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Investigating perennial & annual eelgrass (*Zostera marina* L.) in Padilla Bay as potential donor sources for seed-based restoration

By

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FIELD PROJECT

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December 8, 2023

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All images were taken by myself or Sylvia Yang, unless otherwise noted.

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ABSTRACT

This paper explores recovery of eelgrass (*Zostera marina* L.) meadows through seed-based restoration to address declines from natural and anthropogenic disturbances. The study focuses on the Joe Leary meadow site in Padilla Bay, Washington, aiming to (1) determine the spatial distribution of perennial and annual *Z. marina*, (2) understand variation in flowering shoot density and potential seed production, and (3) compare the phenology of flowering shoots. These objectives were investigated using field-based methods to collect presence/absence transect data for delineation, quadrat-based count data, and monitor seed development. Data collection occurred in the summer throughout the flowering life stage of eelgrass in the site. The study found spatial and temporal variation in the distribution, density, and phenology of flowering perennial and annual populations. Delineation methods revealed that annuals have a similar range in the intertidal as perennials, with differences highlighted in response to potential stress indicators such as proximity to the slough, algal mats, or elevation. Quadrat-based monitoring found annual shoots in the transect higher in the intertidal, above the perennial population. Phenology data indicated that perennial shoots flower about a month prior to annual shoots. While the study advises against using perennial flowering shoots for restoration due to low densities, it emphasizes Joe Leary's potential as a donor site for flowering annuals for seed-based restoration. Careful considerations are crucial to avoid unintended introductions of non-native species. The research concludes with recommendations for refining delineation methods, enhancing understanding of eelgrass population dynamics, and optimizing seed collection strategies for genetically diverse and resilient restoration in the face of climate change.

INTRODUCTION

Seagrasses, which are flowering marine plants, perform important ecosystem functions (Koch 2001), making them an important habitat for many marine birds, invertebrates, and fish (Bertelli and Unsworth 2014). Fringing the coastlines of every continent, excluding Antarctica, seagrass is an indicator of marine ecosystem health worldwide (Unsworth et al., 2022). As well as providing habitat, the above-ground foliage of the plant can attenuate energy from waves (Fonseca and Cahalan 1992), which decreases turbidity and increases light availability (Koch 2001). Like terrestrial vegetation, seagrasses photosynthesize and produce oxygen (Dennison 1987). The rhizomes of seagrasses also play a role in sequestering carbon, entrapping it within the sediment (McLeod et al. 2011; Dahl et al. 2016).

Eelgrass (*Zostera marina* L.) is the dominant seagrass species in soft-sediment habitats throughout the Northern Hemisphere. The foliage of eelgrass plays a role both as a rearing habitat and as a spawning ground for fish species, like Pacific herring (Lemberg et al. 1997). Over the last fifty years, there have been accelerated seagrass declines worldwide (Waycott et al. 2009). In the Pacific Northwest USA, areas like the San Juan Islands have displayed disproportionate decreases in the seagrass species *Zostera marina* L. (eelgrass) (Christiaen et al., 2022).

With eelgrass meadows undergoing natural and anthropogenic pressures, it is important to understand natural processes of recovery (Figure 1). In response to a microalgal bloom on the South coast of Korea, a major die-off in perennial eelgrass was re-established via natural seedling recruitment from the existing seed bank (Lee et al. 2007). Following an anoxic event in the French Mediterranean Sea in 1998-1999, a perennial bed was recolonized within a year due

to vegetative recruitment and a high seedling survival rate (Plus et al. 2003). Following another anoxia event in Denmark, 4% of the plants were from the surviving population, and the remaining 96% were seedlings, even though rhizomes were evident below the sediment (Greve et al. 2005). Degrading perennial meadows in the Bohai Sea, China, showed clonal reproduction within the meadow and seedling recruitment in the gaps between meadows (Xu et al. 2020). These studies all show that sexual recruitment is critical in the recovery of eelgrass populations in areas of high disturbance. With increasing frequencies and severities of extreme events from climate change, perennial *Z. marina* populations may be increasingly dependent on the previous years' flowering and recruitment from seed (Johnson et al. 2021).

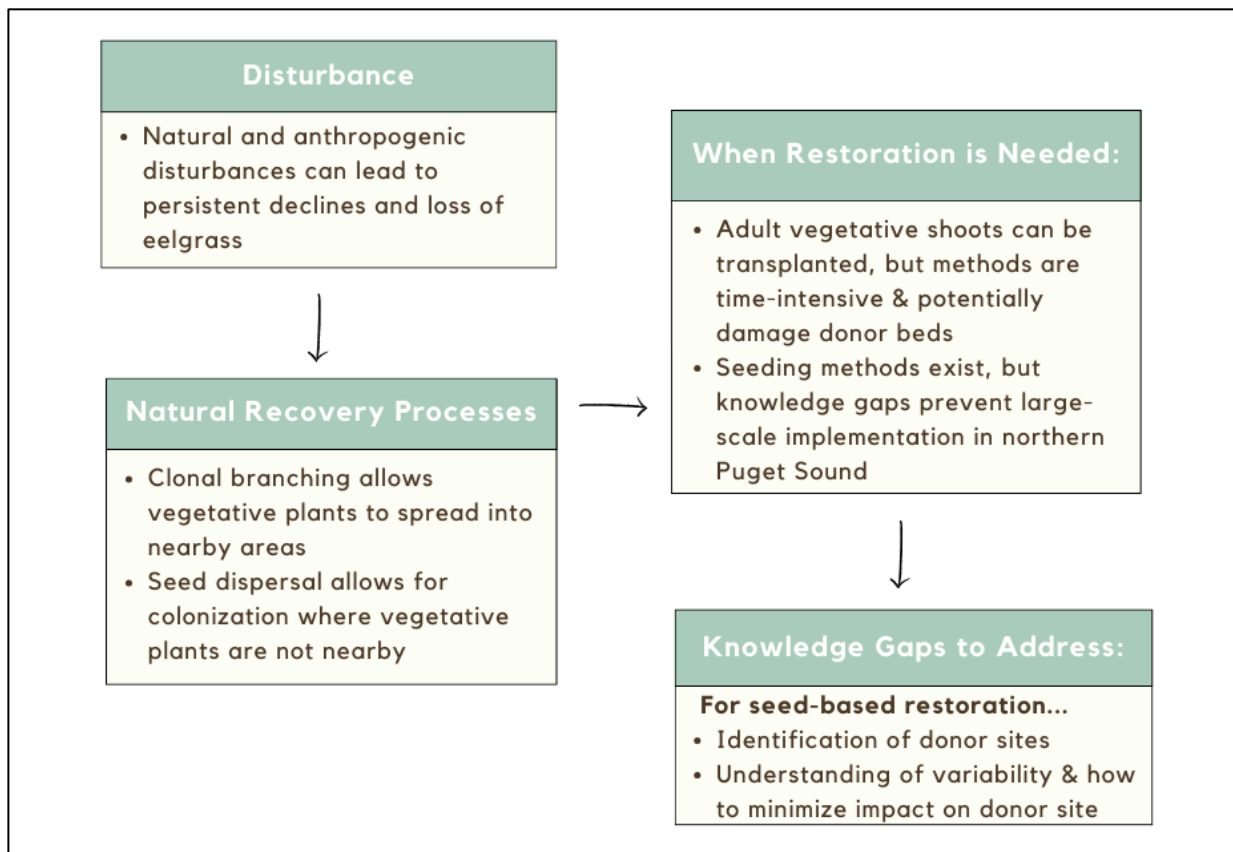


Figure 1. Flow chart showing the status of eelgrass meadows and the process of initiating recovery through large-scale restoration in northern Puget Sound.

Because seagrasses can use both asexual and sexual reproduction strategies to recover from disturbances (Lee et al. 2007), restoration projects may involve both transplanting adult plants and seeding methods. Each restoration method has advantages and disadvantages when it comes to large-scale restoration.

Transplanting adult vegetative shoots is a commonly used method (Davis and Short 1997; Short et al. 2002; Zhou et al. 2014), though it has disadvantages when looking at large-scale restoration because it is consuming in both time and resources and has potentially deleterious effects on donor beds (Orth et al. 2007). Using multiple donor sites to source transplants, however, has been shown to effectively increase the genetic diversity of transplanted meadows. Resilient meadows have been restored successfully using the transplant method (Thom et al. 2012).

Within the last decade, restoration efforts using seeds have become increasingly prominent, also spanning several countries (such as Govers et al. 2022 (Netherlands), Unsworth et al. 2019 (Wales); Infantes et al. 2016 (Sweden); Tanner et al. 2016 (Australia); Zhang et al. 2015 (China); Harwell and Orth 1999 (USA)). Seed dispersal is a necessary part of the expansion and colonization of areas completely devoid of eelgrass (Jørgensen et al. 2019). Colonization through seed propagation increases a population's genetic diversity (Williams 2001; Reynolds et al. 2012), enhancing long-term resilience to stress (Orth et al. 2007; Jørgensen et al. 2019). Areas sown with seeds for restoration have been recorded to be more genetically diverse than natural meadows (Hori and Sato 2021) or sites restored with transplant methods (Williams and Davis 1996).

However, restoration by seeding also has challenges. Seeds might be eaten by predators (Fishman and Orth 1996; Orth et al. 2006b), buried too deep by infaunal bioturbation

(Valdemarsen et al. 2011), removed by hydrodynamic forcing (Statton et al. 2017; Gräfnings et al. 2023) or succumb to disease (Govers et al. 2017). Then, of the remaining seeds, only a percentage germinates. Optimum environmental conditions, such as light and oxygen levels and appropriate burial depth, must be met for successful seedling establishment and growth (Moore et al. 1993). Extensive research is occurring to reduce seed loss and improve these low rates of recruitment (Moore et al. 1993, Blackburn and Orth 2013, Jarvis and Moore 2015, Wang et al. 2016, Jørgensen et al. 2019). Laboratory conditions executed prior to seed planting, like cold stratification and hyper-salinity shock baths, have been shown to increase germination rates, thus leading to higher rates of seedling emergence (Liu et al. 2016; Xu et al. 2016). Burying the seeds in the sediment reduces seed loss and is another method used for more efficient and effective seeding strategies (Jørgensen et al. 2019; Govers et al. 2022).

A limiting factor for seed-based restoration is identifying donor sites to obtain large numbers of seeds because, in the field, seed germination and seedling survival is naturally very low (Robert et al. 2003). Studies show that using the broadcast seeding method, only 1-10% of seeds will become established seedlings (Orth et al. 2006d). Thus, millions of seeds must be used to yield the desired outcome of a self-sustaining population (Busch et al. 2010; Govers et al. 2022), and potential seed donor sites must have sufficient flowering shoots available for collection required for this scale. Seed banks are a substantial contribution to the resiliency of a meadow in the face of adversity, as mentioned previously, with the examples of recovery following major diebacks. The impact on the donor site for seeds is not well known, so seagrass scientists should consider harvesting guidelines for other wild plants when extracting flowering shoots for seed collection (Unsworth et al. 2023).

The density and distribution of flowering shoots within a site are not homogenous (Harwell and Orth 1999; Morita et al. 2007) and are influenced by several factors. For example, meadows with more available light have a higher fecundity (Johnson et al. 2017; Cabello-Pasini et al. 2003). Additionally, the phenology of flowering shoot development and seed production depends on environmental cues like seasonal increases in ocean temperature (Combs et al. 2020) and decreases in salinity (Philips et al. 1983a). These act as environmental indicators for the flowering cycle to begin (Philips et al. 1983; Thom et al. 2003).

These thresholds can vary regionally with later development in colder, northern latitudes (Blok et al. 2018). Different genotypes that have adapted different temperature requirements could also cause the variation in timing between latitudinal gradients (Philips et al. 1983). Tidal depths, temperature, light availability, and soil requirements influence temporal and spatial variations in flowering shoot density between and within populations (Jacobs and Pierson 1981). In one site in France, the upper intertidal flower production started one month earlier than in the lower intertidal, but the development of flowers and seeds occurred more quickly in the lower bed (Jacobs and Pierson 1981). A similar delayed trend followed by a quick maturation can be seen in flowering annuals with a decrease in shoot length (De Cock 1980). Annual flowering shoots have also been observed to peak higher in the intertidal gradient, where there is more exposure to air at low tide compared to areas lower in the intertidal (Keddy 1987).

Understanding the timing behind a population's flowering development is critical to consider when harvesting the flowering shoots to optimize seed yield. Flowering shoots should be harvested when at least 50% of the spathes are observed to be in Stage IV (Infantes 2018; De Cock 1980), which is after pollination and the seeds are maturing but have not yet dropped out (Table 2). However, applying a standardized developmental timeline for flowering shoot and

seed development is difficult because of the inherent variation within each population, between the individual flowering shoots, and even between inflorescences on the same shoot (Jacob and Pierson 1981).

Additionally, while some eelgrass populations are annual and die back in the winter, most populations are perennial and provide year-round habitat (Phillips et al. 1983). Annual populations are often in areas associated with disturbance, such as the intertidal (Qin et al. 2014). This is not always the case, however, and some annual populations have been observed to be in deeper areas, with perennials residing in shallower depths, potentially due to the stress of low light availability in the deeper regions (Kim et al. 2014).

Depending on whether a population is composed of annual or perennial plants, or both, will influence the density and distribution of flowering shoots. In the Gulf of California, for example, of the perennial plants, 10-45% flower (Cabello-Pasini et al. 2003), while 100% of the annual plants flower (Meling-López and Ibarra-Obando 1999; Santamaria-Gallegos et al. 2000; Felger and Moser 2016). Due to the high productivity and prevalence of flowering shoots in nearby meadows, eelgrass seeds are a subsistent part of the Seri (Comcáac) peoples' diet (Felger and Moser 1973). Subtidal perennial populations have been shown to respond to stress by putting energy towards reproductive effort (Qin et al. 2014). An increase in sexual reproductive effort is observed when plants face disturbance or stress (Qin et al. 2014; Cabaço and Santos 2012). Annuals are found in intertidal areas with higher rates of disturbances and stressors, particularly in the upper intertidal (Qin et al. 2014) and have higher flowering rates than perennials. Algal mats are another component that might influence flowering shoot densities and development (Infantes and Moksnes 2018) by influencing light availability.

Seed production varies between perennial and annual populations (Keddy 1987; Morita et al. 2007). Fluctuations in depth have been shown to be an influential factor in seed production, with subtidal perennial plants observed to produce more seeds per shoot than intertidal annual plants (Phillips et al. 1983; Harrison 1993; van Lent and Verschurre 1994). Research in Korea has found that there are more spathes per shoot *and* more seeds per spathe in perennials than annuals. Still, the seed production per area was estimated to be higher in annual populations due to their higher flowering shoot densities (Kim et al. 2014). In eelgrass populations in Nova Scotia, Keddy (1987) counted seven times more seeds in a given area in an annual bed than in the same area in a perennial bed.

Both perennial and annual populations provide different services and attributes to their habitat. Underground rhizomes of perennial eelgrass reduce erosion in sandy sediments (Infantes et al. 2022) comparable to tree root systems. Higher genetic diversity is found in annual meadows than in perennial populations (Muñiz-Salazar 2005), and genetic diversity is positively correlated with increased resilience (Plaisted et al. 2020). Thus, intentionally incorporating seeds from perennial and annual eelgrass meadows may enhance the success of seed-based restoration.

Overview of this Study

In Padilla Bay (Washington, USA), both perennial and annual life histories of *Z. marina* are found. This study's aim is to identify the potential for the Joe Leary site in Padilla Bay, WA, to serve as a seed donor site through the collection of perennial or annual flowering *Z. marina* shoots. Flowering perennial shoots start to develop early summer, but little is known about the timeline for annual flowering shoots develop.

In this study, we seek to use data from 2023 from the Joe Leary meadow site to (1) determine the geographic distribution and areal extent of flowering perennials and annuals; (2) understand the variation in flowering shoot density and potential seed production, and; (3) compare the flowering shoots phenology.

Based on the literature reviewed it is hypothesized that (1) the distribution of the annual *Z. marina* population could be found higher in the intertidal, or potentially lower in the intertidal due to reduced light from macroalgae presence; (2) the flowering shoot density and seed production per area will be higher in annuals than in perennials, even though more seeds will be produced *per shoot* in flowering perennials; (3) the development of annual flowering shoots and seeds will begin after perennials, but the timescale of this is unknown.

METHODS

FIELD SITE

Padilla Bay contains the largest contiguous eelgrass meadow in the lower 48 states of the USA (over 3,000 hectares of *Zostera* sp., Bulthuis 2010). *Zostera marina* areal extent has been stable or increasing over time (Christiaen et al. 2022), and flowering shoots and seedlings are present (Yang et al. 2013). *Z. marina* distribution has been mapped throughout Padilla Bay (Webber et al. 1987; Bulthuis 1995; Bulthuis and Shull 2002, 2006; Christiaen et al. 2022), but these maps do not include spatial distribution or variation in flowering and seed production. Variation in flowering shoot abundance and phenology has only been documented at individual sites (Flem, 2020).

Recently, a dense bed of annual *Z. marina* was observed downstream of the Joe Leary Slough (Figure 2), an agricultural drainage channel that is currently the main freshwater input into Padilla Bay (Tierney-Fife, 2022). A perennial *Z. marina* meadow also occurs at this site. Because of the potential for annual *Z. marina* beds to produce more seeds than perennial beds and provide increased genetic variation, the *Z. marina* beds downstream of the Joe Leary Slough have become a geographic location of interest for research.

The methods of this research will integrate the data from the following four field methods that were conducted during the summer of 2023 (Delineation Transects and Mapping; Quadrat-Based Transects; Phenology of Flowering Shoot Development; and Collection and Ripening of Flowering Shoots for Seed) in the Joe Leary meadow site in Padilla Bay (Figure 2)



Figure 2. Sitemap showing where the Joe Leary site is located in Padilla Bay. This map shows where the delineation transects (JL0-JL6) are and the quadrat-based transects (A, B & C) that were used for density counts and phenology observations of flowering shoots, the results of which guided their collection.

FIELD METHODS

Delineation Transects and Mapping

To map the spatial distribution of perennial and annual *Z. marina*, ground-based delineation methods were adapted from the Washington State Department of Natural Resources' Submerged Vegetation Monitoring Program (WA DNR's SVMP).

WA DNR's SVMP consists of towing an underwater video camera behind a boat, traveling along transects across an eelgrass meadow. Staff observers review the video footage and have been trained to identify the following seagrass species: *Z. marina*, *Z. japonica*, and *Phyllospadix sp.* Presence of each species is recorded every 1 meter along each transect. From these data, the area distribution and depth limits of *Z. marina* are estimated, and from which change is calculated.

However, near-shore edges of Padilla Bay's seagrass meadow cannot be delineated using this method because depths are too shallow for the SVMP vessel, and analysis of the video footage does not dictate whether the eelgrass is in a vegetative (flowering) or non-vegetative state.

Although these two states can be distinguished phenotypically, this distinction is not relevant to their monitoring and is not recorded.

Because annual *Z. marina* grows in a shallower depth range than perennial *Z. marina*, an analogous, ground-based method was developed to delineate the Joe Leary seagrass meadow at low tide. Seven transects were set up in 2023 at the Joe Leary meadow site to provide the same meter-by-meter resolution and similar presence or absence data as the SVMP methods, with additional cover types of macroalgae (Figure 3) and if *Z. marina* plants were annual versus perennial. The transects were set up 100 m apart, perpendicular to shore (Figure 2). Delineation

data was collected on foot during low tide (-0.7 feet MLLW). For ease of distinguishing perennial and annual types, the timing of sampling coincided with peak flowering of annual *Z. marina* after the perennial *Z. marina* flowering shoots had disappeared.



Figure 3. Image of macroalgae (one of the cover types recorded in delineation methods) in Padilla Bay on July 30, 2023.

Ground-based delineation protocol: From the start of each transect, the recorder and observer walked along a 50-meter transect tape, recording the presence of each of 4 cover types (*Z. japonica*, *Z. marina* perennial, *Z. marina* annual, macroalgae) in every square meter along the south side of the transect line. The 50-m transect tape was then slid forward into the next 50-meter segment, following a set compass heading to ensure a straight line was followed for the whole transect line. Compass headings were set for 270°W when walking away from shore or 90°E when heading towards shore. Landmarks were also selected to orient the recorder in a straight line when walking (Figure 4).

Each transect's location (latitude, longitude) was marked with a GPS waypoint at its end so that the waypoint of the previous section had already marked the start of the next one. Seven

transects were made (JL0, JL1, JL2, JL3, JL4, JL5, and JL6), ranging from 519 to 1142 meters long, with an average length of 805 meters. Although the percent cover of each cover type varied every square meter, only presence-absence was recorded (presence = “1”, absence = “0”) to produce similar data as WA DNR’s SVMP so that the same calculations could be used to estimate area.



Figure 4. Ben Molenhouse walking away from shore towards a selected landmark (e.g., the small, exposed beach on Hat Island) with compass bearing set to 270°W from a starting point marked by a PVC pipe.

Quadrat-based Transects

To quantify flowering shoot density, perennial and annual *Z. marina* was counted in 0.25 m² quadrats along 50-m long transects, using methods adapted from the SeagrassNet seagrass monitoring protocol (Short et al. 2015). Three permanent transects (A, B, & C) were established parallel to the shore in the Joe Leary site in 2022 at different tidal elevations (Figure 5).

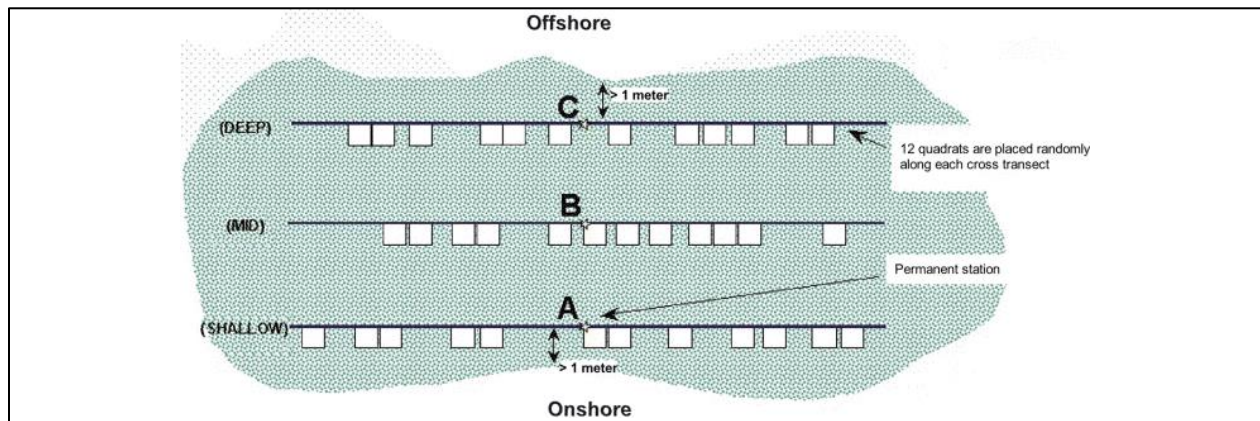


Figure 5. Monitoring design from SeagrassNet (from Short et al. 2005). Three fixed transects run parallel to the shore. Squares represent random, pre-selected 0.25m² quadrats from which seagrass composition, abundance, and density are some recorded measurements.

Running the transects parallel to shore ensures that the elevation within each transect is relatively similar and that the population of seagrasses at each of the three depths is represented. During the surveys, researchers laid out a 50-meter transect tape. At pre-selected distances between zero and fifty (as per the SeagrassNet protocol), 0.25m² quadrats were laid out starting at the right corner of the selected meter distance of the transect. Researchers recorded a visual estimate of the percent cover of algae, detritus, bare substrate, and seagrass species. The two species present in Padilla Bay are *Zostera marina* and *Zostera japonica*. *Z. marina* length and eelgrass wasting disease cover on the 2nd youngest leaf (of 3 haphazardly selected *Zostera marina* shoots, Groner et al. 2016) and vegetative and flowering shoot densities were also recorded from each quadrat

(Figure 6). HOBO pendant temperature loggers were placed on the benthos at the 0-m mark and shaded with a cover, logging every 15 min.



Figure 6. Alexis Jordan measuring the length of Z. marina shoot for methods established by SeagrassNet (Short et al. 2015) in Padilla Bay, June 17, 2023.

The quadrat-based transects at the Joe Leary site in Padilla Bay were established in 2022; however, no distinction was made between perennial and annual plants. Starting in 2023 for this study, flowering seedlings (annuals) were counted separately from perennial flowering shoots, as determined by gently feeling the base of the flowering shoot and whether a long rhizome connection was present (perennial) or absent (annual). Monitoring occurred quarterly in 2023, but only data from the summer is included in this study because that is when flowering occurs.

Phenology of Flowering Shoot Development

The phenology of flowering shoot development was quantified by categorizing each spathe on ten flowering shoots haphazardly collected along each transect (A, B, C) using the developmental stages (I, II, III, IV, V) described by De Cock (1980) (Figure 7). Additional stages (0, VI) were added for spathes that differentiated or decomposed after dispersal (respectively). Flowering shoot length and type (annual or perennial) was also recorded (Figure 8). This survey was conducted along each transect approximately every two weeks, starting on June 6th, for as long as flowering shoots were present. The final observation date was July 30th, the same day the annual flowering shoots were harvested.

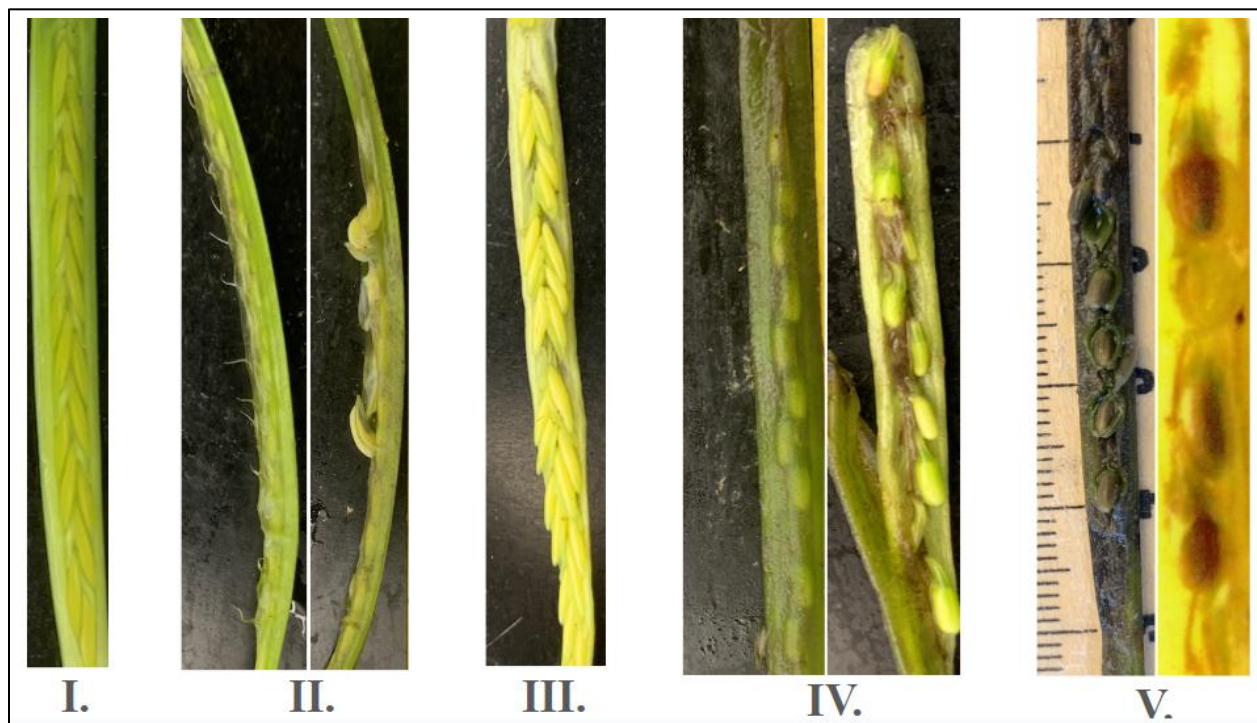


Figure 7. The five stages of development (De Cock 1980) Images by Elinor Tierney-Fife (2022).



Figure 8. Ian McBride examining the spathes to categorize developmental stage in Padilla Bay, June 21, 2023.

Collection & Ripening of Flowering Shoots

From the Joe Leary meadow, annual flowering shoots were collected June 30, 2023, and flowering perennial shoots were collected a month later on July 30th (Figure 9). Collection took place when most of the spathes of each type were observed to be in Stage IV of development (as per De Cock 1980; Infantes and Moksnes 2018). The number of flowering shoots was recorded (Table 3 in Results).

The collection areas were pre-determined through observations of where each type of flowering shoot (annual or perennial) seemed to be most abundant during monitoring sessions from years previous. The perennials were collected between transects B and C, and the annuals were

collected near transect A (Figure 2). The flowering shoots were broken off above the sediment, leaving the rhizome intact underground. Back in the lab, the flowering shoots were divided into mesh bags (window screen, approx. 1-2 mm x 1 mm opening size). The bags were labeled and stored in ambient saltwater flow-through tanks at Shannon Point Marine Center to allow the flowering shoots to ripen and decompose vegetative matter.



Figure 9. Alexis Jordan collecting flowering perennial shoots in Padilla Bay, June 30, 2023.

During the week of September 18, 2023, the seeds were sieved from the mesh bags and rinsed clean of decaying detrital matter with seawater. The number of seeds for each site and type was estimated by measuring the total volume of seeds and counting seeds in subsampled volumes (either 1 mL or 2 mL, n=5). Because seed hardness is associated with viability (Fishman and Orth, 1996; Marion and Orth, 2010), hard and soft seeds were counted separately. From each

site & type, 20 hard seeds were measured for length and width using a dissecting microscope (20x magnification) with a calibrated reticle. Viability testing was conducted on these 20 hard seeds by splitting each seed's seed coat and then leaving it in a solution of tetrazolium chloride (TTC) for 18 hours following Conacher et al. (1994). Staining occurred in different parts of the seed (cotyledon, base, curve, and tip) and was marked as either being "white," "tan," "pink," or "dark red." Overall, if the seed showed any pink or dark red, the seed was counted as potentially viable for this study.

DATA ANALYSIS

Data from the above field methods were synthesized to address each of the questions and objectives as follows:

QUESTION 1. What is the geographic distribution and areal extent of perennial & annual *Z. marina*?

The ground-based delineation data were entered and processed to map spatial distribution and areal extent of the cover types recorded. Using ArcGIS Pro (v3.1.2; Esri 2022), two tables were used to create a feature class that illustrated the data on a map. The first table consisted of the ground-based Delineation data, which had 6,812 meter-by-meter points with their associated cover types present (*Z. japonica*, *Z. marina* annual, *Z. marina* perennial, macroalgae), transect number, and GPS waypoint ID numbers for each 50-m segment. The second table, the Waypoint data, had the GPS coordinates (latitude and longitude in decimal degrees) from the 152 GPS waypoint ID numbers associated with each 50 m transect segment. The coordinate system used

to map the delineation data was NAD 1983 StatePlane Washington North FIPS 4601 (US Feet), which is consistent with other maps from Padilla Bay. Raster data, provided by Suzanne Shull, was used as the base map.

The Delineation and Waypoint tables were tidied and merged in ArcGIS Pro. All null numeric values in the Delineation dataset for each cover type were replaced with 0 (compared to 1 = present). A column was added to the delineation dataset called “MeterMark,” which was calculated within transects with numerical values using the “Sequential Numbers” option in the “Calculate Field” tool. The numbers ranged consecutively from the start (1) to the end of the transect (between 518 and 1142). Depending on the shore orientation, these numbers would be ascending (Shallow to Deep) or descending (Deep to Shallow). The reason was that for the entire length of the transect, each data point representing each square meter had a number representing its place in relation to the other data points. The two tables (Delineation and Waypoint) were then “joined” using the WaypointID field as the common field. The “Coordinate Table to Point” tool was then used to display the data from the joined tables on the map using the Degrees, Minutes, Seconds two-field option. This data was then separated into seven feature classes representing each transect: JL0, JL1, JL2, JL3, JL4, JL5, and JL6.

To create x and y coordinates for each data point, the “Features to Equidistant Point (Fixed Number)” tool was used. The fixed *number of equidistant points* for each section between the waypoints was 50. Some sections did not have 50 as the fixed number because they either ended early because of channels or because it was the end of the transect. These were identified easily within the dataset as the “Notes” section denoted “Short segments,” and the highest number in the corresponding “Distance” column could be used as the fixed number instead of 50. Twelve

data entries were excluded because they marked either the end of the transect, or the start of a channel, so they did not have any corresponding eelgrass data.

Each of the seven equidistant point feature classes were joined with each of the seven transect feature classes. The separate datasets for each transect were merged to create a comprehensive dataset displayed on the map with 6,800 points, all with the fields from the data collected, including the presence of the four cover types. Feature classes for four of the cover types were made: *Z. marina* Perennial, *Z. marina* Annual, *Z. japonica*, and macroalgae.

A polygon of the total site area was created to include all data points (Figure 10). This means the edges to the north and south of the site are composed of transect JL6 and JL0, respectively. The boundary lines are connected in straight lines, except where the polygon meets the channel in the southwest. Here, it is curved to include fewer water bodies in the estimate of the total polygon area. For the north channel that bisects JL6, a channel polygon was created so that the area of the channel running through the total area could be erased.

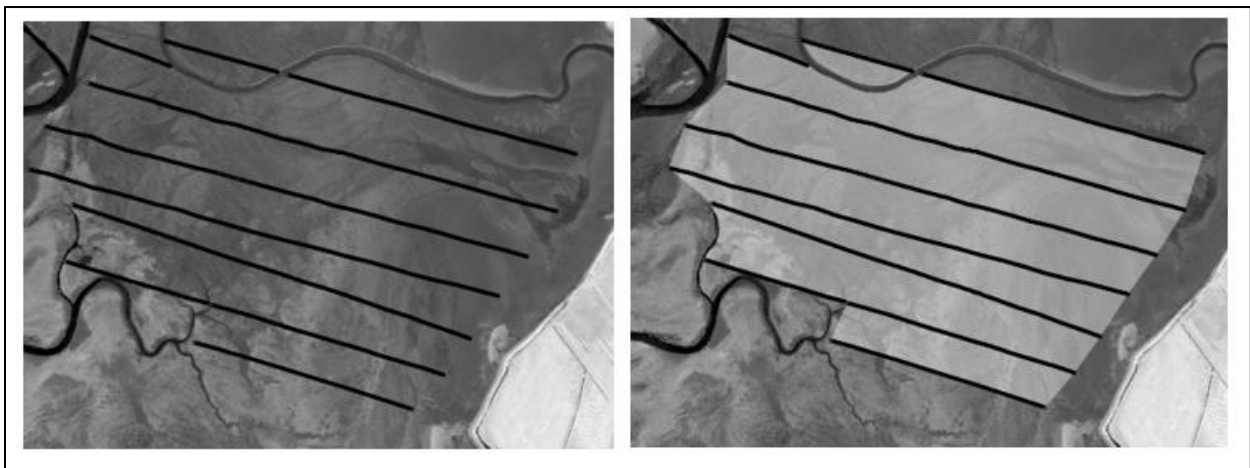


Figure 10. Steps showing the delineation of the site polygon for Joe Leary access site, using transects (left) to create a reference for the perimeter of the polygon (middle), of which the area of the entire polygon feature (right) could be measured to get an estimated area for the site.

The black lines shown in Figure 8 are not lines, but 6,800 points representing a square meter, and the data collected for each meter. The length of each transect was: 519 m for JL0; 891 m for JL1; 951 m for JL2; 1101 m for JL3; 1101 m for JL; 1101 meter for JL5; 1143 m for JL6.

A polygon feature class was made for each cover type using a “Definition query” to select records with the presence of each species. Using the outermost points depicted on the map, a polygon was drawn to include all the data points where the cover type was observed. The northern channel polygon was erased from each of the cover-type polygons so as not to be included in the area estimates. Similar to the total site polygon, the smaller channels were not removed. The area of the three cover-type polygons and the total site polygon were calculated using the “Measure features” tool.

The following steps were done based on delineation methods found in the 2018-2020 Puget Sound Monitoring Report by WA DNR (Christiaen et al. 2022, pg. 12)

- Determine the polygon area for each cover type (*Z. marina* perennials, *Z. marina* annuals, and *Z. japonica*).
- Calculate the proportion of records denoting presence to total records for the three cover types, along each Delineation transect.
- Evaluate the average proportion of cover for each transect and its corresponding variance, considering the length of each transect. Multiply this proportion by the cover area to get a percentage of cover for each eelgrass type.
- Predict the total area (m²) for each eelgrass cover type for perennials and annuals by multiplying the percent cover calculated above by the total site area.

QUESTION 2. How does flowering shoot density and seed production vary for perennials and annuals?

2.1 Estimate the number of viable seeds per flowering shoot for perennials and annuals.

The average number of viable seeds per flowering shoot was calculated as follows for perennial and annual *Z. marina* at the Joe Leary site: The total number of hard seeds from each batch was divided by the number of flowering shoots that were collected for each type (annual, perennial) to estimate the number of hard seeds per flowering shoot. Then, the number of hard seeds per flowering shoot was multiplied by the viability percentages determined with a tetrazolium chloride test on hard seeds to estimate the number of viable seeds per flowering shoot (Table 1).

2.2 Determine flowering shoot densities for flowering perennial and annual populations

The flowering shoot densities collected from the quadrat-based transects were highly variable. A statistical test is necessary here to determine whether there are significant differences between the flowering shoot counts. Because the data included many zero counts, the data is not normally distributed and is heteroskedastic. Therefore, a non-parametric Kruskal-Wallis test was performed to look at the differences in mean flowering shoot counts between transects and between dates. A Dunn test, using the Bonferroni correction for p-values, was performed as a post-hoc test to further investigate the differences in flowering shoot counts between transects. Because each transect represents a different elevation, and the phenology of flowering shoots changes over time, these would likely contribute to the variability of flowering shoot counts. The

quadrats used for monitoring were 0.25 m² so all the counts were multiplied by four so that the densities would represent the number of flowering shoots per m².

The counts of *Z. marina* perennial and annual from the quadrat-based transect data were analyzed using R Statistical Software (v4.3.1 4.3.1; Posit Team 2023) to calculate summary statistics, like the mean and standard error, to show variation in density. Boxplots were made using the ggplot2 package.

2.3 Determine estimates of viable seeds per m² and sitewide for perennials and annuals

The viable seeds per flowering shoot and the flowering shoot densities were multiplied to determine approximate seed densities (per m²). Seed density was multiplied by areal estimates of perennial and annual cover types (based on Delineation mapping) to obtain sitewide estimates of viable seed production within the Joe Leary study site boundary.

Flowering shoot collection for perennials occurred in the middle of the two monitoring dates. So, an average of perennial flowering densities from both dates was used as the input for the mean flowering shoot count per m² for perennials because the densities were not significantly different between the two dates. For flowering annual densities, the count per m² from July 14th was chosen because it was the date closest to when annual flowering shoot collection happened (July 30th) and because the counts were shown to be significantly different between the two dates.

Table 1. Equations for calculating flowering shoot density and seed production for the Joe Leary site. Calculations were completed separately for perennials and annuals.

Calculations	Output
Total est. seed count/Flowering shoots collected	<i>Avg. Seeds Per Shoot</i>
Total est. hard seeds/Total est. seed count	<i>Percent Hard Seeds of Total</i>
Viability score * % hard seeds * Avg. seeds per shoot	<i>Viable Seeds Per Flowering Shoot</i>
Mean flowering shoot density counts * 4	<i>Mean Flowering Shoots per m² ± Std. Error</i>
Mean flowering shoots per m ² * Avg. seeds/shoot ± Mean flowering shoots per m ² * Std. error	<i>Seeds per m²</i>
Mean flowering shoots per m ² * Avg. viable seeds per shoot	<i>Viable Seeds per m²</i>
Avg. seeds per shoot * Est. flowering shoots in JL ± Avg. seeds per shoot * Std. error	<i>Total Seeds in JL</i>
Avg. viable seeds per shoot * Est. flowering shoots in JL ± Avg. viable seeds/shoot * Std. error	<i>Total Viable Seeds in JL</i>

QUESTION 3. How does the phenology of perennial and annual flowering shoots vary?

Using the Phenology dataset, the proportion of spathes of a certain stage was calculated by dividing the number of spathes in each stage (I-V, Figure 10) by the total number of spathes in stages I-V for each flowering shoot replicate. Line plots were made using the ggplot2 package to show the mean proportion of each stage per flowering shoot over time for each transect. Data is shown to be continuous, but this is not the case, and the trendlines are not predictive but are a visual aid for looking for peak stages in development. The samples are the means from total spathe counts from ten flowering shoots.

Observations from transect B were omitted from this study because a mixture of annuals and perennials were present but not distinguished.

The timeline from DeCock (1980) (Figure 11) is based on perennial plants from Grevelingen in the Netherlands. The number of days between monitoring times of perennials and annuals from Joe Leary are compared to one another. Day 1 refers to the first observation date but not the first day of spathe development. Data collection happened every two weeks with different flowering shoots. The timeline from De Cock shows hour-by-hour and day-by-day development of individual flowering shoots and is used as a reference. According to De Cock's observations, the entirety of seed development, from pollination to the release of the seeds, lasts just over a month. This timeline will be used to guide phenology observations from this study.

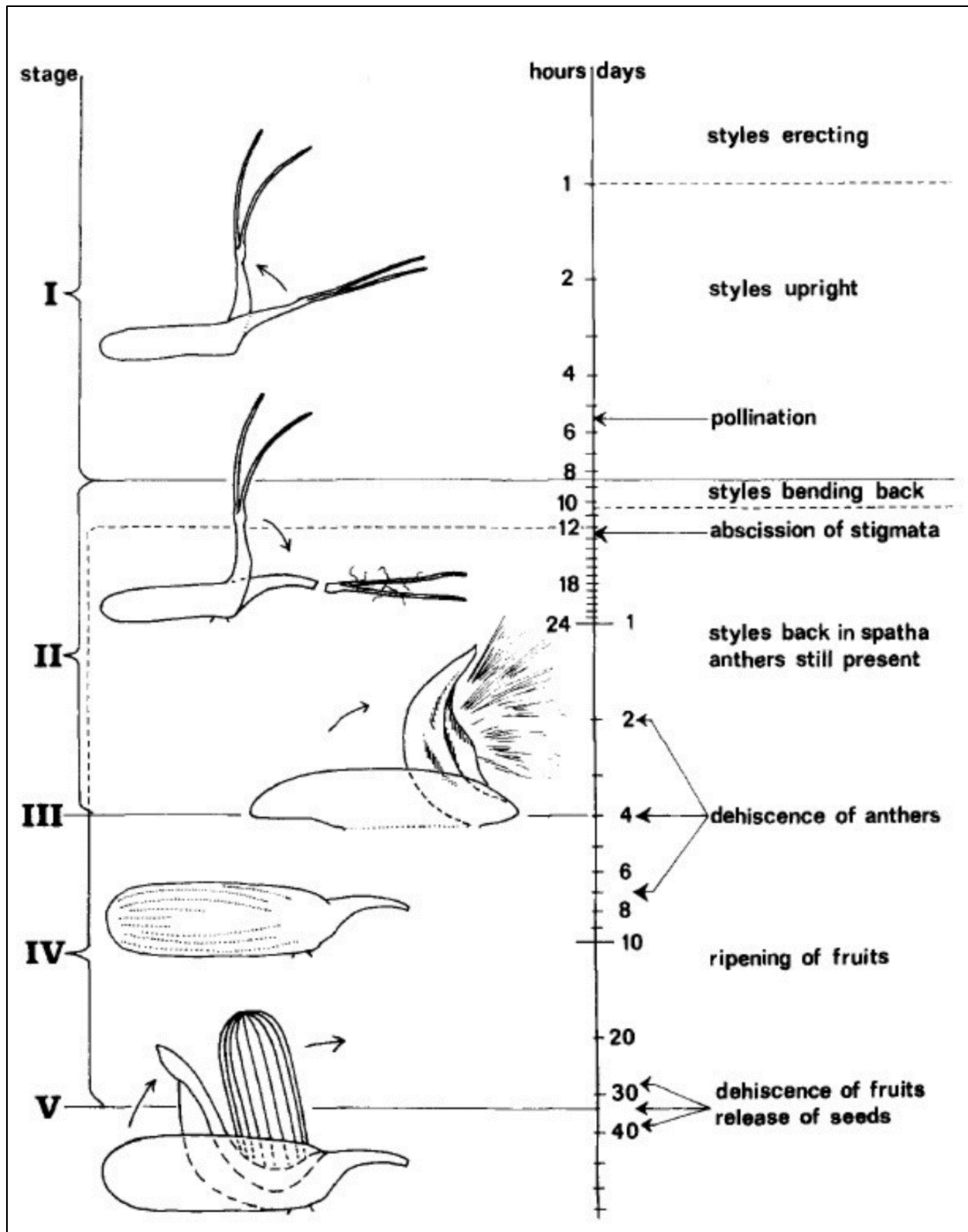


Figure 11. Developmental stages (I-V) for flowering and seed development from De Cock (1980).

RESULTS

QUESTION 1. What is the geographic distribution and areal extent of perennial and annual *Z. marina*?

Geographically there is an overlap in the distribution of perennial and annual *Z. marina* and *Z. japonica*. Within the Joe Leary meadow, *Z. japonica* has the largest area covered, followed by perennials, then flowering annuals (Table 2). *Z. japonica* is present consistently in the upper intertidal and in JL4 annual presence also seems to be consistent, whereas in a similar latitudinal location along the tide flat on JL1 perennial presence is patchy (Figure 12). There are a few sections in the lower intertidal where only perennial shoots exist (JL1, JL2, JL3, and JL4 in Figure 13). Macroalgae distribution overlaps with the presence of the lower annual flowering shoots (Figure 14). The dashed transect line depicts bare substrate with no presence of any eelgrass cover type or macroalgae.

Table 2. The areal estimates for eelgrass cover types in the Joe Leary meadow site with \pm referring to variance.

	<i>Z. marina</i> . Perennial	<i>Z. marina</i> Annual (flowering)	<i>Z. japonica</i>
Total Area of Polygon (km ²)	0.391	0.296	0.466
Mean % of Total Transects	50% \pm 9%	59% \pm 8%	85% \pm 2%
Avg. Area of Polygon Cover (km ²)	0.196	0.175	0.396
Total Area of Site (km ²)	0.622	0.622	0.622
% of Entire Site Covered	32% \pm 6%	28% \pm 4%	64% \pm 1%

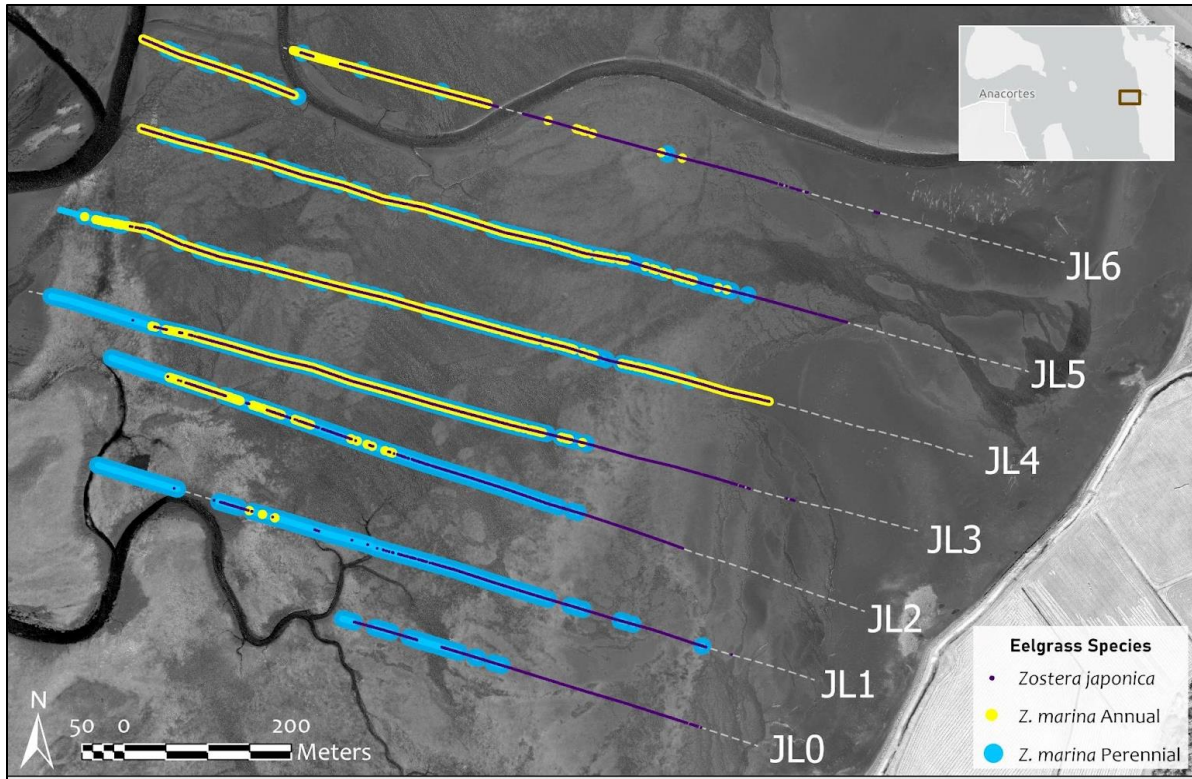


Figure 12. Map showing eelgrass distribution at the Joe Leary meadow site, Padilla Bay.

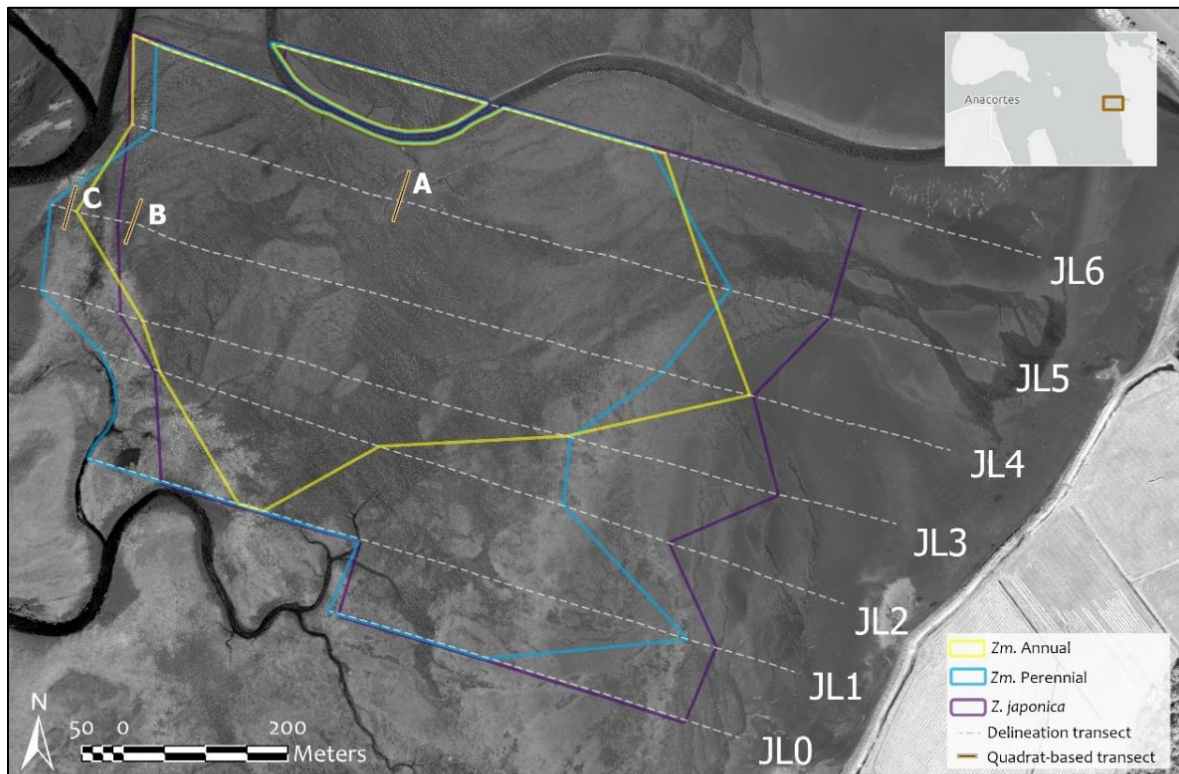


Figure 13. Map with three polygons for the eelgrass cover types within the total site polygon.

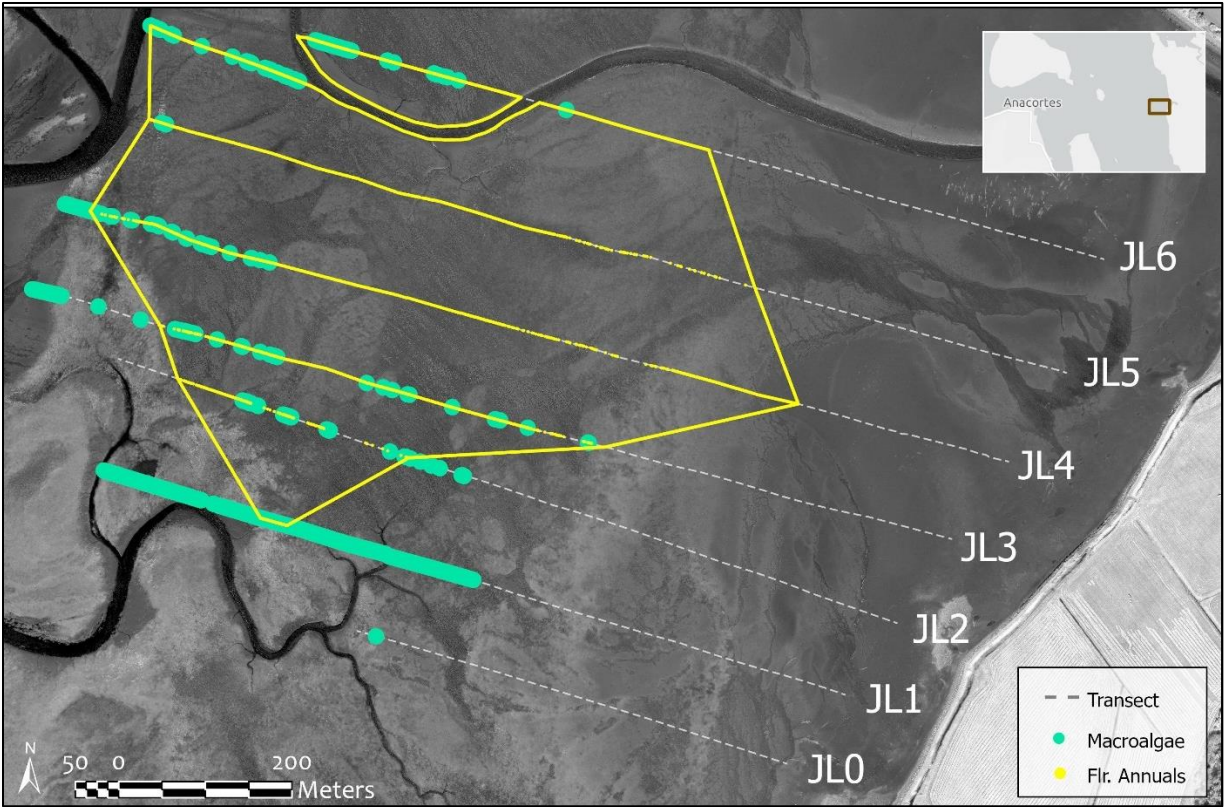


Figure 14. Map showing distribution of macroalgae and flowering annuals.

QUESTION 2. What is the estimated flowering shoot density and seed production for perennials and annuals?

2.1 Estimate the number of viable seeds per flowering shoot for perennials and annuals.

The estimated number of viable seeds per shoot was higher in perennials than annuals (Table 3), but the mean flowering shoot densities are much higher than in perennials (Figure 14).

Therefore, the estimated seed production for annuals is much higher overall than for perennials.

2.2 Determine flowering shoot densities for perennial and annual *Z. marina*

The estimated flowering shoot densities varied between population, transect, and date of observation. The highest densities of annual flowering shoots were observed in transect A, and much lower densities are observed in B and C for the June and July monitoring dates. Densities of perennial flowering shoots are much lower in C and are not observed in A or B (Figure 15).

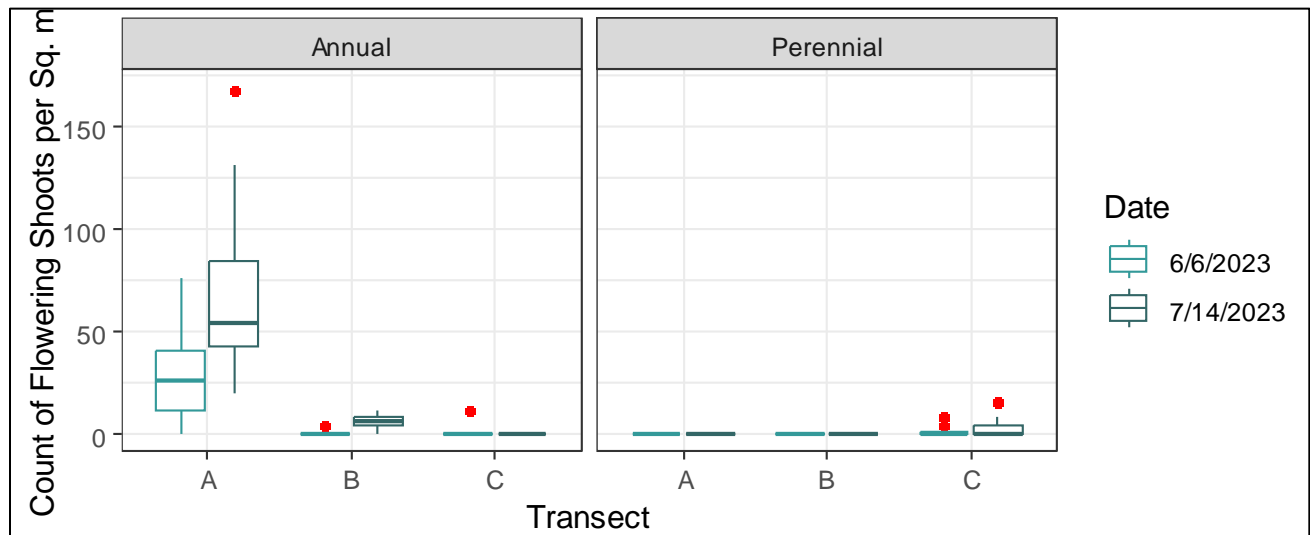


Figure 15. Flowering shoot count data is categorized between perennials and annuals by transect and date.

There was a significant difference in flowering shoot counts between perennials and annuals ($X^2_1 = 27.518$, $p < 0$) and between the three transects ($X^2_2 = 14.39$, $p < 0.001$). The post-hoc test showed that there was the highest significant difference between transects A and C ($p < 0.001$) with a smaller difference between A and B ($p < 0.05$) and no significant difference between B and C ($p = 1.0$).

Within the annual population found in Joe Leary, there was a significant difference between the observation dates ($X^2_1 = 5.5878$, $p < 0.05$). In contrast, this was not the case within perennials ($X^2_1 = 0.584$, $p = 0.445$).

2.3 Estimate quantity of viable seeds for perennials and annuals

Perennial flowering shoots had more viable seeds per flowering shoot, as determined by TTC testing on the hard seeds (Figure 16). Annual flowering had a higher flowering shoot density, so the seed production per m^2 and for the site is higher (Table 3).

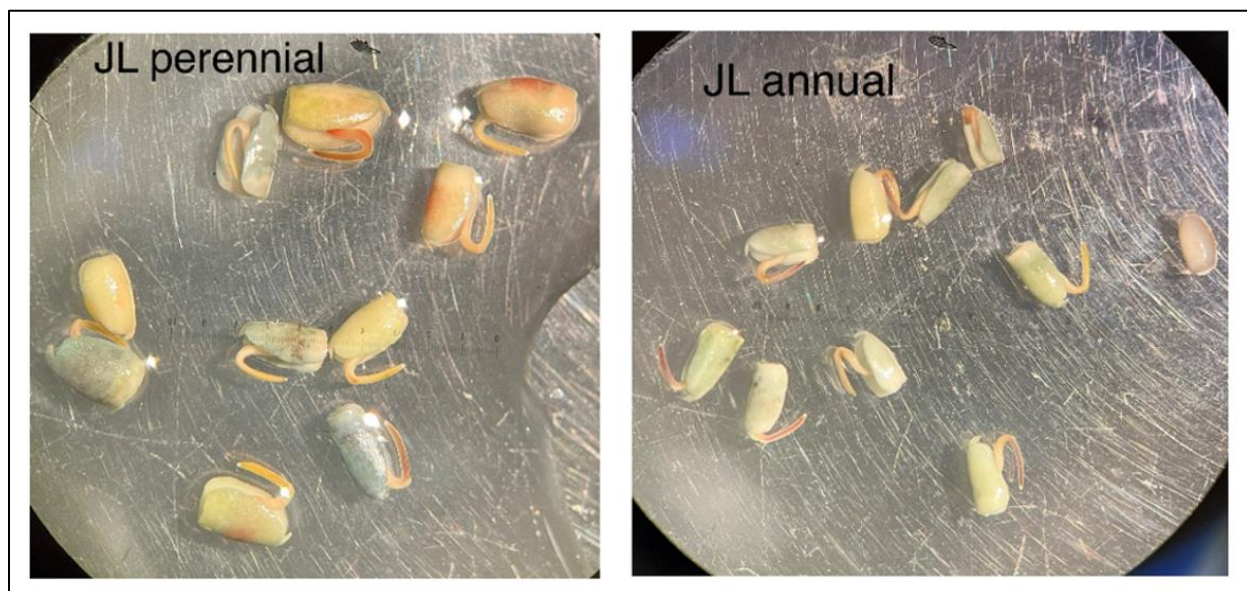


Figure 16. Perennial & annual seeds under 20 x magnification dyed with TTC (Sept. 18, 2023).

Table 3. Estimates of viable seed count estimates and flowering shoot densities with \pm referring to standard error.

	Perennial	Annual
<i>Collection Date</i>	21-Jun-23	30-Jun-23
<i>Flowering Shoots Collected</i>	220	671
<i>Total Est. Seed Count</i>	6,377	18,964
<i>Avg. Seeds Per Shoot</i>	29	28
<i>Percent Hard Seeds of Total</i>	83%	90%
<i>Percent Viable of Hard Seeds</i>	95%	72%
<i>Viable Seeds Per Flowering Shoot</i>	23	18
<i>Mean Flowering Shoots per m² ± Std. Error</i>	4 ± 1	68 ± 13
<i>Seeds per sq. m</i>	116 ± 29	1,904 ± 364
<i>Viable Seeds per sq. m</i>	92 ± 23	1,224 ± 234
<i>Polygon Cover Area (m²)</i>	196,908	175,346
<i>Total Seeds in JL</i>	~ 23 million	~334 million
<i>Total Viable Seeds in JL</i>	~18 million	~215 million

QUESTION 3. How does the phenology of perennial and annual flowering shoots vary?

The phenology data showed transect B records having both a perennial and annual presence. The collection of flowering annuals occurred near transect A, and the collection of flowering perennials occurred near transect C. The data presented in all four data sets shows that within the cover area, there are places where only annuals, only perennials, or a combination of both, exist (for example, transect A, C, and B, respectively).

Annuals flowering stages of development are delayed relative to flowering perennials. On the first day of data collection (June 6, 2023), 100% of annual spathes were in Stage I, showing zero counts of any of the other stages (Figure 17). In comparison, on the same day, perennial spathes already had 50% of the total spathes observed in Stage IV (Figure 18). Annual flowering shoots did not reach 50% of spathes in Stage IV until day 25 (June 21) (Figure 17). Observations started at the same time for all three transects but were continued longer at transect A than at transect B or C due to the later development of the annual population in transect A. Stage IV in annuals took about five and half weeks to go from 0% to the observed peak at 75%. This was also when Stage V was first recorded. Stage III was observed to start 2 weeks into monitoring, which coincided when Stage II peaked at 25%.

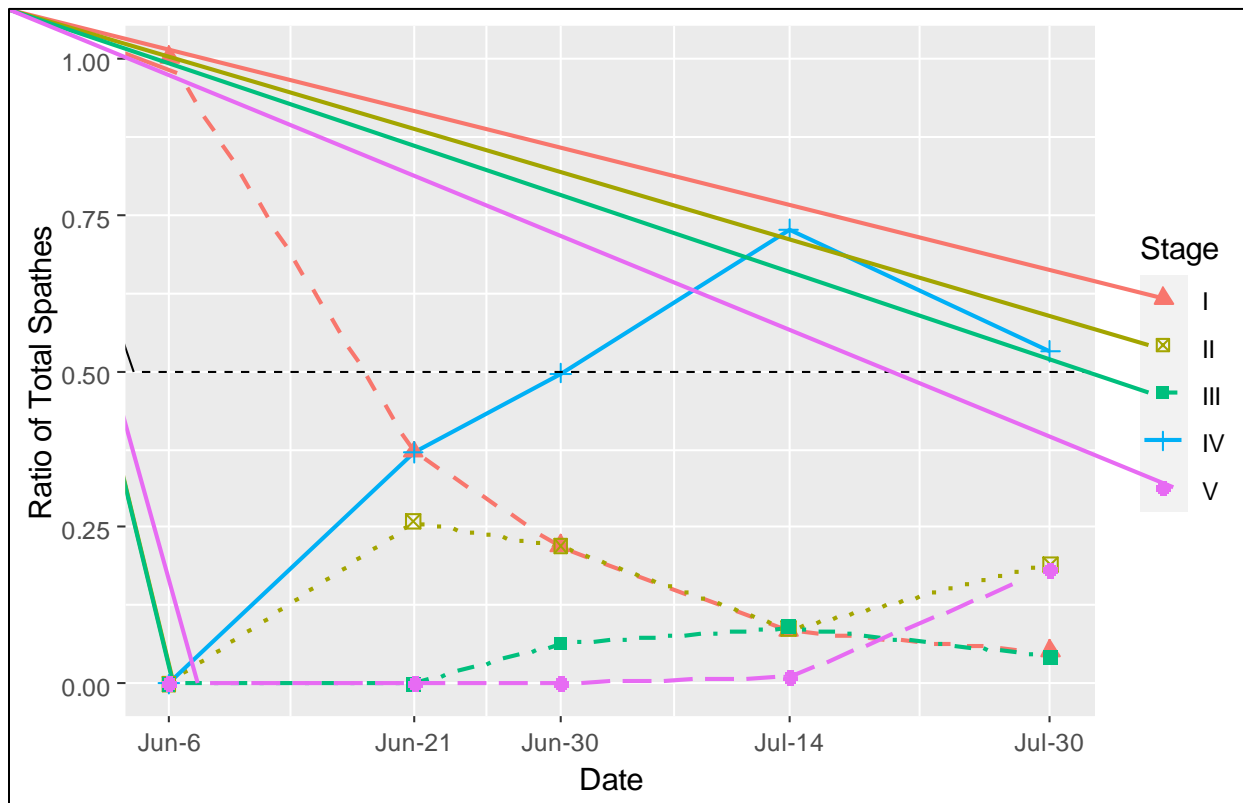


Figure 17. Annual lowering shoot development of spathes from transect A, from the Joe Leary meadow in 2023. Data collected June 6th, June 21st, June 30th, July 14th and July 30th, flowering shoots collected on July 30th.

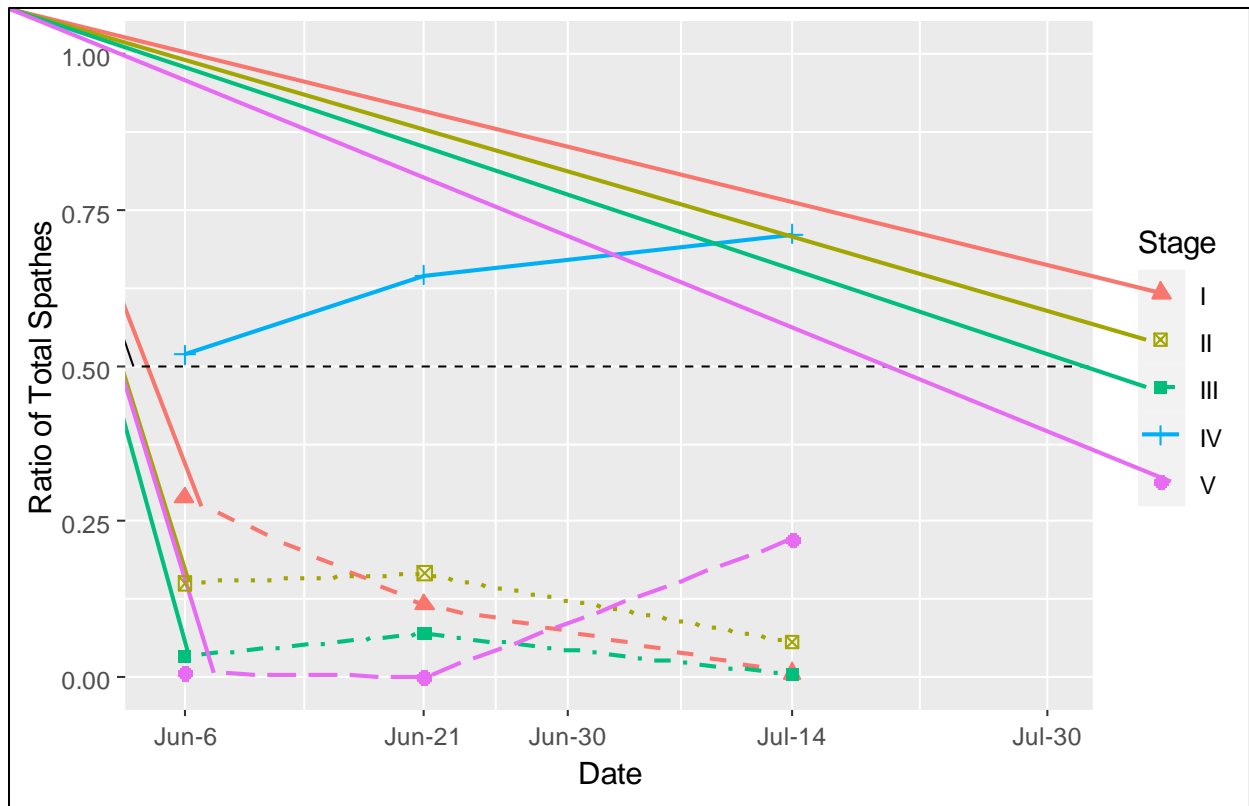


Figure 18. Perennial flowering shoot development of spathes from transect C from the Joe Leary meadow in 2023. Data collected on June 6th, June 21st and July 14th, flowering shoots collected on July 30th.

DISCUSSION

In this study, we used data from 2023 from the Joe Leary meadow site to (1) determine the geographic distribution and areal extent of flowering perennials and annuals; (2) estimate flowering shoot density and potential seed production, and; (3) compare the flowering shoots phenology.

QUESTION 1. What is the geographic distribution and areal extent of perennial & annual *Z. marina*?

The delineation maps (Figure 11 & 12) show that the geographic distribution between perennial and annual *Z. marina* plants overlaps until transect C, after which only perennials are flowering. Transect A was established higher up on the intertidal, with transect C established lower in the intertidal. Flowering annuals were observed almost exclusively in transect A, and flowering perennials were observed, almost exclusively, in transect C. This supports the literature that has observed that annuals reside higher on the intertidal than perennials (Qin et al. 2014). From the delineation data it looks as though macroalgae and annuals have an inverse relationship to elevation. Macroalgae is observed lower in the intertidal, and annuals are observed higher in the intertidal. Other indicators of disturbance that could contribute to the distribution of flowering annuals could be proximity to the slough. Water moving down the slough could be a disturbance, as could any agricultural run-off that might potentially be transported via the slough.

QUESTION 2. What is the estimated flowering shoot density and seed production for flowering perennials and annuals?

The estimated viable seed production per shoot was higher for flowering perennials than for flowering annuals (Table 3). However, as reported in other literature (Keddy 1987; Morita et al. 2007; Kim et al. 2014; Qin et al. 2014), the seed production per unit area was higher for annuals than perennials because the density of flowering annuals was higher than flowering perennials.

Although the annual flowering shoot densities seem high in comparison to perennial flowering shoot densities, this is not the case compared to previous studies. Overall, other studies show higher densities of flowering shoots and number of seeds per shoot (Table 4).

Table 4. Summary table, comparing flowering shoot densities, seed count and viability data (with SE) between perennials and annuals, from literature from different latitudes and countries.

Year	Time period	Site	Type	Flowering shoot density m ⁻²	Potential seed production m ⁻²	Viability	Reference
2007	May	Tategami Bay, Japan	Annual	117 ± 29	6000 ± 2400 m ⁻²	70% germination	Morita et al. 2007
2009	NA	Jindong Bay, Korea (Shallow)	Perennial	30 ± 6	7,114 m ⁻²	NA	Kim et al. 2014
2009	NA	Jindong Bay, Korea (Deep)	Annual	179 ± 17	9,981 m ⁻²	NA	Kim et al. 2014
2009-2013	Mid-July	Yellow Sea, China	Annual	NA	386 per liter of flowering shoots	91% TTC (after storage)	Pan et al. 2014
2014-2017	Aug-Sep	Wadden Sea, Netherlands	Annual	NA	8,459 ± 2,134 kg ⁻²	92% ± 4%	Govers et al. 2022
1995	June	Chesapeake Bay, USA	Perennial	0.09% ± 0.61%*	55-6,160 m ⁻²	40-58%	Harwell & Orth 2002
2023	End of June	Padilla Bay, USA	Perennial	4 ± 1	116 ± 29 m ⁻²	95% TTC	This study
2023	End of July	Padilla Bay, USA	Annual	68 ± 13	1,904 ± 364 m ⁻²	72% TTC	This study

*Proportion of flowering shoots from total number of shoots.

All studies in Table 4, except for Harwell and Orth (2002), used similar methods to this study, estimating seed production by counting the number of seeds per shoot and using flowering shoot densities when applicable. Harwell and Orth (2002) counted seeds from sediment cores sampled from 105 different sites across twelve “zones”.

Lower densities of annual flowering shoots in the Joe Leary meadow compared to sites from other studies (Table 3), could be because non-flowering perennial shoots and *Z. japonica* are also intermingled with the annual population (Figure 19, Supplemental Figures) and are in competition for resources. The presence of perennials has been observed to reduce the reproductive potential of annuals in some sites, except for in drain depressions (Keddy 1987). Establishing more transects within the annual polygon, in depressions and mounds, could lead to potential patterns in flowering annual shoot densities, and perhaps greater densities could be found higher up on the intertidal (Keddy 1987).

QUESTION 3. How does the phenology of flowering perennials and annuals vary?

Based on the phenology data, the subsequent collection of flowering shoots was timed well with the observations done during monitoring. The developmental stages occurred later for annual plants than for perennial plants, with the peak ratio of spathes in Stage IV observed a month later. De Cock’s timescale showed a total period of 40 days from Stage 0 to Stage V.

Observations from this study showed all stages were present within the 54 days of monitoring for both perennials and annuals, although some developmental stages were recorded at 0% at points for annuals but not perennials. Perennials would need to be monitored earlier if the total duration of all the stages were to be observed. Both plots show that all the flowering shoots do not start

flowering and developing at the same time, and the data shows that even within a single shoot, multiple stages can be present at once. Relative to flowering shoot collection, Stage IV for annuals lasted five and a half weeks, but was only above the harvesting threshold proportion for a month. Because the data was collected every two weeks and is not continuous, making predictions of the differences between stages is not very accurate.

Continuing to track the development of flowering shoots and maturation of seeds each year is important. The optimal date for collection will not be the same year-to-year because of climatic conditions. More data is needed to find other places of high flowering shoot densities.

Temperature and elevation could be possible predictors for the observed variation (Harrison 1993; Johnson et al. 2017; Flem 2020). This data could be overlaid with flowering shoot density data in the delineation map to see whether a relationship exists.

Areas for Further Study

Geographic distribution of flowering perennials and annuals

Further investigation at the Joe Leary meadow site is imperative to address existing knowledge gaps pertaining to these somewhat enigmatic annual plants. Future research endeavors should incorporate an assessment of elevation changes across the entire site, aiming to identify potential factors contributing to distinct shifts in population types.

Moreover, establishing a JL7 transect could be insightful as to what the population dynamics are on the north side of the slough. Another recommendation for the delineation methods would be to incorporate a measure for flowering shoot density. Introducing an ordinal ranking system, wherein the density is categorized as either absent, sparse, moderate, or abundant, would

contribute to a more nuanced understanding of the distribution of flowering shoots. The current delineation methods equate a single shoot per square meter with a hundred shoots per square, which, while it meets the SVMP monitoring protocol, does not contribute to describing variation within each cover type. On the other end of the spectrum are the flowering shoot counts from the monitoring of the quadrat-based transects, which, if scaled up to the delineation transects, would be too time-consuming.

Finding a middle ground of producing more accurate data without having to individually count shoots holds the potential to (1) illustrate the patchiness inherent in the distribution of flowering shoots, thereby depicting their variability, and (2) yield more precise estimates of flowering shoot densities. Consequently, modifying this protocol would enhance the accuracy of subsequent flowering shoot and seed production estimates within the covered area.

Another recommendation for additional investigation study would be to revisit where the annual shoots were in the winter. The perimeter of bare substrate could be recorded in the field, using Esri's FieldMaps software, for example. Subsequently, during the summer, follow-up monitoring of the same area could be conducted to further delineate regions where annual shoots have populated the polygon and where the substrate remains bare. This approach would provide a more precise assessment of locations that are exclusively characterized by the presence of annual populations.

Monitoring during winter would also contribute valuable insights into the spatial boundaries of perennial patches since they persist year-round. Areas devoid of eelgrass in both seasons could be subject to longitudinal observation to ascertain whether these regions eventually undergo colonization and if so, by which population. Additionally, integrating remote sensing, like drones

for example, would give greater detailed imagery that would enhance the capacity to monitor the variability of temporal and spatial dynamics of eelgrass cover over time.

Flowering shoot and seed production estimates of flowering perennials and annuals

The exploration of the annual meadow at the Joe Leary site could benefit from establishing additional transects to discern potential variations in density within areas where annuals are more exclusively present. By strategically placing more transects across the meadow, researchers can assess what is considered a higher density relative to a lower density. This spatially explicit approach can provide a high-resolution understanding of the meadow's distribution patterns and ecological dynamics.

Furthermore, a comparative analysis of sediment cores would be a valuable methodology to delve into seed production variations between perennial and annual populations. Sediment cores have been used by other literature (Morita et al. 2007) for quantifying the number of seeds in a meadow. Cores could be extracted from areas distinctly representative of perennial and annual populations to evaluate and compare the quantity of the existing seed bank. Further monitoring could reveal a site that is exclusively annuals with potentially higher flowering shoot densities than what has been observed in 2023. For seed-based restoration, flowering shoot densities and seed yield need to be optimized to make the methods efficient, and the time and resources the most effective while minimizing impact on the donor site.

Comparison of the phenological development of flowering perennials and annuals

Observations in this study were *in situ* and did not follow the development of the same plants (instead, it followed a mean of a sample of 10 plants that were different over time). Therefore, the data is not comparable. A mesocosm experiment could be done that would observe the

development of individual flowering shoots to make the phenology data comparable to De Cock's timeline. Incorporating both perennial and annual shoots into this experiment would allow for a more precise timeline for each population.

Implications for Seed-Based Restoration

This section seeks to bring the following five questions to the forethought of considering Joe Leary as a donor site for seeds:

1. Is there enough known about the Joe Leary meadow to conduct collection of flowering annuals for seed collection?
2. How can the impact of collection be monitored?
3. What resources exist to help determine what percent of the flowering annuals should be harvested?
4. Should annuals be seeded in restoration sites where annuals potentially did not exist before?

In evaluating the suitability of the Joe Leary meadow as a potential seed donor site, it is not advisable to collect perennial flowering shoots due to the low densities observed. Conversely, annual flowering shoots may be selectively collected in limited quantities for further scientific investigation.

Although transect A has a comparatively high density of flowering shoots in contrast to the other two transects, it is relatively low compared to other studies in similar latitudes in the literature (Table 4). Results from this study indicate the potential for increased densities along the slough, and ongoing monitoring during spathe development could pinpoint areas with greater relative

abundance of flowering annuals compared to the mean counts observed at transect A. This approach could effectively optimize seed yield within the same year of collection. Given the recent discovery of this patch of annuals, accumulating data and knowledge could occur while progressing toward the subsequent steps in seed-based restoration.

Understanding potential impacts on donor sites is an aspect that remains largely unknown in the context of seed-based restoration (Unsworth et al. 2023). In the event of a significant die-off in Padilla Bay, seedling recruitment would become crucial for recovery. To mitigate adverse effects on the Joe Leary meadow from potential over-extraction of flowering shoots, tracking targeted extraction areas post-collection is recommended. Establishing monitoring plots to assess flowering shoot densities in the harvested areas can prevent overharvesting at the same locations in following years. Implementation of specific metrics could quantify the impact of flowering shoot removal from the plots. Worth further consideration is following the protocol for seed collection recommended in terrestrial harvesting guides for optimal management (Unsworth et al. 2023).

A significant consideration in seed-based restoration revolves around whether annuals should be considered in areas where this population type has not been documented. In the San Juan Islands, for instance, perennial *Z. marina* is the predominant native eelgrass species, without recorded instances of annual *Z. marina*. Given the phenotypic and genetic similarities between the two populations (Gagnon et al. 1980; Muñoz-Salazar et al. 2005) and their overlapping ranges, the possibility exists that annuals may have existed, or do exist, without recorded presence. Seed-based restoration still requires time and resources, and the ecological niche for annual eelgrass, specifically in the Puget Sound, is not comprehensively understood due to its lack of either presence and/or documentation.

The risk of introducing non-native *Z. japonica* area could occur because of the overlap with annual *Z. marina*. If *Z. japonica* seeds are gathered accidentally during flowering shoot collection of annual *Z. marina*, the non-native eelgrass could potentially colonize the restoration sites where annuals are being seeded. This *japonica* is much more similar in size to flowering annuals than flowering perennials. Currently, *Z. japonica* has only been observed in very few locations in the San Juan Islands (Christiaen et al. 2022). Methods, like considering the timeline of *Z. japonica*'s flowering shoot phenology, could mitigate the accidental introduction of this non-native species during seed-based restoration in sites where it has not been introduced.

In the face of large-scale restoration, methods need to be adapted for managing time.

Maximizing seed yield is, necessary to effect seed-based restoration and processing thousands of flowering shoots. In the Wadden Sea in the Netherlands, annual flowering shoots are collected, and instead of being counted individually, are weighed, thus producing the estimated number of seeds per unit of weight (in this case, it was 2.5 million seeds per 420 kg, Govers et al. 2022).

Other methods that suspend the flowering shoots in situ after collection to prevent desiccation, measure flower shoots in liters. Adopting these methods would be beneficial because it would be less time consuming to not count the number of flowering shoots harvested in the field or after collection.

Summary

- Annuals have a similar range in the intertidal than perennials in the Joe Leary meadow, but there does seem to be some potentially stress induced distinction where flowering annuals end, and flowering perennials begin.

- Flowering perennials developed earlier than flowering annuals.
- Joe Leary is not a good site for flowering perennials to be collected and more monitoring is needed to find more pockets of annuals with potentially higher densities.
- Discussions need to happen around whether, considering the benefits of annuals for restoration used in other places, whether annuals should be used for restoration in areas that don't have a recorded presence of annuals.
- If flowering shoot collection of annuals is to take place, more exploration is needed to determine densities relative to the mean flowering shoot count observed at transect A.
- If flowering shoot collection of annuals is to take place in the Joe Leary meadow site, then setting forth a protocol for monitoring subsequent flowering shoot densities following removal is needed. This would provide knowledge on the effects of flowering shoot harvest on annual beds and would provide seeds for restoration.
- Terrestrial harvesting protocols should be adhered with the lack of an alternative framework.
- Methods for measuring flowering shoots should be considered if collection is scaled up.
- Warming ocean temperatures could negatively affect seed production (Qin et al. 2020).

SUPPLEMENTAL FIGURES

To capture the spatial variation in density between June and July, a similar approach used in the delineation mapping methods was implemented, using the equidistant points in ArcGIS Pro for each quadrat-based transect (Figure 19). Fifty sequentially numbered points were spread equally across where each of the three transects were. The flowering perennial, flowering annual, non-

flowering seedlings and non-flowering perennial shoots were fields selected from the dataset and merged with the point data. Only targeted values were kept and therefore the values from the twelve selected quadrats from each field transect, were assigned points along the mapped transects depending on the pre-assigned distance column. These results can be found in Supplemental Figures because it includes non-flowering shoot data and this study's focus is on flowering shoots.

Non-flowering perennial shoots exist in high densities in transect B. Flowering and non-flowering shoots are shown for perennials, annuals, and non-flowering seedlings in the figure to visually illustrate that there is a high density of non-flowering perennial shoots in transect B and C, but A is still predominantly flowering annuals (Figure 19).

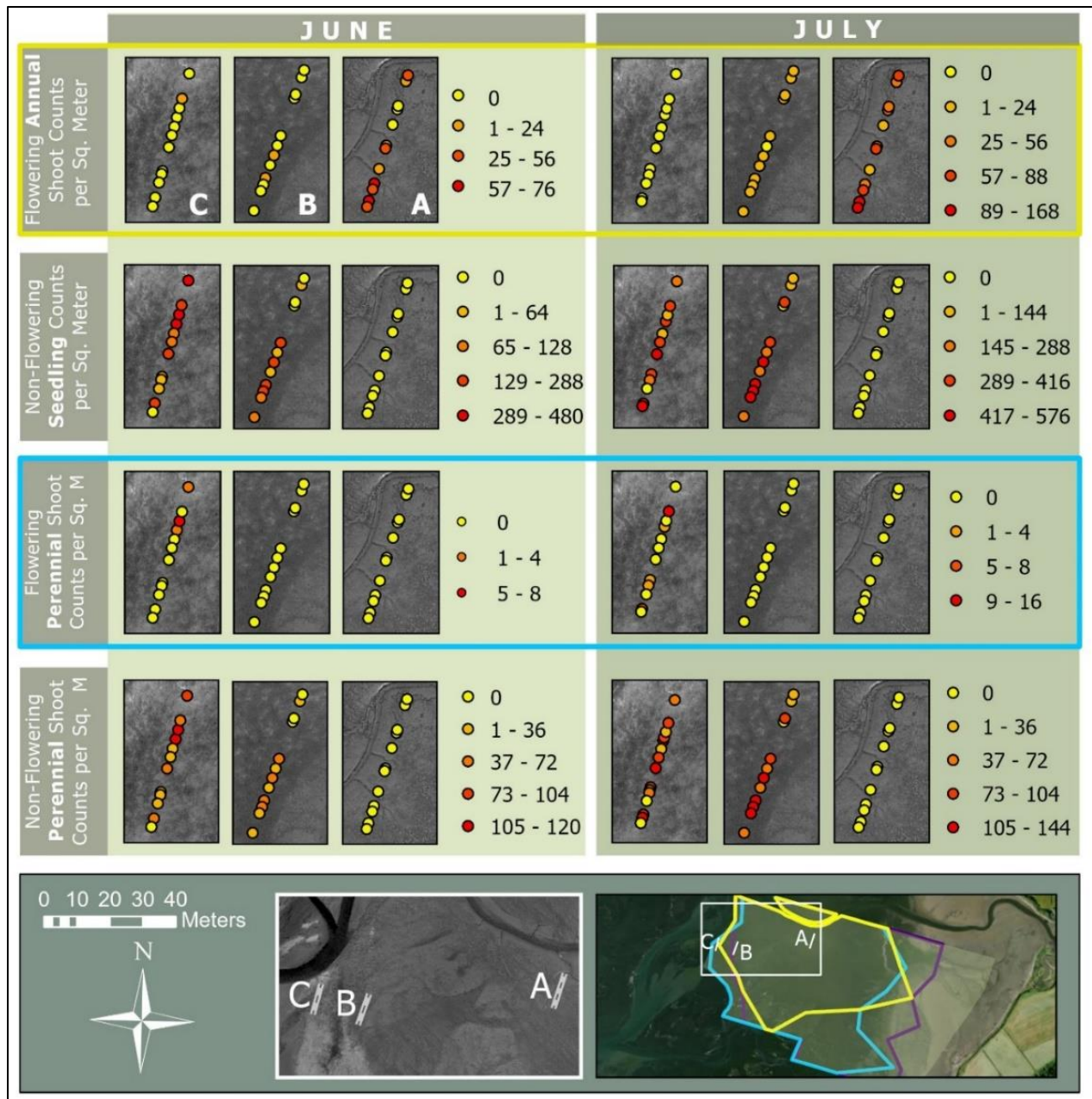


Figure 19. Densities of flowering and non-flowering perennials, annuals and seedlings across quadrat-based transect (A, B, and C) for the monitoring dates in June (6/6) & July (7/14), 2023.

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