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PRACTICE AND TECHNICAL ARTICLE

Optimizing seed injection as a seagrass restoration method

Max L. E. Gräfnings^{1,2,3} , Jannes H. T. Heusinkveld⁴, Dieuwke J. J. Hoeijmakers⁴, Quirin Smeele⁵, Henk Wiersema⁴, Maarten Zwarts⁴, Tjisse van der Heide^{1,2,6}, Laura L. Govers^{1,6} 

Due to the major declines of seagrasses worldwide, there is an urgent need for effective restoration methods and strategies. In the Dutch Wadden Sea, intertidal seagrass restoration has proven very challenging, despite numerous restoration trials with different restoration methods. Recently, however, the first field trial performed with a newly developed “dispenser injection seeding” method (DIS) resulted in record-high plant densities and seed recruitment. Here, we present the further development of the methodology and consequently improved restoration results. During two consecutive growing seasons, we honed the seeding technique and experimentally investigated how seeding depth (2/4 cm), injection density (25/100 injects/m²), and seed amount (2/20 seeds/inject) affected restoration of intertidal annual *Zostera marina*. We found that all variables had a significant impact on plant establishment. Seeding deeper (4 cm) had the largest positive effect on restored plant densities, while lowered seed densities (2 seeds/inject) had the largest positive impact on seed recruitment. The optimized DIS method, combined with an altered placement of the seeding hole, resulted in a 50-fold increase in restored plant densities (from approximately 1 to 57 plants/m²) and a simultaneous increase in seed recruitment (from 0.3 to 11.4%). These improvements stem from the method’s ability to counteract a recruitment bottleneck, where seeds are lost through hydrodynamic forcing. The methodological improvements described here are important steps toward restoring self-sustaining seagrass populations in the future and our study demonstrates the high potential of the seed-based DIS method for seagrass restoration.

Key words: bottleneck reduction, dispenser injection seeding method, seagrass restoration, seed-based restoration, Wadden Sea, *Zostera marina*

Implications for Practice

- The novel dispenser injection seeding method is a promising tool for seed-based seagrass restoration. The method’s adaptability allows it to be used effectively in differing environmental conditions and with differing seed qualities.
- Annual eelgrass plants can reliably be restored with low seed amounts (approximately 5 seeds), if high seed quality is secured with pretreatment of seeds and overwinter mortality is reduced with seed storage.
- Identifying bottlenecks hindering restoration success is key, as bottleneck reduction (e.g. with innovative restoration methods) can greatly enhance restoration success.

Introduction

Seagrass meadows can be found globally in coastal waters, where they function as important ecosystem engineers and hotspots of biodiversity (Hemminga & Duarte 2000). During the last century, approximately 29% of the global seagrass area was lost and this trend is still ongoing (7% loss/year; Orth et al. 2006a; Waycott et al. 2009). To halt and reverse seagrass losses, numerous seagrass restoration attempts have been undertaken globally, with the first trials performed already over half a

century ago (Van Katwijk et al. 2016). Seagrass restoration is not merely about restoring the actual plants, but chiefly about restoring the whole associated ecosystems, including their functions and services (e.g. Reynolds et al. 2016). A recent review even showed that seagrass restoration should be considered as an integral part in global efforts to rebuild marine life (Duarte et al. 2020). However, success rates of seagrass restoration

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attempts are generally low (<40%) (Bayraktarov et al. 2016; Van Katwijk et al. 2016), with early plant establishment and survival being major hurdles hindering successful seagrass restoration. Fortunately, once successfully established, restored seagrass have in several areas been found to have very high long-term persistence rates (Statton et al. 2017; Rezek et al. 2019), highlighting the need for more effective restoration methods and strategies that can reduce bottlenecks hampering seedling establishment and survival.

Ecological feedbacks have been found to play a major role in the structure, functioning, and fate of seagrass ecosystems (Maxwell et al. 2016). Growing evidence suggests that including positive feedbacks and other ecological interactions in restoration designs can improve the restoration success of seagrasses (Valdez et al. 2020). Previous studies have shown that large-scale (both in amount of transplanted donor material and restored area) seagrass restoration trials perform better than small-scale trials (Van Katwijk et al. 2016; Paulo et al. 2019). The improved performance is attributed to spreading of risks and to the activation of self-sustaining feedbacks that can transform the local conditions for the better. Similarly, establishing high plant densities has become a common objective of seagrass restoration projects, because it has been shown that high plant densities can induce density-dependent self-facilitative feedbacks, e.g. by reducing turbidity (Van Katwijk et al. 2016), increasing seed retention (Bos & van Katwijk 2007), and enhancing sexual reproduction (Furman et al. 2015).

Most seagrass restoration attempts have thus far been performed on small scales (Van Katwijk et al. 2016), with upscaling being out of the question due to insurmountable operational costs and/or by the use of unscalable restoration methods. Seed-based restoration strategies are generally thought to be more scalable, because this is logistically more feasible and cost effective than transplanting plant fragments and large amounts of donor material can be collected without seriously harming the donor population. In some areas, seed-based restoration efforts have been very successful (see Orth et al. 2012), but overall seed-based restoration attempts are still scarce, with only approximately 8% of all seagrass restoration trials worldwide being performed with seeds (Van Katwijk et al. 2016).

Most *Zostera marina* (hereafter eelgrass) has vanished from the Dutch Wadden Sea since the 1930s, which has prompted numerous seagrass restoration trials during the last decades (Govers et al. 2022). These restoration efforts have focused on intertidal areas, because subtidal seagrass restoration has been deemed extremely challenging in the highly turbid waters of the Wadden Sea. Several different seed-based methods have been tested, but with overall poor results (Govers et al. 2022). Recently, however, two major breakthroughs have resulted in increased restored plant densities and seed recruitment rates in the Wadden Sea (Govers et al. 2022). First, overwinter seed mortality was greatly reduced by storing the seeds overwinter in a controlled environment and by treating the seeds with copper to negate a prevalent water mold (*Phytophthora* spp.) infection (Govers et al. 2017). Second, an effective seeding method,

the dispenser injection seeding (DIS) method, was developed. During the first field trials in 2017, restoration experiments seeded with the new method yielded up to 100× higher plant densities than any previous experiments in the Wadden Sea (Govers et al. 2022). Although the first trial was deemed a success, both plant densities (>10 plants/m²) and seed recruitment (0.3%) remained low.

The main objective of this study was to further develop and optimize the DIS method, so that: (1) target densities of >10 plants/m² could reliably be established; (2) seed recruitment could be increased over 10%, making the method less taxing on donor populations and more economically viable; and (3) large-scale restoration attempts can be conducted in the future. To achieve our goals, we conducted two field trials in the intertidal Dutch Wadden Sea. First, we investigated with a field experiment how seeding depth, injection density, and seed amount affected plant densities and seed recruitment (%). For the second field trial, minor but significant adjustments were made to the method that boosted our restoration results and the method's reliability.

Methods

Study Area and Restoration Sites

The Wadden Sea is a shallow coastal sea that harbors the world's most extensive intertidal mudflat system and extends from northwest Netherlands to the southwest coasts of Denmark. Seagrass restoration trials were performed at two intertidal sites in the Dutch Wadden Sea: northeast of the island Griend (53.2692°N, 5.2949°E) and Uithuizen (53.4663°N, 6.6883°E) (Fig. 1). Uithuizen was selected because the site had proved most suitable for seagrass restoration in previous trials (Govers et al. 2022) and Griend was selected because of the spontaneous establishment of intertidal dwarf eelgrass (*Zostera noltii*) and *Ruppia maritima* patches at the site between 2014 and 2017. No common eelgrass (*Zostera marina*) grew naturally at either site before the onset of these restoration trials.

Seagrass Restoration Method

Seeds of annual intertidal eelgrass (*Z. marina*) were collected in autumn the year before the restoration trials from two large and healthy meadows in the German Wadden Sea, Sylt (54.7835°N, 8.2950°E, 2017) and Hamburger Hallig (54.5986°N, 8.8111°E, 2018). Seed harvest, treatment, and storage were performed in similar fashion as described in Govers et al. (2022), including seed treatment with copper sulfate (0.2 ppm; Govers et al. 2017). With the DIS method, a sediment–seed mixture is injected into the mudflat sediment with a sealant gun (for detailed description of methodology and characteristics of used sediment see Govers et al. 2022). Metal grids (size: 1 × 1 m, grid size: 0.1 × 0.1 m) were used during seeding to space injections evenly and in desired quantities (see Fig. S1). The nozzles of the sealant guns were marked with tape (demarking depth), so that the seeding could be accurately performed at the desired injection depth.

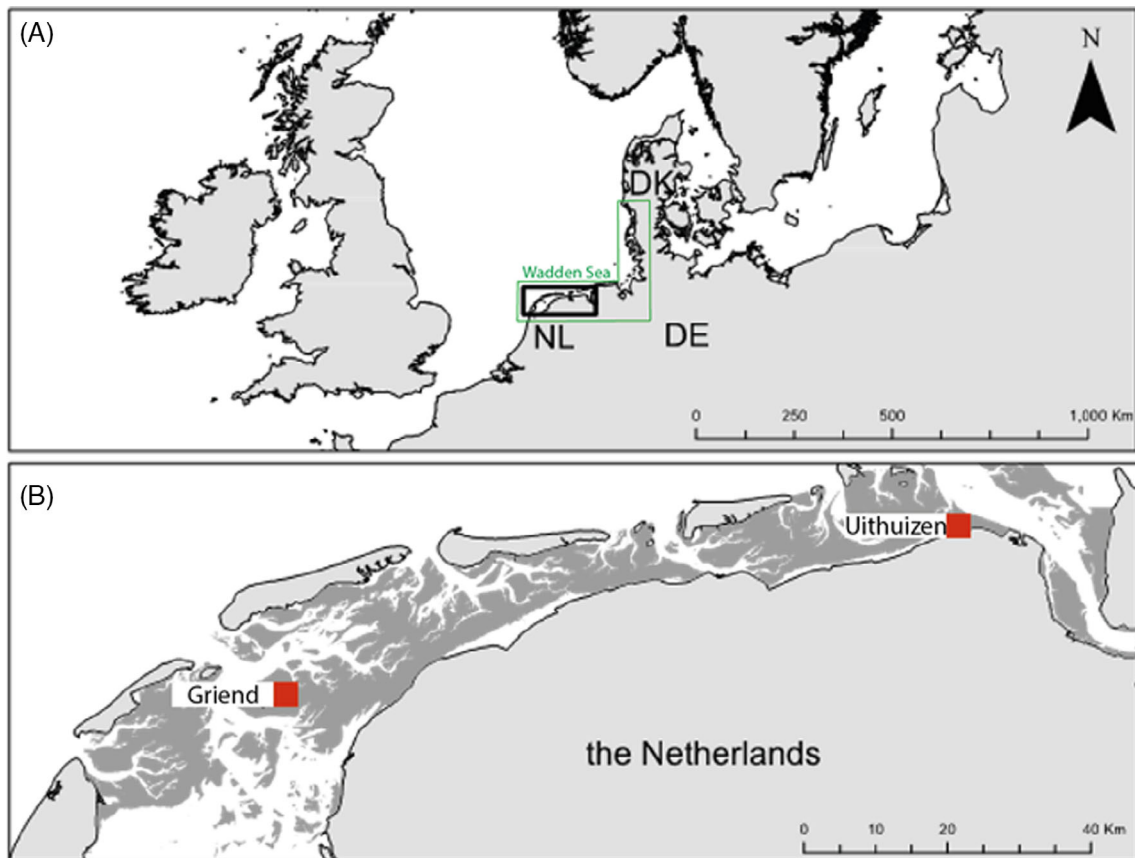


Figure 1. (A) Location of the Wadden Sea in North Western Europe and (B) location of the two restoration sites in the Dutch Wadden Sea.

Restoration Trials

In 2018 we conducted a field experiment with the aim to improve the DIS method, increasing the method's reliability and effectiveness. We tested the effect of seed amount (two levels: 2 or 20 seeds/injection), injection depth (two levels: 2 or 4 cm), and injection density (two levels: 25 or 100 injections/m²) on emerged eelgrass plant densities and seed recruitment (%). The full factorial field experiment had 8 treatments replicated in 6 blocks, thus resulting in 48 plots/site (plot size: 4 m²). The experiment was constructed in March at the two restoration sites, Griend and Uithuizen. The number of adult eelgrass plants (defined as plants with flowering shoots) in the experimental plots were counted at both sites in July 2018. Total seed recruitment rate (% of seeds that germinated and survived to adults) was determined for each plot by dividing the amount of emerged adult plants by the initial number of seeds injected. The calculated seed recruitment rate (%) does not take into account seed viability.

In a follow-up restoration trial, we seeded five additional plots at both restoration sites in 2019. Here, we based our seeding design on the previous year's most successful treatment and implemented it on a larger scale (plot size: 7.3 m²). Seeding parameters used were: injection density: 100 injections/m²; seeding depth: 3 cm; and seed amount: 5 seeds/injection.

Additionally, we altered the position of the seeding hole from the tip of the nozzle to the side (Fig. 3), because in 2018 it was observed that the seed mixture was easily pulled out of the sediment due to vacuum when the nozzle was removed after injection. Thus, by switching the position of the seeding hole we aimed to reduce loss of the injected seed mixture. The number of plants in each plot was counted in May 2019 and seed recruitment (%) was determined as described earlier.

Statistical Methods

To investigate how the different seeding parameters affected restoration success in field experiment (2018), we used a negative binomial model for established eelgrass plants per plot (glmmTMB, glmmTMB package) and a linear mixed-effect model for seed recruitment % (lmer, lme4 package), with "seed amount," "injection depth," and "injection density" as fixed factors (interactively tested) and "block" as a random factor. Residuals of the linear mixed-effect model were checked for normality. Based on Akaike information criterion, a backward stepwise regression was used to find the minimal adequate for both models. All statistical tests were performed in R version 3.5.3 (R Foundation for Statistical Computing, 2019). All figures show untransformed data.

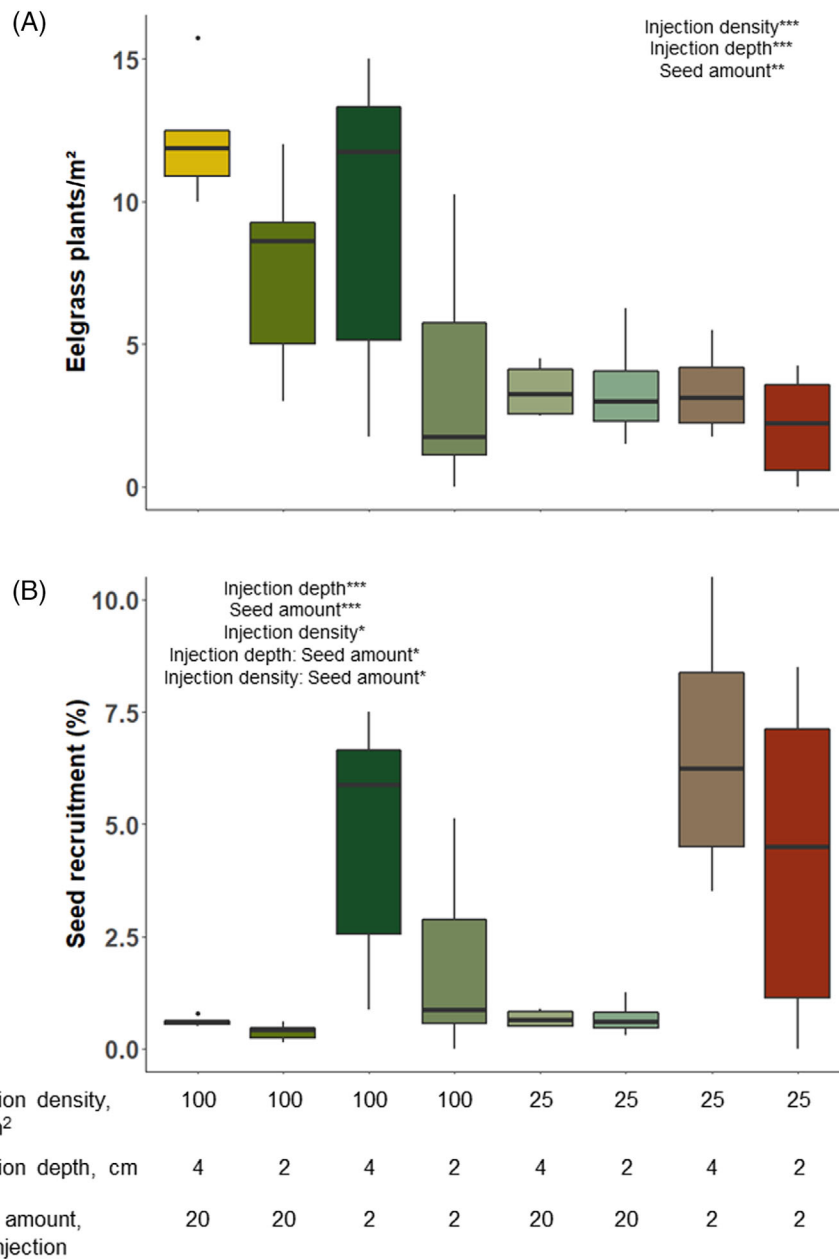


Figure 2. Restored eelgrass plants per square meter (A) and seed recruitment % (B) for the experimental treatments in July 2018 at Griend (The Netherlands). Boxplots show median (line in box), upper and lower quartile (vertical line), 1.5 × interquartile range (vertical line), and outliers (circle). The variables significantly explaining observed differences between treatments are included (statistical models: generalized linear mixed-effect model [Negative Binomial] for plants per square meter and linear mixed-effect model for seed recruitment %). The stars indicate significance: ***, <0.001; **, <0.01; and *, <0.05.

Results

Field Experiment

In July 2018, a total number of 1,081 and 4 eelgrass plants were recorded in the experimental plots at Griend and Uithuizen, respectively. Due to the very low plant establishment at Uithuizen, only Griend data was further analyzed. The number of restored adult plants were positively affected by deeper injection depth ($\beta = 0.99$, $SE \pm 0.28$, Incidence Rate Ratio (IRR) = 2.69, $p < 0.001$), higher seed amount ($\beta = 0.92$,

$SE \pm 0.28$, IRR = 2.50, $p < 0.01$), and higher injection density ($\beta = 0.85$, $SE \pm 0.17$, IRR = 2.34, $p < 0.001$) (Table S1; Fig. 2A). Seed recruitment % was increased by lower seed amount ($\beta = -3.50$, $SE \pm 0.88$, $p < 0.001$), deeper injection depth ($\beta = 2.68$, $SE \pm 0.72$, $p < 0.001$), and lower injection density ($\beta = -2.18$, $SE \pm 0.72$, $p = 0.002$) (Table S2; Fig. 2B). In addition, there were significant interactions of injection depth and seed amount ($\beta = -2.56$, $SE \pm 1.01$, $p = 0.011$), as well of injection density and seed amount ($\beta = 2.00$, $SE \pm 1.01$, $p = 0.049$) (Fig. 2B). The overall best performing combination

of seeding variables was deemed to be: 100 injections/m², 4 cm depth, 2 seeds/injection, since the combination produced high plant densities (9.5 plants/m²) while seed recruitment was recorded to be second highest (4.75%; Fig. 2).

Follow-Up Restoration Trial

In May 2019, 0 seedlings were found in the experimental plots at Uithuizen. The seeds were most likely washed away or buried during a storm that occurred a week after seeding. At Griend the target plant density was strikingly overreached, as the plots had on average 57 eelgrass plants/m² (SE ± 9.7) in May, which translates to an average seed recruitment of 11.4% (SE ± 1.93). This corresponds to a 5× increase in plant densities compared to 2018 and a 50× increase compared to the first field trials with the DIS method in 2017 (Govers et al. 2022) (Fig. 3A). Simultaneously, seed recruitment (%) was increased by over 10% in 2 years (Fig. 3B) (0.3% seed recruitment in 2017; Govers et al. 2022; 4.4% in 2018). After May, the eelgrass cover grew

very dense in the trial plots, hindering the count of individual plants.

Discussion

Very few seagrass restoration trials have been conducted in the intertidal and generally trials in this dynamic zone show the lowest success rates (Van Katwijk et al. 2016). However, here we showed that, with a suitable restoration method and proper site selection, seagrass restoration can be successful even in this dynamic zone. Through an adaptive development process of the DIS method, we discovered that injecting seeds at optimal seeding depth, with higher injection densities, with few seeds per injection and through the side of the nozzle increased seagrass restoration yields. Translating these findings into practice resulted in very high restored eelgrass densities (57 plants/m²) and significantly higher seed recruitment (from 0.3 to 11.4%, uncorrected for seed viability) at our restoration site Griend in the Dutch Wadden Sea. With the DIS method, we managed to

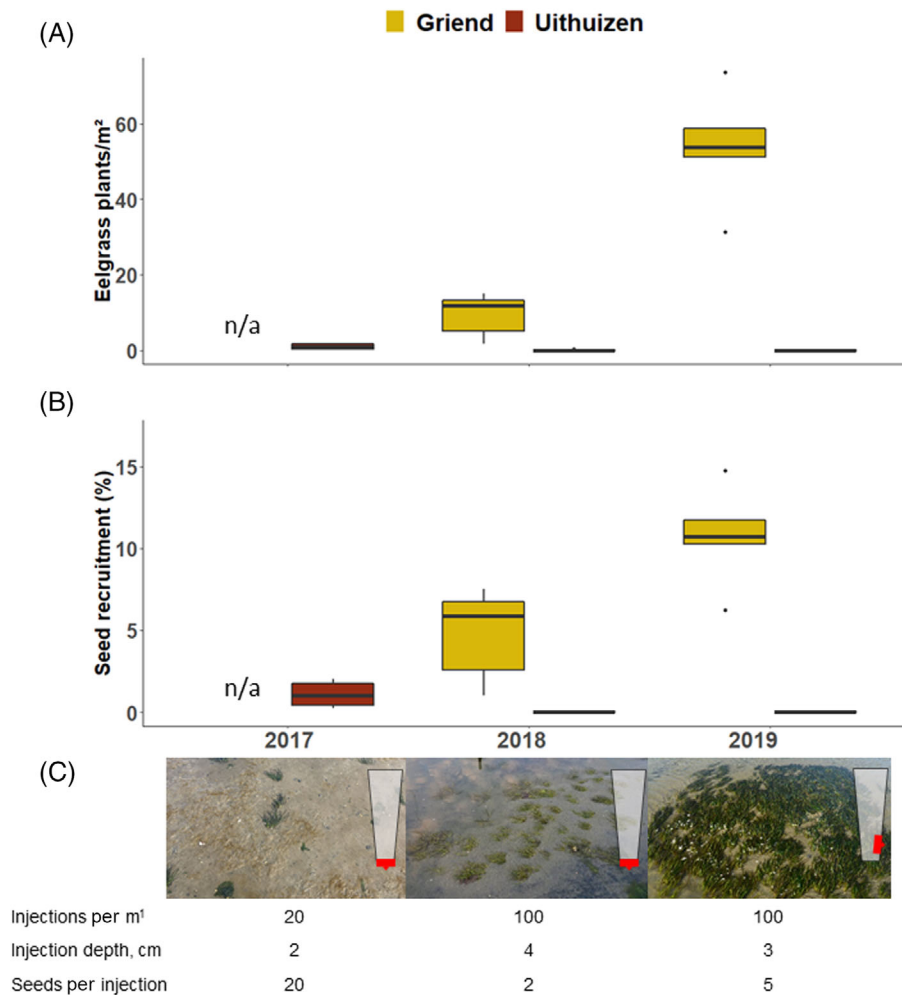


Figure 3. Restored eelgrass plants per square meter (A) and seed recruitment % (B) of the most successful treatments in 2017, 2018, and 2019 at the two experimental sites in the Dutch Wadden Sea (Griend and Uithuizen). Of note, no seeding was done at Griend in 2017. Boxplots show median (line in box), upper and lower quartile (box), 1.5× interquartile range (vertical line), and outliers (circle). (C) Pictures of the experimental plots with the highest restored eelgrass densities in 2017 (Uithuizen), 2018 (Griend), and 2019 (Griend) with the placement of injection hole (red) on the nozzle depicted on the right side of the pictures.

achieve a 5,000-fold increase in restored eelgrass densities compared to previously used seed-based restoration methods in the Dutch Wadden Sea (Govers et al. 2022), highlighting the method's high potential and suitability for intertidal seagrass restoration.

Seed-based restoration trials often suffer from low seed recruitment rates (e.g. Golden et al. 2010; Eriander et al. 2016), reducing the efficiency and reliability of many seed-based restoration methods. Low recruitment rates have been attributed to a number of site-specific reasons (e.g. bioturbation—Valdemarsen et al. 2011; predation—Orth et al. 2006b; hydrodynamics—Statton et al. 2017; and disease—Govers et al. 2016). In the intertidal Wadden Sea, the failure of previous seagrass restoration trials has been mainly attributed to the dislocation of seeds due to hydrodynamic forcing and seed mortality due to *Phytophthora* spp. infection (van Duren & van Katwijk 2015; Govers et al. 2016). The seed mortality bottleneck has successfully been addressed through overwinter storage and copper treatment (Govers et al. 2017, 2022). Here, we show how the repression of both bottlenecks simultaneously resulted in record-high restoration yields. By injecting the seeds directly into the sediment, dislocation effects were actively counteracted, resulting in one of the highest recruitment rates (11.4% of all injected seeds produced an adult plant; seed viability not accounted for) documented for any seed-based restoration method to date (see e.g. Marion & Orth 2010; Eriander et al. 2016; Unsworth et al. 2019). Seeds planted closer to the surface generally have higher seed survival and seedling emergence (optimal: 2 cm—Jørgensen et al. 2019; 2–3 cm—Zhao et al. 2016), but here we show a trade-off between closeness to surface and increased chance for dislocation. At our site, we assumed that at the shallower seeding depth (2 cm) seeds were more likely to wash away, leading to increased seed losses and consequently lower plant densities. Recently, in the subtidal, Marion et al. (2020) obtained the best seagrass restoration results at deeper seeding depths similar to ours (3–4 cm) and also linked the increased success to lowered probability for dislocation. Hence, optimal seeding depth should be considered site-specific. Additionally, we found that changing the placement of the seeding hole increased seed retention and this small practical adjustment is believed to be one of the largest reasons for the huge improvement in restoration success between the 2018 and 2019 trials.

Scalability is becoming an increasingly important requirement of seagrass restoration methods, since restoration success has been found to increase with larger restoration trials (Van Katwijk et al. 2016). A major advantage of seed-based restoration methods is that donor material can be collected in large quantities without seriously harming donor populations. Additionally, by increasing a restoration method's efficiency the burden on donor populations can be even further reduced. In our 2018 restoration experiment, seed recruitment was increased most by injecting fewer seeds per injection (2 rather than 20), leading to 3.5% higher seed recruitment. In the second field trial (2019), seed recruitment was increased above the target value (>10%) for the first time. The fact that only 5 seeds/injection was able to restore plants reliably (60% of all injections produced an adult plant), decreases the method's burden on donor

populations and paves the way for sustainably upscaling seed-based restoration trials. Additionally, despite requiring manual seeding, large areas can be seeded with the DIS method relatively quickly (approximately 25 m² hour⁻¹ person⁻¹, 100 injections/m²; personal observation), enhancing the method's scalability also from a practical point of view. The restoration trials performed with the DIS method have thus far been relatively small in size, but large-scale trials are a logical next step considering the improved methodology and consequent restoration successes shown here.

Positive ecological feedbacks play a major role in the structure and functioning of seagrass ecosystems (Maxwell et al. 2016) and growing evidence suggests that including positive feedbacks in restoration designs can improve the restoration success of seagrasses (Valdez et al. 2020). For example, through positive density-dependent feedbacks, seagrasses have been found to facilitate their own fertilization, survival, and growth (Valdez et al. 2020). The spatial arrangement of seagrass restoration designs can potentially also affect outcomes, as seagrass establishment can likely benefit from aggregated rather than dispersed planting arrangements under stressful conditions, similarly to what has been found in salt marshes (Silliman et al. 2015). In the 2018 experiment at Griend, we reached for the first time the project's goal of >10 plants/m². The following year, with the optimized DIS method we were able to restore plant densities (57 plants/m²) that are extremely high for a seed-based seagrass restoration method. A useful attribute of the optimized DIS method is that the densities and assemblages of restored plants can easily be altered, enabling the development of intricate restoration designs that aim to trigger self-sustaining positive feedbacks. For instance, designs leading to high restored seagrass densities might facilitate higher seagrass survivability or sexual reproduction (Bos & van Katwijk 2007; Valdez et al. 2020).

Several previous studies (e.g. Van Katwijk et al. 2016; Statton et al. 2017) have noted that suitable site selection is key for successful seagrass restoration. The failure of two consecutive restoration trials at Uithuizen reflects poor site selection, but also demonstrates the limits of the DIS method. Still, the complete failures at Uithuizen were surprising, as previous restoration trials had performed best at the site (Govers et al. 2022) and habitat suitability models have predicted the larger area to be highly suitable for seagrass (Folmer et al. 2016). The apparent unsuitability of the site most likely spurs from high vulnerability to storms that lead to periodic high sediment dynamics. Powerful winter storms are relatively common in the Wadden Sea and as long-term restoration success of annual plants relies on good seed retention, we deem the Uithuizen-site too unstable for further restoration efforts. Here, we have shown that the DIS method can be successfully used to counteract seed dislocation bottlenecks, but this is not applicable if the sediment itself is washed away. Hence, it is important to acknowledge that different sites, seagrass species, and life strategies (annual vs. perennial) might require different restoration methods and that choosing a suitable restoration method is highly context dependent.

This study shows that seed-based seagrass restoration can be successful in the dynamic intertidal, when an appropriate

restoration method is used (DIS seeding). Our results highlight the importance of a continued and adaptive optimization process of restoration methods. Without an iterative trial and error loop, bottlenecks hindering successful seagrass restoration in the intertidal would have remained unclear and the DIS method directly targeting these bottlenecks could not have been developed. To unravel the methods' full potential, additional experiments need to be conducted subtidally. We believe that the DIS method can be very useful especially in shallow subtidal areas, where passive restoration methods cannot counteract recruitment bottlenecks (e.g. dislocation by hydrodynamics). Additionally, the success of the DIS method hinges on good seed quality and quantity, which might limit the methods applicability for some seagrass species.

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Supporting Information

The following information may be found in the online version of this article:

Figure S1. Using a 1×1 m metal frame to space injections with the DIS method evenly in the field.

Table S1. Results of generalized linear mixed-effect model.

Table S2. Results of linear mixed-effect model predicting eelgrass seed recruitment.

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