Standard Operating Procedure: Assessing Eelgrass Flowering Density and Seed Maturity

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Purpose: *There is great interest in using eelgrass (Zostera marina) seeds for restoration efforts, but little is known about the optimal location and timing of harvest activities. This field protocol was developed to address a regional data gap and provide a standardized approach to data collection across several National Estuary Programs and NGO organizations located in New England. The protocol can be implemented from shore or boat, and via snorkel, wading or scuba, by professional or trained volunteer scientists.*

Rationale/background

Traditionally, eelgrass restoration in New England has been predominantly done by adult shoot transplants. The actual method of deploying the uprooted shoots at the restoration site may vary (e.g., horizontal rhizome method, TERFs, tortilla method), but these just represent a minor variation on a theme. Success rates have for the most part been low and unpredictable. Adult shoot transplanting is labor intensive and as a result expensive. Due to the labor and costs involved, most practitioners are attempting to restore areas of < 1 acre over a period of 1-3 years, often not long enough to result in success. This track record has led to some funders no longer supporting eelgrass restoration projects.

In the Chesapeake Bay region, eelgrass restoration is no longer attempted by adult shoot transplants, and all restoration efforts are carried out via seeding. In the coastal bays of Virginia, close to 10,000 acres of eelgrass have been restored after a persistent large scale seeding effort, involving the deployment of over a million seeds a year for a decade. From year to year, they had highly variable rates of success. After a decade, they had accumulated enough success that the surviving restoration areas become seed sources spurring natural expansion.

Using a seeding approach for restoration has some benefits and some challenges. The challenges include having sufficient infrastructure to hold the reproductive shoots and an efficient way of separating seeds from the rest of the plant material. Benefits include easy transport and deployment of seeds to restoration sites and a relatively easy way to increase genetic diversity by using seeds from multiple meadows. In order to initiate seed-based restoration at the scale needed to combat regional declines in eelgrass, key data gaps must be filled to inform restoration planning.

This protocol was developed to fill knowledge gaps while accommodating programs with varying resources and capacity for field work. Programs may elect to conduct one, two, or all of three assessments described herein.

Site Selection

Many states have online-accessible eelgrass maps derived from aerial surveys. These maps are a good initial step to determine the current distribution of eelgrass in your geographic area of interest. From the mapped meadows in your area, consider these factors to select target sampling meadow(s):

> *Logistics:* Does the site have easy public access? Is there parking? Can you swim to the meadow from the shore (if needed)? Is a boat required? Does water depth dictate a sampling method (i.e. scuba, snorkeling, wading) available to you? Is the site close enough to allow for every-other week visits?

Safety: Is the site far removed from substantial of boat traffic or sewage outfalls? Are the tidal currents excessively strong?

Data Collection

Beginning May 1 of any year and continuing until seed release has ended, visit each site and conduct the following assessments:

(**A**) Phase of seed maturation (seed scoring), *at least every-other week*, and/or
(**B**) Flowering shoot density, *every-other week, or at least once per year when at least 50% of spathes reach stage 4*, and/or
(**C**) Seed density, *at least once per year when at least 50% of spathes reach stage 4*.

If sampling every-other week, approximately 8-10 visits are anticipated per site. Weekly records are useful if capacity allows, especially as seeds reach the dehiscing stage. Once on site, the assessments are expected to take 0.5 to 2 hours.

Assessment A: Seed maturity field sampling *(every-other week)*

Reproductive shoots are morphologically very distinctive. They tend to grow taller than the rest of the meadow canopy and are often a lighter green, almost yellowish in color, with a spindle-like stem (Figure 1).

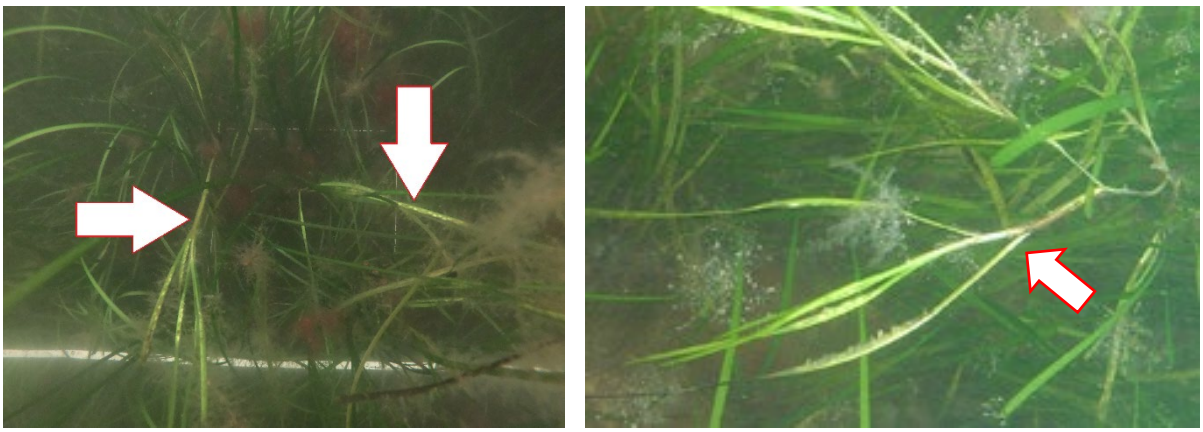

Fig 1 : Examples of reproductive shoots in the field. Source: MA DMF (left), SeagrassLI.org (right)

The seeds on a reproductive shoot are contained within spathes, which protect the developing seeds until they dehisce or separate from the plant. Spathes are clustered in branches called rhipidia (Fig 2). Immature seeds are green in color, and mature seeds tend to be dark brown or almost black in color. The timing of seed maturation can extend over a number weeks in one meadow, and is a critical piece of information to gather for restoration planning purposes. We would like to know the earliest date when seeds reach maturity and when most seeds have dropped.

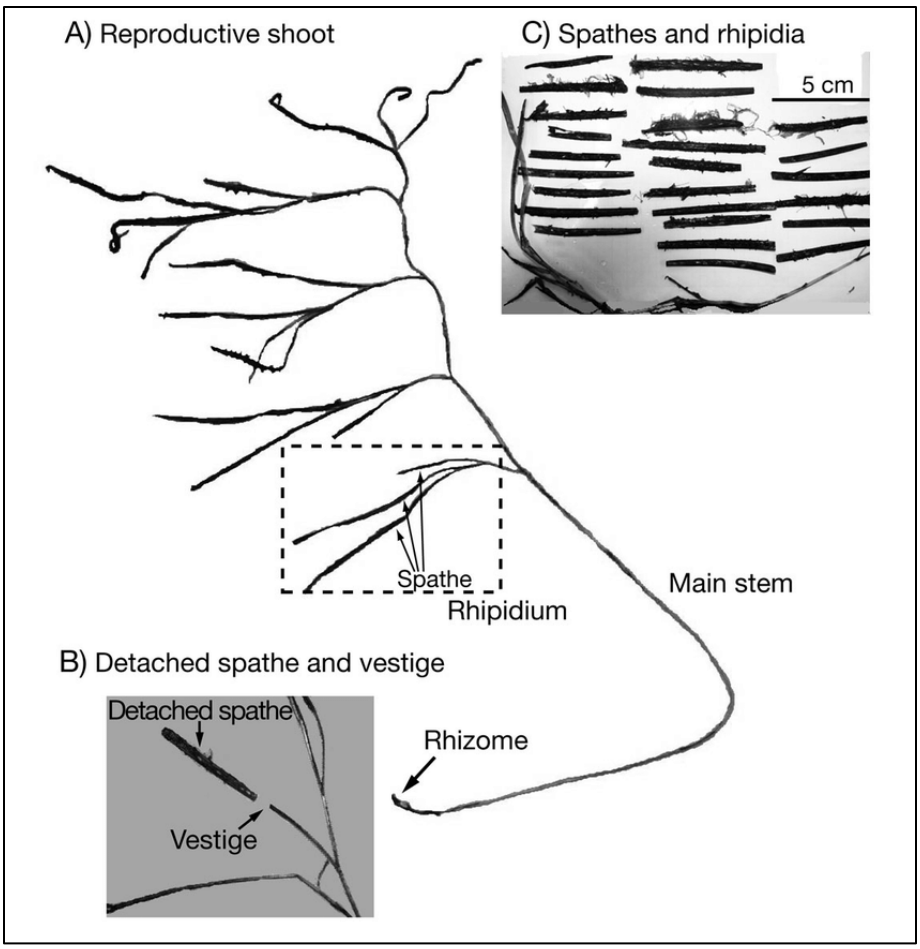

Fig 2. Eelgrass reproductive shoot morphology. Source: Hosokawa et al 2015

It is important to note that seeds on the same flowering plant do not mature uniformly. Spathes lower on the plant, within older rhipidia, tend to contain mature seeds sooner than those higher on the plant (De Cock 1980, Kuo and McComb 1998). Thus, sampling will include multiple parts of the plant, which will be scored using a key to describe the stage of seed maturity.

Field Protocol

1. Record site details on the Site Information Datasheet.
2. From each site, collect **five flowering shoots** from locations spread across the sampling area, by reaching to the bottom of the plant and pinching / snapping the stem where it meets the sediment, and give a gentle pull. Collect shoots at least 1 m apart, ideally spacing samples out over 10-20 m sampling area. Avoid sample collection within quadrats used for density sampling (Assessment B), if applicable.
3. Combine all samples from the site into one zip-close bag and keep in a cool and dark place until you can score the plants, ideally within 24 hours. Scoring at the site is acceptable.

Plant Scoring

1. Identify the reproductive components of the plant (Fig 2).
2. Find the first (lowest and oldest) rhipidium. Record this as rhipidium #1 in your datasheet. For each spathe on that rhipidium, in any order, identify the maturity stage (0-6) using Figure 3. Enter UNS if unsure. Consider taking a photo if unsure and ask for a second opinion.

3. Repeat step 2 for the next rhipidium moving up the plant, which will be #2. Continue working upward to the youngest, uppermost rhipidium.
4. Complete for each of the five shoot samples. Record stages on the field sheet.
5. For each sample, take a representative photo of the stages observed. This will help QA/QC data later.
6. Collect additional seed data (Assessment C) once per year when *at least 50% of the spathes* are in stage 4. Otherwise, discard samples.

Flowering stages of *Z. marina*. Stage 0: Spathes have developed, but styles have not yet erected; stage 1: Styles erect out of spadix; stage 2: Styles bend back into spathe after pollination; stage 3: Half-anthers release pollen; stage 4: Half-anthers have been released, seeds maturing; stage 5: Seeds are starting to release; and stage 6: Post-seed release and the flowering shoot begins to wither. Stages 1–6 are described in more detail in De Cock (1980)

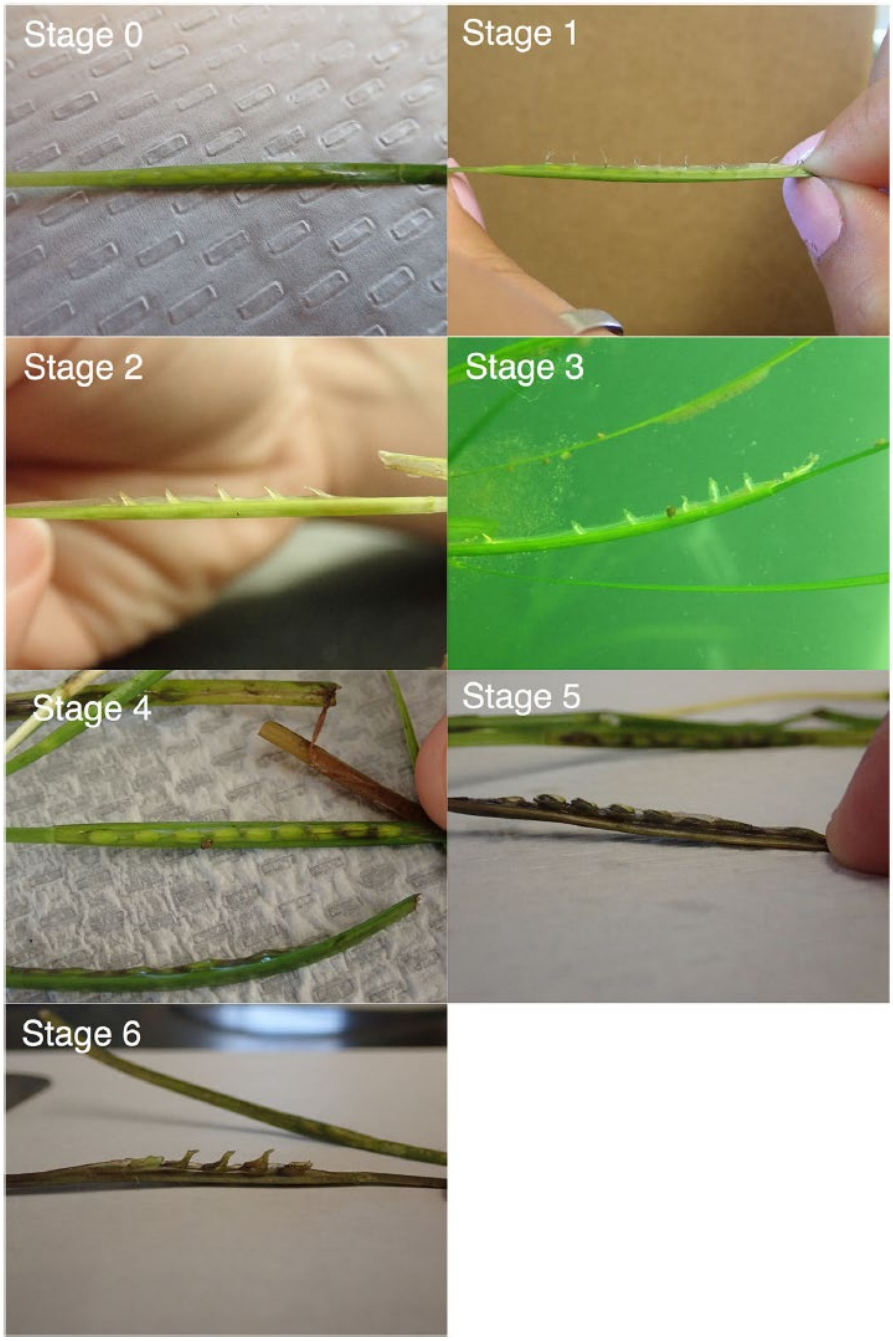

Fig 3A. Stages of eelgrass seed development (von Staats et al. 2021).

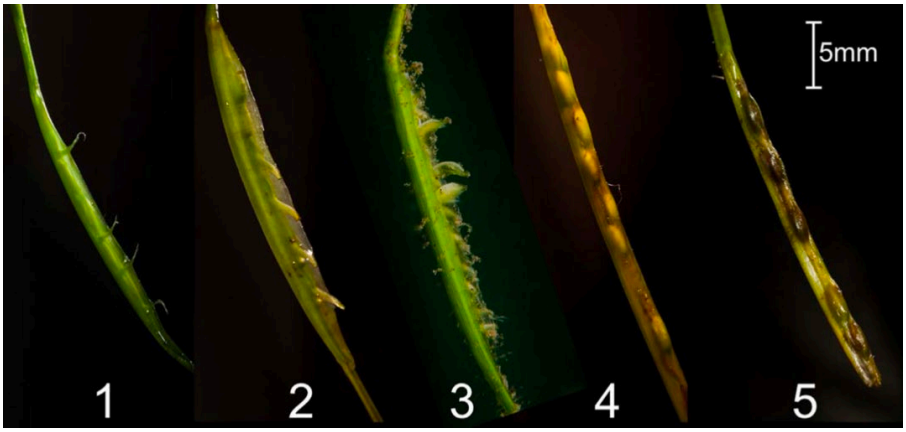

Fig 3B. Stages of eelgrass seed development (Infantes and Moksnes 2018).

Assessment B: Flowering shoot density

(every-other week, or at least once annually when at least 50% of spathes are in stage 4)

Establish sampling design & equipment

To determine flowering density, the number of reproductive shoots are counted in a fixed area as defined by a square shaped quadrat. Quadrats come in many sizes, designs, and materials. The largest quadrats use for seagrass assessments are generally 1 m^2, with other common quadrat sizes being 0.25 m^2 ($1/4^{th}$) or 0.0625 m^2 ($1/16^{th}$). *The 0.25 m^2 size is preferred* for ease of maneuvering and efficiency when performing shoot counts, though any size may be used as long as quadrat size is recorded in the data. If you do not own a quadrat, they can be inexpensively built with PVC pipes and PVC elbows. Most home improvement stores will cut the pipe to size for you (e.g., into four 1 m, 0.5 m or 0.25 m segments), and then you must glue the segments to the elbows to form a square. It is recommended that you drill several holes in each pipe segment to allow for water flow and reduce buoyancy of the quadrat. PVC of diameter 1" to 2" works well.

Aim to sample at least 3 square meters of eelgrass per site (e.g., 12 x 0.25 m^2 samples (*preferred)*; but if needed, can sample 3 x 1 m^2 samples or 36 x 0.0625 m^2 samples).

There is flexibility in approaches to spacing of the quadrat samples, depending on site conditions and access. Attempts should be made to sample quadrats separated at least 1 m from each other.

1. Completely random sampling: Throw the quadrat in completely random distance and direction. The advantage of this approach is it can save time. The disadvantage is you might miss areas of specific interest and you can't define the exact locations sampled.
2. Directed sampling: After doing some initial reconnaissance, one can target areas of a meadow that may appear to have higher flowering rates. Timing and quantity of flowering will vary spatially within individual meadows. This approach will ensure flowering shoots are captured. The disadvantage is this might result in an overestimate of the actual flowering rate throughout the entire meadow, however, literature has already documented that different parts of the meadow flower at different rates, a phenomenon that is largely depth-driven.
3. Transect sampling (*preferred*): A transect is simply a measured line laid out and quadrat samples are taken at regular *predetermined* intervals (e.g., every two meters). By taking GPS coordinates at the

beginning and end points of the transect, fairly precise sample locations can be revisited over time. Resampling sections of the meadow through time is a valuable approach. If one is trying to define the time of maximum flowering and seed ripening, it is best done by resampling the same area through time. This approach does take more time to complete. To expedite subsequent sampling visits, one can deploy semi-permanent markers (e.g., metal screw anchors, wooden stakes) at the beginning and end points of the transect.

Field protocol: Quadrat data collection

1. Record site details on the Site Information Datasheet.
2. Access the meadow by snorkel, scuba or wading. If wading, be mindful of impacts caused by footsteps.
3. If possible, collect a GPS point of the sampling location. You can get coordinates using phone apps like Google Maps. Otherwise, interpolate the location as accurately as possible from a map (e.g., Google Earth, ArcGIS).
4. Place the first quadrat per the sampling design chosen, above. Count the number of reproductive shoots that are rooted within the quadrat. It is best practice to go around the outside edge of the quadrat and ensure the shoots rooted outside the quadrat are not laying down and included incorrectly in the count.
5. *Optional:* if time and capacity allow, also count vegetative (non-reproductive) shoots in each quadrat.
6. Carefully lift the quadrat and move on to the next, until all are completed. Complete the field data sheet as you work.

Assessment C: Seed density *(once annually when at least 50% of spathes are in stage 4)*

Once per year, collect data on the number of rhipidium, spathes, and seeds per spathe using a 5-shoot sample from each site. This is best done when at least 50% of the spathes are in stage 4 (Fig 3) for accuracy and ease of observation. The timing of this is likely mid- to late-summer but will vary by location. Information about seed density per plant is useful for restoration planning and a helpful tool in donor bed prioritization. The more sites you can assess, the better for your local restoration planning. This assessment can take place while at the site or in the lab.

Field/Lab Protocol:

1. Record site details on the Site Information Datasheet.
2. Use a sample from A above (e.g., 5 flowering shoots from one site).
3. Starting with rhipidia #1 (lowest), count and record the number of spathes.
4. For each spathe, count and record the number of seeds, which can be directly seen and felt through the spathe. Stage 4 seeds are still maturing and are mostly green in color. Use a magnifying glass and a pointing tool or probe if needed to assist counting.
5. Continue for ALL rhipidia on the plant (there may be 4 or more).
6. Note qualitative variations in seed size, condition or color within the sheath in the Notes column.
7. Record using the datasheet, discard samples.

References

De Cock, A.W.A.M., 1980. Flowering, pollination and fruiting in Zostera marina L. Aquat. Bot. 9, 201–220

Hosokawa, Shinya & Nakaoka, Masahiro & Miyoshi, Eiichi & Kuwae, Tomohiro. 2015. Seed dispersal in the seagrass Zostera marina is mostly within the parent bed in a protected bay. Marine Ecology Progress Series. 523. 41-56. 10.3354/meps11146.

Infantes, E. and Moksnes, P.O., 2018. Eelgrass seed harvesting: Flowering shoots development and restoration on the Swedish west coast. Aquatic botany, 144, pp.9-19.

Kuo, J., McComb, A.J., 1998. Zosteraceae. In: Kubitzki, K. (eds) Flowering Plants · Monocotyledons. The Families and Genera of Vascular Plants, vol 4. Springer, Berlin, Heidelberg.

von Staats, D.A., Hanley, T.C., Hays, C.G., Madden, S.R., Sotka, E.E. and Hughes, A.R., 2021. Intra-meadow variation in seagrass flowering phenology across depths. Estuaries and Coasts, 44, pp.325-338.

Site information datasheet

Site Name:_____
Site Address:_____
Lat (dd.dddd°):_____
Long (dd.dddd°):_____
Organization:_____

Access Notes:_____
_____
_____

Site Location Type
Tidal River ____ Embayment ____ Open Ocean____
Other _____

Bottom Type (*select all that apply*)
Mud_____Sand____Silt_____Gravel_____Shell hash____
Other _____

Meadow Characteristics
Sparse___Dense___Patchy___Mixed___ Other:_____
Stressed___Healthy___Other:_____

Describe meadow size, shape, stressors present, etc.:
_____
_____
_____

Sketch of meadow and sampling sites

Assessment A: Seed maturity data sheet

Site Name:_____ Sample **Collection** Date/Time:_____
Sample Scorer Names:_____ Sample **Scoring** Date/Time:_____
Org Name & Contact:_____

Sample (shoot) *Values: 1-5*	**Rhipidium** *Values: 1 - x* (1 is lowest on plant)	**Spathe Stage** *Values: 0-6, UNK* (Separate by comma, include as many spathes as present)	**Notes**
1	*1*	*3, 3, 4, 4*	
1	*2*	*3, 3, 4, 5*	
1	*3*	*4, 5, 4*	
1	*4*	*2, 2, 2*	
2	*1*	*4, 5*	
2	*2*	*3, 5, 3*	

Numbers shown as example.

Assessment B: Quadrat sampling datasheet

Site Name:_____ Date:_____
Sampler Names:_____ Time:_____
Org Name:_____ Water Temp:_____

Quadrat size used: 1 m^2_____ 0.25 m^2_____ 0.0625 m^2_____ Other:_____

Quadrat placement strategy: Random_____ Directed_____ Transect_____
Other:_____

Quadrat Number	Repro Shoot Count	Vegetative Shoot Count *optional*		Quadrat Number	Repro Shoot Count	Vegetative Shoot Count *optional*
1	*8*					
2	*3*					
3	*0*					
4	*3*					

Notes:_____
_____
_____

Numbers shown as example.

Assessment C: Seed density data sheet (once per year)

Site Name:_____Sample **Collection** Date/Time:_____
Sampler Names:_____Sample **Processing** Date/Time:_____
Org Name & Contact:_____

Sample (shoot) *Values: 1-5*	**Rhipidium** *Values: 1-x* (1 is lowest on plant)	**# Seeds per Spathe** *Values: 0-x* (Separate by comma, include as many spathes as present)	**Notes**
1	*1*	*22, 20, 18*	
1	*2*	*14, 10, 19, 20*	
1	*3*	*23, 20, 18*	
1	*4*	*13, 18, 18*	
1	*5*	*22, 21*	

Numbers shown as example.