STUDENT PROJECT Conservation of *Zostera marina*: evaluating the effectiveness of domestic seed storage conditions for restoration

Laurie Thomson[1]

Abstract

Zostera marina is a seagrass species that acts as an ecosystem engineer, creating biodiversity-rich habitats that offer important ecosystem services. The species is, however, in decline across its range owing to environmental change and anthropogenic impacts. Conservation work includes the use of seeds and shoots to restore seagrass meadows, although *ex situ* storage of *Z. marina* seeds is a small area of research and there is no one set protocol. This study investigated the effects of salinity and temperature on the maintenance of dormancy and viability of *Z. marina* seeds during cold storage. Seeds were stored at 1 °C and 4 °C, in a range of salinity solutions (20, 30, 40, 50, 60 and 70 psu) over a period of 112 days. Results were collected by a velocity-based viability test at 28-day intervals, with seeds categorised as either viable, non-viable or germinated. Over the course of the storage period, results indicated that low salinities (20, 30, 40 and 50 psu) would exhibit premature germination during storage as well as loss of viable seeds at either temperature, while 60 and 70 psu groups have zero germinations and the highest viable seed number is found in 70 psu groups. Analysis revealed that overall temperature was only statistically significant in relation to viable seeds at 1 °C, suggesting that this is the better temperature to maintain viability. The study indicates that low salinity should be avoided for *Z. marina* seed storage; instead, a salinity solution of 70 psu at 1 °C storage for up to 4 months could ensure seed dormancy is unbroken and few seeds become non-viable. Overall, results from this study were used to create a simple storage protocol that could contribute to community-based restoration projects.

Introduction

Seagrasses are a globally distributed polyphyletic group of 72 species of marine angiosperms (Den Hartog & Kuo, 2006; Short *et al.*, 2018). They generally live fully submerged on intertidal zones, naturally inhabiting shallow coastal areas and tidal estuaries owing to high light requirements (Potouroglou *et al.*, 2014; IUCN, 2022).

Zostera marina L. (common eelgrass) is one of two seagrass species native to the coasts and estuaries of the United Kingdom, the other being *Zostera noltii* Hornem. (dwarf eelgrass) (Boström *et al.*, 2014; Blok *et al.*, 2018; McKenzie *et al.*, 2020). Eelgrass, like many seagrasses, forms areas ranging in size from lone patches to large meadows (Fig. 1) in the substrate on the seabed or the bed of an estuary (Short *et al.*, 2007; Livernois *et al.*, 2017). These populations can be considered ecosystem engineers and biodiversity hotspots, supporting many marine food

[1] Laurie Thomson graduated from the BSc Horticulture with Plantsmanship (Hons) at Royal Botanic Garden Edinburgh in 2022.
Address: 20A Inverleith Row, Edinburgh, EH3 5LR, UK.
Email: Laurie.A.Thomson@hotmail.com

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Fig. 1 *Zostera marina* meadow in Dunvulaig Bay, Loch Craignish, Scotland. Photo: © Project Seagrass.

webs as a source of food and shelter for other organisms (Peters *et al.*, 2015; Ruiz-Frau *et al.*, 2017; Orth *et al.*, 2020). They provide important ecosystem services including binding sediment which can reduce erosion; nutrient cycling; fish nurseries; and carbon storage via their roots (Bos *et al.*, 2007; Röhr *et al.*, 2018; Qin *et al.*, 2021). The restoration and conservation of declining or lost eelgrass meadows is important, given their status as important habitats, primary producers and providers of ecosystem services.

Threats to seagrasses can include diseases, the spread of predators, an increase in invasive species and anthropogenic impacts such as pollution, physical damage and nutrient loading (Reynolds *et al.*, 2016, 2018; Jones *et al.*, 2020; IUCN, 2022). Nutrient loading can also lead to eutrophic conditions that reduce water clarity and light levels, subsequently stunting growth and interfering with phenology (Hauxwell *et al.*, 2006; Burkholder *et al.*, 2007). Climate change could also affect seagrasses through ocean acidification and water temperature increases leading to changes in the hydrological cycle and a shift in the species range (Short *et al.*, 2016; Shields *et al.*, 2019; Johnson *et al.*, 2021; Lowell *et al.*, 2021; Tang & Hadibarata, 2022).

Conservation and restoration efforts are often labelled as either *in situ* (onsite in the natural environment) or *ex situ* (in a controlled environment offsite) (Volis & Blecher, 2010; Braverman, 2013) and involve a range of plant stages. Seagrass meadows can be restored either by the transplantation of asexually produced vegetative shoots (Salo *et al.*, 2014; Paulo *et al.*, 2019a) or by the collection and dissemination of seeds (Harwell & Orth, 1999; Marion & Orth, 2010). The storage and protection of reproductive plant material such as seeds can play an essential role in species conservation through safeguarding genetic diversity and as a tool for reintroductions (Potouroglou *et al.*, 2014; Paulo *et al.*, 2019b; Johnson *et al.*, 2020). The preservation of marine angiosperm seeds, both *in situ* and *ex situ*, is a small area of research with gaps in the understanding of optimal storage conditions. Nevertheless these techniques may hold the key to an effective and low-cost method of restoring or reintroducing lost seagrass beds (Busch *et al.*, 2010). However, previous studies, such as Marion & Orth (2010), have shown that current conservation and restoration methods are not cost effective and have met with limited success, highlighting the need to develop this area of research.

To aid restoration of this species, this study aims to expand on previous findings and explore the effects of different storage conditions on seed viability and dormancy.

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More specifically, it aims to investigate the relationship between salinity and temperature on maintaining the dormancy and viability of *Zoster marina* seeds during storage while also evaluating the feasibility of the method for future *ex situ* conservation projects, with the objective of creating a usable storage protocol and recommendations for future studies.

Background

The restoration of seagrasses may focus on three main strategies: (1) the restoration of degraded sediment (Bos *et al.*, 2007; Qin *et al.*, 2021); (2) the collection, storage and deployment of seeds; and (3) the collection and transplantation of vegetative shoots and seedlings. In theory, combining a number of methods – from seed storage to growing on then planting out seedlings – may be effective in restoring eelgrass beds in an area of decline or severe dieback, or even as a way of connecting fragmented beds to strengthen the resilience of a vulnerable population.

Eelgrass reproductive strategies and their relevance to restoration projects

The restoration of eelgrass can vary in technique from the transplanting of vegetative shoots to the storage and broadcasting of seed. Understanding the variations of reproductive patterns and strategies of the populations being restored is vital to ensure the right technique is used to achieve the greatest success. If the technique does not correspond to the dominant reproductive strategy of the bed in question, the restoration may not have any long-term effect (Orth *et al.*, 2006; Yang *et al.*, 2016; Johnson *et al.*, 2020).

Eelgrass resilience to disturbance is thought to involve a population's ability to resist and recover from changes that have occurred. According to studies by Unsworth *et al.* (2015) and Vercaemer *et al.* (2021) resilience can be influenced by environmental conditions, latitudinal gradients, adaptations to the physical environment, genetic diversity, energy reserves, continuity of habitat (connectivity and distribution of beds) and reproductive strategy (ratio of sexual to vegetative reproduction). This resilience is probably achieved through affecting phenology and reproductive effort (Meling-López & Ibarra-Obando, 1999; Potouroglou *et al.*, 2014; Qin *et al.*, 2014).

Most eelgrass beds will rely on both asexual and sexual reproduction but will vary in dominance with populations either being annual, where post-dieback rejuvenation occurs through seeds produced in spathes (Fig. 2), or perennial, through clonal shoots from vegetative reproduction, or a mix of both strategies (Phillips *et al.*, 1983; Paulo *et al.*, 2019b; Johnson *et al.*, 2020). The reproductive strategy that dominates within an eelgrass bed can be highly variable across latitudes and environmental conditions, and even within a region (Meling-López & Ibarra-Obando, 1999; Qin *et al.*, 2020; Vercaemer *et al.*, 2021), with Salo *et al.* (2014) suggesting that a population can even alter its reproductive pattern over time to adapt to new conditions. Therefore the distribution of effort placed in either method of reproduction and its effectiveness in maintaining and expanding a population can vary, depending on the environmental conditions and level (intensity and frequency) of disturbance.

Perennial populations tend to grow in more stable conditions and do not die back annually; they can therefore allocate more energy into vegetative shoots. However, they can exhibit some sexual reproduction, which plays a part in recovery when disturbances

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Fig. 2 *Zostera marina* spathes containing seeds. Photo: © Project Seagrass.

create gaps, allowing for the emergence of seedlings while primarily relying on clonal shoots to expand the populations (Jarvis *et al.*, 2014; Qin *et al.*, 2014; Vercaemer *et al.*, 2021).

Annual eelgrass populations tend to be found in unstable environments and regions exhibiting long periods of low temperature and light levels, with a reliance on a seedbank within the sediment for rejuvenation each spring and summer (Meling-López & Ibarra-Obando, 1999; Bos *et al.*, 2007). Eelgrass seedbanks are transient, with viable seeds lasting up to 12 months, as well as varying in size and over time, and with patchy distribution and abundance of seeds, particularly in perennial beds (Orth *et al.*, 2000; Jarvis *et al.*, 2014). A healthy seedbank is key for the long-term stability of the population and resilience to disturbance, allowing for the natural regeneration of beds during periods of dieback (Burkholder *et al.*, 2007; Johnson *et al.*, 2020). Studies by Greve *et al.* (2005) and Jarvis *et al.* (2014) identified that extended periods of stress and disturbance on a population could lead to the depletion of viable seeds within the seedbank, due to a decline in the population's reproductive rate.

Techniques used for eelgrass restoration

In situ restoration using seeds

Seeds can be broadcast by hand or using bags suspended in the water (Pickerell *et al.*, 2005; Eriander *et al.*, 2016), or mechanically, where seed is fired from the stern of a boat (Marion & Orth, 2010). Using a bag suspended in water instead of hand- or machine-sowing doubles as an *in situ* storage technique. This method is cheaper and less labour intensive, and can focus the dispersal of seed in a specific area of clear sediment so as to reconnect fragmented habitats (Pickerell *et al.*, 2005; Busch *et al.*, 2010; Marion & Orth, 2010; Yang *et al.*, 2016; Livernois *et al.*,

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2017). This process involves packing freshly harvested spathes into mesh bags and relies on the dispersal of the mature seed through the mesh.

However, the survival and establishment rate of seedlings using these techniques appears to be quite low (Pickerell *et al.*, 2005; Eriander *et al.*, 2016) due to their vulnerability to predation (Fishman & Orth, 1996; Infantes *et al.*, 2016a), bioturbation (being buried by other organisms in the sediment) and biotic dispersal via lateral currents (Sumoski & Orth, 2012; Sousa *et al.*, 2017). For example, burial depth in the sediment can affect a seed's ability to germinate and emerge. Studies by Marion & Orth (2010) and Infantes *et al.* (2016b) determined that the ideal depth for seed was between 2 and 4 cm, with anything lower resulting in germinated seeds dying before they reached the surface or seeds failing to germinate at all.

Armed with an understanding of some of the mechanisms that reduce the survival and establishment rate of seeds, research has gone into methods of protecting the seeds within the natural environment. These can involve storage in net or hessian bags (Harwell & Orth, 1999), using a protective sediment coat around a number of seeds that forms a core to shield them when dispersed (Xie *et al.*, 2020), or embedding seeds into a core fibre mat pegged to the seabed (Sousa *et al.*, 2017). The results of these studies have shown a marked reduction in the loss of seeds. However the survival and establishment rates of seedlings are still highly variable. Results indicated a higher rate of seedling establishment (12–64 per cent, with an average of 30 per cent) when compared with the use of hessian bags such as those of Unsworth *et al.* (2019), where the subsequent total seedling establishment was 3.5 per cent of the bags, with several bags also being lost because of storms.

Ex situ storage of seeds

Storing eelgrass seed in a controlled *ex situ* environment can take advantage of the seed's secondary dormancy to maintain viability over a period of time (such as the winter months), taking the place of the seedbank (Orth *et al.*, 2000; Jørgensen *et al.*, 2019). However, eelgrass seeds are desiccation-sensitive and therefore cannot be stored in the usual dry cold storage of terrestrial angiosperms; instead, they need to be stored in a saline solution (Hay *et al.*, 2000; Yue *et al.*, 2019a).

Salinity and temperature have been recognised as main factors in ensuring seeds remain viable and dormant, but the optimal levels of these conditions are still unclear. It has been suggested that salinity lower than the 35 practical salinity units (psu) average salinity of seawater will break dormancy (Conacher *et al.*, 1994; Dye *et al.*, 2013), while high salinity can keep most seeds viable and dormant (Pan *et al.*, 2011; Yue *et al.*, 2019b; Xu *et al.*, 2020). In the right conditions, the loss of viable seeds can be reduced; however it is difficult to compare results from the few studies focused on this area as they exhibit a broad range of testing conditions, timeframes and sample sizes (Marion & Orth, 2010; Pan *et al.*, 2014; Xu *et al.*, 2016).

Ex situ storage avoids the risks involved in *in situ* storage methods that lead to a loss of seeds such as predation, bioturbation and fluctuating environmental conditions. Additionally, *ex situ* storage could be combined with protective *in situ* dispersal techniques, such as the protective sediment core by Xie *et al.* (2020) or the coconut fibre mats of Sousa *et al.* (2017), to reduce seed loss via burial and other factors.

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Fig. 3 Dunvulaig Bay, Loch Craignish. Photo: © Lewis M. Jefferies.

Method

Zostera marina seeds were supplied by Project Seagrass[2] and were collected in August 2021 from several populations in Dunvulaig Bay, Loch Craignish, a sea loch on the coast of Argyll, Scotland (Fig. 3), where seagrass restoration work is planned. The experiment itself started in late November 2021, when seeds were received, and ended in March 2022. *Z. marina* seeds were stored in different conditions for a period of 112 days to test the effects of temperature and salinity on seed dormancy and viability.

Setting up the experiment

The experiment comprised several parts, including 13 storage treatments with various salinity levels, temperatures and viability tests. The experiment was set up in a private home in order to test whether seed storage is possible away from a laboratory facility, as would be the case with community-based restoration projects.

Viability testing

For this study, viability testing followed the approach described in Marion & Orth (2010) where a velocity test was used involving separation via velocity using a linear water flow in a flume in a flow-through tank. The heavier (likely viable) seeds had a fall velocity of less than 22 cm within 5 seconds (> 5 cm/second) and were therefore separated from the lighter non-viable seeds. This approach was adapted for this study by marking a 15 cm tall container on the side at the 10 cm position and filled with a 30 psu solution. The time a seed took to pass the 10 cm distance was measured with a stopwatch; if the seed took ≤ 2 seconds, it was considered viable. A preliminary viability test was conducted on 30 November 2021 to remove non-viable

[2] www.projectseagrass.org

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seeds, ensuring that the experimental treatments started with seeds that were 100 per cent viable. After the initial viability test at 0 days, further tests were conducted at 28, 56, 84 and 112 days during which the number of viable, non-viable and germinated seeds (Fig. 4) were counted and recorded in MS Excel. Additionally, any presence of white filamentous growth on seeds, referred to as mould, was recorded.

Storage conditions and treatment groups

Following the first viability test, 2,028 viable seeds remained for the experiment. These were split randomly between 39 petri dishes each containing 52 seeds. A set of salinity solutions (20, 30, 40, 50, 60 or 70 psu) and two storage temperatures (1 °C and 4 °C) were used. Each treatment group (Table 1) consisted of three replicates. In addition, a control group (Group X) was included with an ambient temperature and salinity solution of 35 psu (the expected salinity of seawater in Scotland) to observe the effects of fluctuating temperature on seed viability during storage.

Fig. 4 Close-up of a germinated *Zostera marina* seed. Photo: © Project Seagrass.

Storage conditions were created using two fridges (Fig. 5) controlled by thermostats that maintained the set temperatures at 1 °C and 4 °C respectively with a ± 0.2 °C margin. A Styrofoam box was used for housing the control at an ambient temperature, which averaged 5.6 °C over the experimental period with a minimum of 0.9 °C in January–February 2022 and a maximum of 12.3 °C in March 2022. Eelgrass seeds need flowing water to prevent them from becoming mouldy. To imitate this as best as possible in petri dishes, the water in each petri dish was changed regularly, with a sieve used to prevent loss of seeds. Each change of water required specific volumes of salt to be added to chilled water, with the ratio of salt to water determined by using the calculator tool on Hamzasreef.com, which factors in water volume, temperature and desired parts-per-thousand (ppt), which equates to psu. Tropic Marin® Reef Salt was used, and salinity solutions were changed every three days, with 300 ml of solution for each salinity concentration split between the 1 °C and 4 °C petri dishes. A salinity refractometer was used to ensure the correct concentration.

Table 1 Treatment groups, each with three replicates (A1, A2, A3 etc.). Treatment X will act as a control group.

	Salinity						**Control**
Temperature	20	30	40	50	60	70	35
1 °C	A	B	C	D	E	F	
4 °C	G	H	I	J	K	L	
Ambient							X

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Fig. 5 Petri dishes within the 1 °C treatment fridge, with a temperature probe connected to a thermostat. Photo: Laurie Thomson.

Statistical analysis

For the purposes of this study and statistical analysis, seeds deemed of good quality were classed as 'viable', those of poor quality (unlikely to germinate) as 'non-viable' and those that had split coats and visible cotyledons as 'germinated'. Additionally, the presence of mould on viable and non-viable seeds was counted separately. The effectiveness of each storage treatment was assessed by the number of viable seeds remaining in each petri dish. Treatments resulting in germination or more than a couple of non-viable seeds were deemed less successful.

The effects of the independent variables were measured by the collection and comparison of seed viability and germination numbers per dish, with statistical analysis conducted across all 39 data points collected per viability test (instead of on the calculated means per treatment). Graphs and tables were created in MS Excel using the means determined by the 39 data points per viability test and data analysis conducted using the Minitab program. Histograms and the comparison of mean and medium values were used to determine whether data were normally distributed. A paired T-test was used to compare the final number of viable and germinated seeds (at 112 days) with the results of the first test (at 28 days) for each treatment group. One-way and two-way analysis of variance (ANOVA) tests were used to ascertain the p-values of the resulting number of viable and germinated seeds from each of the combinations of factors between the two independent variables. Additional one-way ANOVA analysis was conducted on the mouldy viable and non-viable seeds that had been noted in the 112-day viability test to investigate any relationship between the mould and the non-viability of the seeds. For the purposes of this study, all analysis will be conducted with a significance (alpha) level of 0.05 (5 per cent).

Results

Effect of salinity and temperature over time

An indication of which conditions led to the most seeds remaining viable and dormant can be found by observing the mean difference in viability between 28 and 112 days.

Viable seeds

The average number of viable seeds for all treatments declines over the 112-day period from the starting count of 52 (Fig. 6). Of these, the 20 psu salinity groups show the greatest decline across both temperatures, which is mostly due to the larger number of seeds that germinated. The drop in viable seeds in 20 psu also appears to occur at a steeper rate at 4 °C than at 1 °C, but while the 1 °C group has a mean difference of 12.37 ± 2.89 and p-value 0.017, the 4 °C group has 13 ± 5.69 and p-value 0.053, and is only marginally significant (Table 2). In comparison, the lowest decline in viable seed numbers occurs within the 70 psu groups,

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Table 2 Results of paired sample T-tests conducted separately for viable and germinated seed counts, comparing results from the 28-day viability test with those of the 112-day test. St dev = standard deviation; SE mean = standard error of mean; ~ = no seeds counted to analyse; * = p-value: < 0.05.

Seed type	Temperature (°C)	Salinity (psu)	Mean	St dev	SE mean	T-value	P-value
Viable	1	20	12.67	2.89	1.67	7.6	0.017*
		30	2.333	1.155	0.667	3.5	0.073
		40	4.33	0.577	0.333	13	0.006*
		50	1	1	0.577	1.73	0.225
		60	1.67	2.31	1.33	1.25	0.338
		70	2	1	0.577	3.46	0.074
	4	20	13.67	5.69	3.28	4.16	0.053*
		30	7	2	1.15	6.06	0.026*
		40	8	3.61	2.08	3.84	0.062
		50	4.667	0.577	0.333	14	0.005*
		60	5	3.61	2.08	2.4	0.138
		70	1.667	0.577	0.333	5	0.038*
	Ambient	35	6	1.73	1	6	0.027*
Germinated	1	20	–10	1	0.577	–17.32	0.003*
		30	–1.667	0.577	0.333	–5	0.038*
		40	–1.667	0.577	0.333	–5	0.038*
		50	–0.333	0.577	0.333	–1	0.423
		60	~	~	~	~	~
		70	~	~	~	~	~
	4	20	–9	5.2	3	–3	0.095
		30	–1.667	0.577	0.333	–5	0.038*
		40	–1.667	1.528	0.882	–1.89	0.199
		50	–0.667	0.577	0.333	–2	0.184
		60	–	~	~	~	~
		70	~	~	~	~	~
	Ambient	35	–1.333	0.577	0.333	–4	0.057

with the largest number of viable seeds (51) in the F2 petri dish (1 °C) at 112 days, reflected by a lack of significance between means of 28 and 112 days (t-value 3.46 and p-value 0.074).

The other salinities show less definite declines. During the first two months for salinities 50 and 60 psu, the decline is greater at 1 °C than at 4 °C but this switches at 84 and 112 days. The p-values in Table 3 imply that

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Table 3 Two-way ANOVA results from 112-day viability test for viable and germinated seeds. DF = degrees of freedom; Salinity-Temperature = analysis of the interaction effect between salinity and temperature; * = p-value: < 0.05.

			DF	T-value	P-value
Viable	Salinity (psu)	Overall	5	53.07	0.000*
		20		–15.65	0.000*
		30		1.79	0.078
		40		0.37	0.708
		50		2.60	0.013*
		60		4.02	0.000*
		70		4.02	0.000*
	Temperature (°C)	Overall	1	13.72	0.001*
		1		3.70	0.001*
		4		–3.70	0.001*
	Salinity-Temperature			0.79	0.569
Germinated	Salinity (psu)	Overall	5	73.68	0.000*
		20		18.78	0.000*
		30		–0.80	0.431
		40		–2.18	0.040*
		50		–4.58	0.000*
		60		–5.61	0.000*
		70		–5.61	0.000*
	Temperature (°C)	Overall	1	0.59	0.450
		1		–0.77	0.450
		4		0.77	0.450
	Salinity-Temperature			0.16	0.979

the differences seen over time in 50 and 60 salinities only decrease significantly for 50 psu at 4 °C (t-value 14 and p-value 0.005). Values increase instead of decreasing in the 1 °C 50 psu at 56 days. The reason for this increase is unclear, as values of all three replicates increase, and not just one or two, which would indicate an error in the testing process. While the initial decline of 30, 40, 50 and 60 psu at 1 °C appears more pronounced than those at 4 °C, after 84 days the decline starts to level off.

Germinated seeds

The largest number of germinated seeds occurred in 20 psu (Fig. 7), with most occurring in the G3 petri dish (4 °C) and 16 having germinated. However, only in the 1 °C group was the difference between 28-day and 112-day means statistically significant (t-value –17.32 and p-value 0.003) (Table 2). The smallest number of germinations (0 seeds germinated) occurred at 60 and 70 psu for both temperatures. Those with a salinity

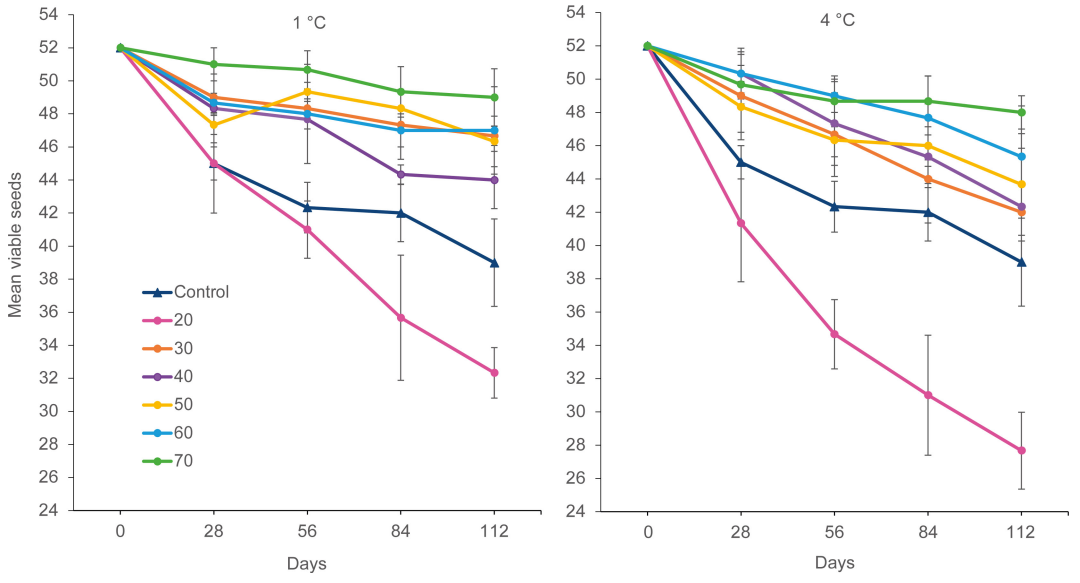

Fig. 6 Mean number of viable seeds over 112 days. The first graph displays 1 °C groups and the second 4 °C groups. Error bars represent the standard deviations.

of 50 psu had no germinated seeds until the period between 56 and 84 days (Fig. 7). The control group had a higher average number of germinated seeds across the 112-day period compared with those at 30 and 40 psu salinities, but the mean difference of the control is only marginally statistically significant (t-value –4 and p-value 0.057) (Table 2).

ANOVA analysis on effect of salinity and temperature

Two-way ANOVA tests were conducted to ascertain the p-values of the resulting mean values of viable and germinated seeds from each combination of the independent variables (Table 3). These were carried out using the 'General Linear Model' analysis in Minitab.

Viable seeds

Figs 8 and 9 show the mean values of viable and germinated seeds respectively at the 112-day viability test, as a close-up comparison. The interaction between salinity and temperature on viable seeds (Table 3) is not statistically significant (t-value 0.79 and t-value 0.569). The lowest viability is in 20 psu and 4 °C treatments, resulting in a markedly lower mean, as seen by the lack of overlap in the error bars. Only the means in salinities 20 and 30 psu show a marked difference in viable seed numbers between the two temperatures but overall temperatures have a significant effect on means (t-value 13.72 and p-value 0.001). Salinities 40, 50, 60 and 70 psu display overlap between the 1 °C and 4 °C means. Overlap with the control group's error bars occurs in the 30, 40 and 50 psu salinities at 4 °C.

Germinated seeds

The largest mean number of germinations occurred at 20 psu, with 4 °C producing more, though the error bars are large and overlap between temperatures (Fig. 9). All salinity pairs had overlapping standard deviations (error bars), corresponding to

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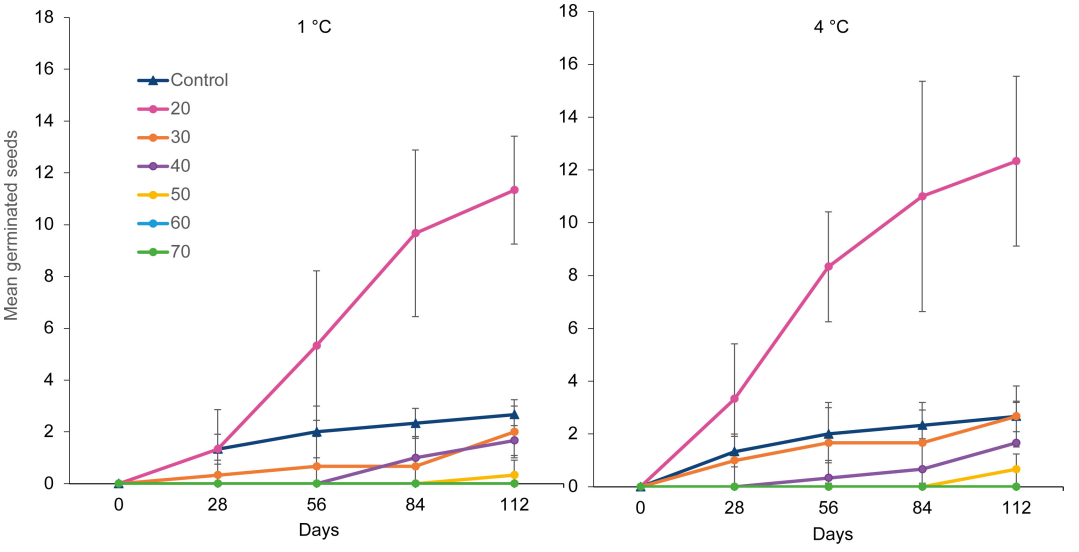

Fig. 7 Mean number of germinated seeds over 112 days. The first graph displays 1 °C groups and the second 4 °C groups. Error bars represent the standard deviations.

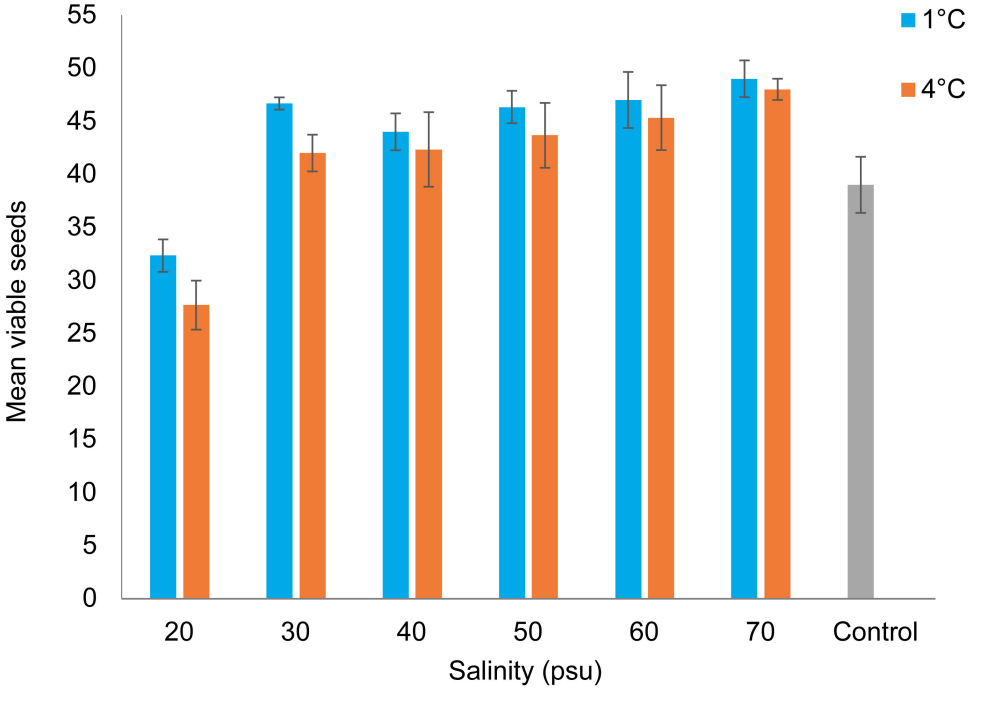

Fig. 8 Mean number of viable seeds at 112 days. Error bars represent the standard deviation of samples. The blue bars represent the 1 °C group and the orange bars the 4 °C group. Control group treatment variables were ambient temperature and 35 psu.

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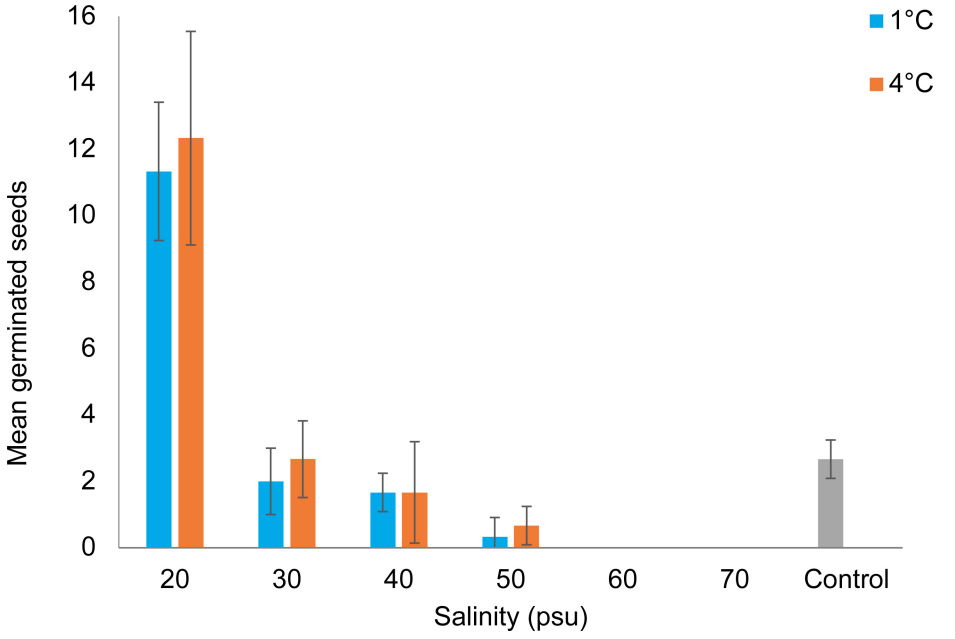

Fig. 9 Mean number of germinated seeds at 112 days. Error bars represent the standard deviation of samples. The blue bars represent the 1 °C group and the orange bars the 4 °C group. Control group treatment variables were ambient temperature and 35 psu.

the overall non-significant result of two-way ANOVA in Table 3 (t-value 0.59 and p-value 0.450). Salinities 30 and 40 psu overlap with that of the control group and 50 psu 1 °C has the smallest number, with only a single germinated seed in D1. Out of temperature and salinity, only salinity produces significant results (t-value 73.68 and p-value 0.000).

Presence of mould on seeds

Filamentous white growth, referred to as mould, was found to be present on seeds in every petri dish to some degree. Over both temperatures, salinities 20, 30 40 and 50 psu appeared to have the most.

Overall, at 112 days, 164 seeds, not including germinated seeds, showed signs of mould, with 24 seeds remaining viable. One-way ANOVA analysis comparing non-viable seeds with temperature and salinity separately (Table 4) indicated that salinity had a statistically significant (f-value 7.09 and p-value ≤ 0.000) effect on the occurrence of mouldy non-viable seeds, with the highest mean occurring in the 20 psu groups. The effect of temperature on the presence of non-viable mouldy seeds is marginally significant (f-value 3.16 and p-value 0.055). In addition, most of the germinated seeds became mouldy either soon after germination occurred or later.

Discussion

Purpose of the study

Using seeds for the restoration of plant species can be crucial for large-scale projects (van Katwijk *et al.*, 2009; Busch *et al.*, 2010; Yang *et al.*, 2016) and for increasing genetic diversity and thus resilience to disturbances and stresses (Paulo *et al.*, 2019b; Johnson *et al.*, 2020; Vercaemer *et al.*, 2021). Restoration projects that use eelgrass seeds

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Fig. 10 Close-up of G3 petri dish, displaying germinated and mouldy seeds. Photo: Laurie Thomson.

can benefit from storage methods that maintain dormancy during storage, since once germination has started seeds can deteriorate quickly. Cold storage in a saline solution as an *ex situ* conservation technique is a potential method of maintaining dormancy and viability of desiccation-sensitive eelgrass seeds for use in restoration projects (Conacher *et al.*, 1994; Orth *et al.*, 2000; Pan *et al.*, 2011; Kaldy *et al.*, 2015; Xu *et al.*, 2016).

For this study, the effect of the independent variables salinity and temperature on seed viability and dormancy was assessed using 12 treatment combinations (plus the control group).

The effects of treatments on seed during storage

Effect of temperature and salinity on viability of seeds

The two-way ANOVA analysis on the variability of seed viability during the experiment indicates that there is no significant interaction between salinity and temperature overall. This is reflected by the variation of significant results in 30 to 70 psu across both temperatures in the paired T-test.

The lack of significant difference between the starting number and final number of seeds is a positive outcome in the context of this study. The smallest overall decline in viable seeds came from the 70 psu group at 1 °C (Fig. 6 & Table 2) with 50 and 60 psu also exhibiting no statistical significance, while only the 40 and 60 psu groups in 4 °C were

Table 4 Results from one-way ANOVA data analysis on non-viable mouldy seeds. St dev = standard deviation; Tukey grouping = Tukey pairwise comparison, where means that do not share a letter are significantly different.

		Mean	St dev	Tukey grouping		F-value	P-value
Temperature (°C)	1	2.89	1.49	A	Overall	3.16	0.055
	4	4.06	1.98	A			
	Ambient	5.00	1.00	B			
Salinity (psu)	20	6.17	1.47	A	Overall	7.09	0.000
	30	2.50	1.38	B			
	40	4.00	1.41	AB			
	50	3.33	1.21	B			
	60	2.67	1.21	B			
	70	2.17	1.17	B			
	35	5.00	1.00	AB			

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not significant. For comparison, the results of this study can best be considered against those from a study by Xu *et al*. (2020), where long-term (3 to 12 months) cold storage was tested. That study was conducted using temperatures of 0 °C and 4 °C alongside a range of salinities from 30 to 70 psu in increments of 10 psu. The study found that the smallest loss of seeds occurred at 0 °C in 40, 50 and 70 psu, which partly supports the results of the current study (Table 2). In addition, Xu *et al.* (2020) indicated that 0 °C produced the best results compared with storage at 4 °C across the entire 12-month period, as well as implying that a longer period of storage may have been necessary to achieve a significant difference between temperature variables.

It is difficult to compare this information with other studies owing to wide variations in the sample size, the storage time, and the salinities and temperatures involved. Often there is no indication as to why the variable ranges were chosen. One such study, by Pan *et al.* (2014), comprised nine treatments using salinities 30, 37.4 and 44.5 psu and temperatures 4, 14 and 20 °C. The results showed that after 7 months the highest number of viable seeds (86 per cent) were found in the 4 °C 44.5 psu group. Another study, by Marion & Orth (2010), looked at a storage period of 3 months, using salinities 12, 20 and 30 psu at 4 °C, 21–24 °C and 23–28 °C. Their results indicate that the highest number of viable seeds occurred in the 4 °C 30 psu group. Taking the findings of previously mentioned studies and others (see Conacher *et al.*, 1994; Xu *et al.*, 2019) it can be assumed that a salinity of ≥ 50 psu (with 70 psu potentially being the best) and temperatures below 5 °C ensure a high percentage of viable seeds during a storage period of 1 to 12 months.

Implications of low salinity on early germination

It is thought that eelgrass seeds can remain dormant for up to 12 months (Conacher *et al.*, 1994; Dooley *et al.*, 2013). Orth *et al.* (2000) alongside other studies highlighted that eelgrass seeds exhibited secondary dormancy that was controlled by environmental factors (mainly salinity) rather than physical ones such as the seed coat (Kaldy *et al.*, 2015; Infantes *et al.*, 2016a).

For this study a wide range of salinities were used, which aided the testing process to determine when dormancy breaks and thus avoid undesirable early germination. The greatest loss of viable seeds in this experiment was within the 20 psu groups (Fig. 6) owing to the high number of premature germinations. This is supported by results from Xu *et al.* (2016) that also found greater germination occurring in salinities of 10–20 psu, with over 80 per cent occurring at 0 °C and 15 psu (88.67 ± 5.77 per cent) compared with natural sea water (< 15 per cent).

Germinations also occurred at 30, 40 and 50 psu, in lower quantities in both temperatures. Additionally, the results of the paired T-test between day 28 and day 112 indicated that only 20, 30 and 40 psu produced significantly higher germination rates over the 112-day storage period at 1 °C, compared to 50, 60 and 70 psu. At 4 °C, only the 30 psu group had significantly increased germination during that time. The overall effect of temperature on germination, however, had no statistical significance. This therefore helps to support the statement that to prevent breaking dormancy, salinities of 60 to 70 psu should be used as they displayed no germination over the 112-day period, while 50 psu and lower should be avoided.

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Overall, the two-way ANOVA test (Table 3) determined that the effect of temperature on germination had no statistical significance and therefore does not affect dormancy. However, individual results from the paired T-test suggest that there is a temperature effect on individual salinity levels that may not be linked to a gradient, i.e. going from high to low salinity. This highlights the need for further studies into the relationship between salinity and temperature and its effect on germination.

While vernalisation is not needed to break dormancy (Lamounette, 1977; Probert & Brenchley, 1999), several studies have found that after a short period of cold storage seed germination can be enhanced (Conacher *et al.*, 1994; Kaldy *et al.*, 2015; Yang *et al.*, 2016). Tanner & Parham (2010) indicate that a period of cold storage such as at 4 °C is enough to enhance germination over 1–8 weeks. However, Marion & Orth (2010) looked at germination alongside viability during storage and found that seeds at 4 °C had higher rates of premature germination during cold storage compared with the 21–24 °C and 23–28 °C groups (with salinity ranges of 12, 20 & 30 psu) over a period of 2–3 months. This could be an indication that cold storage is beneficial for enhancing germination after storage but the longer it lasts the more seeds are lost to premature germination or general decline in quality.

Presence of mould on seeds

Of the 164 seeds (8 per cent of the total seeds used in the study) that were mouldy by 112 days, 140 were deemed non-viable. The Tukey grouping (Table 4) indicates that out of 12 treatments (plus the control group), 20 psu had the largest number of mouldy seeds and the overall salinity gradient was the factor that significantly increased the presence of mould on seeds as salinity decreased.

It is unclear if the mould amassed due to the deterioration of the seeds or if the infection caused the seeds to decline in viability. It was also observed that germinated seeds would quickly become mouldy and deteriorate if left in the fridges (Fig. 10), which has implications for maintaining ideal *in situ* storage conditions. The limited space in the fridges used meant that separating out mouldy or germinated seeds was not practical; however, not removing mouldy seeds may have affected the results of this study, particularly for the 20 and 30 psu dishes at both temperatures, as they seemed most affected.

The suggestion that microbial infection reduces the quality of seeds is supported by Xu *et al.* (2019), in whose study solutions of nano-silver or copper sulphate were applied as an antimicrobial prewash before storage. Results indicate that using either antibacterial agent in a range of salinity and temperature storage conditions resulted in less than 10 per cent of seeds lost during storage and high levels of seed viability (> 80 per cent) when stored at 0 °C for 6 months. Govers *et al.* (2017) also found that copper sulphate could be used to reduce infection by *Phytophthora gemini* and *Halophytophthora* sp. pathogens that are becoming a threat to eelgrass restoration. They found that the application of copper sulphate at any volume between 0.2 and 2.0 ppm could reduce infection in seeds by up to 86 per cent during storage. These studies suggest therefore that either nano-silver or copper sulphate could be used in improving storage conditions as a prewash or soak.

Results in relation to restoration and conservation

Treatments that resulted in the lowest numbers of non-usable seeds (non-viable

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and germinated) can be considered the best suited for a storage period of 4 months. While the results of the two-way ANOVA test (Table 3) indicated that there is a lack of statistical significance in the interaction between salinity and temperature for viable and germinated seeds, high salinity on its own appears to have the best outcome in keeping the most seeds viable and maintaining dormancy.

The results of this study imply that 70 psu is the best salinity for maintaining seed viability and dormancy, though, as demonstrated in other studies, this may vary by sample size and length of storage (Conacher *et al.*, 1994; Marion & Orth, 2010; Xu *et al.*, 2019). Evidence also suggests that low salinity (≤ 20 psu) will increase germination rates and should therefore be avoided (Pan *et al.*, 2011, 2014; Xu *et al.*, 2016). The effect of temperature is less clear, but 4 °C or below would be better than an environment with fluctuating temperatures. Further studies are needed.

Effectiveness of viability testing methods

The method for testing seed viability used in this study followed Marion & Orth (2010) (see above) which was developed from an earlier study by Harwell & Orth (1999). Harwell & Orth found that at least 85–90 per cent of seeds were of good quality. This is an example of a separation technique that can be applied to large- and small-scale restoration projects alike to reduce the waste of cost and effort created by non-usable seeds (Busch *et al.*, 2010; Marion & Orth, 2010; Pan *et al.*, 2014).

Destructive methods include staining, which involves cutting the seed coat and applying the chemical tetrazolium chloride which turns a viable embryo from white to red. However, the seed coat must be cut to carry out the test, rendering the seed unusable (Conacher *et al.*, 1994; Pan *et al.*, 2014). While potentially more accurate, this technique would require a large sample size, particularly if multiple testing points occurred, as each test would reduce the final number of seeds in the sample groups. Another viability test is the squeeze test, where a soft seed coat indicates non-viable or bad seed. While this method should not damage the seed if carried out carefully, it has the potential to rupture the seed coat if too much pressure is applied and there is little indication in the literature on the accuracy of the test (Tanner & Parham, 2010; Xie *et al.*, 2020). The velocity viability test was useful for identifying viable seeds without adversely affecting them, but it was time consuming, as it could take one individual 10–11 hours to test all the samples.

Alternatively, instead of testing seed viability a vigour test could be carried out, where germination is induced at low salinity (usually < 10 psu) and high temperature (≥ 20 °C), as has been done in other studies (Xu *et al.*, 2019, 2020; Yue *et al.*, 2019a, 2019b). For this study, a vigour test after the final viability test would have been useful to ascertain the reliability of the testing method, but this was not possible within the timeframe of the project.

Limitations, errors and future recommendations

The study was limited by sample size and the number of replicates, making the results less reliable and harder to compare with those of other studies such as Infantes & Moksnes (2018), who used around 90,000 seeds. The volume of seed may also affect the outcome of the restoration project, which highlights the need for large quantities of

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eelgrass seeds to be stored. For example, van Katwijk *et al.* (2016) found that seeds and shoots planted in the context of small-scale restoration projects had a 22 per cent survival rate, whereas those forming part of large-scale projects had a survival rate of 42 per cent after 23 months. In these terms, future projects should use a threshold of more than 10,000 seeds or shoots. Van Katwijk *et al.* (2016) estimate that 55 per cent of previous trials carried out around the world used fewer than 1,000 seeds or shoots. They conservatively estimated the survival rate to be 37 per cent after 36 months. Therefore, using a larger number to start with can improve the overall success of a restoration project as well as potentially increasing the accuracy. Additionally, while the viability testing method used in this study may not be as accurate as destructive methods, a larger sample size could allow for more accurate testing in future studies.

However, the small sample size used in this study did make it manageable for one person to do the testing (taking a full 11 hours to carry out the test on all seeds), particularly in the restricted space of the home setting in which the experiment was conducted. The limited number of people involved in handling and testing the seeds (i.e. a single person) means that errors could go undetected. For example, the increase in viable seeds in the 1 °C 50 psu group at the 52-day viability test (Fig. 6) indicated that a measuring error potentially occurred during the test, but this is uncertain as the number of viable seeds increased for all three replicates.

Overall, the technique used demonstrates that it is feasible to store eelgrass seeds away from a fully equipped lab facility. This study can act as a foundation for developing protocols for future small-scale community projects as well as contributing to reducing the gaps in knowledge in eelgrass seed storage on a larger scale. There is also potential to combine seed-storage techniques like this with a dispersal method such as the core designed by Xie *et al.* (2020), where a mix of sediment and sand is used to form a protective coating around seeds. After a period of drying, with the seeds still protected from desiccation, the core can be scattered in an open area near other eelgrass beds where they will slowly dissolve as the seeds germinate and grow. This method reduces loss of seed via predation, bioturbation and lateral drift and could potentially be a low-cost way of encouraging interest within communities and other groups to participate in the restoration of local eelgrass beds.

Conclusion

The first objective of this study was to investigate the relationship between salinity and temperature in maintaining the dormancy and viability of *Zostera marina* seeds during storage while also evaluating the feasibility of the method for future *ex situ* conservation projects.

The null hypothesis for the study was that salinity and temperature have no effect either on the viability of *Zostera marina* seeds during storage or on seed dormancy. Of the variables, only salinity had statistical significance in the difference in means between first and last viability tests via the paired T-test (Table 2) and the two-way ANOVA analysis (Table 3). Therefore, the null hypothesis can be rejected, but only partially: the temperature variable and the temperature-salinity comparison were not overall statistically significant, unlike the statistical analysis results from the individual variable. This implies that while salinity on

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its own may affect dormancy and viability, the same cannot necessarily be said for temperature. Therefore, we can conclude that salinity is the main factor influencing the number of seeds that remained dormant and viable over the 112-day storage period. Salinities of 50 psu or lower should be avoided, and further tests could determine if a temperature of 1 °C should be used instead of 4 °C for the cold storage of eelgrass seeds.

The second objective was to evaluate whether a home set-up is sufficient to store *Zostera marina* seeds for conservation. The conclusion is that it is a feasible method, though future studies would benefit from increasing the storage capacity for seeds to allow for an increased sample size and potentially more accurate viability testing methods. Additionally, future studies may also consider avoiding mould on seeds through the use of antifungal agents like nano-silver and copper sulphate (as seen in Govers *et al.*, 2017; Xu *et al.*, 2019). However, as these compounds can be harmful to other living organisms they may be less suitable for use in a home environment, where there is no guarantee of safe storage and disposal.

The use of seeds in restoring *Zostera marina* populations is an important area of study as seeds are a vital source of genetic diversity that can aid a species' resilience to disturbance and can be dispersed in large quantities with less effort compared with the transplanting of shoots and seedlings. However, storage techniques need to be perfected and it is hoped that this study can assist future research in determining the optimal seed storage conditions so that eelgrass meadows can be restored and protected before they become too vulnerable or are lost completely.

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