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Clonal and sexy

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Zipperle, A. M. (2012). *Clonal and sexy: The dynamics of sexual and asexual reproduction in dwarf eelgrass, Zostera noltii Hornemann in the northern Wadden Sea*. s.n.

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Clonal and sexy:
The dynamics of sexual and asexual reproduction in dwarf eelgrass, *Zostera noltii* Hornemann in the northern Wadden Sea

Andreas M. Zipperle

This PhD research was carried out in the Marine Benthic Ecology and Evolution group (MarBEE) within the Centre for Ecology and Evolutionary Studies (CEES) Institute, University of Groningen, The Netherlands and in collaboration with the Wadden Sea Research Station, List, Sylt, Alfred Wegener Institute, Bremerhaven, Germany. This thesis was prepared following the requirements of the Graduate School of Science, Faculty of Mathematics and Natural Sciences, University of Groningen;

Andreas Zipperle was supported by an *Ubbo Emmius* PhD stipend from the University of Groningen (Project-Nr. Xxxx ?)

Layout and Figures:	Andreas Zipperle
Cover Design:	Andreas Zipperle
Printed by:	Mails & More, A-3441 Baumgarten
ISBN:	978-90-367-5637-2 (e-Book)

RIJKSUNIVERSITEIT GRONINGEN

Clonal and sexy: The dynamics of sexual and asexual reproduction in dwarf eelgrass, *Zostera noltii* Hornemann in the northern Wadden Sea

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 1 oktober 2012
om 14.30 uur

door

Andreas Michael Zipperle
geboren op 13 juli 1976
te Karlsruhe, Duitsland

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Chapter 1

General Introduction

My interest in seagrasses stems from 2002 during my diploma thesis research on the Island of Sylt, one of the German Wadden Islands (Fig. 1). Although working on a different topic at the time, I was impressed by the resistance of *Zostera noltii* (dwarf eelgrass, klein zeegras) to the changing physical rigors of the upper intertidal and the observation that some meadows (Box 1) seemed to persist over several years, whereas others in the same habitat area appeared and disappeared. These observations were not new, as a number of meadows have thrived on Sylt continuously since the 1920s (Nienburg 1927, Wohlenberg 1935, Reise unpubl.), whereas others have waxed and waned on a (sub-) decadal time scale (Reise et al. 2005, A. Schanz pers. communication). In talking with various ecologists, however, it soon became clear that the underlying mechanisms of meadow persistence were not completely understood.

Box 1: Terminology – Meadow, Patch, Population?

Seagrasses are variously referred to as beds, meadows, patches or populations with no consistency in the various terms with respect to spatial scale or density. In this thesis, I use the terms meadow and patch interchangeably. To get an idea of a meadow/patch in the Wadden please see Figure 7 A1.2+A2.2. I do not use "population" as a synonym for meadows or patches, as several meadows or patches may be part of the same population (Chapter 6, Waples and Gaggiottti et al. 2006).

On one hand, meadow stability and growth are affected by physical parameters such as water flow, turbidity and other disturbances (Hemminga and Duarte 2000, Duarte et al. 2006); while on the other hand, meadow persistence is affected by biological parameters related to epiphitism, grazing, bioturbation and the reproductive and mating systems (Valiela et al. 1997, Reusch 2001a, Neal and Anderson 2005, Valentine and Duffy 2006). Aspects of vegetative and sexual reproduction, however, such as the type of mating system, the presence or absence of a seed bank, recruitment and dispersal had not been investigated at a fine spatial and temporal scale using molecular markers.

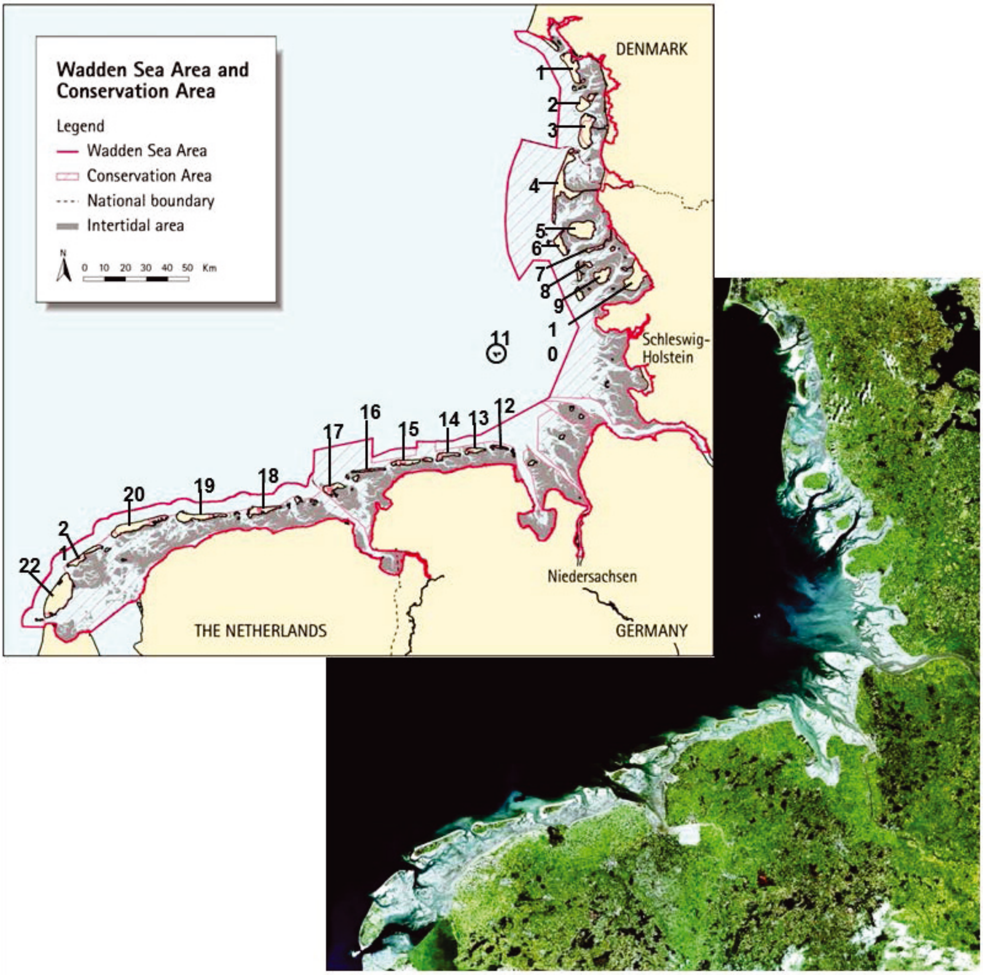

Figure 1.The Wadden Sea from Denmark to The Netherlands. Upper panel, the most important Wadden Sea Islands enumerated from north (DK) to south (NL). Lower panel, satellite image of the Wadden Sea: DK: 1 = Fanø; 2 = Mandø; 3 = Rømø; GER: 4 = Sylt; 5 = Föhr; 6 = Amrum; 7 = Langeneß; 8 = Hooge; 9 = Pellworm; 10 = Nordstrand; 11 = Helgoland ; 12 = Wangerooge 13 = Spiekeroog; 14 = Langeoog; 15 = Norderney; 16 = Juist; 17 = Borkum; NL: 18 = Schiermonnikoog; 19 = Ameland; 20 = Terschelling; 21 = Vlieland; 22 = Texel (adapted from Common Wadden Sea Secretary).

Thus, the overarching questions in this thesis are: How does the reproductive (mating) system facilitate persistence and resilience of dwarf eelgrass in the Wadden Sea and how does this information affect conservation efforts?

To approach the topic, I divided the general question into subsets, which are addressed separately in the different chapters of this thesis. In **Chapter 2**, I address the relative importance of life-history parameters (overwintering of rhizome, gap recruitment, fitness advantage of large clones) for meadow persistence and the clonal architecture (clone size, age and turn over) of *Z. noltii* meadows over time. **Chapter 3** contains a detailed mating system analysis to determine the degree of inbreeding vs. outcrossing, occurrence of multiple paternities, the pollen dispersal distance, and patterns of small scale relatedness. Whether a *Z. noltii* seed bank exists, how long seeds are viable in the sediment and what the contribution of the seed bank is to recruitment on an annual basis is addressed in **Chapter 4**. **Chapters 3** and **4** jointly examine the contribution of seeds produced within a patch vs. seeds imported from outside sources. The effect of disturbances in the intertidal, specifically waterfowl grazing on seedling recruitment is examined in **Chapter 5**. In **Chapter 6**, I expand the results for the small-scale (meadow) life-history processes to the large-scale Wadden Sea with respect to meadow/patch connectivity, gene flow and the determination of management units. The importance of the results for conservation and management of Z. noltii is briefly discussed in **Chapter 7**.

An overview of seagrass ecosystems

Seagrass ecosystems rank among the most valuable ecosystems on Earth, surpassing the economic value of tropical rainforests and coral reefs (Costanza et al. 1997, Hemminga and Duarte 2000) despite covering only 0.1-0.25% of the coastal area (Duarte 2002). Seagrasses enhance the productivity in coastal areas dominated by soft sediments (Heck et al. 1995, Bruno and Bertness 2001) and also provide numerous ecosystem services (Box 2). For example, seagrass meadows provide habitat, shelter and breeding ground for numerous coastal resident and migratory fish species and other ecologically and economically important marine organisms (Heck et al. 2003, Polte et al. 2005a,b; Polte and Asmus 2006a,b). Migratory geese and ducks directly feed on seagrass leaves and rhizomes (Jacobs et al. 1981, Ganter 2000) and epiphytes colonizing the leaves provide food for micrograzers, such as mollusks and crustaceans (van Montfrans et al. 1984, Schanz et al. 2002). Hence, *Z. noltii* contributes to various trophic levels of the soft-sediment coastal ecosystem and enhances overall productivity and biodiversity (Heck et al. 1995, Bruno and Bertness 2001). From an abiotic standpoint, the extensive root and rhizome networks of *Z. noltii* meadows prevent coastal erosion by reducing current velocities, which facilitate sedimentation (Widdows et al. 2008). Neal and Anderson 2005 Enhancement of sedimentation in concert with utilization of dissolved nutrients and oxygenation of the water column also directly and indirectly improves coastal water clarity (Asmus and Asmus 1998, 2000). Thus, seagrasses are true ecosystem engineers (Wright and Jones 2006, Peralta et al. 2008).

Box 2: Ecosystem services of seagrasses

Provisioning Services Products obtained from ecosystems	**Regulating Services** Benefits obtained from regulating ecosystem processes	**Cultural Services** Non-material benefits
Food – fisheries resources	Runoff regulation	Recreation value
fiber	Water purification (nutrient fixation)	Aesthetic value
Genetic resources	Erosion regulation	Educational function
		Cultural heritage

Threats to coastal ecosystems have never been greater than they are today (Alongi 1998, Lotze et al. 2006, BBVA Foundation 2007). It is currently estimated that 65% of the world's seagrass ecosystems have been impacted by human activities and up to 30% have been lost over the past 20 years (Duarte 2002, Orth et al. 2006a). The greatest anthropogenic threats to seagrass ecosystems are eutrophication, coastal construction leading to habitat loss and/or fragmentation, destructive fishing practices and, of course, global climate change (Short and Neckles 1999, Ralph et al. 2006, McGlathery et al. 2007).

The majority of northern hemisphere seagrass ecosystems consist of only one species and even mixed-species stands are typically dominated by a single species. Consequently, the loss of only one seagrass species might initiate a negative cascade of effects for the whole biome (Duarte 2000, Hemminga and Duarte 2000) that may occur quite suddenly. Such cascading effects or "tipping points" create regime shifts (van der Heide et al. 2007) and are difficult or impossible to reverse.

For this reason, conservation is always preferable to restoration (Hawkins et al. 1999, Orth et al. 2006a). Under the EU Water Framework Directive (Directive 2000/60/EC), seagrass habitats have been legally recognized as key ecosystems and as Biological Quality Elements (BQEs), thereby fulfilling a primary requirement of the directive for the mandatory use of organisms as bioindicators for the assessment and evaluation of ecological status.

Classification systems for EU coastal waters are currently under study and member states have installed programs for the conservation (and restoration) of seagrass ecosystems (de Jonge et al. 2000, Borum et al. 2004, UNEP-WCMC). In the Wadden Sea and adjacent areas conservation/restoration is mediated through the Rijkswaterstaat (RIKZ) in The Netherlands, the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety in Germany and Miljøministeriet in Denmark.

Seagrasses

Seagrasses are not true grasses (Poaceae), but part of the Alismatales. Collectively, they form an ecological rather than a taxonomic group as they are a polyphyletic assemblage of ~60 species (13 genera and 5 familes) (Les et al. 1997, 2002, den Hartog and Kuo 2006, Waycott et al. 2006). They have successfully colonized nearly all muddy to sandy coastlines world-wide and occur intertidally and subtidally to >60 m in depth (den Hartog 1970, Coles et al. 2000, Green and Short 2003).

Although seagrasses evolved in three or four separate lineages - each making a separate transition from terrestrial to marine habitats - all species have been exposed to similar evolutionary pressures (Les et al. 1997). Their existence in a wide range of salinities, coupled with growth, reproduction, and dispersal in an aquatic environment, have fostered convergent evolution of many growth and reproductive characteristics (e.g. strap-like leaves, filamentous pollen, hydrophilous pollination of much reduced flowers, pronounced root and rhizome system, overdominance of dioecy) (Fig. 2).

Zostera is the most widespread seagrass genus throughout the world with nine species currently recognized (Green and Short 2003). The most widely distributed species in the northern hemisphere is *Z. marina* (eelgrass, groot zeegras) (Fig. 3 B1, B2) with a North Pacific and North Atlantic distribution. Along European Atlantic coastlines, *Z. noltii* (dwarf eelgrass, klein zeegras) is the dominant intertidal species (Fig. 3 A1, A2), although it also occurs subtidally in some areas. Dwarf eelgrass ranges from southern Norway/Kattegat to Mauritania including the Mediterranean, Black, and Azov Seas, but is rapidly expanding throughout Europe (Green and Short, 2003), especially along the coasts of the Wadden Sea (Borum et al. 2004, Reise and Kohlus 2008).

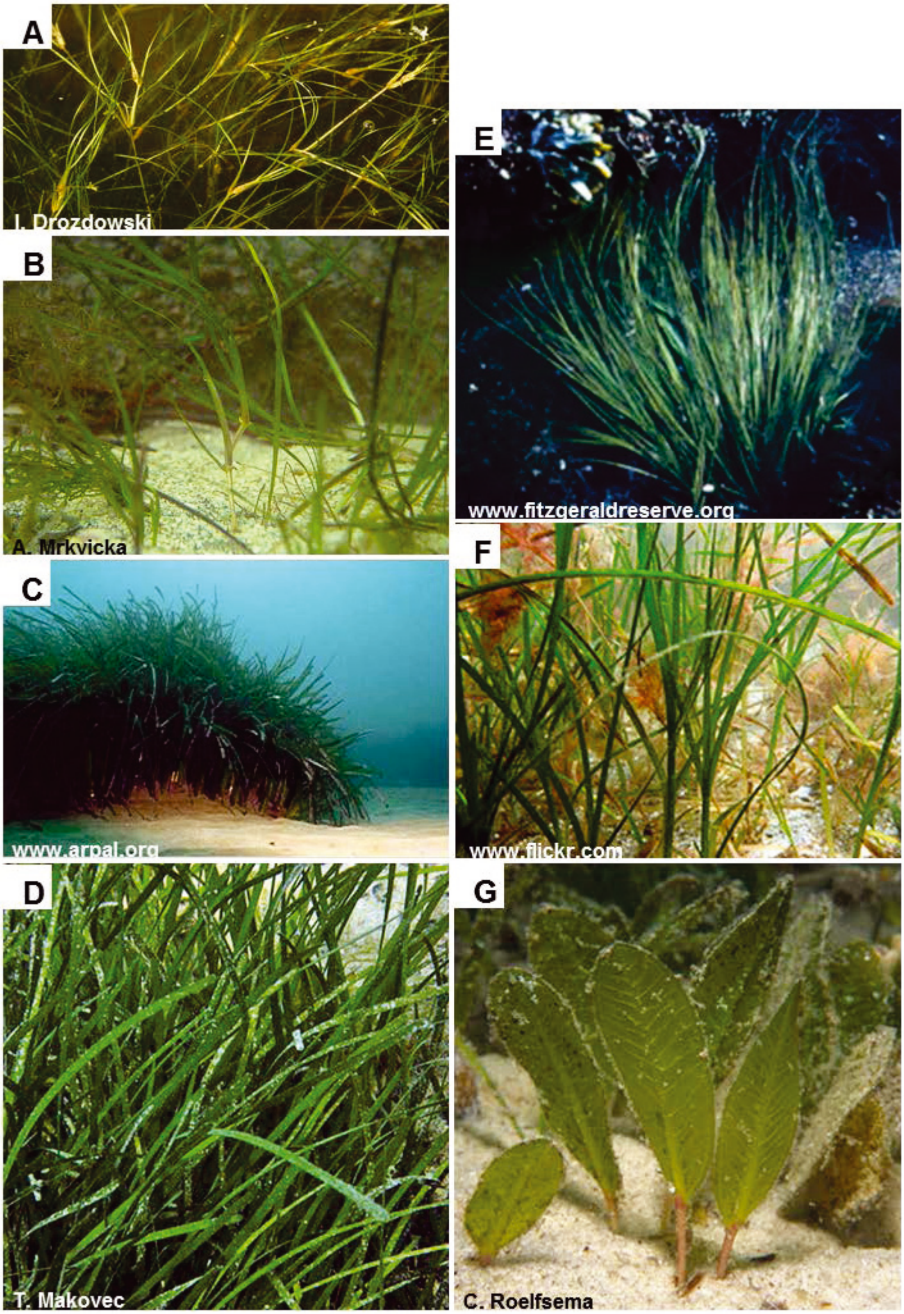

Figure 2. Seagrass diversity. A, *Ruppia maritime*; B, *Zostera noltii*, C, *Posidonia oceanica*; D, *Cymodocea nodosa*; E, *Phyllospadix torrei*; F, *Zostera marina*; G, *Halophila ovalis*.

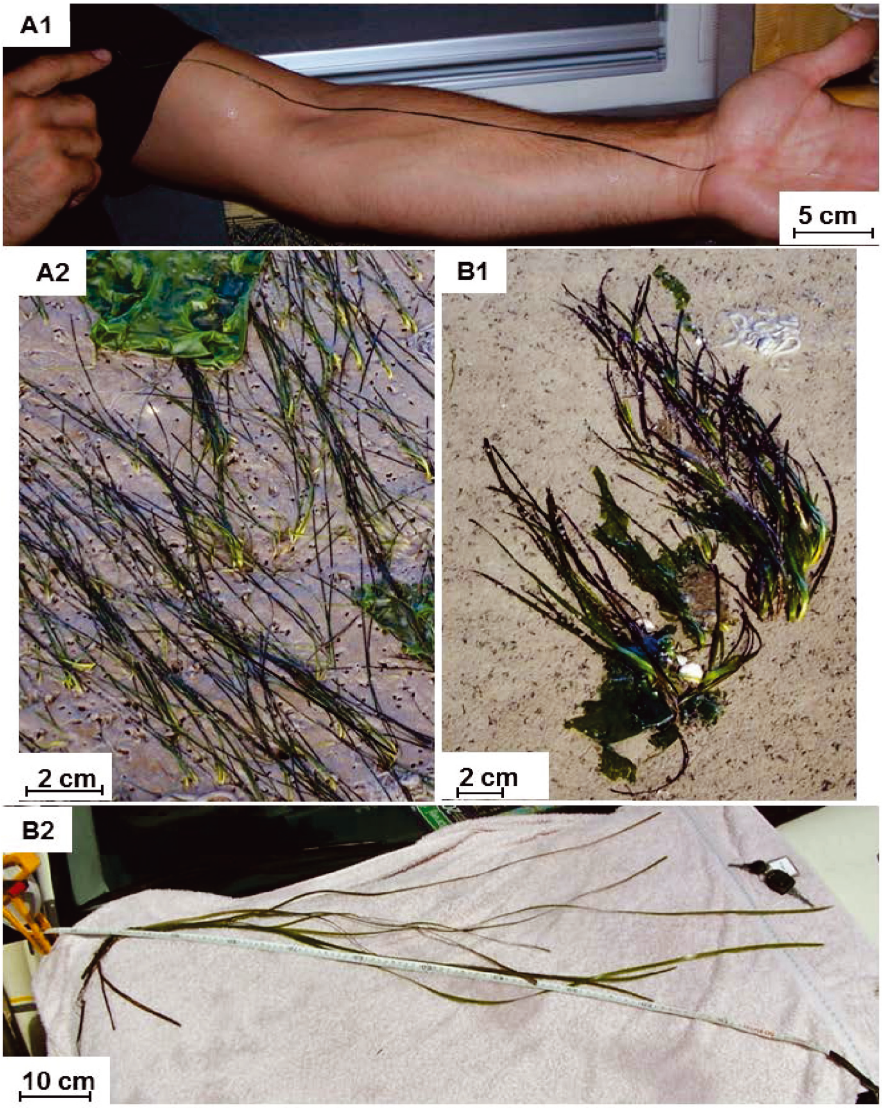

Figure 3. Morphological and size differences of *Z. noltii* and *Z. marina*. A1*. Z. noltii* leaf from a perennial meadow in the upper intertidal, but constantly submerged during low tide (Rømø, Denmark). A2. Perennial, intertidal *Z. noltii* in a sheltered, sandy location (Kampen Sylt) during low tide. B1. Solitary annual *Z. marina* plants from the upper intertidal in the German Wadden Sea. B2. Perennial, subtidal *Z. marina* (Limfjord, Denmark).

Seagrasses and the seagrass ecosystem in the Wadden Sea

The Wadden Sea comprises one of the world's largest marine wetland reserves covering 9,000 km² along the North Sea coast of The Netherlands, Germany, and Denmark. Because it supports a unique assemblage of invertebrates (including commercially important fishery species), fish, migratory birds, and marinemammals, it has been designated as part of the Natura 2000 network of the EU (European Council, Habitats Directive 92/43/EEC 1992) and a World Natural Heritage Site in June 2009 (CWSS 2008).

The Wadden Sea was formed about 7500 yrs BP (Fig. 1) and has been subjected to nearly continuous reshaping by rising sea levels, coastal uplifting, and constant exposure to tides. The last major morphological changes occurred in 1362 (Second Marcellusflood or Mandränke) and 1634 (Burchardiflut oder Second Grote Mandränke) when severe storm floods inundated large areas of diked marsh and fen (in present-day Germany) behind the barrier islands to the North Sea, leaving the Wadden Sea approximately as it is today (Schroor and Kühn 2001). Over the last millennium, humans have further modified the coastline with dikes and dams, effectively altering hydrodynamic conditions (Reise 2005). Construction of the afsluitdijk, separating the former Zuiderzee (present-day IJsselmeer) in The Netherlands from the Wadden Sea, is the most dramatic example.

Three species of seagrass are found in the Wadden Sea: *Zostera marina* (eelgrass, groot zeegras), *Z. noltii* (dwarf eelgrass, klein zeegras) and *Ruppia maritima* (widgeon grass, snavelruppia). *Zostera* species have experienced the heaviest losses and have been listed as "endangered to vulnerable" on the "Red list" of vascular plants in the Wadden Sea" (Wind et al. 1996).

Until the 1930s, *Z. marina* was the dominant seagrass and principally occurred in the subtidal. Leaves were harvested from extensive beach wrack for animal fodder and building insulation. Beginning in the 1930s, massive mortality occurred throughout the North Atlantic due to the "Wasting Disease" caused by the protist *Labyrinthula zosterae* (Rassmussen 1977, Vergeer and den Hartog 1991). Gradual recovery occurred throughout the North Atlantic over the subsequent decades with the exception of the Wadden Sea (Wohlenberg 1935, den Hartog and Polderman 1975, den Hartog 1987). Recovery failure, however, coincided with the closure of the afsluitdijk (creating the Ijsselmeer) and other hydrographic modifications that took place in that period, strongly suggesting that physical rather than biological causes have impeded recovery. At present, *Z. marina* occurs mainly as a small annual ecotype on the tidal flats sometimes interspersing with *Z. noltii* meadows, although monospecific perennial and annual meadows still exist in the intertidal.

The dominant seagrass in the Wadden Sea today is the dwarf eelgrass, *Z. noltii*, which forms large meadows primarily in the intertidal. It was largely unaffected by the wasting disease (presumably because of its intertidal habitat), although demonstrated (Vergeer and den Hartog 1991). Dwarf eelgrass was never commercially important and relatively few ecological studies have been conducted, in sharp contrast to the amount of research devoted to *Z. marina*. Scientific interest in *Z. noltii* increased only in the 1960s and 1970s when meadows declined due to eutrophication and increased water turbidity (den Hartog and Poldermann 1975, Phillippart 1994, Philippart and Dijkema 1995).

Recovery of dwarf eelgrass has been extremely uneven. For example, meadows declined in the southwestern and central portions of the Wadden Sea (Philippart and Dijkema 1995, Kastler

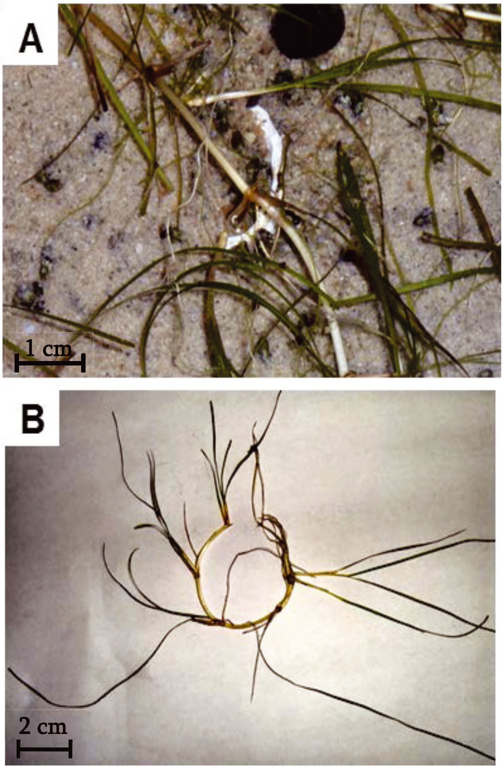

Figure 4. *Zostera noltii* on the Island of Sylt. A. Creeping *Z. noltii* rhizome in the field. B. Rhizome fragment of *Z. noltii*.

and Michaelis 1999), but have steadily increased over the past 20 years in the northern portion (Reise et al. 2005, Reise and Kohlus 2008). On the German Wadden Island of Sylt, *Z. noltii* is abundant in many areas and forms a crucial food supply for migrating waterfowl such as Brent geese (*Branta bernicla* L.) and Widgeons (*Anas penelope* L.) in the Wadden Sea (Figs. 4, 5) (Jacobs et al. 1981, Vermaat and Verhagen 1996, Ganter 2000).

Habitat loss and overexploitation represent the most severe anthropogenic footprints in the Wadden Sea (Lotze et al. 2005). Although direct habitat conversion was halted in the late 1980s (Wolf 1992, Reise 2005), construction of dikes and embankments has continued for the purpose of coastal protection (German Stade declaration 1998, Reise 2005). In the specific case of Sylt, creation of the Sylt tidal basin as intertidal bay from the open Wadden Sea has influenced the formation and mobility of mega-ripples in the sand, which can bury *Z. noltii* meadows over a decadal time scale (Fig. 6C, 7A1.2) (Reise and Kolhus 2008, Dolch and Reise 2010).

Physical impacts on *Z. noltii* include eutrophication, which limits light penetration (Fig. 6A, B) (Philippart 1994a, Philippart 1995c, Cardoso et al. 2008), and current velocities leading to increased turbidity, erosion, uprooting and burial (Fig. 6C-E) (Schanz et al. 2000, Cabaçao and Santos 2007, Dolch 2008). Biological impacts include smothering by algal mats and epiphytes (Fig. 6A, B) (Reise and Siebert 1994, Philippart 1995a, Schanz et al. 2002), negative interactions with bioturbating infauna such as lugworms (*Arenicola marina*) (Philippart 1994b) and grazing waterfowl (Fig. 5, 6 F) (Jacob et al. 1981) (see below).

What may be even more important to survival of coastal marine species, however, is that climate change models forecast dramatic increases in the frequency and intensity of extreme climatic events, such as the European Heat Wave of 2003 (Schär and Jendritzky 2004, Beniston et al. 2007, IPCC 2007).

Climate change also is expected to impact coastal systems through increased storm activities, which in turn, are expected to increase turbulence, decrease water clarity, and decrease stability (Short and Neckless 1999, Duarte 2002, Reise and van Beusekom 2008). Eelgrass die-offs due to unusually high (and sustained) sea surface

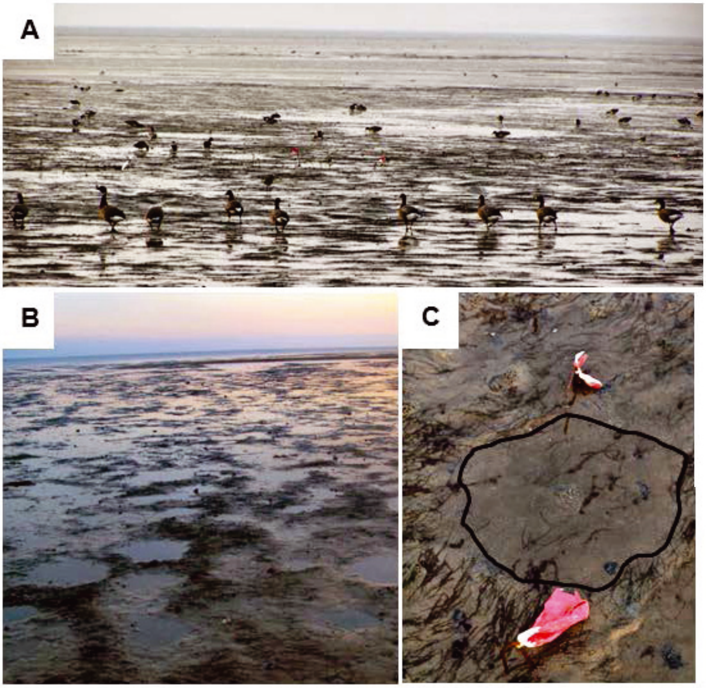

Figure 5. Waterfowl grazers of *Z. noltii* off the Island of Sylt. A. Brent geese (*Branta bernicla bernicla*), foreground and widgeons (*Anas penelope*), background. B. Characteristic feeding pits from geese foraging in the same meadow as A. C. Close- up of a sediment-filled feeding pit after 7 days (delineated by black line).

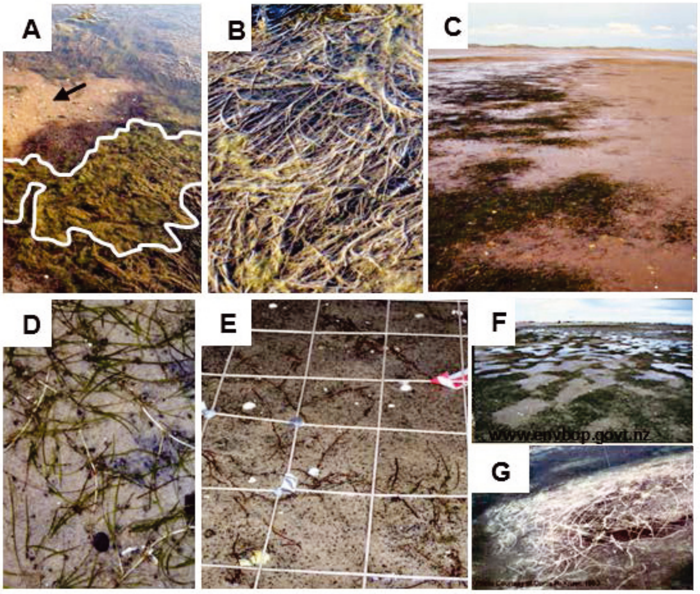

Figure 6. Disturbances characteristic of eelgrass meadows. A. Green algal mat (*Enteromorpha* sp.) on *Z. noltii* meadow (white line) generating gaps due to anoxic eelgrass die off (black arrow). B. Stressed *Z. noltii* due to epiphyte growth. C. Burial of *Z. noltii* meadow off Sylt by a mega-ripple. D. Rhizomes of *Z. noltii* exposed due to hydrodynamic sediment removal early in the growing season. E. Decaying *Z. noltii* rhizome as a result of exposure. F. Feeding trails of swans in a *Z. capricorni* bed (New Zealand). G. Boat propeller scars in a mixed seagrass bed (*Thalassia testudinum*, *Syringodium filiforme*) (Florida Keys, USA).

temperatures have already been reported for *Z. marina* (Greve et al. 2003, Reusch et al. 2005, Ehlers et al. 2008).

Conservation and restoration of seagrasses in the Wadden Sea remains difficult. Despite the presence of apparently suitable seagrass habitat, the few restoration efforts of *Z. marina* in the Dutch portion of the western Wadden Sea have hadlittle success (de Jonge et al. 2000, Bos et al. 2005, van Katwijk et al. 2009), consistent with low restoration success (<30%) world-wide (Orth et al. 2006).

However, a limited amount of self-reestablishment has been reported from earlier transplant experiments that were initially thought to have failed. Natural recruitment in the western Dutch Wadden Sea is probably constrained by the predominantly south-to-north current patterns, which isolate the western end (Bos et al. 2005, Ferber et al. 2008, van Katwijk et al. 2009). Identification of at least five management units (Palsbøll et al. 2007) for conservation of existing *Z. marina* in the Wadden Sea has been discussed by Ferber et al. (2008), but similar studies do not exist for *Z. noltii* and are addressed in this thesis (**Chapters 6 and 7**).

Reproductive strategies, demography and mating system

Vegetative reproduction (clonal growth) is extremely important for seagrasses and involves the iteration of a basic modular unit (the ramet or shoot) along a horizontal growth axis (the rhizome) (Fig. 4A, B). All ramets on a rhizome are genetically identical and comprise the genetic individual (the genet) (*sensu* Harper 1977). Genets can be physiologically integrated over large distances (Alpert and Stuefer 1997, Jónsdóttir and Watson 1997), but the breaking of a rhizome, with subsequent formation of independent genet fragments after a few months (or years) is common in seagrasses (Vermaat and Verhagen 1996, Duarte and Sand-Jensen 1990, Hemminga and Duarte 2000).

Along southern European coasts, intertidal *Z. noltii* exhibits clonal integration and apical dominance comparable to other seagrasses (Brun et al. 2006, Duarte et al. 2006, Brun et al. 2007), whereas intertidal meadows in the Wadden Sea regularly experience rhizome fragmentation. As the end of the growing season approaches during autumn, leaves of dwarf seagrass are shed and rhizome connections decay. Vegetative regrowth starts from successfully overwintered rhizome fragments in mid-April (Jacobs 1982, Jacobs et al. 1983, Philippart 1995b, Vermaat and Verhagen 1996), which often is mistaken for new recruitment (from overwintering seeds) that in actuality begins the end of March to early April and lasts at least until May (Fig. 8 C, D1-3). Vegetative expansion is an energetically inexpensive way to hold space, colonize new areas and optimize resource allocation (Alpert and Stuefer 1997, Oborny and Cain 1997), especially in resource-poor and stressful marine environments (Barrett et al. 1993, Philbrick and Les 1996, Jónsdóttir and Watson 1997). Hence, clonal propagation ensures survival and longevity of genets (Eriksson 1993, van Groenedael et al. 1996, Eriksson 1997).

The relative contribution of sexual reproduction in seagrasses, however, is highly variable. In annuals (e.g., some *Z. marina*), it is essential, whereas in perennials (most seagrasses, including *Z. noltii* and most *Z. marina*) it may be sporadic. In *Z. marina*, for example, the importance of vegetative reproduction is greater than sexual reproduction at meadow edges and at the distributional margins in southern Portugal (Reusch 2002, Billingham et al. 2003).

In contrast, studies of *Z. noltii* in the Wadden Sea (intertidal) and the Mediterranean (subtidal) concluded that vegetative (= clonal) propagation was the key to meadow maintenance and that sexual reproduction was of minor importance based on the observation of large seed-sets against little to no seedling recruitment (Jacob 1982, Hootsmans et al. 1987, Loqués et al. 1988, Buia and Mazzella 1991, Harrisson 1993, Vermaat and Verhagen 1996, Laugier et al. 1999). Tellingly, most of the aforementioned studies were based on single or short-term observations and none utilized genetic markers, which provide the only reliable way to identify clones.

Spatial mapping of genets, in combination with estimated rhizome growth rates and multilocus microsatellite genotypes allowed the first estimate of clone size and age in *Z. marina* (Reusch et al. 1998, Hämmerli and Reusch 2003a). Subsequent studies on different species and locations (*Z. marina* in the Baltic; *Posidonia* *oceanica* in the Mediterranean) have revealed the presence of one or a few giant clones covering >1000 m^2 and are estimated to be >1000 years old (Reusch et al. 1999, Ruggiero et al. 2002, Olsen et al. 2004, Arnaud Haond et al. 2012). In *Z. noltii*, no comparable mega-monoclonal meadows have been detected in the subtidal (up to 60 m^2) (Coyer et al. 2004a, Ruggerio et al. 2005) and only a few intertidal clones have exceeded 10 m^2 (Coyer et al. 2004a). In general, most intertidal clones of *Z. noltii* were ≤3 m^2 and the presence of numerous smaller clones suggests that sexual reproduction is more important for patch maintenance than previously suggested (Coyer et al. 2004a, Diekmann et al. 2005).

It should be clear that the clonal aspect of seagrasses complicates traditional demographic approaches (recruitment, mortality, growth rate, patch turnover) because of the long (to very long) life spans of genets, overlapping generations and the technical difficulty of identifying and following specific genets, including new recruits, in dense meadows. Such studies have been even more difficult in physically small seagrasses such as *Z. noltii*. Nevertheless, more detailed understanding of spatial and temporal dynamics of clones has been made possible with the development of microsatellite markers for *Z. marina* (Reusch 2000a) and *Z. noltii* (Coyer et al. 2004b). In **Chapter 2**, *Z. noltii* clones were mapped (physically and genetically) at two intertidal locations (Fig. 7) and followed over four years in population 1, and over two years in meadow 2 in order to estimate genet number and size, age, turnover rate, and new recruitment.

The mating system in the majority of seagrass species is dioecious (separate sexed individuals), a trait that has evolved multiple times in plants as a mechanism to counter selfing (Barrett 2002, Dorken et al. 2002). Nearly 20% of seagrass species (including *Zostera* spp), however, are monoecious (specifically, protogynous hermaphrodites) with each individual bearing male and female flowers contained in an infructescence or spathe (Fig 9 A, B) (Waycott and Les 1996). Pollen release and transfer are entirely aquatic (hydrophilous) and the negatively buoyant mature seeds sink immediately to the sediment upon release. Thus, pollen and seed dispersal distances (see below) are typically within a radius of 3 to 4 m with most <2 m (Orth et al. 1994, Ruckelshaus 1996, Hammerli and Reusch 2003b).

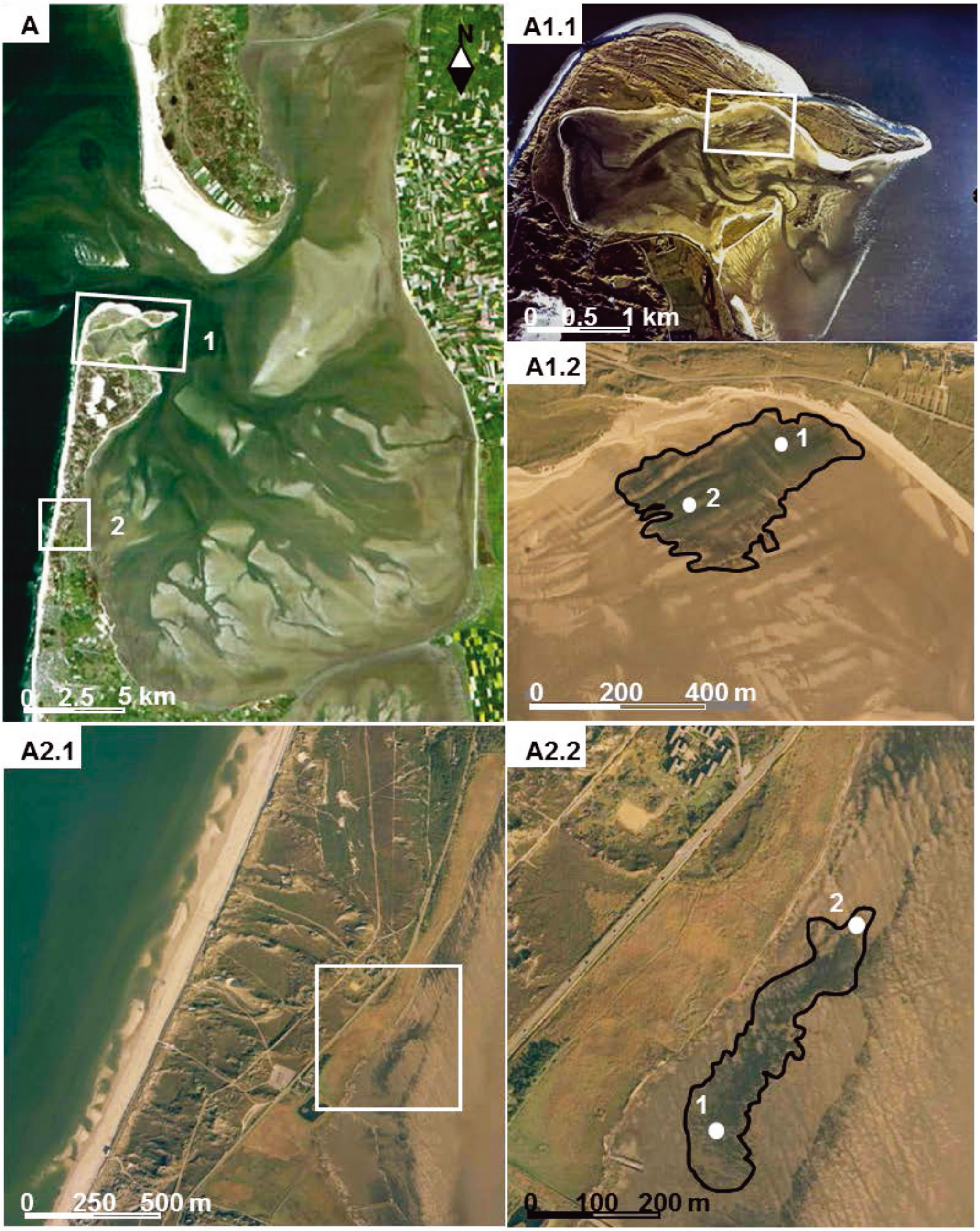

Figure 7. Study sites off the Island of Sylt. A. Königshafen (square 1) and Kampen (square 2) *Z. noltii* meadows. A1.1. Enlarged view of Königshafen. A1.2. Delineated Königshafen meadow with location of plots 1 and 2. A2.1. Enlarged view of Kampen. A2.2. Delineated Kampen meadow with location of plots 1 and 2.

Possibilities for selfing are high, especially as the size of large clones can easily exceed pollen dispersal distances (Waycott 1995, Reusch et al.1999, Reusch 2000b). Uncertain pollination conditions have been proposed as a driving force for the evolution of selfing (Barrett 2002, 2003), which may also trigger mating system evolution in self-compatible plant populations (Barrett 1998, Reusch 2001a).

Temporal separation of male and female sexual function within one spathe may facilitate self-incompatibility as suggested for *Z. marina* (Hämmerli and Reusch 2003b), although pollen from a male flower on one spathe may fertilize a female flower on another spathe distant from (but part of) the same genet (geitonogamous selfing), thereby resulting in selfing (Handel 1985, Charpentier 2001).

Outcrossing appears to dominate in both *Z. marina* and *P. australis* (Ruckelshaus 1995, Waycott and Sampson 1997, Reusch 2000b, Hämmerli and Reusch 2003a), even in large meadows, which suggests that strong selection against selfed individuals must occur early in the recruitment or post-recruitment process. Given the much smaller clone size and shorter lifespan of *Z. noltii*, especially in the intertidal, selfing may provide insurance against pollen limitation. Thus, in order to characterize the mating system of *Z. noltii* inbreeding vs. outcrossing, multiple paternity occurrence and pollen dispersal were evaluated in **Chapter 3**.

Seed banks

A seed bank ensures the survival of the population and the transmission of parents' genes from one generation to the next (Silvertown and Charlesworth 2001). Seed banks are also highly advantageous as a reservoir for genetic and genotypic diversity over time and provide a way to increase effective population size (N_e) (Tempelton and Levin 1979, Vitalis et al. 2004). For rhizomatous plants, such as seagrasses, in which just a few genotypes may dominate an area (= small N_e), a seed bank provides flexibility and insures maintenance, especially where environmental fluctuations are common (Thompson and Grime 1979, Thompson et al. 1998, Bekker et al. 1998). Three types of seed banks are generally recognized:

Type 1 (transient), seeds persist in the sediment for less than one year; Type 2 (short-term persistent), seeds persist for at least one year, but less than five years; and Type 3, (long-term persistent), seeds persist for at least five years (Thompson et al. 1997).

A strong mismatch has been observed in *Z. noltii* between the theoretical output of seeds (up to 10,000 seeds/m^2) and persistent measures of low recruitment (e.g. Hootsmans et al. 1987). Low recruitment has been attributed to massive seed loss due to tidal currents and/or the lack of suitable germination conditions—low salinity, anoxic sediments, temperature, light (Hootsmans et al. 1987, Harrison 1993). Such discrepancies between observed production and actual germination are not unusual for most plant species, but such observations do not provide evidence one way or the other for the presence of seed banks. However, Alexandre et al. (2006) estimated *Z. noltii* fertility (defined as the probability of a seed to establish a new plant) to be among the highest expected for seagrasses based on measured seed release and experimental germination data.

Most evidence for seagrass seed banks is indirect, as finding seeds in the sediment does not demonstrate the existence of a viable seed bank. Viability must be demonstrated and this has come almost exclusively from laboratory germination experiments rather than field observations (Hootsmans et al. 1987, McMillan 1991, Conacher et al. 1994). Moreover, quantification of seeds in the sediment using core-sampling methods typically leads to extremely high sampling variances because of natural patchiness. Orth et al. (2006b) reviewed the literature on seagrass seed banks and concluded that information on presence, quantity, viability and longevity to population maintenance remains ambiguous for most species.

For *Z. noltii*, seed banks have been thought to be Type 1 or transient - if existent at all - based on observations of seedling recruitment in the Wadden Sea (Jacob et al. 1981, Hootsmans et al. 1987, Vermaat and Verhagen 1996) (Fig. 9), although the studies were conducted at only two sites in the Netherlands (Zandkreek on the island of Terschelling). In the Mediterranean subtidal, the absence of a *Z. noltii* seed bank was reported by Buia and Mazzella (1991), while frequent seedling recruitment was observed by Goubin and Loques (1991). Genetic mapping of clone structure on a local scale also concluded that asexual reproduction was dominant and seed banks were absent (Ruggiero et al. 2005).

Such conflicting reports are not unexpected when studies occur at a single site at a single time. In order to resolve whether seed banks are present or absent in *Z. noltii* in the Wadden Sea, I combined temporal sampling of fixed areas over a 3-year period with genetic assignment tests to establish viability, contribution and longevity of a *Z. noltii* seed bank in the intertidal (**Chapter 4**).

Recruitment

Introduction of new genotypes into a plant population depends on successful gamete fertilization, seed germination, and seedling establishment (Fig. 8). The greatest benefit of seed dispersal is the escape of seeds and seedlings from the high density-dependent mortality that results from pathogens, seed predators, and/or herbivores directly under the parent plants (Janzen-Connell-Hypothesis) (Wang and Smith 2002, Fenner and Thompson 2005).

Recruitment strategy underlies the genotypic diversity of clonal plant populations where initial seedling recruitment (ISR) and repeated seedling recruitment (RSR) represent the endpoints of a continuum (Eriksson 1989, 1993). An ISR strategy is marked by a single recruitment event establishing a plant population with subsequent declining genotypic diversity due to mortality of genets. Under RSR conditions, however, seedlings are continuously added to the adult population thereby maintaining or increasing genotypic diversity over time.

Despite high seed production in many instances, translation into successful recruitment is typically low and appears to mainly depend on the presence of gaps in the canopy, in which recruit/adult competition is low (Fig. 5C, 6 D1+2) (Eriksson and Fröborg 1996, Kalamees and Zobel 2002). In more sparse canopies, seedling recruitment is expected to be higher, either from the seed bank or dispersal from nearby.

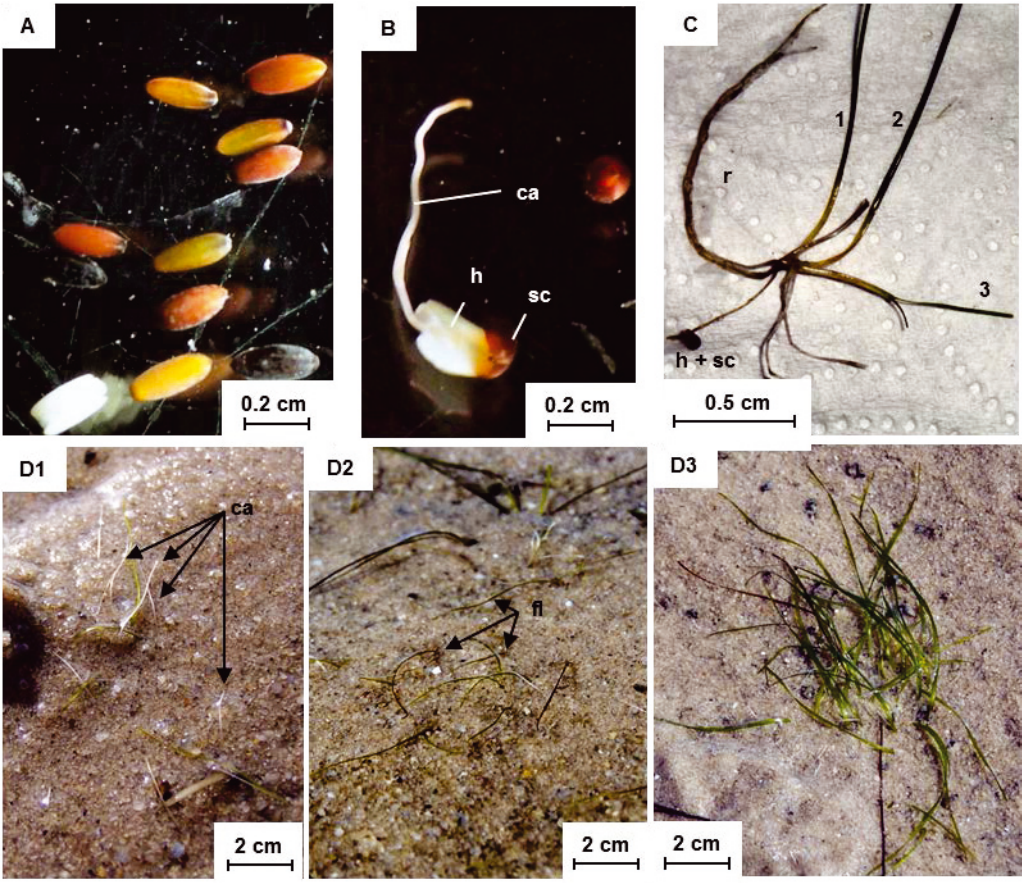

Figure 8. Seeds and seedlings of *Z. noltii*. A. variation in seed color. B. germinating seed. C. June seedling with three leaf shoots (1-3), roots, and the attached hypocotyl and seed coat. D1. First appearance of seedlings in late April. D2. Seedlings with first leaf in early May. D3. Older seedlings ready to propagate rhizomatously in mid-May (caulicle has been shed). ca = caulicle; fl = first leave; h = hypocotyl; s = shoots, r = root, sc = seed coat.

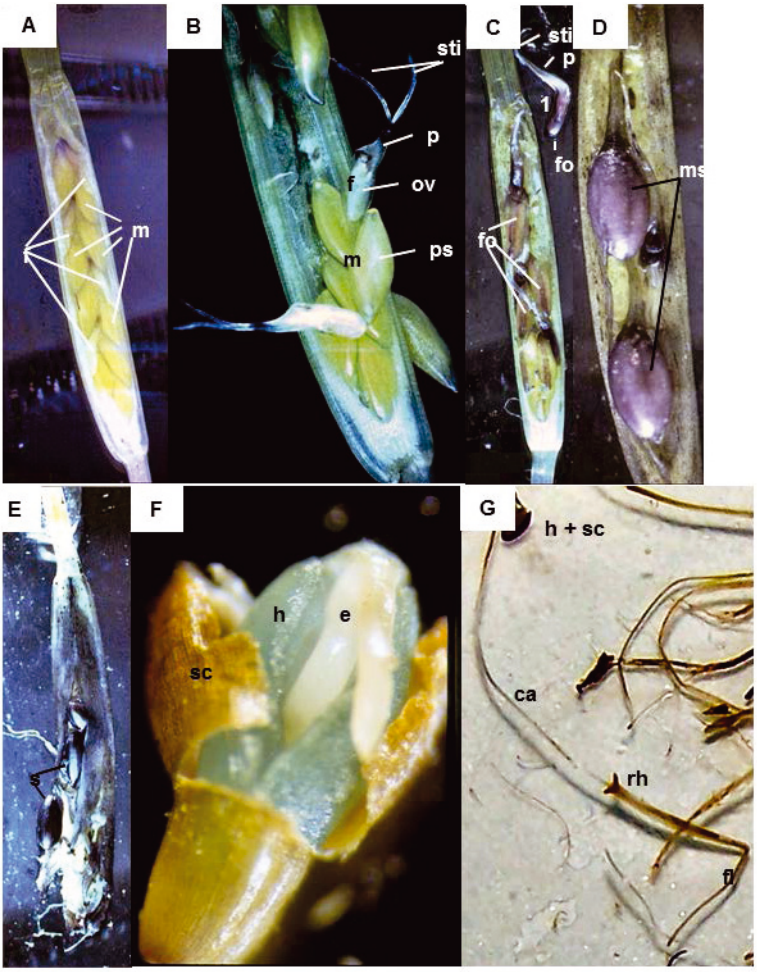

Figure 9. Flowers and seeds of *Z. noltii*. A.Closed spathe containing female (f) and male (m) flowers in alternating rows. B. Reduced flowers of *Z. noltii*; male flowers (m) consist of the pollen sack (ps) and female flowers (f) include ovary (ov), pistil (p) and bifurcated stigma (sti). C. Fertilized ovaries (fo) with a female flower still in receptive position; pistil (p) attached to the fertilized ovule projects the stigma (sti) into the water column for pollination (fo). D. Maturing seeds (ms) with reduced pistils (p). E. Mature seeds (s) ready to be shed from the spathe. F. Germinating seed with seed coat (sc), hypocotyle (h) and embryo (e). G. Early seedling of *Z. noltii* with ruptured seed coat (sc), caulicle (ca), first leave (fl), and primordial rhizome (rh).

Evidence suggests that seedling establishment in *Zostera* species is fostered by environmental forcing (Robertson and Mann 1984, Peterken and Conacher 1997, Diekmann et al. 2005) in which the canopy is reduced in density or removed altogether by disturbances, thereby forming gaps of variable sizes. For example, a *Z. marina* meadow that was extensively grazed by swans had a higher clonal diversity than an ungrazed meadow (Hämmerli and Reusch 2003a) and experimental manipulation of canopy density resulted in enhanced gap recruitment (Reusch 2006). In order to develop a better understanding of recruitment in *Z. noltii*, recruitment was followed both empirically and using temporal, genetic-assignment tests (Chapters 4 and 5).

Dispersal-gene flow

Seed dispersal is typically leptokurtic, i.e., with the highest percentages of seeds found at short distances from the parent plant and smaller percentages found at increasing distances along the flat tail of the distribution (Harper 1977, Nathan andMuller-Landau 2000). Short-distance dispersal of pollen and seeds in seagrasses, including *Zostera,* is <4 m but most often within 1-2 m and results in kinship structure (Ruckelshaus 1998, Hämmerli and Reusch 2003c, Coyer et al. 2004a). In some meadows of *Z. marina*, for example, strong kinship structure resulted in selfing rates of up to 65% in seeds in a geitonogamous neighborhood, i.e., fertilization of female flowers by males from different spathes on the same clone (Reusch 2001b). Such results suggest that inbreeding with or without loss of fitness may affect some meadows, especially where water flow is weak. At a fine-scale, knowledge of kinship and paternity can provide information about potential fitness (**Chapter 3**), about seed banks and recruitment (**Chapter 4**), and meadow isolation from gene flow.

Intermediate and long-distance dispersal may involve any combination of three mechanisms. First, seed-bearing shoots can drift in the local currents resulting in dispersal at the meadow-scale (De Cock 1980, Orth et al. 1994, Ruckelshaus 1996) and beyond. At these distances, however, direct methods of estimation (e.g. monitoring tagged plants) become less reliable and indirect measures based on genetic data are essential. Using microsatellite data, intermediate and long-distance dispersal of *Z. noltii*, as well as *Z. marina*, has been estimated at as little as 2 km and up to 150 km in the Wadden Sea (Coyer et al 2004a, Erftemeijer et al. 2008, Ferber et al. 2008). Using genetic assignment tests, Reusch (2002) was able to assign drift material to a source 30 km distant.

Second, long distance dispersal may involve floating and reattachment of rhizomatous shoots (Jacob 1982, Noten 1983). Genetic evidence has confirmed this mechanism in *Z. marina* and *Z. pacifica* populations off the coast of southern California*,* although how common it is remains unknown (Coyer et al. 2008).

A third mechanism of dispersal is zoochory or the dispersal of seeds by animals (Nathan and Mueller-Landau 2000, Jordano et al. 2007). In temperate *Zostera* meadows, migrating waterfowl have been proposed as seed dispersal vectors, but the temporal mismatch of bird migration and seed production, as well as inconclusive evidence for successful germination of seeds following gut passage, renders this dispersal strategy unlikley (Clausen 2002, Ehlers 2002). Long-distance connectivity via gene flow has been examined for *Z. marina* in the Wadden Sea (Ferber et al. 2008), but not *Z. noltii.* With this in mind, I examine population differentiation and connectivity in **Chapter 6**.

Disturbance and meadow fitness

Disturbances are a key factor in structuring many marine communities (Dayton 1971, Sousa 1984, Sousa 2001), including seagrass meadows (Larkum et al. 2006). However, the nature of the disturbances and the distinction between normal, low-level disturbance and high-level disturbance (causing stress) is only loosely defined (Rykiel Jr. 1985). In this thesis, I refer to disturbance as a process that causes damage, displacement or mortality due to physical agents or incidentally by biotic agents (Sousa 1984, 2001). Accordingly, disturbance almost always removes biomass, whereas stress reduces an organism's potential for growth (Grime 1977). For example, broad fluctuations in seawater temperatures continuously affect seagrasses in which short-term acclimation with the potential for adaptation is present (Short and Neckless 1999, Duarte 2002, Reusch et al. 2005). In contrast, however, sustained, hot-summer-events like the 2003 and 2005 European heat waves represent detrimental pulses to seagrass populations in which not only biomass is lost, but reestablishment from seeds and new genotypes is required (Reusch et al. 2005, Cardoso et al. 2008, Massa et al. 2008). Additional negative effects such as eutrophication and/or overgrowth by epiphytes can lead to biomass loss and meadow death due to light limitation (McGlathery et al. 2007), as can unusually severe winter storms and ice scour (Jacob et al 1981, Robertson and Mann 1984, Vermaat and Verhagen 1996, Reise 1998).

Disturbance, however, is not necessarily negative, as highest levels of species diversity are often associated with intermediate levels of disturbance (= intermediate disturbance hypothesis) (Connel 1978, Sousa 2001). Consequently, intermediate levels of disturbance have been suggested as a driving force of population prosperity and genetic diversity of seagrass populations (Hemminga and Duarte 2000).

Biological sources of disturbance also are important. For example, the lugworm, *Arenicola marina*, is an important bioturbating, intertidal infauna species that increases surface roughness and sediment permeability, thereby increasing sediment oxygen and grain-size, as well as altering chemical sediment properties (Volkenborn et al. 2007, Monserrat et al. 2011). Early work noted the inverse relationship between densities of the sediment-disrupting lugworm and the sediment-stabilizing *Z. noltii* (Philippart 1994b); subsequent work has revealed that the lugworm-eelgrass mosaic is more complicated and maintained by scale-dependent interactions between habitat-mediated facilitation, competition and disturbance (Eklof et al. 2011). Additional negative effects of biological disturbance occur as eutrophication and/or overgrowth by epiphytes can lead to biomass loss and meadow death due to light limitation (McGlathery et al. 2007).

Finally, disturbance brought about by canopy removal via, e.g., clam digging, can also have effects. In Portugal, *Z. noltii* increases sexual output in response to rhizome disintegration (Alexandre et al. 2005). In the Wadden Sea, grazing by waterfowl at the end of the growing season facilitated vegetative regrowth of *Z. noltii* in the following year (Nacken and Reise 2000) and gap formation had positive effects on seagrass growth and recruitment (Duarte et al. 2006, Reusch 2006). Additionally, the feeding pits created by grazing Brent geese and widgeons have been hypothesized to act as seed traps, thus enhancing seed bank formation and potential seed recruitment. A search of the accumulated sediment in aged geese pits revealed some seeds, but the link between feeding pits and seed traps could not be established (Ehlers 2002). With these results in mind, I conducted a manipulative experiment to compare seedling recruitment between artificial grazing pits and controls over a two-year period. These results are reported in **Chapter 5**.

Management and conservation

In the early 1990s, the World Conservation Union (IUCN) recognized the need to conserve biodiversity at all levels, including genetic diversity (McNeely et al. 1990). To date, however, conservation genetics (Hedrick 2003, Frankham et al. 2010) has focused mainly on endangered species, where extremely low numbers of individuals and inbreeding depression can rapidly lead to an extinction vortex (Frankham 1995, Blomquist et al. 2010). The concept of "genetic health" (Lowe et al. 2005) and its monitoring in non-endangered species has come into play in the context of general threats to biodiversity - overharvesting, habitat loss and fragmentation, pollution, invasive species and climate change - and the ability to evaluate and track population-level processes using widely available genetic markers.

In the case of seagrasses, we have the opportunity to combine two levels of population-level diversity. While genetic diversity (allelic richness, heterozygosity) provides insight into the evolutionary history of a population over many generations (hindcasting), genotypic diversity allows inferences about current ecological processes affecting the mating system, extent of clonal propagation, inbreeding depression, and gene dispersal that may also be important for forecasting potential range shifts and adaptive potential (Procaccini et al. 2007).

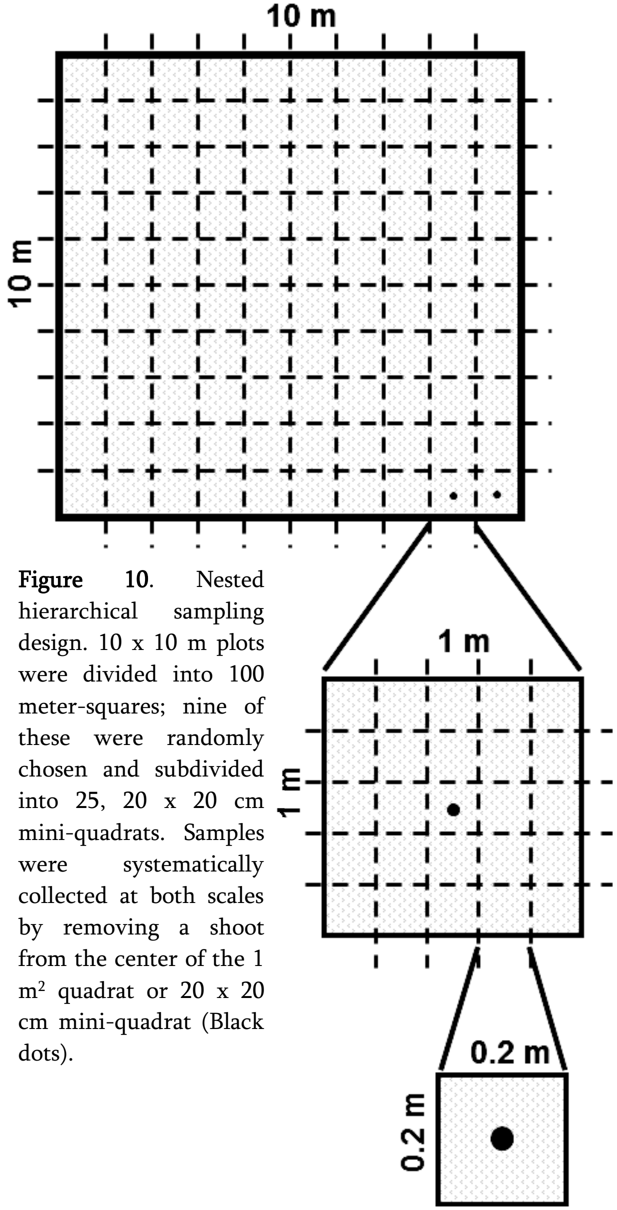

Figure 10. Nested hierarchical sampling design. 10 x 10 m plots were divided into 100 meter-squares; nine of these were randomly chosen and subdivided into 25, 20 x 20 cm mini-quadrats. Samples were systematically collected at both scales by removing a shoot from the center of the 1 m^2 quadrat or 20 x 20 cm mini-quadrat (Black dots).

There is now strong evidence that genetic and genotypic diversity contribute to population resistance, resilience, and recovery on the genetic level, as does species diversity on the community level (Frankham 2005). In seagrasses, this has been demonstrated in *Z. marina* (Hughes and Stachowicz 2004, Reusch et al. 2005, Reusch and Hughes 2006). As part of the overarching question in this thesis, we provide specific recommendations for the role of genetic data in the place-based conservation/restoration of seagrasses in the Wadden Sea and how this can best be carried out through EU and National initiatives that are already in place (**Chapter 7**).

Technical Issues

Sampling. Sampling of *Z. noltii* meadows was conducted using a nested hierarchical design (Fig. 10). Replicate 10 x 10 m plots were divided into 100 meter-square quadrats and sampled systematically by taking one sample in the middle of each quadrat. In turn, replicate 1 x 1 m quadrats within a plot were divided into 25, 20 x 20 cm mini-quadrats and sampled systematically by taking one sample in the middle of each mini-quadrat. Systematic sampling was used instead of random sampling to assess heterogeneity of plots within meadows. Based on an estimated clone size of ≤3 m^2 (Coyer et al. 2004a) it was concluded that the appropriate sampling scale, i.e., the scale that captured the genetic diversity was 100 m^2 and that collecting one individual per m^2 was appropriate for genetic diversity.

However, numerous unique multi-locus genotypes (MLG) (Coyer at al. 2004a, Diekmann et al. 2005) and a large variability in clone size and number are common. To capture clonal architecture, therefore, individuals were also collected on a scale of 0.2 x 0.2 m.

DNA extraction. DNA was extracted from silica-dried leaf material following standard protocols (Coyer et al. 2004b). A method for high-throughput DNA extraction based on a self-made silica suspension as a binding matrix was developed (Box 3) modifying the protocol described in Elphinstone et al. (2003). The self-made silica suspension was later replaced by commercially available glass-fiber plates for more consistent DNA yield.

Microsatellites. Microsatellite loci were developed for *Z. noltii* by Coyer et al. (2004b). Nine loci were used with a diversity ranging from 11 to 25 alleles/locus for a total of 149 alleles. Following Reusch et al. (1998 it was determined that a total of 1.8 x10^{19} unique multi-locus genotype (MLG) were theoretically possible in the genotyped set using the nine markers. Thus, the probability of obtaining identical MLGs by chance was low. For each pair of samples sharing the same MLG the probability of two individuals sharing the same MLG by chance P_{sex} (F_{IS}) was calculated (Arnaud-Haond et al. 2005). Using P_{sex} (F_{IS}), all samples sharing a MLG in this thesis were identified as true clone mates.

A new multiplex PCR strategy was developed involving 3 PCR reactions of loci triplets in 20 µl reaction mix (Table 1). Conditions for a 20 µl PCR reaction were 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.

Box 3: Inexpensive, fast DNA extraction (Ferber S, Zipperle AM)
(After Elphinstone et al. (2003) Mol Ecol Notes 3:317-320)

Preparations:

Digestion buffer (100mL):
- a) 2.5mL 4 M NaCl
- b) 5.0mL 1 M Tris HCl (pH 8.0)
- c) 2.0mL 0.5 M EDTA
- d) 2.5mL sodium dodecyl sulphate (SDS)
- e) Deionized water to 100mL
- f) 40mg proteinase K (add fresh daily)
  20µL/100mL

Wash buffer (100mL):
- a) 1.0mL 1 M Tris HCl (pH 7.4)
- b) 100µL 0.5 M EDTA
- c) 1.25mL 4 M NaCl
- d) 50mL 100% ethanol
- e) Deionized water to 100mL

Binding buffer (100mL)
- a) 2mL NaI 90.8 g
- b) Na_2SO_3 1.5 g
- c) Deionized water to 100mL
- d) Filter (use conventional paper filters)

Elution buffer (100mL):
- a) 1.0mL 1 M Tris HCl (pH 8.0)
- b) 200µL 0.5 M EDTA
- c) Deionized water to 100mL

Silica suspension:
1. Add 10g silica fines(silica gel 60, 15-40µm, 115111, Merck, Germany) to 200 mL 5 M nitric acid (HNO_3) (63.3mL of 65% HNO_3-solution, with deionized water to 100mL)
2. Carefully heat to near boiling in a fume hood
3. Let mixture cool down for 30min.
4. Discard supernatant
5. Resuspend silica in 100mL distilled water and incubate 10min. @ RT
6. Discard supernatant and repeat washing 3 times

7. Add 100 mL distilled water and buffer suspension to pH 7 with 1 M Tris.HCl (pH 8.0)
8. Incubate 10min. @ RT
9. Discard supernatant
10. Resuspent silica in equal volume of milliQ water
11. Autoclave the suspension

Procedure:

1. Place tissue samples in tubes from collection tube rack (96 well plate) and add one thungsten carbide bead in each individual well
2. Grind tissue samples with the Retsch Mixer Mill MM301 1min at 20Htz
3. Turn tube rack 180 degrees and grind 1min at 20Htz
4. Add 500µL digestion buffer
5. Incubate 2h @ 55°C
6. Centrifuge 5min @ 3000 rpm
7. Mix binding buffer (150µL/ind.) and silica suspension (20µL/ind.) in an individual tube in and add 170µL of the mixture to individual wells of the filtration plate (or use commercially available 96-well glass-fiber filter plates (Millipore MultiScreen HTS))
8. Add 50µL aliquot of each supernatant in individual wells of the filtration plate
9. Mix and incubate for 3min (binding of DNA to silica)
10. Centrifuge filtration with waste plate for 5min @ 3000 rpm
11. Add 200µL ice-cold wash buffer in individual wells of the filtration plate
12. Centrifuge filtration with waste plate for 5min @ 3000 rpm
13. Repeat wash step *(go to step 11)*
14. Centrifuge filtration plate for 1min @3000 rpm to remove the last drops of wash buffer
15. Install a sterile 96-well PCR titer plate under the filtration plate
16. Add 200µL elution buffer (60°C)
17. Incubate for 3min
18. Centrifuge filtration plate for 5min @ 3000 rpm

Method tested successfully for: vascular plants, algae, fungi, crustaceans, and fish.

Table 1: Multiplexing conditions for microsatellite loci.

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

Paternity and assignment. Gamete dispersal measures using molecular markers involve the determination of parents' genotypes and the genotypes of a representative sample of the offspring (Ouborg et al. 1999). Three different approaches are typically used for parentage analysis (reviewed in Jones and Ardren 2003):

1. Exclusion: based on incompatibilities between parent and offspring to reject particular parent-offspring hypotheses. The method is most powerful with few candidate parents and highly polymorphic genetic markers. Reliability of a strict exclusion approach decreases with increasing number of loci needed for complete parent exclusion because genotyping errors, null alleles and mutations would lead to false exclusions.

2. Categorical and fractional likelihood
    a. Categorical allocation: selects the most likely parent from a pool of non-excluded parents by calculating a logarithm of the likelihood ratio (LOD) and assigning offspring to the parent with the highest LOD.

b. Fractional allocation: Requires the calculation of a *prior* probability of paternity. Likelihoods are than calculated same as for the categorical allocation. A fraction between 0 and 1 is assigned to all non-excluded candidate parents.

3. Genotypic reconstruction: uses the MLG of parents and offspring to reconstruct the genotypes of unknown parents contributing gametes to a progeny array for which one parent is known (Jones 2001). Computational intensity limits the number of fathers per array to six and accuracy is hampered by genotyping errors, null alleles and mutations.

To assess small-scale pollen dispersal, I used a likelihood-based categorical paternity assignment (2a) including the determination of the statistical confidence of the assignments (**Chapter 3**). This method is superior to exclusion based approaches (1) when multiple males are non-excluded, which was to be expected in *Z. noltii* due to an expected small-scale kinship structure (Marshall et al. 1998).

Fractional likelihood methods (2b) are superior to categorical paternity exclusion due to the possibility of population scale assessment of paternity with markers of low discriminatory power and the maximum use of information provided by the data. However, the method systematically underestimates variance in the male reproductive success and does not represent the biological truth, since fractional parentage does not exist (Smouse and Meagher 1994, Jones and Ardren 2003). The categorical likelihood-based paternity assignment was preferred in this thesis because of the increase in determination power using highly polymorphic microsatellites and the small scale of the study. Additionally, the available software has frequently been updated to improve the quality of the assignment (e.g. allowing for inbreeding and self-fertilization, consideration of genotyping error rate) (Kalinowski et al. 2007). Because MLG similarity of *Z. noltii* is to be expected on small scales due to kinship structure caused by limited seed dispersal, the approach of genotypic reconstruction (3) was deemed inappropriate for paternity assignment in this thesis.

In the categorical approach, applied paternity is inferred by determining the male with the highest log-likelihood ratio (LOD-score). The LOD-score is the likelihood of paternity of a particular male relative to the likelihood of paternity of an arbitrary male. Confidence of assignment is defined by the log-likelihood statistic Δ (i.e. the difference in LOD scores between pairs of the most likely fathers). Paternity inference and Δ are based on simulations of generated parental genotypes and derived offspring genotypes from the allele frequency of the data set. Simulations are used to determine LOD-scores for the real data set, as well as to generate genetic data. The generated parental genotypes are then used for paternity inference of a large number of simulated offspring in order to generate Δ-distributions. The simulation considers the number of candidate males, the proportion of males sampled, the proportion of loci typed, and the error rate in the real data set. In the final step, the simulation determines critical values for Δ to test significance of Δ values found in the real data set. Distributions of Δ-scores are compared for cases where the most-likely father was the true father relative to cases where the most-likely father was not the true father. Confidence is derived from the overlap of Δ-distributions. Considering a 95% confidence criterion, the Δ value is chosen such that 95% of Δ-scores exceeding this particular value are obtained by true fathers (Marshal et al. 1998, http://www.fieldgenetics.com/pages/aboutCervus_Method.jsp.).

Genetic assignment methods can be used for structure and relationship analyses at an individual level and generally aim for an assignment or exclusion of reference populations as origin for an MLG (Estoup and Angers 1997). More recently, assignment methods were used for the assessment of real-time dispersal by the detection of immigrants into populations (Paetkau et al. 2004, Coyer et al. 2006).

In order to characterize a *Z. noltii* seed bank as transient or persistent, I assigned MLGs of seedlings originating from a patch and sampled in different years to MLGs of the adult population from the same patch sampled in different years (Chapter 4). The applied assignment method is based on a Bayesian approach (Rannala and Mountain 1997, Paetkau et al. 2004). A Monte Carlo resampling is used to calculate a statistical threshold for exclusion of an individual from a population. A distribution of genotype likelihoods of the reference population is generated and compared to the likelihood of the individual in question.

Box 4: Working with small plants in the muddy intertidal:
Nature, 1; Field biologist, 0.

Ecological fieldwork is sometimes frought with so many obstacles that it is hard to imagine why anyone would want to do it. The soft-sediment marine intertidal is one of those habitats that pose a special challenge to the species who live there and the scientists who try to study them. While the species have evolved adaptions to the alternating physical conditions during tidal emersion and immersion, scientists must fight the tides (which often occur too early or too late and are not sufficiently low) and slog through glue-like sediment (Reise 1985) or "whipped-cream mud" up to the knees.

In many cases, it was virtually impossible to manipulate a *Z. noltii* meadow without destroying it in the process. Intertidal forms of *Z. noltii* are small (5 - 25 cm) with narrow leaves (0.5 – 2 mm) and slender rhizomes (0.5 - 2 mm dia) (Den Hartog 1970). Marking individual seagrass shoots and leaves, therefore, is difficult under the best of conditions; and even if successful, subsequent monitoring is like looking for a needle (= marked shoot) in a haystack (= dense and thick carpet of green leaves) (Fig. 4C).

Marking study sites or plots adds another dimension of uncertainty, as tides, currents, sediment mobility, and wave action collectively remove most external markers (straws, sticks, metal pins, flags etc.) not big enough to be rigidly fixed (even rebar poles pounded in to 75 cm deep where sometimes not enough). Increasing the size of marker usually is not feasible, as the bigger the marker, the higher the probability of directly or indirectly impairing proximal shoots by whiplash activities caused by tidal drift (algae, seagrass, plastic bags etc.) snared by the markers.

Marking and relocating individual shoots are unnecessary, however, if one focuses on the whole clone as the evolutionary individual and uses molecular markers. The caveat is that the process of selecting 100 individual shoots from each of the plots entailed a minimum of 400 deep knee bends in the soft mud (e.g., repeated kneeling with bended back and face in the mud, and walking (not crawling) from site to site so as to not to destroy/disturb the patch). Temporal sampling, of course, requires the whole process to be repeated on a monthly basis, regardless of weather.

Clone sizes determine the scaling of field experiments because experimental units need to exceed clone (genet) size in order to include more than one genet. Establishing and maintaining the various and essential structures (e.g. cages, marking stakes, lines) at the appropriate scale over a 2-4 yr period was labor intensive and required nearly daily monitoring/cleaning, as every solid structure rising above the sediment level captures tidal drift that can/will smother experimental units (Fig. 11 B1, D2).

Assessment of *Z. noltii* reproduction presented yet another set of challenges. The small fruits (spathes) bearing male and female sexual organs cannot be distinguished in the field as identification requires a 25x stereomicroscope (Fig 9). The small (1-2 mm) and negatively buoyant brown seeds are nearly invisible in the sediments (Fig 8A). Seeds germinate about one month prior to the start of the growing season and seedlings are easily confused with overwintering shoots (Fig. 8 D1-3). Seedlings are characterized by a white, threadlike caulicle (<1 mm width), but the first reliable indication of a seedling is the first leaf (Fig. 8 D1). Once the caulicle is shed, seedlings cannot be distinguished from young vegetative shoots (Fig. 8C, D3).

And finally, even the best experimental design and the most careful maintenance through all sorts of weather conditions, can prove irrelevant in the face of extreme natural events. The 2003 pan European heat wave, coupled with a long-term increase in sediment mobility, nearly exterminated my study sites between 2004 and 2006 and substantially influenced the intial goals and some of the outcomes of this thesis.

“Murphy’s Law” (=anything that can go wrong will go wrong) also played a role. A large-scale geese-exclusion experiment was established in late summer 2004 to determine the effects of waterfowl grazing in autumn on clone structure and genotypic diversity in the subsequent spring growing season.

Each experimental unit consisted of a cage (treatment), an open cage (treatment control) and an untouched control (Fig. 11 A1-3). Each treatment and control was 3 x 3 m in size. Experimental units were replicated 8 times and randomly placed in an intertidal seagrass bed dominated by sandy sediments. Density of vegetative and generative shoots was assessed at each of several time points and tissue was collected for molecular genotyping. However, in the winter of 2005, ice scour

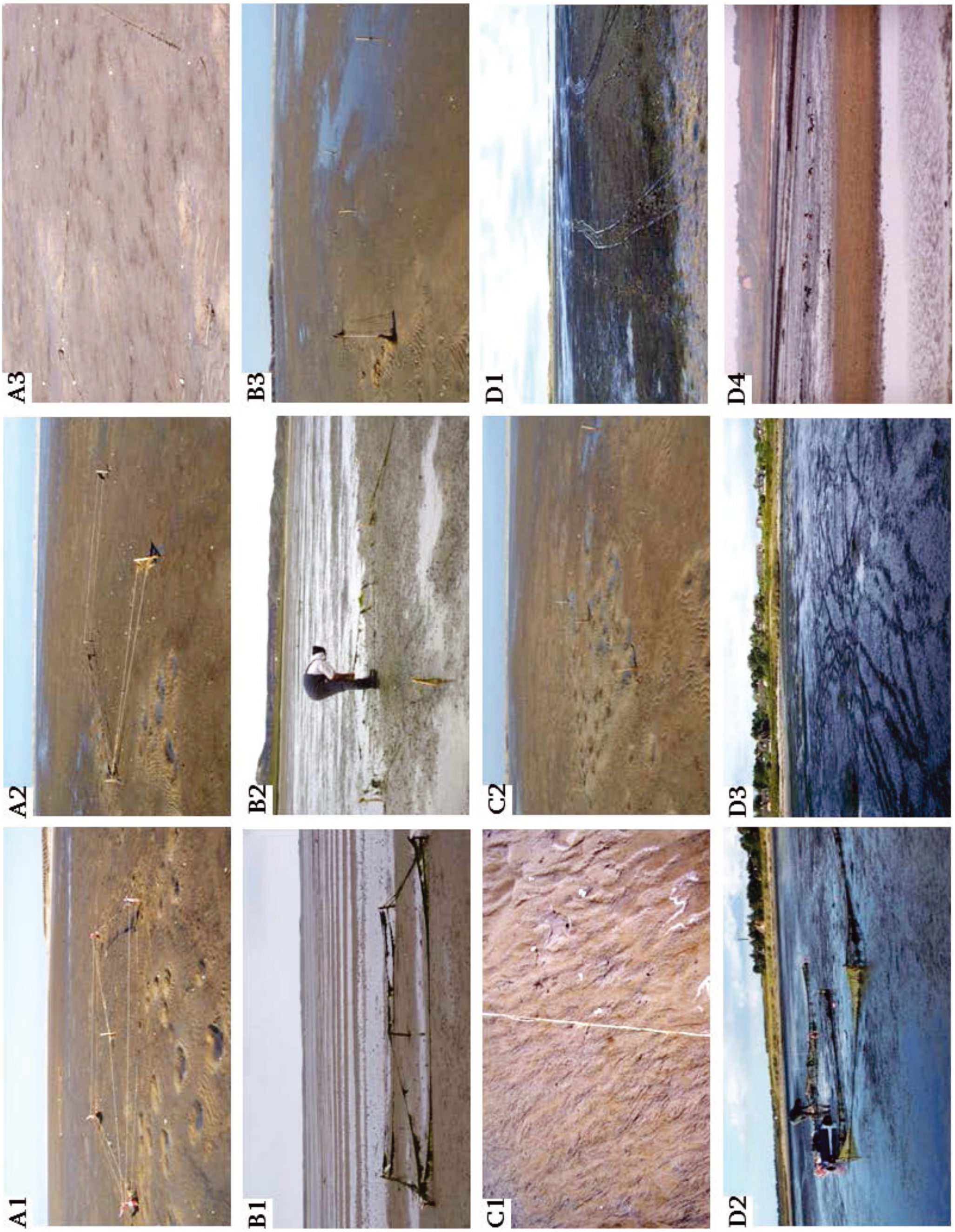
A1
A2
A3
B1
B2
B3
C1
C2
D1
D2
D3
D4

Figure 11 (previous page). Geese exclusion experiment. A1-3, experimental set up: A1= treatment cage, A2 =Treatment (cage) control, A3 = control. B, maintenance of damaged cages after the winter: B1, algae-smothered cage; B2, cage cleaning; B3, Cage destroyed by winter storm. C, Success and loss: C1, density differences in spring between inside (left) and outside (right) cages; C2, full-scale removal of seagrass in an unrepaired cage (3 low tides). D, second attempt of exclusion experiement in 2005: D1, experimental sites located in a sheltered, muddy-sediment seagrass patch; D2, maintenance of experimental structures; D3, Trails of waterfowl; D4, comparison of site in October 2005 to D2, with grazing geese and widgeons in seagrass bed adjacent to experimental site.

destroyed two experimental units and severely damaged several cages (Fig.11 B1-3).

Nevertheless, seagrass density was significantly higher within the remaining cages compared to the grazed controls in March 2005 (Fig. 11 C1). As weather conditions and the tidal cycle prevented cage repair, grazing geese (which were not supposed to be present) removed biomass from the majority of cages, thereby ruining the experiment (Fig. 11 C2). In fact, the entire experimental area failed to grow back in 2005. In an effort to persevere, the experiment was set up again in the Fall of 2005, this time in a very dense and sheltered *Z. noltii* patch partly submerged during low tide and growing in very muddy sediments (Fig 11 D1-2). But this time, the geese ignored the patch entirely (most likely due to the muddy sediments and the partial submergence), instead focusing on and nearly eliminating an adjacent patch on mostly emerged sandy sediment (Fig. 11 D3-4). Final score: Nature 1; field biologist 0.

Outline of the thesis

Using fine-scale genetic mapping in **Chapter 2**, I examine the contribution of clonal vegetative and sexual reproduction to meadow structure through time. I use temporal sampling in a hierarchical nested design to determine clone size and number, as well as to assess genet turnover rates during a 4-yr period. Temporal clonal mapping has rarely been attempted in seagrasses and has never spanned two growing seasons, primarily because of long life spans and large clone sizes in large, slow growing seagrass species that have been studied.

Average clone size of *Z. noltii* is >3 m^2 throughout its distributional range. Because of the strong seasonality and stochasticity of the intertidal habitat, I expected a highly dynamic clone structure and increased contribution of recruitment from seeds to fill gaps resulting from natural mortality and disturbances (both biotic and abiotic). Several aspects of *Z. noltii* (size, life span, etc.) provide a unique opportunity to directly track demographical processes in a clonal plant. The extended assessment of genet turnover will give an estimate for the resilience of established clones and the introduction of new clonal individuals through sexual reproduction. The molecular evidence is supported by direct observation of seedlings.

The determination of clone size, number, and turnover, served as a baseline for a subsequent investigation of the mating system (**Chapter 3**) and dynamics of the seed bank (**Chapter 4**). I examined the mating system in **Chapter 3**, by assessing the role of kinship and possible inbreeding. Here, I conducted a detailed mating system analysis using MLG of mother genets and a corresponding set of seeds. As inbred seeds foster inbreeding depression, thereby influencing mating system evolution, the relative contribution of selfing vs. outcrossing was determined. More importantly, the rate of self-fertilization influences the genetic diversity and gene flow among seagrass patches. Therefore, I distinguished between self-fertilization (geitonogamy) and bi-parental inbreeding to reveal the impact of a kinship structure on pollination. In addition, I assessed patterns of multiple paternities and the number of pollen donors as a proxy for pollen availability from the genetic neighborhood.

Using paternity analysis, I attempted to locate potential sires to individual seed-mother pairs, incorporating the fine-scale mapping of genotypes on two hierarchical scales. By spatial localization of father genotypes relative to mother-seed-pairs, I estimated pollen dispersal distance and assessed the relative importance of pollen import from outside the studied area. Finally, mating system and pollen dispersal on a patch scale were correlated to the spatial and temporal clone structure of *Z. noltii* in order to explain patterns of genetic diversity and gene flow evident on the Wadden Sea scale.

Also in **Chapter 4,** I determined the presence and persistence of seed banks over a 3-yr period and their contribution to recruitment and meadow turnover. Sediment cores collected monthly over a 2-yr period were analysed to establish the permanent presence of sediment seeds as a first indication of a seed bank. I then examined seed germination in both the laboratory and field to demonstrate seed bank viability and to estimate the proportion of the seed bank that germinates and its annual contribution to the patch.

To determine whether the seed bank was transient or persistent, I used assignment tests based on the likelihood that a particular seedling genotype originates from a particular patch. Seedling cohorts and adults from a source patch were sampled in subsequent years and genotyped. The temporally sampled cohorts of seedlings were then assigned to the adults of different years to reveal: 1) whether or not the seedling originated from the source patch, and 2) the year the seed most likely was produced in the patch. These analyzes determined the longevity of *Z.noltii* seeds and characterized the seed bank as transient or persistent.

Given the substantial impact of grazing by migratory geese and other waterfowl, I explored the effects of grazing disturbance on seagrass recruitment and meadow growth in **Chapter 5**. In a field experiment, I simulated feeding pits created by waterfowl grazing on *Z. noltii* (leaves, shoots, rhizomes) in autumn to evaluate the impact of feeding pits on seedling emergence in the subsequent spring. Feeding pits created by grazing Brent geese (*Branta bernicla* L.) disrupted rhizome connections and generated gaps in the meadow that are essential for subsequent seedling recruitment. Additionally, a previous study indicated that *Z. noltii* seeds accumulated in the geese feeding pits in autumn prior to being buried by sediments. Therefore, I expected geese feeding pits to act as seed traps in autumn and that there would be a higher rate of spring germination within experimental pit treatments compared to ungrazed control treatments.

Given that local dynamics interact to create regional dynamics, population connectivity was examined for the entire Wadden Sea in **Chapter 6** and compared with a similar Wadden Sea-wide study of *Z. marina.* Population structure and gene flow among 13 and 10 populations of *Z. noltii* and *Z. marina*, respectively, throughout the Wadden Sea and were analysed using a Bayesian approach implemented in the software STRUCTURE 2.3 (Falush et al. 2003). Using the determined population structures for an inter-species comparison, I examined the importance of population history, life-history, and the physical environment, as driving forces for observed patterns of connectivity and gene flow in the two congener species sharing the same habitat in the Wadden Sea. Further, I attempt to relate the regional population structure and gene flow to the small-scale population dynamics described earlier in this thesis. Recommendations for management and the establishment of management units for *Z. noltii* in the Wadden Sea are proposed.

In **Chapter 7** I summarize my results and conclude with considerations about how management and conservation of intertidal seagrass meadows can be informed by a genetic evaluation and propose specific recommendations for the Wadden Sea. I also suggest further experiments that may contribute to a fuller understanding of the complex population dynamics influencing persistence/resilience of intertidal seagrasses.

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Chapter 2

Clonal architecture in an intertidal bed of the dwarf eelgrass Zostera noltii in the Northern Wadden Sea: persistence through extreme physical perturbation and the importance of a seed bank

Abstract - Genotypic structure and temporal dynamics of the dwarf seagrass, *Zostera noltii*, were studied in an intertidal meadow that has persisted since prior to 1936 near the Wadden Sea island of Sylt. Samples were collected from two 10 x 10 m plots separated by 250 m from May 2002 to June 2005 and from four 1 x 1 m plots from June 2003 to September 2004. All the samples were genotyped with nine microsatellite loci. No genotypes were shared between the plots separated by 250 m. Genetic diversity was higher in the Wadden Sea than in the other regions of its geographic range. The average clone size (genets) (SD) in the two plots was 1.38 (0.26) and 1.46 (0.4) m^2, respectively, with a range up to 9 m^2 and <20% persisted for >4 years. A high genetic and genotypic diversity was maintained by annual recruitment of seedlings despite a dramatic decrease in ramet density that coincided with the severe heat stress event of 2003. Fine-scale (1 m^2) analysis suggested that extensive loss of seagrass cover precluded space competition among the genets, while a persistent seed bank prevented local extinction. Long-term persistence of *Z. noltii* meadows in the intertidal Wadden Sea was achieved by high genet turnover and frequent seedling recruitment from a seed bank, in contrast to the low diversity observed in large and long-living clones of *Z. noltii* and other seagrasses in subtidal habitats.

Zipperle et al. (2009) Clonal architecture in an intertidal bed of the dwarf eelgrass *Zostera noltii* in the Northern Wadden Sea: persistence through extreme physical perturbation and the importance of a seed bank. *Marine Biology*. 156, 2139-2148

Introduction

The dwarf eelgrass, *Zostera noltii*, is the most important seagrass in the sandy to muddy intertidal of the NE Atlantic, where it forms meadows spanning tens to thousands of square meters in size (Fig. 1). Some meadows consist of several distinct genets (genetic individuals or clones), whereas others are comprised primarily of a dominant genet with high numbers of ramets (morphological individuals arising from vegetative reproduction sensu Harper 1977). Clone size and number are important measures for reproductive efficiency of seagrasses in general, as limited pollen dispersal, combined with large clone sizes and few neighboring clones, may reduce the outcrossing potential within meadows (Barrett and Harder 1996, Charpentier 2001, Reusch 2001a). In the self-compatible genus Zostera, limited outcrossing increases the rate of inbreeding and may lead to inbreeding depression and degradation of population fitness (Ellstrand and Elam 1993, Reusch 2001a, Hämmerli and Reusch 2003). Clone size and density also directly affect recruitment success from seeds through space and resource competition, and hence genetic and genotypic diversity (Eriksson 1993). Consequently, persistence of extensive and locally adapted genets can lead to a reduction of evolutionary potential to changing environmental conditions (Silvertown and Charlesworth 2001, Hämmerli and Reusch 2002).

Determination of clonal or patch architecture requires variable molecular markers such as microsatellites, which provide the only reliable means of distinguishing genets (clones) among the sea of ramets (shoots) (Reusch 2001b, Arnaud-Haond et al. 2005). Molecular population genetic studies on several seagrass species have not only uncovered clonal structure, but revealed patterns of gene flow and population connectivity, neighborhood size, local adaptation, and the mating system (reviewed in Waycott et al. 2006, Procaccini et al. 2007), thus providing a snapshot of pattern and process at the landscape scale.

Temporal studies of seagrass clonal architecture, however, are not common, as the natural genet longevity of the most intensively studied large seagrass species (some *Zostera marina*, most *Posidonia australis*, and all *Posidonia oceanica* populations) ranges from decades to centuries or even millennia (Waycott 1995, Reusch et al. 1999, Ruggiero et al. 2002). To our knowledge, no study has examined temporal architectural changes of seagrass genets in a fixed area for more than 1 year.

An important component of temporal stability of plant populations is the presence/absence of a seed bank. Seed banks constitute reservoirs of genetic and genotypic diversity in established populations and may enhance recovery after population disturbance. Furthermore, recruitment from a seed bank can strongly influence population structure and genotypic diversity (Koch et al. 2003, Barrett et al. 2005, Greve et al. 2005). Seeds of *Z. noltii* are produced annually through subaquatic pollination of protogynous, hermaphroditic flowers. The four to six male and four to six female flowers are combined in a single spathe and release four to six negatively buoyant seeds (den Hartog 1970). Seeds enter the seed bank either directly upon release or after dispersal. While seeds typically are dispersed on the scale of a few meters (Orth et al. 1994, Ruckelshaus 1996), seeds may be more widely dispersed via floating spathes or rafting shoots of *Z. noltii*, thereby ensuring gene flow among distant populations (Coyer et al. 2004a, Erftemeijer et al. 2008) (Reusch et al. 2002, Ferber et al. 2008 for *Z. marina*).

Seed banks of *Z. noltii* persist for at least 3 years near the island of Sylt in the northern Wadden Sea and contribute up to 20% of the annual new recruitment (Zipperle et al. 2009). Therefore, an established meadow or large patch of *Z. noltii* could persist more or less indefinitely and indeed, decades-old meadows are known (Nienburg 1927, Wohlenberg 1935, Reise and Kohlus 2008). However, even large meadows wax and wane over the course of years (Philippart and Dijkema 1995, Zipperle, personal observation). Meadow persistence depends in part on large-scale stochastic physical factors such as the direct effects of increased hydrodynamics due to storms (Schanz and Asmus 2003), or indirectly by sediment mobility (burial, erosion) (Hemminga and Duarte 2000, Cabaço and Santos 2007, Reise and Kohlus 2008), as well as fine-scale biological disturbances associated with grazing by waterfowl (Jacobs et al. 1981, Nacken and Reise 2000). Intrinsic instability of *Z. noltii* patches also may be caused by a demographic factor. For example, once a few large clones have achieved dominance (the closely spaced ramets exhibit phalanx growth, Brun et al. 2006), the low genotypic/ clonal diversity may render them more susceptible to new stress events. In order to gain insight into the ecological and evolutionary implications of temporal changes in the large- and fine-scale genetic and genotypic composition of seagrass meadows, an intertidal meadow of *Z. noltii* was repeatedly mapped at two permanent plots at two spatial scales over a 4-year period. We examined: (1) spatial and temporal stability of meadow composition, (2) the relative importance of sexual/clonal input, and (3) the importance of a seed bank in maintaining genotypic diversity through physical perturbations.

Materials and methods

Study site and sampling

The study was conducted within an intertidal meadow of *Z. noltii* in the shallow bay of Königshafen on the island of Sylt, Germany (55°2´N, 008°25´E) (Fig. 1). The semidiurnal tides (mean tidal range is 1.8 m) leave the meadow exposed for 6–8 h during the low-tide period and covered with a mean water depth of 0.5 m during high tide. Large-scale mapping of clonal architecture was determined in two permanent 10 x 10 m plots, separated by 250 m (Fig. 1: Plots 1 and 2). Samples were collected at the start of growing season (May/June) and at the end of growing season (September) over seven time points from May 2002 through June 2005.

Samples were collected at four time points in May and September of 2003 and 2004. Each of the four 1-m^2 subplots was further subdivided into 0.2 x 0.2 m quadrats, and one sample was taken from the center of each quadrat for a maximum of 25 samples. Again, if no plant was present in a 10-cm radius around the center of a quadrat then no sample was taken. All plant material was stored in silica crystals. Seagrass density was estimated as percent-cover at low tide at the

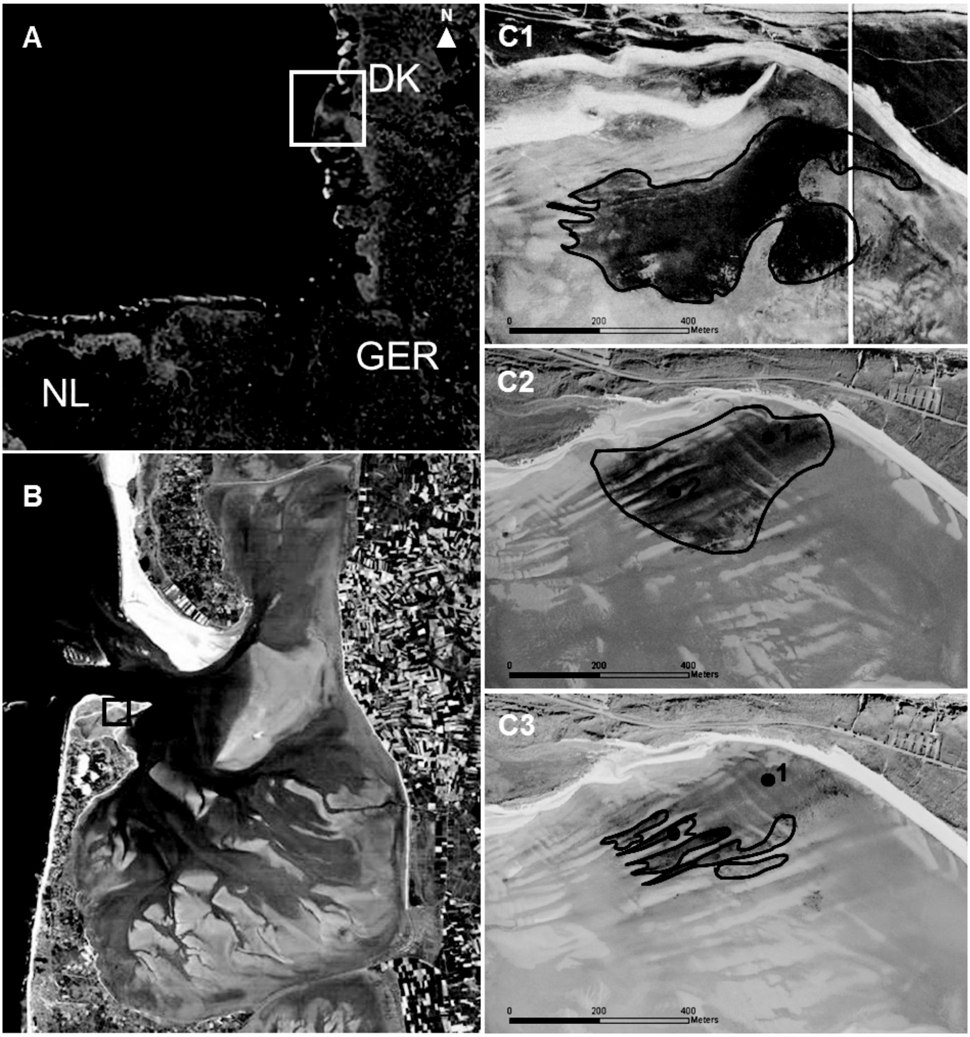

Figure 1. Study site. A. Island of Sylt (white square) in the Northern Wadden Sea. B. List tidal basin with study site (black square) in Königshafen off the northern tip of the island. C. *Z. noltii* meadow in Königshafen: C1, 1936, C2, 2003, and C3, 2004 after the large decline. Seagrass bed boundaries are outlined. Locations of Plots 1 and 2 are indicated in C2 and C3 (black dots). (Aerial photographs C1–3: AWI-Bremerhaven, Germany)

time of plant collection. Seedlings, identified by the emerging caulicle and/or the still attached seed as described by Zipperle et al. (2009), often were collected from the center of a subplot/quadrat during the early growth season (June 2004, 2005). Sampled seedlings displayed only restricted vegetative propagation in June (Fig. 2).

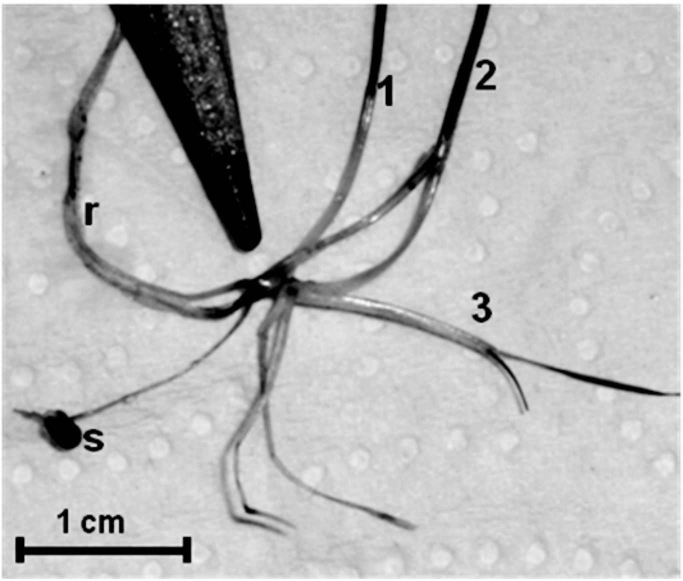

Figure 2. Vegetative propagating *Zostera noltii* seedling in June. Attached seed, three vegetative shoots (1–3), and roots (r) are visible. Elongated rhizome internodes are lacking.

DNA extraction, microsatellite amplification and genotyping

Five mg of plant tissue was pulverized with a Retsch Mixer Mill MM 301 (Qiagen) and DNA was extracted following the protocol of Elphinstone et al. (2003). Nine microsatellite loci (Coyer et al. 2004b) were amplified as three triplets (plex-1: ZnB1, ZnH10 and ZnB3; plex-2: ZnB8, ZnH8 and ZnD6; plex-3: ZnE7, ZnF8 and ZnF11) using multiplex PCR in 20 µl reactions (2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U *Taq* (Promega), 0.01% bovine serum albumin, 1x reaction buffer (10 mM Tris HCl, 50 mM KCl, 0.1% Triton X-100,) (Zipperle et al. 2009). PCR reaction conditions were the same for each primer triplet: initial denaturation 3` at 94°C, followed by 35 cycles 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. Multiplexed primer triplets were visualized by gel electrophoresis (ABI 377 gene analyzer, Applied Biosystems) and scored against an internal lane standard (ROX 350, Applied Biosystems) using the GENSCAN software (Applied Biosystems).

Data analysis

Probabilities of identity by chance (P_{sex} (F_{IS})) were calculated for each sample to avoid false assignment of individual shoots (ramets) sharing the same multilocus genotype (MLG) by chance (GenClone 2.0, Arnaud-Haond and Belkhir 2007) to the same genet (clone). P_{sex} (F_{IS}) accounts for departure from Hardy–Weinberg and provides the most conservative estimates of clonal identity (Arnaud- Haond et al. 2007). Clone maps were generated with GenClone 2.0 (Arnaud-Haond and Belkhir 2007) using xy-coordinates of individual samples collected within the 10 x 10 m plots and the four 1-m^2 subplots. GenClone 2.0 also assessed genetic and genotypic diversity (R) for each sampling. R was calculated as $R = G/N$; where G is the number of genets present and N is the number of ramets sampled.

The genets were tracked from May 2002 through June 2005 in the 10 x 10 m plots to estimate their survival time and mean size. Only the genets found on multiple occasions were used to estimate genet longevity, since they were considered the dominant genets within the quadrat. The mean genet size (per plot) of *Z. noltii* was estimated by averaging sizes of genets found multiple times for each sampling date and calculating the grand mean over all samplings. A minimum estimate of genet survival was achieved by averaging the survival times of individual genets in the study period. Genotypic diversity (R) and genetic diversity were calculated for each sampling date (GenClone 2.0) (Arnaud-Haond and Belkhir 2007). Genetic diversity was estimated as allelic richness (N_c) normalized to a sample size of 28. Fine-scale clone maps of the 1-m^2 subplots were used to estimate the mean number of genets m^{-2} by calculating the mean over quad 1–4 for individual sampling dates. R was calculated for each quadrat individually for all sampling dates.

Results

Large-scale clonal architecture (100 m^2)

Seagrass cover ranged from 50 to 100% in 2002 and 2003 and declined to <10% in 2004 and <1% by the end of the study in June 2005 (Fig. 3, 4). Ramets sampled from Plot 1 (n = 570) and Plot 2 (n = 419) revealed 392 and 272 genets, respectively; of these, 41 and 44 were found more than one time (Fig. 3). Several genets (Plot 1, n = 39; Plot 2, n = 41) found in May 2002 were tracked for growth and survival. Estimates of P_{sex} (F_{IS}) confirmed clonal identity for all ramets sharing the same MLG on both the large- and fine-scale. The mean genet size (SD) in Plot 1 (1.46 [0.4] m^2) was equivalent to Plot 2 (1.38 [0.26] m^2) (*t* test, p = 0.34). However, genets exceeding 3 m^2 were restricted to 2002 and 2003 in both plots (Fig. 3). The 1-m^2 size class was dominant; but genet sizes ranged up to 9 m^2.

The mean genet survival time (SD) was 2.1 (0.61) years in Plot 1, significantly less than in Plot 2 (3.14 [0.9] years) (*t* test, $p < 0.001$). Only one genet (2.4%) survived for 4 years in Plot 1 while 18 (41%) genets survived the study period in Plot 2 (Figs. 3, 4). Genotypic diversity (R) and allelic richness remained high throughout the study period and invariant among plots. Average R was 0.84 in both Plot 1 and Plot 2 (ranging from 0.64 to 0.96 and 0.68 to 0.95, respectively) (Figs. 3, 4). The mean allelic richness was 6.2 in Plot 1 and 6.4 in Plot 2 (ranging from 5.8 to 6.3 and 6.2 to 6.6, respectively) (Fig. 4).

Fine-scale clonal architecture (1 m^2)

Seagrass cover was 10–35% in June 2003 and increased to 100% in all four quadrats in September 2003 (Fig. 5). In June 2004 the seagrass cover was <1 to 3% and 1% in September 2004 (Table 1). A total of 106 genets were identified among the 269 ramets sampled in the four subplots over the 2 year study period, but only nine were found on multiple occasions. Genet density (grand mean m^{-2} [SD]) was

7.6 [1.4] for the whole study period and did not significantly differ between sampling dates (Table 1).

Genotypic diversity, however, was significantly lower in 2003 as compared to 2004 (*t* test, $p < 0.001$) (Table 1). In September 2003, quadrats 2 and 3 were entirely dominated by one individual clone (Fig. 5). Both of these genets were dominant by June 2003 and also survived until September 2004. In quadrats 1 and 4, two dominant genets were encountered in 2003 and September 2004 but not detected in June 2004 (Fig. 5).

Table 1. Fine-scale clonal architecture of *Z. noltii*.

	1	2	3	4	Mean No. of Genets x m^{-2}
June 2003					
No. of ramets (N)	23	18	20	23	
No. of genets (G)	7	4	7	10	
Genotypic diversity	0.30	0.22	0.35	0.43	
Seagrass coverage (%)	10	23	35	11	7.0 ± 2.45
Sep 2003					
No. of ramets (N)	25	25	25	26	
No. of genets (G)	10	2	2	11	
Genotypic diversity	0.4	0.08	0.08	0.43	
Seagrass coverage (%)	100	100	100	100	6.3 ± 4.92
June 2004					
No. of ramets (N)	17	11	10	1	
No. of genets (G)	14	8	8	1	
Genotypic diversity	0.82	0.73	0.8	1	
Seagrass coverage (%)	3	1	1	< 1	7.8 ± 5.32
Sep 2004					
No. of ramets (N)	13	8	18	6	
No. of genets (G)	9	7	17	5	
Genotypic diversity	0.69	0.88	0.94	0.83	
Seagrass coverage (%)	1	1	1	1	9.5 ± 5.26

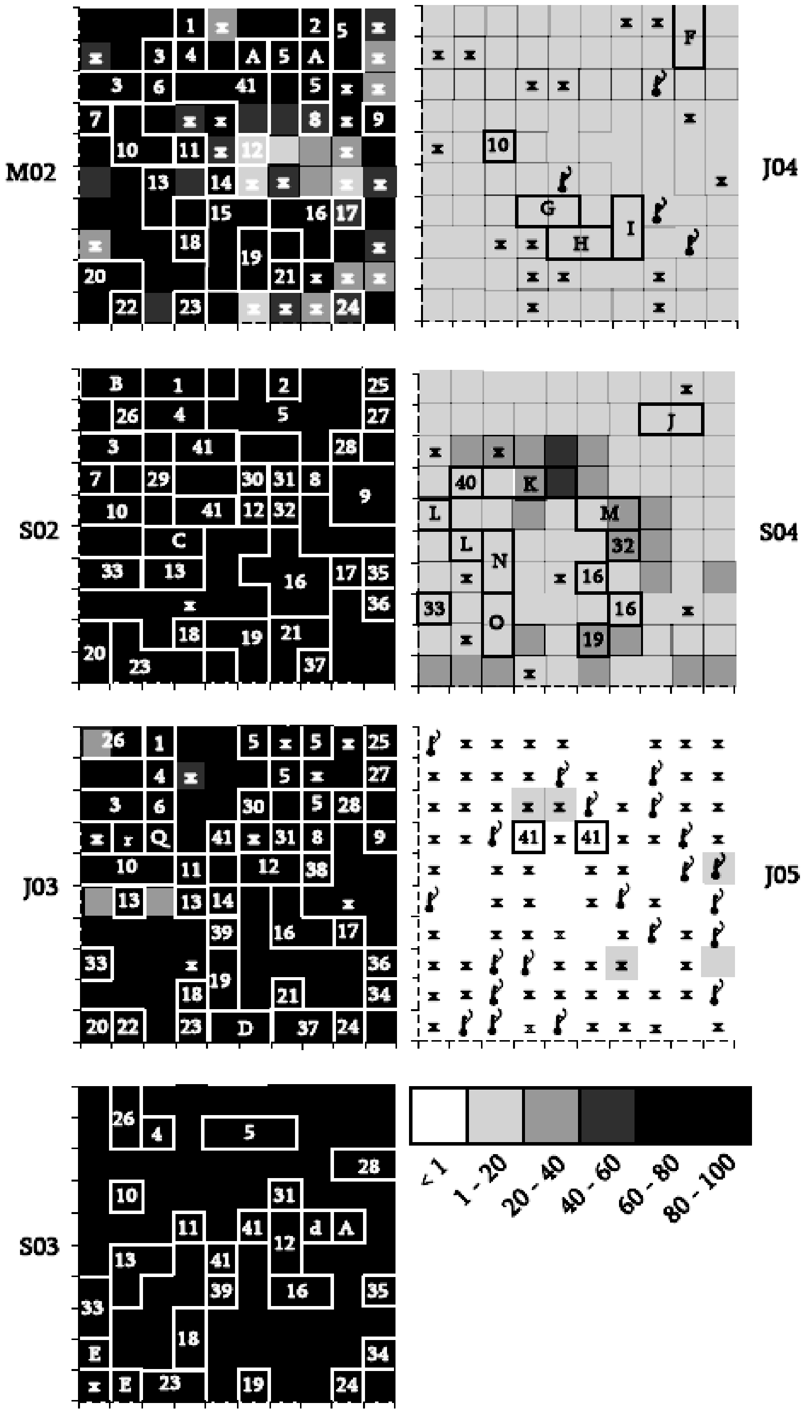

Figure 3. Large-scale clonal architecture (10 x 10 m) from May 2002 to June 2005 (M02 to J05). Clone maps of Plot 1 sorted by sampling date from top to bottom and left to right. Genotypes found multiple times are marked with numbers. Genotypes found in more than 1 m^2 but only at one sampling time are marked by capital letters. Genets are framed in white for better visualization. Blank areas represent spatially and temporally unique genotypes. x, no sample taken. Background coloring indicates seagrass cover in % for each 1 m^2 from low (white) to high (black) density (see scale).

Discussion

Seagrasses have two levels of genetic variation: genetic/allelic and genotypic/clonal (reviewed in Procaccini et al. 2007). Consequently, meadows may be genetically diverse but genotypically depauperate and vice versa. Understanding clonal architecture, therefore, provides a basis for understanding the dynamics of a seagrass bed in relation to habitat.

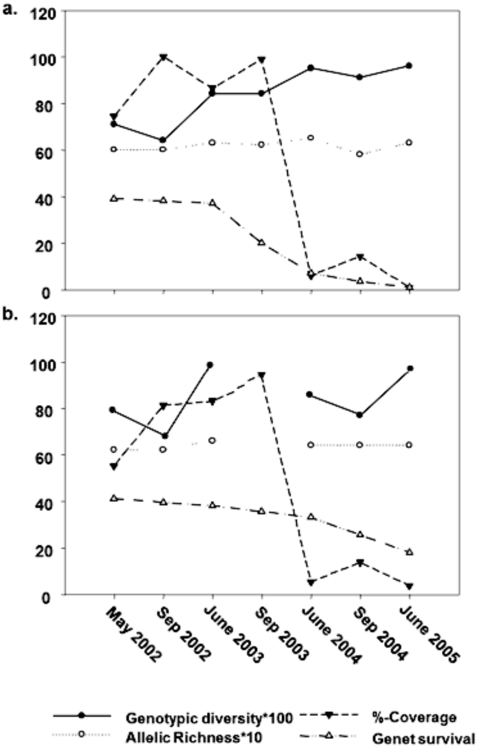

Figure 4. Genet survival, seagrass cover, genetic and genotypic diversity in Plot 1 (a.) and Plot 2 (b.) for the study period (May 2002 to June 2005).

Clone size and longevity

The genet size of *Z. noltii* determined by microsatellite analysis during our 4-year study averaged 1.4 m^2, and ranged from 0.04 to 9.0 m^2. The genet size range is comparable to the range determined by physical excavation of whole genets and by digital image analysis of an intertidal meadow at Cadiz Bay in Spain (0.04–4.0 m^2) (Brun et al. 2007). The largest clone in Brun et al. (2007) measured 5 m in length, a size attainable in 8 months based on internode production along the main axis and apical dominance, but requiring 7 years based on an average rhizome elongation rate of 0.68 m $year^{-1}$ (Marbà and Duarte 1998). In the present study, most clones detected on the large-scale persisted for 2–3 years, although ~20% of them were present throughout our 4-year study suggesting that genet age was not correlated with size in the intertidal. Decades-old clones of *Z. noltii*, however, have been reported from the

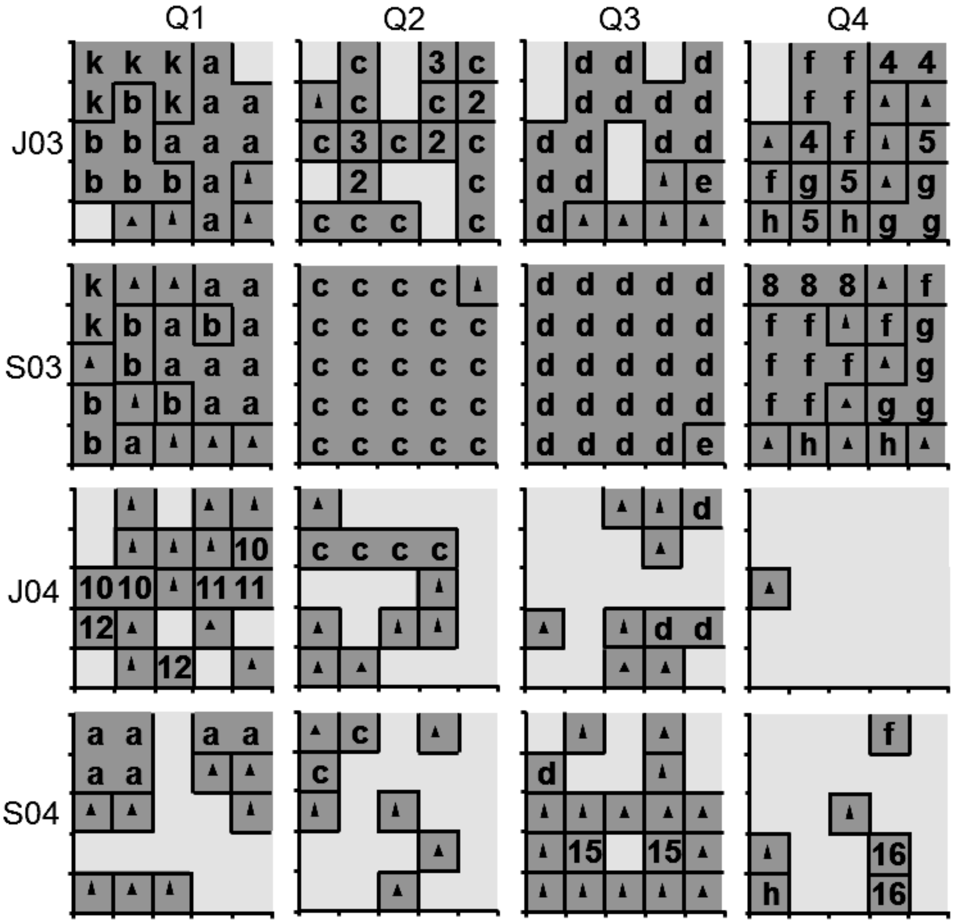

Figure 5. Fine-scale clonal architecture of subplots (1 m^2) within Plot 1 from June 2003 to September 2004. Clone maps of subplots 1–4 are shown from left to right and temporally sorted from top to bottom. Genotypes found on multiple sampling times are identified with letters. Genotypes found in more than one 0.2 x 0.2 quadrat but only on one sampling time are identified by numbers. Genets are framed in black for better visualization. Small triangles (filled triangle) indicate unique genotypes found only once in time. Light gray areas indicate bare sand.

shallow subtidal in the Black and Azov Seas, and genet size may be a better proxy for genet age under less disturbed subtidal conditions (Coyer et al. 2004a). For example, each of five 1 x 3 m subtidal plots of *Z. noltii* off Ischia (Naples, Italy) was densely covered by only two genets and a fragmented clone extended almost 60 m^2 with an estimated age of 14.7 years (Ruggiero et al. 2005a). Genet persistence and size positively correlated in *Z. noltii* on the fine-scale, as clones with >50% cover at the beginning of the growing season were competitively dominant and persisted for at least 2 years (Fig. 5).

The large, early-season clones undoubtedly arose from vegetative growth of overwintering rhizomes of fragmented clones. Although seedlings generally appear a month before (late April/early May) the start of vegetative propagation of the overwintering, shoot bearing rhizomes (late May/early June) (Vermaat and Verhagen 1996, Zipperle et al. 2009), subsequent vegetative growth of seedlings is not sufficient to outcompete overwintering clones by June (personal observation, Fig. 2).

Genet size of intertidal and annual meadows of the sister species *Z. marina* rarely exceeded 0.01 m^2, while in some subtidal and perennial populations, genets can extend for hundreds of m^2 (Reusch et al. 1999, Olsen et al. 2004, Coyer et al. 2008) and persist for hundreds or even thousands of years (Reusch et al. 1999, Olsen et al. 2004). An emerging pattern for both *Z. noltii* and *Z. marina*, therefore, may be that clone size and longevity range from small and short-lived in the intertidal to large and long-lived in the subtidal, and are further influenced by a sliding scale of annual to perennial lifestyles. The changes of clone size and longevity from intertidal to subtidal also suggest that habitat characteristics shape the clonal architecture of *Zostera* species along a stress and disturbance gradient (e.g., from benign subtidal to harsh intertidal conditions), which ultimately cause a switch from a perennial to an annual lifestyle as an adaptation to stochastic environmental changes (Hu et al. 2003).

Genetic diversity

Allelic and genotypic (clonal) diversity remained high and stable throughout the study in spite of a 99% loss in aerial cover and number of genets in the large-scale plots from 2003 to 2004 (Fig. 4). As has been documented elsewhere (Coyer et al. 2004a), allelic and genotypic diversity of *Z. noltii* for Wadden Sea populations are among the highest recorded throughout its range and connectivity among meadows also is generally high (also for *Z. marina*; Reusch 2002, Ferber et al. 2008).

At our Königshafen site, the high diversity was attributed to a combination of local and regional processes. On the larger Wadden Sea scale, entrainment and admixture of rafting, fruiting shoots and spathes are the basis for seed import, and hence population connectivity and gene flow (Coyer et al. 2004a, Erftemeijer et al. 2008, Zipperle et al., unpublished). However, finescale clonal diversity increased from 2003 to 2004, suggesting the scale-dependence of *R* and the necessity to determine the appropriate scale to answer population biological questions in clonal seagrasses (Table 1). While large-scale sampling suffices to monitor population genetic parameters and stability over time in intertidal *Z. noltii*, assessment of demographical changes such as genet mortality and immigration requires fine-scale mapping.

The persistently high allelic and genotypic diversity observed in Königshafen suggests that disturbance plays an important role and sexual recruitment is frequent. For plants in general, stable habitats should favor vegetative reproduction and foster populations dominated by few large clones, whereas disturbed habitats should be dominated by more and smaller clones due to frequent seedling recruitment (de Steven 1989, Eriksson 1993, Kudoh et al. 1999).

Disturbances by grazing waterfowl, lugworm bioturbation, epiphyte growth, and storm-induced sediment mobility, are common types of disturbances in the intertidal Wadden Sea (Jacobs et al. 1981, Reise 1985, Philippart 1994, Nacken and Reise 2000, Schanz et al. 2000, Zipperle et al. 2010). All facilitate seedling recruitment of *Z. noltii* by creating variably sized gaps in the meadow into which recruitment can occur. Increased sexual recruitment under disturbed conditions is known for flowering plants and seagrasses (Peterken and Conacher 1997, Inglis 2000, Greve et al. 2005) and has been shown experimentally for *Z. marina* (Reusch 2006).

In intertidal *Z. noltii*, small clone sizes coupled with relatively large pollen dispersal distances via tidal currents, supports a predominantly outcrossing mating system, which further promotes high allelic and genotypic diversity (Zipperle et al. 2011). In contrast, large clones can lead to inbreeding depression due to pollen limitation for outcrossing, as has been shown in subtidal *Z. marina* (Hämmerli and Reusch 2003). Furthermore, as seedling recruitment substantially contributes to clonal diversity (Harada et al. 1997), it is significant that *Z. noltii* seedlings were observed in all June sampling periods (Zipperle, unpublished; see also Figs. 2, 3) and that seeds germinated annually from a short-term persistent seed bank (Zipperle et al. 2009). Thus, *Z. noltii* exhibits a repeated seedling recruitment (RSR) strategy as has been demonstrated for other seagrasses (Ruggiero et al. 2005b, Reusch 2006), as opposed to an initial seedling recruitment strategy (ISR) (sensu Eriksson 1993). Consequently, the annual losses of genets from the *Z. noltii* meadow in Königshafen are buffered by seedling recruitment, thereby providing the basis for the stable and high levels of genotypic diversity and allelic richness.

Persistent seagrass meadow

Although genet turnover is on the order of a few years, the intertidal *Z. noltii* meadow in Königshafen has been present since prior to 1936 (Reise et al. 1989, Dolch 2008; Fig. 1). The decades-long persistence in a harsh environment may be explained by the contribution of sexual reproduction and seed bank supply to meadow maintenance. The precipitous decline in seagrass cover from 2003 to 2004 in Königshafen may have been caused by enhanced sediment mobility, as revealed by characteristic megaripple formation (Fig. 1, C2 and 3) (Dolch and Reise 2010). The increased sediment mobility may be attributed to the loss of extensive *Z. marina* beds, anthropogenic alteration of the hydrodynamic regime in the List tidal basin, and/or an increase of mean tidal height due to variation in storm intensity over the last decades (Dolch and Hass 2008).

Alternatively, the 2003–2004 decrease may be due to the severe heat stress event (HSE) in the summer of 2003, which has been associated with biomass and density losses in European populations of *P. oceanica*, *Z. marina*, and *Z. noltii* (Mayot et al. 2005, Reusch et al. 2005, Cardoso et al. 2008). Although the decline of *Z. noltii* recorded at Königshafen was correlated with the HSE, the correlation was not observed at the metapopulation scale (930 km^2) in the North Frisian Wadden Sea, of which Königshafen is a part (Reise and Kohlus 2008). Thus, the HSE disturbance may have acted in tandem with increased sediment mobility to degrade the Königshafen meadow (local scale). Cardoso et al. (2008) invoke a comparable coupling of HSE and eutrophication stress to explain observed decreases in an intertidal *Z. noltii* bed in the Mondego river estuary (Portugual, SW Atlantic).

In spite of the biomass decline, genet survival of *Z. noltii* was 4–18 times higher in Plot 2 as compared to Plot 1. The edge location of Plot 1 relative to the more central location of Plot 2 might explain the difference. Wave impact and sediment mobility may be more severe at the edges of seagrass meadows than in the center where hydrodynamics are reduced and water retention is higher (Fonseca and Koehl 2006). Under heat stress, a center location may also be more suitable for survival because retained water prevents desiccation and hence mortality. However, it should be noted that intertidal *Zostera* populations are adapted to survive short-term desiccation (Leuschner and Rees 1993).

Percent-cover of *Z. noltii* remained low during 2004 and 2005. While sufficient sexual recruitment assures evolutionary flexibility in physically disturbed habitats, it may be insufficient to balance exceptionally high adult mortality (Forbis and Doak 2004, Weppler et al. 2006). A patch of *Z. noltii* in the Königshafen meadow recovered only if the overwintering cover was >10% (at the beginning of the growing season). Because of the small genet size, a reduction in density is nearly equivalent to a reduction in total number of genets in the patch. A reduced number of genets in spring, therefore, might not provide sufficient vegetative growth until the end of the growing season. If too few genets survive the winter to provide sufficient vegetative regrowth in the following season, then seedling recruitment may not balance this loss. Alternatively, an inhibition of regrowth in spring and early summer might also be caused by enhanced sediment mobility.

The importance of a short-term persistent seed bank to maintain allelic richness of *Z. noltii* has been demonstrated over a 3-year period (Zipperle et al. 2009). Possession of a seed bank should help *Z. noltii* overcome periods unfavourable for vegetative recovery, as has been demonstrated for seagrasses and other plants (Silvertown and Charlesworth 2001, Fenner and Thompson 2005, Greve et al. 2005). If seedling recruitment and subsequent vegetative growth can attain a density sufficient to exceed winter mortality of genets, the patch is likely to recover even after exceptionally severe disturbances. The marked increase (up to 20%) of *Z. noltii* cover at the Königshafen study site in August 2008 (Reise, unpublished data), 4 years after the precipitous decline, supports the self-preserving effects of sexual reproduction from a seed bank for *Z. noltii* in a stressful environment.

In conclusion, decadal persistence of intertidal meadows of *Z. noltii* depends on a balance of year-to-year genet survival (vegetative growth), frequent seedling recruitment (sexual reproduction), and a lasting seed bank. This is in sharp contrast to larger seagrass species, where meadow persistence over centuries involves extensive vegetative propagation (*Posidonia* species, subtidal *Z. marina*) or to subtidal *Z. noltii* meadows, where vegetative propagation dominates and recruitment from seeds is rare (Laugier et al. 1999, Ruggiero et al. 2005a, b). While seedling recruitment ensures evolutionary flexibility in face of environmental stochasticity and annual disturbances, vegetative growth of overwintering ramets promotes recovery after winter mortality. An assessment of these two factors provides a metric for predicting patch vulnerability in the intertidal.

Acknowledgments We thank S. Ferber, E. Boon and A. Matthäi for field assistance and J. Veldsink for laboratory assistance. G. Hoarau and M. Chevolot provided helpful comments for data analysis and T. Dolch clarified our understanding of sediment dynamics at Königshafen. This research was supported by an Ubbo Emmius PhD stipend from the University of Groningen to AMZ.

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Chapter 3

An evaluation of small-scale genetic diversity and the mating system in Zostera noltii on an intertidal sandflat in the Wadden Sea

Abstract - The dwarf eelgrass, *Zostera noltii*, is a predominant inhabitant of soft-bottom intertidal regions along the coasts of northern Europe. It is a monoecious, protogynous angiosperm in which the potential for self-fertilization and inbreeding are high, especially if clone sizes exceed pollen dispersal distances. The aim of the present study was to determine the relationship between mating system and clonal structure, examine the relative roles of geitonogamous selfing and biparental inbreeding, measure pollen availability (multiple paternities) and estimate pollen dispersal. A 100-m^2 plot was established in a large, intertidal *Z. noltii* meadow of the island of Sylt in the German Wadden Sea. A total of 256 adult shoots was sampled: one from the centre of 100 fixed 1-m^2 quadrats (large scale resolution) and an additional 156 from within eight randomly selected 1-m^2 sub-quadrats (small-scale resolution). DNA was extracted from seeds and leaf tissue of all samples and genotyped with nine microsatellite loci. Mating system analysis revealed high multilocus and single locus outcrossing rates. Average pollen dispersal distance was nearly the same as the average genet (clone) size. Multiple paternity was common and 20–30% of mature seeds originated from matings within the plot. Among inbred seeds, most resulted from geitonogamy rather than biparental inbreeding. Moderate disturbances intrinsic to the intertidal habitat appear to facilitate seed recruitment by gap formation. Pollen dispersal distances are sufficient to maintain outcrossing and high clonal diversity.

Zipperle et al. (2011) An evaluation of small-scale genetic diversity and the mating system in *Zostera noltii* on an intertidal sandflat in the Wadden Sea. *Annals of Botany*. 107, 127-133

Introduction

Dwarf eelgrass, *Zostera noltii*, is one of two dominant seagrass species along Europe's North Atlantic coastline. It is typically intertidal under marine conditions and subtidal in brackish habitats, and ranges from southern Sweden to Mauritania and parts of the Mediterranean, Black and Azov Seas. In the sandy to muddy intertidal found along the northern European coastlines, *Z. noltii* is perennial, characterized by a high genet turn-over and annual genet replacement from seeds. Intertidal genets studied in the German Wadden Sea are typically, 1 m^2 in size, but can be as extensive as 9 m^2 (Zipperle et al. 2009a, b). In contrast, subtidal *Z. noltii* in the Mediterranean forms larger clones (several square metres) with minimal recruitment via seeds (Ruggiero et al. 2005). Intertidal hydrodynamics (e.g. tidal and storm-induced wave action) and natural disturbances have been invoked to explain a sliding scale of life-history differences between intertidal and subtidal habitats, as well as for the maintenance of high genetic and genotypic diversity in the Wadden Sea (Coyer et al. 2004, Olsen et al. 2004, Zipperle et al. 2009a).

The balance between sexual reproduction via seeds and vegetative propagation by rhizomal spreading greatly influences the spatial genetic structure of a seagrass meadow, its relative diversity, and potential for gene flow. For example, spathes (specialized leaf shoots) of the monoecious *Z. noltii* contain four to six female and four to six male inflorescences. Pollination is hydrophilous (den Hartog 1970) and seeds are negatively buoyant (Orth et al. 1994, Ruckelshaus 1996). The consequences of this hermaphroditic mating system are that large clones can lead to pollen dispersal limitation, which translates into reduced outcrossing opportunities and increased probability of self-fertilization/inbreeding (Handel 1985, Charpentier 2002). Thus, a centrally located ramet in a large clone is not only less likely to receive outcrossed pollen, but is also more likely to have its own pollen fertilize another ramet of the same genet (geitonogamy).

Inbreeding depression is an important post-pollination mechanism affecting evolution of mating systems, as the purging of recessive and deleterious alleles by selection against inbred offspring favours an outcrossing mating system (Lande and

Schemske 1985, Eckert and Barrett 1994). Selfing or outcrossing strategies depend upon the magnitude of inbreeding depression (Schemske and Lande 1985, Jarne and Charlesworth 1993): pure mating systems (selfing or outcrossing) are predominant under abiotic pollination, while mixed mating (selfing and outcrossing) is common in animal-pollinated plants (Goodwillie et al. 2005). If outcrossing is under genetic control, however, disruptive selection occurs for either an exclusively outcrossing or selfing mating system (Lande and Schemske 1985).

Previous studies of *Z. noltii* meadows on sandflats in the German Wadden Sea examined clonal architecture, the importance of seed banks, and the role of geese pits for seed recruitment (Zipperle et al. 2009a, 2009b, 2010). In the present study of a large *Z. noltii* meadow on an intertidal sandflat, the mating system and pollen dispersal of *Z. noltii* were investigated at a small (square centimetre) and large (square metre) spatial scale. The specific aims were to: (1) determine the relationship between mating system and clonal structure; (2) examine geitonogamous selfing vs. biparental inbreeding; (3) measure pollen availability expressed as multiple paternity; and (4) estimate pollen dispersal distance (PDD).

Materials and methods

Study site and sampling

A permanent 10 × 10 m plot (divided into 1-m^2 quadrats) was established within a large *Zostera noltii* meadow (approx. 0.125 km^2) in Königshafen, a shallow intertidal bay of the Island of Sylt (German Wadden Sea; 55°2'46.5''N; 8°26'2.5'E). Water depth during high tide is approx. 0.5 m and the meadow is exposed 6–8 h twice a day. At each sampling period, stakes were relocated (using photographs, landmarks, nylon line) and connected with 10-m transect tapes (Fig. 1). The location of each sampled shoot was mapped using a 20-cm grid (Fig. 2). When samples were collected in early September 2003, seagrass cover within the plot was 75–100% and comparable to adjacent areas.

Seed-bearing shoots were sampled from eight randomly chosen 1-m^2 quadrats within the plot, which were further divided into 25 sub-quadrats (20 × 20 cm). When present, a seed-bearing shoot was collected from the centre of each subquadrat. Due to the patchy distribution of seed-bearing shoots, however, the number of samples per quadrat varied. In dense quadrats, random samples were collected, which resulted in more than one sample collected from a sub-quadrat in some cases. Additional shoots were sampled for the entire 100-m^2 plot by

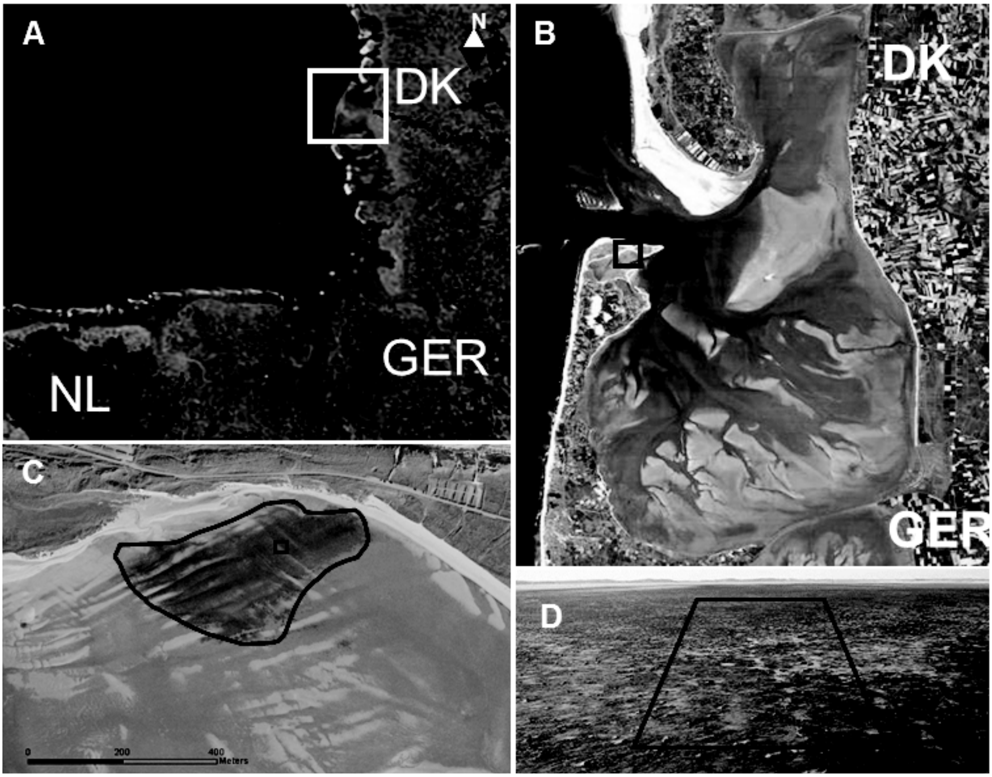

Figure 1. Study site. A. Island of Sylt (white square) in the Northern Wadden Sea. B. List tidal basin with study site (black square) in Königshafen on the northern tip of the island. C. *Z. noltii* meadow in Königshafen 2003. Seagrass bed boundaries are outlined. D. Specific location of the 10 x 10 m study plot (black square).

collecting one shoot from the centre of each fixed 1-m^2 quadrat (n = 100). Thus, a total of 256 adult shoots was sampled and used to determine clone structure and genetic diversity for the larger 100-m^2 plot (Zipperle et al. 2009a). All plant tissue was dried and stored in silica crystals.

The two sampling scales were chosen in order to cover the entire size range of *Z. noltii* genets (square centimetre to several square metres). The dense sampling of 1-m^2 quadrats within the 100-m^2 plot assured: (a) sufficient sampling of mother genets and offspring for mating system analysis; and (b) the representative sampling of potential parents, to assess PDDs over the entire genet size range. The necessity of the dense small-scale sampling rendered replication of plots logistically unfeasible.

DNA extraction and microsatellite genotyping

Seeds were extracted from a spathe and matched with 5 mg of leaf tissue from the reproductive shoot (= mother) for mating system and paternity analysis. Seeds and leaf tissue of all adult samples were pulverized using a Retsch mixer mill MM 301(Qiagen) and DNA extracted as described by Elphinstone et al. (2003). All samples were genotyped for nine microsatellite loci (Coyer et al. 2004) to obtain multi-locus genotypes (MLG).

Three primer triplets (plex-1: ZnB1/ZnH10/ZnB3; plex-2: ZnB8/ZnH8/ZnD6; plex-3: ZnE7/ZnF8/ZnF11) were used in multiplex PCR reactions following the protocol in Zipperle et al. (2009b). The PCR reaction mix was: 2 mM $MgCl_2$, 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 mL per reaction. PCR reaction protocol was the same for each primer triplet: initial denaturation of 3 min at 94°C, 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 GeneAnalyzer (Applied Biosystems). Alleles were scored against an internal lane standard (ROX 350; Applied Biosystems).

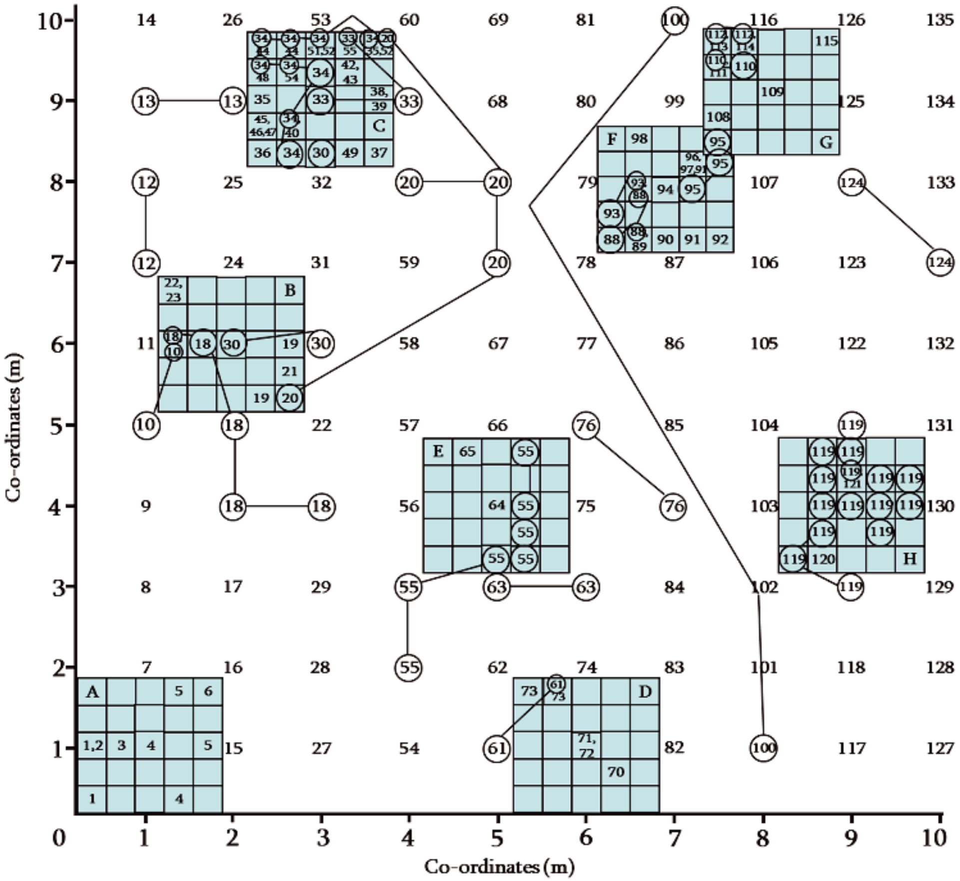

Figure 2. Clonal architecture in quadrats (1-m^2) and plot (100-m^2) in September 2003. Quadrats A-H (grey) indicate the 0.2 x 0.2 m grid. Identical genotypes are indicated by a unique number and connected. Unconnected numbers represent unique multi-locus genotypes.

Genetic, genotypic diversity and clone structure

Genetic and genotypic diversity, as well as clone structure, were determined using GENCLONE v. 2.0 (Arnaud-Haond and Belkhir 2007). Duplicate genotypes of a clone were counted only once in subsequent analyses. Genotypic diversity was separately determined for the 1-m^2 and the 100-m^2 scales. Allelic diversity (*A*) and allelic diversity corrected for sample size (A_c) were determined separately for maternal genets, seeds and all adult genets present in the plot; as were estimates of the global inbreeding coefficient F_{IS}, expected (H_{exp}) and observed (H_{obs}) heterozygosity.

Mating system

Data were analysed with MLTR v.3.3 (Ritland 2002) using the Newton–Raphson method with a mixed mating model. The mating system was characterized by estimating multiband single-locus outcrossing rates (t_m, t_s); biparental inbreeding as the difference between outcrossing rates ($t_m - t_s$); maternal inbreeding as the coefficient F; the correlated selfing rate as rs; and the multi-locus paternity correlation within [$r_{p(w)}$] and between [$r_{p(b)}$] families (genets).

When true selfing is present, the difference between multi- and single-locus estimates of outcrossing, ($t_m - t_s$), is often used to characterize the level of biparental inbreeding: single-locus estimates include all apparent selfing due to biparental inbreeding, whereas multilocus estimates exclude much of the apparent selfing due to biparental inbreeding (see Ritland 2002).

The effective number of pollen donors (N_{ep}) was determined by the reciprocal of r_p ($N_{ep} = r_p^{-1}$) (Smouse et al. 2001, Fernández and Sork 2005). From 32 mother genets, 71 spathes containing 148 seeds were recovered. All seeds retrieved from a genet were grouped in a family. For the analysis of families, default settings for the estimated main parameters ($t = 0.9$, $r_t = 0.1$, $r_p = 0.1$, $F = 0.1$) were used (Ritland 2002). Parameters were jointly estimated from 1000 bootstrap iterations from which confidence intervals were derived.

Paternity analysis and pollen dispersal

Paternity analysis, or determining the most likely father for a given offspring, was performed with CERVUS v. 3.0 (Kalinowski et al. 2007), which employs a likelihood-based approach for categorical assignment of paternity with co-dominant markers. Paternity is assigned to a male if the log-likelihood ratio or LOD score (the likelihood of paternity of a particular male relative to the likelihood of an arbitrary male) is much larger relative to the LOD score of an alternative male.

Confidence of paternity assignment is evaluated using the log-likelihood statistic (Δ) which is defined as the difference in LOD scores between pairs of the two most likely candidate fathers in a simulated data set. A simulation of paternity inference is conducted based on the allele frequencies in the real data set (10 000 iterations). From the simulation, frequency distributions of Δ are generated for two cases: the most likely male being the true father and the most likely male not being the true father. Critical values for Δ are derived from the overlap of the two distributions (Marshall et al. 1998).

To account for self-fertilization and inbreeding, parameters were set at an inbreeding rate of 0.2 and used in all analyses. Inbreeding rate was based on the results of the mating system analysis (correlated selfing-rate r_s = 0.193). Finally, analyses were conducted under strict (95 %) and relaxed (80%) thresholds for the confidence level as suggested by Marshall et al. (1998). Results are shown for the most conservative (95%) confidence level only.

Accuracy of paternity assignment depends upon the simulation parameters used and results are influenced by the genotyping error rate, the number of candidate fathers, and the proportion of parents actually sampled from the population. The genotyping error rate in the data set was low (0.01). Because *Z. noltii* is self-compatible and clonal, all adults sampled were candidate fathers, including mother plants.

For paternity assignment on the 100-m^2 scale, all genets (n = 135) were used as potential parents. The confidence of the assignment depends on the completeness of the sampling of potential parents. This could only be roughly estimated because genets can overlap among quadrats, be linear in shape over several metres, or expand radially. Hence the estimate was made in two ways. Based on Zipperle et al. (2009a), the number of genets for the 100-m^2 plot was 630, thus extrapolation from the eight quadrats in this study yields 720 genets. Consequently, the 135 genets sampled in this study would represent 19–21% of all potential parents present. If every genet was present in at least two adjacent 1-m^2 quadrats, as suggested by a mean genet size of 1.4 m^2 (Zipperle et al. 2009a), then up to 42% of all parents present were sampled. To account for these uncertainties, assignment tests were run for a range of proportions of parents present (0.15–0.95).

PDD was calculated for seed-father pairs assigned under the most strict analysis conditions (95% confidence) and based on the mapped coordinates of seed-bearing shoot and the assigned father. Where a paternal genet consisted of several ramets, the centre of the genet was determined and used to measure the PDD.

Results

Clone structure and genetic diversity

At the 1-m^2 scale, the number of genets varied from 2 to 20 with a mean of 7.2 m^{-2} (Table 1). Not surprisingly, sampling at the scale of 20-cm intervals resulted in repeated sampling of a single genet, i.e. 156 ramets reduced to 58 genets. The very high number of genets (20) found in quadrat C represented closely related individuals, as well as older individuals as judged by their size (Fig. 2). At the 100-m^2 scale, where the sampling was at 1-m intervals, more genets were recovered in relation to ramets sampled. Only about 15% were replicates. Allelic richness was high (Table 1), mirroring levels reported earlier for *Z. noltii* in the Wadden Sea (Coyer et al. 2004). Global F_{IS} values gave no indication of departures from HWE.

Mating system

Outcrossing rates were high (t_m = 0.886; t_s = 0.871) but also indicated a fraction of inbred seeds (Table 2). The majority of inbred seeds resulted from selfing (geitonogamy and autogamy) and only 1.5% of the total inbreeding detected was due to biparental inbreeding (matings between close relatives, e.g. sibs). The coefficient of correlated paternity among outcrossed seeds differed significantly from 0 within [$r_{p(w)} = 0.155$] and between [$r_{p(b)} = 0.118$] genets. Thus, the majority of seeds within spathes of a genet and between different genets had multiple fathers. The number of effective pollen donors N_{ep} was 6.45 within and 8.47 between genets.

Paternity and PDD

A father could be assigned to 34% of all seeds within the eight randomly placed quadrats under strict analysis conditions (95%) using the Δ-statistic. The mother and assigned father were identical in 22% of all assigned seeds indicating self-fertilization (Table 1) and 2–18% of seed-father pairs could be assigned within the 100-m^2 plot depending on the preset proportion of parents sampled from the population (Table 3). Average PDD (SD) ranged from 1.70 (0.79) m to 3.78 (2.44) m with distances ranging from 0.85 to 9.62 m. Because pollen dispersal is commonly leptokurtic, the median provides more explanatory power than the mean. The median of PDD ranged from 1.84 m to 3.22 m (Table 3). Comparisons of *Z. noltii* and *Z. marina* are presented in Table 4.

Discussion

Selfing and outcrossing

In most seagrasses, pollination takes place entirely underwater and early studies hypothesized that reduction of pollen diversity due to extensive clonal growth would result in self-fertilization and inbreeding (Cox 1983, Les 1988, Philbrick and Les 1996). More recent experiments, however, demonstrated high outcrossing in three monoecious species, with rates of 0.89 for *Posidonia australis* (allozymes: Waycott and Sampson 1997), 1.00 for subtidal and 0.90 for intertidal *Z. marina* (allozymes: Ruckelshaus 1995) and 0.97 for intertidal *Z. marina* (microsatellites: Reusch 2000), effectively contradicting the hypothesis of predominant selfing and inbreeding.

Table 1. Genet diversity, genetic diversity and paternity assignments at two spatial scales.

1-m^2 quad-rats	N	G	G/N	Mother Genets (ramets)	No. of Seeds	Seeds assigned to fathers (%)	Selfed seeds, mother=father (%)	Unassigned seeds (%)	Total Genets (combined)
A	9	6	0.67	4 (6)	18	4 (22)	4 (100)	14 (78)	-
B	9	6	0.67	6 (8)	24	13 (54)	0 (0)	11 (46)	-
C	61	20	0.33	8 (44)	53	23 (43)	1 (4)	30 (57)	-
D	7	4	0.57	4 (7)	9	3 (33)	0 (0)	6 (67)	-
E	8	2	0.13	1 (7)	9	3 (33)	3 (100)	6 (67)	-
F	20	11	0.55	4 (12)	10	1 (10)	1 (100)	9 (90)	-
G	11	7	0.64	3 (8)	12	1 (8)	0 (0)	11 (92)	-
H	31	2	0.06	2 (31)	13	3 (23)	2 (67)	10 (77)	-
Total	156	58		32 (83)	148	51 (34)	11 (22)	97 (66)	-
100-m^2 samples				100	84	0.84			
Allelic diversity (A) total				6.8	9	-	-	-	11.6
Allelic diversity corrected for sample size 32 (A_c) total				6.8	6.5	-	-	-	7.2
Expected heterozygosity (H_e) total				0.5986	0.6042	-	-	-	0.6488
Observed heterozygosity (H_o) total				0.6027	0.6014	-	-	-	0.6435
Inbreeding coefficient (F_{IS}) total				-0.0069	0.00468	-	-	-	0.00825

Number of ramets sampled (N), genets identified from ramets (G) and genotypic diversity (G/N). Paternity assignments are based on 95% CI. Other diversity measures are as indicated.

Table 2. Mating system analysis.

Parameter	Value (SE) [95% confidence level]
Parental inbreeding F	- 0.030 (0.001) [- 0.0324; - 0.0276]
Multilocus outcrossing rate t_m	0.886 (0.001) [0.883; 0.888]
Single-locus outcrossing rate t_s	0.871 (0.002) [0.867; 0.874]
Biparental inbreeding $t_m - t_s$	0.015 (0.0005) [0.141; 0.162]
Correlated selfing rate r_s	0.193 (0.004) [0.186; 0.201]
Multilocus paternity correlation within genets $r_{p(w)}$	0.155 (0.002) [0.151; 0.158]
Multilocus paternity correlation between genets $r_{p(b)}$	0.118 (0.015) [0.089; 0.148]
Number of effective pollen donors N_{ep}	6.45 – 8.47 (range)

Table 3. Pollen dispersal distance (PDD) based on assigned seed-father-pairs at the 100-m^2 scale from a sequence of paternity analyses (CERVUS 3.0, Kalinowski et al., 2007) using different assumptions of the proportions of parents sampled from all parents present in the population.

P	N	Mean PDD (SD) [m]	Median PDD [m]	PDD_{max} [m]
0.15	3	1.70 (0.79)	1.84	2.4
0.25	4	2.41 (1.57)	2.12	4.56
0.35	5	2.33 (1.37)	2	4.56
0.45	6	2.23 (1.25)	1.92	4.56
0.55	6	2.23 (1.25)	1.92	4.56
0.65	7	2.87 (2.1)	2	6.99
0.75	11	3.79 (2.96)	2.4	9.62
0.85	16	3.59 (2.79)	2.2	9.62
0.95	26	3.78 (2.44)	3.22	9.62

Table 4. Comparison of *Z. noltii* and *Z. marina* growth and reproduction characteristics

	Zostera noltii	*Zostera marina*	Reference
Plant height (m)	0.1 – 1	0.2 – 2	Den Hartog, 1970
Clone size (m^2)	<1 - 1960	<1 - 6400	Reusch et al. 1999, Coyer et al. 2004, Olsen et al. 2004
Pollen dispersal distance (m)	0 – 9.6	0-15	This publication, Ruckelshaus 1996
Seed dispersal distance (m)	- (inferred to be on a meter scale)	0 – 10 m	Orth et al. 1994, Ruckelshaus 1996
Rafting shoots (km)	Approx. 150	Approx. 150	Reusch 2002, Ferber et al. 2008, Erftemeijer et al. 2008
Kinship structure (m)	1-2	2-4	Coyer et al. 2004, Hämmerli and Reusch 2003c

However, outcrossing rates might be subject to temporal and spatial variation (Ruckelshaus 1995), and accuracy depends on the chosen marker. In the present study, the outcrossing rate was 0.88, which is high, but not unity. Closer examination of the mating system revealed a high degree of multiple paternity [$r_{p(w)}$ = 0.155; $r_{p(b)}$ = 0.118], both within and between genets of *Z. noltii*. This demonstrates high pollen availability from multiple sources, which is supported by the large number of effective pollinators (8.47 between genets).

At the same time, however, <50% of the ovules within a single spathe were fertilized and similar low fertilization rates have been reported for other inter- and subtidal meadows of *Z. noltii* (Hootsmans et al. 1987, Loques et al. 1988, 1990, Curiel et al. 1996, Alexandre et al. 2006). Ackerman (2002) hypothesized that the low fertility rate was due to low levels of pollen released as 1000 to 10 000 pollen grains are necessary per ovule for successful fertilization in *Z. marina*. In contrast, Hämmerli and Reusch (2003a) demonstrated self-incompatibility in *Z. marina*, where a high number of aborted seeds were the result of selfing. The authors' observations support the latter view. The fact that inbred seeds are not reflected in the adult populations suggests strong selection against inbred offspring, either through seed abortion or seedling mortality (Ruckelshaus 1995, Reusch 2000). Selection acting against selfed-progeny has also been detected in field studies of *Z. marina* where plant fitness (clone size and number of flowering shoots) was positively correlated with heterozygosity (Reusch 2001, Hämmerli and Reusch 2003b). Indeed, Reusch (2001) detected decreasing outcrossing rates with increasing clone area in subtidal and perennial *Z. marina* with pronounced vegetative propagation. In the present study, *Z. noltii* clone size averaged 1.4 m^2, comparable to other reports for intertidal *Z. noltii* (Brun et al. 2003, Zipperle et al. 2009a), whereas the median PDDs ranged from 1.8 to 3.2 m. Even so, the presence of a high proportion of selfing among seeds (0.193) highlights the delicate balance between clonal propagation and successful mating.

Selfed seeds result from within (autogamy) and/or between (geitonogamy) ramet selfing. In protogynous *Z. noltii*, geitonogamy potentially is the most likely selfing mode as dichogamy (temporal separation of male and female sexual function) serves as a mechanism to avoid autogamy. Although dichogamy is never complete and might be impaired by environmental factors (Lloyd and Webb 1986, Ruckelshaus 1995), self-incompatibility is an additional means to avoid autogamy present in the sister species *Z. marina* (Hämmerli and Reusch, 2003a).

PDD estimates are based on few positively assigned seed-father pairs (Table 3). However, the lower the proportion of parents sampled used for analysis, the higher the Δ for positive assignment. Under the strict analysis conditions applied the results presented are most conservative especially in the range of proportions of parents sampled considered most realistic (0.15–0.45) (Table 3).

Zostera species also have negatively buoyant seeds (Orth et al. 1994, Ruckelshaus 1996), which means that seeds settling near the mother plant may be related and form a relatedness cluster (Heywood 1991). Quadrat C (Fig. 2) contains a large number of different genotypes, which most likely are the result of a single recruitment event. Such recruitment clusters have also been documented in relation to geese pits, which accumulate seeds and facilitate spring recruitment (Zipperle et al. 2010). Kinship structure has been demonstrated at the scale of 1–2 m in *Z. noltii* (Coyer et al., 2004; A. M. Zipperle, unpub. res.) and is consistent with the low proportion of biparental inbreeding (1.5%) observed in the present study. PDD and kinship structure for intertidal *Z. noltii* were equivalent to values reported for subtidal *Z. marina* despite a nearly 10-fold difference in shoot and genet size (Table 4).

Influences of the local environment

The study site was characterized by a comparatively high hydrodynamic regime (Schanz and Asmus 2003) and subjected to geomorphologic forcing by mega-sand ripples on a decadal time scale (Dolch and Reise 2010). The shift towards smaller genets increased with increased hydrodynamics (Schanz and Asmus 2003), while larger genets tended to dominate with increasing water depth (Brun et al. 2003) probably reflecting decreased water flow. Burial of *Z. noltii* by mobile sediments can cause plant death as well as forcing small genet sizes. The mega-ripples observed throughout the study site, however, move on decadal time scales making burial effects negligible for the present study.

At the outset of this study, it was hypothesized that the small size of *Z. noltii* plants (i.e. leaf width and length characteristics) compared with the larger size of *Z. marina* plants might account for differences in genet size, pollen dispersal and properties of the mating system. This was not the case as PDD and genet size were essentially the same for both species (Table 4). This suggests that local environmental characteristics play a major role for dispersal. In the most extreme dichotomy, dominance of a few large genets may result in reduced seed set and subsequent recruitment as a consequence of pollen limitation and unavoidable selfing. If conditions remain relatively benign, however, a large genet may prosper for decades or even centuries in some species. At present, it is known that intertidal *Z. noltii* can persist for 4–5 years (Zipperle et al. 2009a) and the giant (up to 60 m^2) Mediterranean clones (Ruggiero et al. 2005) of *Z. noltii* are likely to be much older. At the other extreme, high mortality and annual or bi-annual turnover of genets may actually increase seed set as a consequence of pollen abundance and favourable outcrossing. Recruitment will also be enhanced. This is characteristic of the typically dynamic regimes found in the intertidal of the Wadden Sea. Consequently, intermediate disturbance (Connell 1978, Hemminga and Duarte 2000) is likely to create the best of both worlds – a heterogeneous mix of genets and genet sizes along with high outcrossing rates and intermediate mortality. Disturbance may thus enhance genotypic diversity within intertidal *Z. noltii* meadows by: (a) directly promoting pollen and seed dispersal through vigorous hydrodynamics, thus extending seed and PDD; (b) facilitating sexual recruitment through gap formation in a dense meadow (Zipperle et al. 2010); and (c) indirectly increasing the level of outcrossing due to clone size reduction. There is evidence for all three factors.

In conclusion, the intertidal environment selects for small clone sizes of *Z. noltii*, which indirectly influences the mating system by increasing outcrossing opportunities and allowing for large PDDs and a high degree of multiple paternity. While biparental inbreeding is nearly eliminated, geitonogamy seems to be intrinsic to the clonal life-history of *Z. noltii*. The combination of a predominantly outcrossing mating system, large PDD, regular annual recruitment, and a short-term persistent seed bank in Wadden Sea patches of *Z. noltii* (Zipperle et al. 2009b), collectively maintain high genetic and genotypic diversity on a local scale, thereby enhancing the population's resilience to the stochastic intertidal environment. In *Z. marina*, Hämmerli and Reusch (2003b) also found higher genet diversity in a subtidal population in the Baltic Sea grazed by swans, compared with an ungrazed meadow, and Reusch (2006) determined experimentally that seedling recruitment and genet dynamics in *Z. marina* were enhanced in response to physical disturbance. A similar relationship exists for grazing by geese which facilitates seed recruitment (Zipperle et al. 2010). Gene flow, at the larger scale of the Wadden Sea, occurs by the transport of seed-bearing rafting shoots by tides and/or storms (Coyer et al. 2004, Erftemeijer et al. 2008, Ferber et al. 2008), thereby linking *Z. noltii* meadows to a meta-population via the export of genetic and genotypic diversity created at the local scale. The perennial life-history of *Z. noltii* may explain its better performance compared with annual and semi-annual *Z. marina* in the intertidal of the Wadden Sea.

Acknowledgments We thank M.S.C.O.M. Chevolot for insightful comments;
G. Hoarau and S. Ferber for assistance with data analysis; and J. Veldsink for laboratory assistance. This research was supported by a PhD Scholarship to A.M.Z. from the Ubbo-Emmius Programme at the University of Groningen.

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Chapter 4

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

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 yrs, thereby forming a relatively short-term, but persistent, seed bank.

Zipperle et al. (2009) Evidence for persistent seed banks in dwarf eelgrass *Zostera noltii* in the German Wadden Sea. *Marine Ecology Progress* Series. 380, 73-80

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 and Reusch 2003a), intraspecific density-dependent competitive interactions (Hämmerli and Reusch 2003b, Reusch 2006), low seed production (Loqués et al. 1990, Alexandre et al. 2006), local seed predation (Fishman and 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 yrs) and long-term (>5 yrs) (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 and Hughes 2006).

After the near total loss of subtidal eelgrass *Zostera marina* meadows to a wasting disease in the early 1930s, dwarf eelgrass *Z. noltii* has emerged as the primary seagrass species inhabiting the Wadden Sea, particularly in the northern regions (Reise and 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).

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 three years	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
Golf 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
Island of 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

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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 km^2) 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 and 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)

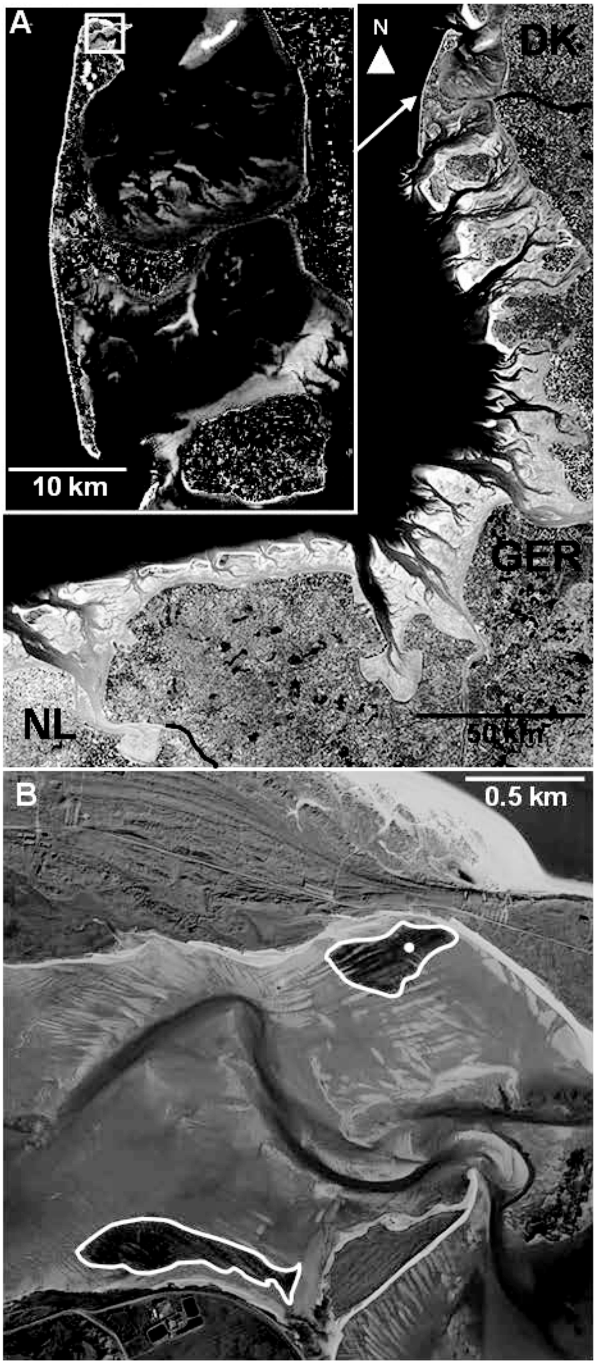

Figure 1. Study area. A. German Wadden Sea. Inset: the shallow bay of Königshafen (small white square) located off the Island of Sylt. DK: Denmark; GER: Germany; NL: The Netherlands. B. *Zostera noltii* meadows in Königshafen (outlined in white) and plot location (white dot)

was established near the centre of the meadow in April 2002. Corners were marked with metal stakes driven completely 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 m^2], 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 meadows. Five seeds were placed in each of 13 (Königshafen) and 6 (southern meadow) 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 wks and considered complete when the green cotyledon was visible. After germination, seedlings were removed and discarded.

The experiment was concluded after 6 wks 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 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 m^2 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 m^2 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 centre of each 1 m^2 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 centre 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 $MgCl_2$, 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).

Table 2. Multiplexing conditions for microsatellite loci

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

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} (F_{IS})) were calculated for shoots sharing the same MLG using GenClone 2.0 (Arnaud-Haond and 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 and 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 shoot density by >99% in subsequent years (2004, 2005) and too few adult shoots were available in 2005 (Zipperle et al. 2009). Values presented are means ± SD.

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 and 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. 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 ± 88.8 m^{-2}, ranging from 0 to 288 m^{-2}. Compared to vegetative shoot density in May (426 ± 228.3 m^{-2}) seedlings represented 19.5% of shoots in the 10 × 10 m plot.

Seed bank and seed germination

Seeds were present in the sediment throughout 2004 (487.5 ± 269.4 m^{-2}) and 2005 (367.3 ± 95.5 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 germination depth was 4.1 ± 0.9 cm in 2004 and 3.0 ± 1.0 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 patch (Table 3), establishing the importance of local recruitment. Additionally, temporal assignment revealed that year-class seedlings arose from seeds originating from up to 3 yrs 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.

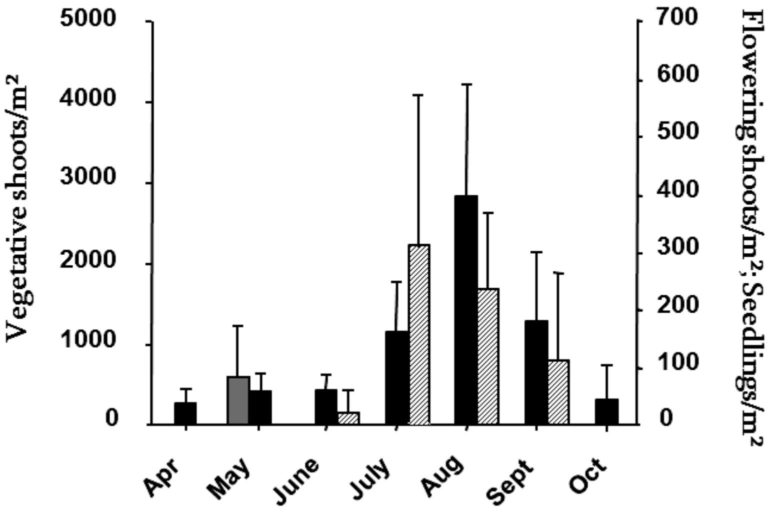

Figure 2. Phenology of the *Zostera noltii* meadow in Königshafen in 2004. Density of vegetative shoots (black bars), seedlings (grey bar) and flowering shoots (striped bars) is plotted by month. Means + SD are shown.

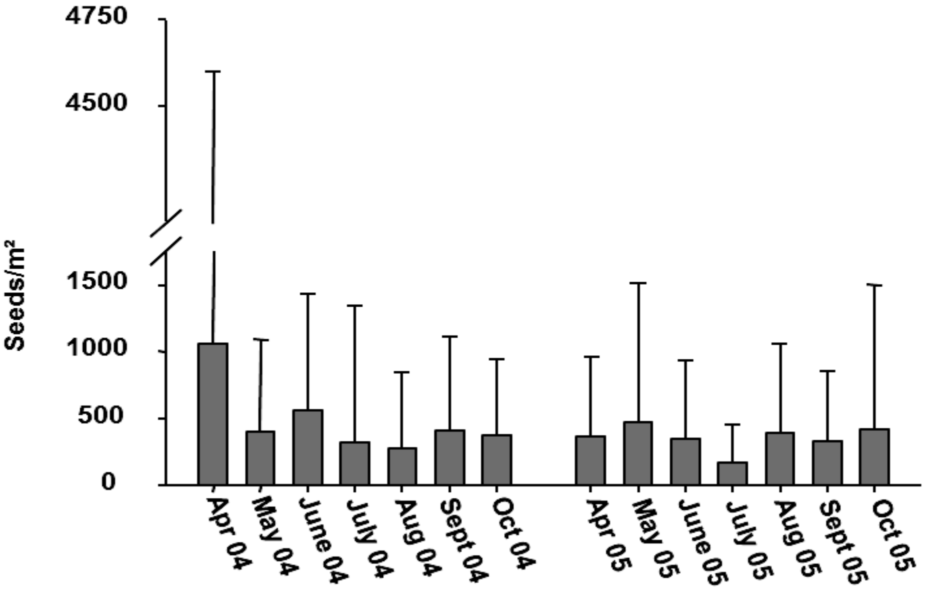

Figure 3. Seed bank densities at the study site of the Island of Sylt from April to October 2004 and 2005. Means + SD are shown.

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%.

Collecting Date	Not assigned (%)	Assigned to adults from 2004	Assigned to pooled adults 2002/2003	Total assigned ver all times
2005	73.2	7.1	19.6	26.8
2006	67.5	2.5	30	32.5

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 yrs 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 better 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 (Zipperle et al. 2009) 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 and Orth 2002, Erftemeijer 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 yrs (Zipperle et al. 2009), a minimal annual recruitment of only 2% of the seedlings (83 m^{-2}) would add 1.5 genotypes m^{-2} into the meadow.

Growth of overwintering rhizomes begins in May–June, nearly a month after the appearance of seedlings (Vermaat and 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 and Verhagen 1996). Therefore, high winter survivorship implies a more extensive amount of vegetative growth (at 68 cm yr^{-1}, Marbà and 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 and 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 (Zipperle et al. 2010). 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 patches 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 yrs 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 and Kohlus 2008), as well as population resilience in highly mobile intertidal soft sediments.

Acknowledgments 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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Chapter 5

Waterfowl grazing in autumn enhances spring seedling recruitment of intertidal Zostera noltii

Abstract - Feeding pits dug by waterfowl in *Zostera noltii* meadows are thought to promote seedling recruitment by accumulating seeds and enhancing germination. We tested the latter hypothesis by creating a series of "treatment pits" (resembling natural feeding pits) in the center and at the edge of two meadows near the Island of Sylt (Germany). Seedling density was monitored from the autumn seed set until the following spring. Seedling density (mean, SE) in treatment pits was significantly higher (4.4, 5.3) than in manipulated (2.4, 1.9) and unmanipulated controls (1.4, 0.4), as well as significantly higher in center (2.8, 0.5) relative to edge (2.5, 1.1) locations. Results confirm a facilitating effect of waterfowl grazing on seedling recruitment in spring due to seed accumulation in feeding pits in autumn. The mechanism could provide a valuable tool for the conservation of intertidal *Z. noltii* meadows in the Wadden Sea.

Zipperle et al. (2010) Waterfowl grazing in autumn enhances spring seedling recruitment of intertidal *Zostera noltii*. *Aquatic Botany*. 93, 202-205

Introduction

Levels of allelic and genotypic (clonal) diversity of dwarf eelgrass, *Zostera noltii* Hornem., populations in the Wadden Sea are among the highest recorded throughout the species' range, including areas that have provided appropriate habitat for a far longer period than the ~6000-y marine history of the Wadden Sea. In a more contemporary timeframe, a recently completed 4-y study of a *Z. noltii* meadow in the Wadden Sea, that has existed since at least 1936, also revealed a high and stable level of diversity, despite incurring a 99% loss in aerial cover and number of genets due to coupling of a heat stress event and increased sedimentation during the summer 2003 (Zipperle et al. 2009a). The persistent high diversity at both scales has been attributed to a repeated seedling recruitment strategy, whereby several modes of biotic and abiotic disturbances create gaps that are colonized by seeds resulting from entrainment and admixture of rafting fruiting shoots/spathes rafting (Zipperle et al. 2009b, Zipperle et al., unpublished).

An important component of the biotic disturbance regime on the Wadden Sea intertidal flats is grazing by waterfowl. Whereas excessive levels of grazing can be destructive, it is also apparent that grazing by Brent geese (*Branta bernicla* L.) and Widgeons (*Anas penelope* L.) on dense *Z. noltii* meadows can be beneficial (Jacobs et al. 1981, Nacken and Reise 2000). For example, grazing facilitates growth by reducing the self-inhibiting effect caused by mud accretion, thereby creating "gaps" in autumn that are colonized by seedlings sprouting from the seed bank during the following spring (Jacobs et al. 1981, Vermaat and Verhagen 1996, Nacken and Reise 2000, Ehlers 2002, Reusch 2006). Although several terrestrial plant species disperse via attachment of seeds to external surfaces (exozoochory) or through the digestive tracts of migrating waterfowl (endozoochory), both processes are unlikely in the waterfowl/*Z. noltii* association (Clausen et al. 2002, Ehlers 2002).

In addition to creating gaps, waterfowl grazing may also help to concentrate seeds. For example, geese create distinct "feeding pits while feeding on subterranean portions (rhizomes, roots) of *Z. noltii* which subsequently can trap and accumulate seeds over a few tidal cycles (Jacobs et al. 1981, Ehlers 2002) (Fig. 1A). Although known for other seagrasses and desert plants (Reichmann 1984, Inglis 2002), the importance of disturbance-related sediment depressions to seedling recruitment has not been tested in *Z. noltii*. In this paper, we experimentally evaluate whether formation of experimentally created feeding pits in autumn (after seed release) enhances germination of *Z. noltii* seeds during the following spring.

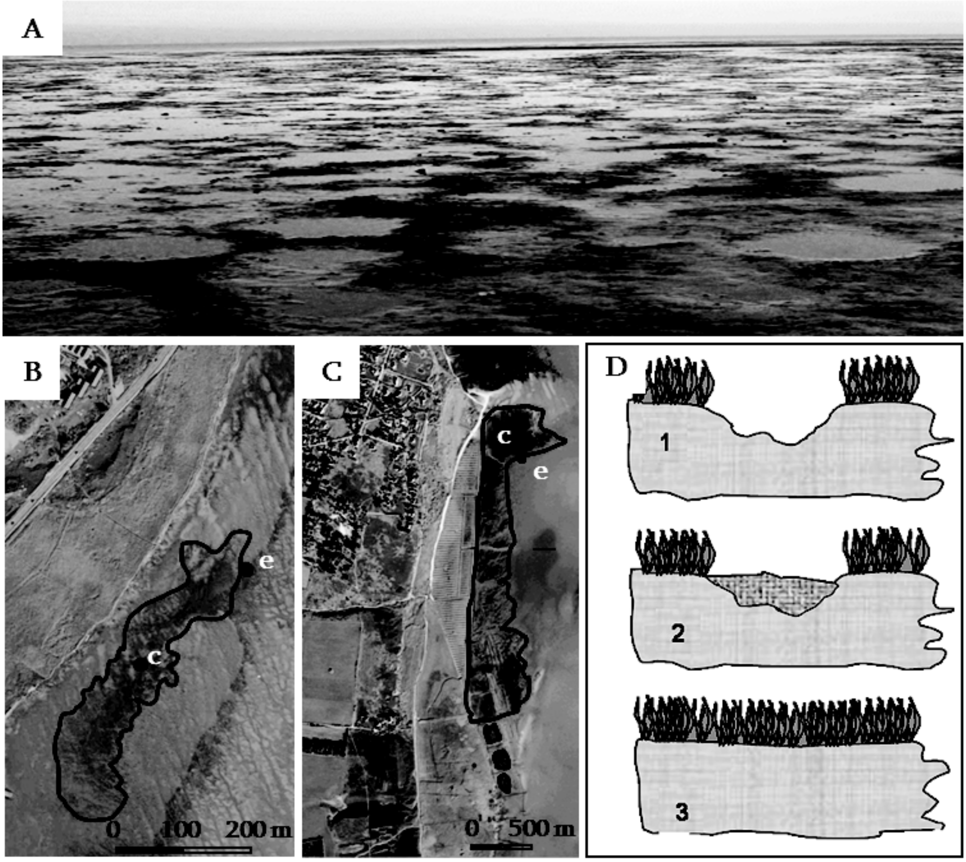

Figure 1. Meadow description and experimental design. A. Characteristic feeding pits of Brent geese in a *Z. noltii* meadow. B. Aerial images of seagrass meadows (black line) at Vogelkoje and C. Leghörn with center (c) and edge (e) locations identified (black dots). D. Each experimental unit consisted of one treatment pit (D1), one manipulated control (D2), and one unmanipulated control (D3). Eight experimental units were established in each meadow.

Materials and methods

The experiment was established in two *Z. noltii* meadows, Vogelkoje (54° 58’ 15’ N, 008° 21’ 22’ E) and Leghörn (57° 23’ N, 008° 21’ 45’ E), off the east coast of the island of Sylt (Germany), where geese graze annually from September through November (Fig. 1B and C). The semidiurnal tides expose seagrass meadows for 6–8 h per tidal cycle with water depth during high tide ranging from 0.5 to 0.7m depending on location and weather conditions. A center and an edge location were chosen in each meadow in late October (end of the growing season) to evaluate the effects of feeding pits in areas differing in shoot density and grazing intensity. Eight experimental units were established at each location in each meadow, with each unit consisting of: 1) treatment pit (mean diameter of 30 cm and a mean depth of 4–5 cm, in which sediment and plant parts were removed); 2) manipulated control (creation and subsequent covering of a pit); and 3) unmanipulated control (Fig. 1D). The treatment pits closely resembled natural feeding pits of geese as described by Nacken (1998).

Experimental units were placed parallel to shore and all were at the same tidal level. A subdivided quadrat (0.25 m^2) was placed over each treatment pit to estimate initial percent cover of seagrass. Corners of the quadrat were marked with flagged metal stakes to frighten (and exclude) geese and prevent foraging. The experiment was monitored regularly from early October 2005 until all geese had left the meadows in late November 2005. In early May 2006, seedlings were counted in a central 0.2 × 0.2 m portion of the 0.25 m^2 quadrat (to avoid edge effects) of treatment and controls, whereas the entire quadrat was used to determine percent cover of vegetative shoots. Also in May 2006, the natural density of seedlings and percent cover of vegetative shoots were evaluated in the surrounding meadow with eight randomly placed 0.25 m^2 quadrats.

Treatment effects on seedling densities were tested with analyses of variance (3-way factorial ANOVA). Treatment (pit, manipulated and unmanipulated control), meadow (Vogelkoje, Leghörn), and position (center, edge) were assigned as fixed factors. Seedling densities were log-transformed to meet the ANOVA requirements. Normal distribution was tested by Lilliefors tests, and Bartlett-tests confirmed homogeneity of variances. Tukey's post hoc tests were adopted to analyze differences between treatments and controls. 'Meadow' was treated as a fixed factor (and results pooled) rather than a random factor, as random factors with <6 levels should be treated as fixed (Bolker et al. 2008). Student's t-tests were used to compare: 1) percent cover of vegetative shoots (square-root transformed) in each control treatment with natural shoot densities of the surrounding meadow in spring using 0.25 m^2 quadrates and; 2) seedling densities in pit treatments at center and edge locations to seedling densities in the corresponding surrounding meadow. All statistical analyses were conducted using the software package STATISTICA 6.1 (Stat-Soft, Inc.).

Results

Seedling density (mean, SE) in May 2006 was significantly higher within treatment pits in both the meadow center and edge (4.4, 1.0; 5.3, 3.1), compared to the manipulated (2.4, 0.7; 1.9, 1.2) and unmanipulated (1.4, 0.4; 0.4, 0.2) controls (3-way ANOVA, $F_{(2,84)} = 7.51$, $P < 0.001$; Tukeys post hoc, $P < 0.05$) (Fig. 2). There was no difference between the manipulated or unmanipulated controls (Tukeys post hoc, $P < 0.05$). There were significantly more seedlings (mean, SE) in the center of the beds (2.8, 0.5) than at the edges (2.5, 1.1) (3-way ANOVA, $F_{(1,84)} = 9.45$, $P < 0.01$) independent of meadow. Seedling densities (mean, SE) did not differ significantly between pit treatment (Vogelkoje, 5.75, 1.78; Leghörn, 3.13, 0.9) and surrounding meadow (Vogelkoje, 3.7, 1.67; Leghörn, 2.6, 1.03) at center locations (Student's *t*-test, $P > 0.1$ for both meadows). However, seedling densities (mean, SE) were significantly higher in pit treatments at edge locations (Vogelkoje, 9.25, 6.02; Leghörn, 1.38, 0.63) compared to the surrounding meadow (Vogelkoje, 0.50, 0.22; Leghörn, 0.20, 0.13) (Student's *t*-test $P < 0.05$ for both meadows). Percent cover of vegetative shoots in control treatments did not differ significantly from natural shoot densities in the surrounding meadow at both locations in May 2006

(Student's *t*-test, $P > 0.5$ for all cases).

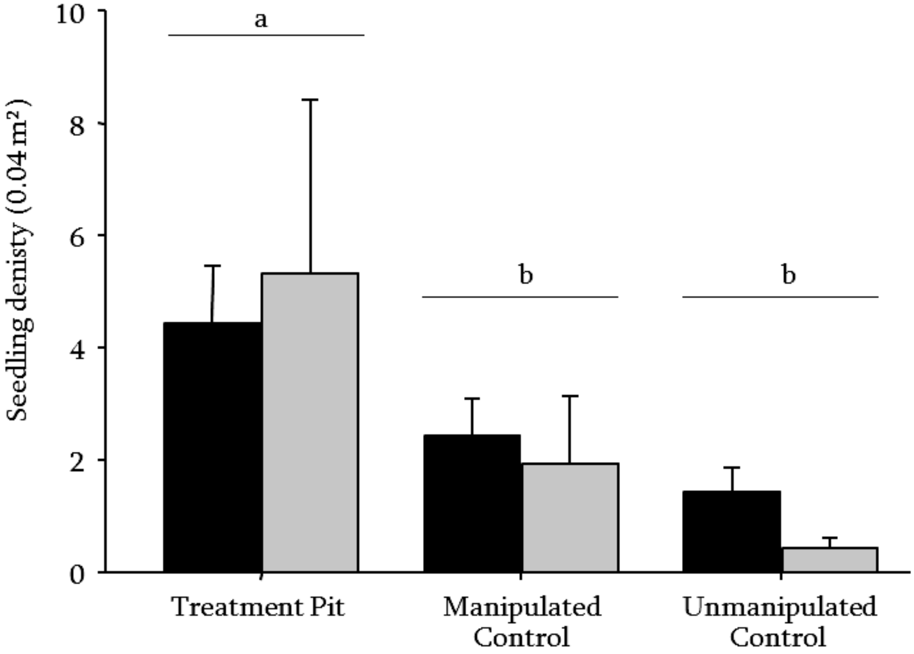

Figure 2. Seedling densities in treatment pits, manipulated controls, and unmanipulated controls. Black bars indicate density at center of the meadow; light grey at edge of meadow. Densities with differing letters (a or b) were significantly different (Tukey post hoc test, $p < 0.05$).

Discussion

Feeding pits created by foraging Brent geese (Fig. 1A) trap and accumulate seeds (Jacobs et al., 1981, Ehlers, 2002) and require up to seven days to be refilled by tidally driven sediment (Nacken 1998). Our study has demonstrated that experimental feeding pits established in autumn had a higher density of *Z. noltii* seedlings in the following spring relative to adjacent non-disturbed areas. Although we did not measure the number of seeds in either natural or treatment pits, Ehlers (2002) showed that seed accumulation in feeding pits was higher than in ungrazed areas. Furthermore, successful germination requires burial beneath 1–7 cm of sediment (Moore et al. 1993, Zipperle et al. 2009b). Consequently, it is likely that both accumulation and germination of *Z. noltii* seeds are enhanced by geese feeding pits. Similar seed-accumulation effects have been reported with grazing dugongs in meadows of *Halodule uninervis* (Forssk.) Aschers and the sister eelgrass species, *Zostera capricorni* Aschers (Peterken and Conacher 1997, Inglis 2002).

The lower shoot density and biomass at edge locations of the meadow are less attractive to foraging geese (Jacobs et al. 1981, Ehlers 2002), resulting in lower grazing activities and fewer feeding pits compared to central meadow locations. However, when treatment pits were placed at the meadow's edge, seedling density was significantly higher than in adjacent areas of the meadow's edge. Hence, germination at meadow edges may be limited by both seed availability and the number of sediment pits for seed accumulation. These results emphasize the importance of feeding pits to seed accumulation and recruitment.

In an earlier study, Nacken and Reise (2000) manipulated the effects of waterfowl grazing under an intermediate disturbance hypothesis and found a higher shoot density and biomass in grazed areas as compared to non-grazed controls. The present study demonstrates the further contribution of grazing Brent geese to the maintenance of *Z. noltii* meadows and their genotypic diversity by facilitating seedling recruitment through seed-trapping. Thus, deposition of seeds in artificially created pits, combined with shoot transplantation, may be a valuable approach for seagrass conservation programs. Indeed, a combination of shoot transplantation and seeding has been proposed for the restoration of *Zostera marina* L., and seeds deposited in meshbags increased germination significantly (Harwell and Orth 1999, Orth et al. 2006a).

World-wide success rates for the reestablishment of lost seagrass beds is only about 30% (Fonseca et al. 1998, van Katwijk et al. 2009) and protection of existing seagrass meadows has been deemed the most successful approach for the conservation of the larger seagrass biome (Hawkins et al. 1999, Orth et al. 2006b).

We propose that deposition of *Z. noltii* seeds specifically at meadow edges will stabilize and facilitate meadow expansion in some parts of the Wadden Sea. The natural losses due to high current and wave regimes could be buffered by seeding from the meadow center or more simply, as our study shows, by establishing artificial feeding pits (randomly or uniformly distributed) to serve as seed traps during the months of seed set (August to October). Enhancement of meadow edges in this manner should increase meadow expansion and ultimately, overall meadow stability (ecological and genetic) through increased area.

Acknowledgments We thank A. Schanz and P. Polte for assistance with the experimental design and B.D.H.K. Eriksson for assistance with the data analysis. Supported by a PhD Ubbo-Emmius-Scholarship to AMZ from the University of Groningen.

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Chapter 6

Two species, one pattern: A genetic perspective on diversity and connectivity of Zostera marina (L.) and Zostera noltii (Hornem.) populations in the Wadden Sea

Abstract - Seagrasses in the Wadden Sea have experienced severe losses, caused first by the 'eelgrass wasting disease' in the 1930s, which nearly eradicated subtidal *Zostera marina* and secondly, by human impact beginning in the 1950s/60s, which affected both intertidal *Zostera marina* (L.) and *Z. noltii* (Hornem.) populations. Over the past 10-15 years, however, both intertidal *Z. marina* and *Z. noltii* meadows have increased in the northern Wadden Sea, although subtidal *Z. marina* meadows remain sparse. With the Wadden Sea declared a World Heritage Site in 2010 and seagrasses declared a bioindicator under the European Habitats Directive, the conditions and possible broader scale restoration of *Zostera marina* and *Z. noltii* are of interest. We assessed genetic diversity, population structure and connectivity for 13 *Z. noltii* and 10 *Z. marina* meadows spanning more than 1,000 km of coastline between the southern Netherlands and northern Denmark. Using nine microsatellite loci, genotypic diversity (R) and allelic richness (A_c) were found to be high for both species throughout the study area. Four clusters of *Z. noltii* and *Z. marina* were identified: southern Netherlands, central Wadden Sea, northern Wadden Sea and north-eastern coast of Denmark. For both species, the Eems-Dollard and Elbe estuaries are partial barriers to gene flow between the west-central and northern Wadden Sea. Consequently, the Wadden Sea should be considered as two management units (in addition to a southern Netherlands unit) and source patches for restoration projects should come from within the management area of interest.

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Introduction

The isolating nature of landscape fragmentation can reduce both the population size and distribution of formerly common species, thereby exposing them to demographic, environmental and genetic stochasticity (Lande 1988, 1998, Honnay and Jacquemyn 2007). Persistence of populations in dynamic landscapes depends upon effective reproduction and dispersal on both a local and regional scale (Hamrick and Godt 1996, Johst et al. 2002, Ingvarsson 2002). In the dynamic near shore marine environment, surface currents (Galindo et al. 2006, Yasuda et al. 2009, White et al. 2010) and other barriers such as estuaries, deep water channels and physically differing water masses (Dieckmann et al. 2005, Hellberg et al. 2009) explain some patterns of isolation and genetic structuring observed among marine populations.

Seagrasses are foundational species in intertidal and shallow subtidal coastal habitats world-wide, where they provide a wealth of ecosystem services (Duarte 2000). Along European coastlines, *Zostera marina* (L.) (eelgrass) occurs in both the subtidal and intertidal (Rassmussen 1977), whereas *Z. noltii* (Hornem.) (dwarf eelgrass) is predominantly an intertidal species, except in sheltered lagoons of the Mediterranean, Black and Azov Seas (den Hartog 1970).

In the Wadden Sea, *Z. marina* occurs as both annual and perennial morphotypes. The annual form is characterized by a “claw foot” rhizome bearing few ramets that are spatially aggregated within a few cm^2 (van Lent and Verschuure 1994) (Fig. 1, Table 1). In contrast, the perennial morphotype bears elongated rhizomes spreading over several m^2 and supports multiple ramets. Both morphotypes may occur intertidally or subtidally and flowering occurs seasonally from May through September, although the frequency of successful recruitment is unknown. Intertidal shoots of both morphotypes are 30-50 cm in length and can form monospecific stands, whereas shoots of the subtidal perennial form are typically longer (30-150 cm) (Fig. 1, Table 1). Since the eelgrass ‘wasting disease’ outbreak of the 1930’s, the perennial subtidal morphotypes have declined and presently are found primarily in permanently inundated tidal runnels bordering

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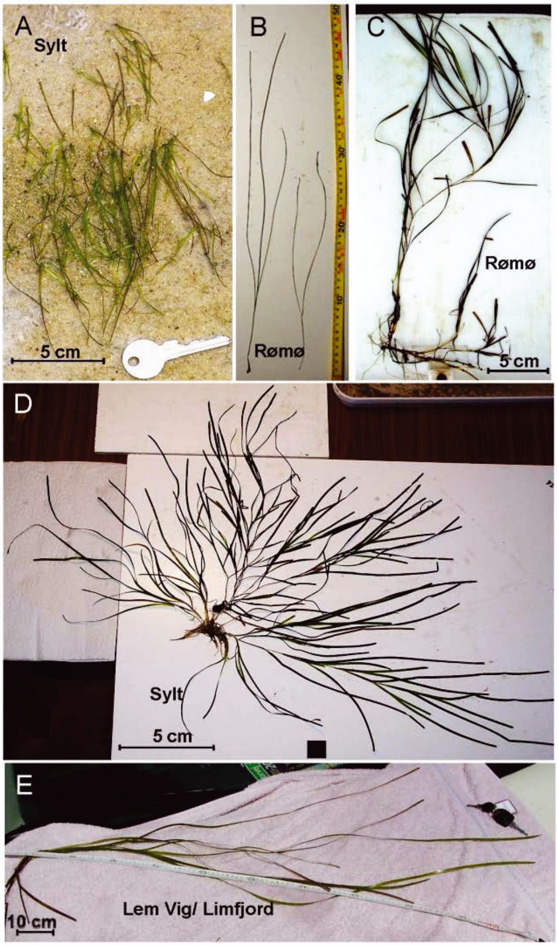

Figure 1. Size differences of intertidal *Z. noltii* and intertidal/subtidal morphotypes of *Z. marina*. A. *Z. noltii*: high intertidal (Königshafen, Island of Sylt, Germany). B. *Z. noltii*: high intertidal with permanent layer of water (Rømø harbour, DK). C. *Z. marina*: perennial, intertidal with permanent layer of water in a large *Z. noltii* meadow (Rømø harbour, Denmark). D. *Z. marina*: annual, intertidal with permanent layer of water in a large *Z. noltii* meadow (Kampen Leg Hörn, Island of Sylt, Germany). E. *Z. marina*: subtidal at 2.5 m depth (Lem Vig, Limfjord, Denmark).

tidal flats (Jacobs 1982, Reusch 2002). Large meadows that in the past were characteristic of deeper portions of the Wadden Sea are now sparse to absent (den Hartog and Polderman 1975).

In contrast, *Z. noltii* was largely unaffected by the wasting disease (Vergeer and den Hartog 1991) and now dominates the intertidal Wadden Sea. Large perennial meadows are densely populated by shoots ranging from 5-25 cm length with extensive vegetative propagation. Flowering occurs from May through September (Zipperle et al. 2009a) (Fig. 1, Table 1) and a seed bank of at least 3-4 years is common (Zipperle et al. 2009b). Shoots die back over the winter; new shoots arise from seeds in the spring, slightly earlier than shoot growth emerging from overwintering rhizomes. Although extensive grazing (rhizomes, shoots) by waterfowl in late summer sometimes leads to local decimation over a short time period (Jacobs et al. 1981) moderate grazing disturbance produces "grazing pits"

Table 1. Life history traits of *Z. noltii* and *Z. marina* morphotypes. Numbers are minimum and maximum values as reported in the literature and our data (parentheses).

	Z. noltii (intertidal)	*Z. noltii* (subtidal)	*Z. marina* (intertidal)	*Z. marina* (subtidal)	References
Life-history	Perennial	Perennial	Annual to perennial	Perennial	den Hartog 1970; van Lent and Verschuure 1994; Vermaat and Verhagen 1996
Reproduction/ Growth	Sexual/ vegetative reproduction	Sexual/ vegetative reproduction	Sexual/ vegetative reproduction	Sexual/ vegetative reproduction	van Lent and Verschuure 1994; Alexandre et al. 2006 Orth et al. 2006; Zipperle et al. 2009
Mating system	Predominantly Outcrossing	No information	Predominantly Outcrossing	Predominantly Outcrossing	Ruckelshaus 1995; Reusch 2000b ; Zipperle et al. 2011
Shoot length (cm)	5 – 25 (50)	< 70	(15) 30 – 50	30 – 150	den Hartog 1970; Borum et al. 2004
Genet size (m^2)	(0.2) < 3 (9)	< 60	(0.01) < 3 (?)	(1.4) 7 – 50 (6400)	Coyer et al. 2004a; Olsen et al. 2004; Ruggiero et al. 2005; Zipperle et al. 2009a

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that act as seed traps and facilitate subsequent recruitment (Zipperle et al. 2010).

Both species of *Zostera* have experienced severe population losses in the central and western parts of the Wadden Sea since the 1950s to 1960s (Philippart and Dijkema 1994, Kastler and Michaelis 1999, van der Graaf 2009), largely due to increased eutrophication, turbidity, and habitat degradation caused by coastal construction (Lotze 2005, van der Graaf 2009). However, a net increase in aerial cover of *Z. noltii* has been reported for the northern portions of the Wadden Sea (Reise and Kohlus 2008), attributed to a sheltered location behind barrier islands, which both reduces sediment mobility and protects against severe wave action caused by the prevailing south-westerly and westerly winds (Reise and Kohlus 2008). Additionally, climate change factors (e.g. increase in CO_2) (Palacios and Zimmermann 2007, Björk et al. 2008) and an increase in water quality parameters may be important for the steady increase of *Z. noltii* in the northern Wadden Sea (van Beusekom et al. 2009), and other areas of its European distribution (Green and Short 2003, Borum et al. 2004).

Information about genetic population structure, mating system, and dispersal across a range of spatial scales assesses standing genetic variation at both the allelic and genotypic (clonal) levels, thereby providing insights about genetic potential, recruitment, dispersal, and importance of seed banks (Reusch 2001, Arnaud-Haond et al. 2005, Zipperle et al. 2009a,b). A number of population genetic studies have been completed for several seagrass species (reviewed in Waycott et al. 2006) and especially for *Zostera* species (reviewed in Procaccini et al. 2007). Understanding diversity, dispersal and connectivity dynamics among locations also provides information about source populations that may be used in mitigation and restoration projects (Procaccini et al. 2007). Additionally, experimental studies integrating genotypic diversity with habitat and disturbance regimes (Hughes and Stachowicz 2004, Reusch 2006, van der Heide et al. 2007, Ehlers et al. 2008, Hughes et al. 2010) reveal insights into physical, ecological and demographic factors that shape the recovery potential, as well as metapopulation dynamics, of *Zostera* species (e.g., Bell 2006).

Despite the restrictive physical environment in the western Wadden Sea (van der Heide et al. 2007) and concomitant low levels of restoration success for *Z. marina* (van Katwijk et al. 2009), a recent genetic analysis revealed high levels of population connectivity and diversity for intertidal *Z. marina* (Ferber et al. 2008). Because similar levels of connectivity and diversity were observed among meadows of *Z. noltii* around the island of Sylt in the northern Wadden Sea (Coyer et al. 2004a), we expanded sampling of *Z. noltii* meadows across the entire Wadden Sea in order to mirror the 2004 survey of *Z. marina* (Ferber et al. 2004a). Our goal was to compare genetic structure of both *Zostera* species in the Wadden Sea in relation to differences in individual size (large *Z. marina*, small *Z. noltii*), life-history (annual intertidal vs. perennial intertidal morphotypes) and recent expansion (*Z. noltii* relative to *Z. marina*)

Materials and methods

Sampling sites and collections

Thirteen *Z. noltii* locations were sampled throughout the Wadden Sea (Table 2, Fig. 2, left) matching as closely as possible nine *Z. marina* locations from Ferber et al. (2008) and Norhold, Randers Fjord (this study). One (Zeeland) of 13 *Z. noltii* meadows and three (St Annaland, Sylt, Mandø) of the 10 *Z. marina* meadows were subtidal (Table 2).

Both species were found on each side of the Elbe estuary. However, no *Z. marina* patches were found in the 300 km between Norden/Norddeich and Nordstrand (Fig. 2, Table 2). Although *Z. noltii* was present at Terschelling, the sparse *Z. marina* meadow reported by van der Graaf (2009) was absent during our sampling period (September 2005) (Fig. 2). Similarly, both species were found north and south of the Eems-Dollard, but not along the high-energy coast between Den Helder and Hoek van Holland, where no suitable habitat occurs (Fig. 2).

Intertidal and subtidal morphotypes were sampled for both species (Fig. 1, Table 1). Tissue was collected from the first leaf of each individual plant (n= 31-46

Z. noltii; 27-70 *Z. marina*) at 1-m intervals along a 50 m random walk within a meadow. Where meadows were patchy, one leaf sample was taken from each patch along the random walk. Leaf samples were placed in tubes with silica crystals for drying and storage.

DNA extraction and microsatellite analysis

Leaf tissue was pulverized using a Retsch mixer mill MM 301 and DNA was extracted using a silica-based method (Elphinstone et al. 2003). Samples were amplified for nine species-specific microsatellite loci and multiplexed for PCR (Reusch et al. 1999, Reusch 2000a, Coyer et al. 2004b, Zipperle et al. 2009a). Sequences were visualized on an ABI 3100 gene analyser (Applied Biosystems) and analysed using Genotyper (Applied Biosystems) software.

Data analysis

Individuals consisting of multiple shoots (ramets) with the same unique, multilocus genotype (clones or genets) were distinguished with GenClone 2.0 (Arnaud-Haond and Belkhir 2007). For every genet, the identity of ramets, P_{sex} (F_{IS}) was tested to avoid false assignment of un-related ramets sharing the same multi locus genotype (MLG) by chance. Genotypic diversity (R as number of genets G over number of sampled ramets N), allelic richness (A) and corrected allelic richness (A_c) for minimum number of genets identified among all locations were also calculated with GenClone 2.0. Expected (H_{exp}) and observed (H_{obs}) heterozygosity, Wright's fixation indices (F_{IS} and F_{ST} as f and θ; Weir and Cockerham 1984), as well as linkage disequilibrium were calculated using Genetix 4.05 (Belkhir et al. 2001).

Population structure was determined in a Bayesian framework implemented in the software STRUCTURE 2.3.3 (Pritchard et al. 2000). The admixture model was used to estimate the log probability Pr(X| K) of each user-determined set of clusters/populations (K = 2, 3, 4 etc.) by genetic assignment of individuals to the

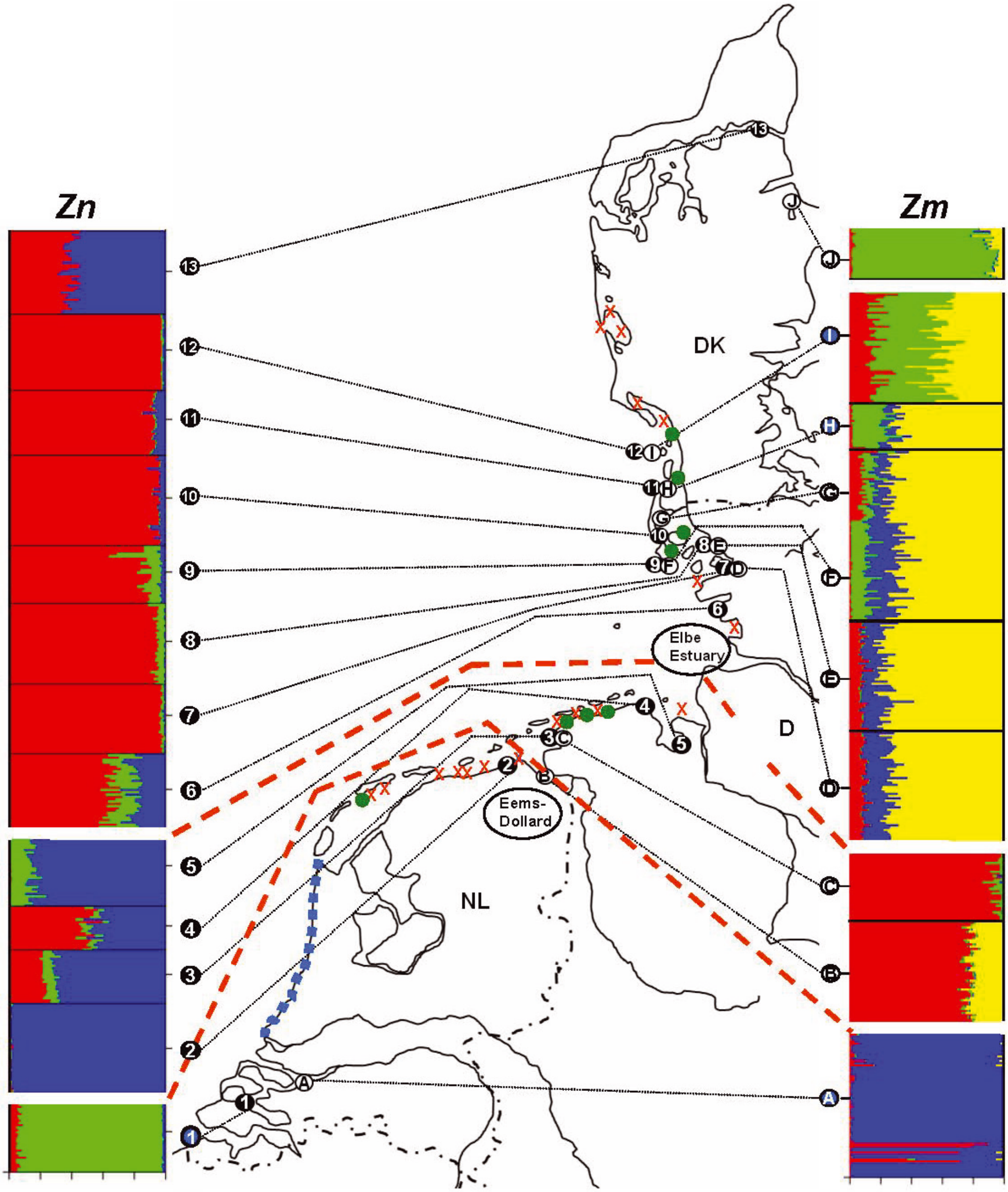


Figure 2. Legend see next page.

Figure 2 (previouse page). Sampling locations and population structure of *Zostera noltii* and *Z. marina* in the Wadden Sea and adjacent areas. STRUCTURE plots are presented for *Z. noltii* (K=3, left) and *Z. marina* (K=4, right). Dotted lines connect populations in the STRUCTURE plot to geographic locations. *Z. noltii*: 1, Yerseke/Zeeland; 2, Gas plant/ North Groningen; 3, Norden/Norddeich; 4, Horumersiel; 5, Seefelder Watt/Jade Bay; 6, Wesselburener Watt; 7, Nordstrand; 8, Hamburger Hallig; 9, Amrum; 10, Rantum/Sylt; 11, Königshafen/Sylt; 12, Mandø; 13 = Hals/Limfjord. *Z. marina*: A, Sint-Annaland/Zeeland; B, Eems-Dollard Estuary "De Paap"; C, Norden/Norddeich; D, Nordstrand; E, Hamburger Hallig; F, Amrum; G, Rantum/Sylt; H, Königshafen/ Sylt; I, Mandø; and J, Norhold/ Randers Fjord.
Closed circles (green), *Z. noltii* present, but not sampled: X (red), neither species present. The Elbe estuary and Eems-Dollard (broken line) are depicted and stem from major breaks as depicted by STRUCTURE analysis (dotted lines). The dashed blue line from Den Helder to Hoek van Holland represents uninhabitable habitat for *Zostera*.

most likely clusters (e.g., Falush et al. 2003). The true number of clusters was estimated under two assumption sets using the web-based STRUCTURE Harvester In the first analysis, posterior probabilities for a given *K*, Pr(X| K) (Pritchard et al. 2000) were determined directly (Fig. 3A, C), whereas in the second analysis the *ad hoc* statistic Δ*K* (Evanno et al. 2005) (Fig. 3B, D) was used; the latter recommended when asymmetrical dispersal patterns exist among given locations. The Δ*K* method is based on the rate of change of Pr(X| K) values between different *K* with the number of sampling locations (n = 13, *Z. noltii*; n = 10, *Z. marina*) used as priors and assigned to the most likely *K*. Each analysis was repeated five times (1 million iterations; Burn-in = 250,000) to avoid dependence on starting values.

Results

Diversity

Zostera noltii. A total of 407 genets was detected from a sample of 501 ramets. Mean number of ramets$^{\text{-genet}}$ (SD) was 1.3 (0.4), ranging from 1 to 5 (Sylt/ Königshafen) (Table 2). Every ramet sampled from Zeeland and Hamburger Hallig had a unique MLG (Table 4); others presented varying degrees of clonal diversity. Average genotypic diversity *R* (SD) was 0.8 (0.2), ranging from 0.5 to 1.0. Four of the 13 locations showed significant heterozygote deficiency (F_{IS}), while the Danish locations (Mandø; Hals) showed significant heterozygote excess (Table 2).

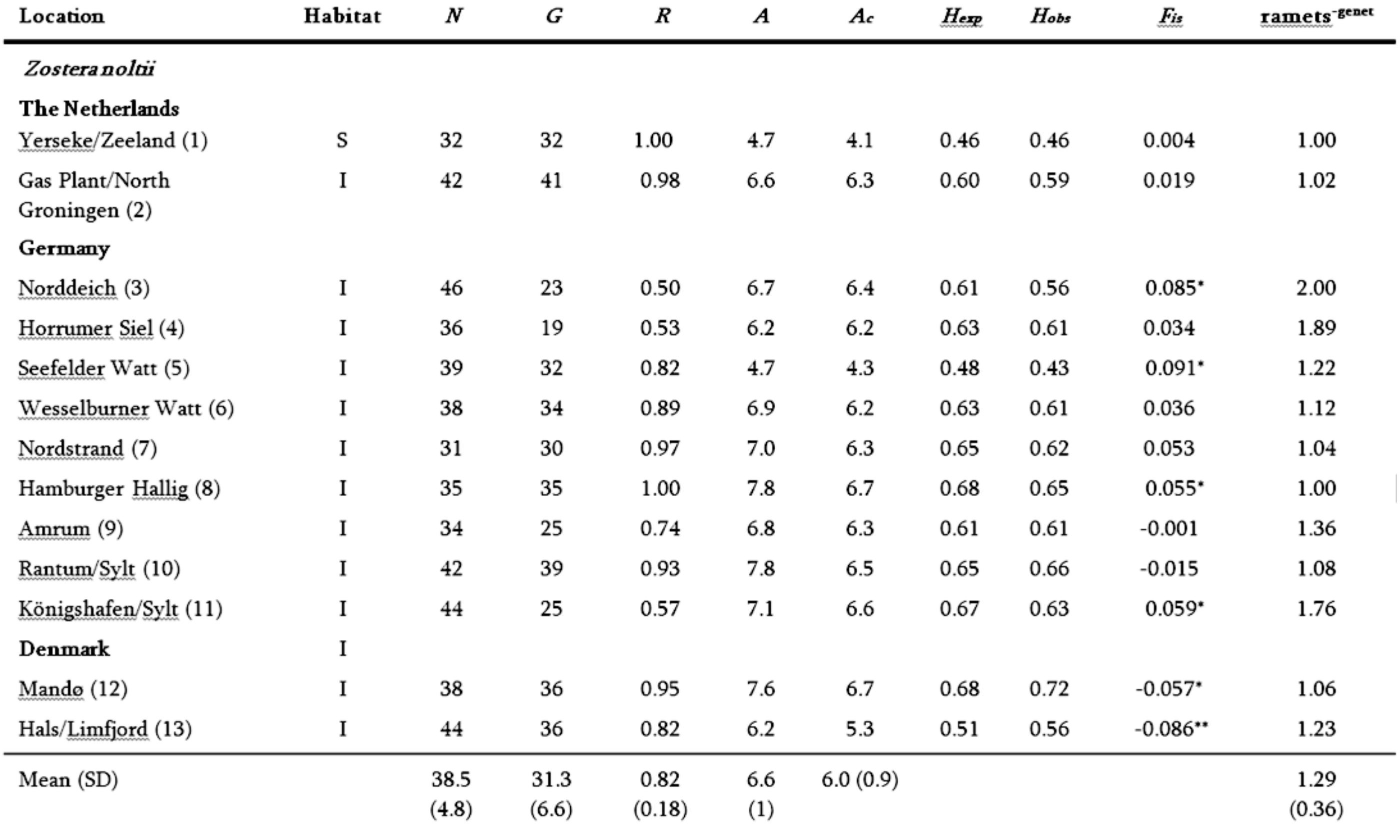
Zostera marina. Analysis of 461 *Z. marina* ramets revealed 416 genets (Table 2). In most locations, few genets were comprised of more than one ramet. Only the northern-most location (Norhold, Denmark) showed a large proportion of clones with genets consisting of up to 9 ramets (mean ramets^{-genet} = 1.88). Average genotypic diversity was high (R = 0.92; SD = 0.14; range = 0.53-1.00) and comparable to *Z. noltii.* All intertidal locations of *Z. marina* showed significant departures from Hardy Weinberg Equilibrium (Table 2); whereas the two subtidal locations did not.

Population structure

Zostera noltii. The Bayesian analysis implemented in Structure suggested $K = 3$ clusters when using the ΔK ad-hoc statistic and $K = 6$ clusters when averaging over the replicates based on the posterior-probabilities (Fig. 3). Under $K = 3$, the distinct clusters corresponded to: 1) northern Wadden Sea (n = 7); 2) central Wadden Sea (n = 4); and 3) Zeeland (Fig. 2). Although included in the northern Wadden Sea cluster, Hals (Denmark) was apparent within the central Wadden Sea cluster. With $K = 6$, a finer partitioning was revealed with clusters two and three under K =3 subdivided into two and three clusters, respectively (data not shown).

Zostera marina. Both methods for determining K revealed 4 clusters, corresponding to the three (+Zeeland) of *Z. noltii* (Fig. 3): 1) northern Wadden Sea (n = 6); 2) central Wadden Sea (n = 2); 3) Zeeland; and 4) Kattegat (Norhold). Clusters were highly distinct in the southern and central regions, but admixture was evident in the northern Wadden Sea.

Table 2. Population genetic characterization of *Z. noltii* and *Z. marina* populations in the Wadden Sea. N = Number of ramets, G = Number of genets, R = Genotypic diversity, A = Allelic richness, A_c = Allelic richness corrected for sample size (n = 19), H_{exp} = Expected heterozygosity, H_{obs} = Observed heterozygosity, F_{IS}= Inbreeding coefficient, ramets^{-genet} = Number of ramets per genet; S = subtidal; I = intertidal; *p = 0.05; **p <0.01. Location numbers/letters (parentheses) refers to geographic location in Figure 2.



Location	**Habitat**	N	G	R	A	A_c	H_{exp}	H_{obs}	F_{is}	**ramets^{-genet}**
Zostera noltii										
The Netherlands										
Yerseke/Zeeland (1)	S	32	32	1.00	4.7	4.1	0.46	0.46	0.004	1.00
Gas Plant/North Groningen (2)	I	42	41	0.98	6.6	6.3	0.60	0.59	0.019	1.02
Germany										
Norddeich (3)	I	46	23	0.50	6.7	6.4	0.61	0.56	0.085*	2.00
Horrumer Siel (4)	I	36	19	0.53	6.2	6.2	0.63	0.61	0.034	1.89
Seefelder Watt (5)	I	39	32	0.82	4.7	4.3	0.48	0.43	0.091*	1.22
Wesselburner Watt (6)	I	38	34	0.89	6.9	6.2	0.63	0.61	0.036	1.12
Nordstrand (7)	I	31	30	0.97	7.0	6.3	0.65	0.62	0.053	1.04
Hamburger Hallig (8)	I	35	35	1.00	7.8	6.7	0.68	0.65	0.055*	1.00
Amrum (9)	I	34	25	0.74	6.8	6.3	0.61	0.61	-0.001	1.36
Rantum/Sylt (10)	I	42	39	0.93	7.8	6.5	0.65	0.66	-0.015	1.08
Königshafen/Sylt (11)	I	44	25	0.57	7.1	6.6	0.67	0.63	0.059*	1.76
Denmark	I									
Mandø (12)	I	38	36	0.95	7.6	6.7	0.68	0.72	-0.057*	1.06
Hals/Limfjord (13)	I	44	36	0.82	6.2	5.3	0.51	0.56	-0.086**	1.23
Mean (SD)		38.5 (4.8)	31.3 (6.6)	0.82 (0.18)	6.6 (1)	6.0 (0.9)				1.29 (0.36)

Zipperle et al. (2012) Two species, one pattern: A genetic perspective on diversity and connectivity of *Zostera marina* (L.) and *Zostera noltii* (Hornem.) populations in the Wadden Sea. *Plant Biology*. Submitted manuscript.

Table 2. (continued) Population genetic characterization of *Z. noltii* and *Z. marina* populations in the Wadden Sea. *N* = Number of ramets, *G* = Number of genets, *R* = Genotypic diversity, *A* = Allelic richness, A_c = Allelic richness corrected for sample size (n = 19), H_{exp} = Expected heterozygosity, H_{obs} = Observed heterozygosity, F_{IS} = Inbreeding coefficient, ramets^{-genet} = Number of ramets per genet; S = subtidal; I = intertidal; *p = 0.05; **p <0.01. Location numbers/letters (parentheses) refers to geographic location in Figure 2.

Location	**Habitat**	***N***	***G***	***R***	***A***	A_c	H_{exp}	H_{obs}	F_{is}	**ramets**$^{-genet}$
Zostera marina										
The Netherlands										
Sint-Annaland/ Zeeland (A)	S	70	66	0.94	5	4	0.44	0.41	0.073**	1.06
Ems-Dollard Estuary "De Paap"/North Groningen (B)	I	50	48	0.98	6.25	6.1	0.51	0.45	0.119**	1.02
Germany										
Norddeich (C)	I	38	32	0.84	5.1	4.6	0.48	0.24	0.500**	1.19
Nordstrand (D)	I	50	49	0.98	8	6.4	0.6	0.53	0.109**	1.02
Hamburger Hallig (E)	I	49	49	1.00	8.6	6.6	0.59	0.59	0.124**	1.00
Amrum (F)	I	46	45	0.98	9.1	6.8	0.59	0.53	0.122**	1.02
Rantum/Sylt (G)	I	33	33	1.00	8.4	7.2	0.64	0.59	0.081**	1.00
Königshafen/Sylt (H)	S	27	19	0.93	6.6	6.6	0.63	0.63	0.011	1.42
Denmark										
Mandø (I)	I/S	51	50	0.98	8.4	6.6	0.62	0.58	0.050*	1.02
Norhold/Randers Fjord (J)	S	47	25	0.53	7.5	6.9	0.56	0.55	0.010	1.88
Mean (SD)		46.1 (11.7)	41.6 (14.1)	0.92 (0.14)	7.3 (1.5)	6.2 (1)				1.16 (0.28)

Zipperle et al. (2012) Two species, one pattern: A genetic perspective on diversity and connectivity of *Zostera marina* (L.) and *Zostera noltii* (Hornem.) populations in the Wadden Sea. *Plant Biology*. Submitted manuscript.

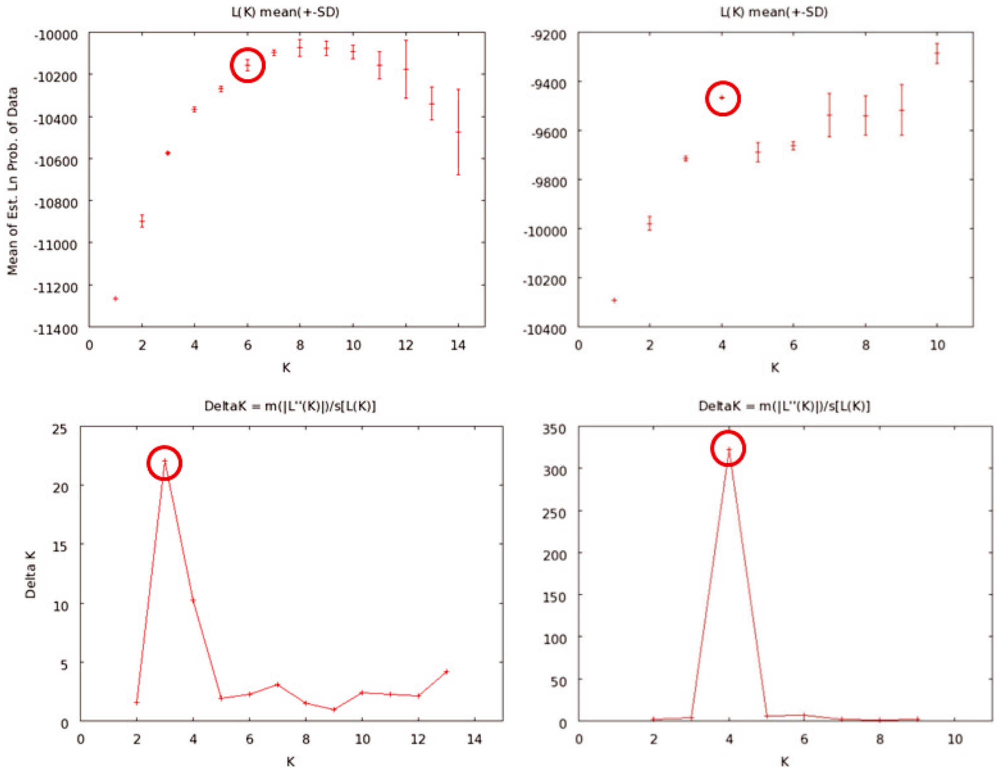


Figure 3. Determination of the number of population clusters (*K*) for *Z. noltii* and *Z. marina* with the software STRUCTURE using two different methods. Top panel based on the posterior probability method Pr(X| *K*); lower panel based on Δ*K* method, respectively. Circled values indicate the most likely *K*.

Discussion

Zostera marina

Despite the severe population bottleneck in the 1930s and the subsequent 70 years of progressive decline of *Z. marina* meadows (both intertidal and subtidal) in the Wadden Sea, genetic diversity remains high. Local (meadow) structure is influenced by some inbreeding, whereas regional population structure is driven by propagule exchange (Ferber et al. 2008).

At the meadow level, most *Z. marina* meadows showed some heterozygote deficiency, perhaps due to local-scale seed dispersal limitations, concomitantly generating closely-related neighborhoods and bi-parental inbreeding in sparse stands (Reusch 2001, Hämmerli and Resuch 2003a). Alternatively, the overall scarcity of *Z. marina* across the Wadden Sea (Fig. 1) might have resulted in a small effective population size (N_e) and an increasing number of matings between relatives on the local scale (Ellstrand and Elam 1993). Although the frequency of selfing is positively correlated with increasing clone size in *Z. marina* (Reusch 2001) and can cause heterozygote deficiency in intertidal clonally propagating meadows, clonality is low within *Z. marina* meadows in the Wadden Sea and clone sizes are accordingly small.

At the regional scale, propagule import most likely sustains the observed high genetic and genotypic diversity, similar to a 'genetic rescue' effect (Ingvarsson 2002, Tallmon et al. 2004). Under this scenario, fitness of distant populations is increased by propagule import as immigrants produce more fit offspring compared to the local population. Outcrossed offspring of *Z. marina* are superior and heterozygosity is positively correlated with fitness (Reusch 2001, Hämmerli and Reusch 2003b). 'Genetic rescue' effects also have been observed in *Sarracenia flava* (yellow pitcher plant; Sheridan and Karowe 2000) and demonstrated experimentally in *Silene alba* (white campion; Richards 2000) and *Brassica campestris* (wild turnip; Newman and Tallmon 2001). The overall high genetic and genotypic diversity of *Z. marina* in the Wadden Sea further supports an earlier study identifying the Wadden Sea as a hot spot for genetic diversity in Europe (Olsen et al. 2004).

Zostera noltii

The 1930s wasting disease apparently did not affect *Z. noltii* and as for *Z. marina*, genotypic and genetic diversity was high, agreeing with an earlier study (Coyer et al. 2004a). At the meadow scale, local inbreeding was minor as compared with *Z. marina*. Only four of the 13 *Z. noltii* meadows revealed a weak homozygote excess and all were either locally isolated (e.g. Seefelder Watt in Jade Bay) or sparsely distributed (e.g. isolated patches with meter-scale distance between patches to single-shoot bundles dispersed over a mud flat). Selection against inbred offspring may alleviate heterozygote deficiency in *Z. noltii* (Zipperle et al. 2011). Furthermore, the intertidal *Z. noltii* meadows are more common and much larger than both intertidal and subtidal *Z. marina* meadows in the Wadden Sea (Fig. 1) (Reise and Kohlus 2008, van der Graaf 2009). Larger meadow size decreases the probability of inbreeding (Young et al. 1996, Spigler et al. 2010), as do small clone sizes, relative large pollen dispersal distances, predominant outcrossing mating system, long-term seed bank and frequent seedling recruitment (Alexandre et al. 2006, Zipperle et al. 2009a,b, 2011). At the regional scale, a population structure and connectivity almost identical to that of *Z. marina* were observed.

Barriers

The Wadden Sea can be considered as two regions or 'management units' (north, central) with respect to *Z. marina* and *Z. noltii*, as the Eems-Dollard and Elbe estuaries form significant barriers to gene flow (Fig. 2). A third unit consists of southern Netherlands. Analysis of microsatellite genotypes with STRUCTURE 2.3.3 reveals very little connectivity among the three clusters. River estuaries have also been identified as a barrier to gene flow among *Z. noltii* populations along the Portuguese coast (Dieckman et al. 2005), as well as for populations of macroalgae (Alberto et al. 1999). In addition to the estuaries, extensive coastal areas do not/cannot harbor seagrass, and as such, provide another effective barrier to dispersal/gene flow. For examples, a 300 km stretch of coastline on either side of the Elbe estuary is devoid of *Z. marina* and the exposed/high-energy North Sea coast of the Netherlands from Den Helder to Hoek van Holland is uninhabitable for either species. Although long-distance dispersal (34-54 km; Harwell and Orth 2002; Reusch 2002) of seed-bearing spathes has been demonstrated for *Z. marina*, absence of appropriate habitat along a substantial section of coast will always be a barrier to gene flow. Seed transport in or on migrating waterfowl (endo- or exozoochory) across large areas devoid of seagrass is unlikely because of the temporal mismatch between waterfowl migration and fruiting of *Zostera* species in the Wadden Sea (Ehlers 2002, Clausen et al. 2002).

Living apart together

Where both species occur together (in the northern Wadden Sea), the meadows remain relatively stable. Recent studies have shown that *Z. noltii* may facilitate *Z. marina* by protecting new *Z. marina* recruits (vegetative shoots or seedlings) (van Katwijk and Wijgergangs 2004; Bos and van Katwijk 2007), although shading of annual forms by perennial forms reduced both survival and reproductive potential (Keddy 1987). Alternatively, the paired occurrence of *Zostera* species might be explained by the availability of suitable habitat. The scarcity of monospecific *Z. marina* meadows and the dominance of *Z. noltii* meadows, however, supports the facilitation hypothesis, with the mechanisms being protection against desiccation during low tide and sheltering new *Z. marina* recruits from currents and wave action (van Katwijk and Wijgergangs 2004, Bos and van Katwijk 2007).

The rarity of Z*. marina* in the central and southern Wadden Sea has been attributed to a combination of initial population decimation due to the wasting disease, followed by progressive deterioration of the subtidal habitat (coastal construction, nutrient enhancement, increased turbidity, etc.) (van der Heide et al. 2007, Reise and Kohlus 2008). It remains to be seen whether the suitable historical conditions can be restored in these regions, because the present regime shift will be difficult to reverse (van der Heide et al. 2007).

Morphotypes equal ecotypes

Correlations between morphotypes and habitat are generally attributed to plasticity (e.g., Keddy and Patriquin 1978, Gagnon et al. 1980, Keddy 1987, van Lent and Verschuure 1994, Peralta 2000, van Katwijk 2000, Vermaat et al. 2000, Schanz and Asmus 2003, Peralta et al. 2005). Common garden experiments (Keddy and Patriquin 1978) revealed that seeds from annual and perennial *Z. marina* each gave rise to both annual and perennial morphotypes despite differences in phenology and distribution in nature. In contrast, however, reciprocal transplant experiments between annuals and perennials revealed that each retained its morphotype, thus supporting ecotypic differences (Keddy 1978).

Assays using neutral genetic markers have also been unable to detect differences between the morphotypes (Gagnon et al. 1980, 17 allozyme loci; Oetjen and Reusch 2010; 25 microsatellite loci); however, genetic analyses based on genome scans of *Z. marina* in the Wadden Sea did find differences in annual intertidal and perennial subtidal ecotypes that were attributed to habitat specific divergent selection (Oetjen and Reusch 2007, Oetjen et al. 2010). As expected, such differences were not detectable in our Structure analysis using neutral markers.

Cast in traditional ecological terms, plasticity is reversible, non-genetically driven and environmentally determined; whereas ecotypic differentiation is irreversible, genetically-hard-wired, and independent of the environment. In terms of contemporary ecological genomics, both ecotypic differentiation and plasticity are genetically controlled but at different levels; the DNA level (detected as genotypic differences) and the RNA level (detected as transcriptional regulatory differences). At present, our knowledge of what controls plasticity of annularity and perenniality in *Z. marina* is not known although clearly rapid and strong habitat selection on a suite of (as yet unidentified) genes is part of the story (Oetjen and Reusch 2010). More generally, it is well-established in plant models that annual and perennial phenotypes occur repeatedly within and among species, and are strongly coupled with meristematic activity and cell death/senescence (Thomas et al. 2000).

Annularity and perenniality thus appear to be a matter of relative gene expression during development. Recent modeling work further suggests that annual and perennial life histories are epigenetically regulated in response to environment-specific selection affecting flowering (Satake 2010). The possible role of epigenetic inheritance, i.e., heritable changes in gene expression and function that cannot be explained by changes in DNA sequences (*sensu* Richards 2006), opens the door to our understanding how phenotypic plasticity interacts with changing environments. For example, epigenetic processes have been shown to induce transition in flowering plants (Bastrow et al. 2004) and flowering time is related to annularity and perenniality. Though speculative for the moment, it appears that the switch to annularity in Wadden Sea populations of *Z. marina* is an epigenetic response to as yet unidentified environmental drivers and is a key challenge.

In conclusion, earlier concerns about loss of genetic potential, isolation, and lack of connectivity among *Zostera* species in the Wadden Sea are unfounded. Restoring physical habitat may be possible in some regions, while radically changed hydrodynamics may preclude successful mitigation in others (e.g the Dutch Wadden Sea).

A joint restoration approach for *Z. noltii* and *Z. marina* to regions of the Wadden Sea, combined with seeding of *Z. marina* into existing *Z. noltii* beds using a recently developed buoy technique (Pickerell et al. 2005) and/or artificial depression seed-traps (Zipperle et al. 2010), offer a realistic 'first step' (a seeding program was initiated at three locations in the Wadden Sea in late 2011 by RIKZ). From a management perspective, the Wadden Sea should be considered as two management units (central, northern) in addition to a third unit (Zeeland, southern Netherlands), because of the low levels of gene flow (based on neutral microsatellite loci) between the units. However, differentiation patterns revealed by neutral loci may reflect differential patterns of selection. Consequently, transplantation and seeding efforts should stem from source patches within a unit, rather than between units, in order to minimize potential negative effects of 'genomic invasions' (*sensu* Mallet 2005).

Acknowledgments We thank Anja Schanz for extensive help in the field and Jan Veldsink for laboratory assistance. AMZ was supported by a PhD scholarship from the Ubbo-Emmius Programme at the University of Groningen.

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Chapter 7

Summary and Conclusions

SUMMARY

Seagrasses are ecosystem engineers providing numerous ecosystem services by adding structure to otherwise flat, featureless, and soft sediment habitats, as well as providing food and shelter for innumerable species. Seagrasses are restricted, like coral reefs, to the shallow margins of the world oceans. It is this restriction that hastens their world-wide decline as coastlines are the most populated areas of the world and pollution from terrestrial run off and fragmentation/destruction from coastal construction are the prime threats. Projected temperature increase and sea level rise under the climate change scenario put additional pressures on seagrass ecosystems. Survival, therefore, heavily depends on their intrinsic resilience to disturbances and on better management practices of these vulnerable coastal habitats.

In the 1930s' the 'wasting disease' (caused by the marine slime mold protist *Labyrinthula zosterae*) removed up to 90% of *Z. marina* (eelgrass) stands along the Wadden Sea coast and these populations have never really recovered, largely due to a changed hydrodynamic regime and concomitant light limitation resulting from increased turbidity. Despite high genetic diversity, which might lend itself to greater intrinsic resilience, and several restoration efforts, *Z. marina* presently is restricted to a few sandy to muddy intertidal regions where it often co-occurs with the smaller and more abundant congener *Z. noltii* (dwarf eelgrass). *Zostera noltii* has emerged as the dominant seagrass species in the Wadden Sea and it too, displays high diversity throughout its distribution. It has also expanded in the northern Wadden Sea and throughout Europe since the wasting disease epidemic (Borum et al. 2007, Reise and Kohlus 2008).

In an attempt to understand factors affecting the different trajectories of the two species, my thesis hypothesized that mating system differences have played a key role. The main question asked was:

- **How does the reproductive system facilitate persistence and resilience of dwarf eelgrass in the Wadden Sea?**

The term mating system combines at least three complex processes of plant reproduction: 1) life-history (monoeocy vs. dioecy); 2) patch maintenance (vegetative vs. sexual reproduction); and 3) fertilization of gametes (outcrossing vs. inbreeding). Interactions occur among all three features. For example, a dioecious plant exhibiting extensive vegetative propagation over many meters might suffer from pollen limitation if male genets in the population are below a crucial threshold level or more generally, if genets exceed pollen dispersal distance, thereby rendering sexual reproduction insignificant (as documented in some mega-clones of *Z. marina*).

Zostera noltii is a monoecious hermaphrodite combining sexual reproduction and vegetative propagation. Earlier studies concluded that sexual reproduction in *Z. noltii* was of little importance relative to vegetative propagation for patch maintenance (but see Alexandre et al. 2006). Additionally, a high potential for geitonogamous selfing exists because of clonality. Both characteristics seem to contradict the observed high genetic and genotypic diversity found in the Wadden Sea.

To study the interacting reproductive processes impacting patch maintenance of *Z. noltii*, my main general question was divided into five sets of sub-questions, each of which comprise a separate chapter of my thesis. Ecological and molecular methods were combined to identify clones in the field and to integrate findings over various spatial scales (the clone, the patch, the Wadden Sea).

Chapter 2 formed the baseline study and addressed two questions:

- **What is the clone structure and patch turn-over rate of *Z. noltii*?**
- **What is the contribution of clonal and sexual reproduction to patch maintenance?**

A previous phylogeographic study of *Z. noltii* throughout northern Europe demonstrated that average clone size for *Z. noltii* was <3 m^2, but ranged from <1 m^2 to >10 m^2 (Coyer et al. 2004). The distinct seasonality of *Z. noltii* in the Wadden Sea, suggests small to medium clone sizes and a fast turn-over rate.

Accordingly, clone structure, patch turn-over rates, and recruitment in *Z. noltii* Wadden Sea meadows were examined with a hierarchical sampling design. Two permanent 10 x 10 m study plots were established in a large *Z. noltii* meadow off the Island of Sylt (German Wadden Sea). Each study plot was subdivided into 1 x 1 m quadrats to examine large-scale patterns of clonality; additionally, four 1 x 1 m quadrats were randomly chosen for further subdivision into 0.2 x 0.2 m sub-quadrats for a fine-scale assessment of clone structure.

For genetic analysis a single shoot was sampled at the center of each 1-m^2 quadrat in spring and autumn for four years and additionally at the center of each sub-quadrat for two years. DNA was extracted and amplified in multiplex-PCR reactions. The extraction method, as well as the PCR multiplex protocol for the nine microsatellite markers employed, was developed in the course of this thesis (Table 1, Box 3).

Clone maps were generated at the 100-m^2 and 1-m^2 scales for each sampling date and combined with data on density expressed as percent cover. Clones were tracked spatially and temporally to deduce average clone size, average clone number and patch turn-over rate. Seedling recruitment was determined from frequency of center-shoot seedlings during the sampling period.

Average clone size ranged from 1.2 to 1.4 m^2, although large clones of up to 9 m^2 were occasionally present. Crucially, ca. 20% of genets persisted through the 4-year study period, demonstrating that *Z. noltii* (at least at Sylt) is a perennial species rather than an annual one. Survival was independent of clone size on the 100-m^2 scale. At the 1-m^2 scale, however, the clone that was dominant in the early season remained dominant through autumn, thereby suggesting early inter-clonal competition and a phalanx growth strategy (rhizomes resulting from clonal propagation are short and long-lived) for *Z. noltii* as opposed to a guerilla strategy (rhizomes are long and short-lived).

A severe heat stress event during the summer of 2003 decimated the seagrass cover, but recovery was swift from new seedling recruitment in the following year. The results showed that long-term persistence of *Z. noltii* in the Wadden Sea is realized by a probable seed bank (see below) and strong Spring recruitment (as opposed to seasonal shoot die-off and recovery from rhizomes); a scenario in sharp contrast to subtidal *Z. noltii* meadows in the Mediterranean, which are characterized by large average clone sizes (Coyer et al. 2004, Ruggiero et al. 2005) and low annual recruitment.

Extrinsic factors further affecting persistence included lugworm disturbance, geese grazing (see below), tidal currents and ice scour. Thus, recruitment alone does not determine clone size or persistence but the fact that the recovery potential exists in the sediment (the seed bank), provides added insurance for a quick recovery when disturbances completely wipe out both below and above sediment biomass. Sexual reproduction and new recruitment are thus of greater relative importance than vegetative expansion and multi-year clonal maintenance in the Wadden Sea.

Chapter 3 analyzed mating system and paternity in order to understand the relationship between clone structure and pollen dispersal distance. Specifically, I addressed the following questions:

- **What is the proportion of outcrossing vs. inbreeding in *Z. noltii* in the Wadden Sea?**
- **What is the relationship between pollen dispersal distance and average clone size; is pollen limiting for outcrossing beyond the border of the average genet?**

Samples were collected within one of the 10 x 10 m plots in a dense *Z. noltii* patch off the island of Sylt. Seed-bearing shoots were sampled from eight randomly chosen 1-m^2 quadrats within the plot, which were further sub-divided into 25, 20 x 20 cm sub-quadrats. When present, a seed-bearing shoot was collected from the center of each sub-quadrat.

All 256 adult shoots were genotyped with nine microsatellite markers and multiple replicate genotypes were removed from the data set. A total of 148 maturing seeds were extracted from 71 spathes collected from 32 distinct mother genets and were genotyped with the same set of markers. Seed and adult genotypes were used in subsequent analyses.

The high outcrossing rate (0.886) was similar to rates for both inter- and subtidal *Z. marina* (Ruckelshaus 1995 subtidal: 1.00, intertidal: 0.90; Reusch 2000: intertidal: 0.96 and 0.97). However, deviation from complete outcrossing was significant indicating the presence of a small fraction of inbred seeds. Geitonogamy was responsible for most inbred seeds, while biparental inbreeding was minor (1.5 % of total inbreeding). Multiple pollen donors (6-8) were determined for each genet indicating that pollen availability did not limit outcrossing.

Pollen dispersal distance (determined by paternity analysis) exceeded average clone size, thus ensuring outcrossing. Additionally, paternity analysis revealed an assignment rate of only 20 – 30% within the plot, indicating that a large fraction of imported pollen from outside the plot contribute to fertilization. Thus, clone size is no greater than pollen dispersal distances and does not restrict outcrossing.

Chapter 4 examined the *Z. noltii* seed bank. Recruitment may stem directly from the previous year seed set or from a persistent seed bank with an accumulation of seeds over multiple years, thereby serving as a permanent reservoir for genetic and genotypic diversity. In general, the existence of seagrass seed banks is controversial. Past studies found few *Z. noltii* seeds in Wadden Sea sediments and depletion of the sparse seed bank before winter, leading to a general conclusion that seed banks were unimportant. My results (**Chapter 2** and **3**) suggest the opposite, namely that a persistent seed bank of *Z. noltii* exists and is important for patch maintenance. Therefore, in **Chapter 4**, I addressed the following questions using ecological and molecular methods:

- **Is there a *Z. noltii* seed bank and if so, what type of seed bank is it?**
- **What is the annual contribution of the seed bank to the patch?**

Sediment cores were collected from the *Z. noltii* meadow on a monthly basis throughout the growing season for two subsequent years. Sediment was oven-dried prior to searching for *Z. noltii* seeds. Phenology of the study meadow was tracked for one year by determining density of vegetative and generative shoots, as well as seedling density in spring. The contribution of the sediment seed bank to annual recruitment was estimated from the germination of seeds under laboratory and field conditions in relation to seed bank size as determined in the field. For laboratory germination, seeds were extracted from sediment cores directly after sampling in spring and placed in a controlled laboratory environment for germination. To determine seed bank dynamics, an assignment test was performed using microsatellite genotypes of seedlings sampled in 2005 and 2006 to adult populations sampled in 2002-2004.

Annual sediment density (SD) of *Z. noltii* seeds ranged from 350 (95) to 500 (269) seeds m^{-2} with extremely high variance, strongly suggesting a highly aggregated distribution. While 16-25% of seeds germinated under laboratory conditions, field assessment of survival to seedling stage revealed a 12% contribution of the seed bank to the existing meadow. Approximately 20% of shoots found in spring were seedlings and 7-33% of the seedlings could be assigned to the local adult population in the current or preceding years. Thus, a seed bank exists and persisted for at least three years and significantly contributed (20% during the study period and up to 100% following the 2003 heatwave) to patch maintenance in *Z. noltii*.

Chapter 5 investigated the role of substrate depressions in seed retention and germination. Seagrass seed banks are patchy. For example, patchiness in tropical seagrasses was caused by seeds accumulating in sediment depressions generated by macrograzers, such as feeding pits made by dugongs. The accumulation and subsequent burial of seeds in the depressions greatly facilitated seed germination.

A similar mechanism may exist for *Z. noltii.* The seeds are ellipsoids, 1-2 mm in size and negatively buoyant. Consequently, they drop immediately to the substratum after release from the spathe and subsequent dispersal is limited to a few meters depending on current velocities as seeds may be swept away immediately if not trapped in the sediment. The presence of depressions caused by grazing of Brent geese (*Branta bernicla*) in autumn was hypothesized to form seed traps. In **Chapter 5** I addressed the question:

- **Does seed accumulation in geese feeding pits and subsequent burial in autumn enhance spring germination of *Z. noltii* seeds?**

The experiment was established in two locations at the center and edge within each of two *Z. noltii* meadows (sites) on the island of Sylt. An experimental unit consisted of three components: 1) a treatment pit (resembling a natural geese pit), 2) a manipulated control (creation of a shallow pit followed by immediate sediment replacement), and 3) an untouched natural control. Experimental units were replicated eight times at each location in each site. The experiment was conducted during winter 2005/ 2006 and was terminated in May 2006 at the conclusion of germination.

A 3-way factorial ANOVA revealed significantly more germination in treatment pits compared to the controls. In the center of the meadow, a comparison of seedling densities between treatment pits and immediately adjacent portions of the natural meadow revealed no differences. At the edges of the meadow, in contrast, there was significantly enhanced germination in the treatment pits. The results suggested that geese feeding pits in *Z. noltii* meadows trapped seeds in autumn, which facilitated spring germination. Accumulation and burial of seeds in geese pits is a potential mechanism for seed bank formation and explains seed aggregation/patchiness. As with the tropical dugong example, moderate grazing disturbance and the formation of the pits, facilitates seed bank formation and subsequent new recruitment at the meadow edges and/or in areas where open space becomes available.

Chapter 6 compared a number of population attributes and parameters between *Z. noltii* and *Z. marina*. Specifically:

- **How do patterns of genetic and genotypic diversity of *Z. noltii* and *Z. marina* vary along the coasts of the Wadden Sea?**
- **What is the spatial scale of a *Z. noltii* 'population' in the Wadden Sea?**
- **How do connectivities compare between the two species?**

- **How does the population structure of intertidal *Z. noltii* compare to the congener intertidal/subtidal species *Z. marina* in the Wadden Sea?**

Zostera noltii and *Z. marina* meadows were sampled throughout the Wadden Sea and selected adjacent areas. Sites were chosen in which both species co-occurred. Samples were genotyped using nine microsatellite loci specific to each species and a Bayesian method was used to infer population structure.

Allelic and genotypic diversity were high for both species, confirming earlier results of a genetic 'hot spot' in the Wadden Sea (Coyer et al. 2004) as compared with an European coastal study. Clone sizes were comparable between species found in the intertidal. Notably, four out of 13 *Z. noltii* patches and eight out of 10 *Z. marina* patches showed significant inbreeding, which corresponds to results suggested by the paternity analyses.

Four clusters of *Z. noltii* and *Z. marina* were identified: southern Netherlands (Zeeland), central-western Wadden Sea, northern Wadden Sea and north eastern Denmark. For both species, the Eems-Dollard and Elbe estuary were partial barriers to gene flow between the west-central and northern Wadden Sea. Consequently, the Wadden Sea is divided into two units (in addition to a southern Netherlands unit).

CONCLUSIONS

This thesis revealed that *Z. noltii* meadows in portions of the Wadden Sea were remarkably resilient to grazing disturbances and temperature extremes over the 4-year period of the study. Although *Z. noltii* meadows are expanding in some areas of the Wadden Sea, they are not in others. For example, the most recent Wadden Sea Quality Status Report (van der Graaf et al. 2009) concluded that seagrasses in the Wadden Sea (*Z. noltii* and *Z. marina*) have expanded in the northern Wadden Sea, but declined in southern and central parts of the Wadden Sea. Additionally, seagrass meadows expanded and contracted around centers of long persisting meadows in the northern regions (Reise and Kohlus 2008), but such centers seem to be missing in the southern and central Wadden Sea (van der Graaf et al. 2009). While eutrophication and turbidity are two of the major drivers of seagrass loss, increased hydrodynamics, diking and construction of drainage ditches also have major impacts on seagrass beds.

The seagrass conservation objective fixed in the Trilateral Wadden Sea Plan (www.waddensea-secretariat.org/management/Plan.html), calls for "…an increased area and a more natural distribution and development of *Zostera* fields". Transplantation programs are one means of achieving the objective and as such, small-scale transplantation efforts have been undertaken to reintroduce subtidal *Z. marina* in the west-central, Dutch portion, of the Wadden Sea. However, transplantation success has been meager due to low survival of transplanted adults and seedlings. For example, small patches of *Z. marina* and *Z. noltii* were transplanted on Balgzand in 1993. While the *Z. noltii* patch increased in size and was still present in 2006 (van Katwijk et al. 2006), the *Z. marina* patch dwindled and eventually disappeared (van Katwijk et al. 2009). Considering that seedlings are the most vulnerable life-history stage (Alexandre et al. 2006), it has been recommended that future transplantation programs for *Z. marina* focus on adult shoots combined with the deposition of seed bearing leaf shoots (Bos and van Katwijk 2005). The most recent attempt by Erftemijer and van Katwijk (2010) utilized this approach. Results are pending.

The variable success of *Zostera* transplantation in the Wadden Sea raises two questions: 1) What is the potential role of the mating system regarding the superior performance of *Z. noltii* compared to *Z. marina* in the intertidal*;* and 2) Might conservation and restoration success of *Z. marina* benefit from a joint strategy with *Z. noltii*? I approach these questions by synthesizing data from my thesis in combination with a recent review of seagrass research in the Wadden Sea over the past 20 years (van Katwijk et al. 2009), which formulated five guidelines to successful seagrass restoration. Below, I incorporate my data with four of the guidelines to address seagrass restoration planning for the Wadden Sea.

1. Reverse habitat degradation, physical habitat quality and connectivity

The most severe threats to Wadden Sea seagrasses are the continuous habitat loss due to diking (which induces changes in the hydrodynamic regime) and drainage ditching, coupled with increased turbidity and eutrophication (Reise 2005, Lotze et al. 2006). As coastal construction is unlikely to be curtailed, associated changes in hydrodynamics are more or less permanent. In some areas, notably the west-central Wadden Sea, such changes have altered the physical conditions to such an extent that restoration may no longer be possible (van der Heide et al. 2007). In other areas, however, coastal turbidity and eutrophication have decreased over the past decades, which may explain some of the increases in the northern Wadden Sea. In addition, decreased storm activity over the past decades and the sheltered location of seagrass meadows behind barrier islands (Reise and Kohlus 2008) may further account for their persistence in the northern Wadden Sea.

A second factor relates to population structure within the seagrass metapopulation. Genetic surveys confirm that *Z. noltii* is predominantly outcrossing, genetically diverse, regularly recruiting and well connected (as opposed to being characterized by a few large clones, low recruitment, patch isolation and poor connectivity). Similar results were found for *Z. marina* by Ferber et al. (2008).

Considering the dominant flow of currents and decreased connectivity between the two Wadden Sea clusters for both species, the west-central Wadden Sea represents a slowly depleting source location with the sweeping northeast current flow. Thus, both *Z. marina* and *Z. noltii* would benefit most from reestablishment of meadows in the west-central Wadden Sea via transplantation of adult plants (with and without reproductive shoots) and/or seeds collected from the more diverse patches within the west-central region (van Katwijk et al. 2005, Erftemeijer and van Katwijk 2010, Giesen et al. 2010) (see Section 3, below and **Chapter 6**).

2. Selection of appropriate habitat

Persistent eelgrass beds are rare in the west-central Wadden Sea and most of the existing meadows are in decline (van der Graaf et al. 2009, van Katwijk et al. 2009). Assuming that the physical conditions can be ameliorated (by, e.g., the addition of sheltered barriers), one means of restoring eelgrass meadows in this area may be to stabilize the few existing meadows as ‘cores’ for reestablishment/expansion into adjacent areas. For example, as the meadow edge is most vulnerable to deterioration by hydrodynamic forces (Fonseca et al. 1982, Schanz et al. 2002, Widdows et al. 2008), restoration may be enhanced by planting seeds along sheltered edges before the onset of winter (**Chapter 5**). My data (**Chapter 2**) also support earlier studies (Vermaat and Verhagen 1996, Giesen et al. 2010) showing that recovery of *Z. noltii* in spring is most successful if winter survival (% cover) is >10%. Thus, increasing shoot density via transplantation of adult shoots and/or seedlings prior to the onset of winter, both in the meadow center and at the meadow edge, may maintain and/or enhance presently low density beds in the Dutch Wadden Sea.

The feasibility of establishing meadows *de novo* is difficult for two reasons. First, a new meadow must be very large in order to overcome the negative impact of adjacent sand flats and to ensure the self-facilitating effect of relatively dense seagrass beds (Bos et al. 2007, van der Heide et al. 2007, 2010). Second, *de novo* establishment requires transplantation of seeds, seedlings and adults, which is expensive and complicated. On a world-wide basis, successful *de novo* reestablishment of seagrass beds has been successful in only about 30% of the cases, even in sites were seagrass historically occurred (Orth et al. 2006a, van Katwijk et al. 2009).

Any transplantation effort will require a careful evaluation of the local environment/habitat (hydrodynamics, sediment composition, nutrients, presence of lugworms, etc.) to maximize success. Although, a habitat suitability map for eelgrass transplantations in the Wadden Sea exists (van Katwijk et al. 2000), it may be necessary to supplement these data with more localized evaluations e.g. environmental similarity analyses.

3. Selection of an appropriate donor area

From a management perspective, the Wadden Sea should be considered as two distinct units (west-central, northern). Whether these two "units" simply reflect isolation on either side of the German Bight or the result of differential selection pressures remains unknown. In this context, therefore, transplantation and seeding efforts should stem from the source patches within a unit, rather than between units, in order to minimize potential negative effects on fitness (*sensu* Mallet 2005).

From a genetic stand point, all *Z. noltii* and *Z. marina* meadows in the northern Wadden Sea are suitable donor locations for transplantation efforts within the northern area because of their high genotypic/genetic diversity and interconnectivity (**Chapter 6**, Ferber et al. 2008). The presence of a seed bank can play an important role for patch maintenance (**Chapters 2, 4**), but sexual reproduction varies across locations on a local scale and predominately vegetative patches definitely exist. Donors from several sexually reproducing sites within each unit are most suitable to ensure overall transplant success. Donors from purely vegetative (often clonal) patches should be avoided because of low genotypic diversity, even with high genetic diversity. In other words, 100 transplants that are genetically diverse, but all from a single or few clones, may have less success than 100 transplants, each of which is genetically distinct.

Although tempting, transplantation programs involving donor material from the northern Wadden Sea to the west-central Wadden Sea, should be avoided under the precautionary principle. Within 'unit' transplantation of *Z. noltii* has been partly successful, within the Zeeland (southern Netherlands) (Giesen et al. 2010), but future transplantations could benefit from a thorough genetic evaluation of donor and recipient sites.

4. Spreading the risks

Spreading the risk over several transplantation sites in space and time is one of the major factors influencing transplantation success (van Katwijk et al. 2009). However, genetic risk spreading potentially is of equal importance. As stated above, pooling transplants from different donor sites with suitable environmental similarity could significantly increase genotypic/genetic diversity in the transplantation site and positively influence reproductive success and/or fitness.

Risk spreading also involves transplantation methods, including what and how to transplant, and any program combining several methods is likely to be the most successful. One method shown to be successful for both *Z. noltii* and *Z. marina* is to transplant whole eelgrass sods with sediment and arrange the transplants in close spatial arrays at the recipient site (Schanz and Asmus 2003, Giesen et al. 2010, pers. observ.). Sods also may contain portions of the local seed bank, which in the case of *Z. noltii* persists for at least three years **(Chapter 3)** and thus provides a reservoir for new individuals and genetic diversity if the original transplants fail. Greve et al. (2005) reported recovery of a subtidal *Z. marina* meadow from a seed bank after a complete die-off caused by sediment anoxia.

The spatial arrangement of transplants at the reception site is crucial as it should consider pollen and seed dispersal distances to ensure outcrossing opportunities and gene flow among transplants and residents. A transplantation program should consider vegetative and reproductive shoots, as well as other life-stages. For example, transplantation of seedlings generally is not successful (van Katwijk and Hermus 2000, van Katwijk and Wijgergans 2004, Alexandre et al. 2006), but transplantation of seeds is inexpensive, logistically feasible, and a reliable method of seagrass restoration (Orth et al. 2006b, Orth et al. 2009, Erftemeijer and van Katwijk 2010). Increasing the eelgrass patch density in the Dutch Wadden Sea by a recently developed seeding buoy technology (Pickerell et al. 2005, 2006) is promising (Erftemeijer and van Katwijk 2010), especially in combination with sod transplantation. In this technique, mesh bags filled with reproductive shoots carrying maturing seeds are attached to floating buoys and floated over transplantation sites, thereby providing a constant seed rain and further increasing restoration success and/or the maintenance and/or expansion of existing sites. It is notable that *Z. noltii* seeds collected from donor sites and dispersed in recipient areas germinated and matured (Giesen et al. 2010). Furthermore, deposition of seeds in mesh bags gave promising results for *Z. marina* (Harwell and Orth 1999); similarly, seeds placed in artificially created pits or depressions yielded the highest number of seedlings (**Chapter 5**).

In conclusion, persistence and resilience of *Z. noltii* meadows in the northern Wadden Sea seems assured as long as habitat quality remains conducive (hydrodynamics, water clarity, nutrients, no invasive species). Recovery and eventual persistence of *Z. noltii* in the western Wadden Sea is unlikely given the current physical dynamics.

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Chapter 8

Samenvatting en conclusies

SAMENVATTING

Zeegrassen zijn ecosysteembouwers die verscheidene ecosysteem diensten verlenen door structuur te geven aan de anders platte en zachte sedimenten, en ook door voedsel en bescherming te bieden aan ontelbare soorten. Zeegrassen komen alleen voor in de ondiepe randgebieden van de oceanen, net als koralen. Deze beperking versneld hun wereldwijde achteruitgang omdat kustgebieden het dichtstbevolkt zijn. Verontreiniging vanaf het land en fragmentatie door bebouwing vormen de belangrijkste bedreigingen. Zeegras ecosystemen staan nog extra onder druk door de voorspelde temperatuur- en zeeniveaustijgingen als gevolg van de klimaatverandering. Overleving hangt daarom heel sterk af van hun intrinsieke veerkracht na verstoring, en van beter beheer van de kwetsbare kustgebieden.

In de dertiger jaren heeft de wierziekte (*wasting disease*; veroorzaakt door de mariene slijmzwam *Labyrinthula zosterae*) bijna 90% van het *Z. marina* (groot zeegras) bestand in de Waddenzee verwijderd en deze populaties hebben zich nooit volledig kunnen herstellen als gevolg van veranderende hydrodynamische omstandigheden en daarmee gepaard gaande verminderde lichtinval door de toegenomen troebeling. Ondanks een hoge genetische diversiteit, wat op zich moet leiden tot een groter intrinsieke veerkracht, en een aantal restoratie pogingen, komt *Z. marina* tegenwoordig slechts voor op een paar zanderige tot modderige gebieden in het intergetijdengebied, vaak samen met de kleinere, meer overvloedige en verwante *Z. noltii* (klein zeegras). *Zostera noltii* heeft zich nu als het meest dominante zeegras in de Waddenzee gemanifesteerd en bezit ook over het hele verspreidingsgebied de hoogste diversiteit. Sinds de wierziekte-epidemie heeft *Z. noltii* zich ook uitgebreid naar de noordelijke Waddenzee en door de rest van Europa (Borum et al. 2007, Reise en Kohlus 2008).

In een poging te begrijpen welke factoren de verschillende loopbanen van de twee soorten beïnvloed hebben, gaat mijn proefschrift uit van de hypothese dat verschillen in voortplantingssystemen een sleutelrol hebben gespeeld. De hoofdvraag is:

- Hoe bevordert het voortplantingssysteem de persistentie en veerkracht van het kleine zeegras in de Waddenzee?

De term voortplantingssysteem combineert tenminste drie processen die bij de reproductie van planten een rol spelen: 1) levenscyclus (eenhuizig vs. tweehuizig); 2) populatie-onderhoud (vegetatieve vs. seksuele voortplanting); en 3) bevruchting van voortplantingscellen (uitteelt vs. inteelt). Tussen deze factoren vinden interacties plaats. Bijvoorbeeld, een tweehuizige plant met uitgebreide vegetatieve verspreiding over vele meters zou kunnen lijden aan stuifmeel limitatie als de mannelijke *genets* in de populatie onder een cruciaal niveau zijn, of meer algemeen, als de *genets* verder verspreid worden dan het stuifmeel, dan wordt de seksuele reproductie van ondergeschikt belang (dit verschijnsel is gedocumenteerd voor een aantal zeer grote klonen van *Z. marina*).

Zostera noltii is een eenhuizige hermafrodiet met een combinatie van seksuele reproductie en vegetatieve verspreiding. Uit eerder onderzoek werd geconcludeerd dat voor het populatie-onderhoud de seksuele reproductie bij *Z. noltii* van ondergeschikt belang was wanneer vergeleken met de vegetatieve verspreiding (maar zie ook Alexandre et al. 2006). Bovendien komt geitonogame zelfbestuiving (buurbestuiving) voor vanwege klonale groei.

Om de interactieve reproductieve processen, die inwerken op het populatie-onderhoud van *Z. noltii*, te kunnen bestuderen, werd mijn hoofdvraag opgedeeld in een aantal deelvragen die in de hoofdstukken 2 t/m 6 van mijn proefschrift aan bod komen. Een combinatie van ecologische en moleculaire methoden werd gebruikt voor identificatie van klonen in het veld en om bevindingen over verschillende ruimtelijke schalen (de kloon, het veld, de Waddenzee) te integreren.

Hoofdstuk 2 vormt het fundament van de studie en behandelt een tweetal vragen:

- **Wat is de klonale structuur en populatie turnover snelheid van *Z. noltii*?**

- **Wat is de bijdrage van klonale en seksuele reproductie aan het populatie-onderhoud?**

Een eerder fylogeografisch onderzoek aan *Z. noltii* uit noordelijk Europa liet zien dat de gemiddelde kloongrootte voor *Z. noltii* < 3 m^2 was, maar kon varieren van <1 m^2 tot >10 m^2 (Coyer et al. 2004). De duidelijke seizoensgebondenheid van *Z. noltii* in de Waddenzee veronderstelt kleine tot doorsnee kloongroottes en een hoge turnover snelheid. Daarom werden klonale structuur, populatie turnover snelheden en rekrutering bij populaties van *Z. noltii* in de Waddenzee bestudeerd met een hiërarchisch bemonsteringsplan. In een groot *Z. noltii* veld bij het eiland Sylt (Duitse Waddenzee) werden twee permanente studie locaties van 10x10 m opgezet. Elk locatie werd onderverdeeld in kwadranten van 1x1 m ter bestudering van grootschalige klonale patronen; daarnaast werden vier willekeurige 1x1 m kwadranten gekozen voor verdere opdeling in 0,2x0,2 m sub-kwadranten voor het vaststellen van kleinschalige klonale structuur.

Voor de genetische analyse werd gedurende vier jaar in zowel het voorjaar als het najaar een enkele scheut verzameld uit het centrum van elk 1-m^2 kwadrant en gedurende twee jaar ook uit elk sub-kwadrant. DNA werd geëxtraheerd en geamplificeerd in multiplex-PCR reacties. Zowel de extractie methode als het toegepaste PCR multiplex protocol werd ontwikkeld in de loop van het promotie onderzoek.

Voor elke verzameldatum werden de klonen in kaart gebracht op de 100-m^2 en 1-m^2 schaal en gecombineerd met gegevens over de bedekkingsgraad (%). Klonen werden zowel in ruimte als tijd gevolgd om daarmee de gemiddelde kloongrootte, gemiddelde aantal klonen en populatie turnover snelheid vast te stellen. Rekrutering via zaailingen werd vastgesteld aan de hand van aantal zaailingen aanwezig in het midden van de scheut gedurende de bemonsteringsperiode.

Gemiddelde kloongrootte varieerde van 1,2 tot 1,4 m^2, hoewel er sporadisch grote klonen tot 9 m^2 aanwezig waren. Essentieel is dat ongeveer 20% van de *genets* gedurende de hele 4-jarige studie aanwezig bleven, daarmee aantonend dat *Z. noltii* (tenminste op Sylt) meerjarig is in plaats van eenjarig. Overleving was onafhankelijk van kloongrootte op de 100-m^2 schaal. Echter, op de 1-m^2 schaal bleef de dominante voorjaars kloon dominant tot in het najaar, waarmee aangegeven wordt dat bij *Z. noltii* klonen onderling competeren en een falanx groei strategie hebben (rhizomen van klonen zijn kort en leven lang), dit in tegenstelling tot een guerrilla strategie (rhizomen van klonen zijn lang en leven kort).

Een hevige hittegolf gedurende de zomer van 2003 decimeerde de zeegras-bedekking, maar herstel was snel door rekrutering van nieuwe zaailingen in het jaar daarop. De resultaten toonden aan dat de persistentie over langere periodes van *Z. noltii* in de Waddenzee waarschijnlijk gebeurd door een zaadbank (zie hieronder) en krachtige rekrutering in het voorjaar (dit in tegenstelling tot seizoensgebonden afsterven van scheuten en herstel vanuit de rhizomen). Dit is in contrast met sublitoraal *Z. noltii* velden in de Middellandse Zee, die gekenmerkt worden door grote gemiddelde kloongroottes (Coyer et al. 2004, Ruggiero et al. 2005) en lage jaarlijkse rekrutering.

Externe factoren die de persistentie beïnvloedde waren o.m. verstoring door wadpieren, grazende ganzen (zie hieronder), getijdestromen en ijsgang. Dus, rekrutering bepaald niet alleen de kloongrootte en -persistentie maar het feit dat er in het sediment een potentieel tot herstel bestaat (de zaadbank), levert een aanvullende garantie voor een snel herstel wanneer de omstandigheden de biomassa boven en onder het sediment vernietigen. Seksuele voortplanting en nieuwe rekrutering zijn in de Waddenzee dus van groter relatief belang dan vegetatieve uitbreiding en meerjarig onderhoud van klonen.

In **hoofdstuk 3** staat de analyse van het paringssysteem en vaderschap ter opheldering van de samenhang tussen de klonale structuur en verspreiding van stuifmeel. Ik stelde daarvoor de volgende vragen aan de orde:

- **Wat is de verhouding tussen uitteelt en inteelt in *Z. noltii* in de Waddenzee?**
- **Wat is de relatie tussen de verspreidingsafstand van het stuifmeel en gemiddelde kloongrootte; is stuifmeel beperkend voor uitteelt buiten de grens van de gemiddelde *genet*?**

Monsters werden verzameld uit een van de 10x10 m locaties in een dicht *Z. noltii* veld bij het eiland Sylt. In deze locatie werden de zaaddragende scheuten uit acht willekeurig gekozen 1-m^2 kwadranten geplukt, die verder werden onderverdeeld in 25, 20x20 cm sub-kwadranten. Indien aanwezig, werd een zaaddragende scheut uit het midden van elk sub-kwadrant geplukt.

Met behulp van negen microsatelliet merkers werd van alle 256 scheuten het genotype bepaald. Vervolgens werden doublerende genotypen uit het gegevensbestand verwijderd. In totaal werden uit 71 bloeiwijzen, behorende bij 32 moederplanten, 148 zaden geïsoleerd. Van deze zaden werd ook het genotype bepaald met dezelfde set merkers. Het genotype van zowel de zaden als de volwassen planten werden gebruikt voor verdere analyse.

De hoge mate van uitteelt (0,886) was vergelijkbaar met getallen gevonden voor *Z. marina* uit zowel het intergetijdengebied als in het sublitoraal (Ruckelshaus 1995 sublitoraal: 1.00, intergetijdengebied: 0.90; Reusch 2000: intergetijdengebied: 0.96 en 0.97).

De afstand waarover stuifmeel zich verspreid (bepaald met een vaderschapsanalyse) was groter dan de gemiddelde kloongrootte, waarmee uitteelt verzekerd is. De vaderschapsanalyse liet ook zien dat 20-30% van het stuifmeel uit de locatie zelf komt, aangevend dat een groot deel van het pollen van buiten aan de bevruchting deelneemt. De kloongrootte is dus niet groter dan de afstand waarover het stuifmeel zich verspreid en beperkt uitteelt niet.

Hoofdstuk 4 behandelt de *Z. noltii* zaadbank. Rekrutering kan direct van de zaadsetting van het jaar daarvoor afkomstig zijn of van een altijd aanwezige zaadbank, waarin zaden door de jaren heen opgehoopt zijn en daarmee dienend als een permanent reservoir voor genetische en genotypische diversiteit. Toch was het bestaan van een zaadbank bij zeegrassen controversieel. Bij eerder onderzoekingen werd in Waddenzee sediment een gering aantal *Z. noltii* zaden gevonden en uitputting van deze schaarse zaadbank voor het aanbreken van de winter, wat tot de conclusie leidde dat zaadbanken onbelangrijk zijn. Mijn resultaten (**Hoofdstuk 2** en **3**) suggereren het tegendeel, namelijk dat er een permanente *Z. noltii* zaadbank bestaat die belangrijk is voor het onderhoud van het *Z. noltii* veld. Daarom stelde ik in **hoofdstuk 4** de volgend vragen, daarbij gebruik makend van ecologische en moleculaire methoden:

- **Is er een *Z. noltii* zaadbank en zo ja, wat voor soort bank is het?**
- **Wat is de jaarlijkse bijdrage van de zaadbank aan het veld?**

Maandelijks werd gedurende twee opeenvolgende jaren tijdens het groeiseizoen sediment kernen uit het *Z. noltii* veld geboord. Voorafgaand aan het zoeken naar *Z. noltii* zaden, werd het sediment in een oven gedroogd. De fenomenologie van de studielocatie werd gedurende een jaar gevolgd door de dichtheid van de vegetatieve en generatieve scheuten te bepalen, en, in het voorjaar, ook de dichtheid van de zaailingen. De bijdrage van de sediment zaadbank aan de jaarlijkse rekrutering werd geschat aan de hand van ontkiemende zaden onder laboratorium en veld condities en gerelateerd aan de omvang van de zaadbank die bepaald werd door een schatting te maken van het aantal bloeiwijzen (= potentiële zaden). Kieming in onder constante laboratorium condities werd gedaan met zaden die onmiddellijk, na bemonstering in het voorjaar, uit de boorkernen werden gehaald. Om de dynamiek van de zaadbank te bepalen werd een toewijzings-test (*assignment test*) uitgevoerd met de microsatelliet genotypen van zaailingen verzameld in 2005 en 2006 naar volwassen populaties verzameld in 2002-2004.

Jaarlijks sediment dichtheid (SD) van *Z. noltii* zaden liep van 350 (95) tot 500 (269) zaden per m^2 met een extreem hoge variantie, die wijst op opeenhoping van zaden. Hoewel onder laboratorium omstandigheden 16-25% van de zaden kiemde, bleek uit de veld-analyses (overleving tot zaailing stadium) dat rond de 12% van de zaadbank bijdraagt aan de bestaande populatie. Ongeveer 20% van de voorjaarsscheuten waren zaailingen en 7-33% van de zaailingen kon worden toegewezen aan de lokale volwassen populatie van het lopend of voorafgaande jaren. Een zaadbank bestaat dus, persisteert gedurende tenminste drie jaren en leverde een belangrijke bijdrage aan onderhoud van het *Z. noltii* veld (20% gedurende de onderzoeksperiode en 100% na de 2003 hittegolf).

Hoofdstuk 5 onderzoekt de invloed van substraatputten op het bewaren en kiemen van zaad. Zaadbanken van zeegrassen kennen een onregelmatige verspreiding. Als voorbeeld de onregelmatige verspreiding in tropische zeegrassen. Deze wordt veroorzaakt door opeenhoping van zaden in sedimentputten gemaakt door grote grazers, zoals eetkuilen gemaakt door zeekoeien. Het opeenhopen en daaropvolgende begraven van zaden in de putten, vergemakkelijkte de kieming van zaden aanzienlijk.

Een vergelijkbaar mechanisme zou kunnen bestaan bij *Z. noltii*. De zaden zijn ellipsvormig, 1-2 mm lang en zinken. Als gevolg daarvan dalen ze direct naar de bodem na het loslaten uit bloeiwijze. Verdere verspreiding is dan beperkt tot een aantal meters, dit afhankelijk van de stroomsnelheden waarbij nog wel de kans bestaat dat de zaden door de stroom meegesleept worden voordat ze de bodem bereikt hebben. Sedimentputten, in de herfst veroorzaakt door grazende rotganzen (*Branta bernicla*), zouden als zaadvallen kunnen dienen. In **hoofdstuk 5** richt ik mij op de volgende vraag:

- **Bevordert het opeenhopen en vervolgens begraven van zaden in gans-eetputten in het najaar de kieming van *Z. noltii* zaden in het voorjaar?**

Het experiment werd uitgevoerd op twee locaties in het midden en aan de rand van elk van de twee *Z. noltii* velden op het eiland Sylt. Een experimentele eenheid bestond uit drie componenten: 1) een behandel put (lijkend op een natuurlijke put van een gans), 2) een gemanipuleerde controle (een zelfgemaakte ondiepe put die daarna onmiddellijk weer met sediment opgevuld werd) en 3) een onaangetaste natuurlijke controle. De experimentele eenheden werden in achtvoud uitgevoerd op elke locatie in elk veld.

Een *3-way factorial ANOVA* onthulde een significant hogere ontkieming in behandel putten dit in vergelijking met de controles. In het midden van het veld zijn er geen verschillen tussen de dichtheid in zaailingen tussen de behandel put en het omringende natuurlijke veld. Dit in tegenstelling tot de randen van het veld waar de kieming in de behandel put significant was toegenomen. De resultaten laten zien dat gans-eetputten in *Z. noltii* velden zaden invangen waardoor kieming in het voorjaar wordt bevorderd. Opeenhopen en begraven van zaden in gans-eetputten is een mogelijk mechanisme voor de vorming van zaadbanken en verklaart de opeenhoping/onregelmatige verspreiding. Net als bij de voorbeeld van de tropische zeekoe, werkt een matige verstoring door begrazing en het maken van putten bevorderend op de vorming van zaadbanken en daaropvolgend rekrutering aan de randen van de velden en/of in gebieden waar open ruimte beschikbaar komt.

Hoofdstuk 6 vergelijkt een aantal populatie kenmerken en grootheden van *Z. noltii* en *Z. marina*. Meer precies:

- **Hoe verschillen de patronen van genetische en genotypische diversiteit van *Z. noltii* en *Z. marina* langs de kusten van de Waddenzee van elkaar?**
- **Wat is de ruimtelijke structuur van een *Z. noltii* "populatie" in de Waddenzee?**
- **Hoe verhoudt de connectiviteit van de twee soorten zich tot elkaar?**
- **Hoe verhoudt de populatiestructuur van *Z. noltii* uit het intergetijdengebied zich tot de verwante *Z. marina* uit het intergetijdengebied/sublitoraal van de Waddenzee?**

Zostera noltii en *Z. marina* velden werden bemonsterd door de gehele Waddenzee en aangrenzende gebieden. Plekken weren gekozen waar beide soorten voorkwamen. Van de monsters werden de genotypen bepaald met negen soortsspecifieke microsatellieten en een Bayesiaanse methode werd gebruikt om de populatiestructuur af te leiden.

Allel- en genotypische diversiteit waren hoog voor beide soorten dit in vergelijking met andere Europese kusten, daarmee eerdere resultaten bevestigend over het bestaan van een genetische "hot spot" in de Waddenzee (Coyer et al. 2004). Kloongroottes tussen de soorten uit het getijdengebied waren vergelijkbaar.

Opmerkelijk was dat vier van de 13 *Z. noltii* plekken en acht van de 10 *Z. marina* plekken significante inteelt vertoonden, hetgeen overeenkomt met de resultaten uit de vaderschapsanalyse.

Vier *Z. noltii* en *Z. marina* clusters werden geïdentificeerd: zuidelijk Nederland (Zeeland), centrale-westelijke Waddenzee, noordelijke Waddenzee en noordoostelijk Denenmarken. Voor beide soorten zijn de Eems-Dollard en Elbe estuaria gedeeltelijke barrières voor *gene flow* tussen de westelijke-centrale en noordelijke Waddenzee. Als gevolg daarvan is de Waddenzee verdeeld in twee eenheden (in aanvulling op de zuid Nederlandse eenheid).

CONCLUSIES

Dit proefschrift maakt duidelijk dat, gedurende een studieperiode van vier jaar, *Z. noltii* velden in delen van de Waddenzee opvallend bestand zijn tegen begrazing en extreme temperaturen. Terwijl *Z. noltii* velden zich in sommige gebieden uitbreiden, gebeurt dat in andere niet. Bijvoorbeeld, het meest recente *Wadden Sea Quality Status Report* (Van der Graaf et al. 2009) concludeert dat zeegrassen in de Waddenzee (*Z. noltii* en *Z. marina*) zich in het noorden van de Waddenzee hebben uitgebreid, maar zijn afgenomen in de zuidelijke en centrale delen van de Waddenzee.

Het zeegras beheersplan, vastgelegd in het *Trilateral Wadden Sea Plan* (www.waddensea-secretariat.org/management/Plan.html), roept op tot *"…an increased area and a more natural distribution and development of* Zostera *fields*". Transplantatie programma's zijn een manier om dit doel te bereiken en, inderdaad, er zijn kleinschalige transplantatie pogingen uitgevoerd om de sublitorale *Z. marina* in het westelijk, Nederlandse deel van de Waddenzee te herintroduceren. Echter met matig succes omdat de getransplanteerde volwassen planten en zaailingen slecht overleefden. Bijvoorbeeld, in 1993 werden kleine veldjes met *Z. marina* en *Z. noltii* op het Balgzand getransplanteerd. Terwijl het *Z. noltii* veldje in omvang toenam en in 2006 nog steeds aanwezig was (Van Katwijk et al. 2006), ging het *Z. marina* veldje achteruit en verdween uiteindelijk (Van Katwijk et al. 2009). Overwegende dat zaailingen de meest kwetsbare fase in de levenscyclus zijn (Alexandre et al. 2006), wordt aanbevolen om bij toekomstige transplantatie programma's voor *Z. marina* zich te richten op volwassen scheuten in combinatie met zaaddragende bladscheuten. (Bos en Van Katwijk 2005). De meest recente poging uitgevoerd door Erftmeijer en Van Katwijk (2010) gebruikt deze benadering. De resultaten zijn onderweg.

Het variabele succes bij *Zostera* transplantaties in de Waddenzee roept twee vragen op: 1) Wat is de potentiële rol van het voortplantingssysteem gezien de superieure prestatie van *Z. noltii* in vergelijking met *Z. marina* in het getijdengebied; en 2) Zou het beheer en restauratie succes van *Z. marina* baat hebben bij een met *Z. noltii* gecombineerde aanpak? Ik benader deze vragen door de gegevens uit mijn proefschrift combineren met een recent review van onderzoek aan zeegrassen in de Waddenzee gedurende de laatste 20 jaar (Van Katwijk et al. 2009), waarin vijf richtlijnen staan voor het succesvol restaureren van zeegrassen. Onderstaand integreer ik mijn eigen gegevens met vier van die richtlijnen om daarmee te kijken naar de restauratieplannen voor de Waddenzee.

1. Tegengestelde habitatdegradatie, fysieke habitatkwaliteit en connectiviteit

Voor de Waddenzee zeegrassen bestaan de meest zware bedreigingen uit verlies van habitat als gevolg van dijkaanleg (induceert veranderingen van het hydrodynamische regime) en lozing van afval, samen met een toegenomen troebeling en eutrofiëring (Reise 2005, Lotze et al. 2006). Het in onwaarschijnlijk dat de bouwactiviteiten langs de kust zullen afnemen waardoor de daarmee samenhangende hydrodynamische veranderingen min of meer permanent zijn. In sommige gebieden, zoals de westelijke Waddenzee, zijn de fysieke omstandigheden dermate vergaand veranderd, waardoor restoratie vrijwel onmogelijk is (Van der Heide et al. 2007). Echter, in andere gebieden is de troebeling en eutrofiëring langs de kust de laatste tiental jaren afgenomen, waarmee een aantal van de toenames in de noordelijke Waddenzee verklaard kan worden. Hun persistentie in de noordelijke Waddenzee valt mede te verklaren door een afnemende stormactiviteit gedurende de laatste tientallen jaren en de beschermde locatie achter de Waddeneilanden (Reise en Kohlus 2008).

Een tweede factor hangt samen met de populatiestructuur binnen de zeegras metapopulatie. Genetisch onderzoek bevestigt dat *Z. noltii* overwegend een uitteler is, genetisch divers, regelmatig rekruteert en goede connectiviteit heeft (dit in tegenstelling tot een karakterisering door een paar grote klonen, lage rekrutering, geïsoleerde plekken en slechte connectiviteit). Vergelijkbare resultaten zijn gevonden voor *Z. marina* door Ferber et al. (2008).

Kijken we naar de overwegende stroomrichting en de afgenomen connectiviteit tussen de twee Waddenzee clusters van beide soorten, dan vertegenwoordigd de westelijk-centrale Waddenzee een langzamerhand uitgeput rakende bron locatie, onderhevig aan de sterke noordoostelijke stroming. Daarom zouden zowel *Z. marina* als *Z. noltii* voordeel hebben van een restauratie van populaties in de westelijk-centrale Waddenzee via transplantatie van volwassen planten (met en zonder reproductieve scheuten) en/of zaden verzameld uit de meer diverse populaties binnen de westelijk-centrale regio (Van Katwijk et al. 2005, Erftemeijer en Van Katwijk 2010, Giesen et al. 2010) (zie Section 3, onderstaand en **Hoofdstuk 6**).

2. Selectie van een geschikt habitat

Persistente zeegrasbedden zijn zeldzaam in de westelijk-centrale Waddenzee en de meeste bestaande velden gaan achteruit (Van der Graaf et al. 2009, Van Katwijk et al. 2009). Even aannemende dat de fysieke omstandigheden verbeterd kunnen worden (door bijvoorbeeld door beschermende barrières), dan is een manier om in dit gebied zeegras velden te restaureren door de weinige bestaande velden te stabiliseren en te laten dienen als "kernen" voor restauratie/uitbreiding naar de directe omgeving. Omdat de randen van een veld het meest kwetsbaar zijn voor afbraak door hydrodynamische krachten (Fonseca et al. 1982, Schanz et al. 2002, Widdows et al. 2008), kan restauratie verbeterd worden door zaden te planten langs beschermde randen voor het begin van de winter (**Hoofdstuk 5**). Mijn gegevens (**Hoofdstuk 2**) ondersteunen ook eerdere studies (Vermaat en Verhagen 1996, Giesen et al. 2010) die laten zien dat herstel van *Z. noltii* in het voorjaar het succesvol is als de winter mortaliteit (% bedekking) lager is dan 10%. Dus een toename in scheutdichtheid via transplantatie van volwassen scheuten en/of zaailingen voor het begin van de winter, zowel in het midden als aan de randen van het veld, zal de Nederlandse Waddenzee bedden met momenteel lage dichtheid kunnen onderhouden en/of verbeteren.

Het vestigen van geheel nieuwe velden is om twee redenen moeilijk haalbaar. Ten eerste, een nieuw veld moet redelijk groot zijn om de negatieve invloed van de omringende zandplaten het hoofd te bieden en om het zelf-faciliterende effect van relatief dichte zeegras bedden te verzekeren (Bos et al. 2007, Van der Heide et al. 2007, 2010). Ten tweede, geheel nieuwe vestigingen vereisen transplantatie van zaden, zaailingen en volwassen planten, en dat is duur en ingewikkeld. Op wereldschaal is het vestigen van geheel nieuwe zeegras bedden slechts in ca. 30% van de gevallen succesvol geweest, zelfs op plaatsen waar zeegrassen in het verleden voorkwamen (Orth et al. 2006a, Van Katwijk et al. 2009).

Om een maximaal resultaat te behalen vereist elke transplantatie poging een zorgvuldige evaluatie van de lokale omgeving/habitat (hydrodynamica, sediment samenstelling, nutriënten, aanwezigheid van wadpieren etc.). Alhoewel er een habitat geschiktheidskaart voor zeegras transplantaties in de Waddenzee bestaat (Van Katwijk et al. 2000), zou het nodig kunnen zijn om deze gegevens aan te vullen met meer lokale evaluaties d.w.z. analyses van overeenkomsten in milieuomstandigheden.

3. Selectie van een geschikt donor gebied

De Waddenzee moet, vanuit het oogpunt van beheer, beschouwd worden als twee duidelijke eenheden (westelijk-centraal en noordelijk). Of deze twee "eenheden" een weerspiegeling zijn van isolatie aan beide kanten van de Duitse Bocht of het resultaat van verschillen in selectiedruk blijft onbekend. Daarom moet bij transplantatie en inzaai pogingen het materiaal afkomstig zijn van bron populaties binnen een eenheid, in plaats van tussen de eenheden, dit om potentieel negatieve effecten op de fitness te minimaliseren (*sensu* Mallet 2005).

Vanuit een genetisch standpunt gezien, zijn alle *Z. noltii* en *Z. marina* velden in de noordelijke Waddenzee geschikte donor populaties voor transplantatie pogingen binnen het eigen noordelijk gebied vanwege hun hoge genotypische/genetische diversiteit en onderlinge connectiviteit (**Hoofdstuk 6**, Ferber et al. 2008). De aanwezigheid van een zaadbank kan een belangrijke rol spelen bij het onderhoud van bedden (**Hoofdstuk 2, 4**), maar seksuele voortplanting kent lokale verschillen en overwegend vegetatieve bedden bestaan zeker. Donoren uit meerdere seksueel voortplantende plekken binnen elke eenheid zijn geven de beste garantie voor een succesvolle transplantatie. Donoren uit puur vegetatieve (meest klonale) bedden moeten worden vermeden vanwege hun lage genotypische diversiteit, zelfs bij hoge genetische diversiteit. Met andere woorden, 100 transplantaten die genetisch divers zijn, maar allemaal afkomstig zijn van een enkele of een paar klonen zullen minder succesvol zijn dan 100 transplantaten die stuk voor stuk genetisch van elkaar verschillen.

Alhoewel verleidelijk, transplantatie programma's met donor materiaal afkomstig uit de noordelijke Waddenzee naar de westelijke-centrale Waddenzee moeten worden vermeden onder het zogeheten *precautionary principle*. Transplantatie van *Z. noltii* binnen een "unit" is deels geslaagd in Zeeland (zuidelijk Nederland), maar toekomstige transplantaties kunnen profijt hebben van een degelijke genetische evaluatie van donerende en ontvangende locaties.

4. Risicospreiding

Een van de belangrijkste factoren die succesvolle transplantaties positief beïnvloed is het spreiden van het risico door meerdere transplantaties in tijd en ruimte (Van Katwijk et al. 2009). Echter, spreiding van genetische risico's is net zo belangrijk. Zoals hierboven al aangegeven, kan het combineren van transplantaten uit verschillende donor gebieden, met passende omgevingsparameters, de genotypische/genetische diversiteit in de transplantatie locatie significant doen toenemen en het voortplantingssucces en/of fitness positief beïnvloeden.

Risicospreiding betreft ook de transplantatiemethoden, inclusief wat en hoe te en transplanteren, en elk programma die verschillende methoden combineert heeft de meeste kans op succes. Een methode die heeft aangetoond succes te hebben bij *Z. noltii* en *Z. marina* is door hele plaggen zeegras met sediment te transplanteren en op de ontvangende locatie de transplantaten in dichte gegroepeerde te planten (Schanz en Asmus 2003, Giesen et al. 2010, eigen waarneming). Plaggen kunnen ook een deel van de lokale zaadbank bevatten, en, in het geval van *Z. noltii*, tenminste drie jaar voortbestaan (**Hoofdstuk 3**) en daarmee een reservoir voor nieuwe individuen en genetische diversiteit vormen indien de oorspronkelijke transplantaten niet aanslaan. Greve et al. (2005) melden het herstel van een sublitorale populatie van *Z. marina* uit een zaadbank na volledig afsterven als gevolg van zuurstofloosheid van het sediment.

De ruimtelijke rangschikking van transplantaten op de ontvangende locatie is cruciaal gezien de afstanden waarover stuifmeel en zaden zich verspreiden en daarmee uitteelt en gene flow mogelijkheden tussen transplantaten en bewoners te verzekeren. Een transplantatie programma moet vegetatieve en generatieve scheuten behelzen, evenals andere stadia uit de levenscyclus. Transplantatie van bijvoorbeeld zaailingen is meestal niet succesvol (Van Katwijk en Hermus 2000, Van Katwijk en Wijgergans 2004, Alexandre et al. 2006), maar transplantatie van zaden is goedkoop, logistiek haalbaar en een betrouwbare methode voor zeegras restauratie (Orth et al. 2006b, Orth et al. 2009, Erftemeijer en Van Katwijk 2010). Bevorderen van de dichtheid van zeegrasbedden in de Nederlandse Waddenzee met de recentelijk ontwikkelde inzaai-boeien technologie (Pickerell et al. 2005, 2006) is veelbelovend (Erftemeijer en Van Katwijk 2010), vooral in combinatie met plaggen-transplantatie. Bij deze techniek worden netvormige zakken, gevuld met generatieve scheuten met rijpe zaden, aan boeien gehangen die over de transplantatie locatie worden getrokken, resulterend in een constante zaadregen en een verdere toename van het restauratie succes en/of het onderhoud en/of uitbreiding van bestaande bedden. Het is vermeldingswaard dat *Z. noltii* zaden, verzameld in donor bedden en verspreid in ontvangende gebieden, ontkiemden en uitgroeiden (Giesen et al. 2010). Het verspreiden van *Z. marina* zaden in netten gaf eveneens veelbelovende resultaten (Harwell en Orth 1999); en net zo leverden zaden geplaatst in kunstmatig gemaakte putten het hoogste aantal zaailingen (**Hoofdstuk 5**).

Concluderend, de persistentie en veerkracht van *Z. noltii* velden in de noordelijke Waddenzee is verzekerd zolang de omgevingsfactoren blijven meewerken (hydrodynamica, helderheid water, nutriënten, geen introducties). Herstel en eventuele persistentie van *Z. noltii* in de westelijke Waddenzee is onwaarschijnlijk gezien de huidige fysische dynamiek.

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Dankwoord/Acknowledgements

When I started my PhD in 2003 nobody ever thought it might end up in such a long stretch. After life took its turns and twists, here I am about to finish and it is highest time to thank all people that, voluntarily or obliged to, accompanied my way. It cost so many of their nerves, hair and life time and I hope I am able to express my deepest gratitude in this brief acknowledgement without forgetting anybody.

Thank you **Jeanine**, for the opportunity to do all this great work and meet all these nice people in your lab and everywhere I went in the course of my thesis. I cannot value high enough that you stood by me all these years. Undisputable we had our few dissents. However, due to your character beyond comparison you repeatedly found the right words to motivate me and push me in the right direction. Much that I owe you.

I would have been lost in the university jungle plenty of times without you **Wytze**, and your administrative and financial skills. No ever so small flaw escaped your eyes in our manuscripts, thereby tremendously improving all my written output. There has been always a relaxing chatter, a nice word or some good advice with you close by. For all that I thank you sincerely.

Karsten, *du hast mir seit meiner Diplomarbeit mit Rat und Ermutigung zur Seite gestanden, wann immer ich diese gebraucht habe. Ohne deinen Anruf wäre diese Arbeit nicht zu Stande gekommen. Vielen Dank. Ich danke Dir besonders dafür, dass ich an "Deiner" Wattenmeer Station die Feldarbeit für meine Dissertation durchführen konnte.*

Colleague, teacher, daily supervisor and - most importantly – good friend: **Jim**. If I would thank you here for everything I need to it would fill another thesis book. Therefore, I confine myself and simply say thank you for everything you did for me and the good times we shared.

For the good atmosphere and working environment in the lab I thank my colleagues at MarBEE Steven Ferber, Malia Chevolot, Galice Hoarau, Katrin Sieben, Henning Reiss, Jos Mieog, Klemmens Eriksson, Deniz Haydar Jan Veldsink, Stella Bos-Boel, Gezien van Roon en Joukje de Vries for assistance, fun and distraction during and after working hours. Special thanks to Steven for the good

times and lively discussions in our shared ‘broom closet’ and in the field. I thank Galice and Malia for fruitful discussions on my raw data and analytical tools. Lots of the lab work would not have been possible without Jan and Stella and I sincerely thank them for their assistance.

Vielen Dank an all meine Kollegen an der Wattenmeer Station für Eure Unterstützung und das familiär Umfeld. Anja Schanz, Patrick Polte, Manuela Krakau, Matthias Strasser, Christian Buschbaum, David Thieltges, Nils Volkenborn, Tina Loebl, Petra Kadel, Georgia Klein, Tobias Dolch, Justus van Beusekom, Christian Hass, Ragnhild und Harald Asmus, Erk Lützen, Elisabeth Herre, Christiane Antrick, Gurdy Bardt, Kristin Kessenich und alle die, die ich nach so langer Zeit in dieser Aufzählung vergessen habe. Besonders Anja, Patrick und Socke danke ich für die wichtigen fachlichen Diskussionen, die Unterstützung bei der Feldarbeit und dafür meine Adoptivfamilie gewesen zu sein. Vielen Dank für Eure Freundschaft. Lilo ich danke dir für deine Aufrichtigkeit, Dein Temperament und all die kleinen und großen Ausrüstungsgegenstände die du mir zur Verfügung gestellt hast.

Thanks to Anett, Thore and Rune for my exciting, happy and filled life.

Publication List

Work in progress

Zipperle AM, van Gils J, van Hattum B, Heise S. Critical evaluation of the Environmental Exposure Assessment of Disinfection By-products generateded by Ballast Water Management Systems as part of the IMO approval procedure G9. **In Preparation**.

Zipperle AM, Coyer JA, Stam WT, Olsen JL. Two species, one pattern: A genetic perspective on diversity and connectivity of *Zostera marina* (L.) and *Zostera noltii* (Hornem.) populations in the Wadden Sea. **Submitted**.

Publications in peer-reviewed journals

Zipperle AM, Coyer JA, Reise K, Stam WT, Olsen JL (2011) An evaluation of small-scale genetic diversity and the mating system in *Zostera noltii* on an intertidal sandflat in the Wadden Sea. *Annals of Botany*. **107**, 127-133

Zipperle AM, Coyer JA, Reise K, Stam WT, and Olsen JL (2010) Waterfowl grazing in autumn enhances spring seedling recruitment of intertidal *Zostera noltii*. *Aquatic Botany*. **93**, 202-205

Zipperle AM, Coyer JA, Reise K, Stam WT, Olsen JL (2009) Evidence for persistent seed banks in dwarf eelgrass *Zostera noltii* in the German Wadden Sea. *Marine Ecology Progress Series*. **380**, 73–80

Zipperle AM, Coyer JA, Reise K, Gitz E, Stam WT, Olsen JL (2009) Clonal architecture in an intertidal bed of the dwarf eelgrass *Zostera noltii* in the Northern Wadden Sea: persistence through extreme physical perturbation and the importance of a seed bank. *Marine Biology*. **156**, 2139–2148

Zipperle AM, Reise K (2005) Freshwater springs on intertidal sand flats cause a switch in dominance among polychaete worms. *Journal of Sea Research*. **54**, 143-150

Published reports

Zipperle AM, van Gils J, van Hattum B, Heise S (2011) Guidance for a harmonized Emission Scenario Document (ESD) on Ballast Water discharge. German Federal Environment Agency Texte 34/2011. http://www.uba.de/uba-info-medien-e/4114.html.

Biography

Biography

Andreas was born on Juli 13th 1976 in Karlsruhe (Germany) where he lived merely a year before moving to the Rhine and close to Cologne where he attended elementary school and Gymnasium. He received his bachelor diploma in 1996 from the Gutenberg Gymnasium (Bergheim/Eft) and started civil service the same year in mobile care for the elderly for ten months. After a year of jobbing and decision taking Andreas started his degree in biology at the University of Cologne. After his basic studies he chose Ecology, Botany and Biochemistry as main subjects. Andreas complemented his main studies by six weeks of voluntary field work on the 'Ecology of Limnic systems' at the Limnologic field station of the University of Cologne (Grietherbusch, lower Rhine area). In parallel to his studies he was an active member of the collegiate self-administration as a member of different boards and committees Finishing his theoretical studies in 2002 he specialized on marine biology/ coastal ecology in his diploma thesis about the 'Impact of freshwater springs on the macrofauna of Wadden Sea soft sediments' under supervision of Prof. Dr. Karsten Reise at the Wadden Sea Research Station of the Alfred-Wegener-Institut für Polar- und Meeresforschung (AWI) on the Island of Sylt. In 2003 Andreas was employed as a researcher for the maintenance of fish ecological experiments on Sylt and in a project studying the formation of different size cohorts and beginning cannibalism among o+- juveniles of *Perca fluviatilis* in a quarry pond (Limnologic field station of the University of Cologne). After a failed grand proposal to continue his work on freshwater springs his supervisor Prof. Dr. Karsten Reise called his attention to a vacant PhD position in the working group of Prof. Dr. Jeanine L. Olsen at the former Department of Marine Biology at the University of Groningen financially supported by an Ubbo Emmius bursal. Keen on broadening his scientific training he accepted the position when it was offered to him presenting him with the opportunity to explore the possibilities molecular tools can provide to an ecologist. Andreas started the PhD thesis in hand in September 2003 under supervision of Prof. Dr. Jeanine L. Olsen, Prof. Dr.Wytze T.

Stam and Prof. Dr. Karsten Reise none of who ever expected such a winding road to the end.

Andreas met his love Anett in summer 2002 on the Island of Sylt, realizing they had done the same study at the same University and shared a couple of courses, but never really took notice of each other till then. Anett searched for a PhD close by Groningen and Sylt finally settling in Hamburg smack in the middle between both of Andreas working places. On the brink of both finishing their theses they decided for Anett to pursue her degree first, bear a child and begin her career. Hence, in 2007 Andreas moved from Groningen to Hamburg and their first son Thore was born. As things turned out well their second son Rune was born in 2010.

To support the family finances Andreas started with project based consulting early and since 2007 works regularly for the Consulting Centre for Integrated Sediment Management (BIS) at TuTech/ Technical University in Hamburg. Starting with literature surveys for ecotoxycological reports Andreas soon started working in the framework of the International Maritime Organisation (IMO) 'Convention for Management of ships' ballast water and sediments (2004)'. He supported a producer of Ballast Water Management System with environmental risk assessments in their attempts for basic and final approval at the IMO and the national authorities and literature surveys on the disinfection capabilities of UV. He was project leader for the German Federal Environmental Agency in preparing a 'Guidance for a harmonized Emission Scenario Document (ESD) on Ballast Water discharge' published in 2011.

Currently, he works for Prof. Dr. Susanne Heise at the Hamburg University of Applied Sciences in the DiPol project `Impact of climate change on the quality of urban and coastal waters´. He is responsible for regular sampling of suspended sediments in the Elbe river to evaluate climate change induced remobilization of contaminated sediments upstream of Hamburg, sampling of macrofauna for ecotoxicological testing and maintenance services (boat/ motor/ trailer).