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Aquatic Botany 76 (2003) 79–86

Aquatic botany

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Technical note

A new method for estimation of *Halophila decipiens* Ostenfeld seed banks using density separation

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Received 23 July 2002; received in revised form 20 November 2002; accepted 13 January 2003

Abstract

Few studies have addressed the spatial and temporal variability in seagrass seed banks. For small-seeded species in particular, seed banks are hard to sample quantitatively because of the difficulty in separating small seeds from sediment. *Halophila decipiens* is a highly fecund and cosmopolitan seagrass species, occupying niches which other larger-sized perennial species cannot utilize. Although many studies have suggested *H. decipiens* meadows are annual and depend on seed banks to re-establish, none have quantitatively examined this necessary life history component. To process the number of samples required to adequately address questions of spatial and temporal variability in *H. decipiens* seed banks, we developed a density separation technique which removes seeds from sediment and allows easier enumeration. Sediment samples were treated with refrigerated Ludox, a colloidal silica. The colloidal silica supernatant and two surface sediment rinses were examined for the presence of seeds. Our protocol removed between 78 and 100% of seeds from sediment, with a mean removal efficiency of 89%. Use of this method increased our sample processing capability from 4 to 20 samples per day.
Published by Elsevier Science B.V.

Keywords: *Halophila decipiens*; Seagrass; Seed bank; Ludox

1. Introduction

Sexual and asexual reproduction have been documented in all but one seagrass species, *Halophila johnsonii* (Jewett-Smith et al., 1997). Even so, sexual reproduction is highly variable and may be rare in some perennial species (Inglis, 2000a,b; Orth et al., 2000), resulting in a long-standing paradigm that most seagrass populations are maintained primarily by vegetative propagation, despite recognition of the unique evolutionary adaptations which enable

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0304-3770/03/$ – see front matter. Published by Elsevier Science B.V.
doi:10.1016/S0304-3770(03)00018-4

them to flower, pollinate, and set seed in the marine environment (Cox, 1993; Philbrick and Les, 1996). Past studies of *Zostera* and *Halophila* spp. suggest there are several exceptions to the paradigm and that the population dynamics of some seagrasses depend almost exclusively on sexual reproduction and seedling recruitment (Keddy and Patriquin, 1978; Robertson and Mann, 1984; McMillan, 1988a; Harrison, 1991; Meling-Lopez and Ibarra-Obando, 1999; Kenworthy, 2000). Members of the genus *Halophila* are among the most fecund and widely distributed tropical seagrasses, with extensive meadows located on deep water continental shelves (Continental Shelf Associates Inc. and Martel Laboratories Inc., 1985; Continental Shelf Associates Inc., 1991; Lee Long et al., 1996), in extreme environments (Sheppard et al., 1992; Kenworthy et al., 1993; Jupp et al., 1996), and in coastal systems where seasonally fluctuating conditions prevent establishment of larger-sized perennial species (Williams, 1988; Hillman et al., 1995; Kuo and Kirkman, 1995; Kenworthy and Fonseca, 1996; Kenworthy, 2000). Many of these *Halophila* spp. meadows are annual and depend on re-establishment by seed and seedling recruitment during temporary or seasonal periods with favorable growing conditions (Kuo et al., 1993; Kuo and Kirkman, 1995; Preen et al., 1995).

The sediment seed bank is an important component of the life history of flowering plants which propagate sexually (McMillan and Soong, 1989; Fenner, 1995; Baldwin and Mendelssohn, 1998; Inglis, 2000a,b; Orth et al., 2000; also see Bonis et al., 1995; Rasheed, 1999). Persistent seed banks may act as a reserve of genetic information affecting the fitness of populations (Harper, 1977). Seeds buried in the sediments avoid the harsh environments present during unfavorable growing conditions. Viable seeds present after over-wintering or remaining after an acute disturbance allow plant populations to recover when conditions are suitable for germination. This life history strategy is suitable for surviving the fluctuating environments where *Halophila decipiens* thrives (Kenworthy, 2000). Morphologically, *H. decipiens* is well adapted to form seed banks. The flowers and fruits are produced at the base of the leaf petiole, on or just beneath the sediment–water interface. As a result, fruits and seeds are easily buried and incorporated into the sediment. Evidence of potentially large seed banks in a *Halophila* species meadow were first suggested for *H. decipiens* by McMillan (1988a) and McMillan and Soong (1989) and later confirmed by observations of the importance of seedling recruitment in several different locations (Josselyn et al., 1986; McMillan, 1988b; Preen et al., 1995; Kenworthy, 2000).

Estimating seed bank densities can be problematic for a number of reasons. First, patchy distribution of seeds results in high spatial variability (Hootsmans et al., 1987; Fenner, 1995; Inglis, 2000a), and this variability may appear higher due to small sample size and small-scale sampling. Second, life history may cause temporal variability in seed banks, so sampling strategies should take life history into consideration. These factors combined necessitate that sampling be conducted at high spatial and temporal resolution to accurately capture seed bank variability (Fenner, 1995; Orth et al., 2000). In many cases, the effort required to enumerate seeds, especially the small seeds of *Halophila* spp. (0.2–1.0 mm), results in small sample sizes. McMillan (1988a) and McMillan and Soong (1989) estimated the abundance of *H. decipiens* seeds in the sediment bank by sieving sediment samples through an appropriately-sized screen (mesh size not given). To count seeds, they placed the sample residue in a petri dish with a small amount of artificial seawater and examined the sample under a binocular microscope. They discovered during subsequent germination

studies, however, that many seeds had gone undetected during this microscopic inspection (McMillan, 1988a; McMillan and Soong, 1989). Thus, these earlier studies may have underestimated the total seed bank. Based on a growing awareness of the function of seeds in the population ecology of seagrasses in general (Inglis, 2000a,b), the widespread distribution of *Halophila* spp. worldwide, and their ecological value as a substitute for larger perennial species, there is a need to develop an accurate and efficient method to estimate the sediment seed reserve (Orth et al., 2000).

Our objective was to develop a more efficient quantitative method to examine the sediment seed bank of *H. decipiens*, for use in large ecosystem studies. To achieve this objective, we developed a density separation technique to remove seeds from sediment so they could be counted with minimal interference. The protocol was refined using sediment "spiked" with *H. decipiens* seeds excised from fruits collected for a related project in the eastern Gulf of Mexico. This technique was intended to decrease the time necessary to process samples and allow sampling in a wider geographic area to better estimate the variability and distribution of the seed bank. To our knowledge, this paper presents the first attempt to use density separation for seagrass seed enumeration.

2. Methods

2.1. Seed bank samples and sediment characteristics

Seed bank sediment samples were collected in *H. decipiens* habitat on the west Florida shelf (25°3.6′N, 81°33.6′W) in June 1999 and January 2000 using core tubes (4.4 cm diameter, 7.5 cm depth, roughly 90 ml in volume). Sediment was transferred to plastic bags and samples were frozen for transport back to the laboratory.

Sediment grain size analysis was conducted on sediment core samples collected for a related west Florida shelf project. Samples were processed using standard wet-sieving techniques.

2.2. Separation test

The density separation technique relies on a difference in specific weight between lighter weight organic matter (e.g. meiofauna, diatoms, seeds) and heavier inorganic sediment. Treatment results in suspension of the organic matter in the extract solution and sinking of the sediment particles (Kropáč, 1966; De Jonge and Bouwman, 1977; De Jonge, 1979).

We initially tested extraction of seeds from sediment with four media: distilled water, supersaturated sucrose solution, supersaturated potassium carbonate solution and colloidal silica (Ludox HS 40, Aldrich; hereafter referred to as Ludox). We attempted to float seeds out of sediment by simply spreading the sediment out in a sorting tray and adding one of the four media until the sediment was submerged by a 2–3 cm layer of media. The sediment was then slowly mixed by hand and particles which floated to the media surface were examined under a dissecting microscope. After draining the test media, we visually inspected the sediment to look for seeds which did not float. Neither seeds contained in the sediment nor seeds added to the sediment from excised fruits floated reliably in distilled

water or sucrose. We therefore conducted subsequent extraction tests only with Ludox and supersaturated potassium carbonate solution.

Although Den Hartog (1970) reported *H. decipiens* seeds to be 0.2 mm long, most authors have described seed diameters of 0.4–0.5 mm or larger (McMillan and Soong, 1989; Aryuthaka, 1993; Kuo and Kirkman, 1995; Kuo et al., 1995). Seeds found in the west Florida shelf sediment during visual inspection had a length of 0.55 ± 0.05 mm and a diameter of 0.46 ± 0.04 mm (mean ± 1 standard deviation; $n = 25$ seeds; Hammerstrom and Kenworthy, unpublished data), while seeds from mature fruits collected on the west Florida shelf had a length of 0.36 ± 0.05 mm (mean $\pm$ 1 standard deviation; $n = 318$ s; Hammerstrom and Kenworthy, unpublished data). Given the seed dimensions we measured, we chose to sieve sediments into three size fractions: 125–250 μm, 250–500 μm, and >500 μm.

Prior to size fractionation, we visually inspected sediment from eight thawed cores to ensure that we started with seed-free sediment. Sediment was rinsed thoroughly with fresh water in a 125 μm sieve and divided into <1 ml volume portions. Each portion was sorted in a gridded petri dish under a dissecting microscope at 60× magnification to remove any seeds present. Each portion was examined multiple times until two visual inspections in a row revealed no additional seeds. Portions were then recombined into the original eight core samples. One hundred seeds excised from mature fruits collected for a related project were added to each seed-free core sample. Spiked core samples with known seed density were then sieved into 125–250 μm, 250–500 μm and >500 μm size fractions. The <125 μm size fraction was rinsed away during sieving. The size fractions were separated into aliquots of approximately 10–20 ml and placed in labeled 50 ml centrifuge tubes. All size fractions were divided into as many as four aliquots. To each aliquot, we added approximately 30 ml of Ludox or saturated potassium carbonate. A total of four core samples were treated with each solution (although each sample was separated into multiple aliquots). All aliquots were shaken vigorously by hand for 15–20 s and centrifuged at 3200 rpm for 10 min. The supernatant was poured through a 125 μm sieve and rinsed with fresh water. The sieve contents were transferred to a petri dish and examined for seeds under a dissecting microscope. All aliquots were then treated a second time with the same separation medium and processed as above. Results from the two trials were pooled by core sample. Mean and standard deviation were computed for the total number of seeds removed by each solution and *t*-tests were performed on log-transformed data using SAS version 8.0. (SAS Institute Inc., 1999).

2.3. Ludox efficacy test

Our comparison revealed that Ludox was the more reliable separation solution and therefore potassium carbonate was no longer used. To test the efficacy of the Ludox separation method, we processed eight sediment cores (same size as above) collected from *H. decipiens* habitat on the west Florida shelf in January 2000. These core samples were sieved into 125–250 μm, 250–500 μm and >500 μm size fractions, separated into small aliquots, treated with Ludox, centrifuged and processed as described. Initial counts focused on seeds floating in the supernatant and resting on the sediment surface. The remaining sediment was transferred into a gridded petri dish in very small aliquots (<1 ml volume) and examined with a dissecting microscope for presence of seeds not removed by the separation technique. The percentage of seeds recovered by Ludox separation was computed for all samples.

3. Results

3.1. Sediment characteristics

Mean sediment organics was 2.35%, largely due to the presence of carbonates. Gravel, sand, and silt/clay values were 12.63, 75.42, and 11.95%, respectively (C. Currin, unpublished data). The gravel fraction was largely composed of shell fragments.

3.2. Separation test

We used fresh water to sieve seed core samples because fresh water was convenient. Although some seagrass seeds will germinate when exposed to low salinity (Orth et al., 2000 and references therein), we did not observe any germinated seeds in our sediment samples, nor did we find burst seeds, as might be expected due to increased osmotic pressure.

Ludox had no discernable effect on the physical characteristics of seeds. Potassium carbonate caused seeds to shrink slightly inside the seed coat, giving them the appearance of a clear bubble around a solid center. This modified appearance made the seeds more difficult to distinguish during sorting. Density separation of seeds from the sediment matrix was more efficient in smaller aliquots of sediment (<10 ml).

No *H. decipiens* seeds were found in the 125–250 μm or >500 μm size fractions of the eight samples tested with Ludox and saturated potassium carbonate solution. Following one treatment of the 250–500 μm size fraction with a separation medium, Ludox removed significantly more seeds from the sediment than potassium carbonate (76 and 54%, respectively; $P = 0.004$, d.f. $= 6$, $t = 2.45$). We then re-treated the sediment with the same separation medium to extract more seeds. Although saturated potassium carbonate solution removed more seeds than Ludox during the second treatment, Ludox proved to be the most reliable and consistent separation medium overall. Ludox removed significantly more seeds in total than potassium carbonate (82 and 70%, respectively; $P = 0.05$, d.f. $= 2.44$, $t = 2.45$). We eliminated the use of saturated potassium carbonate solution and further tested the technique using Ludox.

3.3. Ludox efficacy test

During preliminary tests we discovered that Ludox kept at 4 °C seemed to be more effective in removing seeds from sediment than Ludox kept at 25 °C. Although this observation was not tested, we used chilled Ludox in all efficacy testing. In some instances after separation, *H. decipiens* seeds were removed from the bulk of the sediment but were not suspended in the supernatant. These seeds were easily recovered by washing the sediment surface and the sides of the centrifuge tube with distilled water and pouring the washed contents into a petri dish. Examination of the supernatant and two "rinses" of the surface sediments and sides of the centrifuge tube accounted for recovery of a minimum of 78% ($n = 1$) and a maximum of 100% of the seeds in a sample ($n = 2$). Density separa-

tion using Ludox recovered a mean of 89% of the seeds in the eight samples processed completely.

4. Discussion

We processed samples in which sediment was primarily sand (75%). In samples with a higher percentage of silt/clay, the sieving process might take longer, especially if it were necessary to reserve the smallest sediment size fraction. Sediment with high organic content would cause the Ludox supernatant to contain more particulates, which might necessitate more time at the dissecting microscope. In either case, the density separation method, when modified for the appropriate seed size, would enable researchers to eliminate processing of some portion of the sediment and thus speed up sample processing to some degree.

The density separation technique presented here was developed for *H. decipiens* seeds. Presumably the technique could be adapted for other seagrass species in which small seed size has made enumeration of the seed bank difficult. Although McMillan (1988a) sieved sediment samples, he stated that his visual counts did not account for all the seeds present in the sediment. Our technique reduces the amount of material that must be visually inspected by removing the non-target size fraction of the sediment and decreasing the seed-sized sediment fraction that must be inspected to only the two "rinses." Sorting the entire 250–500 μm size fraction of a core sample (mean volume of approximately 30 ml) with a dissecting microscope required approximately 2 h for one individual. Once our protocol was refined, one individual could process the 250–500 μm fraction supernatant and rinses of eight samples in approximately 2 h, or 20–25 samples in an 8 h work day. Thus, our protocol increased our sample processing ability by seven- or eight-fold. Based on our Ludox efficacy test results, our methods resulted in an average removal efficiency of 89% and a range of removal efficiency of 78–100%. These values allow us to approximate the error in our sampling method and give us the ability to place confidence limits around our estimates of seed bank density, something not done in previous studies (see McMillan, 1988a). Our increase in sample processing capability should enable our sampling regimes to accommodate both temporal and spatial variability without having to sacrifice sample size, an important advantage when sampling large-scale ecosystems such as the *H. decipiens* habitat on the west Florida shelf. Furthermore, this method can be used to examine other habitats where preliminary evidence suggests the ecological role of seed recruitment in the population dynamics of meadows largely dominated by small-seeded *Halophila* spp. (Preen et al., 1995; Kenworthy, 2000).

Acknowledgements

We thank C. Currin and P. Hanson, who participated in discussions that led to the development of this density separation technique. Reviews by P. Marraro, R.J. Orth, G. Thayer, A. Uhrin, J.E. Vermaat and two anonymous reviewers improved this manuscript. Funding was provided by NOAA's National Marine Sanctuary Program and Center for Coastal Fisheries and Habitat Research, NOS, NCCOS, Beaufort, NC, and the Recover Protected Species Program, National Marine Fisheries Service, Silver Spring, MD.

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