

Evidence for persistent seed banks in dwarf eelgrass *Zostera noltii* in the German Wadden Sea

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ABSTRACT: The intertidal dwarf eelgrass *Zostera noltii* is a dominant species in the Dutch and German Wadden Sea. Although numerous studies of its reproductive ecology have been conducted, few have examined the importance of seeds and seed banks for meadow maintenance. We investigated the contribution of a seed bank (size, genetic potential and persistence) to annual recruitment of dwarf eelgrass in the German Wadden Sea using temporal sampling of seeds from the sediment and genetic assignment tests of seedlings to populations of adult shoots from previous years. Annual sediment seed density (SD) was 487.5 m⁻² (269.4) and 367.3 m⁻² (95.5) in 2004 and 2005, respectively, and distribution of seeds in the sediment was highly aggregated. The proportion of over-wintering seeds that germinated under laboratory conditions was 16 to 25%, and field-germination revealed a 12% survival to the seedling stage. Nearly 20% of all shoots present in May 2004 were seedlings. Using 9 microsatellite loci, seedlings sampled in 2004, 2005 and 2006 were compared with adults sampled in 2002, 2003 and 2004; results revealed that 7 to 33% of seedlings could be assigned to the local adult population in current or previous years. Although new recruitment plays an important role in the maintenance of these meadows, considerable new recruitment comes from within the meadow itself. Seeds are viable for at least 3 yr, thereby forming a relatively short-term, but persistent, seed bank.

KEY WORDS: Seed bank · Seagrass · Persistence · Germination · *Zostera noltii* · German Wadden Sea

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INTRODUCTION

The contribution of annual and sexual recruitment from seeds relative to perennial and asexual propagation by rhizomes varies widely in eelgrass *Zostera* sp. Consequently, meadow or patch composition may range from nearly every shoot arising from a separate seed (Coyer et al. 2004a, Olsen et al. 2004) to a single, long-lived clone with little, if any, recruitment (Reusch et al. 1999). Factors affecting this variation include patch isolation (Reusch 2003), poor dispersal (Hämmerli & Reusch 2003a), intraspecific density-dependent competitive interactions (Hämmerli & Reusch 2003b, Reusch 2006), low seed production (Loqués et al. 1990, Alexandre et al. 2006), local seed predation

(Fishman & Orth 1996) and the absence of a seed bank (Hootsmans et al. 1987, Harrison 1993).

Viable seeds in the sediment constitute an age-structured seed bank of which 3 types are distinguished: transient (persisting for <1 yr), short-term (from 1 to 5 yr) and long-term (>5 yr) (Thompson et al. 1997). As seed banks provide a genetic reservoir for changing environmental conditions, subsequent recruitment can strongly influence population structure and genotypic diversity, especially for clonal plants (Eriksson 1989, Morris et al. 2002, Koch et al. 2003, Barrett et al. 2005, Reusch et al. 2005, Reusch 2006, Reusch & Hughes 2006),

After the near total loss of subtidal eelgrass *Zostera marina* meadows to a wasting disease in the early

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Table 1. Reproductive biology of *Zostera noltii*, including seed production, germination success and longevity. Potential seed production is equal to seeds flowering shoot⁻¹ × max flowering shoot density. –: not reported

Location	Seeds per flowering shoot	Meadow seed production (seeds m ⁻²)	Potential seed production (m ⁻²)	Seeds in sediment (m ⁻²)	Seed germination success in laboratory (lab) vs. field conditions	Seed longevity	Source
German Wadden Sea	–	50–1600	–	192–422	16–25% lab (ambient conditions), 5–12% field	Up to 3 yr	Present study
Ria Formosa, Portugal	3	312 ± 66	2633 1062	–	70% lab; 10% to seedling stage	–	Alexandre et al. (2005, 2006)
Venice Lagoon, Italy	2 (spathe ⁻¹)	–	1300	–	No field germination detected	–	Curiel et al. (1996)
Etang de Diana, Corsica, France	–	–	–	–	Field germination 89–7560 m ⁻²	–	Goubin & Loqués (1991)
Golfe Juan, Mediterranean, France	0.71 (vs. 14 seeds shoot ⁻¹)	0–631 ^a ; 95% of seeds never matured	0–3066 ^a	–	0–100% lab (depending on temp., salinity, incision), no field germination detected	–	Loqués et al. (1988, 1990)
Zandkreek, The Netherlands	–	–	–	–	6.5 ± 2.8 m ⁻²	–	Vermaat & Verhagen (1996)
Sylt, Germany	7.8	–	–	996	63% lab	–	Ehlers (2002)
Zandkreek, The Netherlands	–	–	–	157 ± 45.8 ^a	No field germination detected	Unknown, depleted in summer	Harrison (1993)
Zandkreek, The Netherlands	2–3 (spathe ⁻¹)	–	350–9000	0–150	0–80% lab (depending on temp., salinity, incision), no field germination detected	Annual	Hootsmans et al. (1987)

^aEstimated from source information

1930s, dwarf eelgrass *Z. noltii* has emerged as the primary seagrass species inhabiting the Wadden Sea, particularly in the northern regions (Reise & Kohlus 2008). Dwarf eelgrass is usually confined to the upper intertidal zone of sheltered sandy and/or muddy European coastlines, but sometimes it is found in the shallow subtidal zone (den Hartog 1970).

Like most seagrasses, *Zostera noltii* propagates vegetatively by rhizomatous growth and sexually through seeds. It is a protogynous hermaphrodite, with 4 to 6 female and 4 to 6 male flowers grouped in a single floral unit (spathe). Within a single spathe, flowers mature first to avoid self-fertilization, although asynchronous maturation of several spathes from the same genet (sensu Harper 1977) may result in self-fertilization (geitonogamous selfing) (Reusch 2001). Fertilization relies on sub-aquatic pollen transport to receptive stigmas. Mature seeds are negatively buoyant and drop to the sediment surface after release. While some seeds enter the sediment, the vast majority are dispersed away, either as bare seeds or in the spathes of floating leaf wrack (Loques et al. 1988). Nevertheless, seeds that are buried may or may not provide a seed bank and, likewise, seeds that are transported may or may not be lost.

Several studies have directly examined the reproductive ecology of *Zostera noltii* including flowering, seed production, seed density (sediments), seed viability and germination (Table 1). Persistence of seed banks, however, is typically inferred from laboratory studies of seed germination following temporal storage of seeds in seawater under dark and cold conditions (McMillan 1991, Orth et al. 2000) and/or from the presence of seeds in sediment cores sampled in the field (Hootsmans et al. 1987, Harrison 1993). While all of these studies have demonstrated potential seed longevity, none have been able to establish actual longevity. In the present study, we use temporal sampling and genetic assignment tests to determine (1) the size and the genetic potential of the seed bank, (2) seed bank persistence or seed viability over time and (3) the year-to-year contribution of the seed bank to recruitment within *Z. noltii* meadows.

MATERIALS AND METHODS

Study site. We studied a *Zostera noltii* meadow (ca. 0.125 km²) in the shallow bay of Königshafen (55° 2' 50' N, 008° 25' 50' E) off the Island of Sylt in the German Wadden Sea (Fig. 1). Tides are semidiurnal (mean tidal range = 1.8 m), mean annual water temperature is 9°C (seasonally varying between –2 and 19°C) and salinity varies from 28 to 32 psu (for further description see Gätje & Reise 1998). The meadow is

exposed for 6 to 8 h during each low tide and water depth during high tide is typically 0.5 m. A permanent 10 × 10 m plot (ca. 0.1% of the meadow) was established near the center of the meadow in April 2002. Corners were marked with metal stakes driven com-

pletely into the sediment to which a small piece of nylon line was attached and lay exposed on the surface. At each sampling period, stakes were relocated (using photographs, landmarks and nylon line) and connected with 10 m transect tapes. A preliminary ANOVA for sediment seed density over scales of centimetres to kilometres revealed the least variation at the 1 × 1 m level, thus the 10 × 10 m plot is an appropriate size (A. M. Zipperle unpubl. data)

Seed census. Seed density was assessed on a monthly basis from April to October 2004 and 2005. During each sampling period, 40 cores (7.5 cm diam. [0.004 m²], 10 cm deep) were collected at random from within the 10 × 10 m plot. In the laboratory, samples were sieved through 500 µm mesh (*Zostera noltii* seed size = 1 to 2 mm) using ambient seawater. The retained sediment was oven-dried at 50°C for 48 h and then examined under a dissecting microscope for intact seeds. Seeds were collected from the plot (as opposed to throughout the bed) for future spatial and/or genetic matching with seedlings and adult shoots within the plot. Coring-induced disturbance was minimal as the cumulative area cored during 7 mo was only 1% of the plot.

Seed germination. Seeds persisting throughout winter 2004–2005 (corresponding to 2004 and/or earlier seed sets) were collected in April 2005 from the Königshafen meadow (n = 64), as well as another meadow 10 km south (n = 36). Seeds were extracted directly from wet sediment cores for both populations. Five seeds were placed in each of 13 (Königshafen) and 6 (southern population) PVC Petri dishes and covered with 1 cm of sieved sediment collected adjacent to the seed collections. Petri dishes were marked and placed randomly in a flow-through tank with ambient seawater at a depth of 0.5 m and a 12 h light:12 h dark light cycle using white fluorescent light tubes (20 µmol m⁻² s⁻¹). Germination was evaluated on a daily basis for 6 wk and considered complete when the green cotyledon was visible. After germination, seedlings were removed and discarded. The experiment was concluded after 6 wk and all seeds that failed to germinate were discarded.

Field germination and survival to the seedling stage was estimated by the average seedling density and average seed bank size during the germination period (April to June 2004). Germination depth of 100 seedlings was measured in the Königshafen meadow to determine the optimal burial depth. Seedlings were distinguished from overwintering shoots by appearance of a caulicle and a green cotyledon (Dawson 1966); the attached seed and seed coat were revealed after excavation. The cotyledon was cut at the sediment surface level and the seedling was excavated. Germination depth was defined as the distance

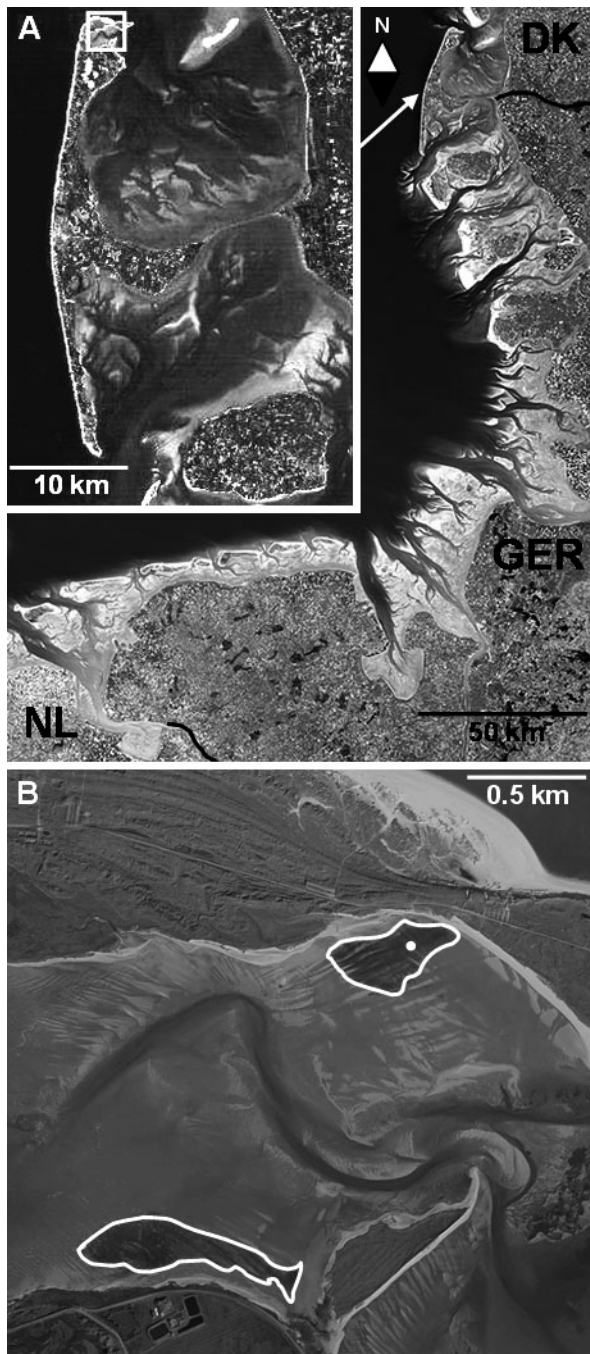


Fig. 1. Study area. (A) German Wadden Sea. Inset: the shallow bay of Königshafen (small white square) located off the Island of Sylt. DK: Denmark; GER: Germany; NL: The Netherlands. (B) *Zostera noltii* meadows in Königshafen (outlined in white) and plot location (white dot)

between the cut edge of the cotyledon and the still-attached seed.

Meadow phenology. Densities of vegetative and flowering shoots were monitored on a monthly basis throughout the growing season of April to October 2004. Shoots were counted in each of ten 0.25 m² quadrats placed randomly within the 10 × 10 m plot. During spring 2004, an additional 10 random quadrats were examined for seedlings on a biweekly basis. With loss of the caulicle (usually 4 wk after seed germination), seedlings could no longer be distinguished from adult and newly produced vegetative shoots.

Sampling of adults and seedlings for genotyping. All tissue samples and seedlings were collected by subdividing the 10 × 10 m into 1 m² quadrats (Coyer et al. 2004a). Sampled seedlings were easily distinguished from newly produced ramets as discussed above. Leaf tissue was removed from one adult shoot at the center of each 1 m² quadrat (maximum of 100 samples) in spring (May to June) and autumn (September) from 2002 to 2004. If no shoot was present within a 10 cm radius around the center of the quadrat, no sample was collected. Intact seedlings were collected in May 2005 (n = 179) and 2006 (n = 40). No samples were collected from November to April because little biomass was present and no flowering or seed germination occurred. All leaf tissues and seedlings were stored in silica crystals prior to DNA extraction.

DNA extraction, microsatellite amplification and genotyping. Whole seedlings and 5 mg of silica-dried leaf tissue from adult shoots were pulverized using a Retsch Mixer Mill MM 301 (Qiagen). DNA was extracted following Elphinstone et al. (2003). Nine microsatellite loci (Coyer et al. 2004b) were amplified using multiplex PCR (Table 2) as follows: 2 mM MgCl₂, 0.2 mM dNTPs, 0.5 U *Taq* (Promega), 0.01 % bovine serum albumin, reaction buffer (10 mM Tris Hcl, 50 mM KCl, 0.1 % Triton X-100) in a total volume of 20 µl per reaction. PCR reaction conditions were the

same for each primer triplet: initial denaturation 3 min at 94°C followed by 35 cycles consisting of 40 s at 94°C, 40 s at 57.5°C and 40 s at 72°C with a final extension step of 10 min at 72°C. Each multiplexed primer triplet was visualized by gel electrophoresis on an ABI 377 gene analyzer (Applied Biosystems). Alleles were scored against an internal lane standard (ROX 350, Applied Biosystems) applying the GENSCAN software (Applied Biosystems).

Data analysis. Individual ramets (shoots) can share the same multi-locus genotype (MLG) either by chance or because they belong to the same genet (clone). To avoid false assignment of adult shoots to clones, probabilities of identity by chance (P_{sex}) were calculated for shoots sharing the same MLG using GenClone 2.0 (Arnaud-Haond & Belkhir 2007). Duplicate MLGs were considered only once in subsequent analyses.

Assignment tests were conducted using the software GeneClass2 (Piry et al. 2004), which uses a Bayesian approach (Rannala & Mountain 1997) to determine the likelihood of assignment of seedlings to adults. Because any given adult clone can persist from year to year, it is possible that a seedling might be assigned to more than one year class. To minimize this possibility, we accepted a particular year class as a source for a seedling only if the assignment likelihood was >95%. Our analysis should be regarded as a minimal estimate because sampling was not exhaustive and unassigned seedlings may come from year classes not included in the sampling or from locations outside of the study area.

Assignment tests utilize both allele frequency differences (F_{ST}) and MLGs of the seedlings and adults. Thus, where F_{ST} values were not significant between years (2002, 2003), samples were pooled. Assignment of 2006 seedlings to the 2005 adults was not feasible because a widespread and extreme heat wave in 2003 reduced population density by >99% in subsequent years (2004, 2005) and too few adult shoots were available in 2005 (A. M. Zipperle et al. unpubl. data). Values presented are means ± SD.

Table 2. Multiplexing conditions for microsatellite loci

Tri-plex	Locus (GenBank accession no.)	Primer concentration (µM)	Fluorescent label (forward primer)
1	<i>ZnB1</i> (AY566799)	0.1	NED
	<i>ZnH10</i> (AY566807)	0.24	6-FAM
	<i>ZnB3</i> (AY566800)	0.72	HEX
2	<i>ZnB8</i> (AY566801)	0.08	6-FAM
	<i>ZnH8</i> (AY566806)	0.1	NED
	<i>ZnD6</i> (AY566802)	0.24	6-FAM
3	<i>ZnE7</i> (AY566803)	0.2	6-FAM
	<i>ZnF8</i> (AY566804)	0.08	HEX
	<i>ZnF11</i> (AY566805)	0.12	6-FAM

RESULTS

Meadow phenology

An increase in the number of vegetative and flowering shoots started in June–July, peaked in August and steadily declined to October (Fig. 2), a phenological pattern typical of other locations in the Wadden Sea (den Hartog 1970, Jacobs et al. 1983, Vermaat & Verhagen 1996). At peak growth in July/August 2004, densities of vegetative and flowering shoots were highly variable at 2842 ± 1373 and 310 ± 263 m⁻², respectively.

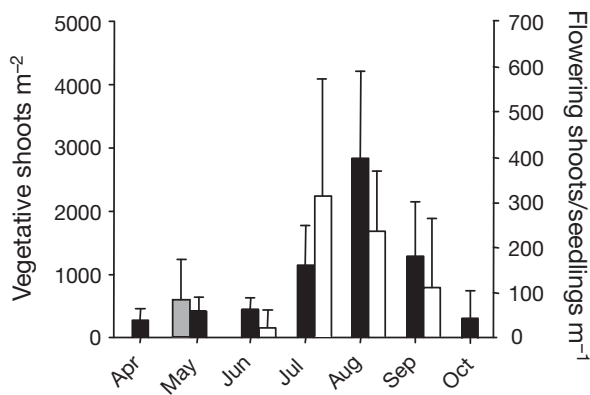


Fig. 2. Phenology of the *Zostera noltii* meadow in Königshafen in 2004. Density of vegetative shoots (black bars), seedlings (grey bar) and flowering shoots (white bars) is plotted by month. Means + SD are shown

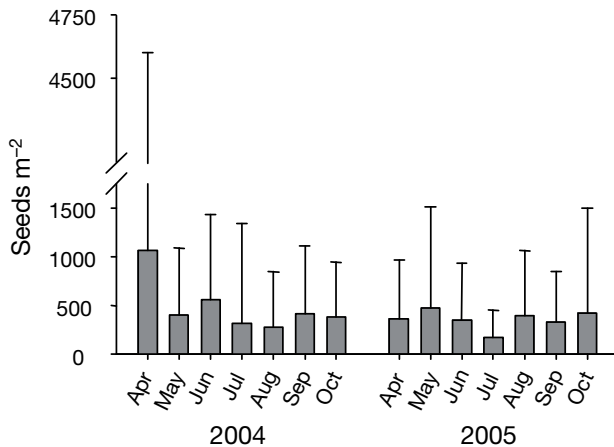


Fig. 3. Seed bank densities at the 2 study sites on Sylt from April to October 2004 and 2005. Means + SD are shown

Flowering shoots accounted for 10% of total shoot density at peak growth. The average seedling density in May 2004 was also highly variable at $83 \pm 88.8 \text{ m}^{-2}$, ranging from 0 to 288 m^{-2} . Compared to vegetative shoot density in May ($426 \pm 228.3 \text{ m}^{-2}$), seedlings represented 19.5% of shoots in the $10 \times 10 \text{ m}$ plot.

Seed bank and seed germination

Seeds were present in the sediment throughout 2004 ($487.5 \pm 269.4 \text{ m}^{-2}$) and 2005 ($367.3 \pm 95.5 \text{ m}^{-2}$) with no discernable seasonal pattern because of the very high variances (Fig. 3). Laboratory germination of seeds collected from the overwintering seed banks showed that 16 to 25% were viable. An estimate of field germination and survival to the seedling stage was 12%. Mean

Table 3. Assignment tests of *Zostera noltii* seedlings. Percent assignment of individual seedlings from 2005 and 2006 to the adult populations from 2002–2003 and 2004. Assignment was accepted only if a seedling was assigned with a likelihood of >95%

Collection date	Not assigned	Assigned to adults 2004	Assigned to pooled adults 2002–2003	Total assigned
2005	73.2	7.1	19.6	26.8
2006	67.5	2.5	30	32.5

germination depth was $4.1 \pm 0.9 \text{ cm}$ in 2004 and $3.0 \pm 1.0 \text{ cm}$ in 2005, but successful germination was observed throughout a 1 to 7 cm depth range. No seedlings germinated from seeds lying directly on the sediment surface.

Assignment

One in 3 seedlings was assigned to their home populations (Table 3), establishing the importance of local recruitment. Additionally, temporal assignment revealed that year-class seedlings arose from seeds originating from up to 3 yr previously (19.6 to 30%), thus establishing that seeds banks were not transient. Most seedlings from 2005 and 2006 were assigned to the pooled adult population of 2002–2003.

DISCUSSION

Earlier studies were unable to confirm the existence of a *Zostera noltii* seed bank using presence/absence data of sediment seeds or seedling emergence because (1) seeds in the sediment are not necessarily viable, (2) viable seeds do not necessarily germinate, and (3) the origin of germinated seeds is unknowable without genetic data (Hootsmans et al. 1987, Harrison 1993). Our estimates of seed density for 2004 (487.5 m^{-2}) and 2005 (357.3 m^{-2}) match well with previous studies conducted in Portugal, the French Mediterranean and the SW Netherlands (Table 1). However, previous studies were not able to assess successful recruitment *in situ* and methodology varied widely. In the present study, new recruitment accounted for nearly 20% of the new shoots each spring and 26.7 to 32.5% of the new seedlings originated from within the plot itself. Among these, 30% were traced to seeds from up to 3 yr earlier, suggesting that the stability and genetic diversity for the northern Wadden Sea *Z. noltii* meadows may be related to the persistent seed banks. Persistent, as opposed to transient, seed banks are bet-

ter able to buffer the stochastic year-to-year variation in seed production, conserve genetic diversity through time and allow the regeneration of meadow-level genotypic diversity after disturbance-related mortality (Honnay et al. 2008).

The strongly negative impact of a severe heat-stress event (HSE) in the summer of 2003 reported for several seagrass populations in Europe (Mayot et al. 2005, Reusch et al. 2005) affected the Königshafen meadow as well. Losses of up to 99% in subsequent years (A. M. Zipperle et al. unpubl. data) undoubtedly reduced both seed output and the number of seeds in the seed bank in the years immediately following the HSE (2004 and 2005). Consequently, most seedlings from 2005 and 2006 were assigned to the pooled adult population of 2002–2003.

Although we have shown that nearly one third of the annual recruitment came from within the meadow, the remaining 70% originated from either outside the meadow or from seeds produced within the meadow before 2002. *Zostera* seeds are heavy and negatively buoyant (Loques et al. 1988, Orth et al. 1994). As direct estimates of seed dispersal suggest distances of only a few metres (Orth et al. 1994), the majority of seeds in the seed bank of our 10 × 10 m plot most likely were produced in the vicinity of the plot. Nevertheless, long distance dispersal must be considered, as detached spathes and whole flowering shoots can be transported by currents, and studies at this level have demonstrated that both seed dispersal (Harwell & Orth 2002, Erfteimeijer et al. 2008, Källström et al. 2008) and gene flow (Reusch 2002, Ferber et al. 2008) occur over 10s of kilometres.

Seedling recruitment also maintains genetic and genotypic diversity (Reusch et al. 2005). As *Zostera noltii* has a genet lifetime of at least 4 yr (A. M. Zipperle et al. unpubl. data), a minimal annual recruitment of only 2% of the seedlings (83 m⁻²) would add 1.5 genotypes m⁻² into the population.

Growth of overwintering rhizomes begins in May–June, nearly a month after the appearance of seedlings (Vermaat & Verhagen 1996, A. M. Zipperle pers. obs.). The amount of vegetative growth is positively correlated with the number of leaf-bearing rhizomes surviving the winter (Vermaat & Verhagen 1996). Therefore, high winter survivorship implies a more extensive amount of vegetative growth (at 68 cm yr⁻¹, Marbà & Duarte 1998) the following spring, resulting in fewer gaps in the canopy for seedling recruitment and more competition from vegetatively expanding adult plants. If regular disturbances, such as grazing waterfowl and storms, significantly open the canopy by the end of the growing season, the number of overwintering rhizomes will be lower, and seedling recruitment in the following spring should be higher. Thus, the proportion of seedlings in a given

meadow is likely to vary considerably from year to year and may be more or less than the 5% suggested by Jacobs et al. (1981).

Grazing by waterfowl creates depressions or feeding pits by removing the canopy and rhizomes. If the pits are formed after seed set they can form seed traps, which will lead to a clumped distribution of seeds in the sediment (Orth et al. 1994, Luckenbach & Orth 1999). On Sylt, dwarf eelgrass seeds accumulate in feeding pits and are buried after a few tidal cycles (Ehlers 2002). As the average depth of feeding pits is 4.5 cm (Nacken 1998), feeding pits provide an ideal habitat for seed germination (Churchill 1992, Moore et al. 1993) and probably account for the clumped distributions (and high variances) we observed for seeds. An experimental study that artificially created pits in autumn showed that germination the following spring was higher in experimental pits than in undisturbed controls (A. M. Zipperle et al. unpubl. data). Taken together, the average germination depth of seedlings (3 to 4 cm) and the mean depth of feeding pits (4.5 cm) suggest that waterfowl grazing facilitates the formation of a seed bank and subsequent seedling recruitment. A similar process has been demonstrated for other seagrasses, where seeds accumulated in feeding trails of dugongs and pits caused by strong hydrodynamics (Inglis 2000).

Zostera noltii has adapted to moderate levels of disturbances in the intertidal zone by combining fast vegetative propagation with high levels of sexual reproduction. As long as intermittent disturbances do not deplete the seed bank, by either disturbing the sediment directly or reducing the meadow seed output, recovery is possible by recruitment. If, on the other hand, the canopy is severely disturbed repeatedly, seed output will decrease, the seed bank will become depleted, and import from nearby donor populations will be reduced or halted.

The present study has partially resolved the paradox of finding extremely high genetic diversity of *Zostera noltii* populations in the German Wadden Sea (as compared with the rest of its European distribution) given their relatively recent colonization since the last glacial retreat several thousand years ago (Coyer et al. 2004a). As 70% of annual recruitment came from outside the study meadow and/or from seeds deposited more than 3 yr earlier, much of the unexpectedly high diversity must stem from admixture from outlying areas (Ferber et al. 2008, A. M. Zipperle unpubl. data). The ability of *Z. noltii* to form short-term persistent seed banks and the seed dispersal abilities in detached flowering shoots may help to explain the increase in *Z. noltii* populations in the Wadden Sea in the past 3 decades (Reise & Kohlus 2008), as well as population resilience in highly mobile intertidal soft sediments.

Acknowledgements. We thank T. Reusch, A. Piquet and M. V. Ruggiero for field assistance and 4 anonymous reviewers for substantial comments. This research was supported by an Ubbo Emmius PhD stipend from the University of Groningen to A.M.Z.

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Dauphin Island, Alabama, USA*

*Submitted: June 25, 2008; Accepted: January 14, 2009
Proofs received from author(s): March 30, 2009*