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# Population dynamics of a fragmented subtidal *Zostera marina* population affected by shell fishing

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## ARTICLE INFO

## ABSTRACT

This investigation illustrates the spatial and temporal dynamics of a *Zostera marina* seagrass meadow affected by clam harvesting. Photointerpretation of satellite imagery corresponding to years 2007, 2013, 2017 and 2018, combined with field monitoring in 2019 allowed assessing the spatial coverage, population dynamics and genetic characterization of the *Z. marina* population in areas impacted and non-impacted by the shellfishing activity. The impacted meadow displayed a highly fragmented and discontinuous seagrass matrix anthropogenically induced by the periodical disturbance associated with bottom raking. A continuous colonization process characterized the seagrass landscape, where the area occupied by the meadow varied by a two-fold factor, with changes even exceeding 86% in some years. Only 740 $m^2$ (ca. 15%) of the seagrass matrix remained vegetated in the four years monitored in this investigation. The number of patches showed a large interannual variability, exceeding 100% in the four years studied, ranging from 58 to 199, while the border effect perimeter/area indicator showed a two-fold variation ranging between 1 and 2. Clearly differentiated patterns were observed in shoot density, biomass, and flowering density between shellfishing-induced patches of different sizes and the long-term non-impacted areas. A significant pattern of genetic differentiation among impacted and control populations were also found. Our results showed that population dynamics varied as a function of *Z. marina* patch-sizes, thus reinforcing the need for a combined approach involving seascape structure and patch dynamics with population dynamics and genetic structure to assess the impact of disturbances on seagrass ecosystems.

## 1. Introduction

Seagrasses are found in coastal areas within the land-marine interface developing dense and continuous meadows or mosaics of vegetated and bare areas (McKenzie et al., 2020). This characteristic spatial structure led to the formulation of the term "seagrass landscape" more than two decades ago (Robbins and Bell, 1994), which refers to a matrix of connected habitat patches showing high spatial and temporal heterogeneity (Boudouresque et al., 2009).

Due to their ecological and socio-economic importance, e.g., fish nursery, carbon sink, protection from coastal erosion, among others (Costanza et al., 1997; Boudouresque et al., 2012), seagrasses are among the first marine habitats studied from a seascape approach (Bell et al.,

2006). However, most of the research in this field has been focused on the seagrass *Posidonia oceanica*, an endangered species in the Mediterranean Sea where it plays an important role as an endemic, habitat-forming species (Abadie et al., 2018), whereas *Zostera marina* is by far the most abundant and widespread species in northern temperate waters (Den Hartog, 1970). There is growing evidence that seagrass meadows are experiencing a worldwide decline due to anthropogenic pressures (Duarte, 2002; Orth et al., 2006). Waycott et al. (2009) reported an alarming loss rate of 110 km² year⁻¹ since 1980, which accounts for a total loss of 30% of the seagrass extent worldwide. In the study area, the trend of seagrass distribution is predominantly negative, and is characterized by the progressive loss of intertidal surface covered by seagrass meadows (https://pradera.ihcantabria.es/).

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### 1.1. Seagrass local scale physical disturbances

The existence of bare areas within seagrass beds is a common phenomenon driven by natural disturbance agents, such as sea turtles, persistent algal mats (Cowper, 1978) or storms (Short and Wyllie-Echeverria, 1996). Anthropogenic impacts such as those related with worse water quality conditions and increased turbidity (Duarte et al., 2004; Orth et al., 2006), anthropogenic land-use modification or and direct physical impacts that remove seagrass plants produce spatial mosaics of unvegetated and vegetated areas.

Among the main drivers causing seagrass bare areas by local scale physical disturbances are boat anchoring and propeller scars (Dawes et al., 1997; Francour et al., 1999; Abadie et al., 2016), and harvesting, raking and dredging activities (Peterson et al., 1987; Boese, 2002; Orth et al., 2002; Alexandre et al., 2005; Cabaço et al., 2005; Neckles et al., 2005; Boese et al., 2009; Barañano et al., 2017, 2018; Garmendia et al., 2021). In Northern Spain, seagrasses coexist with several marine activities that include small-scale fisheries (e.g., cuttlefish, eel and sardines) and shellfishery (e.g., clams and cockles) as well as recreational activities such as windsurfing, kite surfing, bathing, canoeing and recreational fishing (Bas Ventín et al., 2015), with related threats due to the effects of navigation, anchoring and fishing practices. Such interactions severely impact seagrass meadows, as anchoring and chain drag create deep holes which may increase erosion. Shellfishing and boat anchoring disturbances affecting seagrass beds not only reduce the extension of the seagrass but also affect its spatial structure (Montefalcone et al., 2010), leading to habitat fragmentation (Boström et al., 2011). This process is associated not only with diversity loss, but also with an alteration of the ecosystem's functions, in which a series of interrelated changes affect habitat structure (changes in the number, shape, size and patch quality) as well as the associated ecological processes (Boström et al., 2011; Rielly-Carroll and Freestone, 2017).

### 1.2. Seagrass patch effects and growth after disturbances

Habitat fragmentation drives continuous landscape to broke into smaller pieces or patches, often resulting in reduced areal coverage and higher proportion of edge habitat (Sweatman et al., 2017). Several studies showed that patch growth rate and patterns differ with patch size due to allometrically scaled growth rules and clone size benefits (Duarte, 1991; Sintes et al., 2005, 2006; Marbá and Duarte, 1998). It has also been shown that mortality rates are greater in smaller patches (Duarte and Sand-Jensen, 1990a; Olesen and Sand-Jensen, 1994). Consequently, seagrass meadows have been demonstrated to display different behaviour during the colonization process in comparison with the continuous established meadow (Duarte and Sand-Jensen, 1990b; Brun et al., 2003). Physiological and morphological variations between stable and recovering meadows have also been reported (Peralta et al., 2005), although changes at the population level have not investigated.

Seagrass colonization mainly occur through the growth of patches driven by the development of existing rhizome networks as well as by the recruitment of new seedlings to the patch (Kendrick et al., 2005), whereas patch spread mainly depends on clonal growth because of the low reproductive effort of most seagrass species (Hemminga and Duarte, 2000). Three main pathways have been reported to describe seagrass meadow development from discontinuous seagrass patches after an existing patch become fragmented: a) root of displaced ramets imported via tides or currents, b) recovering from the seed bank or surviving rhizomes in recently denuded areas and c) dispersion of a single or set of seeds from the seed bank to new locations (Furman and Peterson, 2015). Seagrass patches formed *de novo* are strongly influenced by differences in clonal growth and clonal integration combined with the frequency and scale of disturbance or environmental patchiness (Diaz-Almela et al., 2008).

### 1.3. Flowering patterns, genetic diversity, and habitat fragmentation

Previous studies on the relative importance of sexual *vs* asexual mechanisms following disturbances reported contrasting results (Macreadie et at. 2014 and references therein). Some empirical results showed that seagrass fragmentation might negatively affect the reproductive output of seagrass meadows because of a reduction in pollen concentration, low reproductive effort, low germination rates (Livernois et al., 2017) and high seedling mortality (Duarte and Sand-Jensen, 1990a). Consequently, asexual recolonization through rhizome growth has been suggested as the dominant recovery mechanism. Other studies, however, stressed the importance of sexual mechanisms of recovery (Macreadie et at. 2014 and references therein). It has been shown that under severe environmental conditions, seagrasses usually tend to allocate more energy into sexual reproduction (Kim et al., 2018), what is consistent with the observation that seagrass meadows often recover at a faster rate than by vegetative propagation alone (Orth et al., 2006). These contrasting results suggest that the capacity of the meadows to recolonize the lost habitat and adapt to the expected global environmental impacts will be strongly dependent on the genetic diversity of these populations (Hughes and Stachowicz, 2004; Ehlers et al., 2008). Indeed, habitat fragmentation has been related to decreasing genetic diversity as reductions in remnant population size and increase inbreeding and bottleneck formation may erode both allelic richness and heterozygosity (Young et al., 1996). Thus, as the intensity of disturbances increase, the un-vegetated areas become more extense and frequent, thus decreasing the connectivity among patches and the stability of the meadow, potentially leading to a decrease in their evolutionary potential as a consequence of reductions in genetic diversity (Unsworth et al., 2015). Nevertheless, it has been also suggested that not all fragmentation events may lead to a reduction of the genetic variability of patches (Kendrick et al., 2017) and even moderate disturbances have been related to enhanced genetic (genotypic) diversity (Hammerli and Reusch, 2003). It has been also reported that those sites with high levels of genotypic diversity prior to the disturbance had the greatest number of new genotypes recruiting during the recovery period (Macreadie et al., 2014).

In the Galician coast, shellfishing constituted an important sector with a strong influence in coastal activities, as important natural clam harvesting areas occur all over the coast supporting employment and income for the coastal communities. However, these harvesting areas often coexist with seagrass meadows as both share habitat in shallow and shelter soft-bottom sediments. Shellfishing activity in the studied area is focused mainly on the clams *Dosinia exoleta* (Linnaeus, 1758) and *Venerupis corrugata* (Gmelin, 1791), which are harvested from afloat shellfish through manual traction gear. Clam harvesting by hand-raking has been shown to affect not only the sustainability of the target exploited species, but also the associated faunal and the vegetated community, due to substratum removal (Barañano et al., 2017). The clam-harvesting season in this area extends from October to April, while the recovery season for the exploited stocks of bivalves runs from April to September. The stock assessment for the annual delimitation of the extraction quotas does not consider the ecological viability of the seagrass meadow that coexists within the extraction zone.

The impact of these activities on seagrass meadows has been reported to reduce their distribution, fragment the habitat, and decrease the density and cover of the meadow (Boese, 2002) and reduce their carbon storage capacity (Barañano et al., 2018), but low attention has been paid to the effect on genetic diversity. Previous research carried out by our research group showed that the meadow under study is highly resilient to physical disturbances caused by clam harvesting, as reflected by the rapid response in the density and biomass of seagrass and the associated fauna in the impacted area, reaching values like those measured in areas non affected by shellfishing activities four months after the disturbance (Barañano et al., 2017). Yet, genetic characterization of the seagrass populations and assessment of the effect of patch

size on population dynamics were not assessed, although potential isolation and genetic divergence of the populations would be expected because of long-term shell fishing activity and the associated meadow fragmentation.

In this study, we hypothesized that habitat configuration across the seascape, standing stocks, flowering density, and genetic diversity of *Zostera marina* populations inhabiting fragmented seagrass meadows associated to clam harvesting would differ between a seagrass bed impacted by shellfish harvesting and two nearby populations located in continuous, established meadows non impacted by shellfishing activity.

## 2. Material and methods

### 2.1. Study area

The Ria of Vigo, located in the southwestern margin of the Galician coast (NW Spain), is characterized by the coexistence of urban uses and shellfishing activity in a highly productive wind-driven upwelling ecosystem. The study was carried out on two seagrass meadows close to the Toralla Island (Fig. 1). The Toralla meadow is distributed from the lower intertidal zone at a depth of 1.2 m (MLW) on the SW part, to the subtidal zone, reaching 5.1 m (MLW) depth in the deepest part, located at the NE part of the meadow. The Canido meadow shows a similar distribution, from the lower intertidal zone at a depth of 1 m (MLW) to the subtidal zone, reaching 4 m (MLW) depth on the deepest part.

Sampling was conducted monthly from April to August 2019 in two *Z. marina* meadows located on the southern and western area of the Toralla island, where the marine biological station of the University of Vigo (ECIMAT) is located. The Canido meadow, not impacted by shell fishing activity, is located 1.500 m southwest of the Toralla meadow, thus both sharing similar environmental conditions, such as depth, temperature, salinity, nutrients, and substrate features. In the case of the Toralla meadow, two areas were defined by the different degree of affection by the physical disturbance associated with shell fishing activity: high impact with patchy distribution and control zones, not affected by clam-harvesting activity, hereafter named as Toralla Control. The Canido meadow (control or established meadow) display a homogeneous coverage with low bare areas within the meadow, whereas the Toralla impact meadow display a considerable fragmented and discontinuous covering showing a clear matrix of seagrass mats with unvegetated areas, these being more important in the highly impacted area. Both areas share similar human-driven activities, except for clam harvesting.

The *Zostera marina* meadows are protected from S and SW winds and swell by the shape and location of the island (Toralla) and the pier of the fishing port (Canido). In addition, the orientation of the Ria of Vigo and the Cíes Islands located at its mouth provide an effective barrier against westerly winds and waves. Consequently, the area is characterized by a low hydrodynamic intensity, where tides and groundswell are the main agents.

### 2.2. Spatial distribution of seagrass meadows

The spatial distribution of the *Z. marina* meadows affected by shell fishing was determined by photointerpretation of four selected Google Earth images, corresponding to years 2007, 2013, 2017 and 2018 (Fig. 2). Seagrass distribution was verified in the field though scuba diving. The area of the control Canido seagrass meadow was also estimated. The four images corresponded to the summer period (June–August) and were selected based on their quality to allow accurate delimitation of the seagrass patches and to illustrate the amplest range of the fragmentation process.

Dark green color in the photograph was interpreted as *Z. marina* patches in contrast with turquoise color, which was identified as sea-bottom sandy sediments. Photointerpretation was restricted to the area affected by shell fishing, as previous diving operations carried out in the area confirmed the presence of rocky reefs dominated by green and brown algae adjoining to the low-impact *Z. marina* meadow. Photointerpretation of this transition zone is likely to introduce a significant methodological bias due to the difficulty to identify both communities.

Polygons were created based on the photo-interpreted images using the Google Earth "Create polygon" tool (Fig. 2) and exported to the geographic information system (GIS) in.kmz files. The ArcGis 10.3
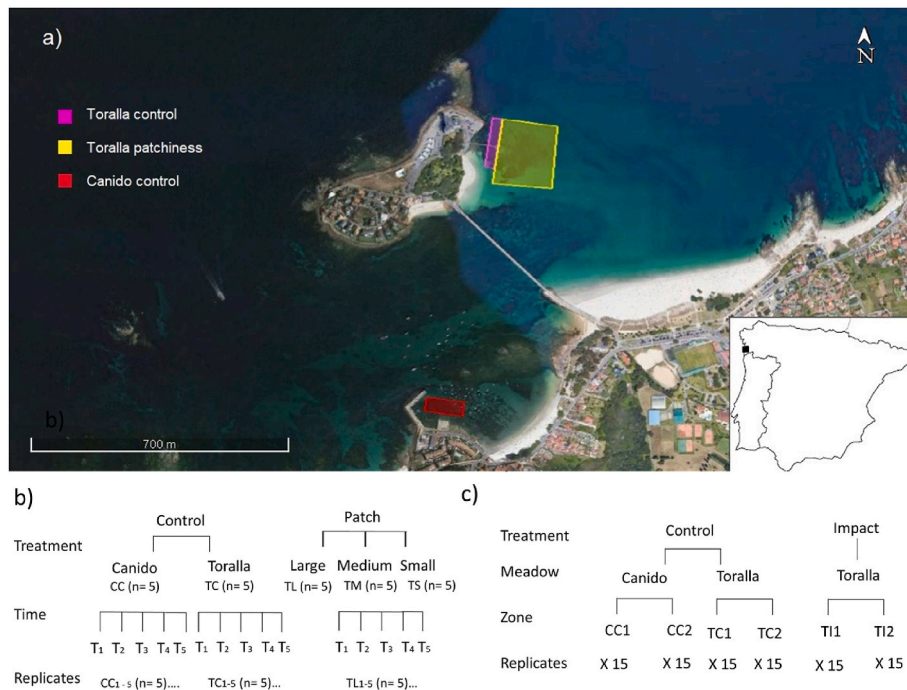


**Fig. 1.** Map of the study area (Toralla island, Vigo, Northwest Spain), with the location of the sampling areas and the experimental designs corresponding to (b) the habitat fragmentation and population dynamics study and (c) the genetic diversity study.
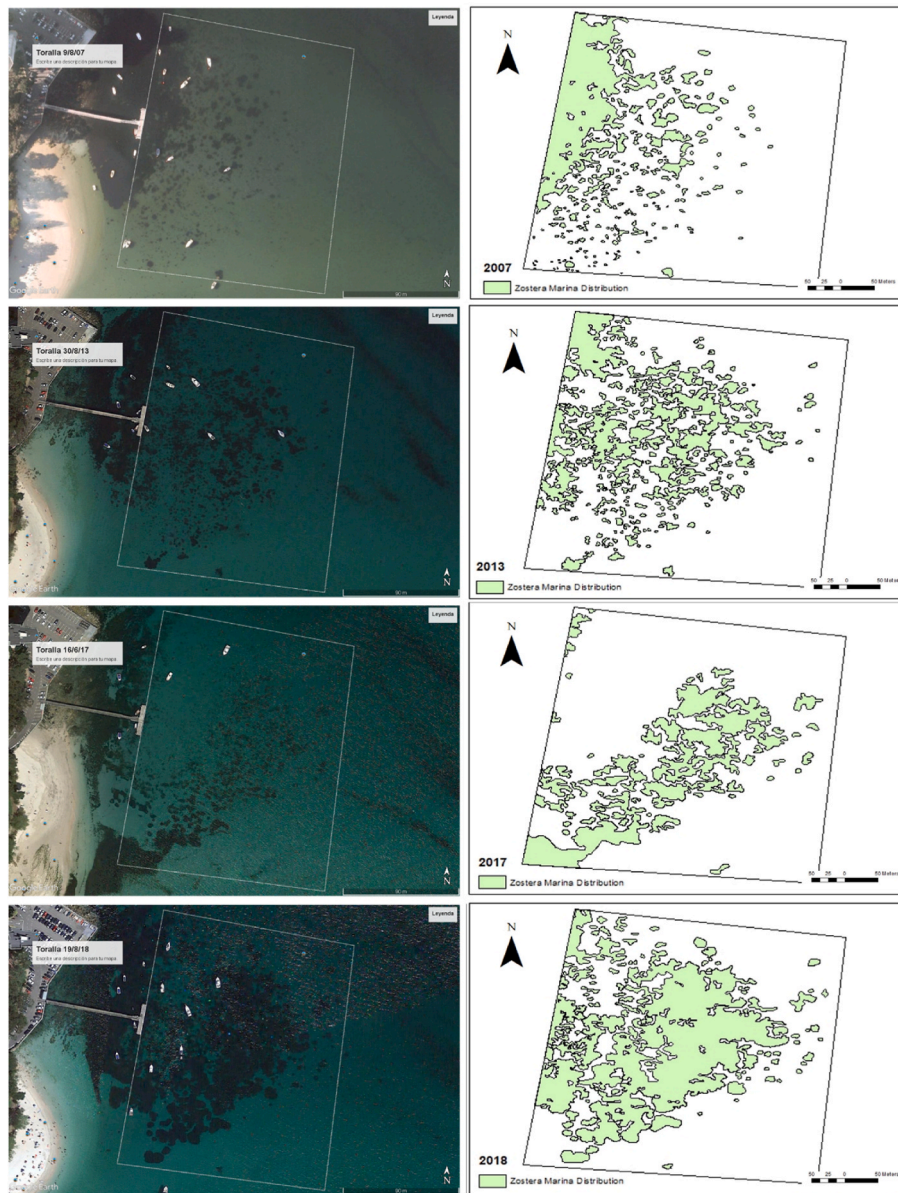
**Fig. 2.** Google Earth images selected for photointerpretation corresponding to years 2007, 2013, 2017 and 2018 (left column) and polygons of seagrass meadow and patches created based on the photo-interpreted images (right column).

software was used. The spatial distribution of *Z. marina* was obtained from.shp files using the "Calculate Geometry" and "Statics" tool. The "Feature to Point" tool was used to determine the centromeres for each polygon and the "Generate Near Table" tool to obtain the average distances between all patch centromeres.

### 2.3. Sampling design and sample collection: population dynamics

Previous investigations in the area and the information derived from satellite imagery processed in this study revealed that the seagrass bed at the impacted area typically displayed small patches ranging from very small patches, typically of 4–10 plants within less than 1 m² patch to large patches ranging from 2 m² to 7 m². Thus, three patch sizes were defined in this study; small-sized patch of less than 1 m in diameter, medium-sized patch of above 3 m in diameter and large-sized patch, greater that 5 m in diameter. The sampling strategy adopted in this investigation considered ACI (after-impact-control) design, with two factors, *treatment* control (established – unpatched meadow) and impacted (affected by clam harvesting) and *time*, as sampling was

repeated monthly from May to August, with a blocked strategy to enable estimations to be made of the effects of patch size on the population variables (Fig. 2). Thus, two control zones (Toralla and Canido) with 5 replicates per zone and three-patch sizes (small, medium, and large) with 5 replicates per size were sampled (n = 25 samples).

At each randomly defined sampling site, a 50 × 50 cm quadrat was haphazardly dropped and the number of reproductive shoots within was counted. Seagrass samples, 20 × 20 cm quadrats down to 8 cm deep, were collected to measure plant morphological characteristics (number of leaves per shoot, leaf length), shoot density, biomass (above and below ground), and reproductive phenology. Sampling was repeated monthly from April to August 2019.

The samples were washed in the field to remove sediments and epiphytes, transported to the laboratory, and further analysed. In each sample, the number of vegetative and flowering shoots was counted to estimate shoot density (vegetative and reproductive). The shoots and the rhizome-roots system were separated and dried for 48 h at 60 °C for biomass estimates.

## 2.4. Sampling design and sample collection: population genetics

To evaluate the effect of shellfish harvesting on the genetic characteristics of the *Z. marina* populations, asymmetrical ACI design was adopted. Genetic sampling was carried out on 3 and July 4, 2018. Two treatments (harvesting-impacted and control) with two control meadows (Toralla and Canido) and one impacted (Toralla). At each treatment, two zones were randomly selected to measure the genetic characteristics. At each site 15 mature shoots were randomly collected individually, separated by at least 1 m to reduce the probability to take ramets from the same genet.

Genomic DNA was extracted from homogenized samples with NZY Plant/Fungi gDNA (NZY) kit according to manufacturer's instructions. AFLP methodology represented a modified version of Vos et al. (1995). For each individual, 50 ng of DNA were digested and ligated using 5 U of *EcoR*I and 3 U of *Mse*I (New England Biolabs), 5 pmol *EcoR*I adaptor, 50 pmol *Mse*I adaptor and 0.4 U of T4 DNA ligase (Roche) in 20 μl total volume of 1X NEB buffer #2 (50 mM NaCl; 10 mM Tris-HCl; 10 mM MgCl2; 1 mM DTT; pH 7.9) and 2X ligation buffer(Roche) (660 mM Tris-HCl; 50 mM MgCl2; 50 mM DTT; 10 mM ATP; pH 7.5) supplemented with 2.5 μg of BSA for 2 h at 37 °C.

Preselective PCR reactions were then performed in 4 μl of 1:10 ligation dilution in 20 μL volumes containing 2.5 mM of $MgCl_2$, 187.5 μM of each dNTP, 20 pmol of E*coR*I-A and *Mse*I-C preselective primers and 1 U of Taq polymerase (Bioline) in 1X PCR buffer (Bioline). PCR conditions for preselective PCR were as follows: 72 °C for 2 min, 20 cycles of 94 °C for 20 s, 56 °C for 30 s, 72 °C for 2 min, and a final step of 60 °C for 30 min.

Selective PCR reactions were performed in 4 μL of 1:10 preselective PCR dilution in 20 μl volumes containing 2.5 mM of MgCl2, 187.5 μM of each dNTP, 8.3 pmol of *EcoR*I-ACT and *Mse*I-CAC selective primers and 1 U of Taq polymerase in 1X PCR buffer. Cycling conditions for selective PCR were as follows: 94 °C for 2 min, 10 cycles of 94 °C for 20 s, 66 °C for 30 s, and 72 °C for 2 min, followed by 20 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min, ending with 60 °C for 30 min.

PCR products were loaded simultaneously with a GeneScan 500 ROX size standard (Thermofisher). into an ABI Prism 310 Genetic Analyzer Fragment analysis and AFLP scoring was performed using GeneMapper v.3.7 software (Thermofisher). Loci were scored as dominant binary markers, as usually done for AFLP markers (1 and 0, for fragment presence and absence, respectively).

## 2.5. Statistical analysis

Differences in the data sets were investigated using two-way ANOVA. The Levene test was conducted to verify the assumption of equal variances and the Kolmogorov Smirnov's test to check for normal distribution. Those variables not accounting for these statistical requirements were log transformed. The non-parametric Kruskal-Wallis test was applied for those variables that did not meet homoscedastic requirements. Post hoc comparisons (Bonferroni) were performed following a significant interaction to explore the exact nature of the interaction, by testing the effect of one independent variable within one level of the second independent variable.

Relationships between variables were described using linear least squares regression analysis, and the strength of the relationships was described using Pearson correlation coefficients. Variables were log-transformed when necessary to comply with the requirements of these analyses.

Analysis of molecular variance (AMOVA) based on PhiPT (analogue of FST) value was performed using GenAlEx 6.5 (Peakall and Smouse, 2012). Expected heterozygosity ($H_E$), Shannon's information index (I) and Nei's genetic distance among populations were also calculated using GenAlEx.

The program STRUCTURE ver. 2.2 (Pritchard et al., 2000) which implements a Bayesian Markov Chain Monte Carlo method, was used to estimate the most likely number of populations (K), each of which is defined by a set of allele frequencies at each locus. The program was run for K = 2 to K = 5 using 100.000 iterations for burn-in and 200.000 MCMC replications. This was repeated three times for each K. The range of K values with the highest probabilities was identified and the program was run again for these K values using 200.000 iterations for burn-in and 400000 MCMC replications. This was repeated five times for each K and the most probable value of K was determined.

## 3. Results

### 3.1. Seascape structure

The spatial distribution of the Toralla seagrass meadow in the four years monitored in this investigation clearly shows the high degree of fragmentation and the very high interannual variability of this anthropogenically impacted ecosystem (Fig. 2). The total area occupied by the meadow varied by a two-fold factor ranging from slightly more than 5000 m$^2$ in 2007 to almost 11000 m$^2$ in 2018 (Fig. 3), with an annual average of 7227 ± 2550 m$^2$. The area of the control Canido meadow showed a much lower temporal variability, ranging from 3076 m$^2$ in 2013–3773 in 2007, with an average of 3448 ± 355 m$^2$.

The total area covered by *Z. marina* in the impacted zone was close to 15000 m$^2$ when the four photointerpreted images were jointly considered. It is worth mentioning that the area permanently covered by seagrasses in the four years analized only occupied 740 m$^2$, ca. 15% of the minimum area covered by *Z. marina* in this area, The interannual variability of the number of *Z. marina* patches in the Toralla seagrass meadow exceeded 100% in the four years studied. The spatial dispersion of patches, as shown by the mean distance between patch centroids, which depend on the total area of the meadow, and the size-structure and spatial location of patches, ranged from values close to 11–85 m.

In summer 2007, the meadow showed the smallest extension and registered 191 patches with an average area of 28 ± 259 m$^2$. The most common patch size was about 8 m$^2$ and showed the highest perimeter/area. In 2013, the seagrass coverage increased by 25% showing new patches, being the year with the highest number of patches. However, in 2017 the meadow surface was 12% lower than in 2013, showing the disappearance of most of the smaller patches (<3 m diameter), decreasing from 50 patches in 2013 to 10 patches in 2017. Accordingly, the meadow reached the lowest perimeter/area ratio (1.0 ± 0.4), indicating a more compacted structure, where the largest patches dominated the frequency distribution, being consequently their dispersion the smallest in the entire study period. However, a year later (2018), the meadow presented a new spatial configuration, doubling the area of the meadow, which was characterized not only by a remarkable higher total area but also by an increase in the number of patches and in mean patch area.

### 3.2. Population dynamics

A significant effect of the main factors, treatment and time was found on the total, above and below ground biomass, shoot density and on the above:below ground biomass ratio (ag:bg ratio; Table 1 and Supplementary Table S1). Most variables showed statistically significant differences between sampling treatments over the five months of study. All the *Z. marina* population variables measured showed higher values in the control than in the patches at the beginning of the study period (April) except the ag:bg ratio and flowering density.

### 3.3. Biomass and shoot density

A clear seasonal trend was found in *Z. marina* total and above-ground biomass in the Toralla and Canido control meadows, displaying a peak in late spring-early summer (454 ± 29 g DW m$^{-2}$ and 334 ± 22 g DW m$^{-2}$, respectively) and lower values in late summer (p > 0.05) (Fig. 4A
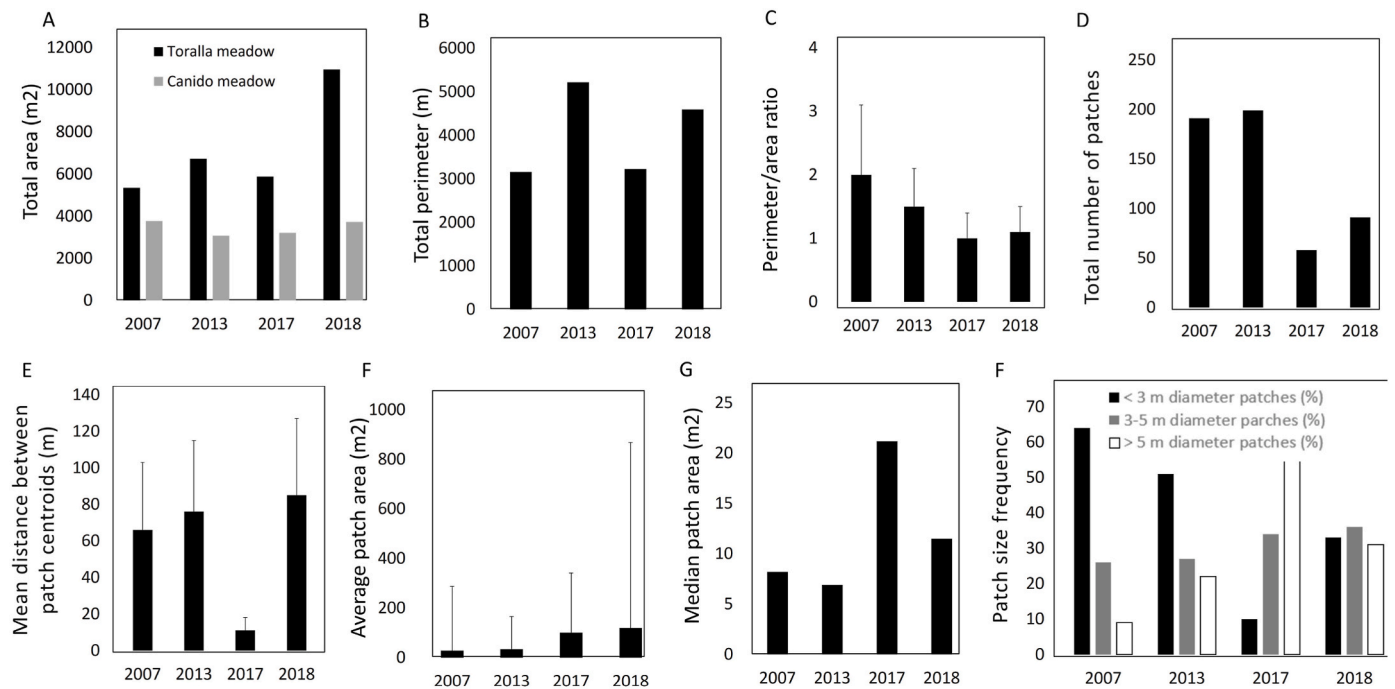
**Fig. 3.** Interannual variability of seascape indicators of the *Zostera marina* meadow (A) Total area of the meadow (B) Total perimeter (C) Perimeter to area ratio (D) Total number of patches (E) Mean distance among patch centroids, (F) Average patch area, (G) Median patch area and (F) Patch size frequency of the three main patch sizes established for the population dynamics study.4.

**Table 1**

Summary of ANOVA for *Zostera marina* variables with F-statistics for total biomass, above-ground biomass, below-ground biomass, Ag:Bg ratio, total shoot density, Flowering density and leaf length. The model include two factors, (1) treatment, with two levels, impacted and non-impacted and (1) time, with four levels (may, june july and august). Degrees of freedom: time (4), treatment (4), time*treatment (16), total (124).

| Variables | Treatment | Time | Treatment x Time |
|---|---|---|---|
| Shoot density | 30.19*** | 38.44*** | 2.24** |
| Total biomass | 95.35*** | 70.65*** | 3.74*** |
| Aboveground biomass | 34.84*** | 74.80*** | 2.85** |
| Belowground biomass | 129.22*** | 28.58*** | 2.62** |
| Ag:Bg ratio | 28.84*** | 10.7*** | 3.76*** |
| Leaf length | 73.38*** | 27.33*** | 5.37*** |
| Flowering density | 1.22 | 1.73 | 12.18*** |

*P < 0.05, **P < 0.01, ***P < 0.001.

and B). Total biomasses in the control meadows were significantly higher than in the patches (p < 0.01), except for the large-size patch (p > 0.05). The medium-size patches (204 ± 17 g DW m$^{-2}$) registered slightly lower total biomass values than the large-size patches (252 ± 14 g DW m$^{-2}$; p > 0.05), but significantly lower with respect to both the Toralla and the Canido control sites (340 ± 19 g DW m$^{-2}$ and 269 ± 14 g DW m$^{-2}$ respectively), and significantly higher than the small-size patches (136 ± 9 g DW m$^{-2}$) which presented the lowest total values (p < 0.05).

The highest total biomass values were measured in Canido throughout the study. At the beginning of the study period, Canido presented similar although slightly higher values (20% higher) than the Toralla control and the large-size patches, while the total biomass of the medium and small size patches was 40% and 60% lower. At the end of the period, the differences between the Canido and Toralla control zones were less than 5%. In turn, seagrass biomass in the large and medium-patch size reached values only slightly lower (9–15%) than in the Canido meadow. However, the smaller patch, maintained biomass values 50% lower than Canido and Toralla control. Likewise, the differences

between the large and medium-size patches compared to the small patch increased at the end of the study period, being the differences between the medium and small patches especially marked; total biomass in the medium patch was 26% higher than in the small patch in April and 50% in August.

The aboveground biomass showed a similar pattern in the controls and in the large-size patch, although no significant differences were found between the Toralla and the large-sized patch (p > 0.05 (Fig. 4B). The Canido Control site presented the highest aboveground biomass throughout the study period (151 ± 18 g DW m$^{-2}$; p < 0.05). Differences between patches were only significant between the smallest patches (80 ± 7 g DW m$^{-2}$) and the medium and large patch sizes (114 ± 10 g DW m$^{-2}$; p < 0.001), being similar between the medium and large patches (137 ± 9 g DW m$^{-2}$; p = 0.06).

The belowground biomass was similar in the two control sites (p = 0.320), and significantly higher than in the patches (Fig. 4C). It was almost two-fold higher in the Canido control (189 ± 10 g DW m$^{-2}$) than in the large and medium-sized patches (115 ± 6 and 91 ± 8 g DW m$^{-2}$, respectively), and three-fold higher than the small-sized patch (56 ± 3 g DW m$^{-2}$). Thus, significant differences were found for the large size (p = 0.04), medium size (p < 0.001) and small size patches (p < 0.001). Statistically significant differences were not found between the large and medium patch size (p = 0.126) but were detected between the small and medium patch (p = 0.014) and the large patch (p < 0.001).

A distinct trend was observed in the aboveground biomass/belowground biomass ratio (Ag:Bg) between the control zones and the patches (Fig. 4-D). Canido and Toralla showed a similar evolution of the ratio throughout the period, with values slightly lower or close to 1, indicating an equal distribution of biomass between the epigeal and hypogeal compartments. On the contrary, this ratio largely exceeded 1 in the patches, which reflects a higher proportion of foliar tissue with respect to the hypogeal biomass. Likewise, the medium and small patches registered a significant peak in July and August, respectively, reaching values close to 2.5, indicating that the fraction of foliar tissue almost doubled that of belowground tissue.

Shoot density showed different patterns in Canido control, Toralla
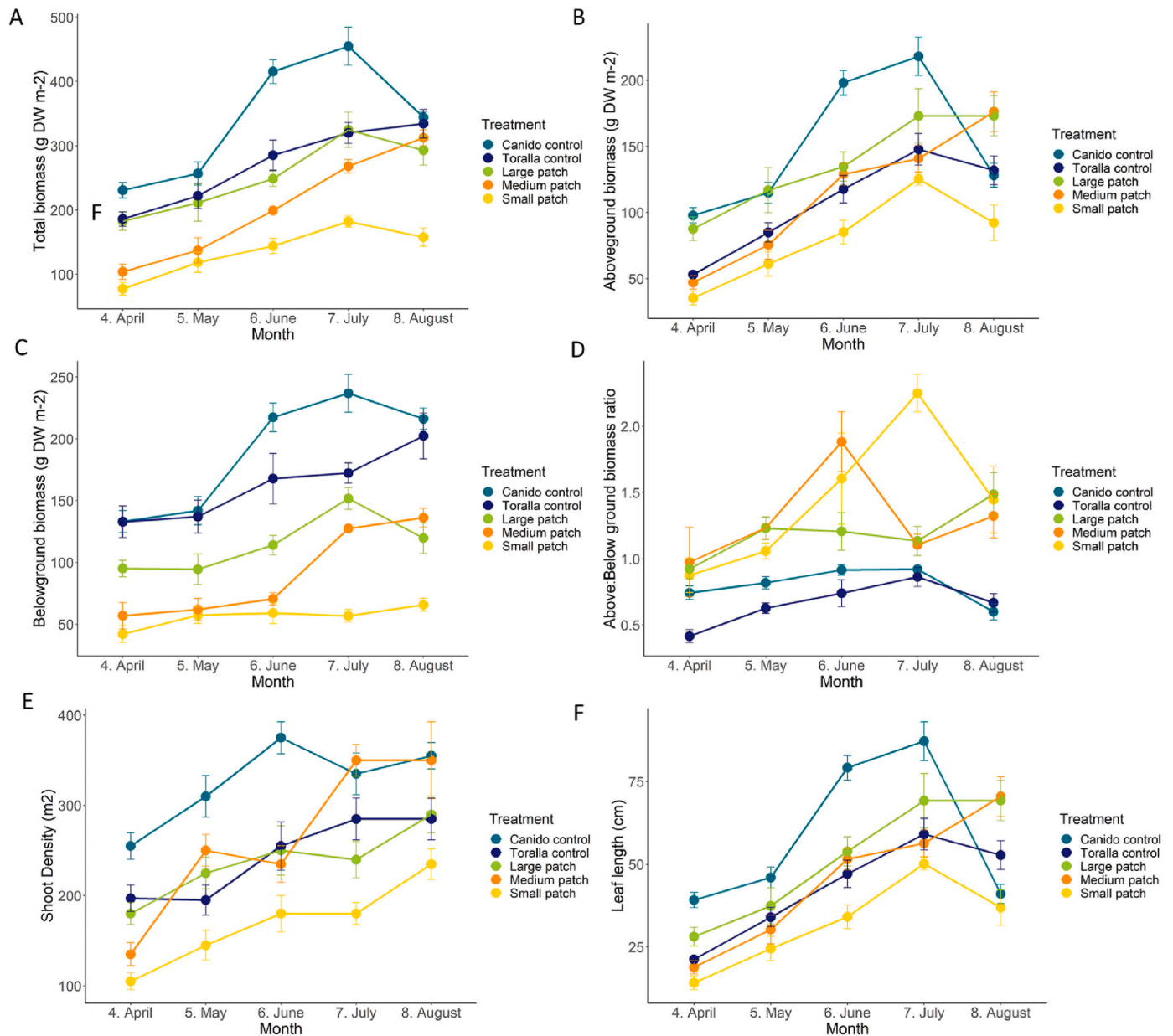
Fig. 4. Temporal variation of A) Total biomass, B) Aboveground biomass, C) Belowground biomass, D) Above:belowground biomass E) Mean *Zostera marina* shoot density and F) leaf length in the control meadows and at each patch size of *Zostera marina*. Error bars represent standard errors.

control and the three patch treatments (Fig. 4-E). The Canido meadow registered the highest values during the entire study period. However, it showed the lowest net increase during the growing season (25%), reaching a maximum in June with $375 \pm 23$ shoots m$^{-2}$. The Toralla control, showed a similar net increase (30%), reaching the maximum density a month later, in July, with $285 \pm 21$ shoots m$^{-2}$. On the contrary, shoot densities in the large, medium, and small patch-sizes registered higher net biomass increases, reaching values 38%, 60% and 55% higher by the end of the sampling period, respectively.

The highest shoot density among patches was recorded in the medium-sized patch by the end of the sampling period, matching the density of the Canido control meadow in July, with a peak of $350 \pm 42$ shoots m$^{-2}$, exceeding by 20% the density of the Toralla control treatment. The small-sized patches registered the lowest density values throughout the study period ($169 \pm 14$ shoots m$^{-2}$), significantly lower than in the medium and large-sized patches and in the controls.

Significant differences in shoot density were recorded between the

Canido control and the rest of the treatments ($p < 0.05$). On the contrary, no significant differences between the Toralla control treatment and medium and large sized-patches were noted. A different pattern was also documented in the small sized-patches, displaying significantly lower values ($p > 0.05$), both with respect to the medium and large sized-patches, as well as with the Canido and Toralla controls (Supplementary Table S1).

The Canido control showed higher leaf length values than the rest of the treatments during the entire study period, except during late summer when a significant drop of leaf length was recorded in August, reaching similar values to those of April ($41 \pm 4$ shoots m$^{-2}$) coinciding with the senescence of the plant (Fig. 4F). In turn, Toralla control and the medium and large size patches showed similar leaf lengths and temporal patterns, whereas the small-sized patch maintained significantly lower values throughout the study period; both with respect to the controls ($p < 0.05$) and to the medium and large-sized patches ($p < 0.05$).
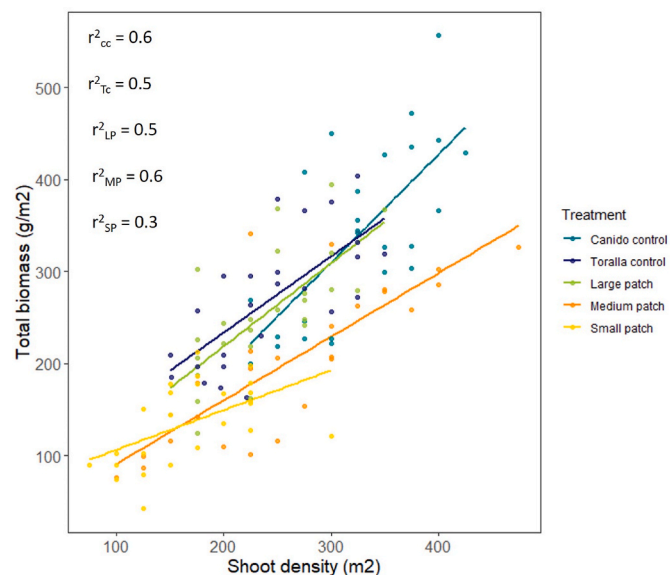
In all cases, a significant increase in mean leaf length was observed between April and July, when the highest foliar length was recorded both in Canido (87 ± 6 cm) and in Toralla control (59 ± 5 cm), the large-sized (69 ± 7 cm), medium-sized (56 ± 7 cm) and small-sized patches (50 ± 4 cm).

The biomass of *Z. marina* in the different treatments increased linearly as a function of shoot density (Fig. 5). The biomass–density relationship described a positive and significant trend for both the control and the three patch sizes but showed different slopes and correlation coefficients.
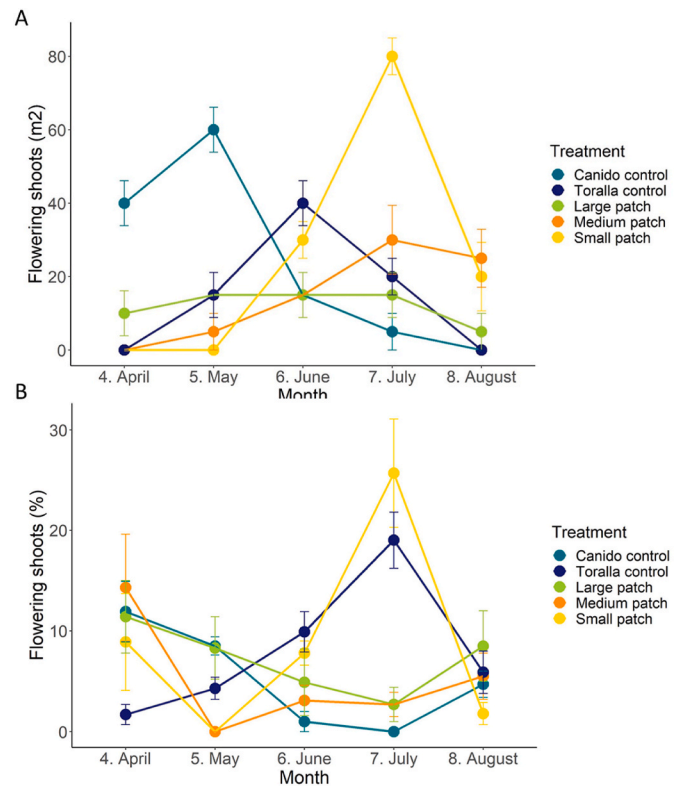
The slopes of the biomass–density relationships were higher in Canido, Toralla Control and the medium and large patches, whereas the lowest slope was recorded in the small patches. A similar pattern was shown for the strength of the correlation, where the average correlation coefficient was higher in Canido control, Toralla control and the large and medium patches ($r^2 = 0.6$, $p < 0.01$; $r^2 = 0.5$, $p < 0.01$; $r^2 = 0.6$), while the relationship between total biomass and shoot density in the small patch-sizes, although still significantly correlated, showed the highest dispersion ($r^2 = 0.3$; $p < 0.05$).

### 3.4. Reproductive effort and genetic diversity

The overall mean flowering shoot density almost doubled in Canido control (24 ± 5 flowering shoots m$^{-2}$) and the small sized patches (26 ± 6 flowering shoots m$^{-2}$) with respect to Toralla control (15 ± 4 flowering shoots m$^{-2}$) and the large and medium sized patches (16 ± 3 and 12 ± 3 flowering shoots m$^{-2}$), respectively (Fig. 6A). However, when the overall proportion of flowering shoots for each population was considered, the small sized patches reached the highest proportion (9% ± 2) along with Toralla control (8% ± 1), while Canido control and the medium and large patches presented overall flowering densities close to 5% in the study period. The timing of the flowering events also differed among treatments. The flowering peak in Canido control and the medium and large patches were recorded in late spring when 11% ± 4, 11% ± 3 and 14% ± 5 of the population displayed flowering shoots, respectively. By contrast, the small patches and Toralla control treatments reached the highest flowering densities showing maximum values

**Fig. 6.** Temporal variation of A) flowering shoots and B) mean flowering shoots (%) for each treatment. Error bars represent standard errors.

two months later, during mid-summer, with a 25% ± 5 and 19% ± 3 of sexual reproductive shoots.

To assess whether these differences in flowering patterns reflect differences in genetic patterns, Molecular Analysis of Variance (AMOVA) was performed on AFLP-derived data (Table 2). This analysis shows that the genetic variation of the Canido, Toralla Control and Toralla impacted populations are statistically different (P (perm) = 0.001), where 89% of the observed variability (P < 0.001) may be attributed to within-population variability while the remaining 11% was explained by among-populations variability.

Accordingly, the highest number of polymorphic loci (96.31%) was found in Canido, also showing the highest values of the Shannon Diversity Index (0.484) and of expected heterozygosity (0.35) (Table 3).

To assess if the dispersion of the samples within the groups drives the significance of the AMOVA test, a dispersion homogeneity test was performed using the PRIMER routine, PERMDISP. The analysis also showed that the dispersion of the three groups was statistically different (F = 87,152, P (perm) = 0.001). Statistically significant differences in the dispersion of groups were found between Canido and Toralla populations, although no differences in the level of dispersion between Toralla populations (control vs impacted) were found (F = 0.678, P (perm) = 0.44). However, the similarity test ANOSIM, pointed out to significant differences between the two Toralla populations (p = 0.001,



**Fig. 5.** Relationship between total biomass (gDW m$^{-2}$) and shoot density (m$^2$) for each treatment. On the left the Pearson correlation coefficients are shown. The linear regressions corresponding to each treatment are: $Y_{(CC)} = 42 + 1.2X$ ($p < 0.01$); $Y_{(TC)} = 67 + 0.8X$ ($p < 0.01$); $Y_{(LP)} = 38 + 0.9X$ ($p < 0.01$); $Y_{(MP)} = 22 + 0.7X$ ($p < 0.01$); $Y_{(SP)} = 63 + 0.4X$ ($p < 0.05$). CC: Canido control; TC: Toralla control; LP: Large patch; MP: Medium patch; SP: Small patch.

**Table 2**
Summary of AMOVA for exploring the genetic difference within/among sites.

| Source | df | SS | MS | Est. Var. | % | Φ ST | P |
|---|---|---|---|---|---|---|---|
| **Among Pops** | 2 | 522.978 | 261.489 | 4.681 | 11% | 0.112 | 0.001 |
| **Within Pops** | 153 | 5660.490 | 36.997 | 36.997 | 89% | | |
| **Total** | 155 | 6183.468 | | 41.678 | 100% | | |

**Table 3**
Genetic diversity and similarity estimates for the three studied meadows. P, proportion of polymorphic loci Shannon's information index; $H_e$, Expected Heterozygosity; I, Shannon's information index.

| Meadow | P | $H_e$ | I |
|---|---|---|---|
| Canido | 96.31% | 0.35 | 0.479 |
| Toralla Control | 83.91% | 0.27 | 0.41 |
| Toralla Impacted | 86.48% | 0.275 | 0.415 |

R = 0.487).

The Bayesian analysis of population structure (Fig. 7) indicated that the three sampled populations, Canido control, Toralla control and Toralla impacted, represented three main genetic clusters (K = 3). Fig. 7 represents the probability of assignment of every single plant to each cluster, indicating an unequivocal K value of 3 and, therefore, a clear genetic separation of the three populations.

## 4. Discussion

High levels of seagrass fragmentation have been frequently documented in coastal systems influenced by intense human activities (Montefalcone et al., 2010; Tamburello et al., 2012). However, studies on seagrass meadows subjected to perturbations by shell fishing activities mapping losses and gains in seagrass patch distribution have not been addressed so far.

The seagrass ecosystem under study is under continuous transformation, seasonally exposed to bottom raking associated with clam harvesting. The results obtained in this study pointed out to the great interannual spatial variability of the meadow, where only 15% of the seagrass matrix remained vegetated in the four years monitored in this investigation. A continuous colonization process characterized the seagrass seascape, where the area occupied by the meadow varied up to a two-fold factor in some periods.

We are confident that this variability is largely attributable to the clam harvesting activity occurring in the Toralla meadow and not to interannual changes in the natural conditions. Unfortunately, the area under study is one of the 12 "free" shellfishing zone where all shellfishers in the Ria of Vigo are authorized to extract molluscs under a joint exploitation plan, thus preventing to know exactly what fraction of the allocated quota is extracted from each one of the authorized areas.

Nevertheless, a series of observations support our statement. First, the analyses of the seasonal series of temperature, salinity and dissolved oxygen obtained from a submerged CTD permanently deployed in the meadow since August 2013 show a relatively low interannual variability of these environmental variables. Summer seawater temperature in 2017 and 2018, when the seagrass cover increased by almost 100%, differed in less than 1 °C.

In addition, light might also limit seagrass growth. However, the average irradiance levels measured in a nearby location during the study period was 685.2 μmol m$^{-2}$ s$^{-1}$ ± 70.6 (data provided by INTECMAR). These values largely exceeded the photosynthesis irradiance saturation values ($I_k$) for *Z. marina* reported in the literature ranges from 130 to 450 μmol m$^{-2}$ s$^{-1}$ (Lee et al., 2007). This suggests that *Z. marina* growth rates were not light-limited in the study area, and consequently, the observed interannual variability in the *Z. marina* spatial coverage should not be related to differences in light regime.

Other possible explanation may be related to changes in current or wave intensity which might negatively impact on seagrass meadows, and have been recognized to determine their spatial distribution, i.e. upper and lower limits or the minimum patch size of a canopy (Infantes et al., 2009; Barcelona et al., 2021). Although *in situ* measured wave height or current speed data are not available in the study area, we are confident that the observed interannual changes in *Z. marina* cover identified in our study are not expected to be related to parallel changes in these hydrodynamic variables due to the high protection provided by the Toralla Island on the seagrass meadow. The sampling area, located in the leeward eastern side of the island, is highly protected from the characteristic western storms in the ria de Vigo, showing wave heights generally lower than 0.5 m and extremely low surface current velocity (generally <0.05 m s$^{-1}$).

We also assessed the variability of seagrass cover in the nearby Canido meadow in the same four satellite images processed for the Toralla meadow. If natural variability was the main driver of the observed interannual variability measured in seagrass cover, we would expect a similar variability in seagrass cover and a temporal covariation of the area occupied by *Z. marina* in the two sampled meadows. Neither of these premises were observed as shown in Fig. 3A. When the variation in *Z. marina* cover between 2017 and 2018 was considered, a 16% increase was estimated for Canido but it increased by 86% in Toralla. Moreover, when the difference in seagrass cover between 2007 and 2013 was assessed, an 18% decrease was estimated for Canido and a
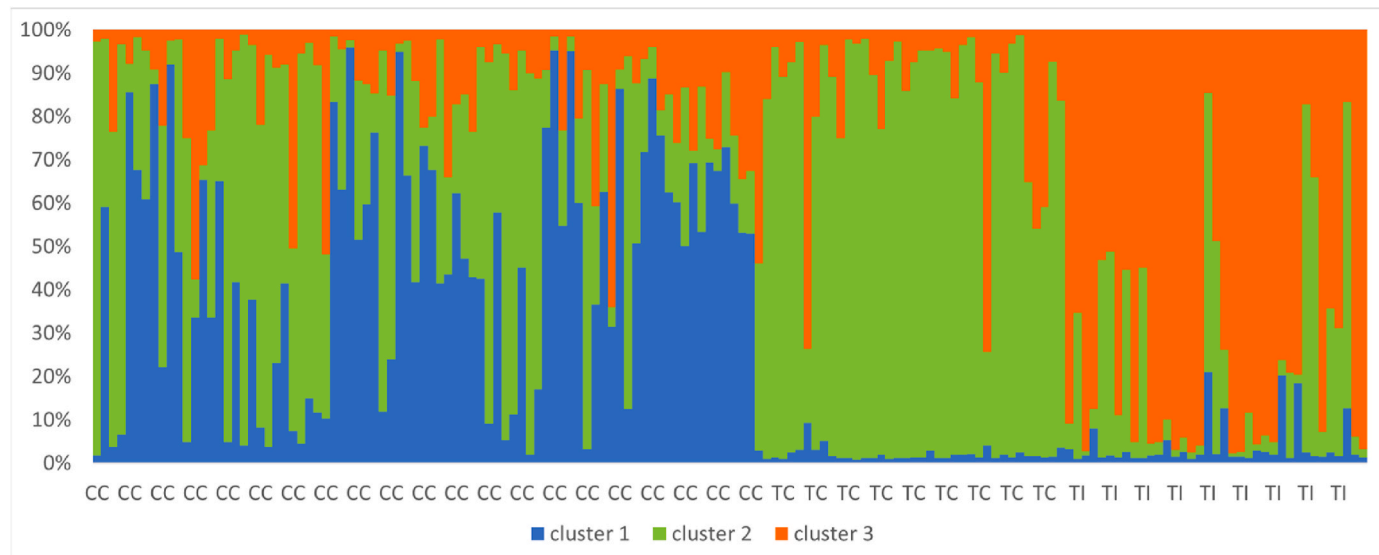


**Fig. 7.** Structure of seagrass populations based on AFLP data revealed by Bayesian analysis implemented in Structure. Each column shows a vertical bar broken into different colored genetic clusters, representing the probability of assignment of every single plant to each cluster. CC, TC and TI stands for Canido control, Toralla control and Toralla impacted.

26% increase for Toralla. In addition, we gathered information on the shellfishing activity in the area from the technicians of the Marine Biological Station of the University of Vigo, located at the Toralla Island, who reported a progressive loss of interest of the area to shell-fishers due to decreasing clam productivity, being especially notable in 2018.

The large differences observed in the total area and in the number of patches derive into two-fold changes in the perimeter versus area ratios, an indicator of the border effect associated with the fragmentation process. The decline in the values of this indicator as the patches become larger may increase the flux of resources from shoots in the inner part of the patches to the rapidly growing rhizomes (Vidondo et al., 1997). The increase in the area occupied by the meadow may be associated with a process of integration of the central area of the meadow, previously fragmented into small patches, into a wide surface of uniform coverage. Nevertheless, the meadow may also expand by colonizing new areas towards the edges, tripling the number of small patches that begin to settle in adjacent areas. This significant increase in area and coverage is also aligned with the above-mentioned decrease in the intensity of shellfish extraction activity observed during the 2017–2019 period.

The adaptability and capacity of the meadow to rapidly restore lost areas is consistent with previous findings, which evidenced the fast-track rate of patch growth with patch size in natural seagrass populations (Vidondo et al., 1997; Marbá and Duarte, 1998; Sintes et al., 2005; Kendrick et al., 2018). The sampled area is seasonally exposed to bottom tracking with a 6-month temporal window of clam extraction closure coinciding with the life-spam of the species. During this period, the surviving vegetated areas regain their original density and coverage (Barañano et al., 2017). *Cymodocea nodosa* In our study, a dynamic equilibrium between patch development and spatial fragmentation, which depended on the intensity and amplitude of disturbance, was evidenced. The capacity of seagrass meadows to expand by clonal growth combined with sexual reproduction and dispersal by seed production and seedling recruitment is a key factor driving seagrass seascape development (Boström et al., 2006). However, variation in size, shape and distribution of patches is mainly constrained by disturbance factors.

Clearly differentiated patterns were observed in *Z. marina* shoot density, biomass, and flowering density between shellfishing-induced patches of different sizes and the long-term non-impacted areas. During the 5 months of the study, the medium and large patches reached total biomass values similar to those of the non-impacted areas, while the small patch did not recover shoot densities and biomass levels of the unaffected areas, thus increasing the differences with the rest of the patches. This growth pattern has been shown to be characteristic of rapid patch formation and mortality in other shallow, highly fragmented seagrasses (Duarte and Sand-Jensen, 1990b; Olesen and Sand-Jensen, 1994; Vidondo et al., 1997). Low disturbance, both in frequency or amplitude, can open temporal windows that allow the development of a continuous seagrass meadow. By contrast, a more intense affection or shorter time intervals between disturbances than those required for patch recovery, may led to a final stage where bare areas prevail (Marba and Duarte, 1995). These studies support the hypothesis of a minimum patch size above which the likelihood of patch mortality decreases (Duarte and Sand-Jensen, 1990a; Olesen and Sand- Jensen, 1994), since growth of large patches are benefited from improved anchoring and mutually sheltering structure (Vidondo et al., 1997).

The different patch dynamics shown in this investigation are consistent with the notion of patch growth and development as a self-accelerating process described in seagrass population models (e.g. Vidondo et al., 1997; Kendrick et al., 2018). *Z. marina.*

The different growth pattern of the different patch sizes studied here is consistent with the observation that patches of different ages showed a 30-fold growth rate variation, despite a similar magnitude of the underlying rhizome (Sintes et al., 2005). The simultaneous density and biomass maximum observed at the end of the growing season in the fragmented meadow, is characteristic of developing meadows where no space limitations constrain the internal packing of shoots during growth, as opposed to the control meadows where density peaked before biomass, facilitating the regulation of ramet formation to prevent the overproduction of shoots.

The different rates of biomass development in the control and patch treatments, particularly the higher rates of the aboveground fraction and the lower investment in belowground tissue, may imply different ecological roles, since faster aboveground biomass turnover and lower belowground structure suggest a more limited capacity to accumulate biomass and, particularly, to retain carbon and nutrients (Evrard et al., 2005).

In this study, no significant overall differences in reproductive effort were found between non-impacted, continuous, meadows and the 3 sampled patch levels. However, a higher contribution from sexual reproduction was reported in the small-patch size. A clear seasonal differentiation was observed in the flowering pattern, where the smaller patches registered the highest density of flowering plants, which occurred two months later than in the rest of treatments. Enhanced reproductive effort following moderate natural and human-induced disturbances has been previously reported in other areas (Marba and Duarte, 1995; Cabaço et al., 2012). In particular, the effect of shell-fishing derived physical disturbance on sexual reproduction was investigated in Ría Formosa (Portugal), revealing that clam harvesting significantly decreased the density of *Zostera noltei*, leading to increasing meadow fragmentation and reproductive effort and the extent of the fertile season (Alexandre et al., 2005), results similar to those reported in our investigation. Flowering may increase the genetic diversity of meadows if successful seed production, survival and development occur (Coyer et al., 2004; Ehlers et al., 2008). Nevertheless, genetic diversity is also influenced by factors such as effective population size, spatial pattern of dispersal and recruitment success of immigrant propagules (dependent on competition and local adaptation) and the biogeographical history of populations (Olsen et al., 2004; Becheler et al., 2010).

A significant pattern of genetic differentiation was observed between impacted and control *Z. marina* populations, associated with a reduction in genetic variability in the impacted meadow, possibly related to reduced meadow size, increased genetic drift and a different predominance of asexual reproduction.

Despite the dispersal potential of *Z. marina* populations is restricted to hundreds of meters, the process of genetic differentiation in vegetative-growth plant species is related to genetic patchiness, a paradox that was previously described as the "chaotic genetic patchiness", broadly reported for marine invertebrates, and recently found in seagrasses (Arnaud-Haond et al., 2007; Becheler et al., 2014). Kim et al. (2018) described a similar pattern of genetic differentiation among *Z. marina* populations possibly related to reduced meadow size, increased genetic drift and a high incidence of asexual reproduction. This genetic differentiation among seagrass populations was also reported in San Francisco Bay (Orth et al., 2000), underlining the importance of clonality in favoring fine-grained genetic structure and spatial patchiness in organisms with mixed mating systems.

A higher *Z. marina* genetic variability might be expected associated with the higher density of reproductive plants found in the impacted patch areas, provided they end up in viable seeds. In addition, the reduction of competition for space due to the lower shoot density of adults may improve settlement of seeds and enhance survival of seedlings (Reusch, 2006; Greve et al., 2005; Kim et al., 2019). However, our results are not consistent with this hypothesis.

Most of the studies carried out to date on seagrass responses to disturbances, did not jointly address temporal and spatial variability of *Z. marina* populations and its genetic characterization. Furman et al. (2015) reported the first attempt to accomplish high resolution spatial data over multiyear periods combined with traditional genotypic surveys, showing that pollination distances ranged from 0.57 m to 73.91 m, while seed dispersal varied from 1.85 m to 5.31 m for naked seeds,

**Table S1**

Post hoc – pairwise test comparisons among treatment level interactions

| INTERACTIONS | Total biomass | Aboveground biomass | Belowground biomass | Ag:Bg ratio | Leaf length | Shoot density |
|---|---|---|---|---|---|---|
| CC - TC | 26.58 | 25.86* | 10.21 | 13.2 | 20.78 | 35.58* |
| CC - LP | 20.2* | 5.22 | 39.48*** | 42.14*** | 8.02 | 38.74*** |
| CC - MP | 45.2*** | 20.61* | 55.16*** | 44.64*** | 16.16 | 28.58*** |
| CC - SM | 73.42*** | 47.32*** | 80.36*** | 46.64*** | 42.64*** | 69.41*** |
| TC - LP | 6.38 | 20.64 | 29.27** | 55.32*** | 12.76 | 3.16 |
| TC - MP | 25.00* | 5.26 | 44.96*** | 57.84*** | 4.66 | 7.11 |
| TC - SP | 53.22*** | 21.46* | 29.28** | 59.84*** | 21.65* | 33.88*** |
| LP - MP | 18.62 | 15.38 | 15.68 | 2.52 | 8.14 | 10.16 |
| LP - SP | 46.8*** | 42.11*** | 40.88** | 4.52 | 34.62*** | 30.66*** |
| MP - SP | 28.22* | 26.7* | 25.20* | 2.01 | 26.48** | 40.82** |

supporting the hypothesis of limited exchange between the studied populations. Despite the broadly reported positive effect of physical disturbances on reproductive effort, Larkin et al. (2010), did not find a significant difference in genetic variability between control populations and those impacted by this type of disturbances, most likely due to the lack of effect on population size (removal of ramets vs genets). In that context, a well-established trend is that large populations with positive demographic balance sustain higher genetic diversity than small declining populations (Spielman et al., 2004).

Seagrass habitat fragmentation was reported to diminish by 22% the seed set in *Z. marina* fragmented patches vs continuous meadows, which was related to reduced pollen concentrations and lower opportunity for outcrossing (Reusch, 2003). Indeed, studies assessing the effects of habitat fragmentation on *Z. marina* seed distribution, showed that despite flowering effort was not affected, seed density was significantly reduced in fragmented versus continuous beds (Livernois et al., 2017), suggesting that flowering shoots in isolated patches within fragmented beds could be experiencing pollen limitation, thus reducing seed production per shoot. In this connection, Van Tussenbroek et al. (2016) identified an Allee effect driven by pollen limitation in a fragmented seagrass meadow, reporting 35% lower fruit set in fragmented populations at similar reproductive density than a continuous population.

Three possible, non-exclusive, hypotheses may explain the unexpected genetic differentiation between the two Toralla populations and their lower genetic variability as compared with the Canido population: the small size of the populations, the constrain of pollen limitation associated to fragmented meadows and the possible low recruitment success of the dispersed propagules. The intense sediment physical disturbance exerted by the shell fishing activity in the area, could be possibly isolating the populations thus limiting gene flow by diminishing drifting immigrant propagules and reducing recolonization via seeds from sexual reproduction due to the physical stress, fragmentation of the meadow and vulnerability of seedlings and the seed bank (Unsworth et al., 2015). This suggests that clonal propagation is likely to be the dominant mechanism for the maintenance and expansion of the population where population dispersal accounts at the scale of up to several tens of meters, as previously reported for *Z. marina*.

In summary, this investigation illustrates the spatial-temporal dynamics of a seagrass meadow affected by clam harvesting, demonstrating the high variability in both their distribution and population patterns at very short spatial scales. Our results showed different population dynamics of the patches composing the seagrass seascape as a consequence of clam harvesting disturbance. We concluded that the characteristics of the studied meadow are the result of a recurrent physical disturbance which maintain the meadow in a continuous process of colonization. The genetic structure of the seagrass meadow was probably influenced by seedling recruitment and clonal growth, where pollen and seed dispersal, and especially seedling survival, appear to be the key drivers behind the genetic differentiation of the *Z. marina* populations inhabiting shellfishing impacted areas.

## CRediT authorship contribution statement

**Carlota Barañano:** Conceptualization, Writing – original draft. **Emilio Fernández:** Writing – review & editing, Validation, Supervision, Investigation. **Paloma Morán:** Investigation, Software, Validation, Writing – review & editing. **Pablo Urbieta:** Software, Investigation. **Gonzalo Méndez:** Supervision, Investigation, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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