Salinity and temperature significantly influence seed germination, seedling establishment, and seedling growth of eelgrass *Zostera marina* L.

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**ABSTRACT**

Globally, seagrass beds have been recognized as critical yet declining coastal habitats. To mitigate seagrass losses, seagrass restorations have been conducted in worldwide over the past two decades. Seed utilization is considered to be an important approach in seagrass restoration efforts. In this study, we investigated the effects of salinity and temperature on seed germination, seedling establishment, and seedling growth of eelgrass *Zostera marina* L. (Swan Lake, northern China). We initially tested the effects of salinity (0, 5, 10, 15, 20, 25, 30, 35, and 40 ppt) and water temperature (5, 10, 15, and 20 °C) on seed germination to identify optimal levels. To identify levels of salinity that could potentially limit survival and growth, and, consequently, the spatial distribution of seedlings in temperate estuaries, we then examined the effect of freshwater and other salinity levels (10, 20, and 30 ppt) on seedling growth and establishment to confirm suitable conditions for seedling development. Finally, we examined the effect of transferring germinated seeds from freshwater or low salinity levels (1, 5, and 15 ppt) to natural seawater (32 ppt) on seedling establishment rate (SER) at 15 °C. In our research, we found that:

1. Mature seeds had a considerably lower moisture content than immature seeds; therefore, moisture content may be a potential indicator of *Z. marina* seed maturity;
2. Seed germination significantly increased at low salinity (p < 0.001) and high temperature (p < 0.001). Salinity had a much stronger influence on seed germination than temperature. Maximum seed germination (88.67 ± 5.77%) was recorded in freshwater at 15 °C. In our research, we found that:
3. Seed germination significantly increased at low salinity (p < 0.001) and high temperature (p < 0.001). Salinity had a much stronger influence on seed germination than temperature. Maximum seed germination (88.67 ± 5.77%) was recorded in freshwater at 15 °C;
4. Freshwater and low salinity levels (< 20 ppt) increased germination but had a strong negative effect on seedling morphology (number of leaves per seedling reduced from 2 to 0, and maximum seedling leaf length reduced from 4.48 to 0 cm) and growth (seedling biomass reduced by 46.15–66.67% and maximum seedling length reduced by 21.16–69.50%). However, *Z. marina* performed almost equally well at salinities of 20 and 30 ppt. Very few germinated seeds completed leaf differentiation and seedling establishment in freshwater or at low salinity, implying that freshwater and low salinity may potentially limit the distribution of this species in coastal and estuarine waters. Therefore, the optimum salinity for *Z. marina* seedling establishment and
colonization appears to be above 20 ppt in natural beds; (4) Seeds germinated in freshwater or at low salinity levels could be transferred to natural seawater to accomplish seedling establishment and colonization. This may be the optimal method for the adoption of seed utilization in seagrass restoration. We also identified seven stages of seed germination and seedling metamorphosis in order to characterize growth and developmental characteristics. Our results may serve as useful information for Z. marina habitat establishment and restoration programs.

Subjects  Ecology, Plant Science
Keywords  Zostera marina L., Salinity, Temperature, Seed germination, Seedling establishment, Seedling growth, Seagrass

INTRODUCTION
Seagrass meadows, together with mangroves and coral reefs, are extremely valuable coastal marine ecosystems that are widely acknowledged for both their ecological and economic importance. They provide essential habitat and nurseries for a variety of marine organisms, and play critical roles in primary and secondary production, as well as coastal ecosystem nutrient cycles (Costanza et al., 1997; Hemminga & Carlos, 2000; Ogden, 2006; Orth et al., 2006a; Liu et al., 2013; Connolly & Waltham, 2015; McCloskey & Unsworth, 2015; Tol, Coles & Congdon, 2016). However, seagrass meadows are declining globally (Short et al., 2011) due to anthropogenic activities (e.g. port infrastructure development and dredging) and natural disturbances (e.g. disease and storms) (Cambridge & McComb, 1984; Short & Burdick, 1996; Wyllie-Echeverria & Ackerman, 2003; Kim et al., 2015; Macreadie et al., 2015; Zhang et al., 2014; Lin et al., 2016; Govers et al., 2016). These issues have led to the recent development of restoration efforts to compensate or mitigate seagrass losses, and to enhance the associated ecosystem services (Shafer & Bergstrom, 2010; Suykerbuyk et al., 2016).

In an effort to restore seagrass meadows on large-scales, a seagrass transplanting technique was developed to transplant mature or adult plants (Fonseca et al., 1994; Zhou et al., 2014; van Katwijk et al., 2016). However, such transplanting is highly labor- and cost-intensive, and harmful to the donor meadows, especially in a large-scale restoration project (Orth et al., 2006a; Paling et al., 2009). Given these drawbacks, large scale seed broadcasting, evolved out of a long-term trialing process since the end of the last century, could be a relative efficient and cost-effective approach to restoring seagrass habitats. Seed broadcasting is one of the most promising approaches (Harwell & Orth, 1999; Orth et al., 2003; Olesen et al., 2004; Orth, Harwell & Lnglis, 2006; Shafer & Bergstrom, 2010); it has been successfully applied to a few seagrass restoration programs (Paling et al., 2009), such as the restoration of Zostera marina habitats in the United States (Orth et al., 2006b). Unfortunately, broadcast seeds have a very low germination rate in the field (compared with transplanted adult plants) because some seeds do not germinate or may be predated on by other animals leading to seed loss (Wassenberg, 1990; Orth, Luckenbach & Moore, 1994; Fishman & Orth, 1996; Orth et al., 2009). However, broadcasting germinated seeds and transplanting artificial seedlings are both important in
seagrass restoration and could largely increase the seedling establishment rate (SER) (Wassenberg, 1990; Christensen, Sortkjaer & McGlathery, 1995; Fishman & Orth, 1996; Kirkman, 1999; Balestri, Piazzini & Cinelli, 1998; Orth et al., 2009).

Eelgrass (Z. marina) is the most widespread species throughout the temperate northern hemisphere of the Atlantic and Pacific (Green & Short, 2003; Olsen et al., 2016). Growth and physiology of adult plants have received considerable amounts of attention over the last three decades (Phillips, 1972; Phillips, McMillan & Bridges, 1983; Hemminga & Carlos, 2006; Touchette & Burkholder, 2000; Phillips, Milchakova & Alexandrov, 2006; Lee, Park & Kim, 2007; Leoni et al., 2008; Collier, Waycott & McKenzie, 2012; Dooley et al., 2013; Dooley et al., 2015; Kaldy et al., 2015); however, there is by far less information available concerning the key environmental factors, such as salinity and temperature, that influence seed germination, seedling establishment, and seedling growth (Orth & Moore, 1983; Hootsmans, Vermaat & Van Vierssen, 1987; Conacher, Poiner & O’Donohue, 1994; Abe, Kurashima & Maegawa, 2008; Tanner & Parham, 2010; Salo & Pedersen, 2014; Park, Lee & Son, 2014; Kaldy et al., 2015). Moreover, results to date have been inconsistent as to whether or not the salinity significantly effects seed germination, which might be influenced by the design of salinity levels (Orth et al., 2000). We hypothesized that salinity levels might significantly influence seed germination, seedling establishment, and seedling growth of Z. marina. In this study, we investigated the effects of salinity and temperature on seed germination, seedling establishment, and seedling growth of Z. marina. L. (Swanlake Lagoon, northern China) in order to determine optimal conditions for this species. This study intends to provide fundamental information for creating adequate seed germination conditions and developing seedling establishment methods for eelgrass habitat establishment and restoration.

MATERIALS AND METHODS

Ethic statement

The collecting of the reproductive shoots of Z. marina from Swan Lake of Weihai was permitted by Peiliang Wang, manager of Mashan Group Co. Ltd. Ethical approval was not required for this study because no endangered animals and plants were involved. However, specimen collection and maintenance were performed in strict accordance with the recommendations of China Society of Plant Protection.

Seed collection

Between July 17 and 19, 2015, reproductive shoots of Z. marina with inflorescences containing developing or developed seeds were collected by hand from Swan Lake, a marine lagoon in the northeast of Rongcheng city, northern China (37°20’58.7”N, 122°34’26.9”E). Reproductive shoots were placed in coolers and transported to lab within 24 h of collection.

In the laboratory, reproductive shoots were stored in a 600 µm mesh bag, held in a circular, aerated flow-through tank (1 × 1.2 × 1.5 m) in natural sunlight. The mesh bag, containing reproductive shoots, was stirred by hand daily and held in the tank until the shoots degenerated and the seeds were released. Floating leaf material was removed,
while seeds and plant materials were retained. Accumulated detritus was passed over a 2.5 mm sieve to remove large debris, and detritus material was separated from *Z. marina* seeds through a 1.0 mm sieve (Wyllie-Echeverria et al., 2003). Following this, all seeds were kept in high salinity artificial seawater (50 ppt) (Pan et al., 2011b) at 5 °C (Zarranz et al., 2010; Dooley, Wyllie-Echeverria & Van Volkenburgh, 2013) until initiation of our tests. With the maturation of seeds, the oyster white seeds (immature) become cyan, then brown or black (mature) (Pan et al., 2014). Only mature seeds in dark color were used in this experiment.

According to their color (oyster white, cyan, and black), seeds were classified as one of three degrees of maturity (immature, medium, and advanced maturity, respectively). To analyze the moisture content of *Z. marina* seeds at different degrees of maturity, 30 seeds were selected of each color and randomly divided to three replicates. Seeds were spread to dry on soft paper towels to remove water, and the wet weight (WW) of seeds was measured. Seeds were then placed in a drier for 72 h (60 °C), and their dry weights (DW) were measured. Moisture content percentage (MCP) of the *Z. marina* seeds was calculated using the equation:

\[
\text{MCP} = \frac{(\text{WW} - \text{DW})}{\text{DW}} \times 100\%
\]

**Seed germination rate at different salinities and temperatures**

This experiment was initiated on November 3, 2015. Seeds used in this experiment were mature and black in color. Germination of seeds was explicitly defined as, not just the rupture of the seed coat, but also the emergence and growth of the cotyledon (Churchill, 1983; Brenchley & Probert, 1998). Seeds were placed in 12 cm plastic petri dishes containing 50 ml of freshwater or artificial seawater with different salinities (5, 10, 15, 20, 25, 30, 35, and 40 ppt) at 5, 10, 15, and 20 °C for a period of four weeks. Each treatment involved three replicate petri dishes containing 50 seeds each. Germinated seeds were counted after 4 weeks. Petri dishes were placed in an incubator, and artificial seawater was changed every 3 days.

**Seedling growth and establishment at different salinities at 5 °C**

To examine the effect of salinity on seedling growth and establishment *Z. marina*, seeds and seedlings were cultured at different salinities (0, 10, 20 and 30 ppt) at 5 °C for six weeks. One hundred seeds were placed in 12 cm plastic Petri dishes containing 50 ml of freshwater or artificial seawater of different salinities (10, 20 and 30 ppt) at 5 °C, with three petri dishes per salinity treatment. Petri dishes were placed in an incubator, and artificial seawater was changed every three days, as described above. All the germinated seeds in each petri dish were transferred to each container with artificial seawater of corresponding salinity to continue culture. The seedling maximum length, weight, number of leaves, and maximum leaf length were measured in the container at the 3rd and 6th week. Any other morphological changes in *Z. marina* seeds and seedlings during and after germination were also recorded.
Seedling establishment rate for seeds germinated at reduced salinities and transferred to natural seawater (32 ppt)

This experiment was conducted to examine the effects of transferring the germinated seeds from low salinity to natural seawater on seedling establishment. Fifty seeds were placed in 12 cm plastic petri dishes containing 50 ml of diluted seawater of different salinities (0, 1, 5, and 15 ppt) at 15 °C, with three petri dishes per salinity treatment. Petri dishes were placed in an incubator, and artificial seawater was changed every three days, as described above. Germinated seeds were immediately transferred to natural seawater (32 ppt) to continue culture at 15 °C. This is because prolonged exposure to freshwater is likely to result in increased incidence of rotten seeds and poor subsequent seedling survival (Kaldy et al., 2015). After four weeks, the number of seedlings from the germinated seeds was counted, and the SER was calculated using the equation:

\[ \text{SER} = \frac{n}{N} \]  

where \( n \) is the number of seedling from germinated seeds; \( N \) is the total number of seeds used for germination.

Data analysis

A two-way analysis of variance (ANOVA) was used to compare the effects of temperature and salinity. When the interaction was significant, one-way ANOVA was used to compare the temperature effect at each salinity, and the salinity effect at each temperature (Zar, 1999). Moisture content of seeds, SER, and seedling maximum length, weight, and maximum leaf length were also analyzed by one-way ANOVA. The effect of salinity on seedling establishment was statistically analyzed by one-way ANOVA.

In the analysis, homogeneity of variance was tested using Levene’s test (Zar, 1999). When ANOVA identified a significant difference, the Tukey test was applied to identify specific treatment differences; when it has no significant difference, the Dunnett T3 test was applied to identify specific treatment differences. Analysis was based on the data collected in the experiment. R version 3.2.1 and SPSS 18.0 for Windows 8.1 were used for all data analyses. Differences were considered significant at a probability level of \( p < 0.05 \).

RESULTS

Moisture content of seeds

*Zostera marina* seeds were classified by seed color (oyster white, cyan, or black), representing the various degrees of seed maturity (immature, medium, and advanced maturity, respectively). The moisture content and WW of seeds at different degrees of maturity both exhibited a significant difference (\( F = 1,055, \text{df} = 17, p < 0.001; F = 1,253, \text{df} = 17, p < 0.001 \), respectively). As shown in Table 1, the moisture content of seeds significantly decreased with increasing maturity; in contrast, the WW of seeds significantly increased with increasing maturity. Therefore, moisture content, together with seed color and weight, may be a potential indicator of *Z. marina* seed maturity. The average
WW of *Z. marina* mature seeds selected for germination was 12.50 ± 0.04 mg, and the mean moisture content was 37.41 ± 0.74% (Table 1).

**Seed germination at different salinities and temperatures**

Germination rates differed significantly ($F = 53.713, df = 107, p < 0.001$) at different temperatures (Table 2). Germination rates also differed significantly ($F = 266.596, df = 107, p < 0.001$) between different salinities. Seed germination rates were significantly higher at higher temperatures and seed germination rates decreased with increasing salinities. Temperature and salinity had significant interactive effects on germination rates ($F = 3.634, df = 107, p < 0.001$).

Germination rates (80.67–88.67%) in freshwater were significantly higher than in artificial seawater at high salinities ($F = 82.094, df = 107, p < 0.001$). There was no significant difference in germination rate between different temperatures in freshwater ($F = 1.076, df = 11, p = 0.412$). The highest germination rate (88.67 ± 5.77%) among all treatments was recorded at 15 °C in freshwater, and ANOVA detected significant differences between the remaining treatments at 15 °C (6–69%) at high salinities. At higher salinities (30, 35, and 40 ppt), germination rates were below 16%, but increased considerably when salinity decreased below 20 ppt (Fig. 1).

Generally, germination rates increased considerably with increasing temperature. Above 10 ppt, germination rates at 15 and 20 °C were significantly higher than at 5 °C (Fig. 1).

**Seedling growth and establishment at different salinities at 5 °C**

Salinity appeared to play an important role in seedling establishment and growth (Figs. 2 and 3; Table 3). After six weeks, the highest germination rate (85.67 ± 4.93%) among all treatments was recorded in freshwater, consistent with the results of the seed germination experiment. The germination rates at lower salinities (0 and 10 ppt)
Figure 1 Germination rates (%) of *Zostera marina* L. seeds subjected to the different salinities and temperatures after four weeks. Different letters above error bars indicate significant differences at p < 0.05 (one-way analysis of variance) (mean ± standard error (SE), n = 3) (bars represent SE).
were significantly higher than at higher salinities (20 and 30 ppt) (F = 94.376, df = 11, p < 0.001). The seedling maximum length, weight, number of leaves, and differentiation of *Z. marina* differed significantly (p < 0.001) between the lower salinities and the higher salinities at 5 °C after three and six weeks. At salinities of 0 and 10 ppt, the cotyledon broke through the seed coat in the first week and the mean length of seedlings were 0.56 and 1.22 cm, respectively at three weeks; these increased to 1.72 and 3.8 cm, respectively, at six weeks. However, leaf differentiation had not occurred at 6 weeks.

![Figure 2](image_url)

**Figure 2** Seed germination, length, and weight of *Zostera marina* L. seedlings subjected to different salinities at 15 °C after three and six weeks. Different letters indicate significant differences at p < 0.05 (one-way ANOVA) (means ± SE, n = 3).
Figure 3 Morphology of Zostera marina L. seedlings after six weeks (bar represents 1 cm) at 5 °C. The germinated seeds were incubated in freshwater (A), 10 ppt (B), 20 ppt (C), and 30 ppt (D). (A) Germinated seeds with a cotyledon exceeding 1 cm; (B) Cotyledon exceeds 3 cm; (C) Leaf and adventitious root differentiation with the leaf length exceeding 4 cm; (D) Leaves exceed 5 cm in length.

Table 3 Ecological morphology of Zostera marina L. seeds and seedlings.

| Time   | Ecological morphology                          | Salinity 0 ppt | Salinity 10 ppt | Salinity 20 ppt | Salinity 30 ppt |
|--------|-----------------------------------------------|----------------|----------------|----------------|----------------|
| 1st week | Germination performance with rate (%)          | Day 1          | First appearance of germination | First appearance of germination | — | — |
|        |                                                | Day 2          | 2.33 ± 0.58   | 0.67 ± 0.58   | First appearance of germination | — |
|        |                                                | Day 4          | 11.67 ± 0.58  | 2.00 ± 1.00   | 0.67 ± 0.58   | First appearance of germination |
|        | Other morphological changes                    | Whole embryo breaks through the seed coat all at once     | Cotyledon breaks through the stoma and then the embryo breaks through the seed coat |
| 3rd week | Germination rate (%)                          | 81.67 ± 10.21a | 72.67 ± 6.35ac | 25.67 ± 5.86bc | 9.67 ± 4.04c  |
|        | Seedling length (cm)                          | 0.56 ± 0.21a   | 1.22 ± 0.33ac | 2.54 ± 0.34b  | 3.32 ± 0.28b  |
|        | Number of leaves/seedling                     | No leaf        | No leaf        | First true leaf appears; the leaf is shorter than the cotyledonal blade |
|        | Other morphological changes                    | Top of cotyledonal blade becomes brown | Cotyledonal blade becomes longer | Occurrence of adventitious root differentiation |
| 6th week | Germination rate (%)                          | 85.67 ± 4.93a  | 74.67 ± 7.23bc | 32.33 ± 4.73bc | 11.33 ± 4.51c |
|        | Seedling length (cm)                          | 1.72 ± 0.48a   | 3.80 ± 1.10bc | 4.82 ± 0.56bc | 5.64 ± 0.96c  |
|        | Seedling wet weight/shoot (mg)                | 8.5 ± 0.5a     | 10.5 ± 2.5a   | 19.5 ± 2.0bc  | 25.5 ± 3.5c   |
|        | Number of leaves/seedling                     | 0 (No leaf)    | 0 (No leaf)   | Second true leaf was observed; the cotyledonal blade is shorter than the leaves |
|        | Leaf length (cm)                              | 0a             | 0a            | 2.34 ± 0.12bc | 4.48 ± 0.34c  |
|        | Other morphological changes                    | The brown part decays continually | Occurrence of leaf differentiation | The cotyledonal blade wizens; the leaves elongates |

Note: Different letters indicate significant differences at p < 0.05 (mean ± standard error).
At 20 ppt and 30 ppt, the cotyledon broke through the seed coat in the first week and after
three weeks the seedlings elongated to 2.54 and 3.32 cm in length, respectively, and
4.82 and 5.64 cm, respectively, after six weeks. Leaf and adventitious root differentiation
occurred at week 6. The highest weight of seedling (25.5 ± 3.5 mg) among all treatments
had reached at 30 ppt after six weeks, and ANOVA detected significant differences
between the remaining treatments at 5°C. In the low salinities (0 and 10 ppt), a
significantly higher seed germination rate was detected; however, these seedlings had a
lower number of leaves, shorter seedling length, and lower WW. This suggested that
*Z. marina* seedlings exposed to low salinities exhibited slower development that may
reduce their eventual survival over longer exposure times than those tested in the present
study. In contrast, the seedling survival rate was greater at higher salinities.

Seedling establishment rate for seeds germinated at reduced salinities and transferred to natural seawater (32 ppt)
The seedling establishment differed significantly (F = 143.089, df = 11, p < 0.001)
among salinities ([Table 4; Fig. 4]). Salinity had a large influence on seedling establishment.
The SER for seeds germinated at 0, 1, 5, and 15 ppt at 15°C and then transferred to
natural seawater (32 ppt) at 15°C, were 10.00 ± 0.00, 28.67 ± 1.15, 39.33 ± 4.16,
and 90.67 ± 9.02%, respectively. The SER at 15 ppt was significantly higher than the other

**Table 4** One-way analysis of variance of salinity on the seedling establishment rate of *Zostera marina* L. seeds.

| Variable     | Df | Sum Sq | Mean Sq | F value | P (> F) |
|--------------|----|--------|---------|---------|---------|
| Salinity     | 3  | 2,682.92 | 894.306 | 143.089 | < 0.001 |
| Residuals    | 8  | 50     | 6.25    |         |         |

**Note:**
Data are not transformed and assumption of homogeneity of variance is met by Levene’s test (P = 0.069).

![Figure 4 Seedling establishment rate for *Zostera marina* L. seeds germinated at reduced salinities and transferred to natural seawater (32 ppt). Different letters indicate significant differences at p < 0.05 (mean ± standard error).](image)
levels of salinity ($F = 61.793, \text{df} = 11, p < 0.001$) and seedling establishment decreased with decreasing salinities ($F = 143.089, \text{df} = 11, p < 0.001$). Elevated salinity increased seedling establishment at 15°C ($F = 143.089, \text{df} = 11, p < 0.001$).

**Morphological changes in germinated seeds and seedlings**

The metamorphosis of *Z. marina* L., at various growth stages during seed germination and seedling establishment, was observed in the seed germination experiment. Seed germination and seedling growth were divided into seven stages using a method modified method after *Sugiura et al. (2009)*. Stage 0 (Fig. 5A) was defined as the pre-germination stage when the seeds were ripe, plump, and intact. At Stage 1, the seeds broke through the seed coat in one of two ways, either via the cotyledon through the stoma with the embryo then breaking through the seed coat (Fig. 5B), or the whole embryo breaking through the seed coat all at once (Fig. 5C). At Stage 2 (Fig. 5D), the cotyledonary blade (CB), cotyledonary sheath (CS), and axial hypocotyl (AH) of the seedlings were found to be elongate from the central sulcus of basal hypocotyl (BH). At Stage 3 (Fig. 5E), the
process of leaf differentiation occurred. The first true leaf (L1) was elongated from the basis of the CS and the first to emerge was the epidermis, followed by the mesophyll, and finally the vein. At Stage 4 (Fig. 5F) adventitious root differentiation occurred. At Stage 5 (Fig. 5G), with the growth of adventitious roots (AR), the second true leaf (L2) emerged from the basis of the CS. At Stage 6 (Fig. 5H), the CB withered away with the growth of the AR and the second true leaf.

**DISCUSSION**

**Salinity effects**

Studies conducted in laboratory and in the field worldwide have shown that salinity, considered a major factor in seed ecology, has a large influence on seed germination (Caye & Meinesz, 1986; Caye et al., 1992; Conacher et al., 1994; Walmsley & Davy, 1997; Orth et al., 2000; Marion & Orth, 2010). The majority of previous studies for seagrasses have demonstrated that optimal seed germination often occurs under hyposalinity conditions and that germination of most species is reduced at elevated salinities, despite the fact that such low salinity is rarely encountered in the field. For example, Conacher et al. (1994) reported a higher germination rate in Z. capricorni seeds at low salinities (< 10 ppt) compared with high salinities (20–40 ppt). Zostera noltii seeds were shown to germinate better in low salinity conditions with maximum germination occurring at 1 ppt (Hootsmans, Vermaat & Van Vierssen, 1987); this was supported with the findings of Loques, Caye & Meinesz (1990).

However, other studies have shown little or no effect of salinity on germination. For example, no difference was found in Z. marina seed germination between 15 and 35 ppt in the laboratory (McMillan, 1983). Orth & Moore (1983), who studied Z. marina seed germination along salinity gradients, also drew a similar conclusion. Salinity stress on germination varies not only among species, but also within a species, with seeds adopting different survival strategies (Kim et al., 2013). Although low salinity has been shown to facilitate germination of Z. marina seeds in several populations, no significant differences correlating with salinity have been reported in the Gulf of California (Phillips, Grant & McRoy, 1983). It seems apparent that salinities affect seed germination differently, depending on population.

In this study, we found that freshwater and low salinities (1, 5, 10, and 15 ppt) were able to significantly facilitate Z. marina seeds germination. Kaldy et al. (2015) reported the same result in Z. japonica. Seed germination in Z. japonica was inhibited at salinities of 20 ppt and above, but germination resumed when seeds were placed in freshwater (Kaldy et al., 2015). Hootsmans, Vermaat & Van Vierssen (1987) found that Z. marina seeds germinated better under conditions of low salinity (maximum germination occurred at 1 ppt), similar to our results. In our study, we recorded the highest germination rate (88.67 ± 5.77%) of Z. marina seeds in freshwater at 15 °C.

Seagrasses began colonizing marine environments from freshwater and terrestrial environments and fully adapted to marine conditions 100 million years ago in the Cretaceous period, establishing in a vast new habitat free of terrestrial competitors and insect pests (Hartog, 1970; Olsen et al., 2016). In the present study, we observed that
maximum *Z. marina* seed germination occurred in freshwater and at low salinities, indicating that freshwater and low salinity facilitate seed germination. This might reflect a relic of *Z. marina* evolution or an evolutionary throwback to its previous freshwater existence, common in the early development of various species (*Arber, 1920; Chen, Wang & Zhu, 2012*).

Given the fact that the germination of some species is significantly reduced at increased salinity or reduced temperature, high salinity and low temperature conditions could be a suitable for the long-term storage of *Z. marina* seeds to maintain germination potential. *Kishima, Harada & Sakurai (2011)* reported the same result in *Z. japonica*. However, increased salinity may result in toxic ion accumulation within the cell membrane, indirectly inhibiting the activities of some enzymes or causing cell death, thereby influencing seed germination (*Katenbe, Ungar & Mitchell, 1998*).

In addition to its large influence on germination, salinity also plays an important role in seedling establishment and growth. Several studies have found differences in the optimum salinity range between seed and seedling stages, implying that the ability of seagrasses to cope with salinity stress may change during their development (*Hootsmans, Vermaat & Van Vierssen, 1987*). In our study, germination increased at reduced salinities, as mentioned above. In contrast, the seedling length, weight, number of leaves, and the progress of differentiation were found to be significantly reduced or inhibited at low salinities. For *Posidonia oceanica* seedlings, higher mortality in hyposaline conditions has been observed, while under hypersaline treatments, a lower seedling mortality occurs, and seedling survival is greater at the highest salinities (*Fernández-Torquemada & Sánchez-Lizaso, 2013*). In the present study, germination of *Z. marina* seeds in freshwater or at low salinities was very high, while very few of the germinated seeds completed leaf differentiation and seedling establishment in freshwater or low salinity, and seedling length was shorter than 4 cm. These results imply that freshwater and low salinity may have a large influence on the establishment and colonization of *Z. marina* in the field and could therefore potentially limit its distribution in coastal and estuarine waters.

Our results indicate that the optimum salinity for *Z. marina* seedling establishment and colonization is above 20 ppt in the field, consistent with study conducted by *Salo & Pedersen (2014)*. The optimum salinity for *Z. marina* seedlings in the present study was consistent with that reported for *P. oceanica* by *Fernández-Torquemada & Sánchez-Lizaso (2013)*. The present study indicated that once *Z. marina* seeds germinate, they grow better at high salinities (20–30 ppt) than at low salinities (< 20 ppt).

Our study showed that germination rate of *Z. marina* seeds increased in low salinity. Germination rates in natural seawater were very low (< 15%). In contrast, germination rates of *Z. marina* seeds in freshwater or lower salinities than natural seawater (10 and 20 ppt) were very high (> 80%). However, very few of these seedlings completed leaf differentiation or established in freshwater or the lower salinities (10 and 20 ppt). In contrast, seeds germinated in freshwater or low salinities (1, 5, and 15 ppt) transferred to natural seawater to accomplish seedling establishment achieved high germination rates (10.00, 28.67, 39.33, and 90.67, respectively). Therefore, these may be the optimal incubating conditions to consider when utilizing seeds in the establishment and
restoration of seagrasses. After seedlings grow to the stage in which they show their second true leaf, they can be transplanted for seagrass colonization or restoration.

**Temperature effects**

Annual temperature plays an important role in controlling site specific seasonal seagrass growth (Lee, Park & Kim, 2007). Zhou et al. (2015) reported that the shoot height of Z. marina from the Swanlake Lagoon was positively correlated with temperature. Similar to seagrass growth, seasonal fluctuations in temperature have shown to control the germination of seagrass seeds from several species (Moore, Orth & Nowak, 1993; Walmsley & Davy, 1997; Probert & Brenchley, 1999; Pan et al., 2011a). It appears that the lowest seawater temperature of the local coastal area is related to the optimal seawater temperature for Z. marina seed germination (McMillan, 1983; Orth & Moore, 1983). Seasonal temperature variations have been found to control the timing of germination in a number of seagrass species (Moore, Orth & Nowak, 1993; Taylor, 1957; Conacher et al., 1994). Zostera marina has been shown to be adapted to autumn/winter germination in Chesapeake Bay (Moore, Orth & Nowak, 1993), and to winter/spring germination in Prince Edward Sound (Taylor, 1957). Seeds of Z. capricorni (Conacher et al., 1994) germinated across a range of temperature treatments (15–30 °C) at low salinities (1, 5, and 10 ppt), while seeds held at higher salinities (20, 30, and 40 ppt) germinated only in lower temperature treatments. Several studies suggest that temperature stratification may be critical for seed germination (Loques, Caye & Meinesz, 1990; Harrison, 1991; Conacher et al., 1994). Brenchley & Probert (1998) found that 16 °C is the optimum temperature for seed germination of Zostera marina, with a 100% germination rate in natural seawater. In the present study, we observed that 15 °C was the most suitable temperature for the germination of Z. marina seeds with the maximum rate of 88.67 ± 5.77% recorded in freshwater, consistent with the aforementioned studies.

**Implications for restoration**

Mature Z. marina seeds exhibited dark color with relatively low moisture content and high weight. The mature seeds can be stored at high salinities and low temperature during long-term storage. Seed germination rates increases with increasing temperature and decreasing salinity. Seeds germination can be conducted in low salinities; and the transfer of germinated seeds from freshwater or low salinities to natural seawater can increase SER for restoration of seagrass habitats.

**CONCLUSION**

In conclusion, the germination rates of Z. marina seeds in freshwater or low salinities were very high, but none of the seedlings completed leaf differentiation or seedling establishment. However, seeds germinated in freshwater or low salinities could be transferred to natural seawater to accomplish seedling establishment and colonization. Therefore, the transfer of germinated seeds from freshwater or low salinities to natural seawater may be the optimal method in the establishment and restoration of seagrass habitats. In addition, high salinity and low temperatures were suitable for the
preservation of *Z. marina* seeds to maintain germination potential. These results may serve as useful data in *Z. marina* habitat restoration.

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**ADDITIONAL INFORMATION AND DECLARATIONS**

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**Competing Interests**

The authors declare that they have no competing interests.

**Author Contributions**

- Shaochun Xu conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Yi Zhou conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Pengmei Wang performed the experiments, contributed reagents/materials/analysis tools.
- Feng Wang contributed reagents/materials/analysis tools.
- Xiaomei Zhang contributed reagents/materials/analysis tools.
- Ruiting Gu contributed reagents/materials/analysis tools.
Field Study Permissions
The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

The collecting of the reproductive shoots of Z. marina from Swan Lake of Weihai was permitted by Peiliang Wang, manager of Mashan Group Co. Ltd. Ethical approval was not required for this study because no endangered animals and plants were involved. However, specimen collection and maintenance were performed in strict accordance with the recommendations of China Society of Plant Protection.

Data Deposition
The following information was supplied regarding data availability:

The raw data has been supplied as Supplemental Dataset Files.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.2697#supplemental-information.

REFERENCES
Abe M, Kurashima A, Maegawa M. 2008. Temperature requirements for seed germination and seedling growth of Zostera marina from central Japan. Fisheries Science 74(3):589–593 DOI 10.1111/j.1444-2906.2008.01562.x.
Arber A. 1920. Water Plants, A Study of Aquatic Angiosperms. Cambridge: Cambridge University Press.
Balestri E, Piazzi L, Cinelli F. 1998. Survival and growth of transplanted and natural seedlings of Posidonia oceanica (L.) Delile in a damaged coastal area. Journal of Experimental Marine Biology and Ecology 228(2):209–225 DOI 10.1016/S0022-0981(98)00027-6.
Brenchley JL, Probert RJ. 1998. Seed germination responses to some environmental factors in the seagrass Zostera capricorni from eastern Australia. Aquatic Botany 62(3):177–188 DOI 10.1016/S0304-3770(98)00089-8.
Cambridge ML, McComb AJ. 1984. The loss of seagrasses in Cockburn Sound, Western Australia. I. The time course and magnitude of seagrass decline in relation to industrial development. Aquatic Botany 20(3–4):229–243 DOI 10.1016/0304-3770(84)90086-5.
Caye G, Bulard C, Meinesz A, Loques F. 1992. Dominant role of seawater osmotic-pressure on germination in Cymodocea nodosa. Aquatic Botany 42(2):187–193 DOI 10.1016/0304-3770(92)90007-6.
Caye G, Meinesz A. 1986. Experimental study of seed germination in the seagrass Cymodocea nodosa. Aquatic Botany 26:79–87 DOI 10.1016/0304-3770(86)90069-9.
Chen X-L, Wang N, Zhu Y-L. 2012. Progress in study of plant atavisms. Acta Agriculturae Shanghai 28(1):102–105.
Christensen PB, Sortkjaer O, McGlathery KJ. 1995. Transplantation of Elgrass. Denmark: National Environmental Research Institute.
Churchill AC. 1983. Field studies on seed germination and seedling development in Zostera marina L. Aquatic Botany 16(1):21–29 DOI 10.1016/0304-3770(83)90048-7.
Coller CJ, Waycott M, McKenzie LJ. 2012. Light thresholds derived from seagrass loss in the coastal zone of the northern Great Barrier Reef, Australia. Ecological Indicators 23:211–219 DOI 10.1016/j.ecolind.2012.04.005.
Conacher CA, Poiner IR, Butler J, Pun S, Tree DJ. 1994. Germination, storage and viability testing of seeds of Zostera capricorni Aschers from a tropical bay in Australia. Aquatic Botany 49(1):47–58 DOI 10.1016/0304-3770(94)90005-1.

Conacher CA, Poiner IR, O’Donohue M. 1994. Morphology, flowering and seed production of Zostera capricorni Aschers in subtropical Australia. Aquatic Botany 49(1):33–46 DOI 10.1016/0304-3770(94)90004-3.

Connolly RM, Waltham NJ. 2015. Spatial analysis of carbon isotopes reveals seagrass contribution to fishery food web. Ecosphere 6(9):148 DOI 10.1890/ES14-00243.1.

Costanza R, d’Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O’Neill RV, Paruelo J, Raskin RG, Sutton P, van den Belt M. 1997. The value of the world’s ecosystem services and natural capital. Nature 387(6630):253–260 DOI 10.1038/387253a0.

Dooley FD, Wyllie-Echeverria S, Gupta E, Ward PD. 2015. Tolerance of Phyllospadix scouleri seedlings to hydrogen sulfide. Aquatic Botany 123:72–75 DOI 10.1016/j.aquabot.2015.02.004.

Dooley FD, Wyllie-Echeverria S, Roth MB, Ward PD. 2013. Tolerance and response of Zostera marina seedlings to hydrogen sulfide. Aquatic Botany 105:7–10 DOI 10.1016/j.aquabot.2012.10.007.

Dooley FD, Wyllie-Echeverria S, Van Volkenburgh E. 2013. Long-term seed storage and viability of Zostera marina. Aquatic Botany 111:130–134 DOI 10.1016/j.aquabot.2013.06.006.

Fernández-Torquemada Y, Sánchez-Lizaso JL. 2013. Effects of salinity on seed germination and early seedling growth of the Mediterranean seagrass Posidonia oceanica (L.) Delile. Estuarine Coastal and Shelf Science 119:64–70 DOI 10.1016/j.ecss.2012.12.013.

Fishman JR, Orth RJ. 1996. Effects of predation on Zostera marina L. seed abundance. Journal of Experimental Marine Biology and Ecology 198(1):11–26 DOI 10.1016/0022-0981(95)00176-X.

Fonseca MS, Kenworthy WJ, Courtney FX, Hall MO. 1994. Seagrass planting in the southeastern United States—methods for accelerating habitat development. Restoration Ecology 2(3):198–212 DOI 10.1111/j.1526-100X.1994.tb00067.x.

Govers LL, Man in ’t Veld WA, Meffert JP, Bouma TJ, van Rijswick PCJ, Heusinkveld JHT, Orth RJ, van Katwijk MM, van der Heide T. 2016. Marine Phytophthora species can hamper conservation and restoration of vegetated coastal ecosystems. Proceedings of the Royal Society B: Biological Sciences 283(1837):20160812 DOI 10.1098/rspb.2016.0812.

Green EP, Short FT. 2003. World Atlas of Seagrasses. Berkeley: University of California Press.

Harrison PG. 1991. Mechanisms of seed dormancy in an annual population of Zostera marina (eelgrass) from the Netherlands. Canadian Journal of Botany 69(9):1972–1976 DOI 10.1139/b91-247.

Hartog C. 1970. The Seagrasses of the World. London: North-Holland Publishing Company Press.

Harwell MC, Orth RJ. 1999. Eelgrass (Zostera marina L.) seed protection for field experiments and implications for large-scale restoration. Aquatic Botany 64(1):51–61.

Hemminga MA, Carlos MD. 2000. Seagrass Ecology. Cambridge: Cambridge University Press.

Hootsmans MJM, Vermaat JE, Van Vierssen W. 1987. Seed-bank development, germination and early seedling survival of two seagrass species from the Netherlands: Zostera marina L. and Zostera noltii hornem. Aquatic Botany 28(3–4):275–285 DOI 10.1016/0304-3770(87)90005-2.

Kaldy JE, Shafer DJ, Ailstock MS, Magoun AD. 2015. Effects of temperature, salinity and seed age on induction of Zostera japonica germination in North America, USA. Aquatic Botany 126:73–79 DOI 10.1016/j.aquabot.2015.06.006.
Katembe WJ, Ungar IA, Mitchell JP. 1998. Effect of salinity on germination and seedling growth of two Atriplex species (Chenopodiaceae). Annals of Botany 82(2):167–175 DOI 10.1006/anbo.1998.0663.

Kim DH, Aldridge KT, Brookes JD, Ganf GG. 2013. The effect of salinity on the germination of Ruppia tuberosa and Ruppia megacarpa and implications for the Coorong: a coastal lagoon of southern Australia. Aquatic Botany 111:81–88 DOI 10.1016/j.aquabot.2013.06.008.

Kim K, Choi J-K, Ryu J-H, Jeong HJ, Lee K, Park MG, Kim KY. 2015. Observation of typhoon-induced seagrass die-off using remote sensing. Estuarine, Coastal and Shelf Science 154:111–121 DOI 10.1016/j.ecss.2014.12.036.

Kirkman H. 1999. Pilot experiments on planting seedlings and small seagrass propagules in western Australia. Marine Pollution Bulletin 37(8–12):460–467 DOI 10.1016/S0025-326X(99)00146-0.

Kishima J, Harada S, Sakurai R. 2011. Suitable water temperature for seed storage of Zostera japonica for subtropical seagrass bed restoration. Ecological Engineering 37(9):1416–1419 DOI 10.1016/j.ecoleng.2011.03.035.

Lee K-S, Park SR, Kim YK. 2007. Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: a review. Journal of Experimental Marine Biology and Ecology 350(1–2):144–175 DOI 10.1016/j.jembe.2007.06.016.

Leoni V, Vela A, Pasqualini V, Pergent-Martini C, Pergent G. 2008. Effects of experimental reduction of light and nutrient enrichments (N and P) on seagrasses: a review. Aquatic Conservation: Marine and Freshwater Ecosystems 18(2):202–220 DOI 10.1002/aqc.842.

Lin H, Sun T, Zhou Y, Zhang X. 2016. Anti-oxidative feedback and biomarkers in the intertidal seagrass Zostera japonica caused by exposure to cooper, lead and cadmium. Marine Pollution Bulletin 109(1):325–333 DOI 10.1016/j.marpolbul.2016.05.062.

Liu X, Zhou Y, Yang H, Ru S. 2013. Elongated detritus as a food source for the sea cucumber Apostichopus japonicus Selenka (Echinodermata: Holothuroidea) in coastal waters of North China: an experimental study in flow-through systems. PLoS ONE 8(3):e58293 DOI 10.1371/journal.pone.0058293.

Loques F, Caye G, Meinesz A. 1990. Germination in the marine phanerogam Zostera noltii Hornemann at Golfe-Juan, French Mediterranean. Aquatic Botany 38(2–3):249–260 DOI 10.1016/0304-3770(90)90009-A.

Marion SR, Orth RJ. 2010. Innovative techniques for large-scale seagrass restoration using Zostera marina (eelgrass) seeds. Restoration Ecology 18(4):514–526 DOI 10.1111/j.1526-100X.2010.00692.x.

Macreadie PI, Trevathan-Tackett SM, Skilbeck CG, Sanderman J, Curlevski N, Jacobsen G, Seymour JR. 2015. Losses and recovery of organic carbon from a seagrass ecosystem following disturbance. Proceedings of the Royal Society B: Biological Sciences 282(1817):20151537 DOI 10.1098/Rspb.2015.1537.

McCloskey RM, Unsworth RKF. 2015. Decreasing seagrass density negatively influences associated fauna. PeerJ 3:e1053 DOI 10.7717/peerj.1053.

McMillan C. 1983. Seed germination for an annual form of Zostera marina from the sea of Cortez, Mexico. Aquatic Botany 16(1):105–110 DOI 10.1016/0304-3770(83)90055-4.

Moore KA, Orth RJ, Nowak JF. 1993. Environmental regulation of seed germination in Zostera marina L (eelgrass) in Chesapeake Bay—effects of light, oxygen and sediment burial. Aquatic Botany 45(1):79–91 DOI 10.1016/0304-3770(93)90054-Z.

Ogden J. 2006. Seagrasses: biology, ecology and conservation. Marine Ecology 27(4):431–432 DOI 10.1111/j.1439-0485.2006.00138.x.
Olesen B, Marba N, Duarte CM, Savela RS, Fortes MD. 2004. Recolonization dynamics in a mixed seagrass meadow: the role of clonal versus sexual processes. *Estuaries* 27(5):770–780 DOI 10.1007/BF02912039.

Olsen JL, Rouze P, Verhelst B, Lin Y-C, Bayer T, Collen J, Dattolo E, De Paoli E, Dittami S, Maumus F, Michel G, Kersting A, Lauritano C, Lohaus R, Töpel M, Tonon T, Vanneste K, Amirebrahimi M, Brakel J, Boström C, Chovatia M, Grimwood J, Jenkins JW, Jueterbock A, Mraz A, Stam WT, Tice H, Bornberg-Bauer E, Green PJ, Pearson GA, Procaccini G, Duarte CM, Schmutz J, Reusch TBH, Van de Peer Y. 2016. The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. *Nature* 530(7590):331–335 DOI 10.1038/nature16548.

Orth RJ, Carruthers TJ, Dennison WC, Duarte CM, Heck KL Jr, Fourquarean JW, Hughes AR, Olyarnik S, Kendrick GA, Kenworthy WJ, Short FT, Waycott M, Williams SL. 2006a. A global crisis for seagrass ecosystems. *BioScience* 56(12):987–996 DOI 10.1641/0006-3568(2006)56[987:AGCFSE]2.0.CO;2.

Orth RJ, Fishman JR, Harwell MC, Marion SR. 2003. Seed-density effects on germination and initial seedling establishment in eelgrass *Zostera marina* in the Chesapeake Bay region. *Marine Ecology Progress Series* 250:71–79 DOI 10.3354/Meps250071.

Orth RJ, Harwell MC, Bailey EM, Bartholomew A, Jawad JT, Lambana AV, Moore KA, Rhode JM, Woods HE. 2000. A review of issues in seagrass seed dormancy and germination: implications for conservation and restoration. *Marine Ecology Progress Series* 200:277–288 DOI 10.3354/Meps200277.

Orth RJ, Harwell MC, Lnglis GJ. 2006. Ecology of seagrass seeds and dispersal strategies. In: Larkum A, Orth RJ, Duarte C, eds. *Seagrasses: Biology, Ecology and Conservation*. Netherlands: Springer Press, 111–133.

Orth RJ, Luckenbach M, Moore KA. 1994. Seed dispersal in a marine macrophyte: implications for colonization and restoration. *Ecology* 75(7):1927–1939 DOI 10.2307/1941597.

Orth RJ, Luckenbach ML, Marion SR, Moore KA, Wilcox DJ. 2006b. Seagrass recovery in the Delmarva Coastal Bays, USA. *Aquatic Botany* 84(1):26–36 DOI 10.1016/j.aquabot.2005.07.007.

Orth RJ, Marion SR, Granger S, Traber M. 2009. Evaluation of a mechanical seed planter for transplanting *Zostera marina* (eelgrass) seeds. *Aquatic Botany* 90(2):204–208 DOI 10.1016/j.aquabot.2008.07.004.

Orth RJ, Moore KA. 1983. Seed germination and seedling growth of *Zostera marina* L. (eelgrass) in the chesapeake bay. *Aquatic Botany* 15(2):117–131 DOI 10.1016/0304-3770(83)90023-2.

Paling EI, Fonseca MS, van Katwijk MM, van Keulen M. 2009. “Seagrass Restoration” in Coastal Wetlands: An Integrated Ecosystem Approach. New York: Elsevier Press.

Pan J, Jiang X, Li X, Cong Y, Zhang Z, Li Z, Zhou W, Han H, Luo S, Yang G. 2011a. Influence of temperature and salinity on germination of eelgrass (*Zostera marina* L.) seeds. *Journal of Ocean University of China* 10(2):147–152 DOI 10.1007/s11802-011-1800-y.

Pan JH, Jiang X, Han HW, Zhang WF, Wang WW, Cong W, Li XJ, Zhang ZZ. 2011b. The research of eelgrass seed collecting and storing. *The Eighth Representative Assemblies at the Chinese Academy of Algae and 16th Seminar Abstract Set. Available at* http://cpfd.cnki.com.cn/Article/CPFDTOTAL-ZGHI201111001191.htm/.

Pan JH, Jiang X, Li X, Han H, Zhang Z, Li Z, Yu S, Song S, Wu R, Jiang Y, Zhao N, Yang G. 2014. An effective method for collecting and storing seeds from *Zostera marina* (eelgrass) in the Yellow Sea, China. *Restoration Ecology* 22(6):716–722 DOI 10.1111/rec.12137.

Park J-I, Lee K-S, Son MH. 2014. Germination rate of *Phyllospadix japonicus* seeds relative to storage methods and periods. *Ocean Science Journal* 49(1):67–72 DOI 10.1007/s12601-014-0007-8.
Phillips RC. 1972. Ecological life history of *Zostera marina* L. (eelgrass) in Puget Sound, Washington. PhD thesis. University of Washington.

Phillips RC, Grant WS, McRoy CP. 1983. Reproductive strategies of eelgrass (*Zostera marina* L.). *Aquatic Botany* 16(1):1–20 DOI 10.1016/0304-3770(83)90047-5.

Phillips RC, McMillan C, Bridges KW. 1983. Phenology of eelgrass, *Zostera-marina* L, along latitudinal gradients in North-America. *Aquatic Botany* 15(2):145–156 DOI 10.1016/0304-3770(83)90025-6.

Phillips RC, Milchakova NA, Alexandrov VV. 2006. Growth dynamics of *Zostera* in Sevastopol Bay (Crimea, Black Sea). *Aquatic Botany* 85(3):244–248 DOI 10.1016/j.aquabot.2006.03.004.

Probert RJ, Brenchley JL. 1999. The effect of environmental factors on field and laboratory germination in a population of *Zostera marina* L. from southern England. *Seed Science Research* 9(4):331–339 DOI 10.1017/S0960258599000343.

Salo T, Pedersen MF. 2014. Synergistic effects of altered salinity and temperature on estuarine eelgrass (*Zostera marina*) seedlings and clonal shoots. *Journal of Experimental Marine Biology and Ecology* 457:143–150 DOI 10.1016/j.jembe.2014.04.008.

Shafer D, Bergstrom P. 2010. An introduction to a special issue on large-scale submerged aquatic vegetation restoration research in the Chesapeake Bay: 2003–2008. *Restoration Ecology* 18(4):481–489 DOI 10.1111/j.1526-100X.2010.00689.x.

Short FT, Burdick DM. 1996. Quantifying eelgrass habitat loss in relation to housing development and nitrogen loading in Waquoit Bay, Massachusetts. *Estuaries* 19(3):730–739 DOI 10.2307/1352532.

Sugiura H, Hiroe Y, Suzuki T, Maegawa M. 2009. The carbohydrate catabolism of *Zostera marina* influenced by lower salinity during the pre-germination stage. *Fisheries Science* 75(5):1205–1217 DOI 10.1007/s12562-009-0156-3.

Suykerbuyk W, Governs LL, Bouma TJ, Gieskes WBT, de Jong DJ, van de Voort R, Giesen K, Giesen PT, van Katwijk MM. 2016. Unpredictability in seagrass restoration: analysing the role of positive feedback and environmental stress on *Zostera noltii* transplants. *Journal of Applied Ecology* 53(3):774–784 DOI 10.1111/1365-2664.12614.

Tanner CE, Parham T. 2010. Growing *Zostera marina* (eelgrass) from seeds in land-based culture systems for use in restoration projects. *Restoration Ecology* 18(4):527–537 DOI 10.1111/j.1526-100X.2010.00693.x.

Taylor ARA. 1957. Studies of the development of *Zostera marina* L.: II. Germination and seedling development. *Canadian Journal of Botany* 35(5):681–695 DOI 10.1139/b57-058.

Tol SJ, Coles RG, Congdon BC. 2016. *Dugong dugon* feeding in tropical Australian seagrass meadows: implications for conservation planning. *PeerJ* 4:e2194 DOI 10.7717/peerj.2194.

Touchette BW, Burkholder JM. 2000. Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology* 250(1–2):133–167 DOI 10.1016/s0022-0981(00)00195-7.

van Katwijk MM, Thorhaug A, Marbà N, Orth RJ, Duarte CM, Kendrick GA, Althuizen IHJ, Balestri E, Bernard G, Cambridge ML, Cunha A, Durance C, Giesen W, Han Q, Hosokawa S,
Kiswara W, Komatsu T, Lardicci C, Lee K-S, Meinesz A, Nakaoka M, O’Brien KR, Paling EI, Pickerell C, Ransijn AMA, Verduin JJ. 2016. Global analysis of seagrass restoration: the importance of large-scale planting. *Journal of Applied Ecology* **53**(2):567–578 DOI 10.1111/1365-2664.12562.

Walmsley CA, Davy AJ. 1997. Germination characteristics of shingle beach species, effects of seed aging and their implications for vegetation restoration. *Journal of Applied Ecology* **34**(1):131–142 DOI 10.2307/2404854.

Wassenberg TJ. 1990. Seasonal feeding on *Zostera capricorni* seeds by juvenile *penaeus esculentus* (Crustacea, Decapoda) in Moreton Bay, Queensland. *Australian Journal of Marine and Freshwater Research* **41**(2):301–310 DOI 10.1071/MF9900301.

Wyllie-Echeverría S, Ackerman JD. 2003. The seagrasses of the Pacific Coast of North America. In: Greene EP, Short FT, eds. *World Atlas of Seagrasses. Prepared by the UNEP World Conservation Monitoring Centre*. Berkeley: University of California Press, 199–206.

Wyllie-Echeverría S, Cox PA, Churchill AC, Brotherson JD, Wyllie-Echeverría T. 2003. Seed size variation within *Zostera marina* L. (Zosteraceae). *Botanical Journal of the Linnean Society* **142**(3):281–288 DOI 10.1046/j.1095-8339.2003.00180.x.

Zar JH. 1999. *Biostatistical Analysis*. Fourth edition. New Jersey: Prentice Hall Press.

Zarranz ME, González-Henríquez N, García-Jiménez P, Robaina RR. 2010. Restoration of *Cymodocea nodosa* (Urchria) Ascherson seagrass meadows through seed propagation: seed storage and influences of plant hormones and mineral nutrients on seedling growth in vitro. *Botanic Marina* **53**(5):439–448 DOI 10.1515/BOT.2010.051.

Zhang X, Zhou Y, Liu P, Wang F, Liu B, Liu X, Xu Q, Yang H. 2014. Temporal pattern in the bloom-forming macroalgae *Chaetomorpha linum* and *Ulva pertusa* in seagrass beds, Swan Lake lagoon, North China. *Marine Pollution Bulletin* **89**(1–2):229–238 DOI 10.1016/j.marpolbul.2014.09.054.

Zhou Y, Liu P, Liu B, Liu X, Zhang XM, Wang F, Yang H. 2014. Restoring eelgrass (*Zostera marina* L.) habitats using a simple and effective transplanting technique. *PLoS ONE* **9**(4):e92982 DOI 10.1371/journal.pone.0092982.

Zhou Y, Liu X, Liu B, Liu P, Wang F, Zhang X, Yang H. 2015. Unusual pattern in characteristics of the eelgrass *Zostera marina* L. in a shallow lagoon (Swan Lake), North China: implications on the importance of seagrass conservation. *Aquatic Botany* **120**:178–184 DOI 10.1016/j.aquabot.2014.05.014.