



Evaluation of light limitation and depth on germinated seeds of two species of water chestnut cultured under experimental conditions

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PURPOSE: This technical note describes the results of a mesocosm experiment to determine the light and depth limitations of growth chamber germinated seeds of two species of water chestnut (*Trapa* spp.) naturalized in the northeastern United States.

BACKGROUND: A cryptic invasion (Morais and Reichard 2018) of water chestnut (Myrtales: Lythraceae: *Trapa*) was discovered in Virginia in the fall of 2014 within the freshwater reaches of the tidal Potomac River (Rybicki, Striano, and Ryan 2015; Dodd et al. 2019; Pfingsten and Rybicki 2021). Following an early detection and rapid response approach, fisheries biologists with the Virginia Department of Game and Inland Fisheries notified federal agencies (US Geological Survey (USGS) and US Army Corps of Engineers (USACE)). The species observed was noted to possess pink petals, red leaf undersides, and 2-horned fruit, which were taxonomic morphological features not present on what was recognized as European water chestnut (*Trapa natans*) known to occur in the United States (EDDMapS 2019; Pfingsten, Cao, and Berent 2021) (Figure 1). As a result of the notification and suspicion that this species was indeed not the naturalized and well-described exotic *T. natans* found throughout the northeastern United States, collaborative research was initiated and funded by the USACE's Aquatic Plant Control Research Program (APCRP) to investigate the genetics and ecology of this species. The work discovered that this new, cryptic introduction of water chestnut is morphologically and genetically similar to another species found in the *Trapa* genus, *T. bispinosa* var. *iinumai* Nakano currently recognized in People's Republic of China - Taiwan (Chorak et. al 2019). Further investigation into the biology and ecology of this cryptic species of water chestnut was warranted considering recognized harmful economic and ecological effects of *T. natans*, which has been present and problematic in the United States since the late 1800's (Gwathmey 1945; Rawls 1964 a and b; Carter and Rybicki 1994; LaManche 2007; Dodd et. al 2019).

Survival and growth of aquatic plant species, regardless if native or invasive, can depend greatly on the ability of seedlings to overcome factors hindering early growth survivability, (i.e., wave energy, suitable substrates, water quality, herbivory, inundation, nutrients, competition, and light availability). Possessing an annual plant growth stage (reproducing by seeds annually), water chestnut requires suitable conditions to attain maturity for seed production to propagate and sustain colonies. While subsurface irradiance is a major factor for seedlings, as photosynthesis is key for accumulation of biomass, the physical aspect of overcoming distance/depth in the early stages of growth can also impact seedling success. For example, stem breakage as a result of morphological limitations as depth increases (regardless of light availability). It is known that *T. natans* can effectively colonize deeper waters, with reports of maximum depths ranging 3.6 to 15 m (Pemberton, 2002; Hummel and Kiviat 2004). In this study, the ability of growth chamber-germinated seeds of two species of water chestnut (*T. natans* and *T. bispinosa* var. *iinumai*) was evaluated under three depth regimes to determine the ability of each species to reach the surface and produce flowers and



fruits in outdoor mesocosms. Results from this study provide valuable biological and ecological information regarding how the cryptically introduced congener *T. bispinosa* var. *iinumai* compares with *T. natans* in response to varying depths that may affect survival and growth of seeds released from floating rosettes (Methé et.al 1993) in transit from parent colonies or transported by vectors and released into deeper waters.



Figure 1. *Trapa natans* flower with white petals (A) and *T. bispinosa* var. *iinumai* flower with pink petals (B); underside of *T. natans* rosettes with green leaves, and both mature and immature 4-horned fruit (C); comparison of underside of rosette for both species of *Trapa* (D, bottom middle picture, *T. natans* left side and *T. bispinosa* var. *iinumai* right side); and 2-horned fruit of *T. bispinosa* var. *iinumai* (E).

MATERIALS AND METHODS: This depth study was conducted in outdoor mesocosms located at the Lewisville Aquatic Ecosystem Research Facility (LAERF) in Lewisville, Texas (33° 4.186'N, 96° 57.242'W). Fruits from two species of water chestnut, *Trapa natans* and *T. bispinosa* var. *iinumai*, were collected in the fall of 2017 for use in this study. Three populations of *T. natans* were collected: Collins Lake, NY (TNCL, 42°49'39.18"N, 73°56'59.35"W), Housatonic River, CT (TNHR, 41°34'8.65"N, 73°24'46.06"W), and the Mohawk River, NY (TNMRC, 42°49'20.10"N, 73°44'8.52"W). One population of *T. bispinosa* var. *iinumai* was collected from a stormwater retention pond in Manassas, VA (TSPMN, 38°45'26.21"N, 77°30'23.87"W). Once collected, fruits were shipped overnight to the LAERF, stored in tap water, and kept in cold storage (4°C) until germinated for this study.

Fiberglass tanks (3.05 m in height x 2.44 m in diameter, 14,000 L capacity) were used to simulate depth profiles that may influence either *T. natans*' or *T. bispinosa* var. *iinumai*'s ability to produce biomass (rosettes and stems) that can reach the water's surface and are able to produce flowers and seeds. Platforms were constructed and positioned at one of three depths (0.305 m, 1.5 m, and 2.9 m)

for each tank in the study (Figure 2). Each platform consisted of polyvinyl chloride (PVC)-coated welded wire (2-in x 4-in nominal size) attached to 2-in (nominal size) diameter PVC glued together resulting in a 1.66 m x 1.66 m square. Platforms were held at the proper depth by cabling the corners to the lip of the fiberglass tanks. Depths were randomly assigned to nine tanks used for the study. Potted plants were randomly assigned to one of twenty-five 0.305-m x 0.305 m positions configured in a 1.56-m x 1.56-m array on each tank platform at each depth (3 levels) (Figure 2).

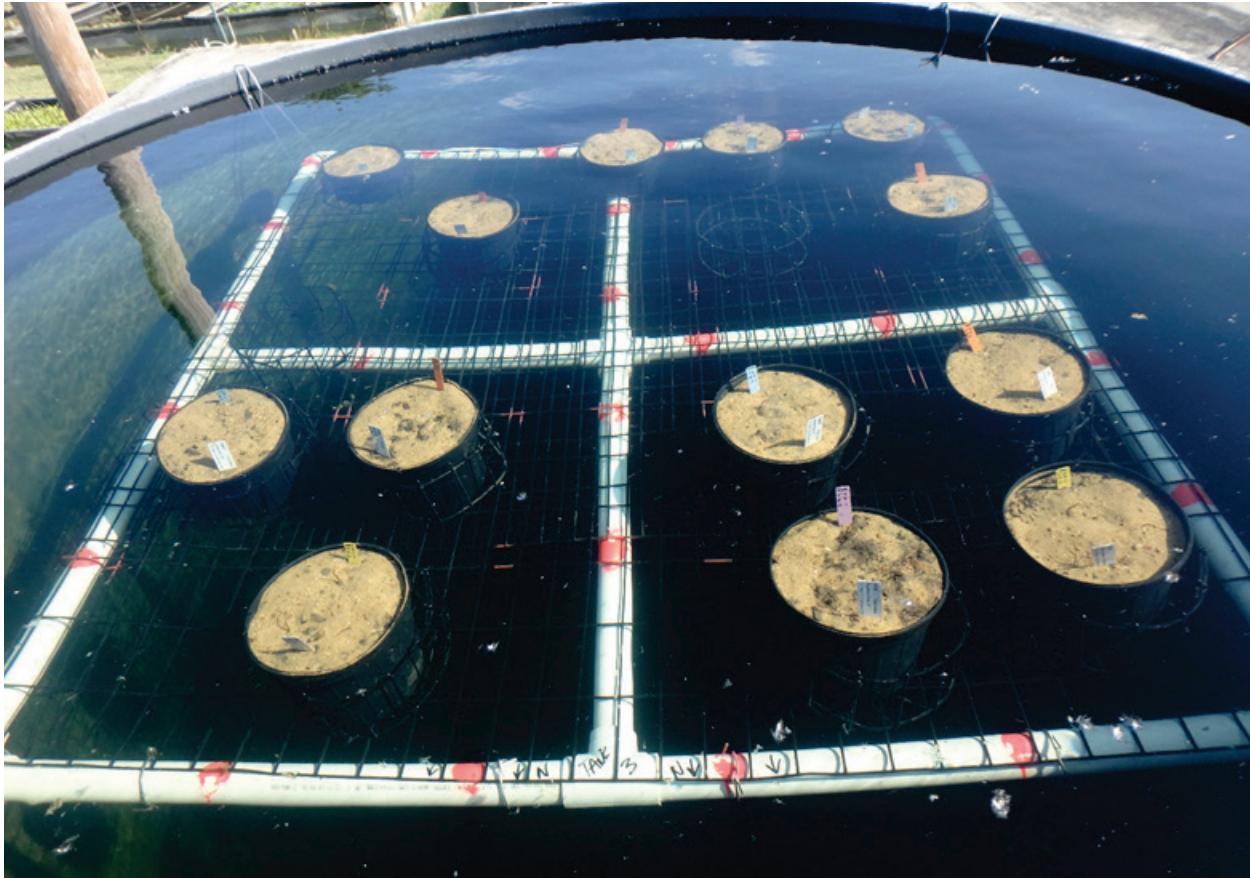


Figure 2. Platform with randomly placed containers of two species of water chestnut; 0.305 m depth pictured; each tank received 13 containers of sown germinated seeds.

Water for the tanks consisted of aluminum sulfate-treated lake water (pH ranging 7.0 to 8.5; measured using an OTT© Hydrolab MS5 - Multiparameter Mini Sonde) and added to the tanks as necessary to maintain water levels throughout the duration of the experiment. Water within the tanks was circulated and filtered using cartridge filters to reduce excess algae growth. Hobo® (Onset®) water temperature loggers were anchored to platforms and data recorded hourly for all tanks (n = 9). Prior to the initiation of the study, light intensity was recorded (as lux converted to photosynthetically active radiation (PAR), $\mu\text{mol m}^{-2} \text{s}^{-1}$) (Thimijan and Heins 1982) with Hobo® Pendant® Temperature/Light Data Loggers for three depths in a subset of three of the nine study tanks.

All plants were grown in blow-molded plastic nursery pots (containers) measuring 22.9 cm in diameter by 20.3 cm in height (volume: 8.3 L). Commercial topsoil (3:1 topsoil: sand) amended with fertilizer cured for two weeks in tap water prior to planting was used as culture medium. Three 4.5 g kg⁻¹ fertilizer tablets (Forestry suppliers, Inc., 20-10-5 NPK plus minors) were placed in the

bottom third of each container equidistant to one another to provide nutrients for the plants for the duration of the study. For each container, three germinated seeds of the same species and population were sown. Seeds were gently pressed into the sediment approximately 2.4 cm equidistant to one another in a triangular arrangement, allowing for epicotyl growth to continue above sediment surface. To reduce sediment suspension and abate nutrient dissolution into the water column, a fine lay of silica sand (1 cm) was placed on the sediment surface. Each 8.3-L container represented one experimental unit (Figure 3).



Figure 3. Three germinated water chestnut seeds of the same population were introduced into each experimental unit.

A total of four populations (TNCL, TNHR, TNMRC, and TSPMN) and three depths (0.305 m, 1.5 m, 2.9 m) were included as experimental treatments. Experimental treatment sample sizes included (sample size dependent on seedling development and availability, and varied among populations) (Table 1): TNCL 0.305 m (n = 9), TNCL 1.5 m (n = 9), TNCL 2.9 m (n = 9), TNHR 0.305 m (n = 6), TNHR 1.5 m (n = 6), TNHR 2.9 m (n = 6), TNMRC 0.305 m (n = 9), TNMRC 1.5 m (n = 9), TNMRC 2.9 m (n = 9), TSPMN 0.305 m (n = 15), TSPMN 1.5 m (n = 15), TSPMN 2.9 m (n = 15). The experiment was initiated 21 May 2018 and plants were harvested 14 August 2018 for a study duration of 12 weeks. At the conclusion of the experiment, the following parameters were recorded for plants found within each container: rosette count and attached fruit count (mature and immature, attached to rosette). Plant biomass was harvested and separated for each container into aboveground (attached fruits, stems, leaves, and flowers) and belowground (roots) biomass (Zobel and Zobel 2002). Aboveground and belowground biomass were then oven dried at 55°C until a constant weight was obtained. Total plant biomass, when reported, included both above and below ground biomass for stems, leaves, flowers, and roots only. Residual fruit (each fruit contained one seed) not attached to rosettes was collected from the bottom of tanks, dried as described above, enumerated, and separated by species (2 spines = *T. bispinosa* var. *iinumai*, 4 spines = *T. natans*). Residual fruit was evaluated separately from attached fruit; total fruit included both residual and attached fruits for each species (but not by population). Fruit per rosette counts were calculated by dividing attached fruit count by rosette count.

Table 1. Populations evaluated as experimental treatments.

Species	Population provenance (CODE)	Water body type
<i>Trapa natans</i>	Collins Lake, NY (TNCL)	Lacustrine
	Connecticut River, CT (TNHR)	Riverine
	Mohawk River, NY (TNMRC)	Riverine
<i>Trapa bispinosa</i>	Manassas, VA (TSPMN)	Lacustrine

Trapa fruit (attached and residual) and rosette data were treated as counts, or as discrete scale variables, and analyzed by creating generalized linear models (GLM) with Poisson as the distribution and Log as the link function. Population/species type and depth were used as between unit predictor variables. Main effects and two-way interactions were included in each Poisson loglinear GLM. Tests for main model effects were done using Wald Chi-Square. Main effect estimated marginal means were also pairwise contrasted for multiple comparisons (with Bonferroni adjustment) to explore differences between levels of factors of interest where appropriate. Correlation analysis (Pearson product moment correlation) was performed to detect intensity of relationships between attached fruit counts and total plant biomass (aboveground and belowground, including stems, flowers, leaves and roots), as well as attached fruit counts and rosette counts. All analyses were done using IBM SPSS version 22; $\alpha = 0.1$. Variance is reported at standard error.

RESULTS AND DISCUSSION: Water temperature in the mesocosms ranged from 27.4 to 35.8° C with a mean of $31.5 \pm 1.3^\circ$ C throughout the study. Mixing of water by cartridge filtration resulted in similar temperatures at all depths. Light intensity was measured for each depth as lux and converted to PAR (Thimijan and Heins 1982): mean PAR values ($n = 3$); 0.305 m = $184 \pm 14 \mu\text{mol m}^{-2} \text{s}^{-1}$; 1.5 m = $79 \pm 3 \mu\text{mol m}^{-2} \text{s}^{-1}$; and 2.9 m = $15 \pm 1 \mu\text{mol m}^{-2} \text{s}^{-1}$). When compared to light irradiance levels recorded at 0.305 m from the surface, light intensity at 1.5 m was 57% lower, and the 2.9 m depth was 81% lower (Figure 4). These light intensity values corresponded with other light effect studies on aquatic vegetation (Smart, Barko, and McFarland 1994; Rybicki and Carter 2002).

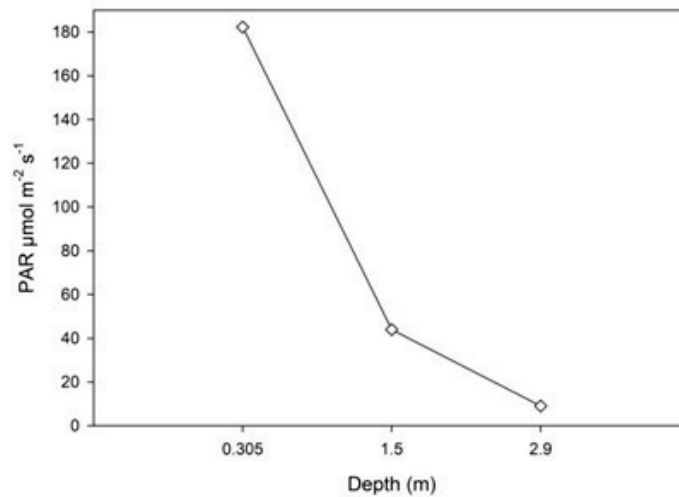


Figure 4. Light intensity recorded as lux and reported as mean for with Hobo® light data loggers (converted to photosynthetically active radiation [PAR $\mu\text{mol m}^{-2} \text{s}^{-1}$]) for 8 weeks from 20 February to 13 April 2018.

Fruit production. When comparing counts of attached fruit from *Trapa* populations and depth, a significant relationship was observed (Poisson loglinear GLM, all $p < 0.001$) (Table 2). Depth, by itself, did not have a significant effect, but population and the interaction between population and depth contributed to the number of attached fruits produced (Table 2). In contrast, species, depth, and the interaction had significant effects on residual fruit production (Table 2). *Trapa bispinosa* var. *iinumai* had less fruit attached at harvest than all *T. natans* populations at each depth evaluated (0.305 m = 35%, 1.5 m = 87%, and 2.9 m = 20%) (Figure 5). Within species, *T. bispinosa* var. *iinumai* had more attached fruit at 2.9 m than compared to 1.5 m (76% less) and 0.305 m (31% less). *T. natans* had approximately 2X more attached fruit at 1.5 m than either 0.305 m or 2.9 m, indicating that its optimal fruit production may occur at or around 1.5 m deep (Figure 5).

Table 2. Results from Poisson loglinear GLM comparing attached fruit counts, residual fruit counts, and rosette counts of two *Trapa* species for main effects depth (m) and population, significant P-values bolded.

	Main effects	Wald Chi-Square	df	P-values
Attached fruit count (immature and mature)	Depth	2.645	2	.266
	Population	170.430	3	.000
	Depth * Population	60.264	6	.000
Residual fruit count	Depth	37.685	2	.000
	Population	65.86	1	.000
	Depth * Population	19.310	2	.000
Rosette count	Depth	26.679	2	.000
	Population	18.689	3	.000
	Depth * Population	16.289	6	.012

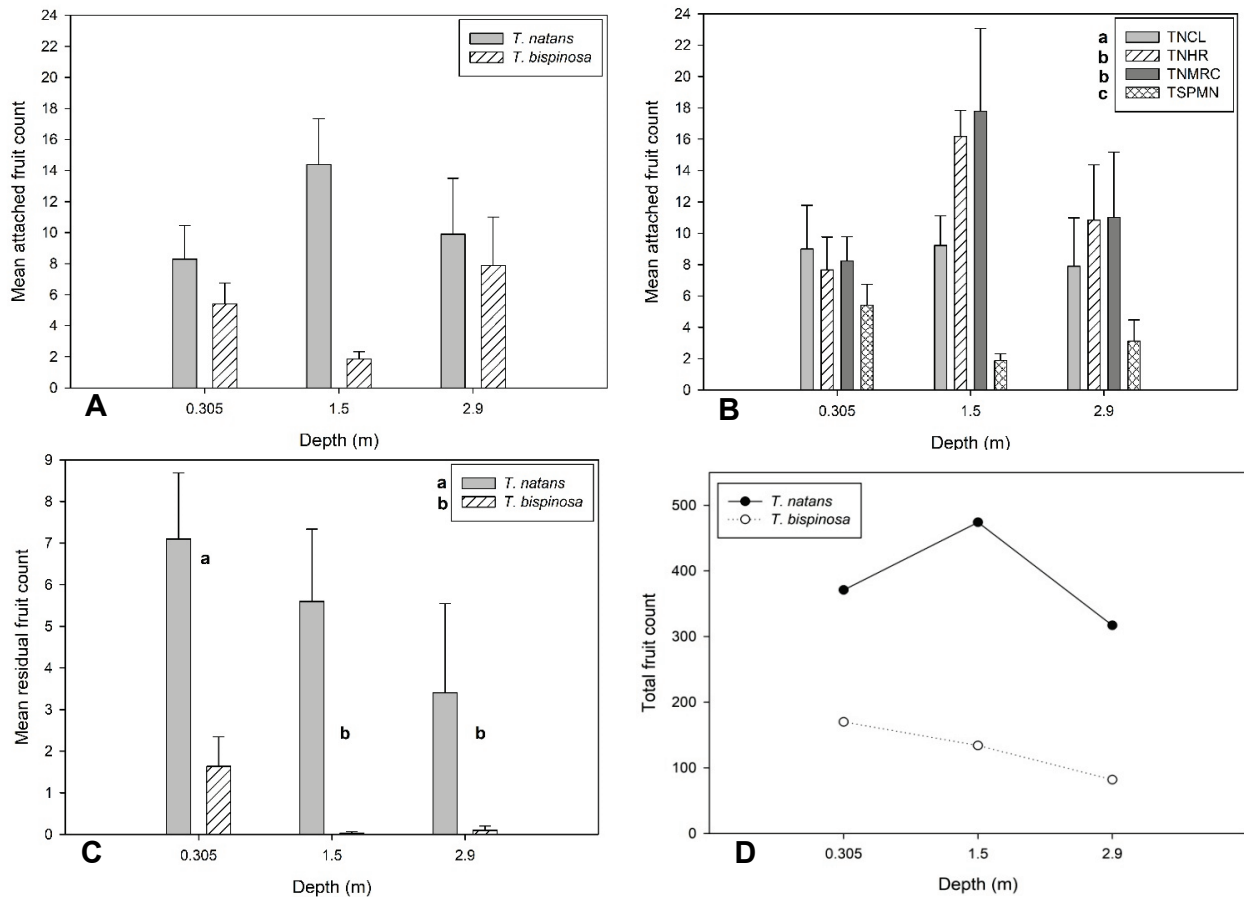


Figure 5. Graph A: Mean counts of fruit attached to rosettes at time of harvest (attached fruit) for each species of *Trapa* at each depth. Graph B: Mean counts of attached fruit by population at each depth (TNCL = *T. natans*, Collins Lake, NY; TNHR = *T. natans*, Housatonic River, CT; TNMRC = *T. natans*, Mohawk River, NY; TSPMN = *T. bispinosa* var. *iinumai*, Manassas, VA) Graph C: Mean counts of fruit collected at the end of the study at the bottom of each tank (residual fruit). Graph D: total fruit counts (attached and residual) for each species. Each bar represents the mean \pm standard error; lowercase letters indicate statistically similar groups (GLM multiple comparisons with Bonferroni adjustment, $\alpha = 0.1$).

Reasons for the contrast between attached fruit counts at 1.5 m (highest for *T. natans*; lowest for *T. bispinosa* var. *iinumai*) are unclear but may be driven by species populations. When comparing differences among *T. natans* populations, TNCL produced relatively the same number of attached fruit at each depth; however, both TNHR and TNMRC had more attached fruit at 1.5 m than either the other two depths (Figure 5). These variations could be attributed to inherent morphological and/or physiological characteristics, or environmental conditions (adaptations to water quality or light histories) to which each of these populations had been exposed. Both TNHR and TNMRC populations were found in tidal riverine environments while the TNCL and TSPMN populations were found in lacustrine waters (< 60 acres) with a mean depth of 2.9 m.

While the total number of rosettes produced was not recorded for the duration of the 12 week study (only number of fruits per rosette observed at time of harvest), it is interesting to note that the mean number of attached fruit was greater than mean residual fruit produced by both species in the 12 weeks prior to harvest (Table 3, Figure 5). For *T. bispinosa* var. *iinumai*, one fruit was collected at

1.5 m and two at 2.9 m, compared to 38 found at 0.305 m, thus indicating *T. bispinosa* var. *iinumai* rosettes at the shallowest depth had reached maturity and dropped fruit earlier than plants cultured at a greater depth. *Trapa natans* followed this trend of producing less residual fruit than attached fruit. However, residual fruit counts were higher than for *T. bispinosa* var. *iinumai* (Table 3). Flower production of *T. natans* was observed by 12 June with mature fruit observed by 17 July (35 days later), whereas *T. bispinosa* var. *iinumai* lagged behind with flowers noted by 17 July and no immature or mature fruit recorded until a week prior to harvest 14 August (28 days later). These findings suggest that the progress of seedling to seed production may be similar between the species, but differences occur between timing and quantity between species, with some dependence upon depth for both. Current methodologies targeting the prevention of seed production (whether mechanical or chemical) for *T. natans* may also be applicable to the control of *T. bispinosa* var. *iinumai*, (field observations of *T. bispinosa* var. *iinumai* phenology in the northeastern United States support these observations, unpublished data). It is also important to note that a third of the total fruits produced for *T. natans* were produced prior to harvest (fruit fallen from the rosettes and collected after harvest from the bottom of the tanks) and targeting this species before flower production is key for control (Table 3).

Table 3. Mean ± SE for rosettes, residual fruit, attached fruit, and total fruit produced for the entire study at each depth by each species of *Trapa*; % difference = 1 – (Residual fruit mean / Attached fruit mean); n= number of replicates.

	Rosettes		Attached fruit		Residual fruit		Total fruit	% difference
	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE		
<i>T. natans</i>								
0.305 m	24	4.9 ± 0.2	8	8.3 ± 2.1	3	7.1 ± 1.6	371	14.4
1.5 m	24	4.0 ± 0.2	8	14.4 ± 2.9	3	5.6 ± 1.7	474	61.0
2.9 m	24	2.6 ± 1.8	8	9.9 ± 3.6	3	3.4 ± 2.2	317	65.7
<i>T. bispinosa</i>								
0.305 m	15	5.6 ± 0.5	15	5.4 ± 1.3	3	1.6 ± 0.7	119	69.7
1.5 m	15	4.5 ± 0.3	15	1.9 ± 0.5	3	0.03 ± 0.03	29	98.2
2.9 m	15	3.5 ± 0.7	15	7.9 ± 3.1	3	0.1 ± 0.1	49	98.7

Trapa bispinosa var. *iinumai* produced less total fruit (collected prior to and at harvest, attached + residual) than *T. natans* at all depths (0.305 m = 68% less, 1.5m = 93% less, 2.9 m = 84% less) but produced more total fruit at 0.305 m than the other depths (4X more than 2.9 m and 2.4X more than 1.5m) within the species (Table 3). Within its species, *T. natans* produced more total fruit at 1.5 m than the other two depths (Figure 5, Table 3). These results indicate that congeners *T. natans* and *T. bispinosa* var. *iinumai* may not share the same depth requirements for optimal production of seeds. This information coupled with the observations that *T. natans* produced flowers and mature fruit nearly a month ahead of *T. bispinosa* var. *iinumai* suggest that these two species differ in regard to phenology. Faster growth to the surface by *T. natans* enabled earlier and higher production of fruits, resulting in substantially greater fruit production than observed in *T. bispinosa* var. *iinumai* under the same conditions. These findings also support those current methodologies that target *T. natans* control earlier in the year also apply to *T. bispinosa* var. *iinumai*, where conversely, if *T. bispinosa* var. *iinumai* produced flowers and fruits before *T. natans* then there would be a greater cause for concern.

Biomass and rosette count effect on fruit production. No intense correlations were observed between attached fruit counts and total plant biomass ($p = 0.167$, $r = 0.129$, Figure 6) or with attached fruit counts and rosette counts ($p = 0.289$, $r = 0.099$, Figure 6). However, a minor non-significant positive relationship was detected in both situations: as total plant biomass and rosette counts increased, the numbers of attached fruit for all populations increased, indicating that biomass and numbers of rosettes produced were only slightly positively linked to fruit production. Therefore, fruit production was prolific regardless of plant biomass and rosette production, providing more evidence for plant invasiveness for both species. Depth and population, along with variable interaction, had significant effects on rosette counts (Poisson loglinear GLM, Table 2) where numbers of rosettes produced by both species decreased as depth increased (Figure 7). At the same time, the number of rosettes produced by *T. bispinosa* var. *iinumai* was on average 16% more than *T. natans* for all depths.

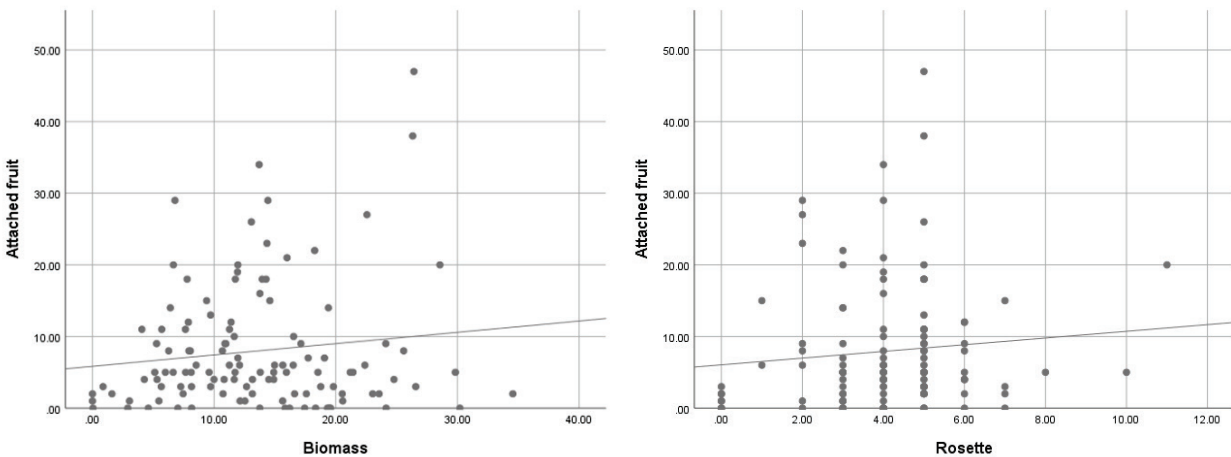


Figure 6. Left - scatterplot of attached fruit and total plant biomass (excluding fruit biomass), for both species for all populations at all depths; right - scatterplot of attached fruit and rosette count, for both species for all populations at all depths; raw data scatterplot, correlation model line, and correlation coefficient given, (correlation analysis - Pearson product moment correlation, $\alpha = 0.1$).

When comparing the different populations of *T. natans*, subtle differences, although non-significant, were detected where numbers of rosettes were similar at shallower depths; however, at 2.9 m, TNHR produced 17% more rosettes than either of the other two populations evaluated (Figure 7).

Numbers of attached fruit per rosette (at the time of harvest) by species increased as depth increased for *T. natans* but was variable for *T. bispinosa* var. *iinumai*. Attached fruit per rosette counts were higher for *T. natans* than *T. bispinosa* var. *iinumai* (an average of 72% more for all depths evaluated) (Figure 7). Within *T. natans*, TNHR produced more rosettes than other populations, but TNMRC produced more fruit per rosette (average of 32% more), highlighting the biological differences displayed by populations of the same species.

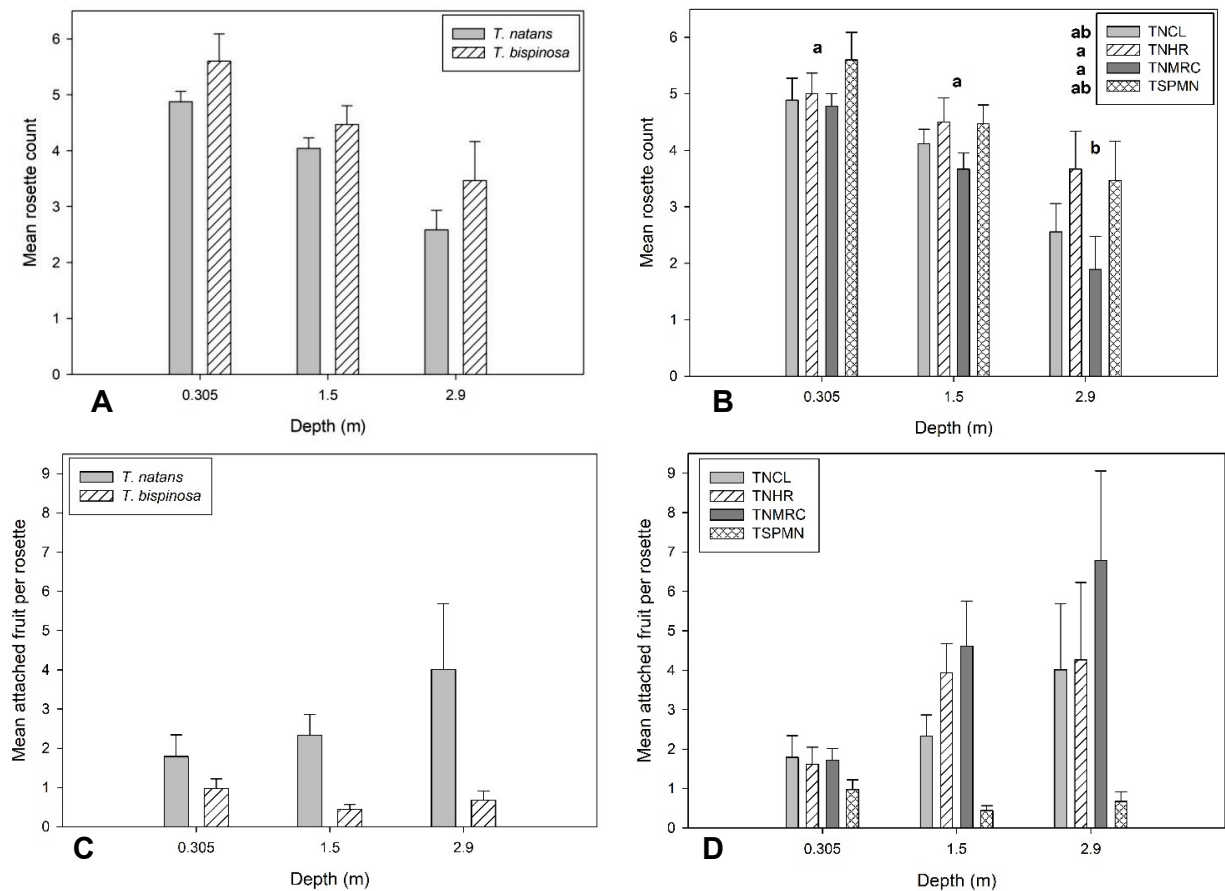


Figure 7. Mean rosette counts (n = 479) by species (Graph A) and by population (Graph B) at each depth. Mean fruits per rosette produced by each *Trapa* species (Graph C) and by population (Graph D) at each depth. TNCL = *T. natans*, Collins Lake, NY; TNHR = *T. natans*, Housatonic River, CT; TNMRC = *T. natans*, Mohawk River, NY; TSPMN = *T. bispinosa* var. *iinumai*, Manassas, VA. Each bar represents the mean \pm standard error; lowercase letters indicate statistically similar groups (GLM multiple comparisons with Bonferroni adjustment, $\alpha = 0.1$).

Field observations of *T. bispinosa* var. *iinumai* have indicated that this species shares invasive qualities of *T. natans*, including rapid growth and coverage, as well as highly mobile seeds for distribution within and across watersheds (personal observation). Although this study revealed that *T. bispinosa* var. *iinumai* may produce less fruit than *T. natans* overall, and that most fruit production occurs at shallower depths than *T. natans*, the species was able to flower and produce fruit and seeds in 2.9 m of water, a cause for concern for its management and distribution potential. Unpublished data indicates that while *T. natans* plants senesce and die in the northeastern United States by September, *T. bispinosa* var. *iinumai* plants were still present with attached fruits in October in Virginia,¹ indicating late season reproduction. Additionally, intraspecific dynamics for *T. natans* populations evaluated in this study revealed subtle growth and reproduction differences that could be attributed to either genetic or environmental factors (or both) that may have implications for management where targeted efforts using certain methodologies (i.e., biocontrol, herbicides, or mechanical harvesting) may result in less than desirable results. Also maybe disconcerting is the overlapping anthesis and potential for distribution for *T. bispinosa* var. *iinumai* in current ranges of *T. natans* in the United States. While Li et al. 2017 indicated that there were

¹ N. Rybicki, personal communication, 2019.

no instances of hybridization among a number of *Trapa* species evaluated in their study for plants growing within the same water bodies within their native range in China, further investigation into this possibility is warranted.

When developing management strategies for either species, it is important to recognize that this study was conducted under optimal, controlled conditions and that it is unclear how other environmental conditions including water clarity (impacted by poor water quality), excess or legacy nutrients, tidal influence and wave action, climate change, and/or predation will impact overall vegetation growth (and ultimately reproductive success).

FUTURE WORK: Existing guidance in management of water chestnut is based upon the understanding that only one species or biotype of water chestnut is present in the United States and that populations of the same species behave similarly; however, the results generated from this study and others indicate substantial differences between plant species. These findings will directly influence current guidance of the management of this species, thereby improving how USACE resource managers address new introductions of water chestnut or extant populations. Improvements to current management strategies may include where to focus or prioritize efforts in the detection of new introductions of water chestnut or determine whether active management is necessary in certain aquatic plant communities to order to reduce adverse impacts to the Nation's water resources (USACE 2015). Additional work to determine what control approaches provide the most sustainable results for both species of naturalized water chestnut are warranted.

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