

Pollen limitation may be a common Allee effect in marine hydrophilous plants: implications for decline and recovery in seagrasses

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Abstract Pollen limitation may be an important factor in accelerated decline of sparse or fragmented populations. Little is known whether hydrophilous plants (pollen transport by water) suffer from an Allee effect due to pollen limitation or not. Hydrophilous pollination is a typical trait of marine angiosperms or seagrasses. Although seagrass flowers usually have high pollen production, floral densities are highly variable. We evaluated pollen limitation for intertidal populations of the seagrass *Zostera noltei* in The Netherlands and found a significant positive relation

between flowering spathe density and fruit-set, which was suboptimal at <1200 flowering spathes m⁻² (corresponding to <600 reproductive shoots m⁻²). A fragmented population had ≈35 % lower fruit-set at similar reproductive density than a continuous population. 75 % of all European populations studied over a large latitudinal gradient had flowering spathe densities below that required for optimal fruit-set, particularly in Southern countries. Literature review of the reproductive output of hydrophilous pollinated plants revealed that seed- or fruit-set of marine hydrophilous plants is generally low, as compared to hydrophilous

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freshwater and wind-pollinated plants. We conclude that pollen limitation as found in *Z. noltei* may be a common Allee effect for seagrasses, potentially accelerating decline and impairing recovery even after environmental conditions have improved substantially.

Keywords Abiotic pollination · Density dependence · Habitat fragmentation · Seed production · *Zostera noltei*

Introduction

Allee effects, or positive density dependence of the fitness of individuals in a population, may accelerate decline of sparse or fragmented populations and may impair recovery of disturbed populations (e.g., Scheffer et al. 2001). Allee effects due to pollen limitation are frequently reported in animal-pollinated land plants. In contrast, surprisingly little is known about water plants. This lack of knowledge becomes even more remarkable if one takes into account that the communities of marine submerged plants (seagrasses) belong to the most valuable ecosystems of our planet (Duarte et al. 2008). Valuable seagrass populations are rapidly decreasing worldwide (Orth et al. 2006; Waycott et al. 2009), and restoration of declining populations is difficult (Valdemarsen et al. 2011; Van Katwijk et al. 2016). Seagrass populations under threat often have decreased densities or may become fragmented or patchy (Bell et al. 1999; Apostolaki et al. 2009). Reduced seed- or fruit-set due to pollen limitation is a demographic mechanism of an Allee effect, causing reproductive impairment (Aguilar et al. 2006). Recolonization of gaps and recovery of such seagrass populations can be partly or fully dependent on recruitment from seed (Ouborg et al. 1999). Allee effects from pollen limitation may thus impair recovery of (meta-) populations as well as recovery from localized damage such as formation of gaps. Hence there is need for more knowledge on Allee effects due to pollen limitation in aquatic species like seagrasses.

Hydrophily is an abiotic pollination mechanism where pollen is transported by water, and differs from pollen transport by biotic vectors such as insects, bats or birds (zoophilous pollination). Abiotic pollination in terrestrial systems occurs by wind (anemophilous) or rarely by rain. Anemophilous plants are thought to suffer less from pollen limitation (sensu Ashman et al. 2004; Knight et al. 2005) than zoophilous ones, because they produce copious amounts of pollen and do not depend on potentially fluctuating populations of pollinators for successful seed-set (e.g., Friedman and Barrett 2009), though pollen limitation may occur in marginal conditions (Davis et al. 2004; Hesse and Pannell 2011). Similar to anemophilous flowers, hydrophilous flowers produce a lot of pollen, resulting in

high pollen:ovule ratios (e.g., $10^4:1$ for *Zostera*: Ackerman 2006). But tremendous amounts of pollen can be lost due to high pollen dispersion in the water, and there is little information whether pollen transport limits seed-set of submersed species. Reduced fruit- or seed-set may be due to failure in pollination due to rapid dilution of pollen and unpredictable hydrodynamic forces (Verduin et al. 2002; Ackerman 2006; van Tussenbroek et al. 2009). Anecdotal evidence suggests that, for plants with hydrophilous pollination, deviations from optimal conditions may result in pollen limitation, as is generally the case for zoophilous land plants (Burd 1994; Ashman et al. 2004). Specifically, pollen limitation in seagrasses has been established for female-dominated populations of the dioecious *Phyllospadix* spp (Shelton 2008; Buckel et al. 2012), populations with limited flowering shoots of monoecious *Zostera marina* (Reusch 2003), and under reduced abundance of male flowers of dioecious *Thalassia testudinum* (Van Tussenbroek et al. 2009). However, it has as yet to be established whether pollen limitation under water is a more common phenomena and, if so, this would have important implications for management of seagrass populations.

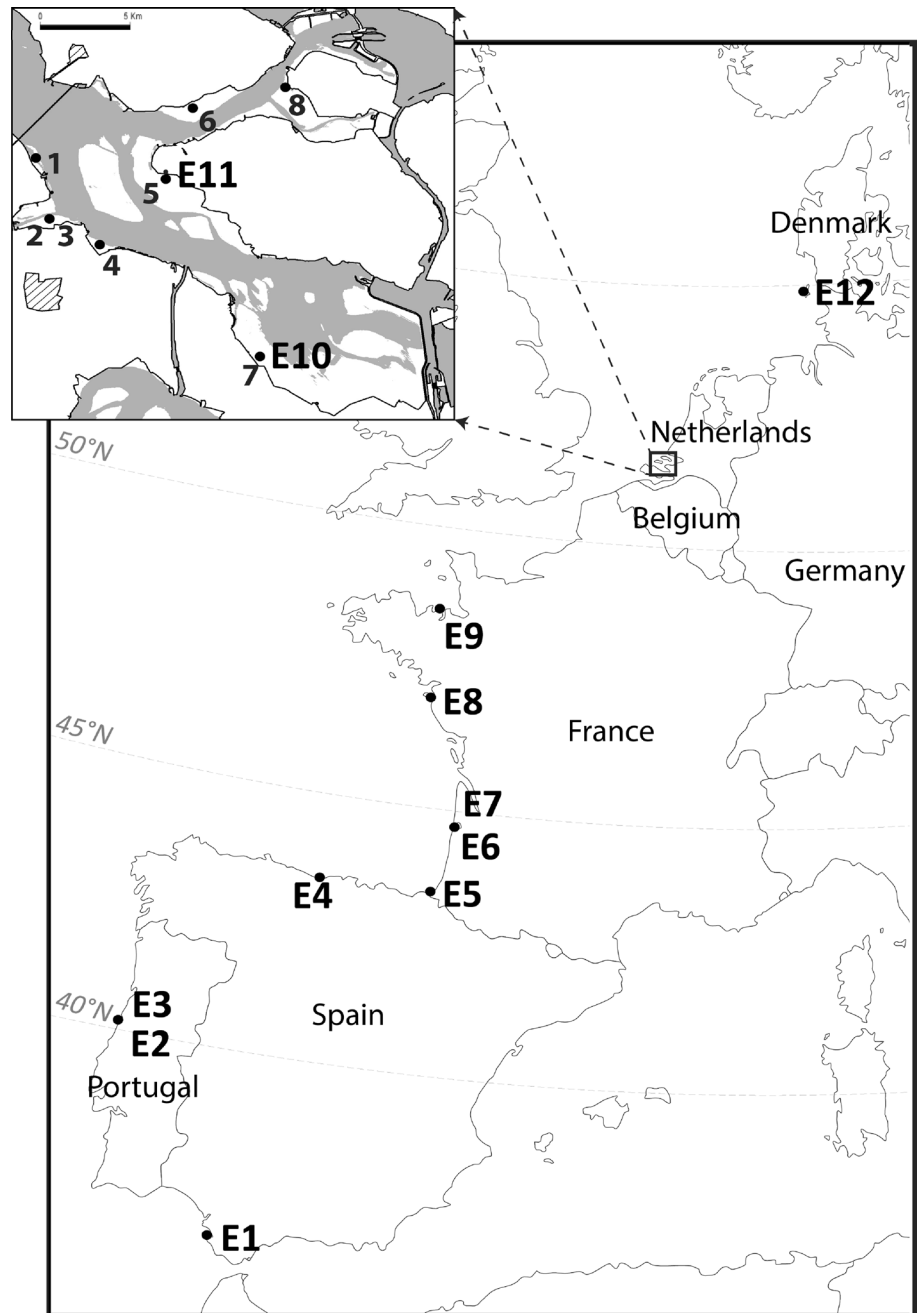
We evaluate, firstly, whether the successful pollination of hydrophilous plants, with the marine angiosperm *Zostera noltei* as a model species, is dependent on the density of reproductive structures (Allee effect). We determine the critical reproductive density for optimal pollination success and whether habitat fragmentation may exacerbate reproductive failure. Secondly, the reproductive density of *Z. noltei* was determined across Europe, to evaluate whether this density is above the critical level for optimal pollination success. We discuss possible consequences of pollen limitation for the preservation of the European *Z. noltei* populations. Lastly, we reviewed the literature to verify whether low seed- or fruit-set is generally common in hydrophilous plants, and may thus contribute to the accelerated worldwide decline of seagrasses.

Materials and methods

Model species: *Zostera noltei*

Zostera noltei typically grows on tidal mudflats or in the shallow subtidal, forming large continuous meadows or growing in patches throughout the European and Northern-African Atlantic coastline and the Baltic, Mediterranean, Black, Caspian and Azov Seas. In its southern distribution area, this species is leaf-bearing throughout the year (Buia and Mazzella 1991; Peralta et al. 2000, 2005; Cabaço et al. 2009, 2012), but in north Atlantic Europe (including the study area Oosterschelde) the plants overwinter as small rhizome fragments with few or no leaves (Vermaat and

Fig. 1 Map of Europe with detailed map of the Oosterschelde indicating sampling sites. See Table 1 for coding of European sites. The *numbers* in the inserted maps of the Oosterschelde indicate the following sampling sites: (1) Slikken van Kats, (2) Zandkreek-area A, (3) Zandkreek-area B, (4) Goesse Sas (continuous meadow), (5) Dortsman, (6) Viane West, (7) Oostdijk, (8) Krabbenkreek



Verhagen 1996). In spring, seeds or overwintering rhizome sections initiate patches through vigorous vegetative expansion, which may eventually form large continuous meadows under favorable conditions. Spathes bearing 6–10 male and 2–5 female flowers are clearly visible later in the growing season (June–September/October: Auby and Labourg 1996; Vermaat and Verhagen 1996; Brun et al. 2003; Zipperle et al. 2009a), with one reproductive shoot producing up to 8 spathes (usually less) per reproductive season. This species is protogynous, and filamentous pollen is taken to the female flowers of neighboring spathes through the water column. Median pollen dispersal distance in the

Waddenzee is 1.8–3.2 m (Zipperle et al. 2011). The fruit is a nutlet with a membranous transparent testa containing one seed. The small seeds (1–2 mm long) are negatively buoyant and form a seed bank, for periods <1 to >3 years (Hootsmans et al. 1987; Zipperle et al. 2009b).

Study areas

Density-dependent reproductive success was determined for intertidal *Z. noltei* populations in the Oosterschelde (the Netherlands) at 8 locations (Fig. 1). The Oosterschelde is a former arm of the river Scheldt delta that became a

Table 1 European sampling: latitude, sampling date (2014), and shoot density for all sites

| Station | Site | Latitude | Sampling time | | | Shoot density (shoots m ⁻²) | | |
|---------|------------------------------|----------|---------------|---------|---------|---|--------------|--------------|
| | | | Start | Peak | End | Start | Peak | End |
| E1 | Spain, Cadiz Bay | N 36°30' | 02-Jul | 09-Sept | 06-Nov | 15643 ± 1525 | 12561 ± 1350 | 5172 ± 732 |
| E2 | Portugal, Mondego Estuary 1 | N 40°8' | 28-May | 13-Aug | 10-Nov | 6548 ± 1243 | 6573 ± 1243 | 7108 ± 458 |
| E3 | Portugal, Mondego Bay 2 | N 40°8' | 28-May | 13-Aug | 10-Nov | 6675 ± 559 | 6420 ± 1357 | 12739 ± 1406 |
| E4 | Spain, Santander | N 43°25' | 13-Jun | 11-Aug | 08-Oct | 2013 ± 277 | 1580 ± 590 | 2191 ± 338 |
| E5 | France, Bidasoa Estuary | N 43°21' | 29-May | 7-Aug | 11-Oct | 2573 ± 283 | 5580 ± 870 | 4892 ± 578 |
| E6 | France, Arcachon-Germanan | N 44°42' | 27-May | 13-Aug | 27-Oct | 6599 ± 1443 | 9146 ± 1061 | 10904 ± 1273 |
| E7 | France, Arcachon-Hautebelle | N 44°43' | 27-May | 13-Aug | 27-Oct | 10293 ± 1246 | 9045 ± 2661 | 9197 ± 588 |
| E8 | France, Noirmoutier | N 46°98' | 27-Jun | 25-Jul | 11-Sept | 5783 ± 1577 | 5478 ± 1554 | 6548 ± 1167 |
| E9 | France, St-Jacut-de-la-Mer | N 48°36' | 25-Jun | 29-Jul | 20-Aug | 3363 ± 1120 | 2752 ± 398 | 2038 ± 593 |
| E10 | Netherlands, Oosterschelde 1 | N 51°26' | 11-Jun | 19-Aug | 26-Sept | 6318 ± 498 | 10318 ± 1052 | 7389 ± 1495 |
| E11 | Netherlands, Oosterschelde 2 | N 51°34' | 12-Jun | 20-Aug | 25-Sept | 3745 ± 640 | 6115 ± 772 | 5427 ± 971 |
| E12 | Germany, Sylt | N 54°54' | na | 30-Jul | 12-Sept | na | 6064 ± 668 | 9809 ± 838 |

na not analyzed

semi-enclosed sea-arm, following large-scale civil engineering work (for more information see Suykerbuyk et al. 2012). These works, consisting of dam constructions and the creation of storm-surge barrier at the mouth of the estuary, reduced the inflow of freshwater and nutrients, thus increasing the salinity of its eastern compartments (Wetsteyn and Kromkamp 1994). The Oosterschelde is a relatively sheltered system with little wind fetch and small waves. The tidal range varies between 2.4 and 3.5 m; maximum current velocities range from around 0.3 m s⁻¹ in the shallow areas to 1–1.5 m s⁻¹ in the tidal channels, and waves are generated within the system by wind (Louters et al. 1998). The air exposure time of the studied meadows ranges between 50 and 70 % of the tidal cycle (see Suykerbuyk et al. 2015 for further information on the 8 study sites). Oostdijk presents at large continuous well-established bed at an average elevation of 0.4 m above sea level, with an average cover of 70–80 % (maximum cover 100 %). The seagrass meadow in Goesse Sas presents an average elevation of 0.35–0.40 m above sea level (Amsterdam Ordnance Datum: NAP) with total surface area of 1.5 ha with an average seagrass cover of 15–20 % with maximum cover of 70 % in the continuous meadow, but this site also presents fragmented meadows.

Spathe density of *Z. noltei* was also determined for 12 seagrass populations across Europe on intertidal flats along the Western-European coast from Southern Spain to Northern Germany (Fig. 1; Table 1).

Density-dependent reproductive success

Density-dependent variability in reproductive success among populations was determined for 8 sites representing

intertidal *Z. noltei* meadows across the Oosterschelde; visited from 28 August until 1 September 2014 (Fig. 1). Between 27 and 56 reproductive shoots were collected haphazardly at each site, and their density was estimated by counting them in 10 haphazardly chosen areas of 10 × 10 cm. The spathes were classified according to reproductive phases as follows: (1) buds (with male and female flower buds), (2) male-anthesis (male flowers opening-up, anthers protruding from the spathe-sheath), (3) female anthesis (female flowers opening-up, fresh stigmas protruding from spathe-sheath), (4) female-post-anthesis (female flowers with oxidized stigmas but without indications of ovule development), (5) fruit-bearing (fruits with unripe or ripe seeds), (6) aborted (spathes with only aborted fruits, often in deteriorated condition). Spathe-set was determined as the proportion of fruit-bearing spathes (phase 5) of the total number of fruit-bearing and aborted spathes (phases 5 and 6).

Density-dependent variability in reproductive success within seagrass populations was subsequently (10-Sep-2014) determined along a tidal gradient in the continuous well-established bed at Oostdijk as follows: (1) channel: minor tributary tidal channels, ≈0.5 m lower than the typical continuous meadow sections, with permanently submersed *Z. noltei*, (2) continuous: typical section of the large well-established continuous meadow at on average 0.38 m above sea level, (3) elevated: elevated section, ≈0.2 m higher than the typical continuous meadow, intersected by minor tidal channels. In addition, we sampled a fourth area that was a fragmented (patchy) meadow section at Goesse Sas, with patch sizes of ≈25 m², at tidal level similar to that of the typical continuous population at Oostdijk. The seagrasses were sampled with a corer

(10 cm diameter). On each site, 5 samples were collected within a more or less homogenous area of $\approx 25 \text{ m}^2$, except in the channels that were usually not wider than 1 m. The samples were collected approximately 2 m apart, as this distance corresponds with the median pollen dispersal distance for this species reported by Zipperle et al. (2011). The samples were placed in a sieve, washed separating the seagrass plants from mud and other materials and placed in plastic bags for transport to the laboratory. In the laboratory, the plants were cleaned and tissues were separated in above-ground (leaves, sheaths and spathes) and below-ground (rhizomes and roots) tissues. For each reproductive shoot, the spathes were classified according to the six reproductive phases as described above, with an additional phase of decayed spathes, which consisted of remnants of the peduncles; occasionally with decomposed scales. All spathes were dissected under a binocular microscope, and the number of developed and aborted fruits was counted to determine fruit-set. Reproductive success was determined as spathe-set (see above) or fruit-set (the proportion of seed-bearing fruits of all fruits either seed-bearing or aborted). Density-dependent successful reproduction was expressed as: (1) spathe-set vs density of reproductive shoots and (2) fruit-set vs density of flowering spathes. The density of flowering spathes (at the time of pollination) was not determined directly, and was considered to be equivalent to the density of the potential seed-bearing spathes at the time of sampling, because these spathes most likely underwent female anthesis at approximately the same time. We assessed annual seed production from the product of total number of spathes of all phases and the seed-bearing fruits per spathe. This assessment assumed that seed production per spathe did not vary throughout the reproductive season, and no more spathes would be formed after our sampling. This is reasonable, because very few reproductive shoots would be formed after our sampling, as the reproductive season finishes in September/October (also see Results: "Spathe density across Europe").

Spathe density across Europe

Zostera noltei plants were sampled with cores of 10 cm diameter ($n = 5$) at the 12 locations at the beginning, the peak and the end of the seagrass growing season (total $n = 15$ per site). The choice of the sampling time was site specific and tuned to the growing season length for each site (Table 1). Following collection, the seagrass samples were washed on site with freshwater and stored in wet tissues for preservation during transportation to the Royal Netherlands Institute for Sea Research (NIOZ) in Yerseke, Netherlands. The number of foliar shoots and spathes was counted to determine, respectively, shoot and spathe

density (no. m^{-2}). Only fully grown spathes with flowers or fruits (phases 2–5) were considered.

Statistical analysis

Potential differences in biomass and density of reproductive shoots among meadow sections were analyzed with ANOVA and post hoc Tukey test. We applied a linear regression to below-ground biomass and number of fruits of the pooled data of the four sampling areas, to verify whether the production of seed-bearing fruits is dependent on internal reserves (which are stored in the rhizomes). Generalized Additive Models (GAM) with a logit link function (for binary data) and polynomial smoothing of x-variable (x being the number of reproductive shoots) were applied to test for significant trends of spathe-set and density of the reproductive shoots for all pooled data. Subsequently, a separate GAM analysis was applied to the data of the three meadow sections along a tidal gradient at Oostdijk (channel, continuous and elevated) with meadow section as a cofactor, to test whether density-dependent probabilities of spathe fertilization differed with the position along the tidal level. A separate GAM analysis was applied to test for differences in density-dependent spathe-set in the continuous population at Oostdijk and the fragmented population at Goesse Sas. These GAM-analyses were also realized for fruit-set vs the density of flowering spathes.

A two-way ANOVA, followed by a post hoc Tukey test, was realized to test for differences in spathe density during the different sampling times and between the different European sampling sites. All statistical analyses were realized with R 2.15.3 (R Core Team 2013).

Literature search

We searched the literature for manuscripts on the reproductive output and success of all plant species known to have hydrophilous pollination and recorded data on fruit- and seed-set.

Results

Density-dependent pollination success in *Zostera noltei*

At the Oosterschelde in between the end of August—beginning of September, *Z. noltei* exhibited spathes at every reproductive phase (Fig. 2a). The number of spathes varied considerably among the 8 sampling sites, and we found a clear positive relation between the densities of reproductive shoots and spathe-set (Fig. 2b).

Zostera noltei had fewer reproductive shoots and spathes in the minor tidal channels than in the typical continuous

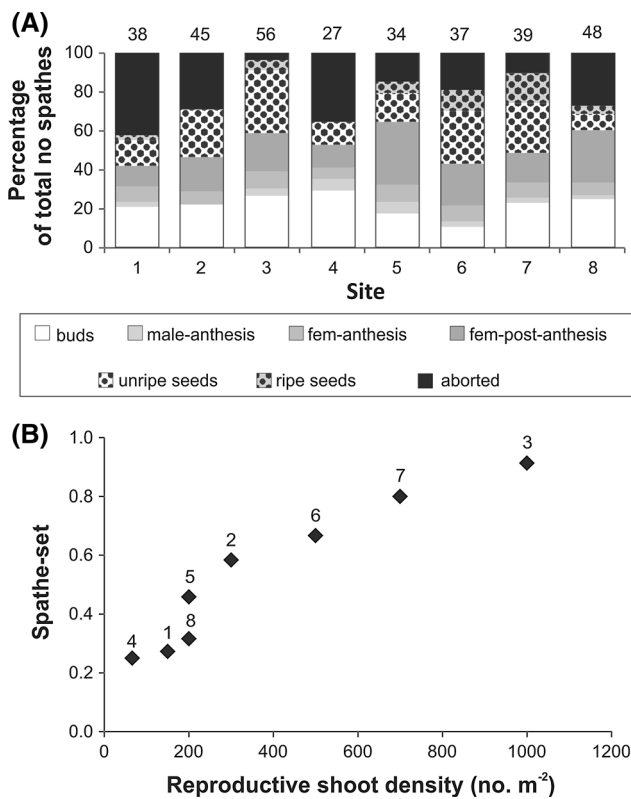


Fig. 2 Reproduction of intertidal *Zostera noltei* throughout the Oosterschelde. Samples were collected in between 28-Aug and 01-Sep-2014. **a** Phases of the spathes per site. The numbers above the bars indicate total number of collected spathes. **b** Spathe-set (proportion of the number of fruit-bearing spathes of the number of flowering spathes) vs. density of reproductive shoots (median density measured in 10 quadrats of 10 × 10 cm). The numbers above the data points indicate the sampling sites. See Fig. 1 for site location map

and elevated meadow sections at Oostdijk, despite the high leaf biomass exhibited (Tables 2, 3, 4; Fig. 3). The fragmented population at Goesse Sas had similar density of reproductive shoots and spathes as those at the typical and elevated meadow sections of the continuous meadow at Oostdijk (Fig. 3). The production of fruits (of the pooled samples) showed no relationship with below-ground biomass ($F = 3.439$, $p = 0.688$, $df = 58$, $R^2 = 0.040$; Fig. 4a).

The GAM of the pooled data showed a significant relationship between spathe-set and density of the reproductive shoots ($X^2 = 43.66$, $p < 0.0001$, $R^2 = 0.657$, $n = 60$).

Table 2 Average (\pm SE) spathe density (no. m⁻²) of *Zostera noltei* in the Oosterschelde on 10-Sep-2014

| Meadow | Place | <i>N</i> | Bud | Male-anthesis | Female-anthesis | Female-post-anthesis | Seed-bearing | Aborted | Decayed | Total |
|------------|-----------|----------|-----------|---------------|-----------------|----------------------|--------------|----------|------------|------------|
| Channel | Oostdijk | 10 | 127 ± 76 | 25 ± 17 | 0 | 51 ± 27 | 51 ± 28 | 89 ± 34 | 38 ± 19 | 381 ± 178 |
| Continuous | Oostdijk | 20 | 407 ± 103 | 45 ± 17 | 45 ± 17 | 324 ± 93 | 859 ± 219 | 134 ± 30 | 1120 ± 295 | 2934 ± 646 |
| Elevated | Oostdijk | 10 | 293 ± 71 | 89 ± 33 | 64 ± 34 | 242 ± 58 | 458 ± 109 | 165 ± 33 | 904 ± 292 | 2215 ± 460 |
| Fragmented | Goese Sas | 20 | 274 ± 47 | 38 ± 23 | 51 ± 24 | 267 ± 41 | 866 ± 77 | 261 ± 20 | 567 ± 206 | 2324 ± 325 |

Table 3 Results of the One-Way ANOVA ($df = 3$) for differences in dry weight and reproductive shoot density of *Zostera noltei* among meadows in the Oosterschelde

| Parameter | <i>F</i> | <i>p</i> |
|-------------------------------|----------|----------|
| Total dry weight | 5.612 | 0.0020 |
| Above-ground/total dry weight | 21.47 | <0.0001 |
| No. reproductive shoots | 2.854 | 0.0452 |
| Spathe-set | 3.185 | 0.0324 |

Results of the post hoc Tukey test are depicted in Fig. 3

The density-dependent spathe-set was similar across all tidal levels (channel, typical continuous and elevated) in the continuous population at Oostdijk (channel vs typical continuous: $z = 1.482$, $p = 0.138$, channel vs elevated: $z = 0.440$, $p = 0.660$, $n = 40$; Fig. 4b). The comparison between typical continuous and fragmented populations showed that spathe-set was significantly lower at similar reproductive shoot density when the population was fragmented ($z = 5.960$, $p < 0.0001$, $n = 40$; Fig. 4b). Similar results were found for fruit-set related to flowering spathe density: the probability of fruit-set for all pooled data increased significantly with increasing density of flowering spathes ($X^2 = 80.79$, $p < 0.0001$, $R^2 = 0.587$, $n = 60$), and fruit-set was significantly lower in the fragmented than in the typical continuous population ($z = 5.311$, $p < 0.0001$, $n = 40$; Fig. 4c). Spathe- or fruit-set approached saturation at 5 reproductive shoots (Fig. 4b) or 10 flowering spathes (Fig. 4c) per sample, corresponding with, respectively, ≈ 600 reproductive shoots m⁻² or ≈ 1200 flowering spathes m⁻². The estimated annual seed production in the channel was low (≈ 200 seed m⁻²), medium in the fragmented population and elevated meadow sections (respectively, ≈ 3100 and 3500 seeds m⁻²), and high in the typical continuous meadow (≈ 5600 seeds m⁻²; Table 4).

Spathe density across Europe

The density of spathes of *Z. noltei* at intertidal flats along the European Atlantic coast showed a significant temporal variation ($F = 21.771$, $df = 11$, $p < 0.001$), exhibiting a clear peak in the growing season (Fig. 5). Spathe densities also varied significantly among sites ($F = 8.227$, $df = 11$,

Table 4 Summary (average ± SE) of *Zostera noltei* populations in the Oosterschelde sampled on 10th September 2014

| Meadow | <i>n</i> | Total vegetative biomass (g dry m ⁻²) | Leaf biomass (g dry m ⁻²) | Reproductive biomass (g dry m ⁻²) | Foliar shoot density (no. m ⁻²) | Reproductive shoot density (no. m ⁻²) | Total Spathe density (no. m ⁻²) | Estimated annual seed production (no. m ⁻²) | Rounded-up values of seed production (no. m ⁻²) |
|------------|----------|---|---------------------------------------|---|---|---|---|---|---|
| Channel | 10 | 106.7 ± 12.9 | 43.2 ± 5.57 | 1.7 ± 1.23 | 1194 ± 1294 | 115 ± 55 | 381 ± 178 | 167 ± 92 | 200 |
| Continuous | 20 | 103.9 ± 13.0 | 32.6 ± 4.6 | 4.6 ± 1.3 | 6958 ± 894 | 687 ± 148 | 2934 ± 714 | 5571 ± 1549 | 5600 |
| Elevated | 10 | 47.1 ± 3.5 | 12.9 ± 1.1 | 2.7 ± 0.5 | 3973 ± 558 | 586 ± 116 | 2215 ± 460 | 3529 ± 943 | 3500 |
| Fragmented | 20 | 75.6 ± 2.4 | 22.4 ± 0.8 | 4.8 ± 0.5 | 4927 ± 395 | 662 ± 82 | 2324 ± 325 | 3064 ± 667 | 3100 |

Total spathe density: density of spathes of all phases (including decayed ones)
n number of samples

$p < 0.001$), and showed a remarkable trend of higher spathe densities at more northern sites, dividing the latitudinal range in two homogeneous subsets (Tukey: $p < 0.001$; Fig. 5); being: Southern locations (from E1 to E8) and Northern locations (from E10 to E12). The highest spathe density was found in the Oosterschelde (Fig. 1, site E11) at 3133 ± 565 spathes m⁻², which was 6–120 times higher than at southern sites.

Seed/fruit-set in hydrophilous pollinated plants

Extensive literature search into the reproductive output of all hydrophilous pollinated plants reveals that the seed- or fruit-set (expressed as Seed:Ovule and Fruit:Flower ratio, respectively) of marine hydrophilous plants (seagrasses) is generally low in comparison with abiotic pollinated land plants with wind as the vector of pollen transport (Table 5). The number of ovules per female flower is low (1–9, but often 1 or 2) similar to anemophilous plants (Friedman and Barrett 2009). The density of the male shoots and ovules varies considerably among and within the species.

Discussion

Pollen limitation may be an important factor in accelerating the decline of sparse or fragmented vegetation, and may impair recovery. We showed pollen limitation in the hydrophilous pollinated seagrass *Z. noltei* at <600 reproductive shoots m⁻², a density that is only reached in northern 25 % of the meadows studied across a large latitudinal gradient during the peak season. In addition, fragmentation contributed to a reduced fruit-set in one of the populations. Literature study revealed that pollen limitation might be a common phenomenon in seagrass species.

Suboptimal reproductive output in *Zostera noltei*

We found that spathe- or fruit-set is generally reduced in *Z. noltei*, and that this decreased reproductive output is related to the density of reproductive shoots or spathes. Reduced seed-set can be due to (1) resource limitation, (2) overproduction of ovules, (3) reduced pollen quality or (4) insufficient pollen production (Ashman et al. 2004; Knight et al. 2005). In our case, we discarded resource limitation, because we did not find a clear relationship between below-ground biomass and the total number of produced seed-bearing spathes or seeds, and rhizomes are the principal storage organs (Vermaat and Verhagen 1996).

An excess of ovule production (bet-hedging) may be a response to stochastic pollination (Burd 1994; Holland and Chamberlain 2007), and water is not always a reliable vehicle for pollen transport as strong hydrodynamics may result

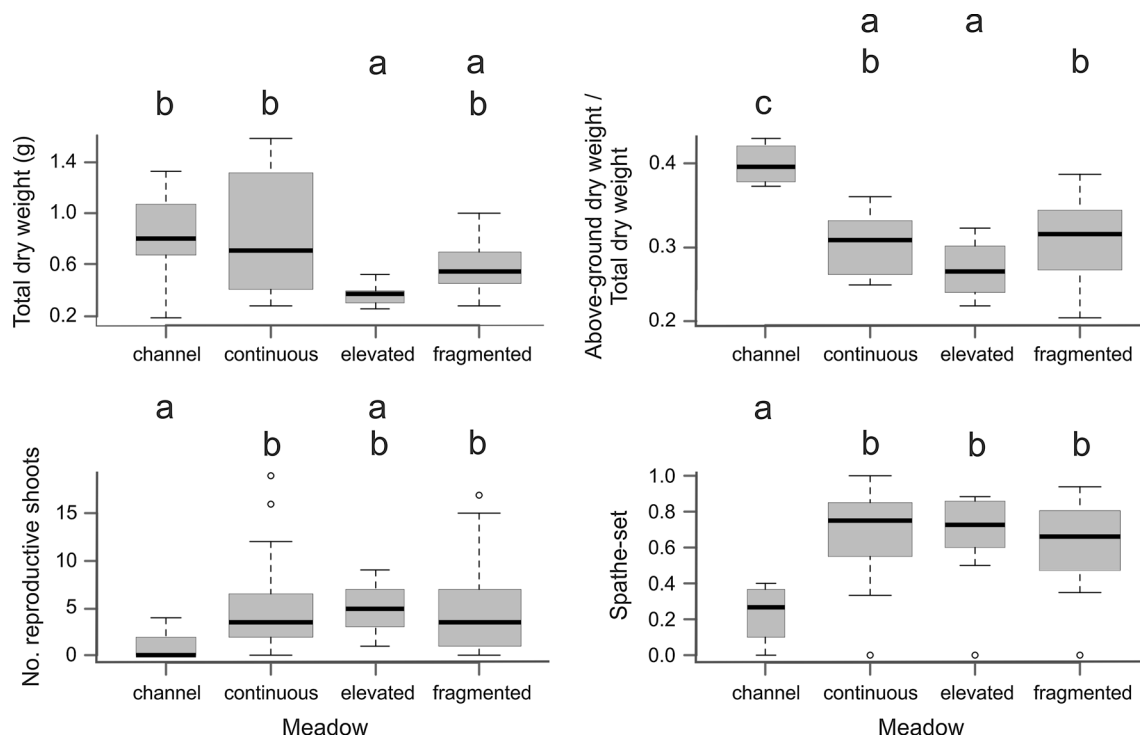


Fig. 3 Boxplot of selected parameters per sample (10 cm diameter, $n = 5$) of *Zostera noltei* in different meadow sections at Oostdijk and Goesse Sas in the Oosterschelde sampled on 10-Sep-2014, indicating median (line in box), upper and lower quartile (box), $1.5 \times$ inner

quartile spread (whiskers) and outliers (circles). The letters above the graphs indicate significantly different groups identified by the Tukey post hoc test (significantly different at $\alpha = 0.05$)

in pollen dilution (Smith and Walker 2002; Verduin et al. 2002; van Tussenbroek et al. 2009). In dioecious *Phyllospadix* species that grow in environments with high hydrodynamics on rocky shores, bet-hedging was suggested as a reason for the high female dominance (Buckel et al. 2012). However, this strategy does not seem necessary on monoecious plants such as *Z. noltei*, as they can rely on self-pollination as a mechanism of reproductive assurance (e.g., geitonogamy has been reported for the congeneric monoecious eelgrass *Z. marina*; Reusch 2001).

Self-pollination may result in reduced pollen quality, which is a third possible cause for reduced seed- or fruit-set. Balestri and Cinelli (2003) reported 87 % of the seed loss due to abortions in the Mediterranean hermaphrodite *Posidonia oceanica*, attributed to a possible combination of limited pollination, resource limitation and inbreeding depression. Insufficient pollen quality due to self-incompatibility (geitonogamous selfing) was registered for the monoecious seagrass *Z. marina* by Reusch (2001). However, in a subsequent study, Hämmerli and Reusch (2003) found that this species had significant outcrossing independent of its genetic neighborhood, suggesting that this species may have a self-incompatibility system. Zipperle et al. (2011) reported that 88 % of the offspring of *Z.*

noltei in the Wadden Sea was outcrossed. More than half of the ovules in their population were aborted, which they assumed were selfed offspring that had failed to develop. But spathe or ovule abortions due to selfing would not decrease at increased flowering density as was found in this study. Seed:ovule ratios of *Z. noltei* in the Oosterschelde were maximally 0.7, and it is possible that geitonogamous selfing was responsible for 30 % of the ovule abortions. But, the increased possibility of spathe- or fruit-set at, respectively, increased reproductive shoot or flowering spathe density in the present study can only be explained by pollen limitation at lower densities, suggesting a demographic Allee effect.

We determined successful reproduction both as spathe- or fruit -set, which may be subjected to different pressures to assure reproduction (Holland and Chamberlain 2007). Holland and Chamberlain (2007) reported that low seed:ovule ratios of cacti were explained by excess (variable) ovule production and not by pollen limitation, whereas fruit:flower ratios were explained by equilibrium between resource and pollen limitation. The ovule production per spathe in *Z. noltei* is fairly constant (Table 4) and we found density-dependent reproductive success for both spathe-set and fruit-set, suggesting that they were both pollen-limited.

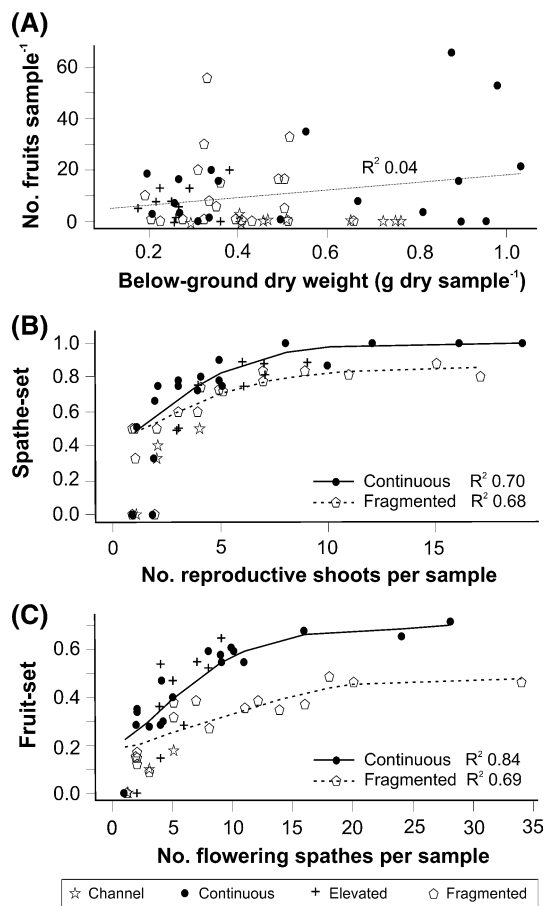


Fig. 4 Reproduction of *Zostera noltei* in the Oosterschelde: **a** relationship between below-ground biomass and production of seed-bearing fruits, **b** density-dependent spathe-set, **c** density-dependent fruit-set. Sample size = 10 cm diameter, $n = 5$

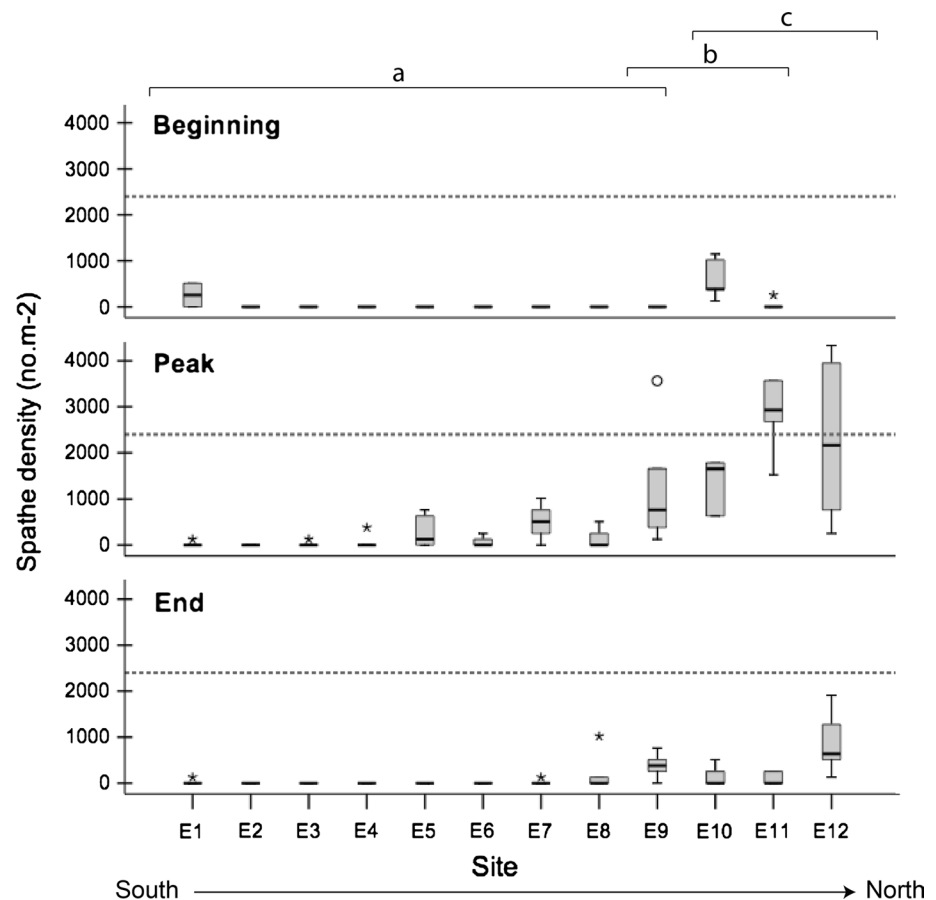
Latitudinal gradient

We found that high floral densities (>1200 flowering spathes m^{-2} or >600 reproductive shoots m^{-2}) were required for optimal pollination in *Z. noltei*. The total mature spathe density (bearing flowers or fruits) throughout western European stations varied from no spathes to 6624 spathes m^{-2} for the upper limit, with an average of 847 spathes m^{-2} during the peak flowering season. Assuming that approximately half of these spathes were flowering at the same time and potentially cross pollinate each other, as was registered for the Oosterschelde (Fig. 2a), only 3 out of the 12 sampling sites (25 %) had upper densities above this critical limit of 1200 flowering spathes m^{-2} (≈ 2400 total spathe density) for optimal pollination during the peak of the flowering season. At other times, flowering spathe densities were almost always below this limit (Fig. 5). Thus, intertidal populations of this species across Western Europe are usually pollen limited.

Spathe density increased from South to North along a latitudinal gradient in Europe (Fig. 5). The more southern populations were evergreen (they had green leaves throughout the year), although seasonal fluctuations in biomass could be considerable (Duarte 1989). Lower reproductive frequency in the more southern *Z. noltei* populations had also been documented by Buia and Mazzella (1991) in Italy, by Peralta et al. (2005) in Portugal, by Peralta et al. (2000) and Brun et al. (2003) in Spain. This difference between northern and southern populations seems to be reflected in their genetic structure. The sizes of the genets tend to be smaller in northern than southern Europe: in N-Europe on the tidal flats they vary between 1 and 10 m^2 (generally $<3 m^2$, Coyer et al. 2004), but in S-Europe they are up to 50 m in length (Coyer et al. 2004; Ruggiero et al. 2005). Thus, the Northern-European populations may be considered to be in a perpetually colonizing phase (sensu Peralta et al. 2000, 2005) with yearly recurrent population initiation by sexual and asexual propagules (seeds and dormant rhizome fragments), and clonal extension of the creeping rhizomes (Vermaat and Verhagen 1996; Zipperle et al. 2009a). In contrast, the southern, evergreen populations of *Z. noltei* depend much more on clonal propagation (Coyer et al. 2004).

The balance between vegetative and generative reproductive modes in clonal plants is thought to be mainly affected by factors suppressing sexual reproduction (Eckert 2002; Silvertown 2008). Models that have examined the conditions under which sexual and asexual reproduction coexist have found that sexual reproduction will only persist if temporal variability in resource supply is such that vegetative growth is (temporarily) not possible (Weeks 1993). This may explain why evergreen populations of *Z. noltei* are more common in the south than in the north with its colder winters, and we found a clear relationship between average air temperature and spathe density in *Z. noltei* (Fig. 6). In a global warming scenario, northern populations may become more clonal when temperatures rise. However, stochastic climate extremes are also expected due to global warming (IPCC 2014), and the reduced sexual reproduction even in the northern intertidal populations may result in less genetic variability and, therefore, a smaller variable genetic pool to allow for genetic adaptations to changing conditions of this seagrass species throughout its whole distribution range. To a certain extent, this may be counteracted by possible increased reproductive effort that plants display under various forms of stress and disturbances (Alexandre et al. 2005, 2006; Cabaço and Santos 2012), but that will depend on the frequency and intensity of the climatic changes.

Fig. 5 Spathe density of *Zostera noltei* in intertidal populations across a latitudinal gradient at different stages of the growing season (beginning, peak and end): *boxplots* represents the spathe density for all sites ($n = 5$); sites are displayed from South to North according to the initials assigned in Table 1. The *dotted gray line* represents the pollen limitation threshold defined at 1200 flowering spathes m^{-2} (≈ 2400 total no. spathes m^{-2})



Are hydrophilous plants generally pollen limited?

Very little is known concerning reproductive success of hydrophilous plants, and we could only find data for seed- or fruit-set for 14 out of the >100 species exhibiting true hydrophily (including ≈ 40 species of *Najas*, Haynes 1997). The low number of ovules per flower in the hydrophilous plants (Table 5; although *Halophila* spp. may have up to 60 ovules: Kuo et al. 1993) may be related with an abiotic pollination syndrome, as Friedman and Barrett (2009) suggested that the low cost of producing flowers in wind-pollinated plants may favor having more flowers per plant with few ovules. This may enlarge the spatial distribution of the flowers and thereby enhancing pollen capture.

Pollination success in the freshwater *Najas marina* (Huang et al. 2001) and seed-set in *Zannichellia palustris* (Table 5) are relatively high; but the seed- or fruit-set of marine hydrophilous plants is generally much lower (Table 5). Although absolute seed-, or fruit-set does not indicate pollen limitation which can be assessed through pollen addition experiments (e.g., Burd 1994; Friedman and Barrett 2009) or density-dependent seed-, or fruit-set (e.g., this study), the generally low value implies that the Allee effect found in our study for *Z. noltei* may be common for

marine angiosperms. Friedman and Barrett (2009) found a seed-set varying between 0.61 and 0.89 for ten herbaceous anemophilous land plants, and only one out of the ten studied plants had increased seed-set after artificial pollen addition, suggesting that these plants are generally not pollen limited. In contrast, Burd (1994) found significant pollen limitation in 62 % out of 258 animal-pollinated species, based on comparisons of natural- and hand-pollinated plants. Further research into the relation between reproductive success and floral density in seagrasses, or pollination experiments is needed to confirm whether pollen limitation in marine environments is common.

Pollen limitation due to habitat fragmentation

In addition to density-dependent pollen limitation, we found that fragmented populations suffered more from pollen limitation than continuous ones at similar flowering density (Fig. 4b, c). Reusch (2003) also demonstrated for *Z. marina* that seed-set was 22 % lower in isolated vegetation patches as compared to continuous populations, and Vermaat et al. (2004) found reduced seed-set in more fragmented meadows of *Enhalus acoroides*. Qi et al. (2014) found reduced seed-set of

Table 5 Summary of successful reproduction (seed:ovule ratio or fruit:flower ratio) of plants with true hydrophilous pollination

| Species | Fl. | Place | No. male reproductive shoots m ⁻² | No. male inflorescence m ⁻² | No. ovules/flower | No. ovules m ⁻² | Seed: ovule ratio | Fruit: flower ratio | References |
|---------------------------------------|-----|-------------------|--|--|-------------------|----------------------------|-------------------|---------------------|-----------------------------------|
| Freshwater | | | | | | | | | |
| Zannichelliaceae | | | | | | | | | |
| <i>Zanichellia palustris</i> | M | Switzerland | na | na | 1–9 | na | 0.56 ^c | | Guo et al. (1990) |
| <i>Zanichellia palustris</i> | M | Utah, USA | na | na | 1–9 | na | 0.91 ^c | | Guo et al. (1990) |
| Seagrasses | | | | | | | | | |
| Cymodoceaceae | | | | | | | | | |
| <i>Syringodium filiforme</i> | D | Mexico | 0–310 (77) | 0–310 (77) | 2 | 0–4972 (845) | 0.02–0.5 | | Van Tussenbroek and Muhlia (2013) |
| Hydrocharitaceae | | | | | | | | | |
| <i>Enhalus acoroides</i> | D | Philippines | 0–2 | | | 0–18 | | 0–0.7 | Vermaat et al. (2004) |
| <i>Thalassia testudinum</i> | D | Mexico | 1–7 | | 2–5 | 3–88 (35) | 0.5–0.7 | | Van Tussenbroek et al. (2010) |
| Posidoniaceae | | | | | | | | | |
| <i>Posidonia oceanica</i> | M | Italy | | 56–137 | 1 | 233–574 | 0.02 | | Balestri and Cinelli (2003) |
| <i>Posidonia australis</i> | M | SW Australia | | 46 | 1 | 736 | 0.27 | | Smith and Walker (2002) |
| <i>Posidonia sinuosa</i> | M | SW Australia | | 42 | 1 | 420 | 0.31 | | Smith and Walker (2002) |
| <i>Posidonia coriacea</i> | M | SW Australia | | 1–3 | 1 | 18–70 | 0.2–0.24 | | Campey et al. (2002) |
| Zosteraceae | | | | | | | | | |
| <i>Phyllospadix torreyi</i> | D | SW-USA | 0–100 | 0–800 | 1 | 2767–9756 | | 0.6–1.0 | Williams (1995) |
| <i>Phyllospadix scouleri</i> | D | NW-USA | <1 | | 1 | | 0.02–0.7 | | Shelton (2008) |
| <i>Phyllospadix ser-rulatus</i> | D | NW-USA | <1 | | 1 | | 0.004–0.37 | | Shelton (2008) |
| <i>Phyllospadix torreyi</i> | D | SW-USA | <1–16 | | 1 | 22–208 | 0.02–0.71 | | Buckel et al. (2012) |
| <i>Zostera tasmanica</i> ^a | M | W-Australia | | 0–5 | 1 | 0–67 | 0.89 | | Campey et al. (2002) |
| <i>Zostera marina</i> | M | NE-USA | 53 | 403 | 1 | 265–2544 | 0.20–0.72 | | Churchill and Riner (1978) |
| <i>Zostera marina</i> | M | NE-USA | 303–424 (353) | 353–2471 | 1 | 1765–15532 | 0–0.87 (0.21) | | Silberhorn et al. (1983) |
| <i>Zostera marina</i> ^b | M | Germany-Baltic S. | 1–27 | | | | 0.48–0.63 | | Reusch et al. (2003) |
| <i>Zostera marina</i> | M | China-Moon Lake | 21–650 | 61–2210 | 1 | 648–56421 | 0.54–0.85 | | Qi et al. (2014) |

Table 5 continued

| Species | Fl. | Place | No. male reproductive shoots m ⁻² | No. male inflorescence m ⁻² | No. ovules/flower | No. ovules m ⁻² | Seed: ovule ratio | Fruit: flower ratio | References |
|-----------------------|-----|------------------|--|--|-------------------|----------------------------|-------------------|---------------------|-----------------------------|
| <i>Zostera noltei</i> | M | Portugal | 126 | 252–1008 (529) | 1 | 2600 | 0.12 | 0–0.75 (0.22) | Alexandre et al. (2005) |
| <i>Zostera noltei</i> | M | Germany-Wadden S | 310 | | 1 | 1240 | <0.5 | | Zipperle et al. (2009b) |
| Land plants | | | | | | | | | |
| Wind pollinated | M&D | Canada | na | na | Often 1 | na | 0.61–0.89 | | Friedman and Barrett (2009) |

Fl. flower type (M monoecious, D dioecious). If the species are dioecious, the density of male reproductive shoots or inflorescences is mentioned. Values within brackets correspond to the average value

^a As *Heterozostera tasmanica* in publication

^b Data for continuous population

^c Part of seed-set due to self-pollination. The data for wind-pollinated land plant are of 10 species of herbaceous plants of the genera *Carex*, *Rumex* and *Thalictrum* (from Friedman and Barrett 2009)

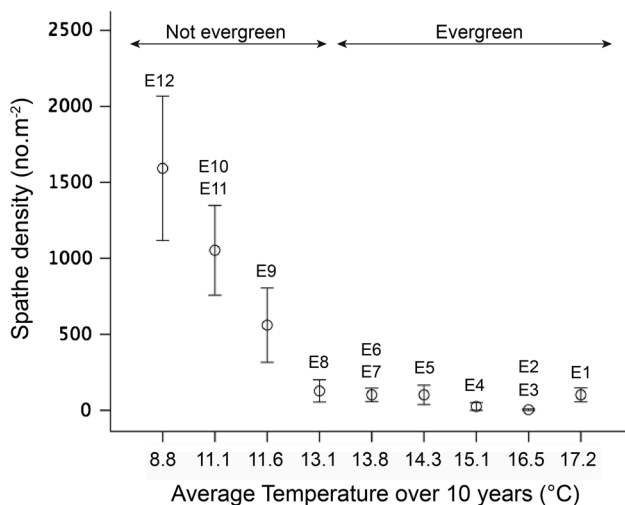


Fig. 6 Spathe density ($n = 5$) and seasonal conditions of the populations (not evergreen = leaves and many rhizome sections dying-off during the winter or evergreen) against the average temperatures at the European sampling sites

Z. marina in fragmented populations and at meadow margins in comparison with the centers of continuous meadows in China. This suggests that seed-set is not only positively affected by proximity of other reproductive shoots at a local (cm) scale, but also at larger scale proximity (patch scale, i.e., 1–10 m). Thus, the recovery potential and colonization capacity will decline more than linearly when populations become fragmented or patchy which is commonplace when seagrass populations are under threat (Bell et al. 1999; Apostolaki et al. 2009). This increased Allee effect in fragmented populations may partially be compensated by higher investment

in reproductive structures (Alexandre et al. 2005; Cabaço and Santos 2012).

Implications for conservation and restoration

Though they are clonal plants, seagrasses depend on generative reproduction for recovery after disturbance, colonization of new areas and to maintain genotypic variability (Ouborg et al. 1999), thus for the longer-term resilience of the populations (Ehlers 2008). For conservation measures, the reproductive density can be considered as an indicator for the reproductive capacity of the population. In our model species *Z. noltei*, below 600 reproductive shoots m⁻² (1200 flowering spathes m⁻²), seed production decreases more than linearly, which may reduce resilience and maintenance of genetic variation.

Reduced successful reproduction due to Allee effects may particularly threaten the conservation of northern *Z. noltei* populations, where recovery and recolonization processes by seed are part of the year-to-year maintenance of the population, though in our studied populations the densities of reproductive shoots were still sufficiently high. In contrast, southern populations depend less on seed recruitment, but are likely experiencing strong demographic Allee effects, making them vulnerable for large-scale disturbances where vegetative recolonization may be slow.

Pollen limitation, shown in our study in the model species *Z. noltei*, will result in non-linear population responses to disturbance, even more so as fragmentation was shown to strengthen this Allee effect. Density-dependent feedback (in our case depending of density of reproductive shoots, but also the ‘density’ of the patches) is self-reinforcing; when the population is decreasing, it leads to accelerated

decline and impaired recovery. It should be realized that in the case of pollen limitation, which is a demographic Allee effect, recovery will be impaired even when environmental conditions improve. This is also the case with genetic Allee effects (shown in *Z. marina* by Reusch 2003; Hughes and Stachowicz 2009). In contrast, environmental Allee effects such as density-dependent reduction of turbidity (Van der Heide et al. 2008; Carr et al. 2010), toxicity (van der Heide et al. 2008; Govers et al. 2014), or nutrition (Williams 1990; Jensen et al. 1998), will disappear when the environment is sufficiently improved. For example, if the water is sufficiently clear, density-dependent reduction of turbidity is not relevant anymore.

Our literature review suggests that a low seed- or fruit-set is likely common in seagrass populations worldwide; this Allee effect may, thus, help to explain the accelerated global seagrass decline and limited recovery as witnessed during recent decades (Waycott et al. 2009; Van Katwijk et al. 2016).

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Author contribution statement BIVT, MvK: conceived and designed and analyzed the section on pollen limitation. LS, TJB, MvK and others (FGB, GP, PGC, TFG, BO, MV, J-MG, FG, LG, JF, PK, RA): design, execution, data analysis of European sampling. BIVT, MvK, LS wrote the manuscript; other authors provided editorial advise

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