersal distance at higher current velocities. This is probably due to the fact that the drag force is directly related with the surface exposed to the current and not to the weight of the seed (Denny, 1988). According to our observations, the current velocity at critical resuspension approached the level necessary to resuspend sandy sediment particles, which may bury the seeds and prevent their further dispersal.

The limited seed dispersal of *Z. noltii* suggests that reproductive fragments are necessary vectors for long distance seed dispersal, as observed for *Z. marina*, (*e.g.* Orth *et al.*, 1994), and reinforces the importance of fragments as seagrass dispersal propagules. Currents had a positive effect on the spathe release from the flowering shoots and, consequently, on the seed release to the bottom, as shown by the change in the number of spathes per flowering shoot subjected to high currents (positive change rate for fragments with only one spathe per flowering shoot after 2 h exposure to 30 cm/s current). More spathes were released during exposure to current in flowering shoots that had initially more spathes, independently of the current velocity. This may be related to the reproductive biology of *Z. noltii*, *i.e.* the successive spathe production within the flowering shoot (Eichler, 1875), which results in presence of spathes of different age and maturation stage in the same flowering shoot. Hence, flowering shoots with more spathes probably carry spathes in advanced stage of maturation, which naturally start to decay and release the ripe seeds. The higher release of spathes from the more developed flowering shoots with increasing current velocity, highlights currents as an important factor for the species dispersal. Previous studies showed that seagrass flowering fragments could travel up to 150 km and for periods of almost a month (Källström *et al.*, 2008), but the importance of flowering shoot dispersal is reduced by the limited flowering season and the low persistence of the spathes on the flowering shoots, which is dependent on their maturation stage (Alexandre *et al.* 2006). Hence, considering that the difference between reproductive and vegetative fragments is the presence of flowering shoots, the potential dispersal of reproductive fragments can only be equal or lower than that of vegetative ones, which are present all year around. In addition, this study showed fragments can stay viable for a prolonged period of time. This may be common in seagrasses, as Balestri *et al.* (2011) found that *Posidonia oceanica* fragments can stay viable for three years without losing the capacity to root and grow. Considering this, we can conclude that the dispersal of vegetative fragments plays an important role for the species’ dispersal and future research should reveal how this dispersal translate into migration.

The considerably higher dispersal potential of asexual propagules of *Zostera noltii* in comparison to that of detached seeds is of fundamental relevance for the classical interpretation of the species life history, and likely to be extended to other seagrasses or other clonal aquatic plants (Gliddon *et al.*, 1987; Fischer and van Kleunen, 2002). The sexual component producing dispersal vectors in the form of pollen and seeds are classically considered as the component that can reach areas beyond the close neighborhood of the parental plant, despite substantial evidence for clonal dispersal across long distances to potentially outreach sexual propagules in many taxa (Highsmith, 1982; Johansson and Nilsson, 1993).

While addressing the importance and maintenance of clonal life history, studies have focused primarily on population level topics, such as phenotypic plasticity (Alpert and Simms, 2002; Bergamini and Peintinger, 2002; Donohue, 2003), foraging strategy and micro-habitat variability (De Kroon and Hutchings, 1995; Poor *et al.*, 2005), and density dependence effects on the balance of clonal versus sexual allocation (Ikegami *et al.*, 2012). In our study, by experimentally replicating the conditions clonal fragments face naturally, we showed that broken fragments are viable for long periods of time (more than 50 days), are able to grow and produce shoots, and can carry and release seeds for long distances. These traits are likely to increase the chances of effective asexual dispersal (*i.e.*, migration). If the fragments establish in new area after the dispersal they will become a part of the dispersal strategy of the plant (Hall *et al.*, 2006). Therefore, these could be adaptive dispersal traits that play a role in the ultimate causes explaining the maintenance of clonal growth. Our findings should attract attention to processes that go beyond the local population in order to understand the evolutionary implications of clonal growth. To the best of our knowledge our study was the first comparison of the sexual and vegetative dispersal capacities of a clonal marine plant species. However, additional research is necessary to understand the probability of rooting and growth of seagrass fragments after dispersal and settlement.

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Chapter 3.

Post-dispersal settlement success of *Zostera noltii* fragments

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Abstract

Floating fragments of *Zostera noltii* have high dispersal potential but their establishment success after dispersal is unknown. In this study we quantified survival, rooting and development of vegetative fragments following dispersal and settlement, in relation to the initial fragment size (3 or 6 shoots) and dispersal regime, *i.e.* different ratios of period of floating and settlement in sediment (0, 2, 4 and 10 weeks). After 10 weeks, survival (considered only when rooting was observed) was significantly higher for fragments which didn't spend any time floating prior to the settlement (~60%) than in those subjected to floating periods (~30%). There was a significant interaction between the effect of dispersal regime treatment and the initial size of fragments on the condition of the apical shoot (intact, broken, 2nd apical). Prolonged floating decreased branching of fragments, relative to the treatment where fragments didn't float. Branching was only observed for settled fragments and was significantly higher for the initially larger fragments. The average number of branches of the branched fragments (1.8) was independent of the floating period but was dependent on the initial size of the fragments. The ratio of above: below ground biomass at the end of the experiment reveals the colonizing-type response in the fragments. Our results indicate that rooting of floating *Z. noltii* fragments is possible, but that prolonged floating period in the dispersal regime decreases their success of establishment, *i.e.* survival, rooting and branching. Applying these results to the Ria Formosa lagoon, Portugal, if several assumptions are met we estimated that successfully dispersed and established fragments could occupy more than 68 ha every year, representing over 5% of the actual *Z. noltii* coverage in the lagoon.

Keywords: seagrass; *Zostera noltii*; dispersal; settlement success; establishment.

3.1. Introduction

Dispersal is a life history trait relevant for both maintaining existing populations and for colonizing new areas. It is defined here as any movement which might lead to gene flow (Ronce, 2007). To fulfil the dispersal role, an organism either moves itself, or it produces propagules. Propagules are units of dispersal, which can be of sexual or asexual origin. Among sexual propagules we recognize spawned gametes, algal spores, plant pollen or seeds and planktonic larvae. Asexual propagules are produced by a single parent, a process most evident in propagation of clonal organisms. Asexual propagules can be special structures (*e.g.* bulbils or rosettes) or simply a fragment of a parental organism. For effective dispersal, propagules need to successfully go through three phases – departure, transfer and settlement (Bonte *et al.*, 2012). Partially clonal organisms rely on both clonal and sexual propagation to maintain and expand their populations. Sexual propagation is advantageous for genetic diversification, a benefit for adaptation to changing environments (Barton and Charlesworth, 1986; Becks and Agrawal, 2012). Asexual propagation is often viewed as a more cost-efficient space occupying strategy in stable environments, by avoiding the need for strategies to ensure gamete encounters. Either way, dispersal is an important process providing avoidance of local competition with relatives, while spreading the risk in dynamic or heterogeneous habitats (Burgess *et al.*, 2015).

Freshwater aquatic plants can disperse via different types of fragments (Riis *et al.*, 2009) such as fragmented parts (*e.g.* Nichols and Shawn, 1986, Johansson and Nilsson, 1993) and dormant apices (*e.g.* Potamogeton crispus; Nichols and Shawn, 1986). Few studies have addressed the topic of dispersal via fragments for marine vegetation, whose drifting fragments show the potential to disperse across large distances (Ceccherelli and Piazzi, 2001; McKenzie and Bellgrove, 2008). An important body of experimental data comes from seagrass restoration ecology. Seagrasses are clonal angiosperms adapted to complete their life-cycle in the marine environment. They encompass less than 60 species (Green and Short, 2003), but are among plant species with widest distribution on the planet (Cook, 1996). While rhizome elongation can explain local space occupation and distribution patterns (*e.g.* Marbà and Duarte 1998), wide distribution and population connectivity, at least to some extent, requires ability to disperse far. Seagrasses are declining worldwide (Waycott *et al.*, 2009) and efforts are made to restore endangered populations (Fonseca, 1998; Orth *et al.*, 2010). Planting of seagrass fragments is a common approach in transplantation for meadow restoration (*e.g.* Fonseca, 1998; Orth *et al.*, 1999). Success of these methods can reach well over 70%, generally increasing with the increase in the size of the planting unit from single fragment, to plugs and sods (*e.g.* Davis and Short, 1997; Uhrin et al, 2009), though it mainly lingers between 35 and 40% (Fonseca et al, 1998). However, the methodology in case of restoration is focused on improving survival and doesn't approximate the natural process of seagrass fragment re-establishment (*e.g.*, the natural release and transfer stages are absent). The majority of studies on seagrass dispersal focused on the role of seeds and pollen (Conacher *et al.*, 1994; Lacap *et al.*, 2002; Orth et al, 2006 and the references therein; Erftemeijer *et al.*, 2008; Ackerman, 2006. and the references therein), but possible dispersal via vegetative propagules has been reported (Berković *et al.*, 2014; Hall *et al.*, 2007). Production and release of seagrass fragments, either natural or human induced, is frequently observed and accounts for high estimated abundance of fragments (Balestri *et al.*, 2011; Cabaço *et al.*, 2005). A recent study on dispersal potential of different types of dwarf eelgrass (*Zostera noltii*) propagules focused on the transfer phase of dispersal, revealing prolonged viability and growth of floating fragments (Berković *et al.*, 2014). Similar findings were obtained for other seagrass species (Hall *et al.*, 2006; Campbell, 2003). To date, estimates of seagrass fragments' dispersal distance extend from about 150 km for *Zostera marina* (Källström *et al.*, 2008), 250 km for *Halodule wrightii* (Hall *et al.*, 2006), up to more than 1,200 km for *Zostera noltii* (Berković *et al.*, 2014), all assuming a dispersal period of one month. In addition, dispersal via plant fragments in some species can last even longer than one month (Berković *et al.*, 2014). While some studies through indirect observation conclude that LDD via plant fragments does happen in seagrasses (Di Carlo *et al.* 2005, Hall *et al.* 2006; Harwell and Orth, 2002), insufficient attention has been given to its success and consequences (Campbell, 2003; Kendrick *et al.*, 2012). Furthermore, it is still a matter of debate whether the success of the re-establishment of dispersed fragments is high enough to be relevant for the species' life history. Some authors argue that these events are unlikely to succeed and happen too rarely to effectively contribute to species spread and establishment in new areas (Ewanchuk and Williams, 1996), at least across large spatial distances in one step (Arnaud-Haond *et al.*, 2012). Although few studies discussing seagrass dispersal strategies overlooked the possibility of fragment detachment and settlement, near or far from the point of origin, or just vaguely mention it (Olesen *et al.*, 2004; Virnstein and Hall, 2009; Harwell and Orth, 2002), others concluded that long distance dispersal (LDD) is the most parsimonious explanation for species' distribution and sudden expansions (Hall *et al.*, 2006). Campbell (2003) observed fragments of two Posidonia species, *P. australis* and *P. coriacea*, establishing in bare sediments, but only *P. australis* fragments were noted to grow after establishment. An experimental study by Hall and colleagues (2006) with *Halodule wrightii* and *Halophila johnsonii* revealed that both species' detached plant fragments have the potential to successfully establish after a period of floating. In brief, although (LDD) in marine plants has been observed (Harwell and Orth, 2002; Hall *et al.*, 2006), our understanding of its effectiveness requires further experimental evidence that fragments can settle and survive after the transfer phase.

Here, we test if successful establishment after the transfer phase of dispersal supports the hypothesis that vegetative fragments are a relevant dispersal propagule type for the dwarf eelgrass *Zostera noltii*. We quantified the survival of vegetative *Z. noltii* fragments after the 10 weeks dispersal period, and their post-dispersal settlement success, *i.e.* rooting and growth, for different plant fragment sizes and dispersal regimes. We mimicked the process in which the plant fragments get entangled while dispersing via floating on the sea surface or drifting along the bottom in shallow waters.

3.2. Material and methods

Fragments of *Zostera noltii* plants were collected during a low tide in a natural intertidal meadow in one of the main channels in Ria Formosa lagoon, South Portugal, close to the Ramalhete field station of University of Algarve (37.00°N, 7.97°W). Plant fragments were maintained in mesocosm facilities in the Ramalhete field station under controlled conditions. They were placed in outdoor tanks (550 L) with low seawater renewal, shaded with plastic mesh to avoid overheating. The experiment was conducted in spring 2013 (March-June), during the *Z. noltii* growth season in this region (Peralta *et al.*, 2005).

We collected over 600 fragments of *Z. noltii* with apical shoots: 350 small fragments, with three shoots, and approximately 250 large fragments, with six shoots (all with roots). All the plant fragments were manually uprooted from the sediment, carefully washed, measured, individually marked with a soft tag (tied coloured cotton threads) on the last (newest) internode and placed in the mesocosm facilities until further processing. During the course of the experiment, fragments were randomly selected from the mesocosm facilities according only to their original shoot number, and assigned to treatment groups. The experimental plots were set up nearby, in the bare sediment of an intertidal pool, partially covered by seagrasses and algae. Presence of seagrass in the pool was taken as a proof of suitable seagrass habitat. We conducted a pilot study to select the methodology for samples placement in the field and tagging. The best approach for tagging was using a thin gardening wire (plasticized to avoid damage to the rhizome) twisted around the rhizome of the last internode into a tiny hook (3-5 mm in diameter), whose end was pushed to the sediment (Fig. 3.1).

Figure 3.1. Gardening wire used to mark last internode of the *Zostera noltii* fragments in the experimental plot.

In order to closely mimic the natural process of fragment entanglement we avoided planting the fragments under the sediment. Therefore we only fixed the plant fragments onto the bottom, adjusting the methods described by Fonseca *et al.* (1998). Fragments were held in contact with the sediment using pins made of 0.9 mm diameter plasticized wire, approximately 10 cm long and bent in half to take the shape of a hair pin (Fig. 3.2). These were placed over the rhizome and gently pushed into the sediment.

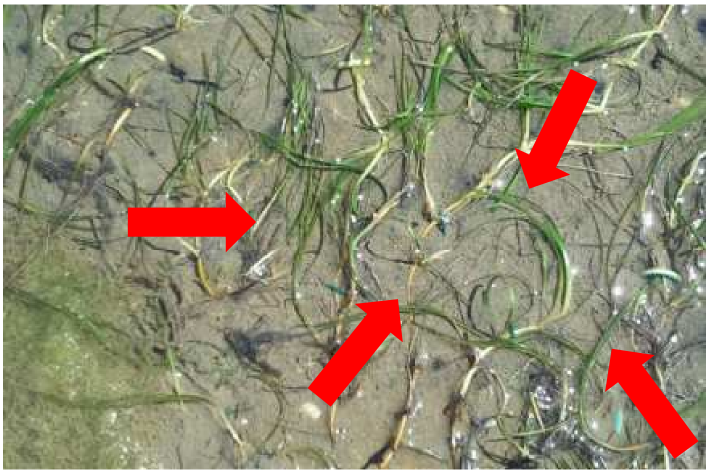

Figure 3.2. *Zostera noltii* fragments held in contact with the sediment using plasticized wire pins (marked with red arrows).

During the weekly monitoring of the experimental plots, pins were checked and pushed back into the sediment if found dislodged or loosened.

The experimental design consisted of four treatments, which differ in the dispersal regime they were exposed to, *i.e.* how long they were floating (F) and were in the contact with the sediment (S). We tested the effect of dispersal regime on survival and rooting with three groups of plant fragments exposed to 0, 2 and 4 weeks floating periods and only on survival for fragments which floated 10 weeks but were not placed in sediment afterwards. Additionally, we had a natural control group. Sixty large and sixty small plant fragments were randomly assigned to each group (Table 3.1), except for the control. For the control thirty apical shoots were selected, marked on the last (newest) internode, and their rhizome was cut approximately 4 cm from the apex without uprooting the plant. Length of the control fragments was chosen as an average length of all the sampled *Z. noltii* fragments (3 and 6 shoots).

Table 3.1. Naming of treatment groups and assignment of fragments by their size.

	Floating time (weeks)	**Time in sediment (weeks)**	**No of small fragments**	**No of large fragments**
Control	NA	10	40 intermediate size fragments	
F0S10	0	10	60	60
F2S8	2	8	60	60
F4S6	4	6	60	60
F10S0	10	0	60	60

Surviving plant fragments in the mesocosm were regularly cleaned from epiphytes, while the dead ones were quantified and removed. The tanks were cleaned weekly from algae growing on the sides and from shed leaves on the bottom. Salinity and temperature were controlled at every visit. Plant fragments in the field and in the mesocosm were monitored weekly for survival, rooting and growth of the rhizome.

In the field, only the fragments which were clearly attached to the bottom (not floating after carefully removing the pin) were considered as rooted. The fragments that were not rooted or dead were quantified. A fragment was considered to be dead if it did not hold any living (green) shoots. At the end of the experiment, after 10 weeks, all the fragments in the field and in the mesocosm were collected carefully to avoid breaking and were transported in seawater to the laboratory for further analysis.

During the third week of the experiment, after the fragments of F2S8 groups were settled in the field, a sudden strong increase in number of crab holes and activity of crabs was noticed along the experimental plots (Berković and Gemelli, pers. observation). This was marked during the regular monitoring and the experiment was continued nevertheless.

3.2.1. Survival and growth of fragments

Survival of the plant fragments was expressed as 0 (dead) and 1 (viable) fragments at the end of the experiment. The ANOVA assumption of normality was not met so to test the effect of the two factors on survival we conducted Permanova in Primer (Clarke and Gorley, 2006). This was preferred to analysis of deviance (binomial response and two factors as predictors, with binomial errors using a GLM) due to imbalanced groups. We define dispersal regime (FL) and size in number of shoots (SH) as two factors, both fixed. We ran 9,999 iterations with unrestricted permutation of raw data. Then we used pairwise test both ways, with SH within FL and FL within SH, again with 9,999 iterations. Further we tested the independence of fragment size and dispersal regime using Chi square test on contingency tables using the frequencies of fragments that survived.

Fragment growth at the end of the experiment was calculated for the surviving fragments. Part of the individual marks was not possible to reliably interpret, so the calculation was performed on group averages rather than per sample. All the initial lengths of the fragments in the group were averaged, and the same was done with the final lengths. These values were then used to calculate growth, in cm/day, following the formula:

(*AVG final length – AVG initial length*) */ number of days*.

This measure does not represent the actual elongation rate, as decay of fragments on their older end is accounted for as well. As observations were brought down to one averaged value, no statistical test was carried out. Same approach was used for growth in number of shoots. In this case both initial and final number of shoots for all the fragments was known, but considering very unbalanced number of samples in each group, again just the group average values were used to characterize the change in shoot number.

3.2.2. State of the apical shoot and branching of fragments

The state of the apical shoot was classified for survived fragments as follows: “main”, when the main rhizome axis had an intact apical shoot; “2nd apical”, if the apical shoot of the main rhizome was broken, but a branch with the respective apical shoot was present; and "broken", if the fragment did not have any apical shoots. State of the apical shoot is relevant for the architecture and growth of the plant. In *Z. noltii*, apical dominance strongly affects clonal architecture (Brun *et al.*, 2007), restricting the branching and regulating the length of the internodes. First we tested for the effect of dispersal regime and initial fragment size on the damage to the apical shoot, noting whether the main apical shoot was damaged or not (binomial response). In the second analysis we looked at what happened to the fragments which had the apical shoot damaged – did the plant develop a new apical shoot or not (binomial response) given the same predictor factors. We used analysis of deviance, with binomial error terms (generalized linear model - GLMs) in R software (R Core Team, 2013) for both analyses.

Initially all the plant fragments consisted of a single rhizome, without branches, so at the end of the experiment we could quantify branching which is relevant for clonal architecture and space occupation (Brun *et al.*, 2007). The percentage of branching fragments and the number of branches per branched fragment was estimated from the total number of live fragments. In the first analysis, we only noted if the fragments branched, ignoring the number of branches. This was used to analyse the occurrence of branching. We used GLM with binomial error terms to model the effect of both variables dispersal regime (FL) and initial fragments' size (SH) on the occurrence of branching, using R software (R Core Team, 2013). We tested if the initial fragment size had an effect on the occurrence of branching using a Mann-Whitney rank sum test because normality for t-test was not met (Sigmaplot v. 11 (Systat, San Jose, CA)). For the effect of treatment on the occurrence of branching we used Kruskal-Wallis one-way analysis of variance. In the second analysis we only included branching fragments and quantified number of branches per fragment. To analyse the number of branches produced per branching fragment we used the GLM in R.

3.2.3. Biomass analysis

The ratio of above: below ground biomass indicates the stage of establishment of newly settled or colonizing fragments (Martins *et al.*, 2005; Cabaço *et al.*, 2012). We looked at the biomass only of the plant fragments with apical shoots ("main" or "2nd apical"), which can potentially grow. Biomass was measured by drying the separated plant compartments (leaves, rhizomes and roots) at 60°C for 48 h, and weighting them (0.001 g precision). The biomass of living fragments without an apical shoot ("broken") was not estimated, because the fragments were not entire. The plants' dry weight was used to assess the biomass allocation in each compartment and to calculate the ratio of aboveground (leaves) to belowground (rhizomes and roots) biomass (A:B ratio). Since only surviving fragments with present apical shoots were used, groups were strongly imbalanced, so we opted to interpret these data without conducting statistical tests.

3.3. Results

3.3.1. Survival and growth of fragments

The highest survival at the end of experiment, after 10 weeks, was observed for the group without a floating period in the dispersal regime and with larger initial fragment size - F0S10 6 shoots (100%). The second highest survival was observed for the control group (80%). The remaining fragments, exposed to dispersal regimes with floating periods of 2, 4 and 10 weeks, *i.e.* groups F2S8, F4S6 and F10S0 of 3 and 6 shoots, had lower survival ranging from 7 to 44% (Fig. 3.3 a). Overall, initial larger plant fragments had significant higher survival in relation to the smaller ones, 51% versus 19% (t-test, $p<0.001$). The two factors (SH and FL) were related (chi-square, $p<0.001$) and an interaction plot showed that survival of initially larger fragments (6 shoots) was always higher than that of smaller ones (3 shoots) but the effect of size was dependent on the level of dispersal regime treatment (Fig. 3.3 b). Strong decrease in survival was visible for the larger fragments (6 SH) if they were exposed to a floating period in the dispersal regime, but the duration of the floating period, if there was one, had a weak effect on the survival of larger fragments. In the case of smaller fragments (3 shoots), highest survival was observed for the group which wasn’t settled on the sediment, but was left floating during whole 10 weeks. Overall average survival of fragments was over 34%.

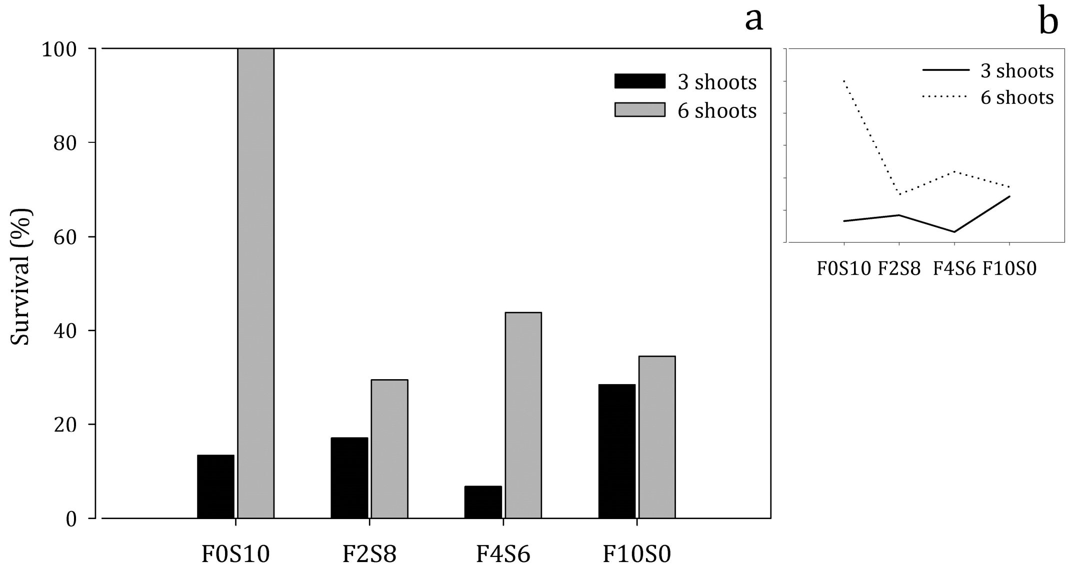

Figure 3.3. (a) Survival percentage of small (3 shoots) and large (6 shoots) *Zostera noltii* fragments, after 10 weeks. (b) Interaction plot for the two levels of size factor SH (3 and 6) and 4 levels of dispersal regime factor FL (F0S10, F2S8, F4S6, F10S0).

The first rooted plants were found two weeks after planting, in group F0S10, for which more than half of the larger plants were rooted. The first plants to root in group F2S8 were observed three weeks after the settlement and in group F4S6 one week after settlement in the sediment.

Surviving plants showed a positive change in rhizome length for both groups of treatment F0S10, and for smaller fragments' in F2S8 and F10S0 treatments. All other groups had a negative change rate (Fig. 3.3 a). A different pattern was observed for the change in number of shoots, where most groups had positive change, while F2S8 6 shoots and both fragment sizes in the group F10S0 had negative changes (Fig. 3.3 b).

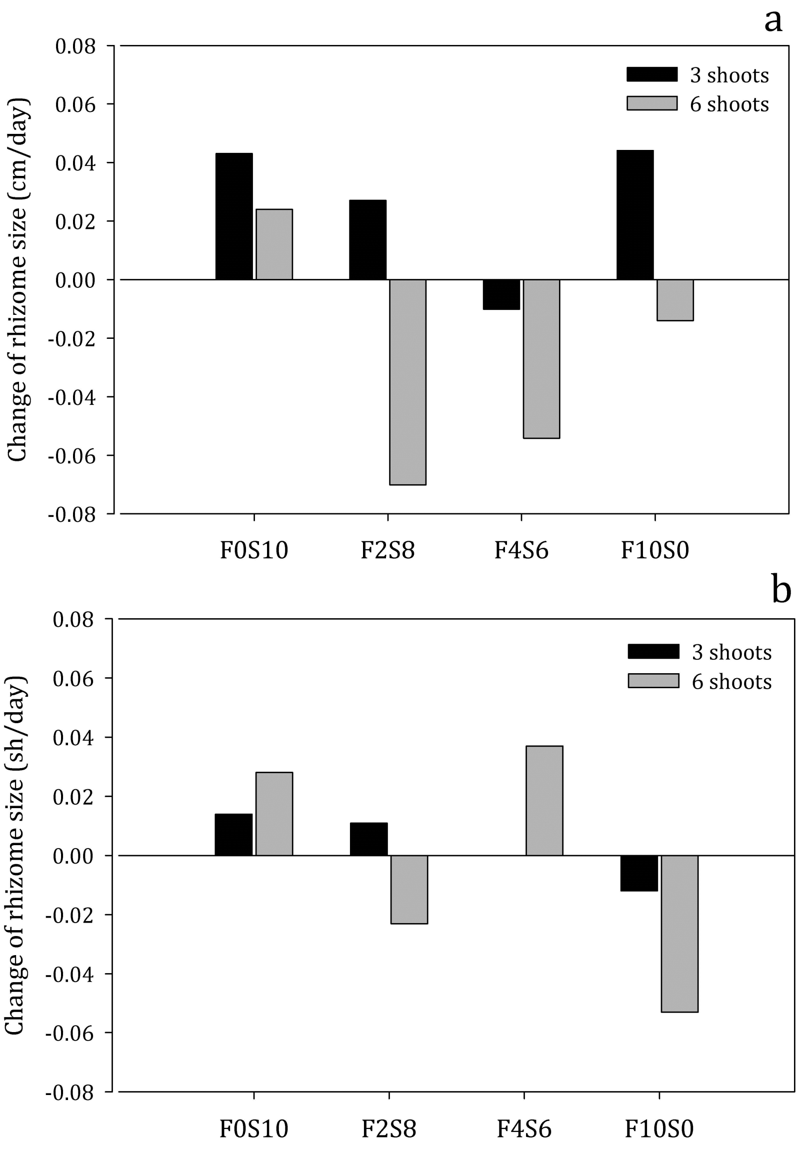

Figure 3.4. Change of rhizome size of *Zostera noltii* fragments for small (3 shoots) and large (6 shoots) fragments (a) expressed as cm per day and (b) expressed as number of shoots per day.

3.3.2. State of the apical shoot and branching of fragments

The main apical shoot remained intact in more than 70% of surviving plant fragments. When the main apical shoot was broken, a 2nd apical shoot was present in approximately 60% of cases. Almost all of the control plants and the F10S0 group had the main apical shoot intact (Fig. 3.3). The effects of initial fragment size and of the dispersal regime were non-independent (GLM, p<0.001). Initially larger fragments generally sustained less damage and recovered in more cases than the initially smaller ones.

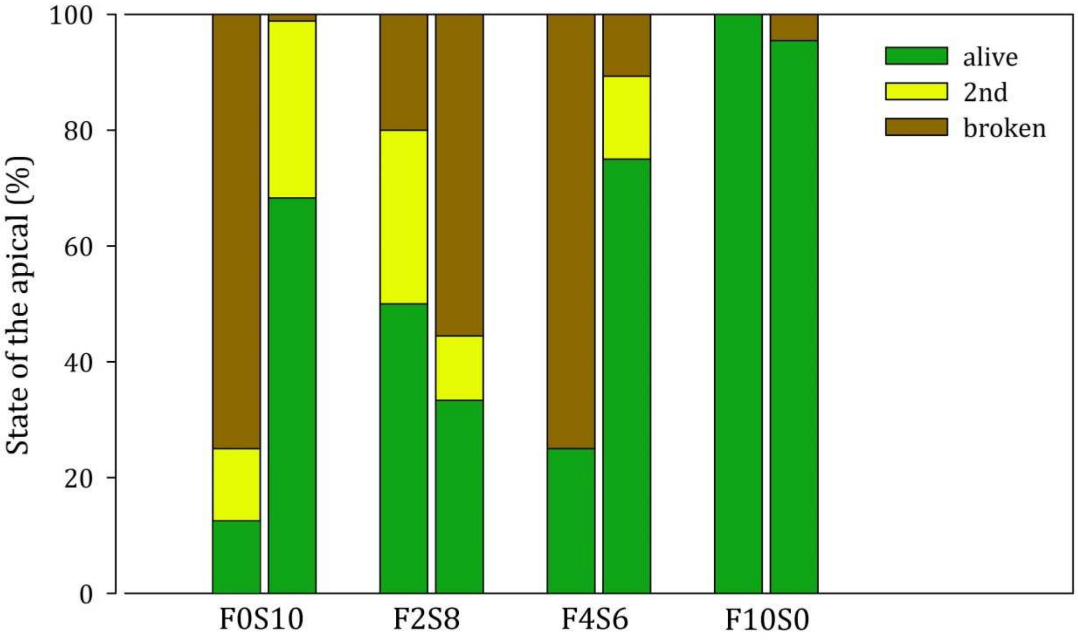

Figure 3.5. State of the apical shoot of small (3 shoots; columns on the left side of each pair) and large (6 shoots; columns on the right side of each pair) *Zostera noltii* fragments.

Branching was highest for the fragments without floating period (F0S10); 45% of surviving fragments developed at least one branch. None of the surviving plants that were floating during the whole experimental period (F10S0) had any branches, *i.e.* branching was only observed for fragments which were settled in the sediment. For the other groups we observed branches on 20-30% of the surviving fragments. GLM didn't indicate interaction ($p>0.05$). Fragment size clearly affected the branching – larger fragments developed branches more often compared to the smaller ones (10% versus 34%; Mann-Whitney, $U=33.88$, $p<0.001$). Dispersal regime treatment had effect on branching but only F10S0 treatment was significantly different from all the others (Kruskal-Wallis, $H=37.1263$, $df=3$, $p<0.001$), but there was no effect on branching when the F10S0 group was omitted from the analysis (Kruskal-Wallis, $H=5.8732$, $df=2$, $p=0.053$). GLM didn't indicate interaction between the factors for the analysis of number of branches ($p=0.823$). The number of branches per branching fragment varied between 1 and 2, and it was independent of the dispersal regime treatment (Kruskal-Wallis, $H=5.50845$, $df=2$, $p=0.064$), but was dependent on the initial size of the plant fragments (Mann-Whitney, $U=72.00$, $p<0.02$). Fragments with initially three shoots developed one branch per fragment, and fragments with initially six shoots developed about two branches.

3.3.3. Biomass analysis

The higher establishment of rhizome/root biomass relative to leaves (A:B ratio) occurred in smaller fragments of dispersal regime without the floating period (F0S10) in contrast to the smaller fragments with shortest possible period in sediment (F4S6). Overall larger fragments (6 SH) had more stable a:b ratio, changing little across the treatments (Fig. 3.4). The control group had an A:B ratio of 0.7 (not shown).

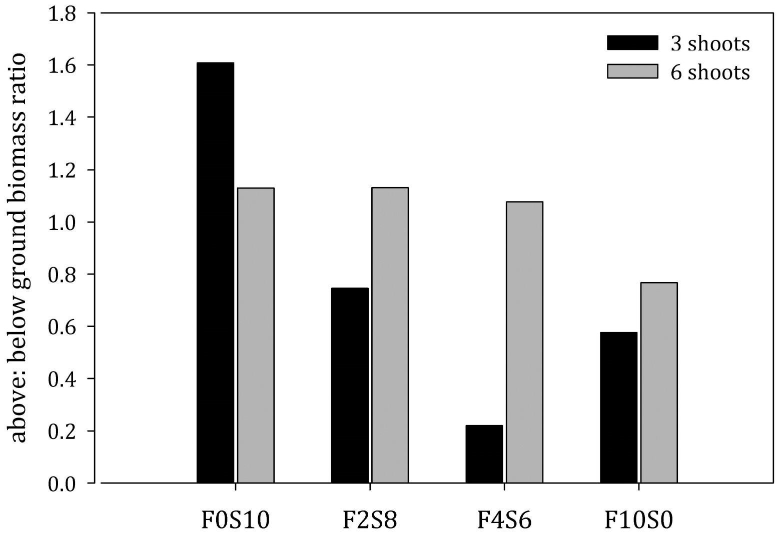

Figure 3.6. Ratio of aboveground to belowground biomass for small (3 shoots) and large (6 shoots) *Zostera noltii* fragments for four treatments at the end of the experiment.

3.4. Discussion

This study showed that fragments of *Zostera noltii* had on average 34% survival after dispersal, reaching 100%, depending on the occurrence of the floating period in the dispersal regime and the initial fragment size. Adding to the previous findings of prolonged viability and growth of detached fragments (Berković *et al.*, 2014), these results support the hypothesis that fragment dispersal and establishment is conceivable and can be a relevant life history trait in dwarf eelgrass.

In our study, survival of the settled seagrass fragments was affected by the presence or absence of the floating period, but not by its duration. Fragments that were uprooted and settled immediately after processing (measuring and tagging) showed the highest survival, apparently even higher than control ones. This might have been the result of increased space availability, also supported by the branching patterns of these fragments, which were the highest among all the treatments. The large fragments (6 shoots) that floated for two weeks prior to settlement (F2S8) showed low survival rates. We observed a bioturbation disturbance in days immediately following the settlement of these fragments in the field: a sudden increase in number of crab (*Carcinus maenas*) holes in the area, which after an initial peak started disappearing and finally almost completely vanished over the course of 3 weeks. This could explain the low survival of the plant fragments set in that period. Even though crabs do not directly feed on the seagrass their presence may cause the uprooting of plants (by adult individuals) and cutting of sections with basal meristems (by juveniles), posing measurable damage to the seagrass meadow (Malyshev and Quijón, 2011; Garbary *et al.*, 2014). Moreover, these fragments had the highest damage of the apical shoots among the large fragments (reaching almost 70%).

The observed discrepancy in growth response metrics between measurements in cm/day or in shoots/day is probably a consequence of the unbalanced growth patterns in the two ends of plant fragments. Namely, the apical growth and production of shoots with shorter internodes on the younger end of the fragment was not synchronised with the dwindling of the longer internodes in the older part of the fragment. This indicates that if the apical part of the rhizome is intact, then the fragment can still successfully establish, even when the plant fragment is seemingly shortening.

The methodology used to evaluate the rooting success of the plant fragments was conceived to avoid damage to the fragments in the experimental plot. As a consequence of this we could only obtain a conservative evaluation of the rooting process, as only the fragments clearly visibly rooted were considered as such. We believe this led to an underestimation of the time necessary for rooting initiation. In general, we can infer that fragments required more time to root if they were exposed to a period of floating prior to settlement. Once settled they can take between one to three weeks to root.

Increasing relative belowground biomass is related with the level of success in seagrass establishment after transplants to a new area (Martins *et al.*, 2005) and the same association is also seen in natural colonizing meadows (Cabaço *et al.*, 2012). In our study, control plants had an A:B ratio similar to the established *Z. noltii* meadow in the same lagoon and at the same time of the year, 1.7 and 1.6 respectively (Cabaço *et al.*, 2012). With the exception of fragments in F0S10 3 shoots group, the A:B ratios estimated for the experimental plant fragments in this study ranged between 0.2-1.2. They were therefore generally lower than those in the natural meadows in the same lagoon (Cabaço *et al.*, 2012) and those measured elsewhere for *Z. noltii* (1.2; Duarte and Chiscano, 1999). This leads us to conclude that uprooting of the plants alone, rather than the dispersal regime, induces the colonizing-type response in the *Z. noltii* plants. The ratio found in this study for the plant fragments which were still floating at the end of the 10 weeks experiment, and were not set in the contact with the sediment, matched that of the colonizing meadows (0.6; Cabaço *et al.*, 2012), indicating that those plants might still be fit enough to initiate growth and space occupation if set in the contact with sediment. It is possible that further decrease of the belowground biomass may lead to the loss of stored carbon necessary for the success of the settled plants (Zimmerman *et al.*, 1995).

We show that the settlement of floating fragments in bare sediment can be successful and lead to the establishment of the plants after asexual fragment dispersal. Some freshwater plant species also settled and successfully re-established within a 10 week-long experimental period (Barrat-Segretain *et al.* 1998). Besides the settlement in new, bare areas, seagrass fragments can also settle within the already established meadows, adding to the local genetic pool. Ewanchuk and Williams (1996) reported the entanglement of *Z. marina* fragments in the canopies of the established meadows, and growing of their roots towards the sediment. Such situation could be equally possible for *Z. noltii* fragments. In our particular study area, tidal movements, bathymetry of the lagoon and the current *Z. noltii* distribution probably even increase the chances of fragments settling in this way.

This study supports our hypothesis that the post-dispersal establishment of *Z. noltii* fragments is indeed possible. We also consider the probability and success of this event to be relevant for the species' persistence in the lagoon. To evaluate the occurrence of this process in our study area, we estimated the number of *Z. noltii* fragments settled along the shores of the channels in the Ria Formosa lagoon. Covering various types and orientations of the shore we focused on *Z. noltii* fragments with a minimum of two shoots, recently broken (evaluated based on the necrosis of the tissue) and stuck in the sand (partially buried) during the previous day and found on average 1 shoot/m^2 of intertidal area of Ria Formosa's channels. We used the available literature data on the seasonal variation in the production (Cabaço and Santos, unpublished data), coverage of *Z. noltii* (Guimarães *et al.*, 2012) and meadow density (Alexandre et al, 2005) and quantified the area new recruits could occupy based on the results of this study. In each step of calculation we opted for the most conservative assumptions and simplifications which we made, *i.e.* we assumed fragments only settle along the main channels, excluding most of the intertidal area; we didn’t consider growth or production of new shoots during the one year period and assumed uniform density of the new meadow. This calculation showed that conservatively we could expect over 68 ha of established fragments each year, representing about 5% of current *Z. noltii* coverage in the lagoon. Because of the above mentioned assumptions this final result might be an underestimation of the real value. We suggest that future studied should focus on measuring each of the necessary variables needed to provide more accurate estimates. Information on the capacity of the system to maintain or even spread is valuable for both the managers of the Natural Park and other stakeholders involved in the use and management of the lagoon area where seagrass meadows are frequently subjected to natural and anthropogenic disturbance (Cunha *et al.*, 2013).

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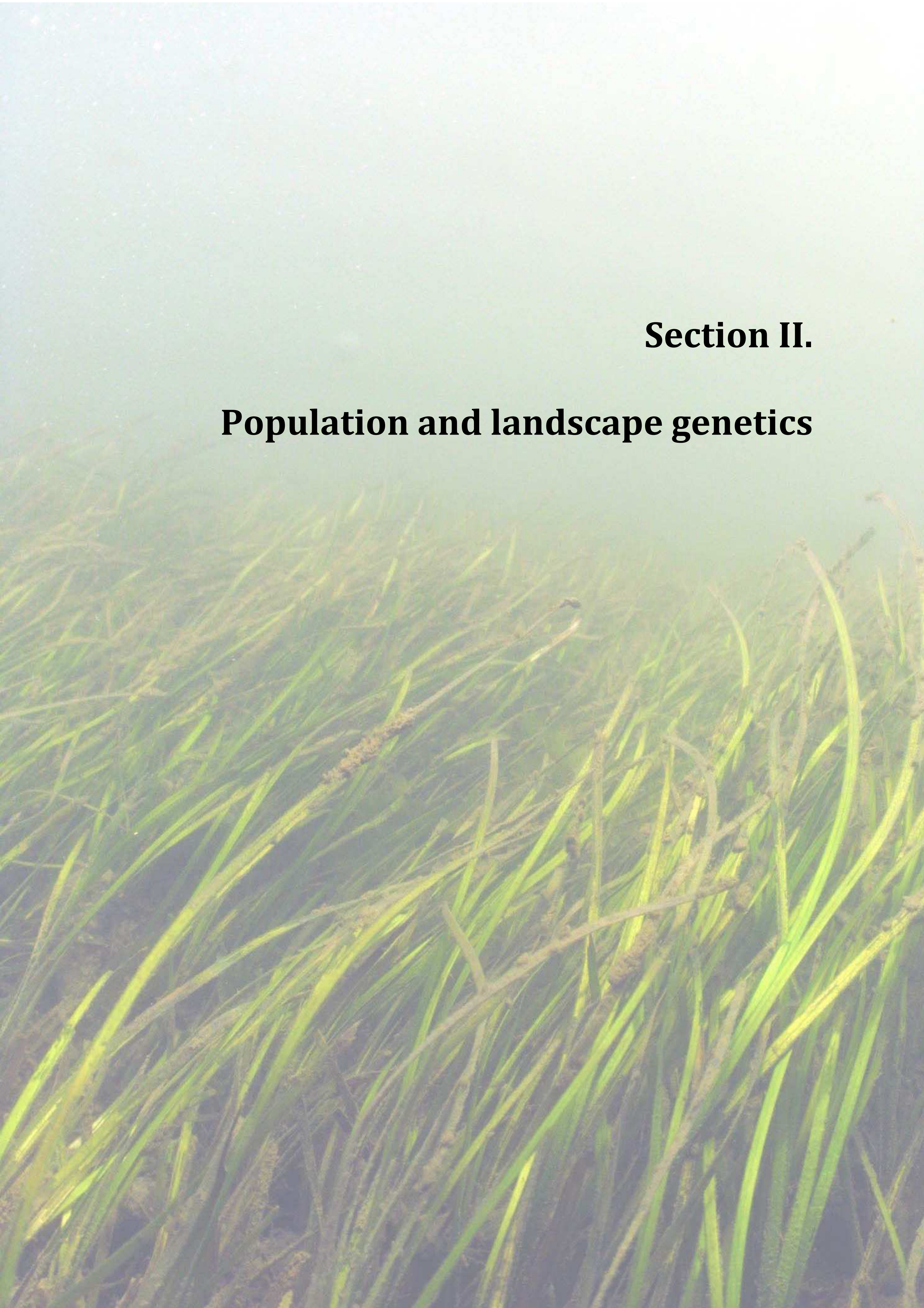

Section II.

Population and landscape genetics

Chapter 4.

Individual based genetic analyses support long distance asexual dispersal and disturbance-clonal richness associations in seagrasses

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Abstract

Clonality occurs in many species and the dispersal role of clonal reproduction is diverse, resulting in dispersal distances from a few centimetres to thousands of kilometres. In this study we use genetic data to describe the clonal structure of seagrass meadows along tens of km within a coastal lagoon and we relate it to dispersal processes. We employed a spatially explicit individual based random sampling design for *Zostera noltii,* across an unprecedented extent (84 km^2). Within the 3,185 genetic samples (63% distinct clones) we identified 1,999 unique multi-locus genotypes using nine microsatellite markers, showing many clones separated by distances as large as the available habitat in the Ria Formosa lagoon (sampled distance up to 26.4 km). To evaluate the possible contributions of dispersal versus clonal growth we estimated clonal age assuming no long-distance dispersal, as if rhizome elongation was the sole spreading means for clones. For this, we iteratively corrected age estimates by combining sampling locations with spatially explicit sea-level data for the time the clone would have been initiated. The majority of the tested clones were found to be over 20, even 40,000 years old, leading us to conclude that more likely explanation of the observed clonal structure needs to include long distance dispersal of asexual propagules, bringing the necessary timescale to tens or hundreds of years. The same analysis was completed for the sympatric seagrass *Cymodocea nodosa*, where estimated age surpassed 53,000 years, supporting similar conclusions as for *Z. noltii*, that clonal dispersal by rafting fragments is necessary to explain the spatial extent of this clone. We also studied the association between a source of disturbance (*i.e.*, factors that cause biomass removal) and increased clonal richness at the landscape scale. Modelling the probability of finding each multi-locus genotype at a minimum of 5, 10 and 15 times in the sample produced a probability map which revealed higher probabilities to sample large clones away from the source of disturbance. This result shows that disturbance is associated to reduced population clonality.

Keywords: asexual dispersal, long distance dispersal, clonal reproduction, seagrass, microsatellites.

4.1. Introduction

Clonal organisms are widespread throughout all domains in the Tree of Life, including multicellular eukaryotes (*e.g.*, about 45% of vascular plant families exhibit some type of clonality (Tiffney and Niklas, 1985), while this proportion is over 70% in animal phyla (Hughes, 1989), but only 0.1% in vertebrates (Avise, 2008)). Most eukaryotic clonal organisms are also capable of reproducing sexually. The circumstances under which organisms benefit from sexual or asexual reproduction and how both systems are maintained have been the focus of much study and debate in evolutionary biology (Barton and Charlesworth, 1986; Butlin, 2002; Silvertown 2008). Besides the evolutionary advantages of sexual reproduction that are relevant at the population level, sexually derived bodies (gametes or pollen, zygotes, seeds, larvae) are often thought of as the relevant dispersal propagules mediating gene flow within and between populations, an added component to the list of important life history traits associated with sexual reproduction. Some of the strategies advanced to justify asexual reproduction are generally constrained to the population spatial limits. For example, resource foraging through clonal extension, physical and physiological integration and population maintenance under mate limitation. In literature, rhizome extension within the population is often the single component of asexual dispersal accounted for (Gliddon *et al.*, 1987, McMahon *et al.*, 2014). However, efficient asexual dispersal is sometimes considered an important trait explaining the rapid and successful spread of invasive species, allowing successful colonization of distant regions by a single or few founder individuals, and permitting populations to colonize habitats where sexual reproduction is physiologically affected (*e.g.*, Tatarenkov *et al.*, 2005).

Clonal reproduction can take many forms and display a wide spectrum of processes (Sibly and Calow, 1982; Fischer and van Kleunen, 2002; Halkett *et al.*, 2005; Cornelissen *et al.*, 2014) that can result in dispersal distances from a few centimetres to thousands of kilometres. In sessile clonal organisms, a necessary condition for an asexual propagule to escape its population boundaries and the classical modular replication pattern is the production, separation or fragmentation of independent units. Throughout this study we use the term asexual as used in Judson and Normark (1996) including, among others, vegetative propagation. Effective asexual dispersal can be generally characterized by a three stage process. First, *separation* from the established clone, second, a *transport* period during which the propagule remains viable, and finally, the capacity for *settlement* and growth at a different location. This is different from the estimation of clonal dispersal as the clonal sub-range that includes clonal growth without separation (Alberto *et al.*, 2005).

Although the separation of vegetative tissue and re-establishment in a new location has long been used by humans in agriculture and horticulture (asexual propagation), the success of this process in natural conditions is unclear, mainly due to dependence on becoming reattached or reburied at a new site by natural processes (but see for ex Tatarenkov *et al.*, 2005). Separation of parts of an organism can be deliberate (Oróstica *et al.*, 2012) or accidental (Balestri *et al.*, 2011), forming a fragment or propagule. The potential to produce fragments or sustain breakage has been observed and demonstrated in a number of animal and plant species (*e.g.*, Crump and Barker, 1985; Wulff, 1991; Sand-Jensen *et al.*, 1999; Smith and Hughes, 1999; Karako *et al.*, 2002). The transport phase of asexual dispersal in nature is often difficult to track, due to practical limitations to observe or experimentally test propagule dispersal, particularly long distance dispersal (LDD). Some authors argue that in case of clonal propagation these distances should be smaller in comparison to those of sexual propagules (Eckert, 2002 and references therein). Some studies have been relying on modelling this process in order to evaluate its occurrence, extent and success (Le Corre *et al.*, 1997; Austerlitz and Garnier-Géré, 2003; Viana *et al.*, 2013). Settlement of the dispersed asexual propagules has been confirmed in several taxa: plants in lentic habitats (De Meester *et al.*, 2002), in streams (Sand-Jensen *et al.*, 1999; Riis and Sand-Jensen, 2006), marine macrophytes (Ceccherelli and Piazzi, 2001; Tatarenkov *et al.* 2005), freshwater bryozoans (Freeland *et al.*, 2000), moss (Cleavitt, 2002), sea anemones (Ayre, 1984), corals (Jackson, 1986; Smith and Hughes, 1999) and sponges (Wulff, 1991), among others. Post dispersal establishment of fragments of submerged freshwater macrophytes is well supported (Johansson and Nilsson, 1993; Sand-Jensen *et al.*, 1999; Boedeltje *et al.*, 2003). Studies which looked into this process in marine macrophytes reported on its success (Mshigeni, 1978; Ceccherelli and Piazzi, 2001; Tatarenkov *et al.*, 2005; Zhang et al, 2011; Umetsu *et al.*, 2012). Meanwhile, in seagrass research no consensus has been reached on the relative success and importance of post dispersal settlement of fragments, although some degree of fragment dispersal was necessary to explain the distribution of very large clones along areas where seagrasses could not have persisted during the low sea levels of the most recent glacial period (Arnaud-Haond *et al.*, 2012).

Seagrasses are marine plants relying on both clonal growth and sexual reproduction to extend their coverage in the habitat or to occupy new ones. Clonal propagation allows for space occupation with lower mortality than observed for seedlings, because the structural integration of rhizomes within a clone ensures resource circulation (Marbà *et al.*, 2002) and promotes resilience by stabilizing the sediments. Dispersal of sexual propagules on their own in the marine environment can often have a limited extent (Orth *et al.*, 2006 a), although long distance dispersal of seeds transported by reproductive structures or fragments floating in the current is likely (Harwell and Orth, 2002; Källström *et al.*, 2008; Erftemeijer *et al.*, 2008; Orth *et al.*, 1994; McMahon *et al.*, 2014). Thus, studies on seagrass dispersal tend to focus their attention mainly on seed dispersal, while asexual dispersal is mainly associated with clonal propagation within the local population (*e.g.*, rhizome elongation).

Production of seagrass fragments is common across species regardless of their size or habitat and can be a natural or human-induced process (Silberhorn *et al.*, 1983; Cabaço *et al.*, 2005; Balestri *et al.*, 2011). The transport of such fragments has been mentioned for the first time in 1908 (Harwell and Orth, 2000) and has since been implied as the most parsimonious explanation for the occurrence of new seagrass patches in previously unoccupied areas (Hall *et al.*, 2006; Diaz-Almela *et al.*, 2008; Virnstein and Hall, 2009) or as a transport vector for seeds (Erftemeijer *et al.*, 2008). Berković *et al.* (2014) experimentally provided strong evidence for long viability of seagrass shoots, and seeds carried in them, during the transport phase of *Z. noltii* fragments. But until today, only a few studies tried to quantify the success rate of fragment establishment after the settlement. Berković *et al.* (Chapter 3 of this thesis) showed post dispersal successful settlement in *Z. noltii* to be biologically relevant, as it can range between 30 and 100% depending on the size of the fragment, the time spent floating in the water surface and the time after settlement. Hence, studies that have looked at dispersal in seagrasses (Ewanchuk and Williams, 1996; Hall *et al.*, 2006; Virnstein and Hall, 2009; Berković *et al.*, 2014; Chapter 3 of this thesis) indicate that at least some species have the potential to disperse and maintain populations via vegetative fragments' dispersal and settlement. From these studies it is clear that dispersal (as in the transport with potential for gene-flow, sensu Ronce, 2007) of seagrass fragments does happen in natural systems. Still, with just few studies focusing on the final stage of asexual dispersal in seagrasses, researchers debate over its importance. While one group of authors argues that seagrass fragment dispersal and establishment is an important process (Hall *et al.*, 2006; Diaz-Almela *et al.*, 2008; Virnstein and Hall, 2009; Berković *et al.*, 2014; Chapter 3 of this thesis; Stafford-Bell *et al.*, 2015), others, although acknowledging the traits that allow for asexual LDD, consider it irrelevant, unlikely or with low frequency (Ewanchuk and Williams, 1996; Arnaud-Haond *et al.*, 2012; McMahon *et al.*, 2014).

The use of hyper-variable genetic markers (*e.g.* microsatellites) in the last 15 years has increased our knowledge on the spatial patterns of clonal structure. However this increased capacity to reveal organismal life-history has not been put to use to test asexual LDD. Unbiased genetic identification of the same clone sampled across distant locations has been explained as a result of rhizome elongation (Reusch *et al.*, 1999). From this assumption, clone age estimation based on available rhizome elongation rates is likely to lead to overestimated ages as LDD is overlooked. Growth rates of seagrasses show great variability between and within the species (Marbà and Duarte, 1998). Reported growth rates largely depend on the area, season and duration of the study (Tab.S1. in Supplementary material). Accordingly, some seagrass species are mentioned among the oldest organisms on the planet. Estimated ages of hundreds to thousands of years or possibly even older in the case of *Posidonia oceanica* (Arnaud-Haond *et al.*, 2012) or on the scale of a thousand years for *Zostera marina* (Reusch *et al.*, 1999) have been advanced. Few of these studies have been done for small seagrass species which tend to have faster growth rates and lower clonal integration (Marbà *et al.*, 2002). These revealed much younger ages, *e.g.*, from clone of *Zostera noltii* at Odessa city beach was found to be between 35 and 70 years old (Coyer *et al.*, 2004) to *Thalassia testudinum* along Atlantic coast of Mexico reaching about 600 years (van Dijk and van Tussenbroek, 2010). We argue that when estimating clone age using this approach, researchers should first try to falsify the hypothesis of asexual LDD, avoiding overestimating age by orders of magnitude.

Studies aimed at understanding the natural occurrence of effective asexual dispersal should complement experimental and population genetic approaches. However, most sampling designs used in population genetics are compromised in their capacity to reveal asexual LDD. When the focus is on comparing different populations most studies still follow a population based sampling. Arbitrarily defined populations are sampled in clusters, often with fewer than 50 sampling units, and leaving a large proportion of unsampled habitat in between. When the focus is on within population processes, like in fine spatial scale genetic structure analysis, sampling densities are higher but the spatial extent of the sample is inherently very limited. To circumvent these limitations the individual based, spatially explicit sampling design, common in landscape genetics (Manel *et al.*, 2003), should be used. In this design, sample units are collected randomly or stratified over the entire habitat under study. Such a strategy, if including a very large sample size, increases the chance of sampling multiple ramets of the same clone spread over large distances in a continuous or disjunctive mode. This sampling design has not been applied in studies of sessile clonal organisms, or used with limited spatial extent.

Besides asexual dispersal, other important questions in the evolution and maintenance of sexual and asexual life histories may also be facilitated by using a landscape genetics sampling design. The conditions that might lead to allocation to one or the other reproduction mode are the focus of many studies (Abrahamson, 1975; Douglas, 1981; Silvertown, 2008 and the references therein). The results are often conflicting and largely context specific, requiring detailed analyses instead of simplified generalizations. Most of these studies are population based; their power to test associations between direct, or indirect, measures of reproductive effort and putative predictors of reproductive response would benefit from sampling both dependent and independent variables over larger spatial extents. Still, a few generalizations can be made in some taxa about the factors controlling reproductive effort. For example, in seagrasses and other aquatic plants, sediment burial and erosion leads to increased shoot mortality (Trémolières, 2004; Cabaço *et al.*, 2008) and a higher proportion of the organism's energy tends to be allocated to sexual reproductive effort in more disturbed or stressful environments in seagrasses (Alexandre *et al.*, 2005; Cabaço and Santos, 2012). Nevertheless, higher sedimentation rates or movement of sandbanks, have also been shown to enhance clonal growth to outgrow the impacted area (Marbà and Duarte, 1994). Another hypothesis advanced to explain variable allocation to sexual and asexual reproduction is the intermediate disturbance hypothesis, rooted in ecological theory (Connell, 1978). According to this simple model, higher species diversity should be observed at intermediate levels of disturbance. For the genetic diversity of partially clonal organisms this translates to a disturbance mediated increase in available space, promoting sexual reproduction and higher clonal diversity (Weider, 1992; Reusch, 2006). Complementary genetic analysis can be used to understand if field measured reproductive effort translates into effective sexual recruitment by estimating genotypic richness (the proportion of different clones in the sample). This type of analysis has revealed contradictory evidence (Arnaud-Haond *et al.*, 2010, Oliva *et al.*, 2014) and is complicated by the maintenance of genotypic richness in the population even when sexual recruitment is rare (Eriksson, 1993) and by its dependency on sample size (Arnaud-Haond *et al.*, 2007). Again, increased power from sampling larger spatial extents and new statistics to measure effective reproductive effort should help testing these associations.

In this study, we used an individual based sampling design, across an unprecedented spatial extent, to study the clonal structure of the seagrass *Zostera noltii*. Given previous experimental work supporting the capacity for asexual LDD in this species (Berković *et al.*, 2014; Chapter 3 of this thesis) we used nine microsatellite markers to unambiguously characterize genetic identity of ramets sampled across tens of km. We show that many clones are found separated by distances as large as the available habitat in the Ria Formosa coastal lagoon. This sampling design also made it possible to test the association between a source of disturbance and increased clonal richness at the landscape scale.

4.2. Materials and methods

4.2.1. Study site

This study was carried out in the Ria Formosa lagoon, in the south of Portugal (37°N 8°W). This intertidal lagoon extends roughly 55 km along the mainland and is 6 km across its widest point, with an average depth of about 2 m and low fresh water input. Separated from the ocean by five islands and two peninsulas, it consists of a complex set of channels, mudflats, saltmarshes and highly dynamic sand barrier islands. Up to 80% of water is exchanged with the open ocean in each tidal cycle. At the spring tides an average of 84 km^2 are under the sea level, but just about 12.5 km^2 are permanently flooded, while up to 67 km^2 are exposed on spring low tides (Andrade *et al.*, 2004). Intertidal mudflats are inhabited by seagrass *Z. noltii*. Subtidal areas are habitat of other two seagrass species *Cymodocea nodosa* and *Zostera marina* (Cunha *et al.*, 2009). Inner channels of the lagoon are dredged along the main navigational channels, and throughout the lagoon there is intensive artisanal bivalve harvesting, especially clam digging along intertidal areas. Changes in the location and topography of channels and sand barriers affect the intertidal population of *Z. noltii*, sometimes causing extinction, migration or colonization of patches (Cunha *et al.*, 2005). Hydrological and geomorphological dynamics of the system are hence an additional force shaping the population genetics of *Z. noltii* within the lagoon.

4.2.2. Study species

Zostera noltii is a small seagrass species distributed along central/southern Europe and NW Africa (Kuo and den Hartog, 2001). In the Mediterranean, as well as in neighbouring seas (Green and Short, 2003), it is mostly found in the shallow subtidal, with lower shoot density, while the Atlantic meadows in most cases inhabit intertidal areas with high density (Marbà *et al.*, 1996; Harrison, 1993; Green and Short, 2003). *Z. noltii* shows highly variable growth rates, but it is generally considered a fast growing species (Tab. 4.S1., supplementary material). Alexandre *et al.* (2006) reported frequent flowering and high production of seeds in the Ria Formosa, for this monoecious species. Seeds can be released directly from the plant or can be transported attached to the flowering shoot (Moore and Short, 2006). Seed transport distance ranges from tens of cm, when released from an attached parental plant, to a couple thousand kilometres when transported by positively buoyant detached shoots and assuming unidirectional current flow (Berković *et al.*, 2014). Detached positively buoyant fragments keep producing new shoots and can carry maturing seeds for more than 50 days (Berković *et al.*, 2014). An experimental study on post-dispersal settlement success, after a floating period of up to four weeks, demonstrated that these fragments once entangled are quickly buried and keep growing (Chapter 3 of this thesis).

Z. noltii is the dominant seagrass species in the Ria Formosa lagoon, covering over 13 km^2 of the intertidal area (Guimarães *et al.*, 2012). Populations of *Z. noltii* along the southern Iberian Peninsula occur in estuaries and coastal lagoons separated by stretches of unsuitable habitat, leading to significant population differentiation among them (Diekmann *et al.*, 2005). Hence, for the purpose of this study, the *Z. noltii* population within the Ria Formosa lagoon can be considered a closed group, with negligible immigration.

A part of the analyses pertaining to clone age estimation (see below) were also completed for the seagrass *Cymodocea nodosa* (Cymodoceaceae) in Ria Formosa. This is a dioecious species, which exhibits fast clonal growth. *C. nodosa* occurs throughout the Mediterranean basin and in the North Atlantic from central Portugal to Cap d'Arguin in Senegal, as well as in the Canary Archipelago and the Madeira Islands (Green and Short, 2003). Barrio (pers. comm.) found that *C. nodosa* fragments have the ability to float and stay viable for over a month and when planted in the sediment exhibited the same growth rates as the non-floating fragments.

4.2.3. Sample collection

To maximize the chances of sampling the same clones at different distant locations we completed an extensive spatially explicit sampling design. We first randomly selected 1,000 sampling coordinates within a 13 km^2 area of *Z. noltii* meadows in the Ria Formosa, based on an available shape file georeferencing their distribution (Guimarães *et al.*, 2012) across more than 84 km^2 of the lagoon. Due to logistic constrains only 899 locations were visited, using boat, kayak and walking within the intricate saltmarsh channels. *Z. noltii* was found at 803 of these coordinates. At each location, 4 sample units were collected at the vertices of a 4 m^2 quadrat. Each individual sample unit consisted of a single ramet with three to four shoots connected by a horizontal rhizome, to ensure that all the tissue belonged to one single genet. Thus, a total of 3,212 individual ramet sample units were sampled. Each sample was individually labelled and stored in separate sampling bags. Back in the laboratory, samples were carefully washed in fresh water, dried on paper and stored dry with silica gel.

The *C. nodosa* data treated here were collected as part of a previous studies (Alberto *et al.*, 2001; Alberto *et al.*, 2008) and consisted of a smaller number of sample units (N=176) collected across 11 arbitrarily defined sites distributed over 75 km^{2} of Ria Formosa.

4.2.4. Genetic analysis of clone identity

Genomic DNA for all ramets sampled was isolated from silica dried tissue (5-10 mg) using an adaptation of the CTAB method (Doyle and Dickson, 1987). All *Z. noltii* samples were genotyped for nine microsatellite loci (Coyer *et al.*, 2004) using fluorochrome-labelled primers on a GeneAmp2700 thermocycler (Applied Biosystems, Foster City, CA, USA). An ABI PRISM 3130xl DNA analyser (Applied Biosystems) was used to determine the size of amplification products (*i.e.*, microsatellite alleles) against a standard (GeneScan 500 LIZ, Applied Biosystems). Raw fragment sizes were scored using STRand v.2.4.59 (http://www.vgl.ucdavis.edu/informatics/strand.php), and inspected for quality and binned into allele classes using the R (R core team 2013) package MsatAllele (Alberto *et al.*, 2009). Details on *C. nodosa* microsatellite genotyping can be found in Alberto *et al.* (2008).

Observed identical multilocus genotypes (MLGs) can either be the result of sampling the same clone/genet twice, or two different genets originated by two distinct sexual reproduction events but sharing the same alleles for all genotyped loci (Arnaud-Haond *et al.*,2005). The probability of encountering the latter depends on the discriminatory power of the set of microsatellite markers used (number of loci and their polymorphism). To ensure our clonal assignments were reliable, we estimated the probability of a given multilocus genotype occurring *n* times as a consequence of different sexual reproduction events (*Psex*), according to Parks and Werth (1993). Detailed description of *Psex* estimation and genet assignment using an appropriate set of markers is reported elsewhere (Arnaud-Haond *et al.*, 2005). In brief, *Psex* is the probability of finding *g* repetitions, or less, of a previous encountered MLG in a set of *N* sample units, under the null hypothesis that all MLG in the set were produced by independent sexual recombination. Rejecting this null hypothesis supports an alternative that identical MLGs are present in a sample because the same clone was sampled twice or more times. We calculated *Psex* using custom written R code, because of sample size limitations (N<=500) in the available software (Arnaud-Haond and Belkhir, 2007).

4.2.5. *Estimating clonal age using rhizome elongation rates*

Typically, a seagrass individual's age is estimated assuming that the sampled spatial extent of a clone was the result of rhizome elongation alone. Using this approach the observed distance between sampled clonemates (samples belonging to the same MLG) is simply divided by the distance horizontal rhizomes can elongate on average during one year (Reusch *et al.*, 1999, Arnaud-Haond *et al.*, 2012). Here we use a similar strategy, but iteratively correct age estimates by combining sampling locations with spatial explicit sea-level data for the time the clone would have been initiated (*i.e.*, subtracting clone age from present time).

Once clonal assignment was determined, we calculated the distance between each pair of clonemates belonging to the same genet. This was achieved using the sampling coordinates and the *distVincentyEllipsoid* function in the geosphere R package (Hijmans *et al.*, 2012). The resulting distance is an "as the crow flies" distance, *i.e.*, shortest distance between the two points on the ellipsoid, not considering possible land obstacles on the way, such as saltmarsh banks that never get submerged. The longest distance between a pair of clonemates of the same genet was used to define a minimum (*i.e.*, sampled) span for that genet. For the purpose of the following methodological description we shall refer to these points as A and B.

We estimated the age for all clones sampled multiple times in the following way: First, assuming a conservative approach we assumed that the clone initiated growth at some middle point (O_0) between A and B. Thus, an initial estimate of clone age (t_0) was calculated by dividing the distance from O_0 to A (half the clone's span) by *Z. noltii* growth rate. We used a mean rate of rhizome elongation of 68 cm/year for *Z. noltii*. This was extracted from data published in a review (Marba and Duarte, 1998), including studies from different areas and seasons. We chose to use species' distribution wide data, instead of estimates for our study area. The rationale was that this is a better representation of growth rate variation through time, reflecting likely changes in habitat and climate. The rhizome elongation rates reported in (Marba and Duarte, 1998) ranged from 10 to 157 cm/year, encompassing the observed rates in Ria Formosa (Tab. 4.S1.). The above methodology to age clones assumes asexual LDD to be impossible, because otherwise age estimation is unworkable.

The estimated age of these clones was then matched with sea-level change reconstruction. The above estimated clone's age, was used as an initial estimate of how many years before the present the clone could have originated (t_0) at the point O_0. Using published paleoceanographic data on sea-level change in the Southern Portugal (Dias *et al.*, 2000.) and the current bathymetry, we mapped sea level changes over the past 20 000 years. This map was then used to verify if the origin point O_0 was, at time t_0, in suitable intertidal habitat (*i.e.* shallow coastal zone). If O_0 was indeed in the coastal zone we assumed this to be the final age estimate. If on the contrary O_0 was above sea level we moved it to the closest coast line, again placing the point at an intermediate distance from A and B, and refer to this new point as O_1. Then we measured the distance to the points A and B and used this distance to update the age of the clone (t_1). We repeated the above process, verifying if at the time t_1, the point O_1 would have been on the coast. If again O_1 was on land, we moved it to the closest reconstructed coast line and marked as O_2, iterating the whole process (Fig. 4.1).

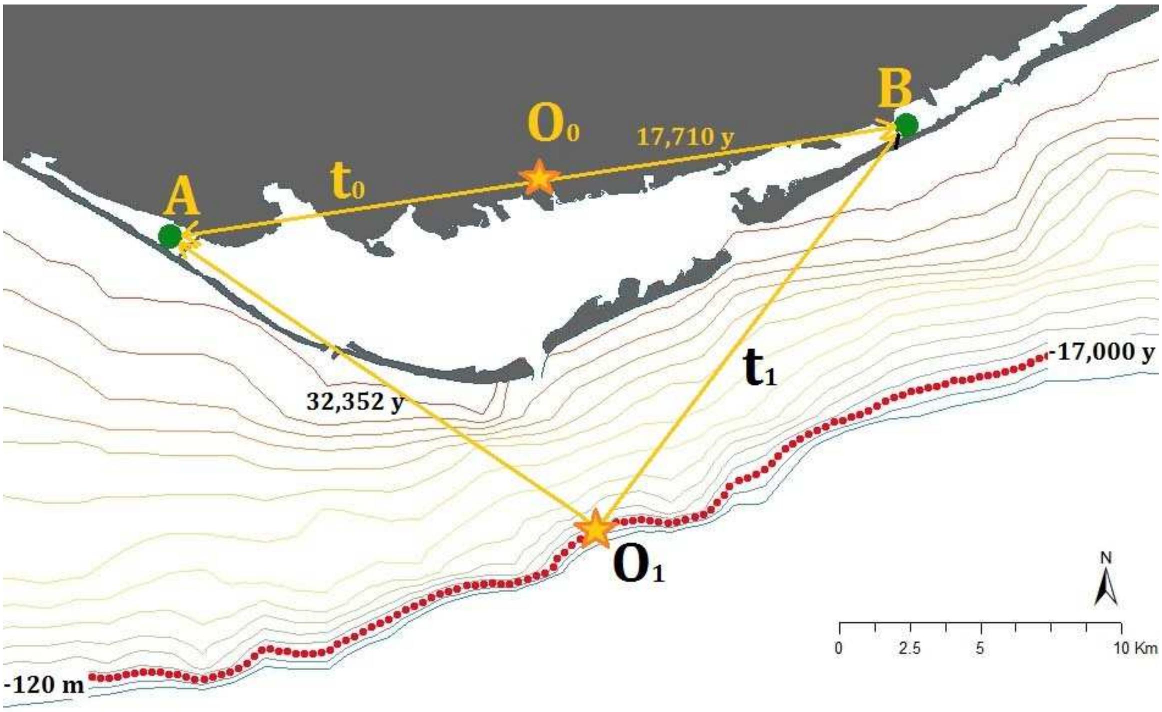

Figure 4.1. Example of one iteration of *Zostera noltii* age estimation based on horizontal rhizome elongation rate and paleoceanographical sea-level reconstitution of the Ria Formosa lagoon area. A and B are locations of ramets belonging to the same MLG. O_0 is the initial origin point, placed in the middle of the distance between points A and B. t0 is the time necessary for the clone to grow from O_0 to A and B, in this case 17,710 years. 17,000 years before present sea level was 120 m lower, marked by the red dotted line. Corrected origin point O_1 is now placed on the coast line, at an intermediate distance from A and B. t_1 is the time necessary to grow from O_1 to A and B, here 32,352 years.

Iterations stopped either after reaching the origin point O_n which was at a certain time t_n before the present in the coastal zone, or when there was no more available detailed data on sea level changes for the region.

We performed the same exercise with available data for another seagrass species in Ria Formosa – *Cymodocea nodosa*, encompassing total of 176 samples. Sampling density was different from that used in this study for *Z. noltii*, because a population based sampling was used for *C. nodosa*. Samples collection, DNA extraction, genotyping and samples analysis details can be found in Alberto *et al.* (2008). The same method was used to evaluate the age of *C. nodosa* clones as described for *Z. noltii*. Like for *Z. noltii*, rhizome elongation rates published in the literature vary (Tab. 4.S1., Supplementary material), so we decided to use the same source of data as for *Z. noltii*, with linear clonal extension rates of 40 cm/year (Marba and Duarte, 1998).

4.2.6. Disturbance and clone size associations

Our data provides an opportunity to investigate the association between the spatial distribution of large clones, expected in areas with lower disturbance regime, and the distance to disturbance source caused by the highly dynamic sand barrier islands separating the system to the ocean (Cunha *et al.*, 2005).

The large coverage of our sampling design resulted in a low density of sampling plots with an average distance between neighbouring sites of 96 m. This unusual sampling design led us to estimate a statistic that would indicate at each sampling plot the probability of finding a clone that was present elsewhere *n* or more times. Because four sample units were sampled in each plot, we asked for each of the four MLGs in a plot if they had been found five or more times in the whole sample. The presence of five clonemates ensured that at least one clonemate would have been found in a different plot. Because neighbouring plots were on average 96 meters apart, such observations represent events where a large clone was sampled. A binomial (one or zero) response was produced for the above condition and averaged for each of the four ramets in a plot. We also produced similar estimates for MLGs observed 10 and 15 or more times. We call these *clonal probabilities*, Pc_5, Pc_{10} and Pc_{15}, respectively. If larger clones are indeed found away from the disturbance source, plots sampled in such areas should on average have higher *Pc*. We binned the average plot *Pc* in distance classes from the disturbance source. We did this by estimating the shortest straight distance from the sampling plot to a shape object contouring the sand barrier island. We also fitted a linear regression of *Pc* on distance from the barrier island. A permutation test was used to test the null hypothesis that *Pc* was not associated to the distance from the barrier island. Null distributions were obtained by repeating a 1,000 times a permutation of plots' sampling coordinates and recalculating each time the *Pc* mean values per distance class and the slope of regression. A particular observed *Pc* mean value or regression slope rejected the null hypothesis if smaller or larger than the 2.5% and 97.5% percentiles, respectively, of the permuted null distributions.

4.3. Results

4.3.1. Genetic analysis of clone identity

The genetic analysis of 3,185 *Zostera noltii* ramets revealed 1,999 unique MLGs corresponding to a relatively high genotypic richness (R=0.63), with mean allelic richness per locus A=15.0, observed heterozygosity Ho=0.62 and unique genotype probability *Pgen* always <0.05. *Psex* calculated multiple times for random 500 samples was always <0.001. Out of the unique MLGs, 504 were sampled more than once. A total of 16 MLGs were sampled more than 10 times and the most abundant MLG (ID 1886) was sampled 59 times (Tab. 1). The largest distance between two samples with identical MLG was 26.4 km. A total of 58 MLGs were sampled across more than 2 km and out of those ten MLGs were sampled across more than 10 km (Fig. 4.2).

For *Cymodocea nodosa*, our analysis included 176 ramets. Only five unique MLGs were found and only one of them was sampled more than once, evidence of an extremely low genotypic richness found for this species (R= 0.03) as shown with RAPD markers (Alberto *et al.*, 2001). The largest distance between the samples of this MLG was 42.9 km. The extremely low number of individuals found for *C. nodosa* precluded an unbiased estimation of allele frequencies that could be used for the estimation of *Psex*. However, the fact that the most common MLG had three heterozygous loci out of eight supports the notion that these ramets come all from the same clone. Otherwise, under Mendelian inheritance, given the large sample size used, we should have sampled MLGs with only one of the alleles (homozygous) present in these loci that were heterozygote for the dominant MLG.

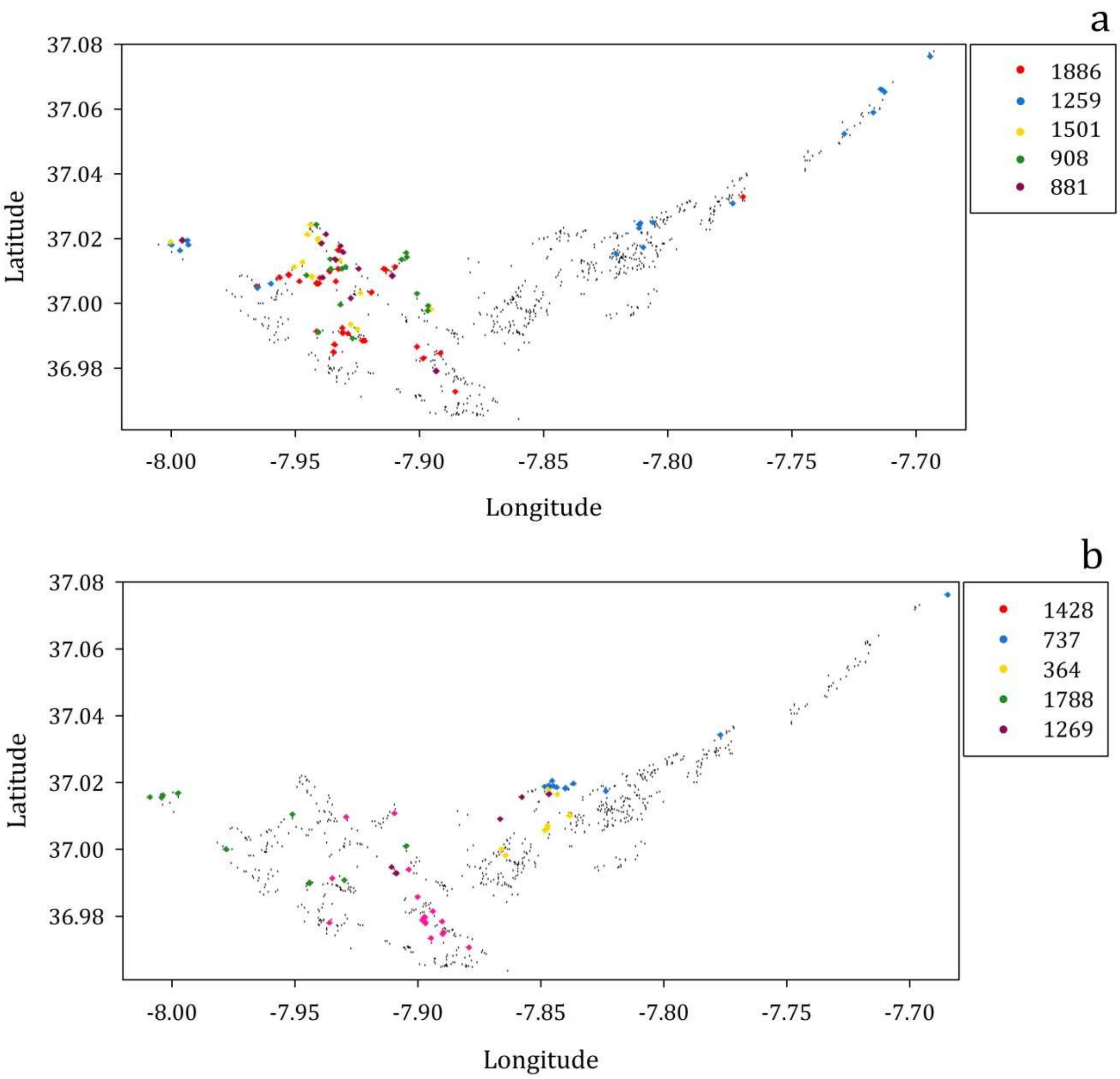

Figure 4.2. Spatial distribution of the largest *Zostera noltii* clones found in Ria Formosa. Similar colours represent samples that had the same multi-locus genotype. Smaller points indicate all sampling locations.

4.3.2. Estimating clonal age using rhizome elongation rates

Age estimation for clones, based on the rhizome elongation rates, for the ten largest (longest distance between clonemates) *Z. noltii* clones, showed that three could be dated to approximately 20,000 years. The other seven clones were estimated to be more than 20,000 years old. Further iterations of our age estimation process previous to that period were precluded, due to the temporal extent of available paleoceanographic data for this region. However, the age of these clones would have to be at least equal or older than the range found at the point where we could no longer proceed (22,058 to 33,088 years depending on the clone, Tab. 4.1). Considering that the longest sampled distance between the two samples of *Cymodocea nodosa* with same MLG was almost 43 km, the estimated age of this clone is over 53,000 years. Again, such age falls far beyond the available paleoceanographic data.

4.3.3. Disturbance and clone size associations

The analysis of spatial distribution of large *Z. noltii* clones within the lagoon revealed higher probability to sample large clones (*Pc*) further away from the source of disturbance (Fig.4.3 a).

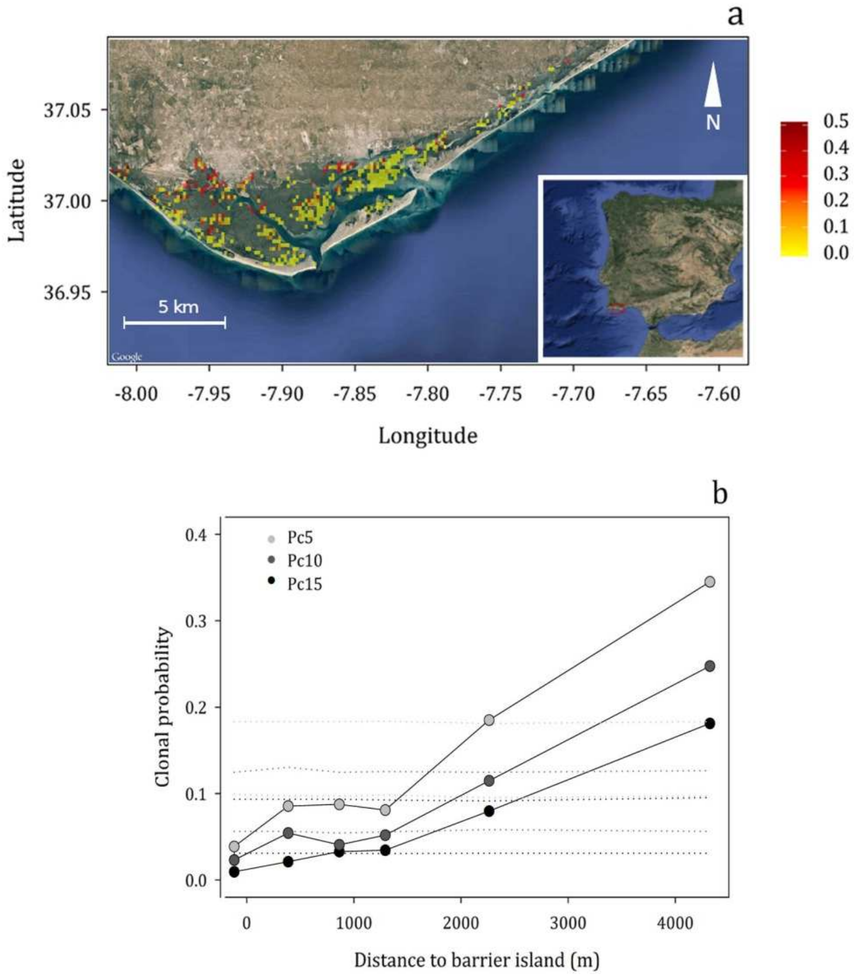

Figure 4.3. Probability of sampling a large clone of *Z. noltii* more than n times (*Pcn*) across the whole sample in each plot. (a) mapped representation of Pc_5 across Ria Formosa. Higher values, representing higher likelihood of sampling a larger clone, were generally found further way from the barrier island. (b) spatial correlogram of Pc_5, Pc_{10} and Pc_{15}. This represents the association between mean Pc and distance from the barrier island. Dotted lines delimit 95% confidence intervals for Pc under the null hypothesis of no association with distance to the barrier island.

This pattern did not change with the number of clonemates used to estimate *Pc* (5, 10 or 15, Fig. 4.3 b). This analysis was not possible for *C. nodosa* because genotypic richness was extremely low.

Table 4.1. Age estimation for ten largest clones of *Zostera noltii* and one clone of *Cymodocea nodosa* found in the Ria Formosa lagoon. N – number of clonemates found in the sample; distance – maximum distance between the two clonemates; O^0…O^4 – shortest distance to the origin point; t_0…t_3 – estimated age of the clone according to the corresponding origin point; * – final age estimation; … – unavailable data.

species	**clone**	**N**	**distance (m)**	**O_0 (km)**	**t_0 (y)**	**O_1 (km)**	**t_1 (y)**	**O_2 (km)**	**t_2 (y)**	**O_3 (km)**	**t_3 (y)**	**O_4 (km)**
Zostera noltii	1259	34	26,413	13.2	19,421	22.5	33,088	…				
	120	6	24,085	12	17,710	22	32,352	…				
	896	11	19,570	9.8	14,389	19	27,941	…				
	1886	59	16,736	8.4	12,306	13	19,117	16.5	24,264	…		
	1480	9	16,155	8.1	11,879	15	22,058	…				
	737	19	15,926	8	11,710	15	22,058	…				
	1386	2	14,962	7.5	11,001	10	14,705	12	17,647	13	19,117*	
	44	3	14,101	7	10,368	11	16,176	12.5	18,382	13	19,117*	
	1524	13	12,335	6.2	9,070	10	14,705	13	19,117	15	22,058	…
	707	6	10,789	5.4	7,993	10	14,705	12.5	18,382	13.5	19,852*	
Cymodocea nodosa	1	9	42,907	21.4	53,635	…						

4.4. Discussion

Our previous research on the potential for asexual LDD in *Zostera noltii* (Berković *et al.*, 2014; Chapter 3 of this thesis), led us to study the spatial genetics of this seagrass in the Ria Formosa lagoon. We hypothesized that the clonal structure of *Z. noltii* population should reveal a signature of long distance asexual dispersal of vegetative fragments. Our results corroborated this hypothesis; despite high genetic and genotypic diversity, multiple identical MLGs were identified separated by tens of kilometres. The presence of these repeated MLGs could not be explained by distinct sexual recombination events.

Given the large spatial extent covered by many clones, we employed the approach used elsewhere in seagrass genetic studies (Reusch *et al.*, 1999; van Dijk and van Tussenbroek, 2010; Arnaud-Haond *et al.*, 2012) to estimate individual age of these clones assuming the absence of LDD. This method considers the species' rhizome elongation rate and the average time it would take, solely by rhizome elongation, to spread across half the sampled MLG extent. Given the extremely old ages found for many of the largest clones sampled, their assumed past starting point, or any other point of the present extent, were on land at the inferred time of clone initiation. This was taken in consideration by adapting the age determination method in light of sea level paleoceanographic data. However, these corrections were limited to a period of 20,000 years before present (YBP) due to the limits on how far back the paleoceanographic data went. Nevertheless, the estimated age of several individual clones within the lagoon surpassed 20, and even 30, thousand years. In addition, for *Cymodocea nodosa* the same approach produced an estimated age of at least 50 thousand years. The estimation of replicated, across species, extremely old organisms is on its own quite interesting and would add both species to the list of the oldest living organisms on the planet. However, assuming no asexual LDD, a necessary condition to apply the above age determination method, is not the most parsimonious model to explain the large spatial extent covered by these clones.

An important observation demonstrating that clonal extent cannot be solely attributed to rhizome elongation is that our conservatively estimated clone ages would precede by up to ten thousand years the age of the Ria Formosa lagoon. The age of the Ria Formosa lagoon itself is still a matter of debate, as different methods used since the beginning of 19th century produced a range of estimated dates for the formation of the lagoon (Andrade *et al.*, 2004). These vary from two thousand YBP (Neves, 1967) to about six and a half thousand YBP (Dias, 1987), with the latest study indicating a period approximately between five and six thousand YBP (C. Sousa, pers. comm.; Andrade *et al.*, 2004). Any clone older than six thousand years could therefore not have evolved inside of the lagoon. Furthermore, clone age estimations based on rhizome elongation rates and spatial explicit genetic sampling are always very conservative given the following shortcomings: stable habitat is continuously available in straight shortest path lines from point of origin to the point of sampling; no physical disturbances occur along tens of kilometres of growth, slowing down or interrupting the rhizome growth; the present spatial extent of a sampled clone corresponds to the widest ever covered by that clone through its millennial history, and that samples captured the furthest away ramets in the whole population of ramets from the same clone. Thus, the certain violation of these assumptions extends clone age even longer.

Any effective asexual dispersal event through fragmentation, transport and re-establishment during the life of a clone would render these age determinations meaningless. In light of the accumulating evidence, from seagrasses and other aquatic species, that support all necessary three stages for asexual LDD, such "old clone" inferences should be reported very cautiously. Fragmentation and dispersal via fragments is known in many partially clonal organisms (Highsmith, 1982; Ayre, 1984; Jackson, 1986; Wulff, 1991; Smith and Hughes, 1999; Freeland *et al.*, 2000; Ceccherelli and Piazzi, 2001; Cleavitt, 2002; De Meester *et al.*, 2002; Tatarenkov *et al.*, 2005; McKenzie and Bellgrove, 2008). Dispersal via vegetative fragments is a well-studied and documented process in freshwater flora. Sand-Jensen *et al.* (1999) found that 90% of new patches in the studied streams developed from the vegetative fragments which settled in the new area and De Meester *et al.* (2002) in their review corroborated this finding with examples from other plant and animal species. Finally, Riis and Sand-Jensen (2006) continued on this work, testing the dispersal distance of the plant fragments. Similar processes have been reported for some seagrass species. Natural detachment, drifting and re-rooting was observed for small *Posidonia oceanica* fragments in the meadows of the Balearic archipelago, Spain (Diaz-Almela *et al.*, 2008), for two other *Posidonia* species in the Western Australia, with varying success (Campbell, 2003), and for *Halophila johnsonii* and *H. decipiens* in Florida, USA (Hall *et al.*, 2006).

For *Zostera noltii,* our previous work provides experimental support for both the dispersal potential of *Z. noltii* asexual propagules, while being transported for long periods in the water surface (Berković *et al.*, 2014), and the success of their post-dispersal establishment (Chapter 3 of this thesis). Combined, these studies strongly suggest that *Z.*

noltii vegetative fragments can indeed disperse over large spatial scales (thousands of km) and establish in new habitat with relatively high success (30-100%). The present study builds on these previous results by identifying the expected genetic signature of such asexual LDD. We consider this scenario to be more parsimonious than assuming that rhizome elongation alone could have produced the observed spatial distribution of so many clones.

As the interest in seagrass dispersal is growing in the last years (Harwell and Orth, 2002; Lacap *et al.*, 2002; Hall *et al.*, 2006; Erftemeijer *et al.*, 2008; Källström *et al.*, 2008; Viernstien and Hall, 2009; Balestri *et al.*, 2011; Kendrick *et al.*, 2012; Berković *et al.*, 2014; Sinclair *et al.*, 2014; Thomson *et al.*, 2014; Stafford-Bell *et al.*, 2015) we propose that asexual LDD might be common in other species if studied at a relevant scale. LDD via fragments has been shown in few seagrass species, though with variable success (previous references). Asexual LDD seems to be possible for a range of seagrass species that cover a range of sizes, from some of the larger species from genus *Posidonia* (Campbell, 2003; Diaz-Almela *et al.*, 2008) to one of the smaller species *Halophila johnsonii* (Virnstein and Hall, 2009). Geography also seems to play no role in this process as areas of study range from Australia to Mediterranean and central Atlantic, both west and east coasts. What makes *Z. noltii* unique among the seagrasses in which asexual LDD was suggested so far, is its intertidal habitat in our study site. This facilitates all important steps for effective LDD; the fragmentation of rhizomes forming asexual dispersal propagules is facilitated by natural and anthropogenic sediment disturbance, the dispersal process made efficient by strong tidal currents and the subsequent reburial of these propagules also possibly facilitated by frequent sediment transport that buries them. The coastal lagoon has conditions that facilitate LDD and it can therefore be speculated that if the lagoon was larger we would have found even larger clones. Fragments carried by currents at the surface of the sea easily settle on the sediments and get buried by sand during the ebb tide, without the need for their buoyancy to change, and root within first weeks of settlement (Berković *et al.*, in prep). Mechanisms of dispersal are rarely explained in detail in the mentioned studies, but a few general ideas are suggested regarding subtidal species. Boudouresque and colleagues (1990) interpreted the case of individual *P. oceanica* shoots wedged within the algal bank as the result of dispersal by drifting fragments after the storm. In the case of *H. johnsonii*, Hall *et al.* (2006) suggested that fragments sink from the surface to the sediment, after a period of dispersal, and subsequently re-establish by rooting. Our field site and available data conveniently allowed us to consider asexual LDD for the subtidal seagrass *C. nodosa*. Barrio (pers. comm.) showed experimentally that fragments are viable while floating for periods that allow for LDD, before sinking to the bottom and rooting with high success. Following the same rationale as presented here for *Z. noltii*, we can infer asexual LDD as the most parsimonious process to explain the wide clonal distribution of a single clone of *C. nodosa* in Ria Formosa. With this accumulating evidence from a range of species, sizes, growth rates and niches occupied along the depth gradient, we believe a synthesis on the importance of asexual dispersal in seagrasses is forthcoming. This should attract the attention of marine ecologists interested in studying the dynamics of seagrass ecosystems.

An alarming rate of seagrass ecosystems decline has been observed in the last twenty years (Orth *et al.*, 2006 b; Short *et al.*, 2006; Waycott *et al.*, 2009; Tuya *et al.*, 2013; Short *et al.*, 2014; Fabbri *et al.*, 2015). The rates of seagrass recovery on their own, assuming the removal of the pressures that led to extinction, may be seriously biased by failing to acknowledge the potential for asexual dispersal. A good example comes from a recent synthesis by McMahon *et al.* (2014) on seagrass dispersal under the movement ecology paradigm (Nathan *et al.*, 2008). In this study, a modelling exercise was conducted to estimate the multiple generation time that different seagrass species would take to disperse over distances ranging from meters to thousands of kilometres. The time needed to disperse over 1 to 10 km distances solely by asexual reproduction (*i.e.*, rhizome elongation) was estimated to be orders of magnitude longer (thousands of years) than by dispersing seeds (weeks). In that review rhizome elongation was considered as the sole asexual dispersal mechanism, although the authors mentioned that "...vegetative fragments of most genera are believed to be neutrally buoyant, but few studies have actually tested this...", no references to these studies were given. Here we presented a review of such studies, besides reporting our own and others' research, on the three necessary stages to accomplish effective asexual dispersal. Evidence from other studies on seagrasses and other clonal aquatic plants support LDD to be more common than previously acknowledged (see above). This means that at least for some species asexual dispersal might be as fast as sexual dispersal. It is noteworthy to remember that the only way a seagrass sexual propagule can disperse as far as an asexual one is precisely by being transported by a floating "vegetative" fragment. However, there has been far more attention to the faith of such sexual propagules than to the faith of the vegetative vector. Moreover, the potential reduction of genetic diversity in patches colonized by asexual propagules after extinction might be counteracted by the migration rate of such propagules. This could be generalized to other seagrasses if their asexual LDD is as frequent as suggested by the large number of replicated spatially disjunct clones in *Z. noltii.* (Although, most sampling designs used so far to study seagrasses and other marine clonal organisms are limited in their capacity to show replicated events of asexual LDD.) Additionally, while our focus here has been in the asexual dispersal component, floating fragments transport maturing fertilized flowers and can release viable seeds (Berković *et al.*, 2014; Barrio *et al.*, *in prep*), further minimizing genetic diversity reductions. These are optimistic news for seagrass ecosystem recovery, but obviously depend on the removal of pressures that led to seagrass local extinction in the first place.

In this study we also studied the effect of disturbance on the spatial distribution of *Z. noltii* clones. By investigating the association between the probabilities of sampling large clones and the distance from Ria Formosa barrier islands, assumed to be a source of physical disturbance through increased burial (Cunha *et al.*, 2005), we found that higher probability of sampling large clones was associated with increased distance from the barrier islands. Our suggestion is that this association can be the result of two non-mutually exclusive processes linked to disturbance regime - different clone survival and different allocations to sexual and asexual reproductive components. Survival and longevity of the seagrasses might be affected by different habitat stability across the gradient of disturbance in the following way: Increased stability in the inner area of the lagoon might allow established clones to survive longer and thus grow larger. Current velocities in this inner lagoon area are generally lower than in the rest of the lagoon (Duarte *et al.*, 2005; Duarte *et al.*, 2008) and fragment dispersal probably doesn't happen across large distances in one dispersal event. If survival is higher each clone has time to grow larger and be involved in more asexual LDD events through their lifetime. On the other hand, in the disturbed areas meadows are frequently buried by increased sedimentation associated to the barrier island inlets and their migration (Cunha *et al.*, 2005). Survival of clones in such a habitat is likely lower, as the conditions are less favourable due to higher burial rates, increased turbidity and disturbance frequency. Simultaneously, higher allocation to sexual propagation is shown for seagrasses in disturbed habitats (Gallegos *et al.*, 1992; Marbà and Duarte, 1995; Cabaço and Santos, 2012). Gallegos and colleagues (1992) showed four-fold increase in flowering for *Thalassia testudinum* after the disturbance cause by a hurricane passage. Looking at *C. nodosa* response to sediment dynamics, *i.e.* sand dune migration, Marbà and Duarte (1995) noted overall higher flowering frequency in the studied disturbed meadow, in comparison to the data for non-disturbed meadows. In particular, fragments which were buried just before the flowering season, showed highest frequencies of flowering. Authors suggest that this kind of moderate burial disturbance can lead to the increase in allocation to sexual production. More recently, Cabaço and Santos (2012) reviewed the published literature showing that in 72% of studies seagrasses responded to disturbance by increasing reproductive effort to 4-fold of the normal levels. The same was shown for terrestrial grasses over 30 years ago (Disraeli, 1984). Thus, our study indicates some interesting directions for future empirical work: the quantification of the relative importance of clone survival, on one side, and directly monitoring allocation to sexual and asexual reproduction as a function of disturbance regime, on the other side.

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4.6. Supplementary data

Table 4.S1. Rhizome elongation rates of *Zostera noltii* and *Cymodocea nodosa* extracted from the literature. Bold are data used in this study.

Zostera noltii	**Site**	**Growth (cm/year)**		**Description**
Marbà *et al.*, 2002	the Netherlands	157		July
Marbà and Duarte, 1998	worldwide	**68**		compilation of studies
Marbà *et al.*, 1996	Spain (2y, monthly)	10	3	2 years monthly study
			29	
Peralta, 2000	RF (optimal conditions)	130	91	large morphotype
			168	small morphotype
Peralta, 2005	RF (full database)	75	88	disturbed site
			62	protected site
Cabaço *et al.*, 2005	RF	11.5	11	natural state
			12	AVG of treatments (diff damage)
Duarte, 1991	worldwide	127		compilation of studies
Cymodocea nodosa	**Site**	**Growth (cm/year)**		**Description**
Marbà *et al.*, 1996	Spain	15	0.3	2 years monthly study
			84	
Marbà and Duarte, 1998	worldwide	**40**	7	compilation of studies
			204	
Duarte, 1991	worldwide	83		compilation of studies
Duarte and Sand-Jensen, 1990	Spain (Alfacs bay)	160		
Cunha & Duarte, 2005	Ria Formosa	20.3		AVG of 5 sites
Terrados *et al.*, 1997	Spain (Alfacs bay)	68		
Olesen *et al.*, 2002	Spain (Alfacs bay)	20	10	0.1 m deep
			30	1 m deep

Chapter 5.

Landscape genetics of *Zostera noltii* in an intertidal lagoon

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Abstract

Landscape genetics analyses link spatial and environmental parameters with population spatial genetic structure (SGS). Here we test the hypothesis that the effects of landscape are surmounted by frequent connectivity during high tide and no strong genetic structure should be visible within the lagoon. We carried out high resolution genetic analysis of *Zostera noltii* in the Ria Formosa lagoon, based on 3,185 genetic samples collected at 803 sampling plots across 84 km^2. Clustering methods didn't indicate any clear spatially defined clusters. Spatial autocorrelation analysis revealed samples within 3 km distance are more genetically related than it would be expected under the null hypothesis of random mating. To test the effect of habitat on gene flow we calculated pairwise genetic distances (GD) between the samples based on Loiselle kinship. Besides Euclidian distance (SD), four additional landscape derived distances were used (cost distance (CD), hydrographical distance (HD) and two tide dependent resistance distances (RW12 and RW21)) as predictors of genetic differentiation. Regression analysis showed RW12 and RW21 seem to be the best predictor of GD, while the other distances were weaker predictors. Genotypic richness (clonal diversity) per sampling plot varied from 0 (a single clone) to 1 (all distinct genotypes). None of the environmental variables indicated any significant association with genotypic richness. The clonal dominance showed only elevation is statistically significant. There was no particular spatial distribution in the distribution of plot specific allelic richness. Modelling the dispersal of propagules in the Ria Formosa revealed possible source and sink areas, three of each within the lagoon. Observed lack of the effect of the landscape on SGS can be explained by previously reported LDD of asexual propagules of *Z. noltii* in the lagoon.

Keywords: landscape genetics, asexual dispersal, *Zostera noltii*, connectivity.

5.1. Introduction

The idea of combining genetic and environmental data is not new in science, but it was only recently that Manel *et al.* (2003) popularized landscape genetics as a new discipline in biology. The development of landscape genetics was made possible by advances in molecular biology techniques and by increased computational power enabling complex geostatistical and statistical analyses in the last 15 years (Guillot *et al.*, 2009). Its main goal is to combine genetic, environmental, and spatial variation to test hypotheses about the biotic and abiotic factors that affect gene flow. This is done by linking spatially explicit ecological and environmental data with genetic information (Riginos and Liggins, 2013). The questions asked cover an array of different time scales and different hierarchical levels of organization, from genes to populations (Riginos and Liggins, 2013). Even though one term is used to describe this field of study, a very wide range of topics and methods are included under the landscape genetics umbrella. Several tools are used to study the spatial genetic structure (SGS) of a population - Mantel test or regression analysis of distances between the samples (Mantel, 1967), spatial autocorrelation (Griffith, 1992), Bayesian clustering (François and Durand, 2010) and multivariate analyses (James and McCulloch, 1990; Jombart *et al.*, 2010), among others.

Manel *et al.* (2003) focused on terrestrial systems when defining the term landscape genetics, but shortly after the popularization of the term similar studies were published focusing on marine systems (Gilg and Hiblish, 2003; Sköld *et al.*, 2003). This change of landscape was coined “seascape” genetics (Selkoe *et al.*, 2008; Amaral *et al.*, 2012). Riginos and Liggins (2013) emphasized the importance of clear distinction between the relevant parameters to study landscape genetics of terrestrial and marine systems. This is visible as the models developed for terrestrial systems don’t translate well for the marine systems (see Galindo *et al.*, 2006). In the review of the first ten years of landscape genetics Manel *et al.* (2013) paid attention to seascape genetics, as an independent and expanding area of research. Authors suggest that seascape genetics lacks extensive large scale sampling efforts, which would help define the connectivity patterns.

Seagrass population genetics have been increasingly studied, particularly after the development of highly variable genetic markers (Procaccini and Waycott, 1998; Reusch, 2000; Alberto *et al.*, 2003 a; Alberto *et al.*, 2003 b; Coyer *et al.*, 2004 a; Ruggiero *et al.*, 2004; van Dijk *et al.*, 2007). Their focus has initially been to describe genetic variation between and within populations of a certain species or a population. After an initial build-up of descriptive knowledge on seagrass population biology as unravelled by population genetics (see Procaccini *et al.*, 2007 and references therein), research diverged in a series of topics. Studies have been done linking genetic diversity with restoration (Reynolds *et al.*, 2013), historical processes (Olsen *et al.*, 2013), population connectivity (Campanella *et al.*, 2015), species resilience to disturbance (Hughes and Stachowicz, 2004; Massa *et al.*, 2013, Oliva *et al.*, 2014), taxonomic relations (Nguyen *et al.*, 2014), restoration efforts (Reynolds *et al.*, 2013; Olsen *et al.*, 2014) and conservation (see Procaccini *et al.*, 2007 and references therein). Seagrasses are clonal organisms, and this has drawn particular research interest into advantages and effects of clonal history (Kendrick *et al.*, 2005; Zipperle *et al.*, 2009; Becheler *et al.*, 2014).

Zostera noltii is a dominant seagrass species in an intertidal Ria Formosa lagoon, southern Portugal, where two other larger and subtidal species can be found – *Z. marina* and *Cymodocea nodosa*. The particularity of studying the landscape genetics of *Z. noltii* in this system comes from the dynamic properties of the habitat, which is drastically changed in every phase of the tidal cycle. On a low tide most of the habitat consists of exposed mudflats and salt marshes criss-crossed by a complex network of channels. On a high tide, the whole lagoon becomes seemingly a homogeneous submerged habitat. This results in frequent changes in connectivity among the seagrass patches four times a day, allowing the whole population putative connectivity to vary dramatically during a single day. Therefore we need to consider both temporal and spatial variation of the habitat. This study also distinguishes itself from others in the unprecedented individual based sampling effort it is built on. Published studies on genetics of seagrasses employed sampling along the transect (Coyer *et al.*, 2004 b; van Dijk and van Tussenbroek, 2010) or random sampling of 30 to 50 samples in one location (Olsen *et al.*, 2013; Nguyen *et al.*, 2014; Campanella *et al.*, 2015). To study genetics on the wider area, several of transects or sampling locations are analysed together (references above). Carrying out a wide spatial scale, *i.e.* landscape scale, analysis of a SGS of a population would probably be more appropriate on a dataset derived from accordingly planned and conducted wide spatial scale sampling design.

The dispersal mechanisms studied in this thesis (Berković *et al.*, 2014; Chapters 2 and 3 of this thesis) suggest LDD of asexual fragments and sexual propagules carried by these. Without barriers to limit connectivity during high tides, *Z. noltii* population in Ria Formosa lagoon might constitute a large panmictic population. Thus, we hypothesize that the effects of landscape are surmounted by frequent connectivity during high tide and no strong genetic structure should be visible within the lagoon. Here we test this hypothesis using the previously acquired large, high resolution data set of spatially explicit genetic data built for *Z. noltii* in the lagoon. We employed several common tools of landscape genetics to look into *Z. noltii* SGS, all indicating little to no spatial organisation of the genetic structure of the population, besides the observed isolation by distance (IBD) across first 3 km, *i.e.* no major effect of landscape on the gene flow.

5.2. Materials and methods

The work reported here was carried out with the same genetic data reported in Chapter 4 (refer to this chapter for sampling design and microsatellite genotyping techniques).

5.2.1. Development of Digital Terrain Model and spatial analysis

To acquire high precision topographic data we contracted a LiDAR survey of the Ria Formosa lagoon, surveying 6,511 ha of the lagoon area. Survey was carried out with Optech Airborne Laser Terrain Mapper-Gemini system on board of an airplane. This dataset was combined with the data of previous LiDAR survey carried out a year earlier, which focused on the 4,300 ha of the barrier islands area and was done with LiDAR Palmer Top Eye MK II B system used on board of a helicopter. Both surveys were performed by BLOM Portugal. Overlap of the two LiDAR surveys was between 12 and 50 m which allowed their interpolation to one continuous surface, carried out in GRASS GIS software v.6.4.0 (GRASS Development team, 2009). In shallow coastal areas expected penetration of laser is limited to about 3.5 m of depth. For this reason we used data from five different bathymetric surveys of the lagoon, the dredged channels and adjacent ocean coastal areas. Available bathymetric map of the Ria Formosa lagoon and adjacent coastal area was developed based on the Portuguese Hydrographic Institute data from 1970’s. Port authority carried out a survey in 1990’s of channels used for navigation, updating the previous dataset. Additional separate datasets are available from the same period, detailing secondary channels, main Faro – Farol channel and western section of the lagoon. Each of these datasets was added to the LiDAR survey results, taking into account the age of the data, so that in each section newest available data was integrated. Final DTM was developed following steps suggested by Brovelli *et al.* (2004). Spatial information was referred to ETRS98 Datum with Portugal TM06 projection and hydrographic zero. The final product was a digital terrain model (DTM) with vertical resolution of 15 cm and horizontal resolution of 1 m. This allowed us to extract the variables of landscape morphology used in this study. Five main variables were extracted from the DTM for each of the sampling points: elevation – in relation to the hydrographic zero; slope – rise relative to horizontal distance; aspect – downhill direction of slope; p curvature – profile curvature, *i.e.* curvature parallel to slope direction; and t curvature – tangential curvature, *i.e.* curvature perpendicular to the slope direction.

Z. noltii in our study site inhabits intertidal areas, because dispersal is dependent on water transport it was critical to characterize the tidal cycles to understand the relative submerged time and relative connectivity at each sample site. We used tidal tables (http://www.hidrografico.pt/previsao-mares.php) published by the Portuguese Hydrographic Institute (Instituto Hidrográfico), specifically the data for Faro – Barra de Faro – Olhão point. This information was combined with tidal harmonics, to calculate period of time each tidal level was present in the lagoon. These times were then expressed as a proportion of the tidal cycle and used as the weight factor for analyses performed on per-tidal-level basis.

5.2.2. Clustering methods

Using the previously acquired genetic data (see chapter 4 of this thesis) we conducted a series of analyses to estimate clusters of individuals sampled based on genetic co-ancestry similarity. First, we used the software STRUCTURE version 2.3.1. (Pritchard *et al.*, 2000), whose algorithm is organising samples into populations with Hardy-Weinberg equilibrium with linkage equilibrium between loci (HWLE). We run the program three times for each K (number of assumed clusters - populations or genetic groups), assuming the admixture model with a burn-in period of 10,000 iterations and 5,000 iterations of a Markov chain Monte Carlo (MCMC), for K 1-75, without providing the prior information on the sampling locations and using ΔK statistic (Evanno *et al.*, 2005) to detect the number of clusters. A separate analysis was done for all 3,185 samples and for the 1,999 samples, representing one copy for each found MLG. A second cluster analysis was run using the *Geneland* package (Guillot *et al.*, 2005) in R software (R Development Core Team, 2014) to estimate the number of clusters, using a similar approach to STRUCTURE. Five runs were done with maximum number of clusters tested ranging from 50 to 70, using the spatial model combined with the correlated frequency model and 100,000 iterations of MCMC with thinning at 100. Finally, we used the Discriminant Analysis of Principal Components (DAPC) analysis in *adegenet* 1.4-1 package (Jombart, 2008) in R software (R Development Core Team, 2014). This analysis serves to identify clusters and describe the relationship between them. Again separate analyses were done for the full dataset and for the unique MLGs only.

5.2.3. Spatial autocorrelation and modelling the distance tables

To look into the association between spatial distance and genetic differentiation between pairs of sample units we used SPAGeDi software (Hardy and Vekemans, 2002). We run a spatial autocorrelation based on multilocus estimates of mean pairwise kinship (F_{ij}), *i.e.* Loiselle kinship coefficient (Loiselle *et al.*, 1995), on mean spatial distance using binned intervals of spatial distance (distance classes). Distances between the samples were first computed automatically, so that the numbers of pairwise data points are evenly distributed among the distance classes. These classes were then adjusted when needed, *e.g.* so that the number of pairs in each distance class was higher than 100, resulting in the following 28 distance intervals: [0.02-0.05], next interval up to 0.075], 0.1], 0.2], 0.3], 0.4], 0.5], 0.6], 1.4], 2.2], 2.9], 3.5], 4.0], 4.7], 5.4], 6.2], 7], 7.8], 8.5], 9.4], 10.3], 11.4], 12.5], 13.7], 15.3], 17.6] and finally up to 22.8] km. We tested that mean Loiselle kinship values per distance class were not different than those expected under the null hypothesis of a random distribution (panmictic population) permuting spatial locations between individuals 10,000 times. Again, two analyses were done for the full dataset and for the unique MLGs only.

Once the GD table was created, we calculated a set of predictor landscape spatial distances and tested their association with GD. First, we calculated the Euclidian distance (SD) between two sampling points, *i.e.* a measure of the "as the crow flies" shortest distance between two points ignoring the landscape features along the way. SD was calculated in *gdistance* package (van Etten and van Etten, 2011) in R (R core team, 2014).

Based on the available fine scale topography of Ria Formosa, provided by the DTM analysis (see above), we modelled the hydrodynamic transport of particles in the lagoon using EcoDynamo (Duarte *et al.*, 2005; Duarte *et al.*, 2008). This analysis produced probabilities of transport between source and destination sites in the system. These probabilities were expressed as the hydrographical distance matrix (HD). EcoDynamo can simulate hydrodynamic processes (water movements), thermodynamic, biogeochemical and anthropogenic processes and is focused on water forcings of the system. For this analysis, DTM was scaled from the original 1 m resolution down to 100 m and 50 m resolution to allow reasonable computational time. This resolution is justified considering the observed period that fragments keep positive buoyancy (Berković *et al.*, 2014). In the first phase only the hydrodynamic model of Ria Formosa was simulated to produce a series of time series files recording average values of water level, mean U and V velocities (U and V corresponding to X and Y directions of Cartesian coordinate system) and mean U and V flows for each 10 minutes interval. During the second phase we integrated a Lagrangian transport routine adapted to seagrass propagules, using the previously saved time series of hydrodynamic simulated data. This model only uses water movements to influence the transport of the propagules, not considering the effect of wind. The model domain was defined by 52,600 cells of 100x100 m, encompassing the whole lagoon, barrier islands and peninsulas delimiting the lagoon and adjacent ocean area. *Z. noltii* distribution mapped by Guimarães *et al.* (2012) was overlaid upon the model domain. A total of 1,253 model cells containing *Z. noltii* (hereafter called seagrass cells) were used as locations of release of particles in the transport model. To track the dispersal patterns, we released 10,000 particles from each of the seagrass cells to avoid the numerical instabilities occurring when we released smaller number of particles. Thus, over 12.5 million particles were simulated dispersing in the model domain. This process was divided in series of simulations each simulating dispersal from up to 100 seagrass cells. From the results obtained in each simulation we calculated the probabilities of particles to travel from point A to point B. According to the model output, only some of the sampling points were connected with some of the other ones. For the pairs of sampling points without the connection shown by the model we assigned the 0.

A set of three additional landscape derived distances were calculated using modelled resistance matrix, *i.e.*, a path from source to destination that implies different landscape resistance to flow through cells in the path. First, we developed resistance surfaces based on Ria Formosa DTM. One resistance surface raster was produced for each tidal level between 0.95 and 2.95 m in 10 cm increments, resulting in 21 different tidal levels. These were chosen according to tidal amplitude in the lagoon, excluding the extreme values of spring tides which only occur few times a year. Resistance matrices were defined so that if the value of DTM raster was lower or equal to the tide level the resistance was set to 0 (*i.e.* point was submerged and connectivity was possible), otherwise resistance was set to 1.

To calculate the cost distances (CD), each resistance matrix was inverted into a conductance matrix where 0 and 1 resistance values were converted to 1 and 0 conductance, respectively. Using the *transition* function of *gdistance* package (van Etten and van Etten, 2011), we then computed the Transition layers, with movement in 8 directions (N, W, E, S and 4 intermediary ones) and applied the geographic correction, correcting for the map distortion and diagonal connections between the cells. Then we employed the *costDistance* algorithm on these Transition objects. In some cases certain points were unconnected, as they were above the sea level, so at the end of the calculation we substituted the INF value of distances to these points with the largest CD for that Transition layer, *i.e.* for that tide. CD matrices we obtained this way were then weighted to account for the occurrence of each tide level (see above). After each CD matrix was multiplied with corresponding weight factor they were averaged to provide one final CD matrix, reflecting the effect of tidal fluctuation on the height from sea level of each sampling point.

Finally, we employed an approach based on electronic circuit theory algorithms using the software Circuitscape (McRae and Shah, 2009). This approach provides a more realistic landscape driven distance between sites allowing for multiple paths to be integrated and has been shown to improve fit when predicting genetic differentiation in heterogeneous landscapes (McRae and Beier, 2007). We used the previously described resistance matrices for each tidal level, and run Circuitscape on each of the tidal resistance matrices, weighted the resulting matrices and then averaged them (as explained above) to obtain one final matrix of weighted resistance distances (RW21). An alternative distance matrix (RW12) was created changing the order of steps. In this approach we first weighted and averaged the resistance matrices and then run the Circuitscape on the final matrix.

Five produced distance matrices (SD, CD, HD, RW12 and RW21) were used to model the GD. We used LM in R (R core team, 2014), GD as a response variable and the five distance matrices as the predictors. These pair-wise distances were averaged by distance class (as shown above for SD), except for HD. As HD actually are probabilities said analysis is not a viable option, due to its dependency on spatial distances. A permutation test was used to test the null hypothesis of no association between GD and each of the predictor resistance distances. Distance tables were tested for correlation. Further we computed linear regressions with restricted ranges, limiting the distances to 5, 10 and 15 km. Restricted regression was not used for HD as the longest connected sampling locations were 5 km apart. Multiple regression was not used because the data is now bind in distance interval in which mean value is calculated, *i.e.* the individuals that fall in each class are different ones.

5.2.4. Modelling genotypic and allelic richness

Besides the association of spatial and resistance distances to genetic differentiation, we also investigated the association between environmental variables and genotypic richness (R), the proportion of different genotypes in a sample. Previous studies suggest that environmental factors directly or indirectly regulate both flowering and seed germination, *i.e.* stress leads to higher allocation to sexual reproduction (Cabaço and Santos, 2012) where elevation can be used as a proxy for stress, either natural (higher elevation brings longer exposure periods, lower elevation ensure prolonged submersion periods) or anthropogenic (physical removal for the purpose of clam harvesting is more common in higher intertidal, than in lower intertidal or subtidal areas). For this purpose, R was estimated at the sampling plot level, *i.e.* using the four ramets collected at the vertices of each 2x2 m plot (see chapter 4 for sampling details). Hence R in each plot varied between zero, when all four sample units were genotypicaly identical (*i.e.* belong to the same MLG), to 1, when each sample unit was genotypicaly unique (*i.e.* belonging to distinct MLGs). R was then modelled as a function of available environmental variables, acquired either through the hydrological modelling with EcoDynamo or extracted from the DTM output (for details see above). These predictor variables were: elevation, slope, aspect, p and t curvature, direction, flow and velocity of the current (described above). Logistic regression was tested to model R after conversion of genotypic richness data into success and failure variable, showing domination of a clone in the plot. If only one MLG was found within the plot (R=0) we labelled it as success (S), *i.e.* one clone dominated the plot, otherwise it was a failure (F). Thus the response variable clonal domination was binomial (success or failure) and was modelled using a logistic regression.

Anthropogenic activities such as clam harvesting, often leading to destruction of seagrass meadows, are generally associated with particular areas of the lagoon, allowing the distinction between disturbed and undisturbed areas. We assessed if the distribution of allelic richness (A), as calculated within each plot, showed any spatial patterns, possibly linked to the hotspots of clam harvesting. We used a custom R (R core team, 2014) script to compute A for each sampling plot, standardizing it by number of valid samples within the plot. These were then viewed on the map of the study area to see if any spatial organisation were apparent.

5.2.5. Transport simulations

The EcoDynamo modelled hydrographical distance matrix (HD) was also used to identify source and sink areas in what pertains *Z. noltii* dispersal in the Ria Formosa. Seagrass cells from which over 20 particles reached other seagrass cells were considered source cells. Seagrass cells which received over 20 particles were defined as sink cells. Cells for which more than 20 released particles did not leave the defined 100x100 m cell border, were considered retention cells. In case a cell met more than one of these conditions it was accounted for in each of the categories. These three cell categories were mapped to identify the different zones of relative *Z. noltii* connectivity within the lagoon.

5.3. Results

Our sampling design resulted in 3,212 individual ramet sample units, providing 3,185 valid genetic samples and 1,999 unique multilocus genotypes (MLGs). 1,495 MLGs were sampled only once. From the remaining 504 MLGs, 16 were sampled more than 10 times and the most abundant MLG (ID 1,886) was sampled 59 times (Fig. 5.1).

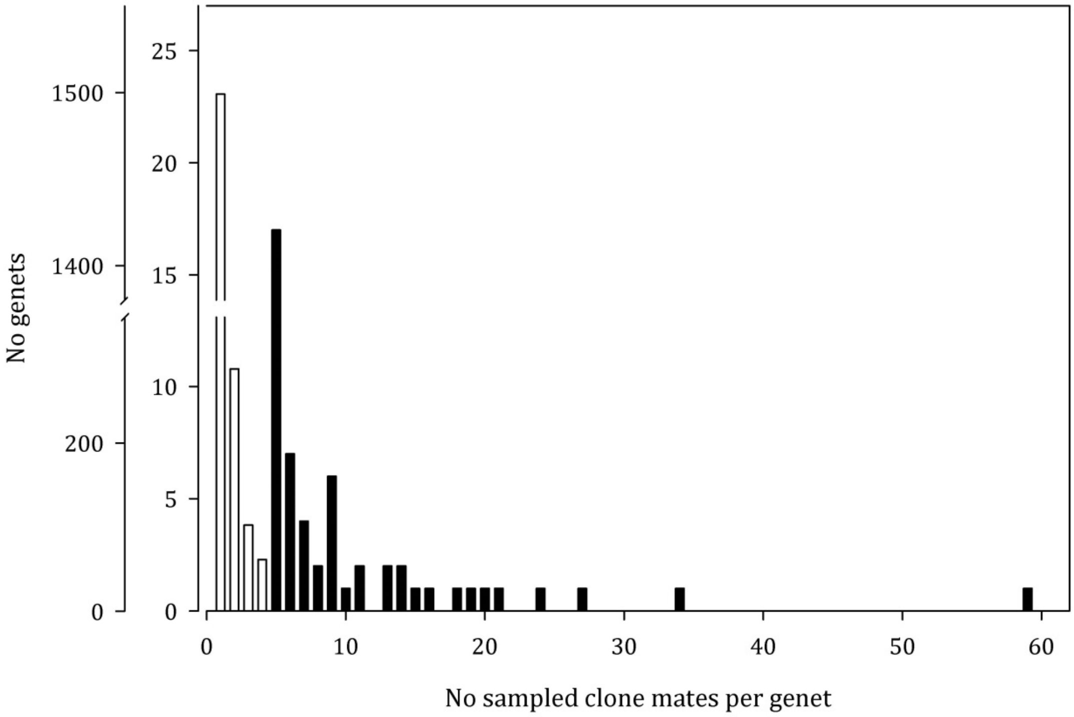

Figure 5.1. Frequency of 1,999 unique MLGs within the samples of *Zostera noltii* from the Ria Formosa lagoon. Double y-axis is input with two scales, to illustrate the full range of observed frequencies. Left y-axis (scale 0-1,500) corresponds to white columns of counts. Right y-axis (scale 0-25) corresponds to black columns of counts.

5.3.1. Clustering methods

Neither STRUCTURE nor Geneland indicated number of clusters in our dataset. DAPC analysis suggested about 77 clusters when done on the whole dataset. Some of them appear to be spatially organized (*i.e.* grouped in certain areas), probably because mostly they correspond to the samples of the same MLG or from other MLGs different by one or a couple of alleles from the dominant MLG in large clones. When the DAPC analysis was done including only a single copy from each unique MLG, 29 clusters were estimated, but they were in no way spatially organised. Clusters 2, 6 and 26 were slightly separated from other clusters in the scatter plot from based on genetic data, but were distributed across the lagoon, not showing any clustering in spatial organisation (Fig. 5.2).

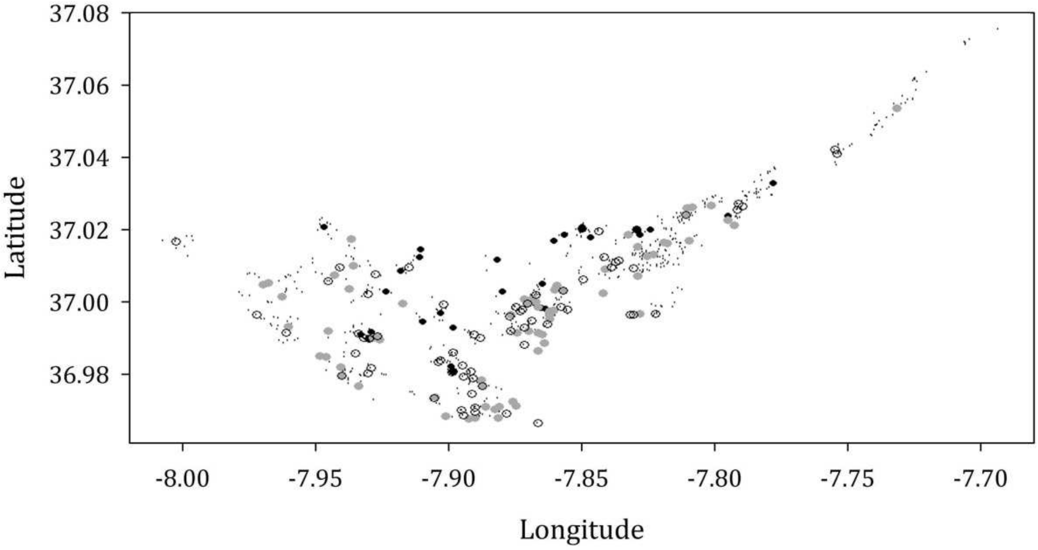

Figure 5.2. Distribution of *Zostera noltii* samples belonging to genetic clusters 2 (black), 6 (gray) and 26 (white) identified by Discriminant Analysis of Principal Components. Dots mark all the sampling plots.

5.3.2. Spatial autocorrelation and modelling the distancee

Spatial autocorrelation analysis of Loiselle kinship coefficient including all ramets generated a significant high mean kinship in relation to the null hypothesis of random mating (red line in the Fig. 5.3). Similar pattern was visible when the analysis was conducted using only the unique MLGs (blue line in the Fig. 5.3). In the latter case, sample units close to each other (*i.e.* within the 30 m radius) were showing less genetic similarity than before, but after this initial discrepancy, the curves followed the same trend, with mean kinship always higher for analysis of all samples.

The different distances used to model genetic differentiation were highly correlated (Tab. 5.1) In particular RW12 and RW21 were highly correlated, indicating small difference in the two methods used for weighting the distances in this case (Pearson correlation coefficient r=0.99, p<0.001).

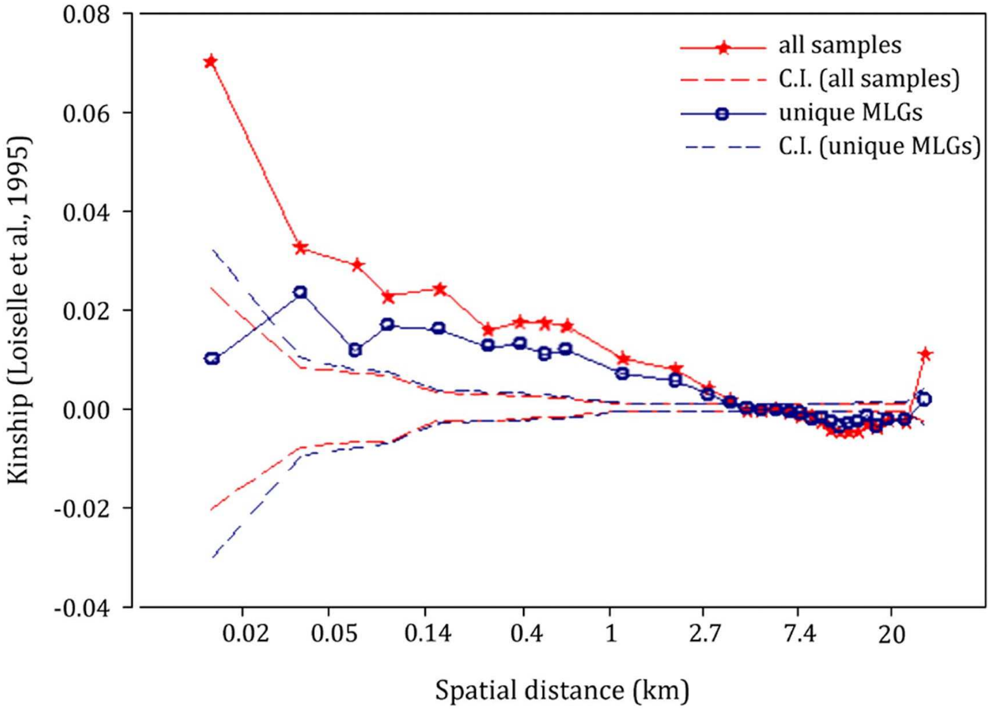

Figure 5.3. Correlogram of Loiselle kinship means (circles and stars) for each distance interval between samples of *Zostera noltii* in the Ria Formosa lagoon. Red line shows the correlogram for the analysis of the full dataset. Blue line shows the correlogram of the analysis including only unique multilocus genotypes (MLGs). Dashed lines delimit 95% confidence intervals (C.I.) for the null hypothesis of random mating, with red line corresponding to full dataset analysis and the blue line to the analysis of unique MLGs only. X-axis scale is logarithmic.

Results of full and spatially restricted regressions, with and without the log data transformation, are shown in Tab. 5.2. RW12 and RW21 seem to be the best predictors of GD (R2=0.80 and R2=0.85, both p<0.001). Other distances showed to be weaker predictors of genetics in *Z. noltii* with HD being the weakest one (R2=0.00004, p<0.001).

Table 5.1. Table of Pearson correlation between the 6 distance tables for *Zostera noltii* samples from Ria Formosa lagoon. GD – genetic distance, SD – spatial distance, CD – cost distance, HD – hydrological distance, RW12 and RW21 – weighted resistance distances with two methods of weighting (see section 5.2.3. for more details). Coefficients in bold are significant at level of p<0.001. ¨ marks p=0.22.

	GD	**SD**	**CD**	**HD**	**RW12**	**RW21**
GD	-					
SD	-0.04	-				
CD	-0.04	0.95	-			
HD	0.002¨	-0.01	-0.01	-		
RW12	**-0.02**	**0.75**	**0.86**	**-0.008**	-	
RW21	**-0.02**	**0.74**	**0.86**	**-0.007**	**0.99**	-

Table 5.2. Results of full and spatially restricted regressions (<5, <10 and <15 km) between genetic distance (GD) and the spatial distance (SD), cost distance (CD), hydrological distance (HD) and two weighted resistance distances (RW12 and RW21). For HD only full regression was done, as maximum distance was 5 km. For each linear model (LM) are given two values: t-value (in bold when p<0.0001, in italic when p<0.05) and R-squared.

Predictor variable	**Full**	**<5 km**	**<10 km**	**<15 km**
Mean SD	**-4.013** 0.3736	*-3.613* 0.4825	**-4.345** 0.4856	**-4.804** 0.4902
Log (Mean SD)	**-12.610** 0.8548	**-8.055** 0.8225	**-10.676** 0.8507	**-12.549** 0.8677
Mean CD	**-4.674** 0.4472	**-4.637** 0.6057	**-5.497** 0.6017	**-5.578** 0.5645
Log (Mean CD)	**-12.74** 0.8574	**-10.67** 0.8904	**-15.15** 0.9198	**-17.38** 0.9264
Mean HD	**-16.456** 0.00004	-	-	-
Mean RW12	*-7.996* 0.8026	**6.877** 0.6507	**6.881** 0.5979	**6.527** 0.5494
Log (Mean RW12)	**6.637** 0.6275	**-7.754** 0.8657	**-7.909** 0.8262	**-7.966** 0.8026
Mean RW21	*-7.246* 0.8529	**6.637** 0.6275	**6.808** 0.5904	**6.491** 0.5457
Log (Mean RW21)	**6.808** 0.5904	**-7.246** 0.8529	**-7.609** 0.8198	**-7.766** 0.7995

5.3.3. *Modelling genotypic and allelic richness*

A single MLG was observed in all four ramets in a plot for 8% of the plots (plot genotypic richness, R=0), 22% had two MLGs (R=0.33), 33% had three MLGs (R=0.66) and 37% had four MLGs (R=1). Genotypic richness was not associated with any of the environmental variables tested. Results of the logistic regression on the clonal dominance showed that from all the environmental variables tested only elevation was statistically significant (p=0.014). Spatial organisation of A per plot was not observed (Fig. 5.4)

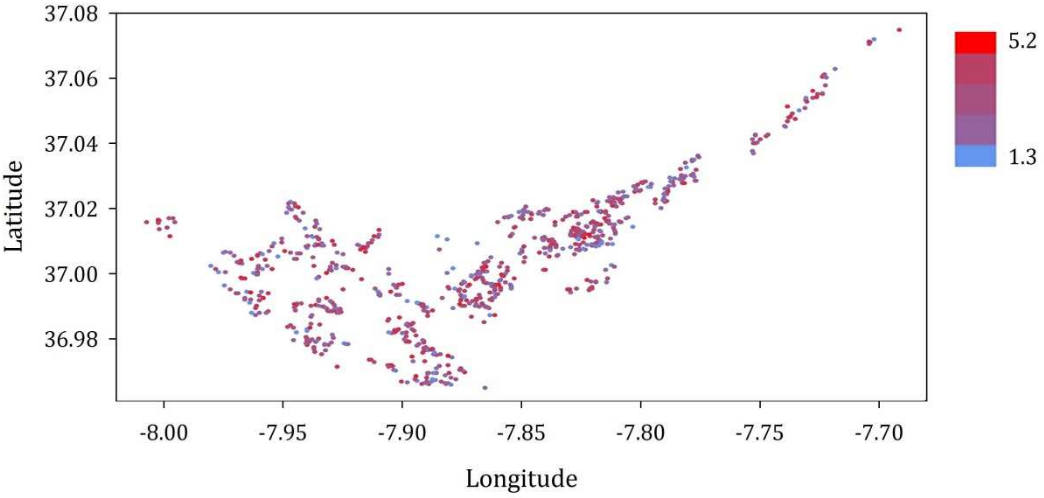

Figure 5.4. Distribution of allelic richness (A) per plot for *Zostera noltii* in the Ria Formosa lagoon. Lowest values of A are showed in blue, and the highest in red. No obvious grouping or organisation is visible from the map.

5.3.4. *Transport simulations*

Transport modelling with EcoDynamo showed that in a system forced only by water movements, out of 12.5 million released particles less than 40,000 stayed within the lagoon (0.33%) and the rest is washed out into the open ocean. Out of these 40,000, 8,500 didn't leave the source cell (*i.e.* stayed within the 100 m diameter). The maximum travelled distance observed within the particles that stayed in the system was 5.45 km. When this transport model data was analysed to infer source-sink dynamics, we observed that source cells were aggregated in three main areas, overlapping with the area of sink cells. This match between the area of source and sink cells corresponds to identification of retention cells in the same areas. The main three areas were the north-western side of Ilha da Deserta, north-eastern end of Ilha da Culatra and northern coast of Ilha da Armona, west from the village (Fig. 5.5). Overall less than 0.5% of 12.5 million released particles stayed inside the lagoon. Out of those more than 78% of particles left the seagrass cell where they were released, *i.e.* travelled more than 100 m.

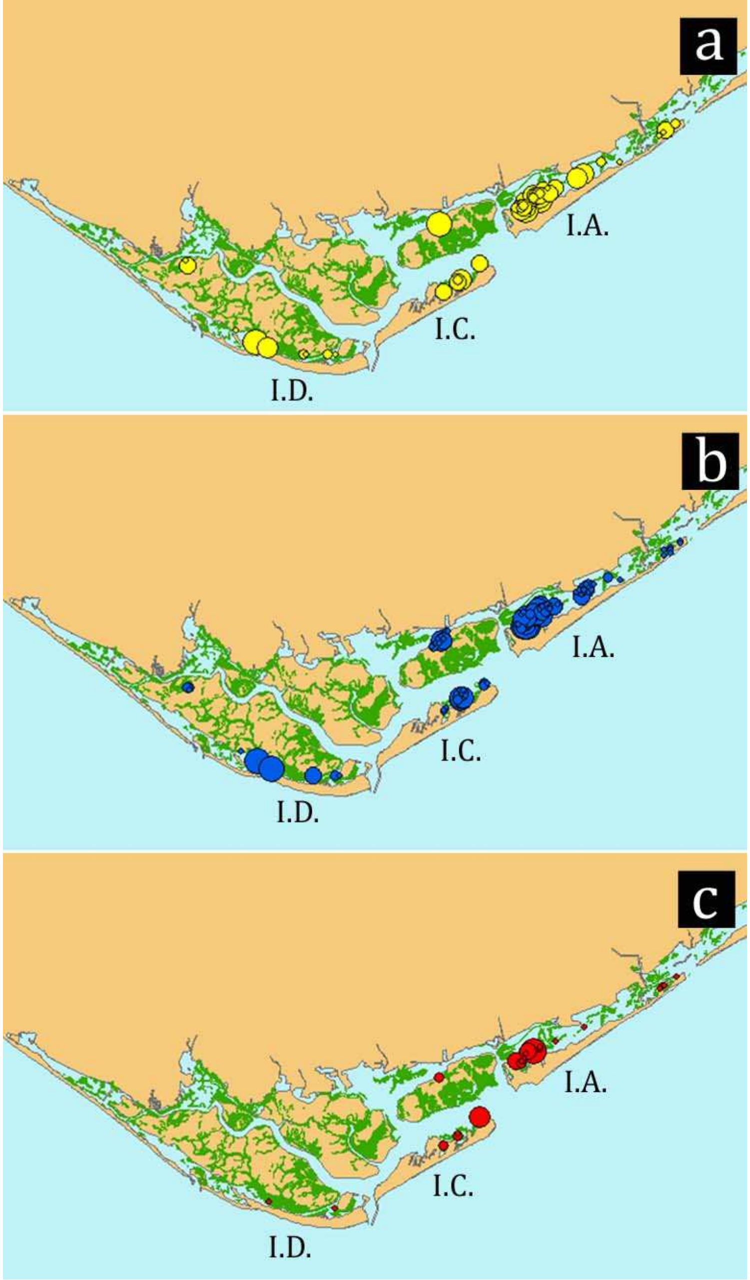

Figure 5.5. Results of EcoDynamo transport model for propagules of *Zostera noltii* in the Ria Formosa lagoon. Islands are labelled as I.D. for Ilha da Deserta, I.C. for Ilha da Culatra and I.A. for Ilha da Armona. (a) Source cells, donating propagules to other cells in the lagoon. (b) Sink cells, receiving propagules from other cells in the lagoon. (c) Retention cells, withholding the dispersal of propagules originating in them. One cell can belong to more than one category at the same time. Size of the circle represents amount of donated, received or retained particles.

5.4. Discussion

Our large scale study showed that there are no spatially organized large (sub) groups within the lagoon revealing lagoon-wide connectivity, but there is IBD revealing spatial restriction of sexually dispersed propagules. Considering the large number of samples and the high variability of the genetic markers used to describe the population's genetic structure, the study design was powerful enough to detect spatial genetic structure. No biologically/genetically relevant groups or other spatial organisation was detected beyond IBD. The spatial fragmentation of the population in a discontinuous series of over 600 patches (Guimarães *et al.*, 2012) doesn't reflect in the genetic structure of the population. There were no abrupt changes in allelic frequencies revealing a border between the genetic groups. Additionally, IBD with long positive kinship distance (approximately 3 km) seems to indicate a continuous population despite the fragmented nature of the habitat yet revealing restricted seed and pollen dispersal. This result can be accounted for by considering previous results where we described the dispersal biology of *Z. noltii* (Berković et al, 2014.; Chapters 2 and 3 of this thesis) and observed a wide spatial distribution of individual clones within the lagoon (Chapter 4 of this thesis) probably achieved via LDD of asexual propagules.

Spatial autocorrelation analysis showed an effect of isolation by distance, based on significantly higher genetic proximity of samples within the 3 km radius than it would be expected under the assumptions of panmictic population. This can be interpreted as a consequence of mutually non-exclusive aspects of the sexual and asexual dispersal, *i.e.* dispersal of pollen or seeds which do not disperse far from parental plant (Berković *et al.*, 2014) and fast rhizome elongation rates observed for this species and in this system (Peralta *et al.*, 2005; Peralta *et al.*, 2008) which would lead to decrease of genetic dissimilarity in the surroundings of a single clone. Clone sizes of *Z. noltii* in the North and Black Sea (Coyer *et al.*, 2004 b) and in Cadiz, Spain (Brun *et al.*, 2007) have been published, based on genetic analysis and plant morphology, respectively. Namely, in the Coyer *et al.* (2004 b) study most identified clones were <3 m^2, with the exception of one location in the Black Sea where the entire sampling area seems to be dominated by a single clone extending 50 m in length. The authors carried out genetic analysis and measured the distance between samples with same MLG. Brun *et al.* (2007) unburied the whole plant from the sediment and performed a detailed analysis of clonal architecture. They found that *Z. noltii* plants maintained physical connection between the modules covering almost 0.5 m^2. In our study we found that less than 10% of the $4\ m^2$ plots, where four ramets were sampled, were dominated by a single MLG. While these might possibly be explained by rhizome elongation, this shows how frequent clone intermingling and high genotypic richness can be found at small spatial scales. In contrast, as is reported in the chapter 4 of this thesis, at large spatial scales almost 60 MLGs were found spread over 10 km or longer, demonstrating long distance fragment dispersal (Chapter 4 of this thesis). Our data supports a hypothetical scenario where both sexual dispersal (seeds and pollen), rhizome elongation and local dispersal of fragments occur on a restricted scale, leading to higher genetic similarity of the samples on a small spatial scales and supporting IBD, while at the same time the events of LDD of fragments lead to lack of distinct genetic clusters within the lagoon.

Spatial distances used in the analysis included Euclidian distance and several least cost and connectivity paths, which reflected the channel network complexity and its fluctuations through the tidal cycle. The best support was provided by the two resistance distance matrices (RW12 and RW21), which combine results of movement through the resistance surface and the change of the resistance of this surface with each tide. On each high tide, most of the locations indeed can be connected by Euclidian distances (SD) as lagoon morphology gets simplified to an almost completely homogeneous surface. This occurs twice a day, greatly diminishing the effect otherwise complex morphology of the lagoon channel network would have on the population connectivity. But these distance measures (RW12 and RW21) represent the connectivity throughout the day, accounting for both very simple and very complex habitat morphologies. This is probably the best way to describe the connectivity, particularly from the point of view of LDD of fragments which can cross considerable distances following the water movements (Berković *et al.*, 2014), as long as they don't get stranded or entangled along the way.

We didn't find any association of the tested environmental variables (elevation, morphology of the terrain or water current characteristics) with genotypic richness. We expected plants at higher elevation, more often exposed to tidal currents and associated sediment movement, to be under more stress, leading to higher allocation to sexual reproduction (Alexandre *et al.*, 2005; Cabaço and Santos, 2012) that would in turn lead to higher genotypic diversity. This might be the result of other limitations associate with the life in the upper intertidal range of the species distribution. Elevated plants are submerged for shorter periods of time, limiting the period for pollination. Also, burial by moving sediments in the intertidal zone could lead to decrease in seed germination rate. Furthermore, canopies of plants which are exposed of prolonged periods of time are fragile and sparse and provide weak shelter for germlings and juvenile plants (Gambi *et al.*, 1990; Peterson *et al.*, 2004). It might be that at the upper edge of *Z. noltii* distribution flowering effort is higher (as reported for *Posidonia australis*; Inglis and Lincoln, 1998), but with lower germination success (due to desiccation during low tide), while at lower edge lower flowering effort (Inglis and Lincoln, 1998) with higher success. These considerations lead us to deemphasize the relative importance of flowering rate and reconsider the assumption of higher genotypic richness at higher elevations as being swamped by effects of fragment dispersal. Throughout this thesis, LDD via vegetative fragments was implied as an important means of asexual propagation. On one hand, along the intertidal gradient, higher areas which are more frequently exposed to tidal currents are likely to receive more fragments entangled or stranded in wreck lines. However, fragments settling in this area are exposed to less stable environment and have fewer chances to stay in place enough time to root and grow. On the other hand, fewer fragments are likely to settle in lower intertidal areas, because they need to reach the sediment while they are still positively buoyant and these areas are more often submerged. However, if plants do settle there they may find more favourable conditions to grow and spread. Therefore, we expect higher input of fragments in the higher elevations, with lower success of establishment, and lower input of fragments with higher success rates in the lower elevations. Similar as with flowering, the two scenarios might balance each other. This shows how important it is to consider both spatial and temporal processes when looking at interaction between the environment and life history of a species.

Coyer *et al.* (2004 b) described the population genetic structure of *Z. noltii* throughout its biogeographic range. The summary statistics reported here show allelic richness standardized for 28 samples was A=5.9. The previously reported results in this thesis (Chapter 4) show non-standardized A for full set of 3,185 samples to be A=15.0. Once standardized to the same number of samples (N=28) A was 7.5, still higher but more comparable to Coyer *et al.* (2004 b). Allelic richness distribution throughout the Ria Formosa seem to be approximately randomly distributed in the Ria, *i.e.*, there was no obvious hot or cold spots of diversity, a pattern different from clonal probability (Chapter 4). Intense clam harvesting is a regular activity during low tides in certain parts of the Ria Formosa lagoon (Fig. 5.6), leading to either complete removal of seagrasses or considerable damage to remaining patches (Cabaço *et al.*, 2005). Population genetics theory predicts the occurrence of bottleneck effects as consequence of disturbance and population size decline which could lead to lower allelic richness in disturbed areas. Our results did not reveal any indication of possible bottleneck effects around the areas of clam harvesting, which indicates that species' fast growth and recovery, in combination with frequent flowering rates and LDD of fragments may cancel out any disturbance effects on genetic diversity. However, we note that disturbance by clam harvesting in Ria Formosa has not been to date thoroughly quantified in a spatial explicit way. Future research could address this shortcoming which would allow testing hypotheses about the association between A and disturbance as well as possible rescue effects from undisturbed areas.

The amount of data acquired in this study often challenged the computational capacities of the used infrastructure and proved to be unusual in population genetic studies, as the majority of classical population genetics software used here had difficulties managing this amount of input data. This indicates the development of methods and analyses of landscape genetics needs to be synchronized with the support for extensive data sets which are increasingly easier to collect.

Figure 5.6. A frequent anthropogenic disturbance to the *Zostera noltii* population in the intertidal areas of the Ria Formosa lagoon is clam harvesting, occurring on low tide on the exposed sandbanks and mudflats.

Synthesizing our results on the effect of landscape, or lack of it, on the genetic diversity of *Z. noltii* it seems clear that the species is highly resilient in Ria Formosa. We argue that this is partially due to the combination of fast clonal growth and LDD with frequent flowering and high seed production rates. This resilience seems to mitigate current anthropogenic and natural impacts, mostly physical removal either for clam harvesting or due to sediment dynamics, and is supported by the observed lagoon-wide *Z. noltii* distribution.

Identification of source and sink patches of seagrass propagules is a step towards identification of relevant areas for the maintenance of the species within the lagoon, aiding managers and interested stakeholders to delimit zones of strict protection which can ensure population maintenance through the lagoon. Our model of connectivity and circulation of propagules within the lagoon pointed out three zones potentially relevant for the transport of seagrass propagules within the lagoon, but also indicated that a very low number of particles stay within the lagoon, while the majority was washed out to the ocean with the ebb tide. However, it is important to point out that this model was driven by water forcing alone. In the field, movement of the floating fragments is strongly driven by wind, sometimes out-competing water current forcing (Erftemeijer *et al.*, 2008; Berković, pers. observation). Hence, future work should model both water and wind forcing. In addition, density of canopy, *i.e.* abundance and morphotype of seagrass in source and sink areas differ greatly (Berković, pers. observation), affecting their. Adjusting the number of released propagules in each cell with the local abundance of seagrass should provide a more realistic estimation of connectivity pathways.

Acknowledgments

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Chapter 6.

General discussion

This study revealed high dispersal capacity of asexual propagules of dwarf eelgrass *Zostera noltii* population in the Ria Formosa lagoon. Following the experimental work estimating the potential for this dispersal, individual based genetic analysis supported asexual long distance dispersal (LDD) as a relevant factor shaping the spatial genetic structure (SGS) of *Z. noltii* in Ria Formosa lagoon. The sampling coverage used allowed to test a significant positive association between distance to physical disturbance and the probability of finding large clones. Finally, landscape genetics analysis revealed isolation by distance (IBD) effect across a scale of 3 km, but no other effect of landscape features on the SGS.

6.1. Dispersal biology of *Zostera noltii*

The first section of this thesis focused on the dispersal biology of *Z. noltii* where I tested the dispersal potential of sexual and asexual propagules (Chapter 2) and the post-dispersal success of vegetative propagules, which had the highest dispersal potential (Chapter 3). The estimated potential dispersal of *Z. noltii* fragments showed fragments could reach 2,300 km over a period of almost two months, under the assumption of unidirectional currents, highlighting that vegetative fragments should be considered an important dispersal propagules. The observed prolonged viability after detachment, essential for the estimated LDD, was lower than previously suggested viability period of *Posidonia oceanica* fragments (Balestri *et al.*, 2011), comparable to that reported for *Zostera marina* (Ewanchuk and Williams, 1996) and higher than reported for *Halophila johnsonii* and *Halodule wrightii* (Hall *et al.*, 2006). This promotes the idea that fragment viability can support LDD in a number of seagrass species. Similar dispersal strategies are suggested in other macrophytes

(McKenzie and Bellgrove, 2008) and clonal marine invertebrates (Highsmith, 1982). In this thesis it is therefore suggested that the seagrass fragmentation leading to production of asexual propagules, and their successive dispersal, should be considered an extension of seagrass life history. Simultaneously, dispersal capacity of individual *Z. noltii* seeds was estimated to be under 30 cm, suggesting that seeds falling from the spathes are not likely to be exported out of the meadow. This suggests that reproductive fragments are necessary vectors for LDD of seeds, reinforcing the importance of fragments as seagrass dispersal propagules. Considerably higher dispersal potential of asexual propagules than of the sexual ones is of fundamental relevance for the classical interpretation of the species life history, and is likely to be extended to other seagrasses or other clonal aquatic plants (Gliddon *et al.*, 1987; Fischer and Van Kleunen, 2002). Suggested LDD of vegetative fragments therefore has an important role for the species' dispersal biology, but in order to evaluate effective asexual dispersal (*i.e.*, migration) we need to understand the reestablishment success of floating fragments.

To the best of my knowledge this thesis was the first comparison of the sexual and vegetative dispersal potential of a clonal marine plant species, which produced data on dispersal potential and opened new questions on the success of LDD and its role in shaping genetic structure of the populations. With these aims in mind I carried out a study of post-dispersal settlement success of vegetative fragments (Chapter 3). This study found 100% survival of settled fragments under certain conditions (short dispersal period and large size of fragments). Success was conditioned by presence or absence of the floating period, but not its duration within the dispersal regime. Even so, overall average success of post-dispersal establishment was 34%, supporting the hypothesis that fragments' dispersal and establishment is a conceivable process, relevant for species' life cycle. Previously, Ewanchuk and Williams (1996) observed that fragments of *Z. marina* get entangled in the canopies start to root after a while, reaching for the sediment underneath them. In my own experimental study, a similar natural entanglement process was manipulated to simulate fragment settlement. My results indicated that fragments settled in this way have relatively high survival rates. In their study Ewanchuck and Williams (1996) suggested that the asexual reproduction via fragments is not relevant for the population due to improbable successful establishment. Applying the results of establishment success found in my study, I estimated, under several assumptions, that asexual LDD could produce about 68 ha/y of new seagrass meadow with the average density observed in Ria Formosa. Such cover represents 5% of the estimated distribution of *Z noltii* in 2002 (Guimarães *et al.*, 2012). This further supports the idea shown in this thesis that asexual dispersal indeed plays a relevant role in the functioning of the population in the Ria Formosa lagoon.

During both experimental studies presented here, on dispersal potential and the post-dispersal settlement success, additional traits were monitored to track the overall state of the fragments either during the dispersal phase or after it. Fragment damage, measured during the transport phase of dispersal, was more frequent in older section of the fragment. This suggests that *Z. noltii* may be adapted to promote protection of the apical shoot and the newer part of the plant. Corroborating this interpretation, Cabaço *et al.* (2005) showed that damage had a significant negative effect on plant growth and survival only when the apical shoot was removed. I believe that due to these mechanisms, even with high fragment damage (approaching 60% in currents of 30 cm/s), the potential colonization capacity of *Z. noltii* fragments does not seem to be diminished. Maintenance of the apical shoot enables longer dispersal phase, lowering the negative impact on the success of the fragments. A previous study on *Zostera marina* showed that the extended viability of positively buoyant fragments allows a dispersal of up to a month (Källström *et al.*, 2008). This is about half of the time found for *Z. noltii* fragments in this thesis, emphasising the importance of vegetative fragments dispersal for this species. Even though *Z. noltii* fragments can carry flowering shoots with fruits or seeds, the importance of flowering shoot dispersal might be reduced by the limited flowering season and the low persistence of the spathes on the flowering shoots, which is dependent on their maturation stage (Alexandre *et al.*, 2006).

Besides reporting high success of post-dispersal establishment of fragments (Chapter 3), this study aimed to evaluate the condition of fragments surviving the transport phase. Fragments required more time to root if they were exposed to a period of floating prior to settlement, but once settled they took between one and three weeks to root, indicating fast adjustment to new conditions. Other measured parameters pertaining to fragments' re-establishment success indicate ability to occupy space after the settlement. Fragments only branched once they settled and rooted, showing that plants do not invest in space occupation during the transport phase, presumably as this makes them more subject to breakage and damage observed in the first experiment (Chapter 3). Across all the experimental groups, more than 70% of fragments maintained the main apical shoot intact, corroborating previous findings about the importance of maintenance of the main apical shoot. Finally, the ratio of above:below ground biomass observed in this study, based on the previously published data for *Z. noltii* in this study area (Cabaço *et al.*, 2012), implies that the settled plants are in a state typically observed in colonizing meadows.

Dispersal biology of *Z. noltii* described in this work does not differ much from dispersal of freshwater aquatic plants, which are known to disperse via fragments (Nichols and Shaw, 1986; Johansson and Nilsson, 1993; Boedeltje *et al.*, 2003), settle (Riis and Sand-Jensen, 2006) and establish after dispersal (Barrat-Segretain *et al.*, 1998). Only a few seagrass species were focus of dispersal studies as thorough as in here, as often the goal is dispersal of sexual propagules (Buia and Mazzella, 1991; Orth *et al.*, 1994; Harwell and Orth, 2002; Lacap *et al.*, 2002). Recently, the body of literature on seagrass dispersal biology is growing and including the dispersal of vegetative propagules (Hall *et al.*, 2006; Erftemeijer *et al.*, 2008; Virnstein and Hall, 2009; Balestri *et al.*, 2011; Berković *et al.*, 2014; Thomson *et al.*, 2014; Stafford-Bell *et al.*, 2015). Virnstein and Hall (2009) suggested fragments displacement and dispersal as a consequence of a storm can explain sudden shift in species distribution for *Halophila johnsonii* and *H. decipiens*. This shift occurred across more than 20 km distance, implying both dispersal of the fragments and their establishment in the new area.

6.2. Landscape genetics of *Zostera noltii* in the lagoon

First two studies in this thesis provided the information on the dispersal which might be taking place within the *Z. noltii* population. I then looked into spatial organisation of *Z. noltii* genetic structure to see if evidence of proposed dispersal processes could be found (Chapter 4). The main hypothesis here was that a signature of asexual LDD should be found in the clonal structure of *Z. noltii* population. Using an extensive individual based sampling I was able to corroborate this hypothesis. I found high genetic and genotypic diversity, but at the same time multiple identical multi-locus genotypes (MLGs) spread across tens of kilometres in the lagoon. *Psex* analysis confirmed that these repeated MLGs could not be explained by distinct sexual recombination events and were thus the copies of the same clone, or clonemates.

In other seagrass genetics studies (Reusch *et al.*, 1999; van Dijk and van Tussenbroek, 2010; Arnaud-Haond *et al.*, 2012), similar observations of repeated MLGs over large spatial extents were used to estimate clones' age, calculating the distance between the samples of same MLG, and relating it with the rhizome elongation rates for their study species. This approach yielded very old clones in some species, reaching hundreds to thousands of years (Reusch *et al.*, 1999; Arnaud-Haond *et al.*, 2012). The Ria Formosa lagoon is a relatively young environment, with a currently estimated time of origin at 6,000 YBP (Andrade *et al.*, 2004; C. Sousa, pers. comm.). For this reason I integrated paleoceanographic data on sea level change for this region when carrying out clones' age estimation. This exercise did not lead to conclusive dates or points of origin for most of the large clones observed, because the available data only allowed tracing sea level changes back to a period of 20,000 YBP. The minimum estimated age of several individual clones within the lagoon surpassed 20, and even 30 thousand years. At that time the current lagoon area was on land, and these clones would have had to evolve in the open ocean and survive during tens of millennia and eventually be surrounded by the lagoon. To support these results this study also looked into another seagrass species found in the lagoon, *Cymodocea nodosa*. For this species, the same age estimation method resulted in a minimum of 50 thousand years for a single dominant clone. Based on this alone, both species would find place on the list of the oldest living organisms on the planet. Due to the assumptions used in this simple age estimation method, *e.g.* undisturbed, straight line growth across homogenous habitat without barriers, any calculated age is a strong underestimation, making these large clones even older. However, I argue that effective asexual dispersal, through fragmentation, transport and re-establishment of fragments during the life of a clone, renders this age estimation method meaningless. Thus, I propose that the most parsimonious explanation for the large spatial extent of these clones is not this age determination model, which requires exclusion of asexual LDD, but instead a model that includes allows for asexual LDD, complemented with rhizome elongation.

Fragmentation followed by dispersal has been reported for a range of clonal organisms (Highsmith, 1982; Ayre, 1984; Jackson, 1986; Wulff, 1991; Smith and Hughes, 1999; Freeland *et al.*, 2000; Ceccherelli and Piazzi, 2001; Cleavitt, 2002; De Meester *et al.*, 2002; McKenzie and Bellgrove, 2008). In seagrasses, detachment, drifting and re-rooting was observed for *Posidonia oceanica* (Diaz-Almela *et al.*, 2008), two other *Posidonia* species in the Western Australia (Campbell, 2003) and for *Halophila johnsonii* and *H. decipiens* (Hall *et al.*, 2006). Information on dispersal is therefore available, even though not in a systematic way. Implications of this type of dispersal on ecology and evolution of the species have not yet been looked at. This thesis links dispersal processes to population and landscape genetics, offering data to re-evaluate importance of asexual dispersal for the species biology.

Asexual LDD has been reported for a range of seagrass species that cover a spectrum of sizes: from some of the larger species in the genus *Posidonia* (Campbell, 2003; Diaz-

Almela *et al.*, 2008) to some of the smaller species like *Halophila johnsonii* (Virnstein and Hall, 2009). Considering the latitudinal distribution of seagrass dispersal studies, there isn't any clear association between of geographic location and the capacity for asexual dispersal. Namely, studies where asexual dispersal was confirmed were carried out from Australia to Mediterranean and central Atlantic, both west and east coasts. The single most characteristic distinguishing *Z. noltii* from other seagrass species for which asexual LDD was reported, is its intertidal habitat in our study site. It is possible that the dynamics of the habitat facilitate the fragmentation and creation of asexual dispersal propagules, the transport by tidal currents and the post-dispersal settlement. Conversely, for subtidal species in most cases the dispersal mechanisms are not investigated, but data can be extracted from general observations. For *C. nodosa*, a larger and subtidal species also found in the lagoon, Barrio *et al.* (*in prep*) observed prolonged viability of fragments in similar experiments. A different aspect observed in that species is that after a certain period of time fragments become negatively buoyant and settle to the bottom where they root. For *P. oceanica* fragments, dispersal is likely achieved by drifting along the bottom seems (Boudouresque *et al.*, 1990; Diaz-Almela *et al.*, 2008) while for *H. johnsonii* Hall *et al.* (2006) suggested that fragments disperse on the surface and later settle to the bottom and root. Mechanisms of settlement might be dependent on the habitat and species, but it seems that the process may occur across a variety of environments.

Decline in seagrass cover worldwide is today a very well documented fact (Orth *et al.*, 2006; Short *et al.*, 2006; Waycott *et al.*, 2009; Tuya *et al.*, 2013; Short *et al.*, 2014; Fabbri *et al.*, 2015). Understanding the dispersal biology of species and the consequences on the new and existing populations can prove beneficial for future management and conservation work. Even though a recent review on seagrass dispersal totally deemphasised the relevance of asexual dispersal and its possible role in species recovery after disturbance (McMahon *et al.*, 2014), this thesis showed that dispersal of vegetative fragments can outcompete dispersal by rhizome elongation. This means that, at least for some species, colonization through asexual dispersal might be as fast as sexual dispersal. There is a risk of possible reduction of genetic diversity in patches developed from established asexual propagules after extinction, but this might be balanced by the migration rate of such propagules. Also, here and elsewhere it was shown that floating fragments can also transport maturing fertilized flowers which in turn can release viable seeds (Berković *et al.*, 2014; Barrio *et al.*, *in prep*), further minimizing genetic diversity reductions. These are optimistic news for seagrass ecosystem recovery, but contingent on the removal of pressures that led to seagrass local extinction in the first place.

The second section of this thesis tested the association between a source of disturbance and the spatial distribution of large clones at the landscape scale (Chapter 4). It was expected that the areas of higher disturbance (*i.e.* sand barrier islands; Cunha *et al.* 2005) will have higher levels of clonal diversity (Weider, 1992; Reusch, 2006). Clonal probability (Pc_n) was used for quantification of probability of sampling same clone *n* or more times in the whole sample. Results of this analysis revealed higher probability of sampling large clones with increased distance from the barrier islands. It is argued that this association can result from two non-mutually exclusive processes linked to disturbance regime - different clone survival restricting how large a clones can grow and different allocations to sexual and asexual reproductive components. This opens up for future research on the mechanisms and relative importance of each of these two hypotheses.

Finally, I used landscape analyses to test the link between the spatially explicit variables putatively affecting dispersal and microsatellite based genetic differentiation of *Z. noltii* population in the Ria (Chapter 5). Spatial autocorrelation unravelled pattern of isolation by distance (IBD) on a scale of 3 km where pairwise genetic kinship was larger than expected randomly. However, most of the other analyses failed to give meaningful or conclusive results, *e.g.*, hindering the identification of any spatially organised clusters of genetic co-ancestry. Besides the characteristics of the habitat which shapes connectivity pathways, mechanism of connectivity itself will play a major role in the formation of genetic differentiation across the space. Landscape dynamics, change in complexity following tidal cycle, and the LDD shown in this study both work towards removal of the effect of landscape and spatial distance on genetics. Namely, intertidal lagoon is a morphologically complex area, providing multiple possible connectivity paths. Twice a day tidal cycle homogenizes this habitat in an uniform continuous landscape where shortest connectivity between any two points can be equal to the shortest theoretical connectivity (*i.e.*, as the crow flies). Dispersal of asexual propagules is facilitated by homogenisation of the habitat, even more so considering the homogenous habitat during high tide is the most suitable for LDD of floating fragments.

Due to an unprecedented sampling effort (approximately 3,200 ramets collected), density of sampling plots in space (average distance to the closest plot 96 m) across the entire lagoon, and use of nine highly polymorphic microsatellite loci, there should be no limitations to the detection of fine scale, changes in the genetic structure. The above mentioned effect of IBD extended over a larger spatial scale indicates natal dispersal, *i.e.* pollen or seeds which are retained close to the parental plant (Berković *et al.*, 2014) or fast rhizome elongation rates observed for this species and in this system (Peralta *et al.*, 2005; Peralta *et al.*, 2008) in proximity of parent organisms, might be causing the observed higher kinship across scales smaller than 3 km. Beyond this scale, LDD of asexual propagules generates gene flow throughout the lagoon, contributing to the observed SGS.

6.3. Conclusions and future remarks

This thesis demonstrated the capacity of *Zostera noltii* to disperse by asexual propagules across large spatial scale (Chapter 2). The observed settlement success rates imply that transport for longer periods can result in effective dispersal (Chapter 3). The clonal structure of *Z. noltii* in the Ria Formosa lagoon is most likely the consequence of long distance dispersal of asexual propagules. At the same time, it is under the influence of the physical disturbances (Chapter 4). Combination of rhizome elongation and short-scale dispersal of propagules leads to higher kinship between the individuals on a scale of 3 km, while long distance dispersal of asexual propagules enhances the gene flow on larger scales (Chapter 5).

It was shown to some extent that dispersal via vegetative fragments is also likely for the sympatric species *Cymodocea nodosa* suggesting further research should be done following a similar framework as carried out in this study of *Z. noltii* for other seagrass species. Studies of (asexual) dispersal are becoming more common, but a shared framework is necessary, defining terms and research questions, which would ease comparison between species and allow generalisation of observed patterns. Based on already available data a synthesis on the importance of asexual dispersal in seagrasses is surely forthcoming and would help direct future research.

There is an ongoing debate over the importance of sexual versus asexual propagation. Approach used in this thesis to study the dispersal traits, could add weight on the balance in favour of asexual propagation. In the light of threats to seagrass meadows, this thesis raises questions important for evaluation of response of these clonal organisms on the disturbance. Results presented here indicate association between the disturbance and the clonal structure, setting the path for further research in this direction. Two interesting questions would be quantification of the relative importance of clone survival and direct monitoring of allocation between sexual and asexual reproduction as a function of disturbance regime

6.4. References

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