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Comparative trials of herbicides for control of *Trapa natans* and *T. bispinosa* var. *iinumai* in the presence of *Heteranthera dubia* and *Vallisneria americana*

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ABSTRACT

Water chestnut is a problematic annual rooted aquatic herb native to Eurasia and Africa. Two species are naturalized in the United States, *Trapa natans* and *Trapa bispinosa* var. *iinumai*. Whereas *T. natans* has been present since the late 1800s, a cryptic introduction of *T. bispinosa* var. *iinumai* was confirmed in 2014 and little is known of its biology or ecology. Aquatic herbicides have been used to control *T. natans*, but the response of *T. bispinosa* var. *iinumai* has not been experimentally evaluated to date. Therefore, repeated greenhouse trials were conducted to investigate the sensitivity of *T. bispinosa* var. *iinumai* to aquatic herbicides under various concentration exposure time (CET) scenarios. Treatment effects on biomass and propagules for both species of *Trapa* and key nontarget (native) species, *Vallisneria americana* and *Heteranthera dubia*, were determined for 2,4-D, flumioxazin, imazamox, and recently registered florpyrauxifen-benzyl. Trial differences were detected and are attributed to environmental conditions of each trial and plant age. Data indicate that both 24-h and static applications of flumioxazin at 429 g ai ha^{-1} resulted in 86 to 96% control and subsurface applications of florpyrauxifen-benzyl at 48 µg ai L^{-1} were highly effective with 89 to 98% control for target species evaluated, while minimally affecting nontarget *V. americana*. Results for *H. dubia* were variable between trials, with higher sensitivity observed for one trial than the other, indicating that plant age may affect efficacy of herbicides evaluated. Both 2,4-D and imazamox resulted in limited control of the target species, 58 to 68% and 35 to 70% control, respectively. This research indicates that the cryptic invader, *T. bispinosa* var. *iinumai*, exhibited similar or higher sensitivity to herbicides and CETs evaluated than its congener, *T. natans*.

Key words: chemical control, concentration exposure time, invasive, native, selectivity, water chestnut.

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INTRODUCTION

Water chestnut (*Trapa natans* L., European water chestnut) is an annual rooted aquatic herb with floating triangular and submerged feathery leaves native to Eurasia and Africa (Muencher 1944, Crow and Helquist 2000) and a problematic invasive in the northeastern United States (Winne 1950, Gwathmey 1945, Hummel and Kiviat 2004). Since its introduction to the United States in the late 1800s (Davenport 1879, Wibbe 1886, Smith 1955), it was believed to be the only species within the genus present in the United States until the discovery of a cryptic congener, two-horn water chestnut (*Trapa bispinosa* Roxb. var. *iinumai* Nakano) in 2014 (Chorak et al. 2019, Dodd et al. 2019). These two *Trapa* spp. are distinguished primarily by fruit and flower morphological differences. *Trapa natans* has white petals as opposed to pink petals for *T. bispinosa* var. *iinumai*, whereas *T. natans* fruit develops with four sharp spines as opposed to the two sharp spines observed for *T. bispinosa* var. *iinumai*.

Typically, *Trapa* spp. are found in slow-moving shallow water, but have also been observed in deeper (3.6 to 5 m) waters (Pemberton 2002, Hummel and Kiviat 2004). *Trapa natans* is found in numerous watersheds in the northeastern United States (Pfingsten et al. 2022), including the Hudson River and Lake Champlain, whereas *T. bispinosa* var. *iinumai* is currently established within the Potomac River watershed in Virginia (Pfingsten and Rybicki 2022).

The U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) has determined *T. natans* to be a high-risk invasive plant (USDA APHIS 2016). Because of its dense growth and ability to establish and spread quickly, *T. natans* can outcompete desirable native aquatic species for available resources in lakes and rivers and can easily exploit disturbed waterways. Observations of *T. bispinosa* var. *iinumai* have indicated similar behavior to *T. natans* in establishment, rapid spread, and dense growth. This suggests that it may have similar negative effects on recreation, navigation, and biodiversity (Rybicki et al. 2019, Dodd et al. 2021, Dodd and Schad 2021). These observations are concerning and warrant further investigation into development of successful control strategies for water resource managers engaging in early detection and rapid response approaches for this recently discovered cryptic invasion.

Methods to control *T. natans* consist of both selective and nonselective means, including biological, chemical, and

Table 1. Date and locations collected, propagule type, and pretreatment culture conditions for *Trapa natans*, *Trapa bispinosa* var. *iinumai*, *Heteranthera dubia*, and *Vallisneria americana*.

Species	Trial	Date Collected	Location (latitude, longitude)	Propagule Type	Planted in Container	Pretreatment Conditions
T. natans	1	28 June 28 2019	Hudson River, Norrie Point, NY	Seedling[1]	1 July 1 2019	Field collected
	2		41°49′55.02″N, 73°56′29.60″W		1 July 2019	Cultured
T. bispinosa var. *iinumai*	1	16 October 2018	Manassas, VA (retention pond)	Seed[2]	19 June 2019	Germinated: 31 May 2019
	2		38°45′26.21″N, 77°30′23.87″W		13 August 2019	Germinated: 17 July 2019
H. dubia	1	17 October 2018	Potomac River, Lorton, VA	Apical fragment	24 October 2018	Field collected; cultivated
	2		38°39.606′N, 77° 9.010′W			
V. americana	1			Bare root		
	2					

[1]Estimated germination occurred during the week of 13 May 2019 (on the basis of field observations).
[2]Seeds remained in cold storage at 4 C before germination.

physical control. Biological control of *Trapa* spp. has included the use of grass carp (*Ctenopharyngodon idella* Cuvier and Valenciennes); however this approach may affect nontarget vegetation (Hummel and Kiviat 2004). Additionally, classical biological control of *Trapa* spp. with the Chinese water chestnut beetle (*Galerucella birmanica* Jacoby) is being researched. However, no agents are currently approved for release within the United States (Pemberton 1999, Ding et al. 2006). Physical methods have been used to remove floating rosettes or whole plants before flowers and fruit develop to control this annual species (Countryman 1977, Madsen 1993, Methe et al. 1993). This is an effective management strategy when properly used and benefits include a reduction in biomass, nutrients, and oxygen depletion (James et al. 2002, Bartodziej et al. 2017). However, *T. natans* seeds can remain dormant for up to 10 yr (Hummel and Kiviat 2004), so physical removal is required on an annual basis until the seed bank is exhausted.

Aquatic herbicides have also shown promise in efforts to control *T. natans*. 2,4-D (Countryman 1978, Hummel and Kiviat 2004, Poovey and Getsinger 2007, GLMRIS 2012, Kishbaugh 2014), glyphosate (NYISI 2019), flumioxazin (N. Hanna, Environmental Scientist, Solitude Lake Management, pers. comm., 2019), imazamox (Anonymous 2019a), and triclopyr (Poovey and Getsinger 2007, Kisbaugh 2014) have demonstrated efficacy in research and field settings. It is unclear whether chemical control methods of *T. natans* can translate into control for *T. bispinosa* var. *iinumai* considering their competitive, morphological, and phenological differences (Chorak et al. 2019, Dodd et al. 2021, Dodd and Schad 2021). For example, *Myriophyllum* congeners have shown varying sensitivity to florpyrauxifen-benzyl at sublethal concentrations and exposures (Beets et al. 2019) and reduced sensitivity to fluridone has been documented in field and laboratory studies (Thum et al. 2012).

To determine if current chemical control methods deployed for *T. natans* result in similar control for *T. bispinosa* var. *iinumai*, we conducted greenhouse experiments to investigate the sensitivity of *T. bispinosa* var. *iinumai* to both contact and systemic herbicides under various concentration exposure time (CET) scenarios and application techniques. Another potentially promising product included in the study was the systemic herbicide florpyrauxifen-benzyl. Registered in 2018 in the United States for the purposes of aquatic plant control (University of Florida 2018, Anonymous 2019b), there have been no empirical studies evaluating its efficacy and CET requirements for *Trapa* spp.

Identifying sustainable integrated control methods for *Trapa* spp. in its introduced range is essential for natural resource managers. It is equally critical to identify those methods that minimize harm to nontarget native submerged aquatic vegetation (SAV). Selectivity is especially relevant when species occur in mixed communities. Two native SAV species of concern with U.S. ranges overlapping with *Trapa* spp. and that require consideration when control is warranted include *Vallisneria americana* and *Heteranthera dubia*. Both provide benefits to aquatic ecosystems by providing food, structure, and improved water quality (Poe et al. 1986, Korschgen et al. 1988, Korschgen and Green 1988, Barko et al. 1991, Moore et al. 2010). As such, we also evaluated chemical effects on both of these perennial species.

MATERIALS AND METHODS

This study was conducted in 2019 at the U.S. Army Engineer and Research Center's Lewisville Aquatic Ecosystem Research Facility in Lewisville, TX (33°4.186′N; 96°57.242′W). We evaluated four aquatic herbicides, 2,4-D, florpyrauxifen-benzyl, flumioxazin, and imazamox, and their effects on *T. bispinosa* var. *iinumai*, *T. natans*, *H. dubia*, and *V. Americana* in two separate trials. These herbicides are the most commonly used according to local municipality and state water resource managers in the Northeast and Virginia (pers. comm.).

Propagation and planting

Table 1 identifies date and location collected, propagule type, and pretreatment culture conditions of each species used in the experiment. *Trapa* seeds were shipped overnight from field sites, immediately stored in tap water, and kept in cold storage at 4 C until germinated for planting (Rector et al. 2015). Field-collected propagules were shipped overnight from field sites; *Trapa* seedlings were quarantined for 3 d and *H. dubia* and *V. americana* propagules for 6 d before planting into 0.946-L blow-molded plastic containers. Each container was filled with 3 : 1 commercial topsoil : sand with one fertilizer tablet[1] (20–10–5 N–P–K including micronutrients; 4.5 g L^{-1}). A 1-cm layer of sand was added

Table 2. Herbicide applications administered to *Trapa bispinosa* var. *iinumai*, *Trapa natans*, *Heteranthera dubia*, and *Vallisneria americana* under greenhouse conditions.

Treatment[1]	Application Technique[2]	Rate or Concentration	Exposure Time (h)
Control[3]	—-	—	—
2,4-D	**Subsurface (granular)**	**3 mg ai L^{-1}**	**72**
Florpyrauxifen-benzyl	**Foliar**	**59 g ai ha^{-1}**	**24**
Florpyrauxifen-benzyl	**Subsurface**	**19 µg ai L^{-1}**	**24**
Florpyrauxifen-benzyl	**Subsurface**	**19 µg ai L^{-1}**	**Static**
Florpyrauxifen-benzyl	**Subsurface**	**48 µg ai L^{-1}**	**24**
Florpyrauxifen-benzyl	**Subsurface**	**48 µg ai L^{-1}**	**Static**
Flumioxazin	*Foliar*	*429 g ai ha^{-1}*	*24*
Flumioxazin	*Foliar*	*429 g ai ha^{-1}*	*Static*
Imazamox	**Foliar**	**561 g ai ha^{-1}**	**24**
Imazamox	**Foliar**	**561 g ai ha^{-1}**	**Static**

[1]Bold indicates systemic herbicide; italic indicates contact.
[2]All herbicide treatment included a nonionic surfactant (0.25% v v^{-1}).
[3]In trial 2, only treatments applied to *Trapa natans*.

to the soil surface at the time of planting to reduce particulate matter and nutrient resuspension into the water column to prevent algal contamination. Before planting, containers were immersed in tap water for 2 wk to leach out excess nutrients and begin hydric soil processes.

For Trial 1, *T. bispinosa* var. *iinumai* seeds were removed from cold storage and placed in growth chambers (20 C/18 C; 14 : 10 photoperiod; 364 ± 55 µmol $m^{-2}s^{-1}$) in shallow glass trays (21.6 by 26.4 by 9.6 cm) using aluminum sulfate-treated Lewisville Lake, TX water (hereafter referred to as lake water). After 20 d, germination occurred, with epicotyls reaching 2 cm in length before transplanting. Seedlings were then planted in containers (one each) and placed in 568-L molded polyethylene tanks with lake water under ambient greenhouse conditions for a period of 12 d. The containers were then moved to aquaria within temperature-controlled water baths, allowing 7 d to acclimate to study conditions before treatment (age 40 d). For Trial 2, *T. bispinosa* var. *iinumai* seeds in storage were germinated and planted as outlined above (age 54 d). Seedling ages for both trials with *T. bispinosa* var. *iinumai* were comparable. *Trapa natans* field-collected seedlings for Trial 1 were planted similarly and resulted in seedlings age 53 d. For Trial 2, *T. natans* seedlings were kept in culture within the same greenhouse until 7 d before the initiation of Trial 2, where they were moved to study aquaria, resulting in immature plants age 115 d. *Trapa natans* immature plant ages resulted in Trial 2 plants being 8 wk older than seedlings collected from the field.

Heteranthera dubia and *V. americana* were sorted by size and planted within 48 h of field collection into separate containers for both trials. Three apical fragments of *H. dubia* (40.4 ± 0.3 cm in length) and three bare-root *V. americana* ramets (27.4 ± 0.5 cm in length, 3.9 ± 0.1 leaves/plant) were each used. Plants were allowed to grow outside until 7 d before initiation of each trial, at which time plants were removed from outdoor tanks and placed into greenhouse aquaria to allow for acclimation to study conditions for experiment initiation on 14 June 2019 for Trial 1 (age 33 wk) and 3 September 2019 for Trial 2 (age 44 wk).

The greenhouse housed 88 72-L polypropylene aquaria (28.5 by 28.5 by 88.5 cm) situated among 11 temperature-controlled 1,000-L fiberglass tanks filled with tap water serving as water baths to maintain temperature (18 to 24 C) under ambient lighting. Each fiberglass tank housed eight planted aquaria filled with lake water and amended with ambient air through diffusers. No water exchange occurred between aquaria and water baths. Basic water quality parameters—pH, temperature, and conductivity—conducive for maintaining healthy plant growth were monitored weekly with an OTT© Hydrolab MS5-Multiparameter Mini Sonde.[2]

Design and herbicide treatments

Foliar or subsurface applications at various CET of 2,4-D,[3] florpyrauxifen-benzyl,[4] flumioxazin,[5] or imazamox[6] were applied to actively growing plants on 9 July 2019 for Trial 1 and 9 September 2019 for Trial 2 (Table 2). To analyze target and nontarget plant effects, each experimental unit consisted of one container of each nontarget species—*V. americana* and *H. dubia*— placed in each unit aquarium with one container of target species—*T. natans* or *T. bispinosa* var. *iinumai* for a total of three containers in each aquarium. In Trial 1, each target *Trapa* spp. was replicated four times for each of all 11 application/CET levels for a total sample size of 44 for each species, which included a nontreated control. For nontarget species, the container number for every treatment level was eight—four individuals grown with each target species, resulting in a sample size of 88 for each species. Trial 2 levels and replicate numbers varied slightly because of the lack of available *T. natans* propagules. Consequently, *T. natans* was included in 3 of the 11 treatment levels at three replicates each (Table 2), resulting in a sample size of nine. At these treatment levels, and as *T. bispinosa* var. *iinumai* replicates and thus sample size remains similar to Trial 1, nontarget species totals were likewise reduced by one to seven for the three treatments evaluating both species of *Trapa*. The remaining eight treatment levels in Trial 2 included one target species of *Trapa*, four each *T. bispinosa* var. *iinumai*, and four each of each nontarget species, resulting in a sample size of 53 for each nontarget species.

Foliar applications were administered to the foliage of target and nontarget plants (where applicable) using a forced-air CO_2-powered sprayer at an equivalent of 935 L ha^{-1} diluent delivered through a single TeeJet®[7] 80-0067 nozzle at 20 psi. Herbicide treatments included a nonionic surfactant[8] (0.25% v v^{-1}). Subsurface treatments of florpyrauxifen-benzyl were pipetted directly from a stock solution into the water column. Granular 2,4-D was evenly distributed across the water surface. At the time of treatment for Trial 1, *H. dubia* leaves had canopied at the surface of the water and were susceptible to foliar application, but for Trial 2, leaves had not yet reached the surface. *Vallisneria americana* leaves and stems were observed to be > 6 cm from the water's surface for both trials, thereby only receiving overspray of target species for foliar applications. All *Trapa* spp. rosettes were floating on the water's surface for both trials. After herbicide applications, plants were subjected to

one of three CET scenarios: static, 24 h, or 72 h. After the assigned CET had been reached, aquaria were flushed with herbicide-free lake water for a period of 20 min, resulting in 3× volume exchange to remove residues. For each species, all viable biomass was harvested 7 wk after treatment (WAT), dried at 55 C to a constant weight, and weighed to the nearest 0.01 g. Counts were recorded for *Trapa* fruits and *V. americana* winter buds.

Statistical analysis

Total dry biomass data of each species, after herbicide treatment, were analyzed with generalized linear models (GLM). Normal distributions with identify link were used for normal distributions, gamma distribution with log link for nonparametric. First, two independent or predictor variables were used, and these included herbicide treatment and trial. Trials were then analyzed separately if trial or the trial × treatment interaction had a significant effect on species total dry biomass. Next, to better understand florpyrauxifen-benzyl's effect, we analyzed it separately for each trial on the basis of two predictor variables: rate and exposure time. *Trapa natans* was analyzed separately for each trial because not all herbicide treatments were tested in both trials, resulting in an unbalanced number of replicates, four replicates in Trial 1 and three in Trial 2.

Trapa natans fruit and *V. americana* winter bud data were analyzed as counts by creating GLM with Poisson as the distribution and log as the link function. Herbicide treatment and trial were independent factors; *T. natans* fruit count was again analyzed separately for each trial. In both GLM analyses (biomass and counts), main effects and two-way interactions were included and 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 test for differences between levels of factors of interest where appropriate. Biomass, fruit, and winter bud analyses were done using IBM SPSS version 22[9] ($P = 0.05$). Water temperature data were analyzed by ANOVA ($P = 0.05$) using STATGRAPHICS Centurion version 16.0.7[10] software.

RESULTS AND DISCUSSION

Significant differences were observed for biomass and winter bud counts for herbicide treatment, trials, and interaction between treatment and trial for *H. dubia* (biomass), *V. americana* (biomass and winter buds), and *T. bispinosa* var. *iinumai* (biomass) (Table 3, Figures 1 and 2). In both trials, *T. natans* biomass and fruit count were significantly different among herbicide treatments (Table 3); however, for fruit count, only Trial 1 was significant (Table 3).

Interactions between treatment and trial were likely attributed to the differences in age of the cultured plants at herbicide application and water bath temperatures. *Trapa bispinosa* var. *iinumai* seedlings, while germinated on two different occasions, were 2 wk older in Trial 2 than in Trial 1 (Table 1), whereas other species were 8 wk older for Trial 2 than for Trial 1. Water temperature was significantly

Table 3. Results from generalized linear models (GLM) for dried mean total biomass data (aboveground + belowground + propagules) and count data (fruit, buds) for nontarget species *Heteranthera dubia* and *Vallisneria americana* and target species *Trapa natans* and *Trapa bispinosa* var. *iinumai*.

		Predictor Variables[1]	Wald Chi-Square	df	*P*-Values[2]
H. dubia biomass (g DW)	A				
		Treatment	92.615	10	**0.000**
		Trial	23.368	1	**0.000**
		Treatment × trial	46.341	10	**0.000**
	B				
		Trial 1 treatment	118.126	10	**0.000**
		Trial 2 treatment	33.383	10	**0.000**
	C				
		Trial 1 rate	8.919	1	**0.003**
		Trial 1 exposure	0.506	1	0.477
		Trail 1 rate × exposure	0.024	1	0.876
	C				
		Trial 2 rate	0.543	1	0.461
		Trial 2 exposure	3.387	1	0.066
		Trail 2 rate × exposure	3.489	1	0.062
V. americana biomass (g DW)	A				
		Treatment	16.569	10	0.084
		Trial	7.141	1	**0.008**
		Treatment × trial	27.197	10	**0.002**
	B				
		Trial 1 treatment	41.774	10	**0.000**
		Trial 2 treatment	14.587	10	0.148
	C				
		Trial 1 rate	8.071	1	**0.004**
		Trial 1 exposure	0.452	1	0.501
		Trail 1 rate × exposure	0.403	1	0.526
	C				
		Trial 2 rate	2.161	1	0.142
		Trial 2 exposure	0.466	1	0.495
		Trail 2 rate × exposure	4.134	1	**0.042**
T. natans biomass (g DW)[1]	B				
		Trial 1 treatment	129.572	10	**0.000**
		Trial 2 treatment	50.926	2	**0.000**
	C				
		Trial 1 rate	49.499	1	**0.000**
		Trial 1 exposure	0.334	1	0.563
		Trail 1 rate × exposure	1.616	1	0.204
T. bispinosa var. *iinumai* biomass (g DW)	A				
		Treatment	202.402	10	**0.000**
		Trial	58.273	1	**0.000**
		Treatment × trial	36.403	10	**0.000**
	B				
		Trial 1 treatment	144.133	10	**0.000**
		Trial 2 treatment	97.731	10	**0.000**
	C				
		Trial 1 rate	22.291	1	**0.000**
		Trial 1 exposure	7.071	1	**0.008**
		Trail 1 rate × exposure	3.224	1	0.073
	C				
		Trial 2 rate	6.423	1	**0.011**
		Trial 2 exposure	0.723	1	0.395
		Trail 2 rate × exposure	3.794	1	0.051
T. natans fruit counts	A				
		Trial 1 treatment	16.029	7	**0.025**
		Trial 2 treatment	3.345	1	0.067
V. americana bud counts	A				
		Treatment	105.483	10	**0.000**
		Trial	5.284	1	**0.022**
		Treatment × trial	116.142	10	**0.000**
	B				
		Trial 1 treatment	243.536	10	**0.000**
		Trial 2 treatment	37.826	10	**0.000**

[1]A = all treatments and trial; B = all treatments by trial; and C = rate and exposure for florpyrauxifen-benzyl biomass only by trial.
[2]Bold indicates significance at $P \leq 0.05$.

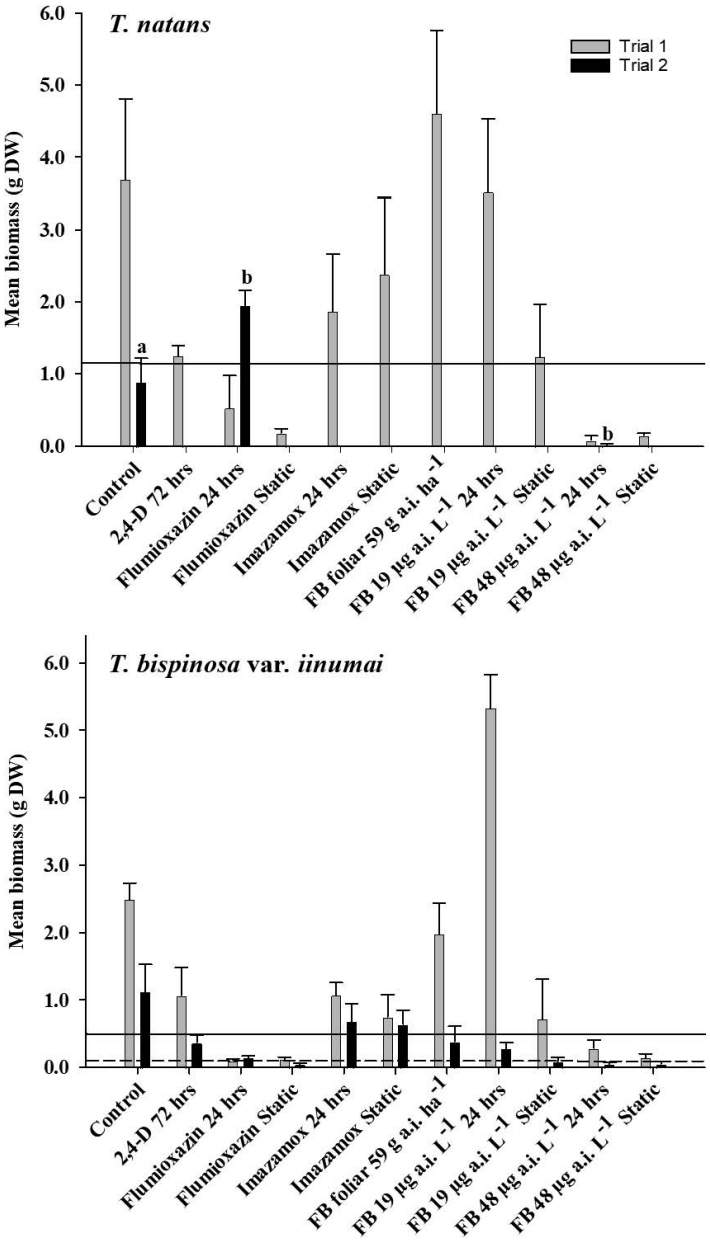

Figure 1. Mean dried total biomass (g DW) ± SE with Bonferroni multiple comparison post hoc test results for target species *Trapa natans* and *Trapa bispinosa* var. *iinumai* control and treatments of 429 g ai ha^{-1} foliar flumioxazin, 561 g ai ha^{-1} foliar imazamox, and 59 g ai ha^{-1} foliar florpyrauxifen-benzyl and subsurface 19 and 48 µg ai L^{-1} florpyrauxifen-benzyl . Exposure time: 24 h, 72 h, or static. Pretreatment mean dried total biomass (g DW) represented by a solid line for Trial 1 and a dashed line for Trial 2. Abbreviations: FB, florpyrauxifen-benzyl. Means within a given trial with the same letter are not different from control according to Bonferroni multiple-comparison post hoc test at $P \leq 0.05$.

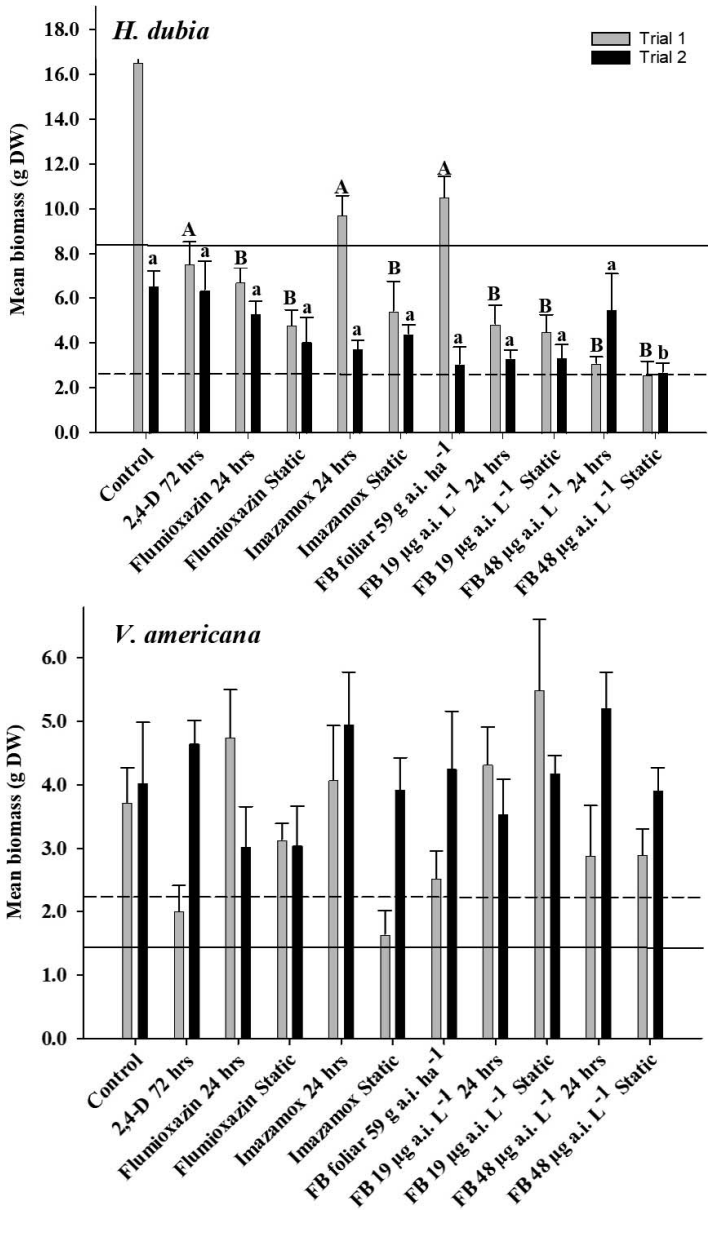

Figure 2. Mean dried total biomass (g DW) ± SE with Bonferroni multiple comparison post hoc test results for nontarget species *Heteranthera dubia* and *Vallisneria americana* control and treatments of 429 g ai ha^{-1} foliar flumioxazin, 561 g ai ha^{-1} foliar imazamox, and 59 g ai ha^{-1} foliar florpyrauxifen-benzyl and subsurface 19 and 48 µg ai L^{-1} florpyrauxifen-benzyl. Exposure time: 24 h, 72 h, or static. Pretreatment mean dried total biomass (g DW) represented by a solid line for Trial 1 and a dashed line for Trial 2. Abbreviations: FB, florpyrauxifen-benzyl. Means within a given trial with the same letter are not different from control according to Bonferroni multiple-comparison post hoc test at $P \leq 0.05$.

different between the two trials ($P < 0.001$). For Trial 1, water bath temperatures ranged from 22.2 to 29.5 C with a mean of 26.3 C. This was attributed to increased ambient air temperatures within the greenhouse during summer months. In Trial 2, water temperatures were easier to regulate from September to October and ranged from 18.6 to 25.5 C with a mean of 22.4 C. Water temperatures were similar to previous *T. natans* research (Poovey and Getsinger 2007, Des Jardin 2015). Water temperatures during both trials were within the range of those recorded for the Potomac River in 2019 (USGS 2019) and considered acceptable for growth of all species evaluated.

Target species response

Biomass. The foliar static application of the contact herbicide flumioxazin was efficacious (Table 3, Figure 1)

against both *Trapa* spp. in both trials. At seven WAT, flumioxazin reduced *Trapa* spp. biomass by 95 to 96%. Additionally, plants exhibited rapid injury with necrotic floating leaves by 3 d after treatment (DAT) and no biomass visually observed at 3 WAT for both target species. Although flumioxazin was applied to the foliage of *Trapa* spp., this contact herbicide has in-water activity on SAV (Mudge et al. 2010) and emergent and floating species (Mudge and Haller 2012), and some overspray that reached the water column was likely taken up by biomass below the surface of the water. The 24-h exposure of herbicide flumioxazin resulted in 86 to 96% reduction in biomass for *Trapa* spp. for Trial 1 and *T. bispinosa* var. *iinumai* in Trial 2. An increase in biomass was observed for *T. natans* in Trial 2, perhaps due to seedlings being 2× older than plants treated in Trial 1; *T. bispinosa* plants were similar in age in both trials and resulted in similar reductions of biomass as Trial 1 *T. natans*. Variation between trials for *T. natans* response and the lack thereof for *T. bispinosa* var. *iinumai* to flumioxazin warrants further investigation, and because of the sensitivity of both *Trapa* spp. to flumioxazin, future research should investigate lower rates, subsurface concentrations, and shorter exposure periods.

Systemic herbicides imazamox and 2,4-D resulted in marginal control of the target species. *Trapa* spp. treated with imazamox displayed visual discoloration on floating leaves at 3 DAT. However, several those same rosettes began to recover, with new growth at the meristem by 7 WAT, including flower production for *T. natans*. *Trapa bispinosa* var. *iinumai* response to imazamox was variable, with moderate reductions of 57 to 70% in biomass observed for Trial 1 but not Trial 2. *Trapa natans* plants exposed to 24-h and static imazamox treatments were not controlled, with only 35 to 49% reductions in biomass for Trial 1. Similarly, 2,4-D showed visual signs of early injury, including wilting and necrosis of leaves by 3 DAT. Advanced necrosis was visually observed for 50% of replicates, although new growth was also observed on remaining rosettes at 3 WAT. 2,4-D data suggested reduced sensitivity, with 58 to 68% reductions in biomass observed for both trials (Table 3, Figure 1). Observed effects for the 2,4-D treatment of *T. natans* in Trial 1 were similar to those found by Poovey and Getsinger (2007), where 2,4-D amine applied at 0.5, 1.0, and 2.0 mg ai L^{-1} for shorter exposures of 24 and 48 h resulted in 60 to 65% reductions of biomass when compared with control treatments.

All systemic florpyrauxifen-benzyl subsurface treatments resulted in injury to both *Trapa* spp., ranging from leaf wilt and stem twisting (i.e., epinasty) to mortality. Overall growth of *T. bispinosa* var. *iinumai* was reduced when compared with control plants for three of four subsurface treatments in both trials at 7 WAT (Table 3, Figure 1). Both subsurface exposures of 48 µg ai L^{-1} florpyrauxifen-benzyl resulted in 93 to 95% reductions of biomass for *T. bispinosa* var. *iinumai* for both trials (Table 3, Figure 1). *Trapa natans* responded similarly in both trials, regardless of plant age, to 48 µg ai L^{-1} florpyrauxifen-benzyl concentrations at 24-h exposure, resulting in 98% reduction in biomass when compared with controls. These results somewhat resemble findings from an outdoor mesocosm study (Beets and Netherland 2018) investigating subsurface florpyrauxifen-benzyl treatments on a floating-leaved invasive plant with similar habitat and morphology, *Nymphoides cristata*. An 89% reduction in aboveground biomass was observed for *N. cristata* after 24-h exposure at 24 µg ai L^{-1}, with 100% control observed at 72 h of herbicide exposure. We observed a 93 to 98% reduction with 24-h exposure for *T. natans* and *T. bispinosa* var. *iinumai* across both trials; however, the herbicide was applied at 48 µg ai L^{-1}. A static exposure of florpyrauxifen-benzyl at 12 µg ai L^{-1} provided 99% control in the same experiment for *N. cristata*, whereas in the present study the static subsurface rate of 19 µg ai L^{-1} resulted in 67 to 92% control for both *Trapa* species across both trials and the 24-h exposure resulted in poor control (Figure 1). The foliar application of florpyrauxifen-benzyl failed to provide acceptable control of *T. bispinosa* var. *iinumai* for Trial 1, but did reduce biomass by 66% during Trial 2, despite plants being of similar age. *Trapa natans* treated with foliar florpyrauxifen-benzyl in Trial 1 exhibited verdant leaves and healthy rosettes with fruit production at the time of harvest 7 WAT.

Fruit. *Trapa bispinosa* var. *iinumai* produced no fruit in Trial 1 for both herbicide-treated and control plants, whereas only one mature fruit was produced by a control plant in Trial 2. The paucity of fruit is not necessarily attributed to herbicide response but is a phenological trait. This species has been observed to produce fruit a month later than *T. natans* (Dodd and Schad 2021) and the 7-wk duration of the greenhouse study most likely did not accommodate fruit production. *Trapa natans*, however, did produce fruit and production was reduced by herbicide treatments compared with controls (Table 3). Significant reductions in *T. natans* fruit (Trial 1) were observed for both systemic and contact herbicides alike: 100% reduction for static flumioxazin, 70 to 90% reduction for both 24-h and static imazamox exposures, 70% reduction for foliar florpyrauxifen-benzyl treatment, and 69 to 100% reductions for both 24-h and static exposures of florpyrauxifen-benzyl at the higher rate of 48 µg ai L^{-1}. In Trial 2, a 70% reduction was noted for the 24-h foliar florpyrauxifen-benzyl treatment and 100% for the 24-h exposure of florpyrauxifen-benzyl at 48 µg ai L^{-1}.

Nontarget species response

Biomass. *Heteranthera dubia* biomass response to the aquatic herbicides tested was variable between trials (Table 3, Figure 2). Significant reductions of 36 to 85% in plant biomass were observed for most treatments when compared with controls for both trials (Figure 2), although visual signs of recovery with production of new leaves were observed for treatments at 24-h exposure times.

Contact herbicide flumioxazin-treated plants exhibited more sensitivity in the first trial over the second, with 65% versus 28% biomass reduction, respectively, with static treatments resulting in higher biomass reduction (Figure 2). Systemic herbicide treatments exhibited the same trend. Rate had more of an influence on plant response over exposure time across systemic florpyrauxifen-benzyl treatments for Trial 1 than in Trial 2 (Table 3). Subsurface

exposure to florpyrauxifen-benzyl with longer exposures resulted in the most injury to plants in Trial 1, with 85% control, over the 59% control observed in Trial 2. *Heteranthera dubia* response to 2,4-D was minimal, with more influence on biomass reduction exhibited in Trial 1 than in Trial 2 (Table 3, Figure 2). The imazamox 24-h foliar treatment elicited similar responses in sensitivity when compared with controls between trials, with 41% control in Trial 1 and 43% in Trial 2; however, the static exposure resulted in 67% control in Trial 1 as opposed to 32% control observed in Trial 2.

Both plant age and water temperature (Barko et al. 1982, McFarland 2006) could account for the variability between the trials for the evaluated products, where *H. dubia* plants in Trial 2 were more mature (7 wk older). Younger plants exposed to warmer water temperatures likely experienced faster growth rates than those in Trial 2. These results indicate that injury to *H. dubia* can occur when treating mixed communities with *Trapa* spp., especially when treatment prescription calls for targeting *Trapa* spp. earlier in the year before fruit production, when photosynthesis may be at its highest and plants are increasing in overall biomass. Mudge et al. (2021) indicated that *H. dubia* was tolerant to lower concentrations of florpyrauxifen-benzyl at reduced exposures under growth chamber conditions; however, these rates may not be sufficient to control *Trapa* spp. and further evaluation of CETs are warranted.

Although significant differences were observed between main effects, trials, and treatment, *V. americana* biomass was not significantly reduced by any treatments when compared with controls (Table 3, Figure 2). Similar to *H. dubia*, *V. americana* plants for Trial 2 were more mature and producing twice the number of winter buds in Trial 2 than in Trial 1 for control plants.

Sensitivity to contact herbicide flumioxazin was variable for *V. americana* from one trial to the next, with less sensitivity noted in younger Trial 1 plants than in Trial 2 plants. Systemic herbicides 2,4-D and imazamox resulted in most injury to plants among treatments; however, these treatments resulted in moderate 46 to 56% plant control for younger Trial 1 plants (Figure 2). The difference in the age of plants or water temperature (Barko et al. 1982) was evident as Trial 2 results were variable and contradictory to Trial 1 with regard to several treatments. For example, sensitivity to 2,4-D in Trial 1 was absent in Trial 2. Similarly, younger plants were more sensitive to a static exposure of imazamox (Figure 2), with visual injury (e.g., necrosis) present 3 WAT. The imazamox-treated plants possessed brittle leaves that disintegrated when disturbed, whereas older plants exhibited minimal injury symptoms regardless of exposure period.

Vallisneria americana plants appeared healthy, with seed production noted and marginal response detected for the systemic florpyrauxifen-benzyl treatments. The difference in the maturity of the plants may have contributed to variability in growth between trials for the various florpyrauxifen-benzyl CETs evaluated, where rate more heavily influenced plant response in Trial 1 and an interaction of both factors (rate × exposure) contributed to response in Trial 2.

The number of perennating structures, or winter buds, produced by *V. americana* for both trials, however, varied significantly between trials and treatments (Table 3). An increase in winter buds produced during Trial 1 ranged from 5 to nearly 200% for herbicide treatments when compared with controls (data not shown), indicating increased production over controls as a response to herbicide exposure for younger plants. However, significantly less production was experienced by younger Trial 1 plants to 24-h and static flumioxazin, 24-h exposure of imazamox, foliar florpyrauxifen-benzyl, and subsurface 24-h and static exposures to 19 µg ai L^{-1} florpyrauxifen-benzyl. The more mature plants of Trial 2 exposed to products and CETs evaluated produced 3 to 39% fewer winter buds than controls. Regardless of mode of action and herbicide application, mature plants treated with static foliar flumioxazin and imazamox and subsurface applications of 48 µg ai L^{-1} florpyrauxifen-benzyl resulted in the least amount of winter buds produced.

Reduced *V. americana* growth because of exposure to herbicides generally resulted in reduced winter bud production; however, this trend did not extend to all treatments (data not shown) and was not limited by mode of action or application. For example, static treatments with imazamox reduced growth by 56% and resulted in the lowest number of buds produced among herbicide treatments for younger plants; however, a modest 3% reduction in biomass of older plants for Trial 2 resulted in the highest reduction of winter buds, at 39%. Similar trends were observed for both static treatments of foliar flumioxazin and subsurface florpyrauxifen-benzyl.

Results for biomass and winter bud production for *V. americana* indicate that those treatments deemed effective in these trials, principally flumioxazin and florpyrauxifen-benzyl, provided selectivity when targeting *Trapa* spp. in mixed communities with *V. americana*. Results are similar to those found by Mudge (2013) where subsurface contact herbicide flumioxazin and systemic imazamox did not result in significant reductions in *V. americana* biomass when compared with controls.

Management of *Trapa* in mixed SAV communities

Smaller floating and floating-leaved plants including duckweed spp., *Salvinia* spp., *Pistia stratiotes*, and *N. cristata* can be difficult to manage using aquatic herbicides because of their size, proximity to water, and growth habits (Thayer and Haller 1985, Willey et al. 2014, Cozad 2017). Maintaining efficacious exposure times can be challenging when using aquatic herbicides for in-water treatment (Mudge et al. 2012), whereas foliar-applied products must also reach emergent plant tissue and remain long enough for uptake and not be washed off floating plant biomass. Control strategies generally aim to reduce standing biomass of *Trapa* before fruit production and maturity.

Data generated in the greenhouse trials indicate that both 24-h and static applications of flumioxazin and subsurface applications of florpyrauxifen-benzyl at 48 µg ai L^{-1} were highly effective against target species evaluated, while minimally affecting nontarget mature and established

V. americana. In addition, these data support field observations (Heilman 2019) where selective control using subsurface florpyrauxifen-benzyl at lower concentrations reduced target species *Hydrilla verticillata*, whereas nontarget *V. americana* coverages/abundances increased after treatment. Results for *Heteranthera dubia* were inconsistent between trials, with more sensitivity to herbicides evaluated against younger plants in Trial 1 than older plants in Trial 2. This indicates that plant age (coupled with water temperature) can affect efficacy of those products evaluated and warrants caution when treatments occur in mixed communities of SAV where timing of application must be taken into consideration.

The phenological and genetic difference between the nontarget species tested and among populations of each *Trapa* spp. in the United States may respond differently to control methods (Chorak et al. 2019, Dodd et al. 2019, Dodd and Schad 2021). This study suggests that products and CETs evaluated here resulted in poor to moderate control for imazamox and 2,4-D for both species of *Trapa*, variable control for both species with foliar and subsurface low-dose, short-term exposures to florpyrauxifen-benzyl, and variable control for *T. natans* to 24-h exposure of flumioxazin. The highest sensitivity observed resulted from static flumioxazin *T. natans* treatments, 24-h and static flumioxazin *T. bispinosa var. iinumai* treatments, and higher rates of subsurface florpyrauxifen-benzyl treatments for both species, regardless of exposure. Overall, *T. bispinosa* var. *iinumai* exhibited similar sensitivity or higher than its congener *T. natans* to herbicides and CETs evaluated. Efforts should continue to investigate herbicide and CETs species response within an integrated pest management framework for water chestnut control in mixed-SAV communities. Additional work to screen other aquatic herbicides against both *Trapa* spp. at various rates and concentrations needs to be undertaken.

SOURCES OF MATERIALS

[1]Forestry Suppliers, Inc., P.O. Box 8397, Jackson, MS 39284.

[2]OTT© HydroMet USA, 5600 Lindbergh Dr., Loveland, CO 80539.

[3]Navigate®, Applied Biochemists, W175N11163 Stonewood Dr., Ste. 234, Germantown, WI 53022.

[4]ProcellaCOR® EC, SePRO Corporation, 11550 North Meridian St., Ste. 600, Carmel, IN 46032.

[5]Clipper™ Herbicide, Valent USA Corporation, P.O. Box 8025, Walnut Creek, CA 94596.

[6]Clearcast® Herbicide, SePRO Corporation, 11550 North Meridian St., Ste. 600, Carmel, IN 46032.

[7]Spraying Systems Co., P.O. Box 7900, Wheaton, IL 60187.

[8]Alligare MSO 1, Alligare, LLC, 13 N. 8th St., Opelika, AL 36801.

[9]IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY.

[10]10504STATGRAPHICS version 16.0.07, Statgraphics Technologies, Inc., P.O. Box 134, The Plains, VA 20198.

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