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Eelgrass seed harvesting: Flowering shoots development and restoration on the Swedish west coast

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ABSTRACT

Eelgrass (*Zostera marina*) flowering development and seed production were assessed along a depth gradient at three sites during 2012 and 2013 to 1) describe the flowering seasonality in the Swedish west coast, and 2) evaluate methods using seeds for large-scale restoration, including harvesting, storage and separation of viable seeds using a vertical flume. Eelgrass flowering shoots were found in the field from June to October, reaching the highest densities in July and August (average 3.8 ± 0.5 shoots m^{-2}). Flowering shoot densities decreased with depth, whereas shoot length, number of spathes, seeds/shoot and seed size increased with depth, resulting in the highest seed production at intermediate depths (2 m) in most bays and years. Because of low densities of flowering shoots, seed production in Sweden (on average 39–126 seeds m^{-2}, Jul–Sep) was an order of magnitude lower than in other studied areas. Results showed that seed production differed 2.5–3.4 times between meadows and years, mainly driven by variation in seed production per shoot. This variation was only partly explained by temperature over the growing season, suggesting that other factors such as light and the amount of filamentous algal mats might also be important in flower development. Results suggest that flowering shoots should be harvested when > 50% of the spathes have developing seeds, and that shoots should not be stored longer than 40 days in tanks to obtain an optimal release of viable seeds. A new mechanized method using a vertical flume to separate large amounts of viable seeds from the harvest is also presented.

1. Introduction

Seagrass habitats are one of the world's most threatened ecosystems and they are disappearing in many parts of the world at an alarming rate. It has been estimated that nearly 30% of the global seagrass area has been lost since the early 1900s, with an accelerating loss (Hughes et al., 2009; Waycott et al., 2009). Along the Swedish northwest coast more than 60% of the eelgrass meadows have vanished since the 1980's (Baden et al., 2003; Nyqvist et al., 2009) with little natural recovery and continuing losses in the southern part of the region (Moksnes et al., 2016). Studies suggest that the primary mechanism behind the decline in this area is caused by eutrophication in combination with overfishing, which has caused a trophic cascade that promotes the growth of algae (Moksnes et al., 2008; Baden et al., 2010, 2012). As environmental conditions are improving (SwAm, 2012), interest to restore the lost eelgrass habitats are growing (SwAm, 2015), but so far, large-scale restoration of eelgrass has not been carried out.

Seagrasses can reproduce by vegetative cloning and sexually through the production of flowers and seeds (Den Hartog 1970; Kendrick et al., 2012; Kendrick et al., 2017). Sexual reproduction is the main way to colonize new areas and it sustains existing beds (Thayer et al., 1984; Marbà and Walker 1999; Greve et al., 2005). Seeds can also be used for restoration, which can be very effective in certain locations. For example, large additions of seeds in Virginia coastal bays in the USA resulted in the development of a 125 ha of eelgrass beds, which increased to over 1700 ha during a 10 year period (Orth et al., 2012). In this successful restoration effort, seeds were produced by harvesting flowering shoots that were stored in tanks until seeds were released. Programs using seeds for large-scale restoration will require assessment of the number of seeds needed for specific planting efforts, and to identify donor sites where flowering shoots can be harvested. It is also necessary to estimate the number of seeds that are available in a donor meadow and the optimal time for harvesting. At the moment, little is known about the seasonality of flower development of eelgrass in Sweden and in Northern Europe it has only been described in Denmark (Olesen 1999; Olesen et al., 2017). Eelgrass flowering, seed development and seed viability have been largely described (Churchill and Riner, 1978; De Cock 1980, 1981) and methods for harvesting

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flowering shoots and seeds are well described (Marion and Orth, 2010). However, a method to select the optimal time for harvesting flowering shoots to maximize the number of seeds collected is currently missing.

Flowering or reproductive shoots mature first at the base of the plant and on the main axis of the stem, then progresses upward and outward toward terminal inflorescences (De Cock, 1980). Flowering and seed development does not occur equally over the entire shoot and some judgment is required to choose a harvest time that ensures the greatest yield of seeds. Harvesting too early might reduce the yield of seeds collected since many seeds might not fully develop. On the other hand, harvesting after the seeds have matured might not be efficient since many seeds could be already released in the field. Selecting the best harvesting time is currently done by experience personal judgment of the donor site bed (Marion and Orth, 2010), but there is not a standard method to select these dates. Temperature appears to be critical for all phases of the flowering event, such as the flower appearance, seed production, seed germination and seedling development (De Cock, 1981; Orth et al., 2000; Ackerman, 2006). This relationship between flowering and water temperature could be further explored to possibly predict seed ripening time. In addition, an effective method to separate large amounts of viable seeds after harvesting is needed.

The aim of the study was to 1) describe eelgrass flowering seasonality in the Swedish West coast, 2) evaluate methods for large-scale restoration such as collection, storage and separation of viable seeds. In addition, we assess if growing degree-days, i.e. heat accumulation during the growing season, could be used to predict seed maturation and the optimal harvesting time.

2. Methods

Eelgrass *Zostera marina* (L.) is the dominant angiosperm throughout the northern hemisphere, extensively distributed throughout Scandinavian coastal waters (Boström et al., 2014). The broad-scale presence of eelgrass in this region follows the 5–30 salinity gradient from the northern Baltic Sea to the Skagerrak (Boström et al., 2014). On the Swedish NW coast, eelgrass is found mainly in muddy and sandy sediments between 0.5–4.5 m depth (Baden et al., 2003). Surface water temperature can range from below 0 °C in winter to 20 °C in summer.

Four large meadows were selected in three regions of the Swedish west coast, the Gullmars Fjord, the Stig Fjord and the Hake Fjord (Fig. 1). In the Gullmars Fjord, two meadows were selected, Lindholm and Gåsö. Lindholm represents a sheltered fjord environment where the water can be more stratified, while Gåsö represents a coastal area well flushed with water form the Skagerrak-Kattegat Sea. The meadow in the Hake Fjord, outside the port of Wallhamn is located near the Marstrand area, which has the largest documented decline of eelgrass in Sweden, where over 90% of eelgrass cover was lost since the 1980's (Baden et al., 2003; Moksnes et al., 2016). The Wallhamn meadow could be potentially targeted as a donor meadow for restoring the area of Marstrand. In the Stig Fjord, Viks Kile was selected since it is a relatively unaffected by the large eelgrass losses. Viks Kile is located near Marstrand and is within a Nature 2000 marine protected area.

2.1. Flowering seasonality and flower development

Flowering shoot densities and the flowering development were measured monthly between Jul-Sep in 2012 at two meadows (Gåsö, Lindholm) and between May-Oct in 2013 at three meadows (Lindholm, Gåsö, Wallhamn) (Table 1). At each site, eelgrass samples were taken along 50 m transects at five different depths from the upper depth limits (1–1.5 m) to the lower depth limits (3–4 m) by snorkeling or SCUBA diving. At each depth-specific transect, the number of flowering shoots were counted in 25 quadrats of 1 m^2 separated by 1 m. Flowering stages were assessed by collecting seven shoots along the transects at each time and classifying all the spathes from each shoot as described by De Cock, (1980). Flowering stages were classified as 1) styles are erect

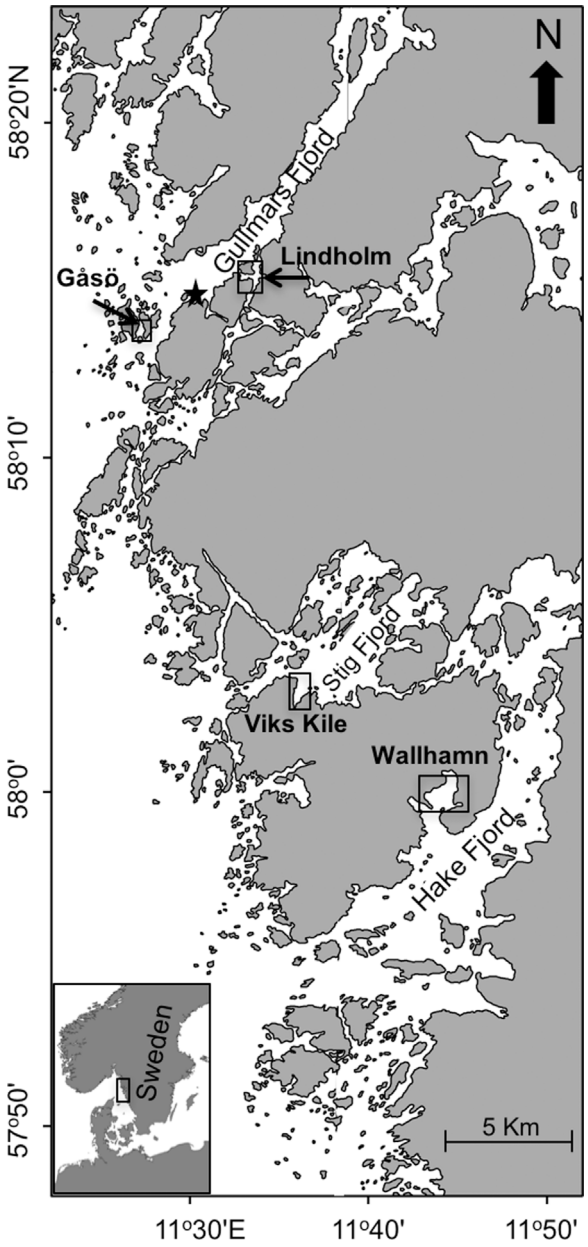

Fig. 1. Map of the study sites in the Swedish northwest coast. Gullmars Fjord and Wallhamn were the main study areas, whereas Viks Kile was used for the large-scale harvesting study. Kristineberg station is shown with a star.

from the spadix, 2) styles bend back after pollination, 3) pollen is released from the anthers, 4) seed maturation during 4–5 weeks and 5) seeds are released (Fig. 2). Morphological characteristics such as shoot length, number of spathes, number of developing and mature seeds per spathe were also measured on every flowering shoot.

Seed maturation and seed release after harvesting was assessed in 2012 and 2013. Flowering shoots were harvested between Jul-Sep and stored in outdoor tanks over 2–3 months (see below for tank storage details). Ten flowering shoots were collected at 1–1.5 m (shallow) and 3–4 m (deep) at all sites (Table 1). Flowering stages were assessed and shoots were placed in mesh bags of 500 µm in outdoor seawater flow-through tanks. Seeds released in the mesh bags after maturation were collected and counted. Water temperature and salinity in the tanks was monitored using data-loggers (HOBO, Onset®).

The relation between water temperature, light and flower development was further explored using growing degree-days (GDD) and the percentage of surface light reaching the bottom. Water temperature and

Table 1
Eelgrass harvesting dates (day of the month) and locations between 2012 and 2014. In 2012, pilot studies were carried out, while in 2013, a larger study was carried with a detail harvest schedule. In 2014, only large-scale harvesting and seed processing was performed.

	Site	2012			2013									2014
		Jul	Aug	Sep	May	Jun	Jul		Aug			Sep	Oct	Jul
Shoot density & Flowering stage	Gåsö	7	10	10	4	6	9	23		10		6	6	
	Lindholm	4	15	11	4	7	9	23		8		6	6	
	Wallhamn				6	11	12	25		9		5	5	
Seed release	Gåsö		10					23	2	10	19	6		
	Lindholm		15					23	2	8	19	6		
	Wallhamn							25	4	9	20	5		
Large scale harvesting	Gåsö									10				24
	Lindholm		15							8				22–23
	Wallhamn									9				
	Viks Kile		14							4				

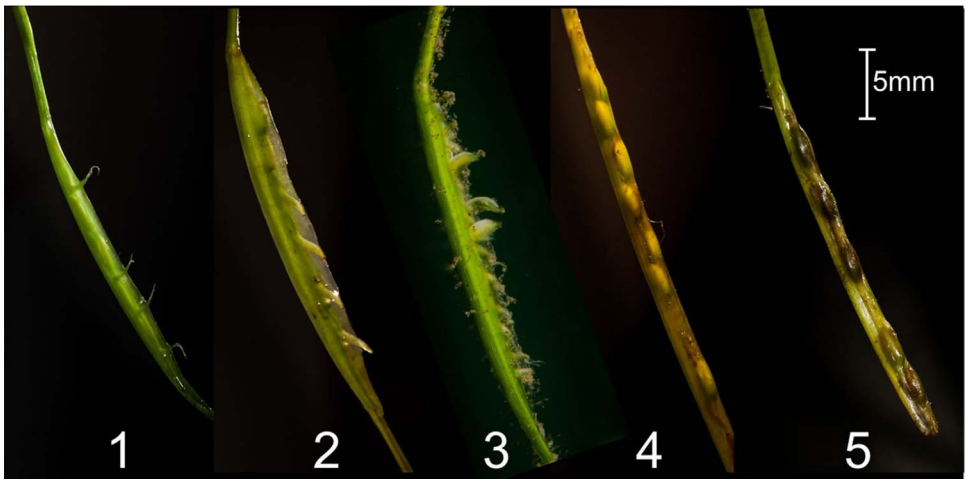

Fig. 2. Flowering stages of *Zostera marina*. 1) styles are erect from the spadix, 2) styles bend back after pollination, 3) pollen is released from the anthers, 4) seed maturation, 5) seeds are released.

light was measured at each site in 2013 using data-loggers (HOBO, Onset®). Loggers were deployed at the shallow (1 − 1.5 m) and deep (4 − 4.5 m) edges of the meadow with a sampling rate of 15 min. GDD is a measure of heat accumulation used to predict or estimate the length of plant development phases such as flower blooming dates or when a crop will reach maturity (McMaster and Wilhelm 1997; Bonhomme 2000; Miller et al., 2001; Schlenker and Roberts, 2009) and was computed as,

$$GDD = \frac{Tmax + Tmin}{2} - T_{base} \quad (1)$$

where T_{max} and T_{min} are the maximum and minimum daily temperatures respectively. If $[(T_{max} + T_{min})/2] < T_{base}$, then $[(T_{max} + T_{min})/2] = T_{base}$. T_{base} is the temperature at which plant development will start and was calculated as 8 °C using a statistical approach where the regression coefficient method was applied (Hoover 1955; Yang et al., 1995). Heat accumulation is represented as a cumulative sum of daily GDD values. The percentage of light reaching the bottom was calculated by using the data from the data-loggers at two different depths for each site. To avoid bias due to sensor fouling, light conditions were calculated for 12 h (n = 48) on 5 dates after the loggers were cleaned, 12-Jun, 10-Jul, 23-Jul, 10-Aug and 5-Sep. Light irradiance was computed by calculating the light attenuation coefficient *Kd* as,

$$K_d = \frac{1}{d_2 - d_1} \cdot ln\left(\frac{I_{z2}}{I_{z1}}\right) \quad (2)$$

where d is depth and I_z is light irradiance at different depths. Then, the light irradiance for a given depth I_x was calculated as,

$$I_x = I_0 \cdot e^{-Kd \cdot x} \quad (3)$$

where I_0 is the irradiance below the surface and x is the depth.

2.2. Large-scale seed harvesting and seeds processing

To assess methods for harvesting and processing large quantities of eelgrass seeds, a total of 25,300 flowering shoots were collected in Jul-Aug in 2012, 2013 and 2014 in four different meadows (Table 1), and stored in outdoor tanks while monitoring the production of mature seeds. In 2013, the quantity and quality of the seeds released in the storage tanks was assessed every 10 days from August to October. Shoots were manually harvested by divers between 1 and 1.5 m depth by keeping them in small bundles of 50 shoots with a rubber band for easy counting and transport. The time needed to collect 100 flowering shoots was measured for 6 divers on 8 different times. Shoots were kept in 1 mm mesh bags during harvesting and transport, and were stored in outdoor tanks of 1500 L with fjord seawater flow-through at the Sven Lovén Center, Kristineberg Station (Fig. 1S, supplementary material). The intake water flow rate was 250 L per hour and the water was renewed every 6 h. Water temperature and salinity in the tanks was similar to natural fjord water between 18 and 20 °C and salinity between 25 and 30. The water column was aerated and mixed using air diffusers at the tanks bottom. Since shoots are positively buoyant, a 5 cm-square nylon net was place on top of the shoots to keep all plants submerged and avoid desiccation. Tanks were kept in the shade of a three-story building to avoid direct sunlight and reduce algae growth. Tanks were weekly maintained to remove fouling from outflow pipes. Matured seeds were naturally released from the flowering shoots and since seeds are negatively buoyant they sank to the bottom of the tanks. Seeds were collected by suctioning the bottom of the tanks and were sieved (1 mm

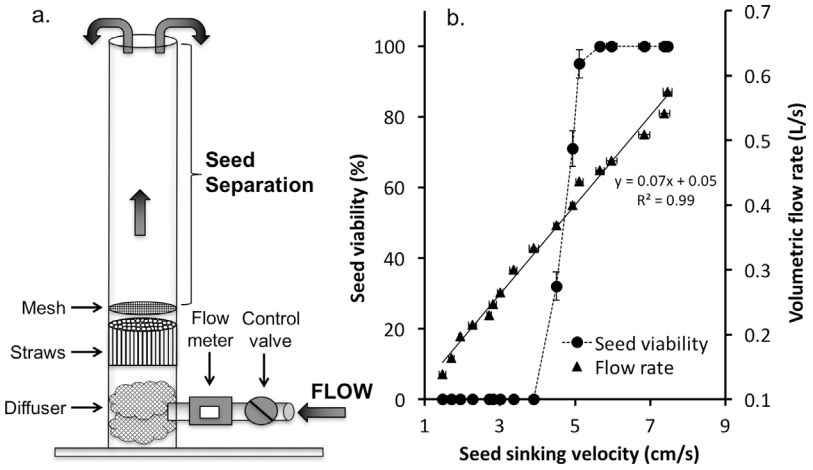

Fig. 3. a) Schematic diagram of vertical flume used for seed separation. b) Relation between seed viability, sinking velocity and volumetric flow rate.

mesh) to remove dead leaves and larger organic material before storage in smaller flow-through tanks in the laboratory. The seed mixture was shaken for 1 min inside the 1 mm mesh to remove the water excess. The number of seeds was measured by counting the number of seeds in 1 gr of the seed mixture and multiplying by the total weight of the seed mixture. In 2013, flowering shoots harvested at Lindholm and Viks Kile (Table 1) was also analysed for variation in seed size. Seed sizes were measured using an electronic calliper.

A key aspect when using seeds for eelgrass restoration is to determine the amount of viable seeds being produced since a large and variable proportion of the seeds released in storage tanks can be immature, damaged or dead and will therefore fail to germinate. Earlier studies have shown that the sinking velocities of seeds are related to seed quality (Marion and Orth, 2010), but mechanized methods to identify and sort viable seeds in large-scale seed processing have been missing. For this purpose, a vertical flume was developed to separate viable seeds from both low-quality seeds and from organic debris that is usually collected with the seeds (Fig. 3a). The flume (75 cm long × 9.5 cm diameter) was used to separate seeds according to their sinking velocities. Details of the vertical flume are available in the supplementary material (Fig. 2S). The flume was calibrated by measuring the relationship between the flow rate and the sinking velocities. Seeds were exposed to different flow rates during 15 min and those seeds leaving the tube by the top were collected and their sinking velocities and viability were measured. Sinking velocities were measured by dropping 10 seeds in a 50 cm vertical × 20 cm diameter glass tube and calculating the time to reach the bottom. Seed viability was tested using the tetrazolium staining method (Lakon, 1949; Sawma and Mohler, 2002). Seed embryos were gently removed from their seed coats and soaked in a 1% tetrazolium blue chloride solution for 24 h. Embryos stained in pink to brown where considered viable.

2.3. Statistical analysis

To assess how density and morphology of flowering shoots varied between sites and depth, data from the sampling dates when shoot densities and developing seeds were highest in 2012 (15-Aug) and 2013 (23-Jul and 8-Aug) were analysed in ANOVA models and regression analyses. Sites (Lindholm, Gåsö and Wallhamn) and depths (1–3 m) were used as random and fixed independent variables, respectively, in mix model ANOVAs testing the average density and length of flowering shoots, number of spathes in stage 4 and 5 per shoot, number of seeds per spathe, and number of seeds per shoot as dependent variables.

To test the relationship between sampling depth and the average density and length of flowering shoots, simple linear regression analyses were carried out using all sampling data when flowering shoots were present from June to September. Regression analyses were carried out using the shoot lengths as independent variables, and the number of spathes per shoot, number of seeds per spathe, and average number of seeds per shoot as dependent variables. In addition, relationship between the shoot length and seed length was analyzed for two sites (Lindholm and Viks Kile) sampled in 2012.

Homogeneity of variance was tested using Cochran's c-test (Sokal and Rohlf, 1995) and heteroscedastic data was square root transformed to meet assumptions of homogeneity. Multiple comparison post-hoc tests were performed using the Student–Newman–Keuls (SNK) procedure.

3. Results

3.1. Flowering seasonality and flower development

Seasonal sampling of eelgrass in three bays in 2012 and 2013 showed a general pattern of flowering stages with flowering shoots first appearing in the beginning of June, seeds maturing at the end of July and being released in August and September, but a large variation was found between sites and depths. Flowering development in Lindholm and Gåsö were similar in 2012 and 2013, and only the results from 2013 are described below. In 2013, 95% of the spathes were in stage 1 in early June, and in early July between 15 and 40% had styles bent back after pollination (stage 2) and pollen started to be released (stage 3; Fig. 4). In late July at Gåsö and Wallhalmn, most spathes were observed with maturing seeds (stage 4), and in early August seeds started to be released (stage 5) at all depths. In contrast, at Lindholm few spathes were maturing in July, where a majority of spathes in stage 4 were found in August and September at 3 m depth (Fig. 4). In September, some seeds were released at all sites but most spathes (5–30%) were already withering. In October, few flowering shoots were observed and most of them were bended and lying horizontally in the bottom with a high epiphytic cover.

Seasonal changes in flowering shoot densities also differed between sites and depths. In both 2012 and 2013, densities of flowering shoots at Lindholm peaked in late July or August at 1 m and 2 m depth, but not until September at 3 m (Fig. 5). At the other two sites, development was more similar between depths with peak densities occurring mid-July to mid-August during the 2012 and 2013. Shoot densities decreased with depth at all sites and years, but the decrease was less clear at Gåsö in 2013, resulting in a significant 'Site × Depth' interaction effect in late July and early August (Table 2). In Lindholm and Wallhamn densities at 1 m were significantly higher, while at all sites, densities were significantly lower at 3 m (SNK-test at $p < 0.05$; Fig. 5a). This difference between sites was also shown in the regression analyses that found a significant correlation between depth and densities in Lindholm and Wallhamn, but not in Gåsö in 2013 (Table 3).

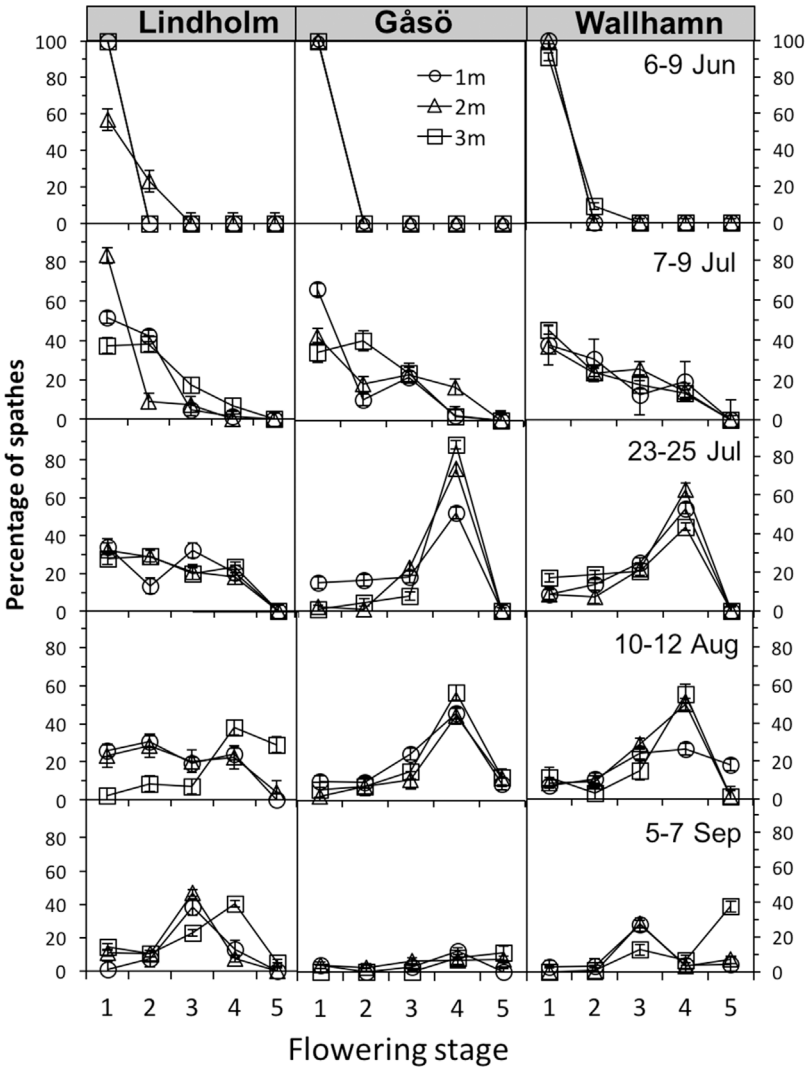

Fig. 4. Flowering shoot stage of development in three meadows from Jun to Sep 2013. Stages between 1–3 indicate pollination, stage 4 is seed maturation and stage 5 is seed release.

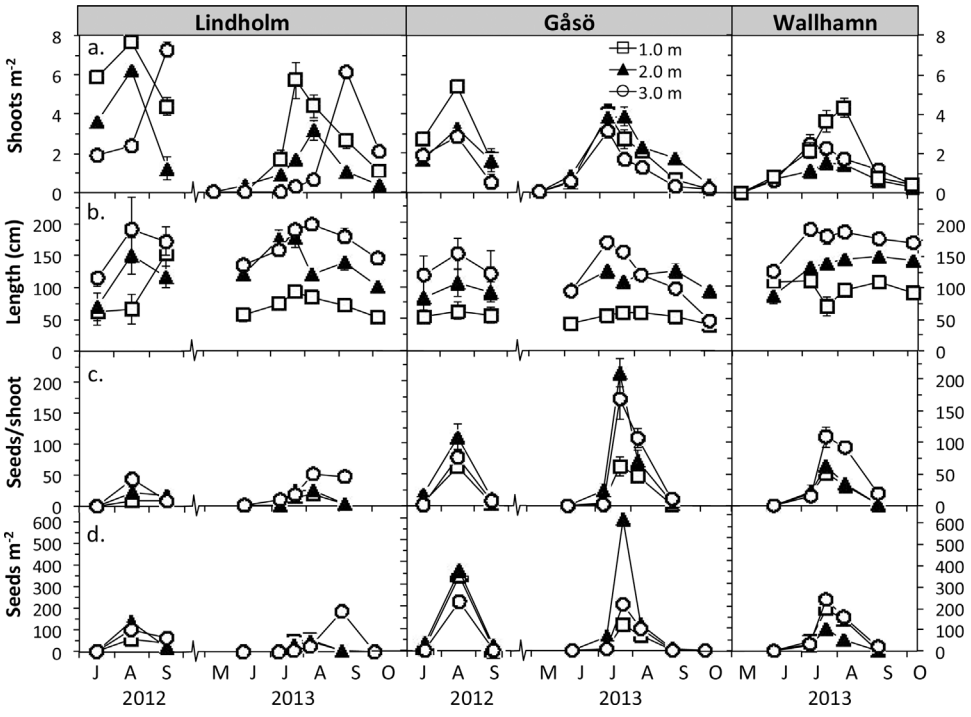

Fig. 5. Flowering shoot distribution by depth and site in 2012 and 2013. a) Flowering shoot densities, b) flowering shoot length, c) number of seeds per spathe and d) estimated number of seed per m^2. Mean ± Std. Error.

Table 2
Two-way ANOVA model testing the effect of site (Lindholm, Gåsö, Wallhamn) and depth (1, 2, 3 m) on flowering shoot morphology in 2012 (20-Aug) and 2013 (23-Jul and 8-Aug). Table shows degrees of freedom (d.f.) and significance (P) level. All data was sqrt-transformed.

Variable	Site	Depth	Site × Depth	Residuals
2012				
d.f.	1	2	2	
Shoot density	1.9	12.8****	2.4	114
Shoot length	124****	471****	46.5****	36
No seeds shoot^{-1}	23.7***	7.5**	8.2*	36
2013				
d.f.	2	2	4	
Shoot density	4.9**	25****	7.5****	735
Shoot length	61***	212****	0.9	111
No spathes (Stg 4 + 5)	50****	16*	0.6	111
No seeds spathe^{-1}	43****	8.6*	1.4	111
No seeds shoot^{-1}	64****	6.2	3*	111

* P < 0.05.
** P < 0.01.
*** P < 0.001.
**** P < 0.0001.

Table 3
Linear regression analyses at three sites from Jul-Sep 2013. Morphological characteristics as a function of depth (1–3 m) and shoot length. (df = 1,95 at Lindholm and Wallhamn; df = 1101 at Gåsö).

	Lindholm		Gåsö		Wallhamn	
	P	r^2	P	r^2	P	r^2
Depth						
Shoot L.	0.0001	0.63	0.0001	0.57	0.0001	0.52
Shoot density	0.0001	0.06	0.0690	0.01	0.0001	0.03
No seeds/shoot	0.0011	0.11	0.0353	0.04	0.0082	0.07
Shoot L.						
No spathes	0.0013	0.11	0.0001	0.18	0.0001	0.26
No seeds/spathe	0.0004	0.12	0.0240	0.05	0.0154	0.06
No seeds/shoot	0.0002	0.14	0.0018	0.09	0.0010	0.11

The length of the flowering shoots were relatively constant over the growth season, but increased with water depth, showing a significant, positive correlation with depth in all bays that explained 52–63% of the variation in 2013 (Fig. 5b, Table 3). In late July and early August of 2012 and 2013, shoot length differed significantly between all depths, being on average 71, 124 and 158 cm at 1, 2 and 3 m, respectively at all sites (Table 3; SNK-test at p < 0.05). In both years, the average shoot length also differed significantly between the sites. In 2012, shoot lengths were similar between Gåsö and Lindholm at 1 m (on average 66 cm), but significant longer at Lindholm than Gåsö at 3 m (on average 217 and 152 cm respectively) with a significant 'Site × Depth' interaction effect (Table 2). In 2013, shoot lengths were significantly longer at Wallhamn than Gåsö at 3 m (on average 190 and 150 cm respectively).

The number of spathes per shoot correlated significantly and increased with the shoot length, explaining 11–26% of the variation in 2013 (Table 3). Interestingly, the number of seeds per spathe and total number of seeds per shoot increased significantly with the shoot length (Table 3). In late July and early August, the number of spathes (stage 4 and 5) and the number of seeds per spathe were significantly higher at 3 m (Table 2; SNK-test at p < 0.05). The number of seeds per shoot also increased significantly with depth, although the regression only explains a minor part of the variation (4–11%; Fig. 5c; Table 2). In late July and early August, the number of seeds per shoot was significantly higher at 3 m at Gåsö and Wallhamn, but not at Lindholm, causing a significant 'Site x Depth' interaction effect (Table 2; SNK-test at p < 0.05). Although a similar decreasing trend with depth was found at Lindholm, the overall lower abundances of seeds at this site made the difference non-significant (Table 2).

The maximum seed density (seeds per m^2) was found from late July to mid August at all depth in both years, except at 3 m depth in Lindholm 2013 where seed production peaked in September. In general, the seed density was lower at 1 m than at deeper parts of the meadow and was often highest at intermediate (2 m) depths (Fig. 5d). However, the average seed production across depths from July to September differed dramatically between sites, being on average 3.4 and 2.5 times larger at Gåsö and Wallhamn (126 and 90 seeds m^{-2}, respectively) compared to Lindholm (39 seeds m^{-2}) in 2013. The lower seed production at Lindholm was a result of both significantly lower number of spathes per shoot (on average 5.3) and seeds per spathe (on average 2.1) compared to the other sites (on average 13.1–16.9 spathes and 3.5–5.2 seeds, respectively), irrespectively of depth in late July and early August (Table 2; SNK-test at p < 0.05).

The number of seeds released by flowering shoots stored in mesh bags showed variations between harvesting dates and depths. In 2012, on average 7 times more seeds were released from shoots harvested in August (62 seeds per shoot) than in early July or September at all sites and depths (Table 4). In 2013, the highest number of seeds collected at Gåsö and Wallhamn were released from shoots harvested in late July and early August at both depths (on average 98 and 73 seed per shoot, respectively). In contrast, shoots harvested at Lindholm at 1 m from late July to late August showed a low and similar release of seeds (8–13 seeds per shoot) whereas shoots collected at 3 m showed the highest release when harvested in late August (on average 106 seeds; Table 4). Shoots at 3 m released between 50 and 65% more seeds than shoots at 1 m at all sites. Plotting the number of seeds released per shoot against the development stage of the flowering shoots indicated a non-linear relationship where the number seeds released approached an upper asymptote when 50–60% of the spathes were in stage 4 (r^2 = 0.75, Fig. 6a). Analysis of seed sizes collected in Lindholm and Viks Kile in

Table 4
Seeds released per flowering shoot. Mean (Std. Error). Harvestings between July to September and shoot storage in mesh-bags for 2 months in outdoor tanks. Average* values are separated for Lindholm/Gåsö and Wallhamn., n = 10 flowering shoots.

Harvesting date		Lindholm				Gåsö				Wallhamn				Average*		
		1 m		3 m		1 m		3 m		1 m		3 m				
2012	**Jul 4–7**	**3**	(2)	**15**	(3)	**5**	(2)	**12**	(3)	–		–		**9**	/	**9**
	Aug 6–14	**19**	(54)	**54**	(7)	**59**	(18)	**117**	(24)	–		–		**36**	/	**88**
	Sep 6–11	**2**	(1)	**18**	(4)	**4**	(2)	**7**	(3)	–		–		**10**	/	**5**
2013	**Jul 23–25**	**8**	(3)	**24**	(8)	**99**	(28)	**127**	(37)	**54**	(19)	**112**	(36)	**16**	/	**98**
	Aug 1–2	**13**	(6)	**43**	(12)	**69**	(26)	**101**	(35)	**28**	(14)	**95**	(26)	**28**	/	**73**
	Aug 8–10	**7**	(4)	**24**	(5)	**26**	(9)	**58**	(22)	**13**	(5)	**61**	(22)	**16**	/	**39**
	Aug 19–20	**10**	(2)	**106**	(62)	**9**	(6)	**36**	(17)	**6**	(5)	**31**	(14)	**58**	/	**21**
	Sep 5–6	**1**	(1)	**21**	(16)	**3**	(2)	**5**	(3)	**3**	(2)	**9**	(3)	**11**	/	**5**
	Average	**8**		**38**		**34**		**58**		**21**		**61**		**23**	/	**42**

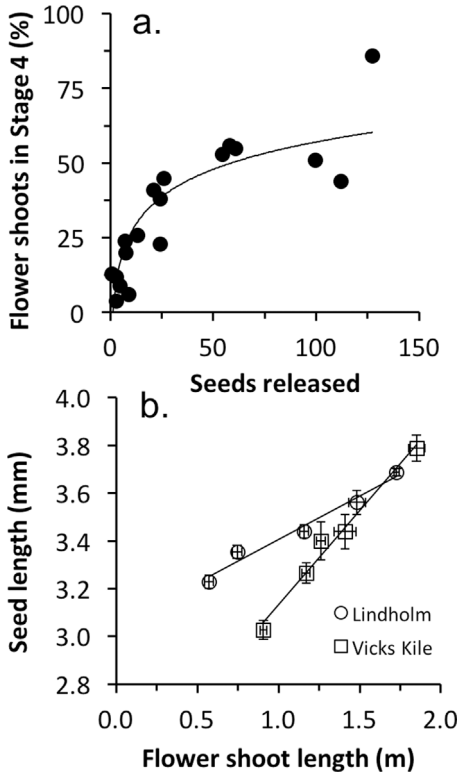

Fig. 6. Relation between the a) percentage of flowering shoots in stage 4 and the number seeds released and b) seed lengths.

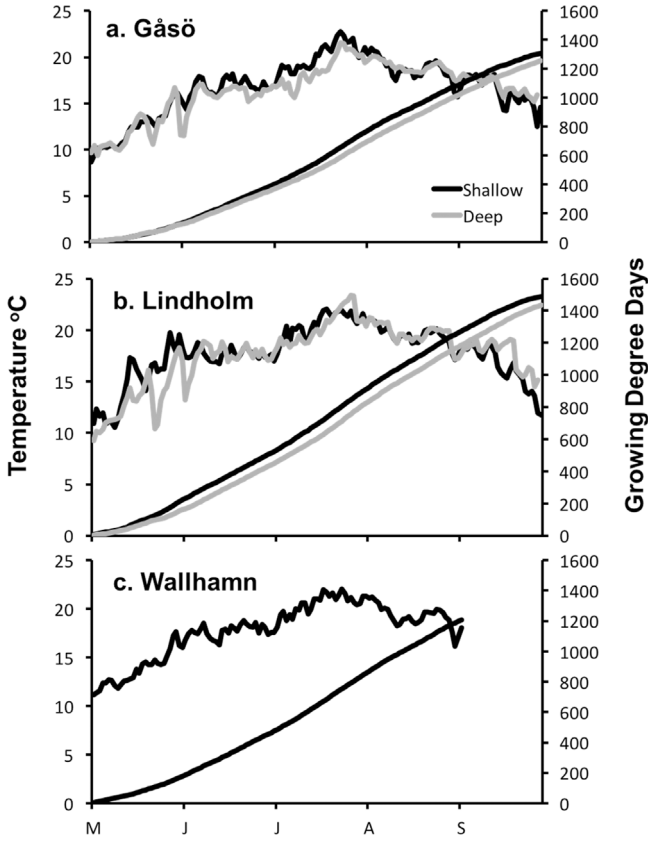

Fig. 7. Water temperature (daily mean) and growing degree-days time series at three bays. Black line is shallow (1 m) and grey line is deep (4 m). Data is missing for Wallhamn deep.

2013 showed similar seed lengths (3.0–3.8 mm) and a positive correlation between shoot length and seed lengths (Fig. 6b; $r^2 = 0.97$, $r^2 = 0.98$, respectively).

Water temperatures increased from daily averages ~10° C in the beginning of May to maximum values of 21.5–23.5 °C on the 25–30 of July 2013 (Fig. 7). From May to July, water temperature was often higher at 1 m than at 3 m, but in August the temperature pattern shifted being higher at 3 m than at 1 m. The cumulative GDD values varied between sites and depth and were on average 21–25% lower at Gåsö than the other two sites, and on average 11–14% lower at 3 m than at 1 m at the end of July when seed development peaked at most sites (Fig. 7). A rather wide range of GDD values were found when different flowering stages were present at the different sites and depths (Table 5). Seeds were visible and developing on the spathes when GDD was in the range of 549–762 and water temperature was 20–22 °C. Seeds were released when GDD was between 769 and 976. The late flowering development and seed release at Lindholm appeared to be only partly explained by lower temperature, since the GDD at 3 m (669) was higher than at Gåsö at 1 and 3 m (612 and 549, respectively) on 23–25 Jul when most flowering shoots had visible seeds (Fig. 4). However, the light conditions also differed between the sites, and the amount of surface light reaching the bottom in Lindholm it was only 59% and 23% at 1 and 3 m, while in Gåsö was 78% and 52%, respectively.

Table 5
Zostera marina flowering stages and growing degree-days (GDD).

		Stage	GDD	Sea °C
Anthesis	Inflorescence become visible	1	132–252	15.4–16.3
Pollen released	35% of styles erect	2	410–581	16.6–18.9
Seeds visible	50% of developing seeds visible	4	549–762	19.9–21.6
Seeds matured	Seeds are released	5	769–976	19.8–19.9
Spathes wither	Spathes separate from peduncle	6	1054–1303	17.9–18.1

3.2. Large-scale seed harvesting and seeds processing

Methods for harvesting large quantities of flowering shoots were assessed to evaluate the efficiency of the collection, storage and separation of viable seeds. Manually harvesting 200 flowering shoots required 1 h per diver (n = 48). In 2013, when shoots were harvested in the beginning of August, seeds were released in the storage tanks up to 2 months after harvesting, although the number and temporal pattern of seed release varied between harvesting sites (Fig. 8). Seed release from shoots harvested at Gåsö was highest during the first ten days (~130 seeds/day) and decreased over time. Seeds released in shoots from Viks Kile and Lindholm peaked (~100 seeds/day) a month later in early September, while in Wallhamn peaked in late September (~125 seeds/day). The quality of the seeds released from all sites was high during the first 30 days after harvesting (80–90% of viable seeds), but decreased after 40 days (60–65%) and after 50 days the quality was low (30–40%), also from sites that were still releasing a high number of seeds in the beginning of October (Fig. 8).

Although shoots harvested between 2012 and 2014 were processed with the same methods and similar time of the year (22-Jul–15-Aug), there was large variation between sites and years in the number of seeds released and the number of viable seeds (Table 6). In 2012 and 2013, the number of seeds released was relatively low from all sites (average 15 seeds shoot^{-1}), with 35–74% lower number of seeds from Lindholm (7.9–10.7 seeds shoot^{-1}) compared to the other sites (average 12.9–30.1 seeds shoot^{-1}). In 2014, shoots were harvested 2–3 weeks earlier and released on average 3.5 times more seeds than previous years (average 54.6 seeds shoot^{-1}), and with a higher seed release at Lindholm (up to 67.9 seeds shoot^{-1}) than at Gåsö (Table 6). The proportion of the released seeds that were classified as viable was fairly

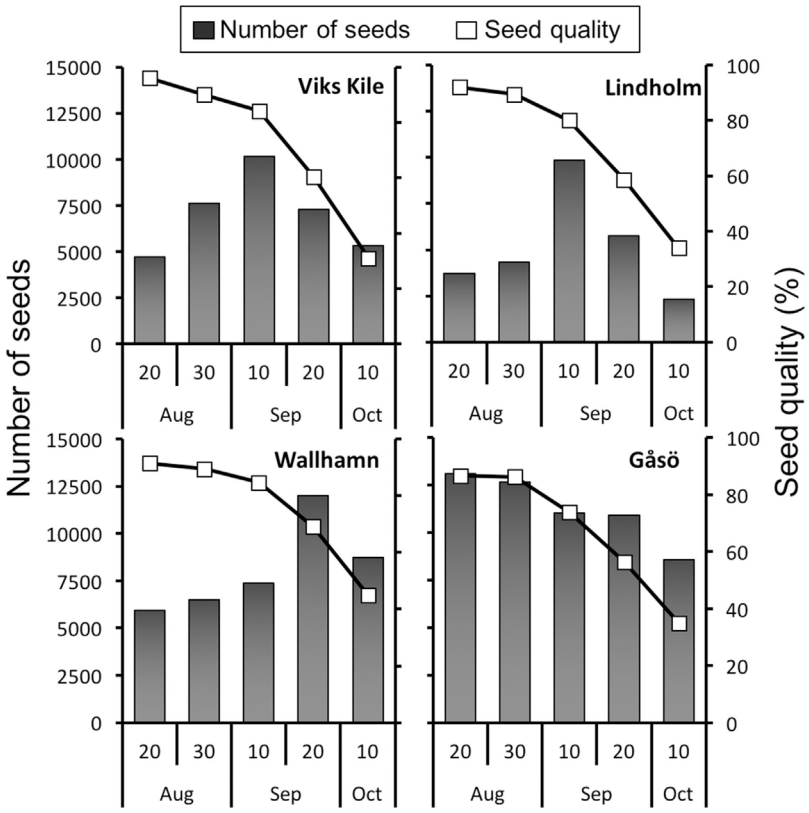

Fig. 8. Seeds released from the flowering shoots during storage in tanks at 10 days intervals during 2013. Seed quality is defined as those seeds with sinking velocities higher than 5.5 cm s^{-1}.

Table 6
Seed harvesting. Flowering shoots harvested and number of seeds collected during three consecutive years.

Site	Fjord	Date	No Shoots	No Seeds harvested	No Seeds viable	Seeds harvested/ shoot (viable)
2012						
Viks Kile	Stig	14-Aug	5050	82,300	60,600	16.3 (12)
Lindholm	Gullmars	15-Aug	1750	18,750	13,200	10.7 (7.5)
			6800	**101,050**	**73,800**	**14.9 (10.9)**
2013						
Viks Kile	Stig	4-Aug	2300	33,600	26,100	14.6 (11.3)
Lindholm	Gullmars	8-Aug	3000	23,700	17,700	7.9 (5.9)
Gåsö	Gullmars	10-Aug	1700	51,200	38,800	30.1 (22.8)
Wallhamn	Hake	9-Aug	2500	32,300	25,800	12.9 (10.3)
			9500	**140,800**	**108,400**	**14.8 (11.4)**
2014						
Lindholm	Gullmars	22-Jul	2500	111,098	82,400	44.4 (33.0)
Lindholm	Gullmars	23-Jul	4150	281,760	223,500	67.9 (53.9)
Gåsö	Gullmars	24-Jul	2350	98,560	81,400	41.9 (34.6)
			9000	**491,418**	**387,300**	**54.6 (43.0)**

constant between sites and dates (70–83%; average 77%) resulting in an average of 10.9–43 viable seeds produced per harvested shoot in the three years (Table 6).

The flume separation method showed that seed viability increased with sinking velocities (Fig. 3b). When sinking velocities were higher than 5.2 cm s^{-1}, 95% of the seeds were viable, but when the sinking velocities were lower than 4 cm s^{-1}, none of seeds were viable (Fig. 3b). The flow rate was related to the sinking velocities (R^2 = 0.99, Fig. 3b) indicating that viable seeds can be separated at flow rate higher than 0.45 L s^{-1} using this specific set-up.

4. Discussion

4.1. Temperature and light on flower development

Seasonal sampling of *Zostera marina* in the northwest coast of Sweden showed that flowering shoots first appeared in the beginning of June, with peak densities in July and August. Although eelgrass showed a high variation in the flowering patterns across environments, where significant differences were found between water depth and some phenological characteristics, the timing of flowering and seed maturation were similar. Mature seeds were released (stage 5) at all sites from August to September during 2012 and 2013. Silberhorn et al. (1983) described an increasing delay in seed maturation along a latitudinal gradient where maturation occurred from April in N. Carolina (latitude 35°) to July in Nova Scotia (latitude 44°). A recent review suggests that this latitudinal trend may be mainly driven by temperature (Blok, 2016). The high latitude of the Swedish west coast (58°) where winters are characterized for short light-hours per day and cold temperatures below zero degrees could explain the late maturation compared to other locations where seed maturation is reached earlier in the season.

Anthesis of eelgrass flowering shoots occurred at 15–16 °C at the study sites and seed maturation first occurred in early August when temperature was 20 °C. This range of temperatures is similar as previously reported for other locations (De Cock, 1981; Phillips and Backman, 1983; Silberhorn et al., 1983; Furman and Peterson, 2015). Since water temperature is a dominant factor controlling flowering shoot development in seagrass (De Cock, 1981; Thayer et al., 1984), a heat accumulation index (growing degree-days, GDD) was used to assess if it could predict seed development and harvesting times for eelgrass restoration. This type of index is widely used in agriculture (Bonhomme, 2000), but as far as we know, has not been applied earlier in seagrass ecology. Although we detected a large variation in flowering shoot development in 2013 (Fig. 4, Table 4), water temperature and GDD appeared to explain only part of this variation. For example, the late development and release of seed from shoots at 3 m depth at

Lindholm compared to the other sites could not be explained by GDD alone since it was similar or higher at Lindholm. Likely other factors such as light, nutrients, salinity or genetics also influenced the observed variation in shoot development. For example, studies have shown a positive relation between sediment ammonium enrichment and eelgrass flowering shoot development (Johnson et al., 2017), and that flowering occurrence can be reduced or inhibited by low irradiance (Backman and Barilotti, 1976; Johnson et al., 2017). In the present study, light attenuation at Lindholm was substantially higher than at Gåsö, and the light conditions at 3 m depth at Lindholm (23% of surface light) is close to the minimum light requirement for eelgrass, around 20% (Dennison and Orth, 1993; Eriander et al., 2016). Thus, low light conditions may have contributed to the late development at Lindholm. In addition, this eelgrass meadow was heavily fouled by mats of filamentous macroalgae in the spring of 2013, which may have further reduced the light conditions, and possibly caused hypoxic stress on the plants, which may also have affected flower development. The unusually low production of seeds in this meadow in 2013 (on average 23.5 seed m^{-2}) was a result of flowering shoots with significantly fewer spathes with fewer seeds (on average 5 spathes $shoot^{-1}$, 2.1 seeds $spathes^{-1}$) compared to the other sites, which may indicate stressed plants. These algal mats have increased dramatically along the Swedish Skagerrak coast since the 1980s (Pihl et al., 1999) as a result of nutrient pollution and loss of large predators along the coast, causing a trophic cascade that release algal mats in eelgrass beds from grazing control (Moksnes et al., 2008).

Although we did not find a strong effect of GDD in the present study, we encourage further studies to test and apply the GDD index when comparing development between regions or during restoration to determine the optimal harvesting time. In the present study, temperatures were rather similar between different sites and depths, and GDD may be more useful when comparing development between regions with stronger temperature gradients. It may be particularly useful when assessing how flower development differs along latitudinal gradients. However, in such comparisons, it is critical to select an appropriate *Tbase* for the calculation of GDD since it could shift the baseline leading to higher or lower values.

4.2. *Zostera marina* flowering patterns

Eelgrass showed a high variation in the flowering patterns across environments, where shoot density, shoot length and seeds production varied strongly between sites and depths. Flowering shoot densities decreased with water depth at all sites, while leaf length increased with depth. Shoot densities peaked with an average of 3.8 ± 0.5 shoots m^{-2} with maximums of 21 shoots m^{-2}. These densities are on several orders of magnitude lower (10–100) compared to other reported locations. For example, in Denmark, densities of flowering shoots peaked in May and June with mean densities between 19 and 79 shoots m^{-2} (Olesen, 1999; Olesen et al., 2017), while in the Cheasapeake Bay, shoot densities varied between 100 and 200 shoots m^{-2} (Marion & Orth 2010). In Mexico, where the annual populations rely on seed production, densities could reach 555–2636 shoots m^{-2} (Phillips and Backman, 1983). Flowering shoot lengths correlated positively with depth, from on average ~70 cm at 1 m depth to on average ~160 cm at 3 m depth, where up to 239 cm long shoots were encountered. This result is consistent with earlier studies of eelgrass in Sweden where transplanted vegetated shoots show a plastic response to light levels, and invest in apical growth rather than lateral growth in low light conditions, and vice versa (Eriander et al., 2016; Eriander, 2017). This is a common response in seagrass to depth, which has been reported on vegetative shoots and flowering shoots as a strategy to increase light availability on the leaves by being closer to the water surface to capture more light and reduce self-shading (Krause-Jensen et al., 2000; Bintz and Nixon, 2001; Koch, 2001).

The number of spathes per shoot, seeds per spathe and the size of the seeds correlated significantly with the length of the flowering shoot. Since the length of the flowering shoot increased with depth, and larger seeds contain higher starch and nutrient content that may increase seedling survival (Moles and Westoby, 2004), the tall shoots at 3 m depth may produce higher quality seeds. In contrast to shoot density, the production of seeds per shoot were more similar to what has been reported in other parts of the world. The number of spathes per shoot (on average 16–23 in Sweden) is in the higher end compared to other areas (2.5–18 spathes per shoot; (Phillips and Backman, 1983; Phillips et al., 1983; Cabaço and Santos, 2010; Olesen et al., 2017). The number of seeds per shoot in Sweden was between 39 and 126, which is also in the range of other studies such as 15–99 seeds per shoot (Phillips and Backman, 1983), 19–41 (Olesen et al., 2017), or 20–100 (Marion and Orth, 2010).

Because shoot density and seed production per shoot showed opposite trends with depth, seed production per m^2 was often highest at intermediate depths in the meadow (2 m), with the exception of Lindholm in 2013 that showed the highest flowering shoot density and seed production at 3 m depth in the fall (Fig. 5). Higher seed production at intermediate and deeper depths within a meadow has also observed in other studies (Cabaço and Santos, 2010; Kim et al., 2014; Olesen et al., 2017) indicating that this intermediate zone might provide stable grounds that increase the resilience of the meadow (Olesen et al., 2017). Due to the unusually low density of flowering shoots in the study areas, the seed production along the Swedish west coast (on average 140–600 seeds m^{-2} during peak release periods) is up to an order of magnitude lower compared other populations. For example in Denmark, South Korea and North Carolina the production was 763–2000, 6000–10,000 and 8500–12,700 seeds m^{-2} respectively (Jarvis et al., 2012; Kim et al., 2014; Olesen et al., 2017) while in Mexico annual populations produced 20,000–37,000 seeds m^{-2} (Phillips et al., 1983).

An optimal strategy for many clonal plants, such as seagrasses, is to allocate resources to both vegetative and sexual propagation (Den Hartog, 1970). Sexual reproduction and seed dispersal could be an adaptive strategy to escape the competitive effect of clonal spread in order to colonise new environments, including bare patches within meadows. The unusual low seed production may suggest that sexual reproduction could be less important along the Swedish NW coast compared to other areas. However, recent analyses of the genetic diversity in eelgrass from the Gullmars Fjord showed an allelic richness and genetic/clonal diversity that was considered average for the East Atlantic region, with an estimated linear clone size of 2–10 m (Eriander et al., 2016). Thus, there is no indication that the low seed production is affecting genetic diversity and connectivity of eelgrass in the study area.

Seed production could be negatively affected by the mats of filamentous algae, which were observed to cover large areas of the eelgrass meadows during the reproductive season. Since water flow is the main driver for pollination in seagrass, but see van Tussenbroek et al. (2016), ephemeral algal mats covering flowering eelgrass shoots could reduce water circulation and pollen dispersal between shoots, and thus reducing the number of ovules that are fertilized. In addition, the low flowering shoot density could by itself affect seed production since Reusch (2003) showed that eelgrass pollination success was dependent on flower density. Since the average number of seeds per shoot in Sweden was similar to earlier studies, algae preventing pollination does not likely explain the overall low seed production in Sweden. However, it may explain the lower production of seeds at Lindholm in 2012 and 2013 compared to the other sites. These plants had a low number of seeds per spathe.

4.3. *Implications for eelgrass restoration*

The demonstrated large variation in flowering shoot density, seed production and development time have several important implications for large-scale restoration of eelgrass using seeds, and show the importance of careful assessment of flowering shoots before harvesting is

started. For example, when assessing a suitable donor bed for harvesting, flowering shoot density alone might be a poor indicator of the number of seeds available in a meadow, since large variations in the number of spathe per shoot, number of seeds per spathe and seed viability were observed. In the study area, shoot density generally decreased with depth, whereas the number of seeds per shoot increased with depth giving the highest production of seeds per surface area at intermediate (2 m) depth in many meadows. Thus 2 m may be the optimal depth for an efficient harvesting. Because seed production could vary between depths, meadows and years, it is still critical to survey each potential donor meadow to determine the optimal harvesting location.

It is also important to assess the development stage of the flowering shoots to optimize the harvest. If the shoots are harvested too early, few seeds will develop during storage, and if harvested too late, few seeds will remain on the shoots. Based on the results in the present study, we recommend that flowering shoots are harvested when more than 50% of the spathes are in stage 4, since seed release was higher at this development stage (50–140 seeds shoot^{-1}; Fig. 6). Along the NW coast of Sweden, this development stage occurred in late July early August in 2012–2014.

Harvested flowering shoots were kept in tanks located on land to produce seeds for restoration, similar to storage facilities used in large-scale eelgrass restoration projects in the USA (Marion & Orth 2010). However, in the present study we carefully monitored the release of seeds to assess how production was affected by harvesting date and time of storage. Estimated seed release per shoot in the storage tanks was similar (± 10%) to seed release from shoots harvested at the same time but kept in net bags with better water circulation, indicating that the storage condition in the tanks did not decrease production of seeds. However, prolonged storage (> 30 d) of flowering shoots resulted in a sharp decline in seed quality, and after 2 month < 50% of the released seeds were viable (Fig. 8), suggesting that longer storage may not be productive. The loss of seed viability over time could be a natural process, but was likely enhanced by the conditions in the tank where shoots were densely packed resulting in low water circulation and nutrient availability, and degradation of the shoots. Nutrients in the sediment such as ammonium have shown a positive relationship with the number of spates per flowering shoot (Johnson et al., 2017). The degradation may also be enhanced by the fact that the shoots were detached from the rhizomes and its supply of nutrients. However, the production of viable seeds was still relatively high during the first 2 month of storage, and the storage densities used (up to 2 shoots L^{-1}) is likely the most economical option since larger tank space will be needed to store the harvested material at low densities.

Although the seed release per shoot varied strongly between harvesting dates and sites (Table 4 and 6), the proportion of viable seeds was fairly constant in each harvest (77 ± 3.7%, mean ± SD). Comparison of the number of immature seeds per shoot measured in the flowering shoots and the number seeds released in tanks from shoots harvested at the same dates, sites and depths, suggest that after harvesting on average 60% of the seeds are released in the tanks, and that 46 ± 15% of the seeds on the shoots at harvest result in viable seeds. These results provide valuable data to be able to estimate the amount of flowering shoots needed to obtain a specific quantity of viable seeds for restoration.

Identifying and separating viable seeds is important for ensuring that the right amount of seeds are obtained for restoration. In the present study, a new method using a vertical flume was assessed. The flume method was efficient in accurately separating large quantities of viable seeds (Fig. 3). Seed sinking velocities were related to volumetric flow rates in the vertical flume. Seeds with sinking velocities above 5 cm s^{-1} had high viability while seeds with a sinking velocity lower than 4 cm s^{-1} were not viable. These values are similar to the ones reported by Marion and Orth (2010), where seeds with sinking velocity higher than 5.5 − 6 cm s^{-1} showed a higher germination rate and produced seedlings, while velocities below 4 cm s^{-1} showed low germination. The vertical flume in the present study was efficient in separating viable seeds from both low quality seeds and the large amount of organic debris accumulating on the bottom of the storage tanks, and can be recommended for large-scale restoration. A horizontal flume was applied in an earlier study to separate eelgrass seeds based on seed density Marion and Orth (2010). In this study, we provide a detailed quantitative relation between flow rate, seed sinking velocity and viability that can be used to separate eelgrass seeds that could be further tested with other seagrass species. However, it is important to note that sinking velocities of viable seeds may differ between regions due to differences in seed morphologies (eg, size, weight) and that the required volumetric flow rates of the flume will depend on its design.

In the present study, shoots were harvested manually one-by-one using snorkelling or diving where a person could harvest on average 200 shoots per hour. This results in ~10,000 immature seeds h^{-1} harvest from the more productive meadows, equivalent of ~4600 viable seeds h^{-1} (60% of the seed being released of which 77% are viable). This seed collection rate is lower than manual harvesting of flowering shoots in the Chesapeake Bay area, USA (on average 16,000 seeds/person h^{-1}), but is still relatively high considering that the density of flowering shoots are about 25–50 times higher in Chesapeake Bay with 100–200 shoots m^{-2} (Marion and Orth, 2010). A substantially higher harvesting yield was obtained using mechanized harvesting system (55,000–132,000 seeds/person-hour) and it was used successfully for large-scale restoration in Virginia, USA (Marion and Orth, 2010; Orth et al., 2012). However, such mechanical harvesters are likely less suitable for Swedish eelgrass meadows considering the substantial lower densities of flowering shoots (on average 6 shoot m^{-2}). Since mechanical harvesters cut all shoots, vegetative shoots will constitute ca. 90% of the harvest in Sweden and require 10 x larger storage facilities for shoots than if they are collected by hand. Moreover, mechanical harvest along the NW coast of Sweden is complicated by sloping bottoms with high amounts of rocks and ephemeral algal mats. In such systems, manual harvesting is likely the most efficient method, which also has the advantage of minimizing the impact on the donor meadow. Recent studies showed no detectable effects on shoot densities one year after all flowering shoots had been harvested by hand from replicate 100 m^2 areas in NW Sweden (Moksnes et al., 2016).

Although the present study demonstrates that viable eelgrass seeds can be produced from Swedish meadows, there are challenges before seeds can be recommended for large-scale restoration in this area. Recent studies show that the loss of eelgrass seeds are very high in Scandinavian waters due to transport by hydrodynamics, burial by lugworms and predation from shore crabs (Valdemarsen et al., 2011; Infantes et al., 2016a; Infantes et al., 2016b). The average seedling establishment rate from over 20 restoration tests along the Swedish NW coast using hand broadcasted seeds has been < 1%, with almost no surviving shoots (Moksnes et al., 2016). However, burying seeds in the sediment increases seed survival (Marion and Orth, 2012; Infantes et al., 2016a; Infantes et al., 2016b), and studies are encouraged to develop methods that can plant large quantities of seeds in the sediment to facilitate large-scale restoration using seeds in Scandinavian waters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the

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