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Eelgrass (*Zostera marina*) restoration on the west coast of Sweden using seeds

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ABSTRACT: Along the northwest coast of Sweden, over 50% of *Zostera marina* L. (eelgrass) meadows have vanished since the 1980s. With the improvement of conditions, there is a growing interest to restore lost habitats, but methods are lacking for restoration of eelgrass beds at high latitudes where long winters create special challenges. We assessed if seed planting could be used for large-scale restoration, with the aim to identify the major causes of seed and seedling loss and to determine which planting method best increases restoration success. In the laboratory, we identified optimal conditions for long-term seed storage and demonstrated that eelgrass seeds can be successfully stored for 8 mo before being planted in the spring. However, field studies did not find an increased seedling establishment in seeds planted in the spring of 2013 compared to those planted in the fall of 2012. Field results suggest that the main causes for the seed loss were seed predation from crabs, seed transport by currents and bioturbation by lugworms, while the main processes affecting shoot development were light availability and physical disturbance. Covering the seeds with a layer of sand increased seedling establishment 2 to 6 times compared to uncovered seeds, probably through the reduction of seed predation and seed transport, and could potentially be developed as a method to reduce losses during restoration. In addition, positive feedback mechanisms (i.e. sediment resuspension and drifting macroalgae mats) may also prevent natural recovery and restoration success. However, high seed loss (on average 98.6%) and high shoot mortality pose a challenge that need to be addressed before restoration using seeds can be recommended for large-scale restoration.

KEY WORDS: *Zostera marina* · Seagrass · Coastal restoration · Seed planting · Seed predation · Seed storage · Feedback

INTRODUCTION

Seagrass meadows and the ecological and economical services that they provide are declining worldwide as a result of human perturbations (Orth et al. 2006). Along the Swedish northwest coast, almost 60% of eelgrass has been lost since the 1980s (Baden et al. 2003). Recent studies suggest that the primary mechanism behind this decline is an increased abundance of epiphytic algal mats caused by overfishing and eutrophication, which results in reduced light conditions and increased anoxia (Moksnes et al. 2008, Baden et al. 2010). Although measures in the last 20 yr have reduced the overall nutrient load to Swedish coastal waters and improved the water quality of the Swedish west coast significantly in most areas (Anonymous 2014), no natural recovery of eelgrass has occurred (Nyqvist et al. 2009). In order to meet minimum environmental standards set by the new EU Marine Strategy Framework Directive (2008/56/EG), Swedish national agencies are presently considering restoration of eelgrass as a measure. However, little information is available regarding methods for eelgrass restoration in high-latitude environments where a short growing season, ice formation, and organic-rich sediments present special

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challenges for restoration. In particular, studies assessing the use of seeds for eelgrass restoration in Scandinavian countries are lacking.

The eelgrass *Zostera marina*, L. is the dominant marine angiosperm throughout the northern hemisphere, extensively distributed throughout Scandinavian coastal waters (Boström et al. 2014). On the Swedish west coast, eelgrass grows mainly in fjords and sheltered bays, forming perennial, subtidal populations on muddy and sandy sediments between 0.5 and 4 m depth (Boström et al. 2003). Surface water temperature can range from 20°C in summer to below 0°C at shallow bays in winter. The upper depth limit distribution of *Z. marina* is mainly determined by ice scouring and hydrodynamics, while the lower limit is determined by light availability (Boström et al. 2003). The winter conditions in the area are characterized by strong winds, low light and low temperatures from November to March when very little growth occurs. In contrast to other locations where eelgrass seeds germinate in the fall (Moore et al. 1993), seeds in Scandinavian waters are thought to lay dormant during the winter and germinate the following spring (Olesen 1999), which may allow for large losses of seeds during the winter period. However, at present very little is known about loss rates of eelgrass seeds during the winter period, the dominant processes causing seed loss, or measures to reduce seed loss during restoration in Scandinavian countries. Laboratory studies (Lillebø et al. 2011) and field studies in the Dutch Wadden Sea (van Katwijk & Wijgergangs 2004, Bos & van Katwijk 2007) suggested that physical erosion and transport of seeds and seedlings are important causes of loss. Studies in Denmark have also shown that high densities of lugworm *Arenicola marina* can affect germination negatively through seeds being buried too deep for successful seedling development (Valdemarsen et al. 2011, Delefosse & Kristensen 2012). Shore crabs *Carcinus maenas* are very abundant in Scandinavian coastal waters and affect eelgrass shoots negatively in the USA where it is non-native (Davis et al. 1998, Neckles 2015), but little is known regarding its importance in seed loss in Scandinavian waters.

Marion & Orth (2010a) showed that storing seeds in the laboratory over the summer and planting them in the fall, just before germination, reduced seed losses. In Sweden, if seeds were planted in spring, just before germination, seeds would need to be stored for 6 to 8 mo. However, to our knowledge there have been no previous investigations on the optimal conditions for long-term storage of eelgrass seed for use inhigh-latitude areas, which may be critical for successful restoration in Scandinavian waters.

The aims of the study were to (1) determine if seeds should be planted in the fall or stored and planted in the spring, (2) identify the optimal conditions for long-term (8 mo) seed storage and (3) identify major causes of seed and seedling losses in different environments and (4) determine which methods increase survival using 3 planting techniques.

We considered seed predation, seed transport by waves and currents, bioturbation by lugworms and light limitation to be the major causes of seed and seedling loss in our system. To be able to separate the different causes of seed loss, we carried out the same field experiments at 4 different depth intervals at multiple sites, where different causes were expected to create depth-specific loss pattern of seeds and seedlings (Fig. 1).

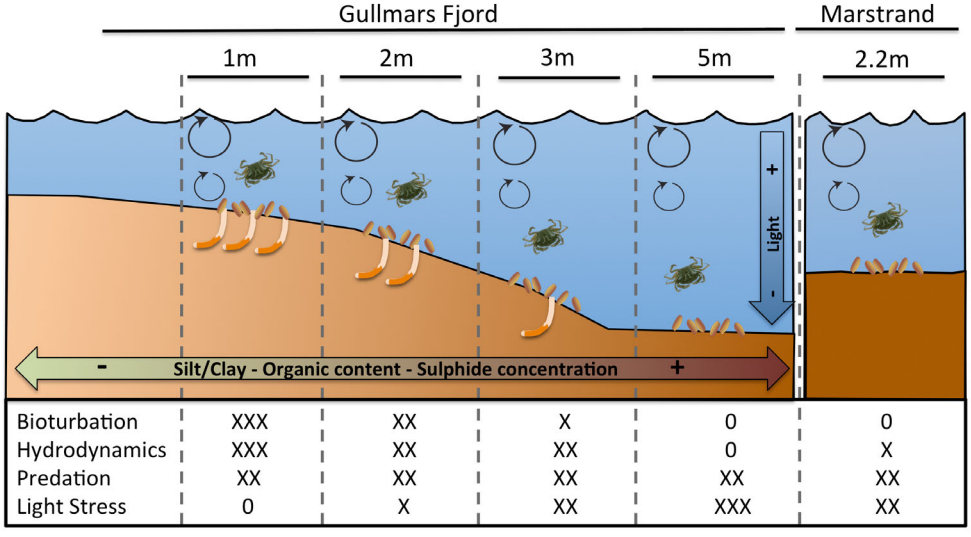

Fig. 1. Schematic diagram of environmental factors and their hypothesized impact on eelgrass seeds and seedling survival depending on depth. Factors include bioturbation by the sand worm *Arenicola marina* (seeds get buried too deep), hydrodynamics (seed trapping), predation by decapods (e.g shore crab *Carcinus maenas*) and light limitation for plant development (0 = no importance, XXX = high importance)

We hypothesised that seed-transport and bioturbation would be the major causes of seed loss at shallow depths, while light availability would limit seedling development in the deep habitats. Seed predators were hypothesised to have similar effects at all depths, but to be lower when seeds were covered with sand.

MATERIALS AND METHODS

Study areas

The major seed planting experiment was carried out in 2 small bays in the Gullmars Fjord on the Swedish northwest coast (Fig. 2). This area has only suffered minor losses of eelgrass since the 1980s (Baden et al. 2003) and at present has relatively good water quality for eelgrass growth (Moksnes et al. 2015). Small eelgrass meadows are found in most sheltered bays of the fjord, typically growing from 0.5 m inside the bay, over the relatively steep slope into the fjord down to 4 or 5 m depth (Fig. 2b–d). In sheltered or deeper areas, the sediment where eelgrass grows typically has a high content of organic material (10 to 25%), water content (>50%) and sulfides (Jephson et al. 2008, Holmer et al. 2009). The seed-planting experiment was also carried out at one site in the archipelago of Marstrand (Fig. 2). This area has suffered more than 80% cover losses of eelgrass (Baden et al. 2003, Nyqvist et al. 2009) and was added to assess eelgrass restoration in a more degraded environment, which is presently targeted for restoration.

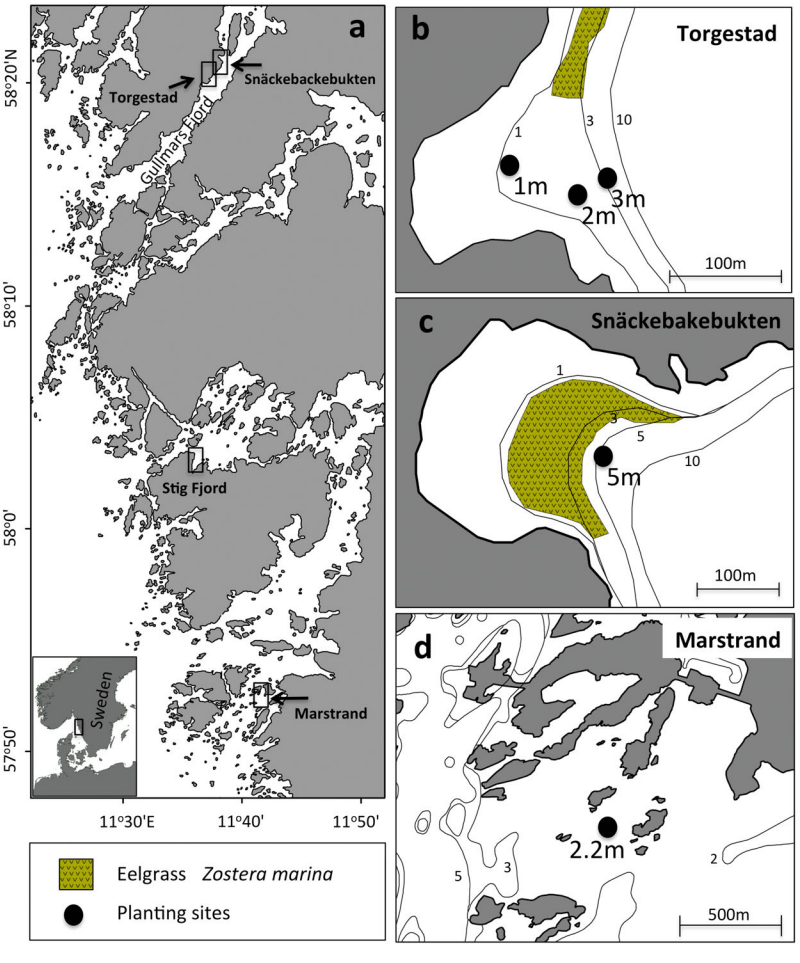

Fig. 2. Location of study sites: (a) Swedish northwest coast, (b) Torgestad, (c) Snäckebackebukten and (d) Marstrand

Seed collection

To obtain seeds for the experiments, reproductive eelgrass shoots with mature seeds were manually harvested from a large eelgrass meadow on 27 Jul 2012 in Stig Fjord (Fig. 2), at 1 to 1.5 m depth and stored in outdoor 1500 l tanks with flow-through seawater at the Sven Lovén Centre for Marine Sciences, Kristineberg, until the seeds matured. Reproductive shoot densities at the collection site varied between 13.6 and 21.0 shoots m^{-2} with an average shoot length of 0.65 ± 0.05 m (mean ± SE, n = 48). Shoots had 17.2 ± 2.1 spathes per shoot with 55 to 63% of them in Stage 4 of development, i.e. seeds were developing within the spathes, but were not yet matured (DeCock 1980).

Seed storage

One of the challenges with long-term storage of seeds is to avoid premature germination. To identify the best storage conditions for avoiding germination of eelgrass seeds, the effects of temperature, salinity and aeration were tested in a short-term trial. Two temperatures (5 and 15°C) and 3 salinities (5, 15 and 30) were assessed, representing natural temperatures over winter and late summer/autumn and the range of salinities present where eelgrass beds are found on the NW coast of Sweden. The effect of water aeration was also tested since

anoxia can induce germination (Moore et al. 1993) and water was either aerated or not aerated. Different light conditions were not included in this study since it does not affect *Zostera marina* germination (Moore et al. 1993).

For the short-term storage experiment, seeds were placed in 0.5 l plastic beakers with covers to avoid evaporation and changes in salinity. Each treatment had 5 replicates with 65 to 75 seeds in each beaker (6000 seeds in total). Seed germination was defined as the moment when the seed coat was opened and the cotyledon became visible (Churchill 1983). The number of seeds germinating was counted every 4 d and seedlings were removed. Water in each beaker was replaced weekly. Seed germination was recorded over 100 d. After that period, the remaining non-germinated seeds were tested for viability by reducing the salinity to 5 and increasing the temperature to 15°C which triggers germination for *Z. marina* (Hootsmans et al. 1987).

Long-term storage (8 mo) of eelgrass seeds was tested by storing seeds in three 10 l tanks with 30 000 seeds at 5°C and a salinity of 30. These storage conditions were selected based on preliminary results from the seed-storage experiment. Tanks were kept in the dark to avoid algal growth, with slight aeration and water recirculation. During storage a bacterial or fungal white slime developed and covered the seeds. Seeds were washed and rinsed and the water changed every 2 wk to improve the storage conditions. Germinated seeds were removed from the tanks. Seed quality was tested at the beginning and end of the 8 mo storage by measuring the sinking velocities of seeds. Good quality seeds were determined as those with sinking velocities higher than 5.5 cm s^{-1}, since these velocities have shown higher germination and seedling success (Marion & Orth 2010b). Low-quality seeds were removed at the start of the storage trial.

Seed planting experiment

Seeds were planted in the Gullmars Fjord and the Marstrand area to identify the main causes of seed and seedling losses, and to determine which planting methods best increase survival of eelgrass seeds. In Gullmars Fjord, seeds were planted using 3 methods at 4 depths (1, 2 and 3 m at Site 1, Torgestad, and 5 m at Site 2, Snäckebackebukten; Fig. 2b,c) during 2 different seasons (fall and spring) in an orthogonal design with 5 replicates. In the Marstrand area, the same planting methods and season were tested, but only at one location and depth (2.2 m; Fig. 2d).

Three planting methods were tested to investigate the effect of seed predation and seed transport by waves and currents. (1) Sand: Seeds were covered with a 2 cm layer of sand (<2 mm grain size) to protect them from predation and transport by currents. (2) Rocks: 15 rocks (ca. 3 × 3 cm) were added to experimental plots before planting the seeds to induce vortices in the flow and scouring holes around the rocks, which could trap and bury the seeds in the sediment. Pilot studies in a flume indicated that seed trapping increases (50 to 70%) with the presence of rocks at undirectional flow velocities above 10 to 15 cm s^{-1} (E. Infantes unpubl. data). (3) Control: Seeds were planted over the sediment surface to mimic the natural reproductive process and to be used as a control. To assess if seed loss was affected by planting season, all treatment combinations were carried out with seeds planted both in the fall (22 to 26 Sep 2012) and in spring (11 to 19 Apr 2013). At each depth, seeds were planted in circular plots of 0.12 m^2 separated by a distance of 2 m. The plots were placed along 30 m transects parallel to the shoreline, with 5 replicates of each treatment. At each plot, 500 seeds were planted, with a total of 75 000 seeds used in the experiment. All seeds used had sinking velocities greater than 5.5 cm s^{-1}, and a mean (±SD) length of 3.03 ± 0.04 mm and width of 1.48 ± 0.03 mm (n = 220).

Light conditions, sediment composition and densities of the lugworm *Arenicola marina* were measured at all depths next to the planting plots (Table 1). The percentage of surface light at the bottom was measured using 2 light data loggers (HOBO, Onset®), calibrated against a PAR meter (Apogee MQ-200) and placed at 2 depths (Dennison et al. 1993). Temperature and salinity were measured at both areas using loggers (HOBO, Onset®). Sediment composition was determined by sampling the top 5 cm layer and used as an indirect indicator of wave and current exposure (Fonseca et al. 1983). Locations with high organic (7 to 11%) and silt+clay content (24 to 50%) were indicative of low flow exposure, while low organic (0.4 to 1%) and silt+clay content (1.3 to 10%) were indicative of higher flow (Table 1). Grain size analysis was measured by wet sieving while organic content (%) was determined as weight loss of dry sediment after ignition (5 h, 520°C). Densities of the lugworm *Arenicola marina* were estimated in fall and spring by counting the number of fecal deposits 6 h after smoothing the sediment in a 0.25 m^2 quadrat next to all the plots. Ice cover is not formed every year along the Swedish west coast and was not present at the field sites during the study.

Replicate sediment cores (6.4 cm diameter; 5 cm depth; n = 3) were taken in April (fall seeding) and in Jun 2013 (spring seeding) to estimate the percentage of seeds remaining in all the plots. The percent seed remaining was calculated by relating the number of seeds found in sediment cores to the surface area of the plot and the number of seeds planted (including visible seedlings). The number of shoots in all plots was counted during 22 to 23 April, 6 to 10 June, 10 to 15 August, 25 to 26 September 2013 and 2 to 4 September 2014. Percent seedling establishment was calculated from the number of seedlings divided by the number of seeds planted. Shoot morphologies were assessed by collecting 7 plants at all depths independently of the planting method in September 2013. The number of leaves, leaf length, rhizome lengths and internode length were measured.

Statistical analysis

The effect of storage conditions on seed germination were assessed using a 3-way fixed factor ANOVA with salinity, temperature and aeration as independent variables and percent germination after 20 and 100 d as dependent variables. Two time periods were analyzed separately to compare short- and long-term storage effects on germination. To assess the factors affecting seed loss and growth in Gullmars Fjord, the effect of planting methods (rock, sand, control), depth (1 to 5 m) and season (fall, spring) were analyzed as independent variables in a 3-way fixed factor ANOVA using percent seeds remaining and number of seedlings per plot in June, and number of shoots per plot in September 2013 and 2014 as independent variables. The data from the Marstrand area were analyzed separately since the light and environmental conditions differed, using the same dependent variables, but in a 2-way ANOVA with methods and season as the independent variables. Before analyses were performed, all data were tested for homoscedasticity with Cochran's *C*-test, and square root transformed to homogenize variances when necessary (Sokal & Rohlf 1995). All figures show untransformed data. *A posteriori* multiple comparisons were carried out with the Student-Newman-Keuls (SNK) procedure.

Table 1. Environmental conditions at the study sites. Light measured between June and September. Data are mean (SE). %LOI: percent weight loss on ignition

Location/ depth (m)	Light conditions (% of surface light)	Organic content (%LOI)	Silt+ clay (%)	*Arenicola maritima* (ind. m^{-2})
Gullmars Fjord				
1	70 (0.1)	0.4 (0.1)	1.3 (0.2)	18.4 (1.7)
2	49 (0.1)	0.4 (0.1)	1.3 (0.2)	9.1 (2.2)
3	35 (0.1)	1.4 (0.1)	10.4 (0.9)	2.5 (1.2)
5	13 (0.1)	11.3 (0.3)	24.7 (2.4)	0
Marstrand				
2.2	20 (0.1)	7 (0.5)	49.9 (3.6)	0

RESULTS

Seed storage

The analyses of the factors affecting germination of eelgrass seeds after 20 and 100 d showed strong main effects of all investigated factors and a weaker interaction effect between salinity and temperature, possibly driven by smaller differences between the 2 temperatures at high salinities (*a posteriori* tests found significant differences between all salinities at all temperatures, and vice versa; SNK-test at $p < 0.05$; Table 2; Fig. 3). In all treatments, germination decreased with higher salinities, lower temperature and higher aeration, but the differences between treatments decreased over time as germination increased in all treatments. Raising the salinity from 5 to 30 decreased the average germination rate after 20 d from 43 to 3.8%, a 91% decrease in germination rate. Lowering the temperature decreased the average germination rate by 54%, and adding aeration lowered it by 21% after 20 d. Since oxygen levels were not measured in the experimental containers we cannot rule out that the relatively small effects of aeration on germination may in part be explained by small differences in oxygen levels between treatments. After 100 d, on average only 7% of the seeds had germinated in the high salinity, low temperature treatments, whereas 75% had germinated in the low salinity, high temperature treatments. At the end of the experiment, the viability of remaining seeds was tested by lowering the salinity to 5 to induce germination. Within 2 wk, 72 to 80% of the seeds stored at high salinity germinated (at 5 and 15°C), and 43 to 57% at medium salinity, whereas no further germination was observed in the low salinity treatment.

Storing seeds for 8 mo at 5°C and salinity 30 led to a 36% loss of viable seeds (32 400 of 90 000 seeds). This loss was caused both by premature germination as well as death and reduced quality of the seeds, determined by decreased sinking velocities (Marion & Orth 2010b).

Table 2. Laboratory seed germination experiment. Three-factor ANOVA model testing seed germination after 20 and 100 d as a function of temperature (5°C and 15°C), salinity (5, 15, 30) and having aeration or not. **p < 0.01; ***p < 0.001; ****p < 0.0001; (ns) non-significant, p > 0.05

	df	20 d			100 d		
		MS	*F*		MS	*F*	
Temperature (A)	1	50.1	240.1	****	10.6	85.4	****
Salinity (B)	2	112.0	536.2	****	118.0	949.1	****
Aeration (C)	1	4.5	21.6	****	1.6	13.3	***
A × B	2	2.1	10.2	***	0.6	5.2	**
A × C	1	0.3	1.6	(ns)	0.04	0.3	(ns)
B × C	2	0.2	0.9	(ns)	0.02	0.2	(ns)
A × B × C	2	0.5	2.2	(ns)	0.08	0.7	(ns)
Residuals	48	0.2			0.1		

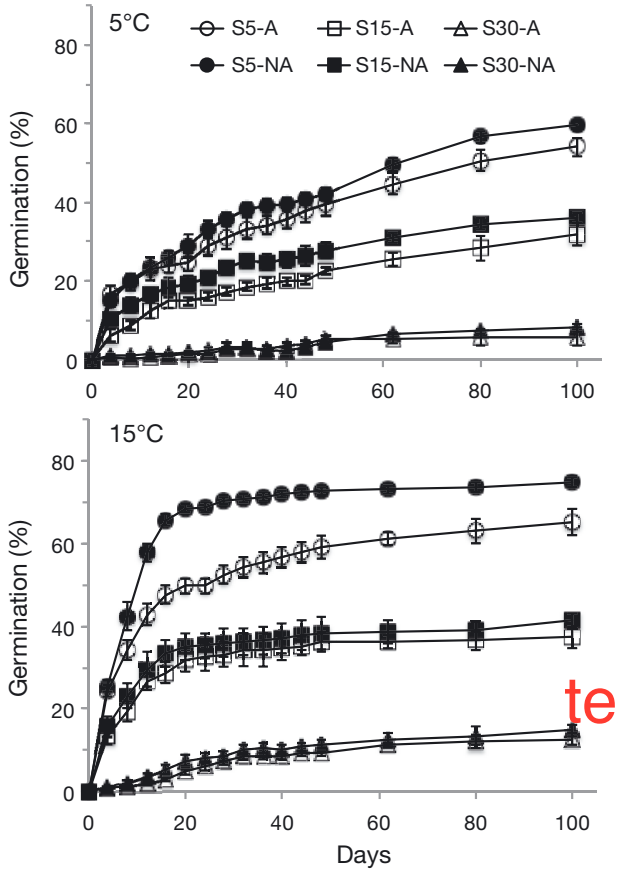

Fig. 3. Cumulative percent of *Zostera marina* seed germination in the laboratory at 5°C and 15°C with salinities 5, 15, 30 (S5, S15, S30), aeration (A) and no aeration (NA). Data are mean ± SE (n = 5)

Planting experiment in Gullmars Fjord

In April, approximately 8 mo after seed planting in fall, no visible seedlings were found in any plots. Sediment core samples indicated that very few seeds (0 to 1.5%) remained in the plots at 1 to 3 m depth, whereas a high proportion (9 to 41%) of seeds remained at 5 m depth, particularly in the sand treatment (Fig. 4a,b). Several of the seeds collected at 5 m depth had had already germinated, with an open seed coat and a visible cotyledon. Seeds planted in April and sampled in June showed a similar pattern, with an estimated 0–1 to 5% remaining at 1 to 3 m depth, and 8 to 22% remaining at 5 m depth. The 3-way ANOVA showed that seed densities were significantly higher at 5 m depth compared to the other 2 depths, but no significant differences between planting methods or planting season were found, although there was a trend of higher number of seeds remaining from the fall planting compared to the spring planting (Fig. 4a,b, Table 3).

In June, an average of 11.3 and 7.6 seedlings per plot were found in fall and spring planting respectively, and a significant 3-way interaction effect was found between planting season, depth and planting method (Table 3). Shoot densities differed between depths in all treatments and was always significantly higher at 5 m, except for the spring control treatment where densities only differed significantly between 5 and 2 m depth (Fig. 4c,d). Seeds covered with sand had significantly higher seedling densities at 2 to 5 m depth in the fall plantings, and at 5 m depth in the spring planting. No significant differences were found between rock and control treatments (Fig. 4c,d). Seedling densities were significantly higher in fall compared to the spring plantings in the 3 m sand treatment and the 5 m sand and control treatments, but spring planting had significantly higher seedling densities at 1 m in the sand and rock treatments. No other significant differences were found between planting seasons (SNK-test at p < 0.05). In June 2013, the addition of sand and rocks were clearly visible on the sediment at 5 m depth, whereas no sand and only a few rocks were visible at 1–2 m depth, indicating high erosion or bioturbation at the latter depths. Seedlings in June had 5–10 cm long leaves independently of the planting method, depth or planting season.

In September 2013, shoot densities were similar in the fall and spring plantings (on average 35.7 and 41.1 respectively) and showed similar treatment effects on all depths resulting in significant main effects of planting method and depth, but not of planting season (Table 3). Densities were now similar in plots at 1 to

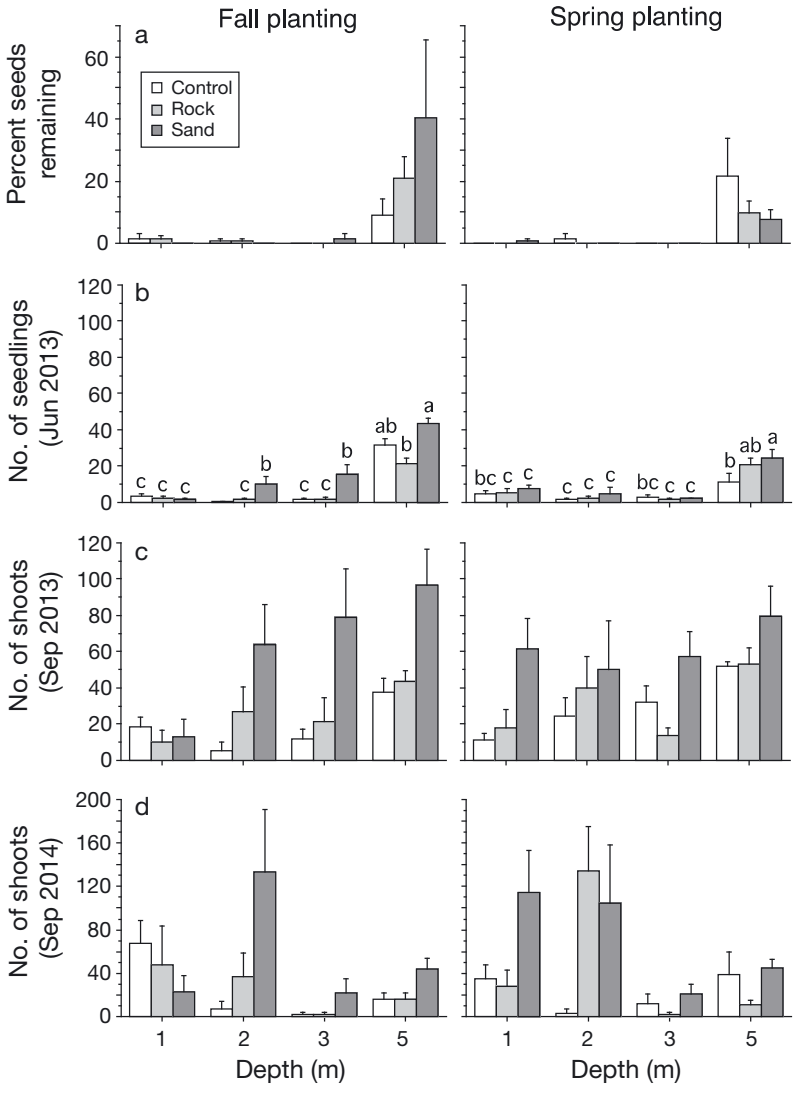

Fig. 4. Results from the planting experiment in Gullmars Fjord. Seeds were planted in the fall (Sep 2012) and spring (Apr 2013) at 4 depths using 3 planting methods (control, rock, sand). (a) Seeds remaining per plot, (b) number of seedlings per plot in Jun 2013, and (c,d) number of shoots per plot in Sep 2013 and Sep 2014 from fall and spring plantings. Data are mean + SE. Different letters above bars in panel b denote significantly different means (SNK-test at $p < 0.05$). For panels a, c and d, there was no significant 3-way interaction and the statistical results are described in the 'Results'

3 m depths (on average 22.2 to 36.1 shoots plot^{-1}), but still significantly higher at 5 m depth (on average 60.5 shoots per plot), in all treatments (SNK-test at $p < 0.05$). Sand treatments had significantly higher shoot densities compared to rock and control treatments at all depths (on average 62.7, 28.5, and 24.1 shoots plot^{-1}, respectively; Table 3; SNK-test at $p < 0.05$). Reproductive shoots were observed in a few plots.

After the second growing season, the pattern in shoot densities had changed markedly in September 2014 with similar or higher densities in the shallow habitats and less clear effects of planting methods compared to the previous year, resulting in a significant interaction effect between depth and methods (Fig. 4d, Table 3). Sand treatments still had higher shoot densities compared to the other planting methods in almost all treatment combinations, but due to high variance, the difference was only significant at 2 m depth compared to control treatments. Shoot densities at 3 m depth were significantly lower than densities at 1 and 2 m depth, but only in control and sand treatments (SNK-test at $p < 0.05$). At the 3 m habitat, located on the steep slope of the bay, 57% of the patches present in 2013 were missing completely in 2014, which explained most of the decreases in shoot density. At 5 m depth only one of the 30 patches were missing and the change in shoot density was explained by a general decrease within patches. In Sep 2014, reproductive shoots were found in some plots at all depths with 3.9 reproductive shoots plot^{-1} at 2 m depth (constituting 8% of the shoots) to 0.8 reproductive shoots plot^{-1} at 5 m and 0.2 reproductive shoots plot^{-1} at 1 and 3 m depth (<1% of the shoots).

The morphology of the shoots sampled in September 2013 differed according to the environment in which the seeds were planted, with larger rhizomal branching and higher shoot densities at shallow depths and a larger vertical growth of leaves at greater depths. The average leaf length increased significantly with depth (1-way ANOVA; $F = 212$; df = 3,19; $p = 0.0001$) from an average of 21.6 cm at 1 m depth to 67.2 cm at 5 m depth, whereas the number of leaves per shoot decreased significantly with depth from average 5.4 cm at 1 m depth to 3.3 cm at 5 m depth ($F = 13$; $p = 0.0001$; Fig. 5a,b). A faster rhizome growth was indicated at 1 to 3 m depth where the average rhizome length and internode distance was around 8 and 2 cm, respectively, compared to 4.8 and 1.1 cm on average at 5 m depth (Fig. 5c,d). However, no significant differences were detected in rhizome length ($F = 1.3$; $p = 0.31$) and internode distance ($F = 2.0$; $p = 0.14$).

Table 3. Planting experiment in Gullmars Fjord. Three-factor ANOVA models testing the number of seeds remaining in the sediment in April, the number of seedlings in June and the number shoots per plot in September 2013 and 2014 as a function of planting season (fall and spring), planting depth (1 to 5 m) and planting method (control, sand cover and rocks). The data were square root transformed to homogenize variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; (ns) non-significant, p > 0.05

	df	April 2013			June 2013			Sept 2013			Sept 2014		
		MS	*F*		MS	*F*		MS	*F*		MS	*F*	
Season (A)	1	0.6	3.6	(ns)	1.3	1.2	(ns)	22.8	2.9	(ns)	32.9	2.2	(ns)
Depth (B)	3	9.4	59.7	****	80.7	79.7	****	84.2	10.9	****	105.0	7.1	***
Method (C)	2	0.1	0.1	(ns)	13.1	12.9	****	105.0	13.8	****	97.3	6.5	**
A × B	3	0.1	0.4	(ns)	8.7	8.6	****	3.4	0.5	(ns)	2.7	0.2	(ns)
A × C	2	0.2	1.2	(ns)	4.1	3.9	*	1.9	0.3	(ns)	7.7	0.5	(ns)
B × C	6	0.1	0.6	(ns)	1.3	1.3	(ns)	5.9	0.7	(ns)	39.7	2.7	*
A × B × C	6	0.2	1.5	(ns)	2.8	2.7	*	13.3	1.7	(ns)	28.7	1.9	(ns)
Residuals	96	0.2			1			7.7			14.8		

Seed planting experiment in Marstrand

At the Marstrand site, results were similar to Gullmars Fjord, although the growth pattern differed. A few small seedlings were detected already in April and sediment core samples in April and June for fall and spring planting, respectively, indicated that a high percentage of seeds remained in the sediment in all treatments (16 to 29%), which did not differ significantly between planting methods or seasons (Fig. 6, Table 4). In June, seedlings were present in all plots, but in low densities (on average 3 to 11 seedlings per plot on average) except for the fall plantings with sand treatment, where densities were significantly higher (on average 72 seedlings per plot), causing the significant interaction between season and planting method (Fig. 6, Table 4). In September 2013, shoots in the sand treatment planted in the fall had almost doubled in numbers (116 shoots plot^{-1} on average) whereas shoot numbers in the other treatments had increased very little or in the spring, rock treatment, decreased to zero, which was now significantly lower than the fall, rock treatment (Fig. 6c, Table 4). The following year, in September 2014, average shoot densities had continued to increase with 76 to 185 shoots plot^{-1} in the fall planting with sand, but decreased to zero in all other treatments except the fall, rock treatment, where 9 shoots remained (Fig. 6d, Table 4). Testing the initial densities of seedlings against the proportional change in shoot density in September 2014 in a simple linear regression analysis showed a significant relationship where no positive growth was obtained at seedling densities below 19 shoots plot^{-1} (simple linear regression, df = 1, 27; p < 0.0001; r^2 = 0.44).

DISCUSSION

Eelgrass restoration using seeds at high latitudes faces special challenges where the long winter period may cause very high losses of seeds during storage or in the field. In this study we identified the major processes responsible for losses of eelgrass seeds and seedlings along the Swedish northwest coast and assessed methods to increase the restoration success. We demonstrate for the first time that seeds could be used for eelgrass restoration in Scandinavian waters, but that very high seed loss and

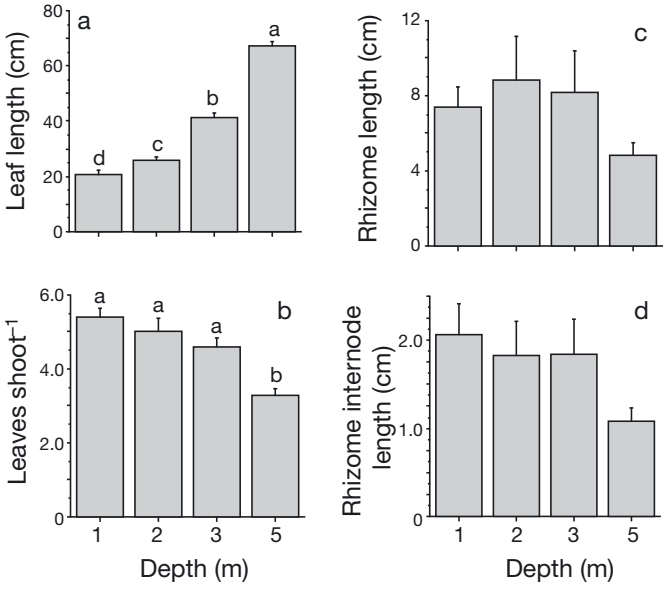

Fig. 5. Shoot morphologies in Gullmars Fjord, September 2013. (a) Leaf length, (b) number of leaves per shoot, (c) rhizome length and (d) rhizome internode length. Data are mean + SE (n = 7). Different letters above bars denote significantly different means (SNK-test at p < 0.05)

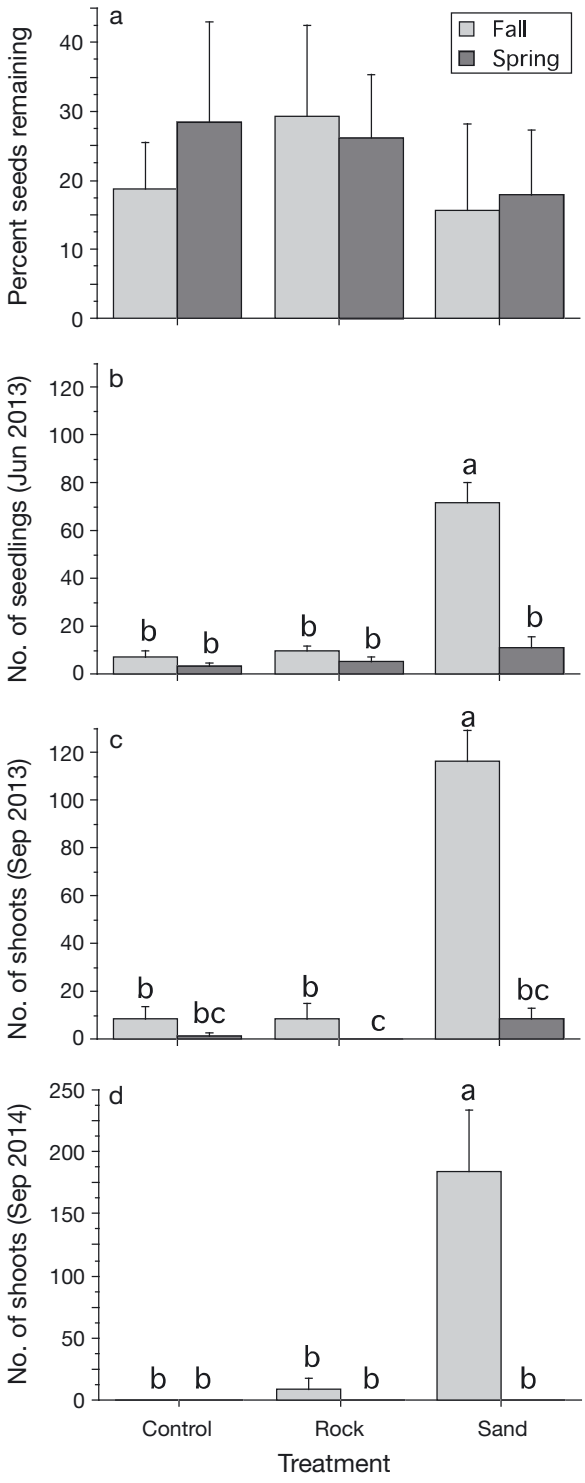

Fig 6. Results from the seed experiment in the Marstrand area where seeds were planted at 2.2 m depth in the fall (Sep 2012) and spring (Apr 2013) using 3 planting methods (control, rock, sand). (a) Seeds remaining per plot, (b) number of seedlings per plot in Jun 2013 and (c,d) number of shoots per plot in Sep 2013 and Sep 2014. Data are mean + SE. Different letters above bars denote significantly different means (SNK-test at $p < 0.05$)

mortality of shoots pose a challenge for large-scale restoration in this region.

Eelgrass seed dormancy and germination at high latitudes

Reproductive behavior and life cycle strategy can vary considerably between populations of *Zostera marina*, depending on climatic factors related to latitudinal distribution. Orth et al. (2000) showed a progressively later seed maturation with increasing latitude (36°–43° N), but with germination and seedling development occurring in the fall regardless of latitude. At higher latitudes, seeds have been suggested to lay dormant during the winter and germinate in the spring (Baskin & Baskin 1998, Olesen 1999). This was supported in the present study in Sweden (58° N), where germination rates of seeds kept at winter conditions in the laboratory were low and where only newly germinated seeds and small seedlings were observed in the field in April. The factors regulating dormancy and germination in eelgrass are not well understood, but studies suggest that cold stratification (i.e. a period with cold temperature) enhances germination (Taylor 1957, Harrison 1991). This is consistent with the present study, where winter temperatures close to 0°C were observed in January–February, and where germination occurred in the spring when temperatures rose above 5°C (Fig. 7). A decrease in salinity also increases germination in seagrass seeds (Caye & Meinesz 1986, Hootsmans et al. 1987, Domínguez et al. 2010). This is consistent with the present laboratory study where salinity was the most important factor inducing germination. Decreases in salinity may also explain the earlier development of seedlings in the Marstrand area, which is affected by freshwater from the river Nordre Älv in the spring, compared to Gullmars Fjord area where the salinity is high and more constant (Fig. 7). Thus, both temperature and salinity appear to be involved in breaking dormancy of eelgrass seeds in Sweden. The results also indicate that many seeds may germinate naturally in fall, as a substantial number of seeds germinated during the first months of the laboratory study when they were kept at temperatures and salinities reflecting early fall conditions along the northwest coast of Sweden. These results demonstrate the importance of performing local studies on germination when developing protocols for restoration using seeds, since local environmental conditions (colder vs. warmer climate) could lead to different reproductive strategies.

Table 4. Seed experiment in the Marstrand area. Two-factor ANOVA model testing the number of seeds remaining in the sediment in April, the number of seedlings in June and the number shoots per plot in September 2013 and 2014 as a function of planting season (fall and spring) and planting method (control, sand cover and rocks). The data of seedlings and shoots were square root transformed to homogenize variance. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; (ns) non-significant, p > 0.05

	df	April 2013			June 2013			Sept 2013			Sept 2014		
		MS	*F*		MS	*F*		MS	*F*		MS	*F*	
Season (A)	1	4.8	0.1	(ns)	42.8	37.5	****	126	50.3	****	170	34.3	****
Method (B)	2	21.4	0.5	(ns)	41.3	36.2	****	94.7	37.8	****	127	25.6	****
A × B	2	7.3	0.1	(ns)	16.5	14.5	****	33.4	13.5	****	127	25.6	****
Residuals	24	44.5			1			2.5			4.9		

Environmental factors affecting seed loss and seedling survival

In this study, seedling establishment was very low when seeds were planted 'naturally' on the sediment surface (on average 1.4% at all sites) and increased significantly from around 0.5% at 1 to 2 m depth to 4.3% at 5 m depth in Gullmars Fjord. Although a large range of seedling establishment rates have been reported from eelgrass restoration studies using seeds in other parts of the world (on average 0.3 to 28%), most large-scale studies have found establishment rates around 5 to 7% (Orth et al. 2003, 2012, Pickerell et al. 2005, Marion & Orth 2012), suggesting unusually low establishment rates in the present study.

In Gullmars Fjord, less than 1.5% of the seeds planted were found in the sediment at 1 to 3 m depth in the spring, demonstrating that the low establishment of seedlings was due to seed losses rather than low germination rates. This is also supported by the high germination rate of seeds in the laboratory study and consistent with other studies reporting >80% germination of viable seeds (Marion & Orth 2012, Orth et al. 2012). Significantly more seeds were found in the sediment at 5 m depth (6 to 41%), reflecting the higher seedling densities found in June. This depth-specific pattern of seed loss in Gullmars Fjord is consistent with physical transport by waves and currents and bioturbation by lugworms being the main processes responsible for the losses in shallow habitats (Fig. 1). Eelgrass seeds can be transported over a flat bottom if flow velocities are higher than 8 cm s^{-1} (Orth et al. 1994) and wind events could have caused large seed losses from the shallow habitats, as was recently demonstrated in a field study in Denmark where 98% of plastic eelgrass seed mimics were lost within 3 wk (Delefosse & Kristensen 2012). Sediment reworking by lugworms likely also contributed to the low establishment rate in the shallow habitats where lugworm densities reached 18 ind. m^{-2}. Lugworms at densities >10 ind. m^{-2} could bury seeds below 6 cm depth over a 10 mo period (Valdemarsen et al. 2011, Delefosse & Kristensen 2012), which is below the maximal depth for successful seedling development (Greve et al. 2005).

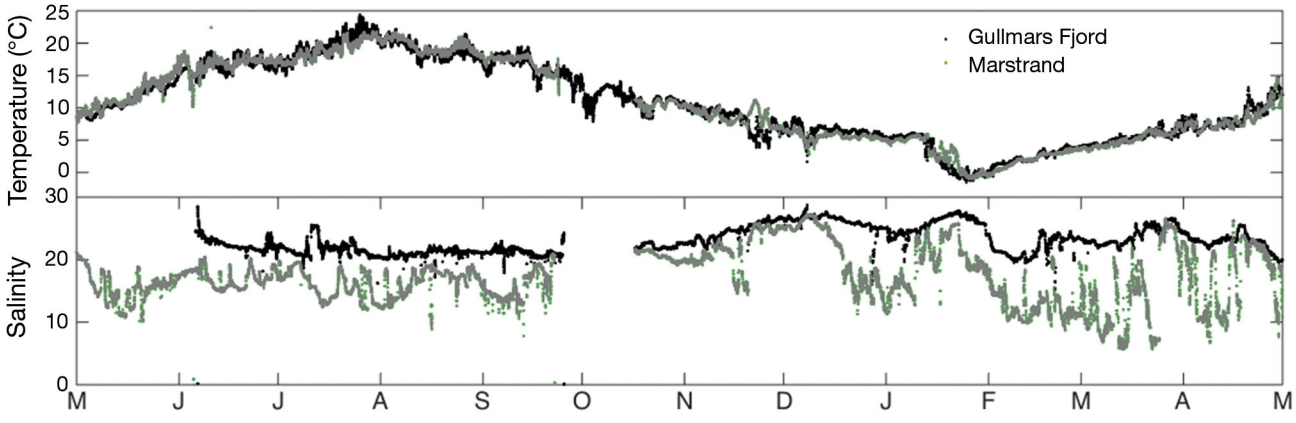

Fig 7. Hourly measurements of temperature and salinity in Gullmars Fjord and Marstrand during 2013–2014 representing the annual variability during the study. Salinity data is missing in May and October

The relatively high establishment rate of seedlings at 5 m depth in Gullmars Fjord was unexpected considering the high organic content (11.3%) and water content (70%) of the sediment. As far as we know, seed plantings have never been studied in this type of sediment, which may present several types of challenges for eelgrass growth and restoration. These sediments are typically anoxic a few mm below the surface with high levels of toxic sulfides (Holmer et al. 2009), which may reduce growth and survival of seedlings if low light or low oxygen concentration in the water result in sulfide invasion (Holmer & Bondgaard 2001, Holmer et al. 2005). The fluid sediments may also decrease the anchoring capacity for seedlings, which may be eroded from the sediment at relatively low flow velocities (Lillebø et al. 2011). However, the results show that seedling growth and survival is possible in these sediments also during low light conditions (15 to 20% of the surface irradiance at 5 m depth). Eelgrass meadows along the Swedish and Norwegian North Sea coasts are mainly found in sheltered areas in fjords and archipelagos where the sediments typically have a high content of organic material (10 to 26%) and water (60 to 90%) at depths over 2 m (Jephson et al. 2008, P.-O. Moksnes unpubl. data). The results suggest that eelgrass is well adapted to grow and reproduce sexually in these environments.

Covering the seeds with sand increased seedling establishment significantly at most planting depths in Gullmars Fjord. Although sand cover can reduce seed-dispersal via protection from hydrodynamic forces, at 5 m depth these hydrodynamic dispersal-effects should be minimal. This suggests that protection of seeds from predators was likely the major contributor to the higher seedling establishment, not just at this depth, but at all depths tested. In support of these suggestions, shore crabs *Carcinus maenas* are efficient eelgrass seed predators and can reduce its abundance by >80% within 1 wk, unless the seeds are covered with a 2 cm layer of sediment (E. Infantes unpubl. data). Shore crabs are very abundant on the Swedish west coast and were present at all depths and locations in the field study. Seed predation has been reported as an important source of seed loss for eelgrass at lower latitudes where species of crabs, fish and terrapin turtles can consume high amounts of seeds (Wigand & Churchill 1988, Fishman & Orth 1996, Sumoski & Orth 2012, Tulipani & Lipcius 2014). To our knowledge, ours is the first study suggesting that seed predation may also play an important role in eelgrass recruitment in northern Europe, with potential negative impacts on restoration.

Interestingly, the positive effect of sand cover on shoot density increased over the summer from 87% to 160% in September (Figs. 4 & 6), suggesting that the sand treatment provided positive effects not only for seed establishment but also for shoot development. The mechanism for this sand effect is not known, but might be related to improved anchoring capacity of the seedlings, preventing uprooting in the shallow habitats that are more exposed to wave and currents. In deeper habitats with high organic sediment content, sand addition could improve sediment permeability and advective exchange processes by increasing the release of anoxic pore-water and oxygen uptake (Huettel & Gust 1992, Janssen et al. 2005).

The growth of seedlings differed between the shallow and deep environments, indicating light-limited seedling development and winter survival. In the shallow (1 to 2 m) habitats, the few established seedlings showed very high growth with lateral branching, increasing shoot densities 8 times to over 50 shoots plot^{-1} in September, with 5 to 6 short leaves (15 to 25 cm) per shoot. The shallow shoots showed high survival over the winter and continuing fast growth in most treatments the second summer, when they doubled their densities to 120 shoots plot^{-1} (Fig. 4). In contrast, the lateral growth was lower at 5 m while the vertical growth was higher with longer shoots (65 to 75 cm; Fig. 5). This allocation of energy mainly to vertical leaf growth is typical in low-light environments (Bintz & Nixon 2001) and demonstrates the ability of seedlings to adapt to a range of environmental conditions. However, at 5 m depth with only ~13% of surface irradiance, the shoots appear to have been growing at the limit of their maximum depth distribution, as suggested by the low number of leaves (on average 3 per shoot), which could indicate light stress (Carr et al. 2012). This was supported by a 50% loss in shoot density at 5 m depth measured after the second summer. This loss likely occurred during the winter as low light conditions may prevent the shoots from storing enough carbohydrates for respiration and survival during the long and dark winter period (Govers et al. 2015). Large losses of shoots after the winter period have been demonstrated for eelgrass shoots transplanted to deep habitats in the study area (Eriander et al. 2016). Large losses were also found after the winter at the 3 m habitat located on the slope of the bay. At this depth, light should not be limiting (35% of surface light). Instead the losses were likely a result of erosion of shoots on the steep slope. The high winter mortality demonstrates the importance of monitoring seed plantings for at least 2 yr before the restoration is evaluated.

The results from the field experiment in the Marstrand area differed from those in Gullmars Fjord. Although the positive effect of sand cover was similar, there was little correlation between seed and seedling densities and most treatments showed a negative growth during the first summer and a complete loss after the winter (Fig. 6), suggesting that factors affecting seedling survival was more important at this site. The Marstrand site has lost a large 140 ha meadow since the 1980s, resulting in a local decrease in water clarity and in maximum depth distribution of eelgrass from about 3 m in the 1980s to 2.2 m today (P.-O. Moksnes unpubl. data), which is close to the planting depth at the Marstrand site. The decreased light conditions are likely a direct result of the eelgrass loss in the area since eelgrass canopy and rhizome-root mats can trap suspended particles, stabilize the bottom and decrease sediment resuspension and turbidity (van Katwijk et al. 2000, Orth et al. 2012). Today, the vegetation in the Marstrand area is dominated by large mats of perennial algae (mainly *Fucus serratus* and *Furcellaria lumbricalis*) that drift on the bottom. These algae mats were not present in the Gullmars Fjord sites. Field studies in Denmark have shown that drifting algal mats of *Fucus* can be responsible for up to 40% of seedling mortality through uprooting and burial (Valdemarsen et al. 2010), and may also increase the resuspension of sediment and the turbidity of the water through physical abrasion (Canal-Vergés et al. 2010). Thus, light limitation and physical disturbance from drift algae may be important factors causing poor survival of seedlings in this area. Physical disturbance may also explain why only plots with relative high seedling densities (≥19 shoot plot^{-1}) showed long-term survival (in contrast to the shallow habitats in Gullmars Fjord). These results suggest that areas along the Swedish northwest coast, that have experienced large losses of eelgrass and which are typically targeted for restoration, are subjected to several positive feedback mechanisms (i.e. sediment resuspension and drifting algal mats) that may prevent the natural return of eelgrass and challenge restoration attempts, similar to what has been described in the Wadden Sea (van der Heide et al. 2007). In the Marstrand area, the relative high content of organic material (2 to 8%) and water (30 to 60%) in the sediment in areas where eelgrass beds have been lost (P.-O. Moksnes unpubl. data) may accentuate the problem by destabilizing the sediments, decreasing the anchoring capacities of seedlings and increasing sediment resuspension (Lillebø et al. 2011).

Restoration using eelgrass seeds in Scandinavian waters

In this study, very high seed densities (equivalent to 4200 seeds m^{-2}) were required to obtain just a few seedlings due to high seed losses. This planting density is approximately 20 to 100 times higher than commonly used for large-scale restoration of eelgrass (Pickerell et al. 2005, Marion & Orth 2010b, Orth et al. 2012) and would make restoration of these habitats using seeds very expensive. Thus, planting methods and strategies need to be developed that reduce the high loss of seeds and seedlings.

One potential way to decrease seed losses is to store them in the laboratory over the winter until they are ready to germinate in the spring and thereby minimize exposure time to various seed-loss factors, e.g. predation, bioturbation and winter storms. In studies from lower latitudes, where eelgrass seeds mature in spring–summer and germinate in the fall, briefly storing seeds before planting in fall increased seedling establishment significantly (Marion & Orth 2010a). In this study we demonstrate that eelgrass seeds can be successfully stored in the laboratory for 8 mo before being planted in the spring and we identified optimal conditions for long-term seed storage to avoid premature germination (i.e. 5°C, salinity 30, with aeration). To our surprise however, planting seeds in the spring did not increase seedling establishment in comparison to planting in fall at either study area. The reason for this is unclear. Apart from decreasing the exposure time to loss factors, April should also be a more favorable time of the year for seed planting with lower frequency of storms compared to September, and with lower activity and densities of seed predators such as shore crabs that migrate to deeper, warmer water over the winter (Pihl & Rosenberg 1982). Our study therefore suggests that it is more cost-efficient to plant seeds in the fall, since winter storage also led to large losses of seeds (36%) without improving seedling establishment. However, although the results were clear and based on experiments performed over multiple depths and areas, they were only tested during one year and more studies are required to assess if spring-planting may increase seedling establishment.

In comparison with seeds planted on the sediment surface, covering the seeds with a 2 cm layer of sand increased seedling establishment by 87% in Gullmars Fjord and over 500% in the Marstrand area on average, likely by reducing both seed predation and transport of seed and seedlings by currents and drift-

ing algae (Fishman & Orth 1996, Koch et al. 2010, Infantes et al. 2011). These results are consistent with earlier studies showing increased seedling establishment when seeds were buried in the sediment (Moore et al. 1993, Marion & Orth 2012). Thus, seed burial could potentially be used as a method to reduce seed losses. In the eastern USA, mechanical planting machines have been developed to bury the seeds in the sediment showing promising results for large-scale restoration (Traber et al. 2003, Marion & Orth 2010b). Similar planting machines could potentially be developed for Scandinavian habitats, but would need to be modified to work on soft sediments with high water content and topographically complex bottoms that dominate in these glacially formed coastal areas.

In the present study, adding small rocks to the planting plots were also assessed as a method to trap the seeds by the vortices in the flow and scouring holes that form around the rocks, which could reduce seed transport by waves and currents. However, we found no consistent positive effect on seedling establishment in any habitat, including the shallow habitats where hydrodynamics were expected to play a larger role. The lack of an effect is not clear. Pilot studies in a flume showed that seed trapping increased with the presence of rocks at velocities above 10 to 15 cm s^{-1} (E. Infantes unpubl. data). One possible explanation is that the current velocities at the study sites were sufficiently strong to transport seeds, but too low to induce trapping. Studies in areas with higher current velocities are encouraged to assess this method.

This study demonstrates that seeds have a potential use for restoration of eelgrass in Scandinavian countries, as planted seeds formed patches that survived in all assessed habitats. However, very high seed loss and high mortality of shoots pose a challenge that need to be addressed before restoration using seeds can be recommended at a large scale in this region. The main processes responsible for the seed loss were seed predation from crabs, seed transport by currents and bioturbation by lugworms, while the main process affecting shoot development was light availability and physical disturbance. In addition, positive feedback mechanisms (i.e. sediment resuspension and drifting algal mats) may also prevent the natural return of eelgrass and challenge restoration efforts. In order to improve restoration success, further work could be focused on the effect of antagonistic types of feedback that prevent seed persistence and seedling development in the field.

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