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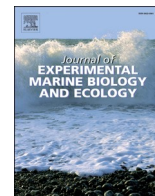
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Ragworms (*Hediste diversicolor*) limit eelgrass (*Zostera marina*) seedling settlement: Implications for seed-based restoration

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ABSTRACT

Seagrasses are globally declining and multiple restoration efforts are undertaken to reverse these losses. However, these efforts have proven to be challenging, facing a variety of bottlenecks. We studied how predation by macroinvertebrates may form a potential bottleneck for seed-based seagrass restoration. Specifically, we questioned if the omnivorous common ragworm (*Hediste diversicolor*) may act as a predator on eelgrass (*Zostera marina*) seeds and whether that could affect seed-based eelgrass restoration trials. In a controlled lab experiment, we studied (1) how seedling establishment was affected by ragworm biomass (0, 2, 8 g DW m⁻²), (2) if the absence or presence of an additional or alternative high-protein food source (Sanikoi® Gold Protein Plus, 52% protein) prevented potential seed predation by ragworms and (3) how ragworm size (small: 0.0029 g and 3.3× bigger: 0.0095 g DW ragworm⁻¹) affected eelgrass seedling establishment. Additionally, we questioned (4) if ragworms may provide a bottleneck for annual eelgrass restoration experiments in the Dutch Wadden Sea by combining data from a large-scale benthic survey (SIBES, Netherlands Institute for Sea Research (NIOZ), Texel) with an existing eelgrass habitat suitability map. We found that >2 g DW m⁻² ragworms completely hampered eelgrass seedling establishment, even when fed an additional, protein-rich, food source. Ragworms only seemed to target sprouted seeds rather than intact seeds. Additionally, sprouted seed consumption by ragworms was size-dependent: sprouted seeds escaped predation by smaller ragworms even when present in high biomass (2 g DW m⁻²). By extrapolating our findings to the field, we showed that 52.8% of the potential eelgrass growth sites in the Dutch Wadden Sea overlap with impeding ragworm biomass (≥2 g DW m⁻²). By consuming sprouted eelgrass seeds, ragworms may consequently strongly impede seed-based eelgrass restoration efforts, especially since both species have highly overlapping distributions. We thus provided novel insights into an unknown bottleneck for seed-based eelgrass establishment, which may have restoration implications. Especially for annual eelgrass that fully depends on successful seedling establishment for their persistence and survival.

1. Introduction

Seagrasses play an important role in nutrient cycling, sediment stabilization, biodiversity amelioration, carbon storage and coastal protection (Duffy, 2006; Van der Heide et al., 2012; Waycott et al., 2009). However, these valuable habitats are declining globally (Van der Heide et al., 2012; Waycott et al., 2009). To prevent and reverse further losses, seagrass restoration efforts, using sods and rhizome fragments, are being undertaken across the world (Valdez et al., 2020). Nonetheless, the

success rates of these efforts remain generally low (Bayraktarov et al., 2015; Valdez et al., 2020; van der Heide et al., 2007) and are mostly focused on decreasing environmental (e.g., hydrodynamic forces) and physiological stressors (diseases) on seagrasses on a small spatial scale (Govers et al., 2016; Marion et al., 2020; Statton et al., 2017). In contrast, biotic interactions, which could also affect restoration success, are less well studied (Gagnon et al., 2020; Statton et al., 2017; Valdez et al., 2020).

Various interactions between seagrasses and associated invertebrates

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have been observed to affect restoration outcomes. For example, epifaunal bivalves (e.g., blue mussels, pinnids) can promote seagrass survival by sediment fertilization and stabilization (Gagnon et al., 2020). Endobenthic lucinid bivalves may in turn engage in a mutualism with seagrasses and promote seagrass growth and survival by removing toxic sulphides from the sediment (De Fouw et al., 2016; Van der Geest et al., 2020; Van der Heide et al., 2012). Inclusion of these positive interactions between bivalves and seagrasses may thus increase seagrass restoration success (De Fouw et al., 2016; Gagnon et al., 2020; Peterson and Heck, 2001; Valdez et al., 2020; Van der Geest et al., 2020; Van der Heide et al., 2012). In contrast, species interactions may also form a bottleneck for seagrass restoration efforts (Statton et al., 2017). Sediment reworking by bioturbating species may negatively affect seagrass growth and may lead to seed loss, forming an early-life stage bottleneck for seagrasses (Marion et al., 2020; Statton et al., 2017; Valdemarsen et al., 2011). For instance, the bioturbating behaviour of lugworms (*Arenicola marina*) may lead to nutrient release from the porewater to the surface water, promoting harmful epiphytal growth on dwarf eelgrass. (Govers et al., 2014; Valdemarsen et al., 2011). Additionally, bioturbating behaviour may also bury seagrass seeds in the sediment. Depending on the bioturbating species, the seeds may be buried too deeply (*Arenicola marina*), preventing the sprouting seed from successfully reaching the sediment's surface (Suykerbuyk et al., 2012; Valdemarsen et al., 2011). Alternatively, bioturbation by *Hediste diversicolor* and *Alitta succinea* may lead to seed burial up to ~0.5 cm depth, which may actually protect seeds from predation by macroinvertebrates (Blackburn and Orth, 2013; Delefosse and Kristensen, 2012).

Macroinvertebrates such as decapod crustaceans are known to be key predators of seagrass seeds. Other seagrass seed predators include mallard ducks and fish, which may consume seeds as part of their diet (Fishman and Orth, 1996; Orth et al., 2006). Especially decapod crustaceans (crabs and shrimp) may predate on seeds when their preferred food is scarce, consequently diminishing the reproductive success and recruitment of seagrasses, forming an early-life stage bottleneck for seagrasses which may negatively affect seed-based restoration attempts. (Darnell and Dunton, 2015; Fishman and Orth, 1996; Infantes et al., 2016; Orth et al., 2006; Statton et al., 2017).

Seed-based seagrass restoration is becoming an increasingly popular seagrass restoration method globally, despite the aforementioned bottlenecks to early-life stage transitions (Govers et al., 2016; Marion and Orth, 2010; Statton et al., 2017). Compared to many traditional approaches, the use of seeds is generally considered to be more cost-effective, easily expandable and allows for the establishment of high genetic diversity in a restored population (Busch et al., 2010; Marion and Orth, 2010; Ort et al., 2014). Eelgrass (*Zostera marina*) is a species that occurs globally in both the subtidal and intertidal (Green and Short, 2003). In the international Wadden Sea, only intertidal populations are left. These intertidal populations consist of an annual smaller-leaved morphotype that occurs in the intertidal from +30 cm to -75 cm mean seawater level (MSL) (Van Katwijk et al., 2000). Because of the annual character of this morphotype, successful seedling establishment is essential for the persistence of these populations. Seed-based restoration trials have been ongoing in the Dutch Wadden Sea since 2011 (e.g., Van Katwijk et al., 2009, Govers et al., 2022). Nonetheless, seed-based restoration projects may increasingly encounter biological early-life stage bottlenecks as a result of changing coastal food webs. For instance, both crustaceans and polychaete numbers seem to be rising in soft-sediment ecosystems (Eriksson et al., 2011; Horn et al., 2021). In the Wadden Sea, similar trends have been found, where polychaete numbers have been increasing since 1989 (Beukema, 1989; Drent et al., 2017; Giesen et al., 1990; Reise, 1982). Additionally, a recent study, Zhu et al. (2016) found that the common ragworm (*Hediste diversicolor*) can act as a seed predator on the sprouting seeds of a salt marsh foundation species of cordgrass (*Spartina anglica*). However, it is yet unknown whether polychaetes, the common ragworm specifically, may also predate on eelgrass (*Zostera marina*) seeds.

We, therefore, questioned in this study how eelgrass seedling establishment is affected by 1) different ragworm biomass (0, 2, 8, g DW m⁻²); 2) the availability of an additional high-protein food source (Sanikoi® Gold Protein Plus, 52% protein) for ragworms; and 3) the size of ragworms (small: 0.0029 g DW ragworm⁻¹ and big: 0.0095 g DW ragworm⁻¹). Furthermore, we questioned 4) if ragworms may provide a bottleneck for annual seed-based eelgrass restoration experiments in the Dutch Wadden Sea. Based on preliminary observations in a mesocosm experiment (i.e., the lack of eelgrass seedling settlement success in presence of ragworms) and the study by Zhu et al. (2016), we hypothesized that sprouted seed/seedling consumption may arise with the scarcity of a more nutritious food source (Fishman and Orth, 1996) and that this would be biomass- rather than size- dependent. Because ragworms are opportunistic foragers, feeding on e.g., debris, algae, diatoms, and other organisms, we provided them with an additional protein-rich food source to prevent food deprivation which has shown in crustaceans to result in seed consumption (Darnell and Dunton, 2015; Fishman and Orth, 1996; Infantes et al., 2016; Orth et al., 2006). In addition, we expected substantial overlap in the areal extent of high ragworm biomass areas with high predicted eelgrass habitat suitability (pers. obs). To answer these questions, we conducted a mesocosm experiment where we measured 1) seedling recruitment during the experiment and 2) seedling biomass and morphology, 3) retrieval of seeds, and 4) ragworm growth after the experiment was terminated. Combining the experimental findings with a unique field survey dataset (SIBES, Netherlands Institute for Sea Research (NIOZ), Texel) on the spatial distribution of *H. diversicolor* and an eelgrass habitat suitability map (De Jong et al., 2005) enabled us to identify potential bottleneck sites for seed-based eelgrass restoration in the Dutch Wadden Sea.

2. Materials & methods

2.1. Study area

The Wadden Sea is a shallow coastal sea with a total surface of 8000 km² that stretches along the coastlines of the Netherlands, Germany, and Denmark (Wolff, 2000). The Dutch Wadden Sea covers approximately 2500 km² of this area of which around 50% consists of intertidal flats with a tidal range between 1.4 and 3.4 m (Compton et al., 2013; Wolff, 2000). Before the 1930s, the Dutch Wadden Sea contained up to 150 km² of eelgrass meadows, which were mainly subtidal eelgrass beds (De Jonge et al., 1996; Van der Heide et al., 2007). After the 1930s, eelgrass declined due to the wasting disease (*Labyrinthula macrocystis*) along the subtidal and intertidal coastal zone of the Northern Atlantic region (Short et al., 1987). Despite the recovery of most eelgrass meadows across the Northern Atlantic range, eelgrass almost completely disappeared from the Dutch Wadden Sea, probably due to the construction of a large dam (Afsluitdijk), connecting the western part to the eastern part of the Netherlands, combined with the simultaneous occurrence of the wasting disease (Giesen et al., 1990). Intertidal eelgrass and dwarf eelgrass (*Zostera noltii*) populations were less affected by the wasting disease but started to decline after the 1970s. Although the subtidal meadows have been extinct ever since, there are still some intertidal populations of *Z. marina* left with extremely low plant densities (11.8 ha, cover density < 5% (Dolch et al., 2017; Govers et al., 2022), although a new population has recently been established after repeated seed-based restoration efforts (Govers et al. unpublished data). Currently, *Z. noltii* is the most common species with varying plant densities (cover density 0–20%) in the Dutch Wadden Sea (Dolch et al., 2017).

2.2. Sediment, worm, and seed collection

Sediment (median grain size = 24 µm, >4% OM) and ragworms (*H. diversicolor*) were collected from the mudflats of Uithuizen, Groningen, the Netherlands (N 53°28'02", E 6°41'17"). Prior to the experiment, the sediment was homogenized by sieving (1 mm) and stored for

9 days prior to the start of the experiment. The ragworms were collected two months before starting the experiment and acclimatized to lab conditions in an indoor mesocosm (120 L sediment, 120 L water, 30 ppt seawater, 15 °C, continuously aerated). These ragworms were fed twice per week with high-protein food pellets (Sanikoi® Gold Protein Plus) to prevent food deprivation. Seagrass seeds were collected in September in the year preceding the experiment on the tidal flats of Hamburger Hallig, Germany (N 54°35'56.2", E 8°48'44.0"). Seeds were then processed and stored under controlled conditions and continuously treated with a 0.2 ppm copper sulphate solution to reduce *Phytophthora* spp. infection (for detailed descriptions, see (Govers et al., 2022, 2017, 2016). Germination data of in vitro germination tests conducted by our project partner (the Fieldwork Company) showed that this batch had in general a low germination rate (only ~20.8% germination). For this experiment, we used 1350 seeds, pre-treated with demineralized water (0 ppt) for 24 h prior to planting, to stimulate germination (Xu et al., 2016).

2.3. Experimental design

To test the effects of ragworms and their feeding preference on eelgrass seedling establishment, we performed a climate-controlled mesocosm experiment at the University of Groningen, the Netherlands (N 53°14'28.7", E 6°32'17.7"). The environmental conditions were set at a constant temperature of 20 °C and a day-night cycle (light: 440 $\mu\text{mol m}^{-2} \text{s}^{-1}$) of 14/10 h. We used cylindrical mesocosms made of transparent Perspex columns of 0.0113 m^2 , 0.4 m high and with a diameter of 12 cm. Every mesocosm was filled up with a 10 cm sediment layer (1.13 L) and topped with 10 cm artificial seawater (30 ppt, Tropic Marin®). The mesocosms were randomly placed in containers that discarded overflow water back to a central basin. After the mesocosms were left to settle for three days, 45 pre-treated seeds were planted at 0.5 cm depth in every mesocosm (~4000 seeds m^{-2}). Six days after planting, ragworms (25–30 mm) were added to the mesocosms creating three ragworm biomass treatments: 0, 2, and 8 g DW m^{-2} . Ragworms were weighed (g WW) and assigned to 2 and 8 g DW m^{-2} ragworm biomass based on a wet weight to dry weight conversion ($\text{DW} = 0.10 \cdot \text{WW}$, Galasso et al., 2018; Gogina et al., 2022). This resulted in adding to a total ragworm weight of 0.25 ± 0.01 g WW and 0.89 ± 0.01 g WW (\pm SE) mesocosm $^{-1}$ (~3 and 16 ragworms per unit, resulting in 265 and 1416 worms m^{-2} , for 2 and 8 g DW m^{-2} respectively). In addition, we applied an 'additional food' treatment with two treatment levels (control vs. additional food). Ragworms in the 2 and 8 g DW m^{-2} treatment were offered 2 and 8 food pellets (0.005 ± 0.0001 g DW food $^{-1}$ mesocosm $^{-1}$ day $^{-1}$ (\pm SE)) twice a week (Sanikoi® Gold Protein Plus) to assure that ragworms received abundant food in the additional food treatments. As a control for the 'additional food' treatment, the no ragworm treatment (0 g DW m^{-2}) received 1 food pellet twice a week. These pellets (52% proteins, 8% fat, and 0.018 g DW pellet $^{-1}$) are considered a high-protein food source in contrast to eelgrass seeds (13.2% protein, 50.9% starch, Fishman and Orth, 1996), 0.0032 g DW seed $^{-1}$). Biomass and food treatments were crossed in a full factorial design resulting in 6 treatments with 5 replicates each.

In addition, two extra treatments were added (2 g DW m^{-2} small ragworms with and without additional food addition, ~ 8 ragworms per unit, resulting in 708 worms m^{-2}) to test the effect of ragworm size on eelgrass seedling establishment. For this, we added an additional 2 g DW m^{-2} ragworm biomass treatment with smaller ragworms (0.029 ± 0.0005 g WW ragworm $^{-1}$ vs. 0.088 ± 0.003 g WW ragworm $^{-1}$ \pm SE in the aforementioned treatments). All other experimental conditions were similar to the other treatments. This resulted in a total of 8 treatment combinations with 5 replicates each (0 g ragworms/no food, 0 g ragworms/+food, 2 g ragworms/no food, 2 g ragworms/+food, 2 g small ragworms/no food, 2 g small ragworms/+food, 8 g ragworms/no food, 8 g ragworms/+food).

During the experiment, microphytobenthos growth formed biofilms

on top of the sediment in the 0 g DW m^{-2} treatment. On day 34, these biofilms were fragmentarily removed by very carefully scraping the top of the biofilm to prevent suffocation of seedlings. The sediment was left undisturbed by biofilm removal. The experiment was conducted between the 22nd of February and the 24th of April 2019 and ran for 57 days.

2.4. Experimental measurements

We visually observed the total amount of germinated seeds in each mesocosm twice a week. The final number of recruited seedlings per mesocosm, seedling morphology and seedling biomass was measured after the experiment was terminated (57 days). Seedling morphology was measured as the maximum leaf length and the maximum root length (mm). The dry weight of aboveground (leaves) and belowground (roots) biomass (g) of each seedling was determined after drying seedlings at 60 °C for 48 h. After seedling collection, living ragworms were sieved (1 mm) back from the mesocosms to measure their number and the total wet weight biomass (g) of the ragworms per mesocosm. In addition, as many seagrass seeds as possible were retrieved from the sediment by sieving the sediment while sieving for ragworms. Upon retrieval, seeds were counted per experimental unit. Ragworm dry weight was measured after drying the ragworms at 60 °C for 48 h.

2.5. Seagrass-ragworm bottleneck map

To study where ragworm biomass could potentially impede seed-based eelgrass restoration trials in the Dutch Wadden Sea, we combined an existing habitat suitability map for intertidal seagrass in the Dutch Wadden Sea (De Jong et al., 2005) with an existing long-term (2008–2014 and 2019) dataset of the NIOZ synoptic intertidal benthic survey (SIBES, (Bijleveld et al., 2012; Compton et al., 2013)) in QGIS version 3.10.3 (QGIS Development Team, 2020). The SIBES field survey is a ~ 4800 benthos core sampling grid design that is sampled yearly on a 500 m resolution in the entire intertidal Dutch Wadden Sea (Bijleveld et al., 2012; Compton et al., 2013). In summer, samples of the total macrofauna were collected at the sampling sites either by foot, sampling a single core of 0.0177 m^2 to a depth of ~25 cm, or by a small boat (when the site was too deep), sampling two cores of 0.0177 m^2 to a depth of ~25 cm, during low tides (Compton et al., 2013). The cores were sieved (1 mm) for macrofauna, and subsequently identified at a species level. Next, macrofauna dry weight (g DW m^{-2}) per sampling site was determined by drying the samples at 60 °C for 48 h (Compton et al., 2013). This unique total macrofauna dataset has both a fine-scale temporal (yearly) and temporal (500 \times 500 m) resolution which allowed us to select for and look at the full spatial distribution of our study species, *H. diversicolor*. Rather than interpolating, whereby values of ragworm biomass are calculated for areas which might not be suitable for them to occur, we have converted the sample site data (biomass g DW m^{-2}) to the same spatial resolution of the sampling grid across all available sampling years (2008–2014 and 2019). For this, we averaged the biomass of all available sample years to assemble an *H. diversicolor* distribution map which covers all intertidal mudflats of the Dutch Wadden Sea in QGIS (Fig. S2). This method did not account for small-scale spatial heterogeneity that may not be picked up by the spatial resolution of the sampling design. The seagrass habitat suitability map (50 \times 50 m resolution) by De Jong et al. (2005) displays areas suitable for seagrass establishment and uses four general predictors for intertidal seagrass habitat suitability: tidal exposure, hydrodynamics, salinity, and ammonium concentrations (in interaction with salinity) (De Jong et al., 2005). Hence, these predictors have been modelled on a different spatial scale which varied between 50 m (tidal exposure and hydrodynamics) and 100 m (ammonium concentrations) but has been converted into a 50 \times 50 m resolution. When the habitat suitability and *H. diversicolor* distribution maps were combined, the overlap between high seagrass habitat suitability ($\geq 50\%$) and high ragworm biomass (≥ 2 g DW m^{-2})

was analysed with the *r.series* tool from the GRASS GIS package version 7.8.3 (GRASS Development Team, 2020) in QGIS on a 50×50 m spatial scale.

2.6. Statistical analysis

Visualization of the data is represented by the mean \pm SE and was performed together with statistical analyses in R version 3.5.3 (R Core Team, 2020).

2.6.1. Seedling establishment and morphology

Seedling recruitment (seedling m^{-2}) has been analysed with the univariate repeated measures ANOVA design to test for the effect of ragworm biomass and additional food and its interaction with time on seedling recruitment using the “car” package v3.1–0 (Fox and Weisberg, 2019). The ragworm biomass and additional food treatments were implemented as independent variables, seedling recruitment (seedling m^{-2}) as the dependent variable and time as a repeated measure. Due to non-normality, a square-root transformation was used. Unfortunately, seedling morphology could not be statistically analysed due to the low number of surviving seedlings with limited leaf length and maximum root length in the 2 g DW m^{-2} and 8 g DW m^{-2} ragworm biomass and additional food treatment (Fig. S1).

Retrieved seeds, as a proxy for predation, were analysed using a generalized linear model (GLM) with a Poisson distribution to test with a likelihood ratio test for differences in retrieved seeds between the ragworm treatments.

2.6.2. Ragworm growth

Survival of ragworms was analysed using a Mann-Whitney *U* test for non-normal data ($W_{2 \text{ g DW}} = 47$; $p\text{-value}_{2 \text{ g DW}} = 0.840$; $W_{8 \text{ g DW}} = 60$; $p\text{-value}_{8 \text{ g DW}} = 0.470$). Ragworm growth (g DW $m^{-2} d^{-1}$) was measured as biomass increase per individual per treatment per day. To calculate the individual growth, the start and final biomass (g WW) were converted to dry weight biomass (g DW) and divided by the number of ragworms per treatment. Subsequently, the final individual biomass (g DW ragworm $^{-1}$) was subtracted from the individual start biomass (g DW ragworm $^{-1}$) and was divided by the number of experimental days. The effect of additional food on ragworm growth was analysed using a linear model (LM) after log transformation due to non-normality.

2.6.3. Ragworm size effect

Survival of the small 2 g DW m^{-2} ragworms was tested with a Mann-Whitney *U* test for non-normal data. The ragworm size effect of 2 g DW m^{-2} big and small ragworms on seedling establishment was analysed using an LM with seedling recruitment as the response variable and ragworm size as the dependent variable. The effect of ragworm size was tested with a one-way ANOVA. Due to non-normality, the model was log-transformed.

3. Results

3.1. Seedling establishment

Eelgrass seedling recruitment was significantly hampered ($F_{(2,24)} = 147.079$, $p\text{-value} < 0.001$) when ragworms were present (Fig. 1A). In the absence of ragworms (0 g DW m^{-2}), a mean of 364 ± 16 seedlings m^{-2} established during the experiment ($n = 57$ days). A ragworm biomass of 2 g DW m^{-2} resulted in 26 times lower seedling recruitment (9 ± 9 seedlings m^{-2}), whereas a ragworm biomass of 8 g DW m^{-2} completely impeded seedling establishment over time ($F_{(30,360)} = 26.18$, $p\text{-value} < 0.001$; Fig. 1).

Surprisingly, additional food provisioning (+food treatment) did not improve seedling establishment in the ragworm treatments ($F_{(1,24)} = 0.237$, $p\text{-value} = 0.631$). Thus, ragworms completely hampered seedling establishment in the 8 g DW m^{-2} treatment regardless of the presence of

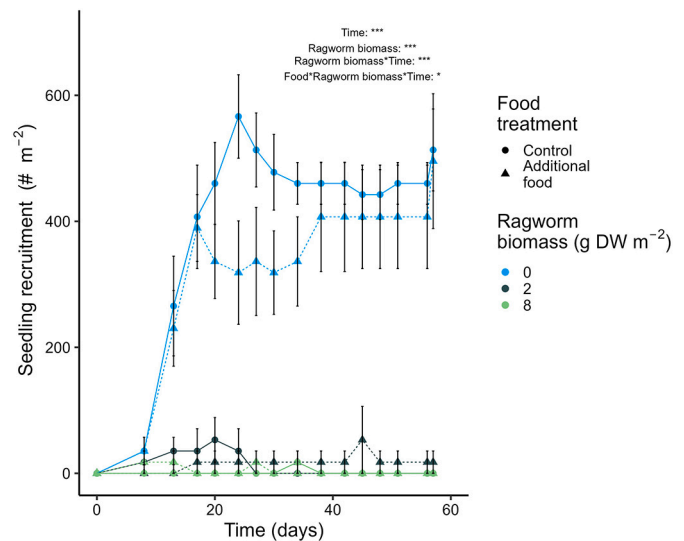


Fig. 1. Eelgrass seedling establishment. Seedling recruitment in different treatments during the experiment ($n = 57$ days). Stars indicate significant treatment effects ($* 0.05 > p > 0.01$, $** 0.01 > p > 0.001$, $*** p < 0.001$) after square root transformation. Error bars represent the standard error.

additional food (Fig. 1).

Furthermore, we retrieved an average of $47.2 \pm 2.4\%$ (\pm SE) of the seeds that were planted at the start of the experiment. Interestingly, the number of retrieved seeds was 1.4 times higher in the ragworm 8 g DW m^{-2} ragworm treatment ($52.7 \pm 2.3\% \pm$ SE) than in the control treatment ($38.2 \pm 4.2\%$; $\chi^2_{(2)} = 7.82$, $p\text{-value} = 0.0201$). The 2 g DW m^{-2} treatment seed retrieval did not differ significantly from the other treatments (Table S1).

3.2. Ragworm growth

In general, ragworm biomass increased on average 8.7 times during the experiment ($F_{(1,16)} = 126.56$, $p\text{-value} < 0.001$; Fig. 2A). Food addition ($F_{(1,16)} = 190.35$, $p\text{-value} < 0.001$) even led to an increase of approximately 10 times in the final ragworm biomass in both the 2 g DW m^{-2} (control = 17.4 ± 0.8 g DW m^{-2} ; additional food = 28.1 ± 2.0 g DW m^{-2}) and the 8 g DW m^{-2} (control = 22.2 ± 2.0 g DW m^{-2} ; additional food = 75.3 ± 3.6 g DW m^{-2} ; Fig. 2A). When looking at the individual ragworm growth (g DW d^{-1}), ragworms in the 2 g DW m^{-2} treatment without food addition ($F_{(1,16)} = 51.42$, $p\text{-value} < 0.001$) grew 4.7 times faster (0.0011 ± 0.000015 g DW d^{-1}) than the ragworms in the 8 g DW m^{-2} treatment without additional food (0.00023 ± 0.000028 g DW d^{-1} ; Fig. 2B). In contrast, ragworms in the food addition treatments had similar growth rates in both biomass treatments (0.0015 ± 0.000095 g DW d^{-1} and 0.0010 ± 0.000017 g DW d^{-1} for 2 and 8 g DW m^{-2} respectively). This indicates that food addition relieved biomass-dependent food competition between ragworms in the 8 g DW m^{-2} treatment (Fig. 2B).

3.3. Ragworm size effect

The additional ragworm size treatments showed that the negative effects of ragworm abundance on eelgrass seedling establishment are size- rather than abundance-dependent (Fig. 3). In the 2 g DW m^{-2} small ragworm treatment (0.0029 ± 0.0002 g DW ragworm $^{-1}$), 22 times more seedlings established (195 ± 8 seedlings m^{-2}) than in the 2 g DW m^{-2} big ragworm (0.0095 ± 0.001 g DW ragworm $^{-1}$) treatment (9 ± 9 seedlings m^{-2}) ($F_{(1,17)} = 10.62$, $p\text{-value} = 0.0046$; Fig. 3A, B). In total, an individual ragworm biomass > 0.0029 g DW ragworm $^{-1}$ seemed to impede seedling establishment almost completely (Fig. 3A).

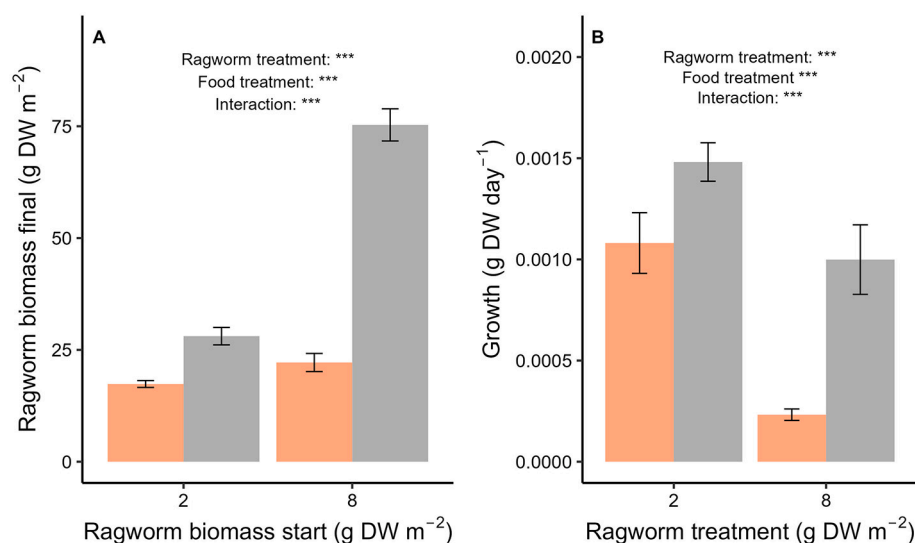


Fig. 2. Ragworm growth A) as the final ragworm biomass (g DW) per treatment at the end of the experiment and B) growth per individual ragworm per treatment per day (g DW day⁻¹). Colours represent the food treatment; salmon is control and grey is additional food. Stars indicate a significant effect of the treatments (* 0.05 > p > 0.01, ** 0.01 > p > 0.001, ***p < 0.001). Growth per individual ragworm was log transformed. The interaction is defined as the interaction between the ragworm biomass treatment and the additional food treatment. Error bars represent the standard error (n_{treatment} = 5).

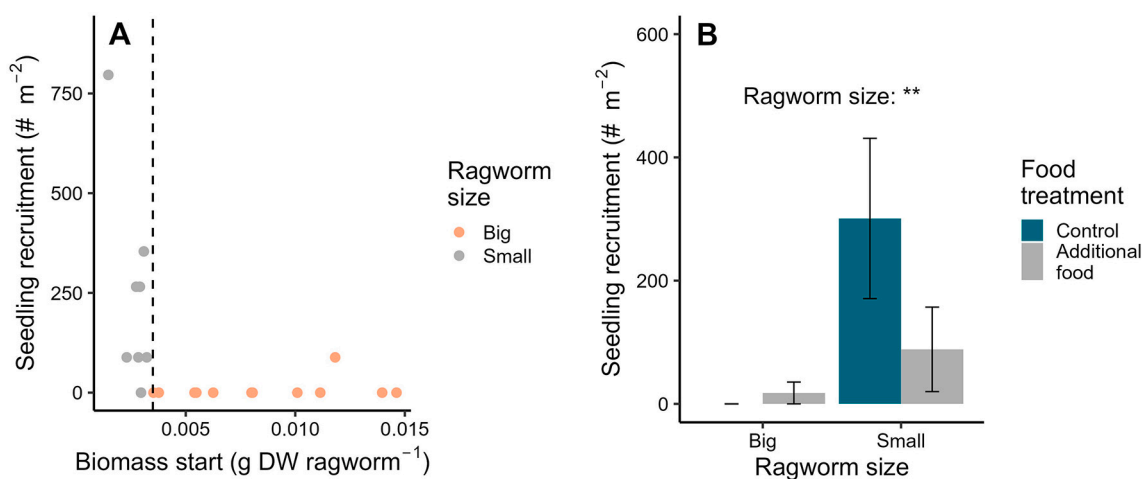


Fig. 3. Ragworm size effect on final seedling recruitment per A) individual ragworm size (g DW ragworm⁻¹), B) 2 g DW m⁻² big and small ragworm treatment with food treatment. Grey dots are the small ragworm 2 g DW m⁻² treatment and salmon dots are the big ragworm 2 g DW m⁻² treatment. The colours of the bars represent the food treatment; salmon is the control and grey is the additional food. Seedling recruitment in the 2 g DW m⁻² big and small ragworm treatment was transformed using a square root transformation. Stars indicate a significant effect of the treatments (* 0.05 > p > 0.01, ** 0.01 > p > 0.001, ***p < 0.001). Error bars represent the standard error (a) n_{treatment} = 10 and b, c) n_{treatment} = 5).

3.4. Seagrass-ragworm bottleneck map

52.8% of the total area indicated as suitable for seagrass establishment (determined as a suitability score of $\geq 50\%$) by the seagrass habitat suitability map of De Jong et al. (2005) may be sensitive to a potential seedling establishment bottleneck based on mean high ragworm biomass. Locations where seagrass restoration seems promising (habitat suitability $\geq 50\%$), but where the ragworm biomass is high (≥ 2 g DW m⁻²) were mostly found at sites (pink squares; Fig. 4) at the eastern coast of Terschelling (N 53°24'40", E 5°19'53") and Balgzand (N 52°53'55", E 4°50'44") in the western part of the Dutch Wadden Sea (Fig. 4A). Additionally, in the eastern Dutch Wadden Sea, sites on the east coast of Schiermonnikoog (N 53°29'21", E 6°12'80"), on Rottumerplaat (N 53°32'30", E 6°28'51") and Rottumeroog (N 53°32'25", E 6°34'55") might be less suitable for eelgrass restoration due to an average high ragworm biomass in the sediment (Fig. 4B). In addition, local eelgrass populations that are still present in the indicated areas (e.g., Rottumerplaat) are all annuals, which makes them sensitive to the ragworm bottleneck that we uncovered in this study.

4. Discussion

Seagrass seedling recruitment is sensitive to specific early-life stage bottlenecks (Statton et al., 2017). We showed that the common ragworm (*Hediste diversicolor*) may impede eelgrass (*Z. marina*) seedling establishment by consuming sprouted eelgrass seeds regardless of being offered an alternative, protein-rich food source. We thus showed that polychaetes may not only hamper seagrass establishment by burial (Suykerbuyk et al., 2016, 2012; Zipperle, 2012), but also by seed predation. A translation of these results to the field indicated that 52.8% of the potential seagrass growth sites in the Dutch Wadden Sea (Rottums, eastern Schiermonnikoog, eastern Terschelling and Balgzand) overlap with high ragworm biomass (≥ 2 g DW m⁻²). Therefore, it is clear that ragworm abundance should be taken into account when assessing habitat suitability for seed-based eelgrass restoration.

4.1. Early life-stage bottlenecks

Seed predation by crustaceans, resulting in a reduction in seedling growth and survival, is a well-studied early life stage bottleneck for

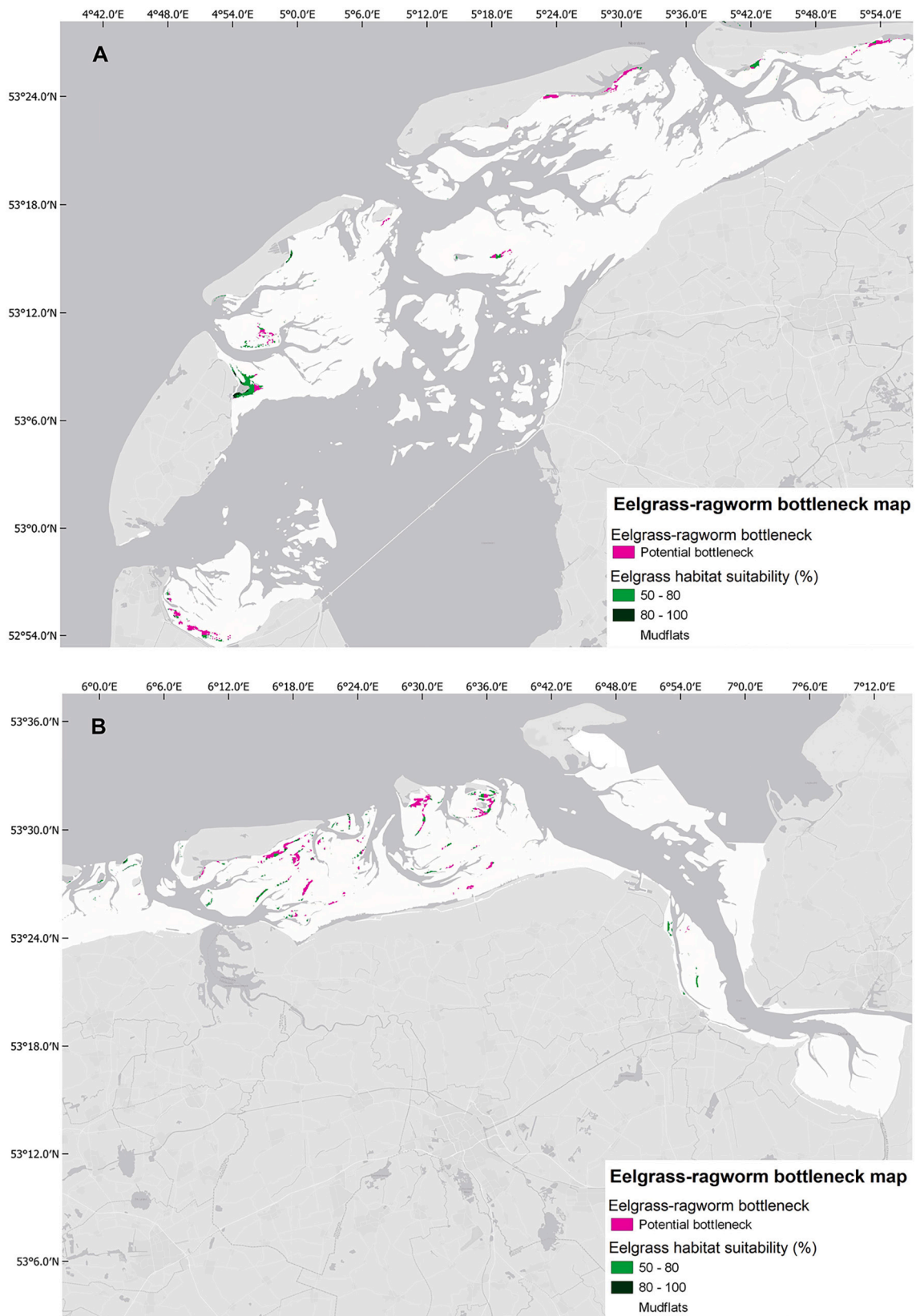


Fig. 4. Eelgrass-ragworm bottleneck map shows sites in the A) Western and B) Eastern Dutch Wadden Sea (pink) where high ragworm biomass ($\geq 2 \text{ g DW m}^{-2}$) overlap with sites with high eelgrass habitat suitability ($\geq 50\%$), forming a potential bottleneck for annual eelgrass recruitment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seagrasses (Darnell and Dunton, 2015; Infantes et al., 2016). However, it is yet unknown whether other species groups, such as polychaetes may also act as seed predators. The results of our experiment suggest that ragworms are very likely unable to consume unsprouted eelgrass seeds, probably due to their sturdy seed coat (Paulsen et al., 2014), as indicated by the high retrieval of intact seeds ($\sim 50\%$) in the 2 and 8 g DW ragworms m^{-2} , the treatments where seedling establishment was completely impeded (Table S1). We conclude this based on an equal number of retrieved seeds in the ragworm treatments and the no ragworm treatments (0 g DW m^{-2}). If we assume that seed germination was similar among all treatments ($\sim 20\%$ based on germination tests in the lab) and that we lost similar amounts of seeds due to sieving or degradation in all treatments (est. $\sim 30\text{--}40\%$; Table S1), the equal/higher amounts of retrieved seeds indicate that these were very likely not accessible to ragworms as a food source. We think that this argument is additionally supported by the voracious feeding behaviour of the ragworms that has been shown in the treatments where an alternative, better nutritional quality food source was provided. This additional/alternative food source did nothing to prevent the complete impediment of seedling establishment. We, therefore, think it is likely that ragworms would have consumed eelgrass seeds if they'd been available to them as a food source. However, the equal/lower amount of retrieved seeds in the no-ragworm control treatment indicates that it is very likely that ragworms preyed upon sprouted seeds instead. This argument is also supported by similar behaviour observed for ragworms feeding on sprouting *Spartina anglica* seeds (Zhu et al., 2016). The same study also showed that ragworm seed handling did not promote the decay of those seeds (Zhu et al., 2016).

4.2. Size dependency

Next to the windows of opportunity created for the establishment of eelgrass by low ragworm biomass, the size of the ragworms might also create these opportunities. Our results show that seedling consumption by ragworms was size-dependent because seedlings did establish in the presence of smaller ragworms, even when present in high numbers. This suggests that small ragworms either require less food or might not be able to collect and consume seeds when they start to sprout. How exactly the ragworms consume the seeds remains unclear because we weren't able to establish their exact behaviour within their burrows. Ragworms are omnivorous but food preference seems to be size-specific: small (juvenile) ragworms are known to obtain plant detritus from the sediment's surface and collect it in their burrow, whereas big (adult) ragworms are able to build a food pellet near the burrow and progressively consume it (Scaps, 2002). Juveniles might benefit more from an algal diet in terms of growth than from a vascular marine plant (eelgrass) diet because the assimilation efficiency and the digestion rate are higher in algal species compared to marine vascular plants (Olivier et al., 1997). Nonetheless, over time, common ragworms may form a predation bottleneck for seagrass species that mostly rely on sexual reproduction for their survival, colonization, and recovery (Zipperle, 2012).

4.3. Effects of additional food

Ragworms can display a variety of feeding strategies to obtain food: filter-feeding, scavenging, predation and deposit-feeding (Scaps, 2002). However, irrespectively of the presence of the additional nutritious bigger (0.018 g DW pellet $^{-1}$) food source, ragworms consumed the sprouted smaller eelgrass seeds (0.0032 g DW seed $^{-1}$). It seems unlikely that the additional food source (pellets) was too big for ragworm consumption and might not have been eaten immediately because these food pellets soften in contact with water and were almost immediately pulled into their burrows when presented (supplementary video). Additionally, the difference in growth between the ragworms who received the additional food and the ones who didn't (+0.00058 g DW d^{-1}) also indicated that they were able to consume the additional food.

Based on the number of retrieved seeds and the study of Zhu et al. (2016) together with the low general germination rate (20.8%), we suggest that all the 45 sowed seeds are not (immediately) available for ragworm consumption due to the intact seed coat but become available for consumption when the seeds start to germinate over time. Therefore, it is very likely that sprouted eelgrass seeds will be consumed when encountered by ragworms, regardless of their size and nutritional situation. Furthermore, the additional food relieved biomass-dependent food competition between ragworms, promoting growth in the high 8 g DW m^{-2} ragworm + food treatment, and potentially also promoting more seed consumption (Fig. 2). This reduced growth due to biomass-dependent competition is supported by other studies investigating intra-specific competition in polychaetes (Nesto et al., 2012; Scaps, 2002). Despite that additional food relieved biomass-dependent food competition in the high ragworm biomass treatment, sprouted seeds were still consumed in all treatments.

4.4. Case study: The Dutch Wadden Sea

Our controlled mesocosm experiments have shown that ragworms may strongly impede eelgrass seedling establishment. This evidence is compelling and suggests that this may also be the case in field situations, which in turn may have potentially negative consequences for seed-based (annual) eelgrass restoration projects. In the Wadden Sea, seed-based eelgrass restoration projects have been conducted since 2011 (Van Duren and Van Katwijk, 2015; Govers et al., 2022; Gräfnings et al. n.d.). On three locations (Balgzand, Schiermonnikoog, Uithuizen) that have previously been identified as successful by a habitat suitability map (Fig. S4, De Jong et al., 2005), eelgrass recruitment was very low (< 0.01 plant m^{-2}) and seed-based seeding trials failed to form self-sustainable populations (Van Duren and Van Katwijk, 2015; Govers et al., 2022; Gräfnings et al. n.d.). This may have been the result of recruitment bottlenecks such as strong hydrodynamics, sediment instability or *Phytophthora* spp. infection (Govers et al., 2016; Statton et al., 2017). However high ragworm biomass (≥ 2 g DW m^{-2}) at these sites may also have contributed to the lack of seed-based eelgrass restoration successes on those sites (Fig. S2 and Fig. 4). Additionally, a small, recently established eelgrass population on the tidal flats of Rottumerplaat has been observed to decrease in recent years (*pers. obs.*) despite the high eelgrass habitat suitability (50–80%) around Rottumerplaat as indicated by the map of De Jong et al. (2005) (Fig. S4). According to the eelgrass-bottleneck map (Fig. 4B), there is high ragworm biomass (≥ 2 g DW m^{-2}) at this location, which may strongly hamper population persistence and survival as indicated by our experiment. Hence, we have taken this number (≥ 2 g DW ragworms m^{-2}) as a cut-off point to determine possible bottlenecks for eelgrass seedling establishment in the field. We consider this a conservative estimate since the bottleneck very likely already occurs with lower ragworm biomass. In addition, many studies have shown that polychaetes (Suykerbuyk et al., 2012; Valdemarsen et al., 2011) may be a bottleneck to seagrass restoration. Based on our findings, and the fact that we have observed low eelgrass seedling emergence in high ragworm biomass areas in the field, we do suggest we can make this extrapolation to the field.

The biomass of the common ragworms is found to differ yearly in the Dutch Wadden Sea (Fig.S3). This natural stochasticity in ragworm biomass might be due to interspecific competition for space and food (e. g., with *Alitta virens* and *Alitta succinea*) and environmental factors such as salinity and sediment sulphide concentrations (Kristensen, 1988; Miron and Kristensen, 1993). This natural stochasticity in worm biomass may create windows of opportunity for annual eelgrass establishment in years with low ragworm densities (Balke et al., 2014; De Jong et al., 2005; Giesen et al., 1990). However, we could not take this interannual variability in ragworm biomass (g DW m^{-2}) in the Dutch Wadden Sea into account when creating our eelgrass-ragworm bottleneck map (Fig. 4). In addition, seed predation by common ragworms might not be the only bottleneck influencing population development of eelgrass at

these locations. Despite these windows of opportunity, the density of eelgrass in the Dutch Wadden see may also be too low ($<1 \text{ plant m}^{-2}$) to produce enough seeds for natural subsistence or population recovery (Van Duren and Van Katwijk, 2015; Dolch et al., 2017; Govers et al., 2022; Gräfnings et al. n.d.). Since the 2000s, the Dutch intertidal eelgrass population (*Z. marina* and *Z. noltii*) has stabilized between 220 and 290 ha of which only 11.4 ha consists of 5% - 20% eelgrass cover beds (Dolch et al., 2017), and the majority of eelgrass growth sites exhibit a plant density with a scarce density coverage $<1\%$.

Both eelgrass habitat suitability maps do not differentiate between intertidal eelgrass and dwarf eelgrass (De Jong et al., 2005; Folmer, 2019). This means that areas suitable for eelgrass may in reality be unsuitable for dwarf eelgrass and vice versa. For instance, intertidal eelgrass is less tolerant to high tidal exposure (+30 cm MSL to -75 cm MSL) than dwarf eelgrass (+100 cm MSL to -75 cm MSL) and therefore, dwarf eelgrass generally occurs higher in the intertidal zone (Dolch et al., 2017; van Katwijk et al., 2000; Wijgergangs and De Jong, 1999; Zipperle, 2012). Therefore, including intertidal exposure would reduce indicated suitable areas for intertidal eelgrass (De Jong et al., 2005). Additionally, dwarf eelgrass may be less vulnerable to seed predation than eelgrass because it mostly relies on vegetative expansion for colonization and recovery (Zipperle, 2012). Since both species differ in sensitivity to certain growth requirements (e.g., tidal exposure) and life-stage bottlenecks, we suggest that future eelgrass suitability maps should differentiate between both species and include interactions between seagrasses and associated invertebrates that may affect restoration efforts.

In conclusion, we found that high ragworms (*H. diversicolor*) densities may present an early-life stage bottleneck for seedling settlement of eelgrass (*Z. marina*), due to the high likelihood of the consumption of sprouted seeds. However, due to this polychaete species' cryptic burrow-dwelling nature, more research on their predatory behaviour is needed to provide more insight into the behavioural mechanism behind the consumption of sprouting seeds. Nonetheless, this early-life stage bottleneck may present real challenges for seed-based restoration of annual intertidal eelgrass that entirely depends on seeds for successful settlement and population expansion. Our study indicates that it is important for seagrass restoration perspectives to identify biotic bottlenecks for seagrass settlement and population expansion.

Author contributions

DJJH and MZ provided preliminary data for the experiment; LLG designed and funded the study; DJJH aided in the set-up of the experiment; CK performed and analysed the experiment; AIB and SH collected and provided the SIBES data; LLG and CK drafted the manuscript; all authors approved of the final version of the manuscript.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jembe.2022.151853>.

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