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AQUATIC PLANT CONTROL
RESEARCH PROGRAM

Growth Assessments of Starry Stonewort (*Nitellopsis obtusa*) in Various Substrate Types for Large-scale Cultivation Studies

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PURPOSE: The purpose of this study was to compare multiple substrate types to optimize cultivation conditions for the invasive macroalga *Nitellopsis obtusa* (Desv. in Loisel.) J. Groves, commonly known as starry stonewort. Large-scale cultivation will allow for tiered approaches to management evaluation research while minimizing the influence of confounding variables.

INTRODUCTION: Submerged aquatic vegetation (SAV), including macrophytes and macroalga, are important components to a healthy aquatic ecosystem as it directly impacts the biogeochemical cycle along with the surrounding biological community structure and provides valuable ecological services (Sand-Jensen and Borum 1991). A diverse SAV community provides food and shelter for aquatic and terrestrial organisms, oxygenates the water column, and stabilizes sediment in the littoral zone (Carpenter and Lodge 1986; Dibble et al. 1996; Jeppesen et al. 1997). These effects are similar for vascular aquatic plant and charophyte populations, which often grow together to form a healthy and diverse community (Kovtun-Kante et al. 2014). However, the establishment of invasive SAV species can greatly diminish these ecological benefits by reducing native plant growth, water quality, and increasing sedimentation and nutrient loading (Madsen 2009). In North America, the charophyte starry stonewort (*Nitellopsis obtusa*) is a prominent example of an aquatic invasive species that induces these detrimental changes to SAV dynamics and threatens the stability of littoral ecosystems (Pullman and Crawford 2010; Larkin et al. 2018).

Starry stonewort is a green macroalgae native to Europe and Asia and is similar in appearance to other macroalgae including other stoneworts and muskgrass (Pokrzywinski et al. 2020a). Starry stonewort is much larger, however, with stem and branch cells about 1 mm in diameter and stems over 80 cm in length (Sher-Kaul et al. 1995). While it is dioecious, only male plants have been described in North America (Minnesota DNR 2015; Wisconsin DNR 2017). Reproduction in the region is achieved asexually through the propagation of star-shaped bulbils attached to the rhizoids, which give the species its common name (Larkin et al. 2018). Reproduction is also thought to occur via fragmentation of the thallus (Kipp et al. 2019). Bulbils can be found in the sediment year-round but are most common when biomass is decreasing in late summer since they serve as the overwintering mechanism for starry stonewort (Midwood et al. 2016). In the spring, bulbils can germinate in as little as three to five days under favorable conditions (Hackett et al. 2017).

Starry stonewort was first documented in the US in 1978 in the St. Lawrence River, NY (Geis et al. 1981). Since then, the invasive charophyte has continued to spread and has been discovered at high densities in six US states and two Canadian provinces as of 2017 (Larkin et al. 2018; Sleith et al. 2018). Ecological niche modeling suggests that 29 additional states have lakes suitable for starry stonewort (Escobar et al. 2016). Similar to other invasive aquatic species, starry stonewort is believed to spread primarily through human activities (e.g., boats, fishing gear, trailers, and ballast water), but can also spread via wildlife and water movement (Pokrzywinski et al. 2020a; Wisconsin DNR/Golden Sands 2016). Once established, it can produce dense beds at depths up to 30 m where it impacts fish habitat and spawning activities, water chemistry, and recreation (Pullman and Crawford 2010; Hackett et al. 2017; Larken et al. 2018). Concern over the increasing distribution of starry stonewort and its damaging impact on aquatic systems has led watershed managers and other stakeholders to call for the development of effective management strategies.

Starry stonewort cultivation requires a controlled setting to properly evaluate management strategies. This allows for observational changes in growth and physiology to be documented as a direct result of implementing a management option while minimizing confounding variables that often cloud the results of field-demonstrations or small-scale studies. This study facilitated the controlled cultivation of starry stonewort by addressing the need for an optimized growth substrate. Building upon cultivation optimization work previously conducted at the US Army Engineer Research and Development Center (ERDC) (Pokrzywinski et al. 2020b), the impact of various substrate types and constituents on starry stonewort sprouting and growth were explored. Combinations of four sediment types, four fertilizer additives, and four amendment conditions were assessed.

The results of this study will demonstrate suitable synthetic substrate alternatives to natural lake sediments for achieving optimal growth. The use of a synthetic substrate is desirable for laboratory experiments as it controls for many variables otherwise inconsistent in lake sediments (e.g., nutrients, contaminants, or microbial communities) and facilitates methodological reproducibility and standardization for follow-on research efforts. The results presented here will inform the appropriate large-scale cultivation conditions for subsequent management studies of starry stonewort.

MATERIALS AND METHODS: Lake sediment was collected from Wells College Bay, Cayuga Lake in Aurora, NY from known starry stonewort sites on June 8, 2018, using a Peterson grab sampler. Sediment was shipped to ERDC in Vicksburg, MS and stored at 4 °C upon receipt. Sediment was sieved (1 mm sieve) to collect bulbils and remove debris. Collected bulbils were then measured to the nearest millimeter using a standard metric ruler and sorted into five size categories: A) $1 \leq 2$ mm, B) $2 \leq 3$ mm, C) $3 \leq 4$ mm, D) $4 \leq 5$ mm, and E) > 5 mm. Sorted bulbils were placed in glass amber vials filled with tap water and a thin layer of Cayuga Lake sediment and stored at 4 °C prior to the start of the study.

The experimental design was a completely randomized 4 x 4 x 4 factorial with ten replicates per treatment combination. Treatments were sediment type, amendment, and fertilizer type. The four sediment types consisted of (1) Cayuga Lake sediment, (2) topsoil,[1] (3) garden soil,[2] and (4) commercial sediment marketed for culturing aquatic plants.[3] Each sediment included one of four

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[1] Gardenese Top Soil, Phillips Bark Processing Co., Inc., 141 Executive Drive Ste. 2, Madison, MS 39110.

[2] Natures Care® Organic Garden Soil, Miracle-Gro Lawn Products, Inc. 14111 Scottslawn Rd. Marysville, OH 43041.

[3] Dyna Dirt Aquatic Planting Soil™, Givhandy's Inc. 3687 HWY N.E., Rydal, GA 30171.

different fertilizers: (1) dolomitic limestone[4] (N-P-K: 0-0-0), (2) bone meal[5] (N-P-K: 6-8-0), (3) slow-release plant food[6] (N-P-K: 15-9-12), or (4) no fertilizer. Lastly, the sediment plus fertilizer was amended with either 20% sand,[7] 20% clay,[8] 20% sand plus 20% clay, or no sand/clay addition.

Soil/sediment classification was performed following the procedures outlined in Thien (1979), which were designed to determine the texture designation of mineral soil materials. In practice, coarse roots and woody debris are removed from soil samples prior to soil texture determinations. In this study, however, debris removal was not performed because the soil compositions were formulated based upon a desired distribution of fertilizers and amendments. As a result, many samples contained organic fragments (e.g., bark, acorn husk) >0.5 cm in diameter. Thus, the hand texture designations shown in Table 1 may be artificially biased toward coarse texture designations, especially in samples containing high proportions of the top soil and compost materials.

Table 1. Soil classifications for sediment types and amendment types, independent of fertilizer type. An "*" indicates a likely artificial bias toward coarse texture designations as result of the necessary modification to the Thein (1979) procedures described in the text.

	No sand or clay	**20% clay**	**20% sand**	**20% clay + 20% sand**
Aquatic Soil	sandy clay loam	sandy loam	sandy loam	sandy loam
Garden Soil	sand*	sand*	loamy sand	loamy sand
Lake Sediment	silty clay loam	sandy clay	sandy clay	sandy clay loam
Top Soil	sand*	sand*	sand*	loamy sand

Sediments were added to a 0.185 m^2 (2 ft^2) container at approximately 5 cm in depth (7.8 L of sediment by volume) to determine appropriate fertilization rates as per label instructions. Dolomitic limestone was added to achieve a 27.2 kg per 93 m^2 (60 lb per 1,000 ft^2) rate, bone meal was added to achieve a 237 ml per 1.85 m^2 (8 oz per 20 ft^2) rate per label recommendations, and slow-release plant food was added to achieve a 22.5 ml per 0.185 m^2 (2 ft^2) rate per label recommendations. Fertilizers were thoroughly mixed into the sediments for 5 min by hand. The 20% sand, 20% clay, and 20% sand plus 20% clay amendments were added to the fertilizer-amended sediments on a volume-by-volume basis and thoroughly mixed for 5 min by hand.

Bioassays were conducted using 5 mL of amended sediment in 50 mL falcon tubes with 35 mL of Forsberg's media II at half strength (Anderson 2005). A single bulbil was planted approximately 1 cm below the sediment surface. Bulbils were randomly distributed by sizes among treatments to account for differences in sprouting rates that may have occurred owing to bulbil size. Bioassays were conducted in an environmental growth chamber with Hydrofarm AgroSun Gold halide bulbs (1,000 W) under 25-35 μmol photons m^{-2} s^{-1} with a 14-hr light /10-hr dark photoperiod at 22 °C

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[4] Pro AgLime, Fine Pulverized Dolomitic Limestone, Austinville Limestone Co. PO Box 569, Austinville, VA 24312.

[5] Burpee® Natural and Organic Bone Meal (6-8-0), W. Atlee Burpee & Co. 300 Park Ave Warminster, PA 18974.

[6] Osmocote® Smart-release® Plus Outdoor & Indoor (15-9-12). The Scotts Miracle-Gro Company 1411 Scottslawn Road, Marysville, OH 43041.

[7] Quikrete® Cement & Concrete Products™ Playsand, The QUIKRETE® Companies, One Securities Centre, 3490 Piedmont Road, Suite 1300, Atlanta, GA 30329.

[8] Hydro CRUNCH expanded clay pebbles. HydroCrunch, 20651 Golden Springs Dr. Suite 115. Walnut, CA 91789.

for 31 days. After 31 days, specimens were harvested, and growth was assessed by measuring the longest axis of the specimen to the nearest millimeter. Bulbil sprouting rate was also determined, where sprouting was considered anything that displayed green vegetative biomass regardless of main axis height. SAS was used to create a generalized linear mixed model using PROC GLIMMIX to test for main effects and interactions for all sprouted bulbils based on (1) sediment type (aquatic soil, garden soil, top soil and lake sediment), (2) fertilizer type (no addition, bone meal, limestone, and osmocote) and 3) additive (no addition, clay, sand, and clay plus sand). A non-parametric Kruskal Wallis test was used to determine statistical differences within treatments, for single factors, independent of other factors at an alpha level of 0.05. A Dunn's multiple comparisons test was used to evaluate differences between conditions. For more complex analyses across treatments, a generalized linear model was conducted using R version 3.6.3 and R-Studio version 1.2.5042 to assess statistical differences between and within groups for soil type, fertilizer type, and amendment.

RESULTS AND DISCUSSION

Soil classification and pH. Soil classification informs how quickly water can infiltrate the sediment (intake rate) and how much water the sediment retains after excess water has drained away (field capacity). Generally, sediment containing a higher percentage of fine particles like clay has a lower intake rate and higher field capacity, while sediment containing a higher percentage of coarser particles like sand has a higher intake rate and lower field capacity. Top soil and garden soil tended to have coarser sediment while lake sediment had finer sediment (Table 1).

Substrate pH measurements are shown in Table 2 for the soil type–fertilizer type combinations. Aquatic soil had the lowest median pH, lake sediment had the highest median pH, and garden soil and top soil were both closer to neutral pH values.

Table 2. Median soil pH for soil types and fertilizer types, independent of amendment type.

	No Fertilizer	Bone Meal	Limestone	Osmocote®
Aquatic Soil	4.37	4.25	5.36	4.43
Garden Soil	6.48	6.19	6.86	5.83
Lake Sediment	8.13	8.18	8.21	7.72
Top Soil	7.11	7.31	7.22	6.60

Intake rate, field capacity, and soil pH are all important factors affecting plant and algae establishment and growth. The soil classification and pH of substrates demonstrating high sprouting rates and axis growth could further explain the preferred growth conditions of starry stonewort.

Growth assessment by soil type. Growth assessment of soil types without added fertilizer or amendments showed there was a statistical difference between soil types ($p < 0.0001$). Sediment collected from Cayuga Lake had the highest percentage of sprouted bulbils and a significantly higher axis height than that of garden soil or aquatic plant soil ($p < 0.001$) (Figure 1). There was no significant difference between the lake sediment and top soil treatments, suggesting that top soil performs comparably to lake sediment in promoting starry stonewort stem growth. All bulbils planted in the lake sediment sprouted, while only half of those planted in top soil, and 40% of bulbils planted in garden soil or aquatic soil sprouted.

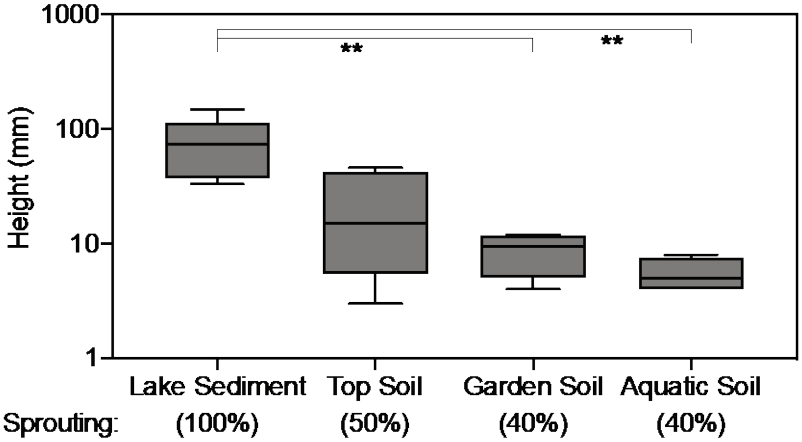

Figure 1. Starry stonewort sprouting rate (parentheses) and height box and whisker plots after 31 days of growth (n=10) incorporating the minimum, maximum, median, and interquartile range of starry stonewort specimens, excluding additives (fertilizer or amendment). Statistical significance is denoted by asterisks where: ** $p < 0.001$.

Growth assessment by fertilizer type. Growth assessment of fertilizer types, independent of soil type and without added amendments, showed there was no significant difference in axis height (Figure 2). The greatest percentage of bulbils sprouted in the limestone fertilizer (75%). Fewer bulbils sprouted in the bone meal and Osmocote® treatments (35% and 30%, respectively) than in treatments where no fertilizer was used (58%).

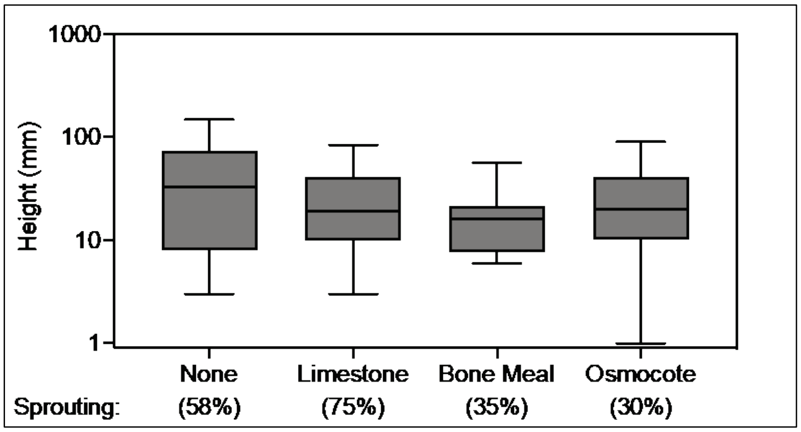

Figure 2. Starry stonewort sprouting rate (parentheses) and height box and whisker plots incorporating the minimum, maximum, median, and interquartile range of starry stonewort specimens by fertilizer type, independent of soil or amendment type after 31 days of growth, n=40 per fertilizer type (no significant differences between means).

Growth assessment by amendment type. Growth assessment of amendment types, independent of soil type and without added fertilizer, showed there was no significant difference in axis height (Figure 3). The greatest percentage of bulbils sprouted in the 20% sand + 20% clay amendment condition (78%), followed by the 20 % clay (63%), no amendment (58%), and 20% sand (50%) conditions.

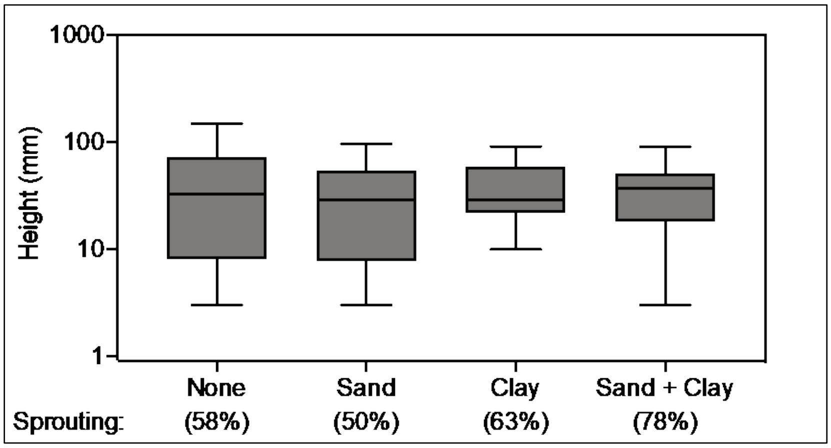

Figure 3. Starry stonewort sprouting rate (parentheses) and height box and whisker plots incorporating the minimum, maximum, median, and interquartile range of starry stonewort specimens by amendment type, independent of soil or fertilizer type after 31 days of growth. Each box plot represents the starry stonewort height for all treatments from each sediment type pooled by amendment, n=40 per amendment type (no significant differences between means).

Optimal synthetic substrate. While lake sediment facilitated the greatest sprouting rate and several of the highest median axis heights observed across the various substrate treatment conditions, a synthetic substrate is more desirable for future lab scale trials. It allows for better control over confounding variables when comparing studies (Richardson and Huag 2018) such as microbial community, contaminants, and nutrients, which can fluctuate significantly in natural sediments collected from lakes. Based on the growth assessment by soil type (Figure 1), top soil was identified as the most optimal synthetic sediment choice for replacing lake sediment in future management trials as there was no significant difference in median axis height between the two sediment types.

Further comparison of the top soil and lake sediment treatment conditions showed that top soil amended with 20% sand and 20% clay without added fertilizer performed better than other top soil treatments, achieving a median axis height of approximately 70 mm which is similar to the best-performing lake sediment treatments (Figure 4). Thus, the most suitable synthetic substrate alternative to natural lake sediments for achieving optimal growth in large-scale, controlled cultivation and management studies is top soil amended with 20% sand + 20% clay without added fertilizer (pH 7.63; loamy sand soil classification). Statistics on specimen height comparisons between sediment, amendment, and fertilizer type could not be performed owing to variable sprouting rates that resulted in a variable *n* for each condition.

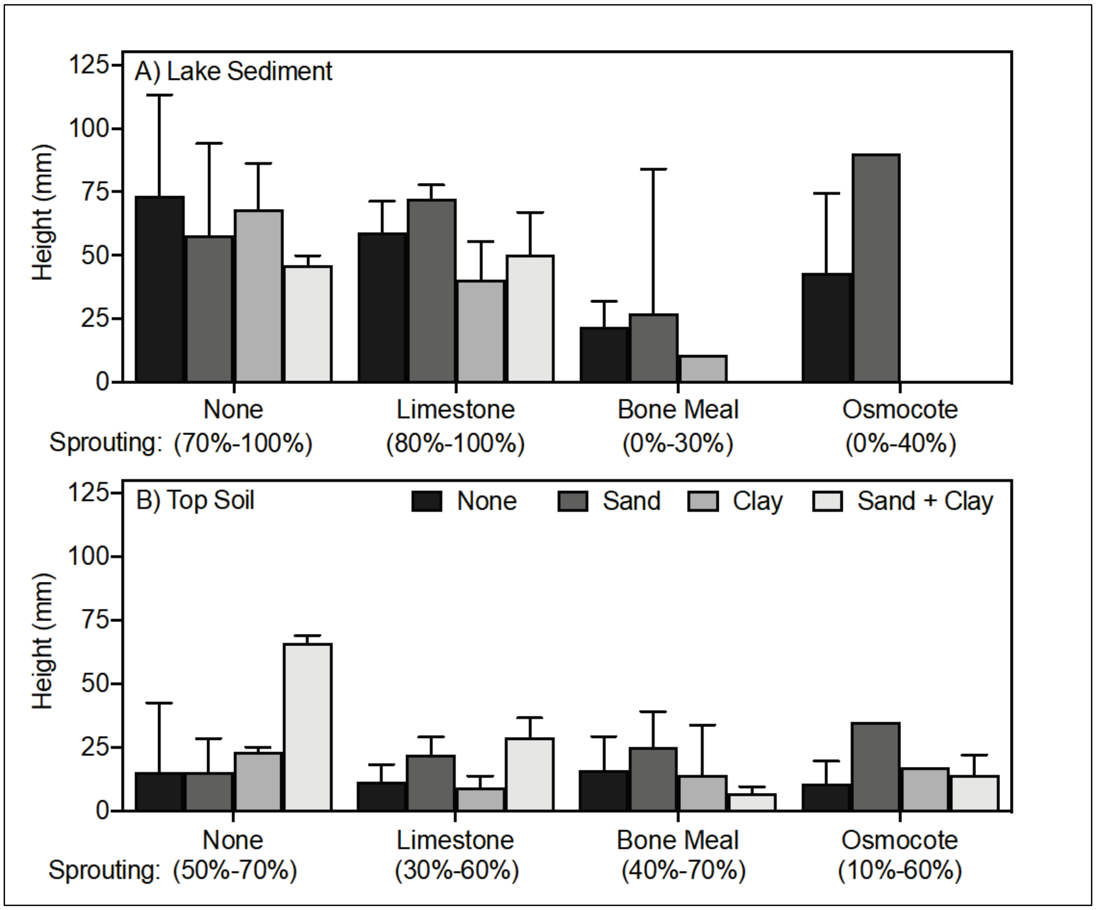

Figure 4. Starry stonewort sprouting rate (parentheses below axis) and median height for all amendment types for specimens grown in lake sediment (A) and top soil (B) after 31 days of growth, n = 10 per condition (no statistics performed due to lack of consistent sprouting).

FUTURE WORK: With numerous products available for the chemical treatment of charophytes, it is important to objectively assess the effectiveness of different treatments on starry stonewort. The specific concentration-exposure times (CET) necessary for successful chemical control should be determined through scaled, replicated management trials before field demonstration. Such a tiered monitoring approach allows for a streamlined identification of chemical control products, their CETs, and their application rates in the absence of confounding variables, ultimately leading to better management outcomes during full-scale field demonstrations and applications. Given the projected spread and future distribution of starry stonewort, the timely identification and field application of a chemical treatment is imperative.

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