ABSTRACT

BEETS, JENS PATRICK. Phenology, Growth, and Herbicide Management Strategies for *Hydrilla verticillata* in Lotic Systems. (Under the direction of Drs. Robert J. Richardson and Ramon G. Leon)

Invasive aquatic plants can have significant negative consequences to the water systems they invade. Hydrilla (*Hydrilla verticillata* (L.f.) Royle) has been particularly problematic in the United States, invading water bodies across the country. Two biotypes of hydrilla have historically been invasive in the U.S. Monoecious hydrilla has spread from central Georgia to Maine, with populations as far west as California. Dioecious hydrilla is predominantly in the southeast U.S. with limited populations in Idaho. Recently, a third biotype has been documented invading a section of the Connecticut River, CT. Additionally, hydrilla has become increasingly problematic in lotic system where research is lacking compared to lentic systems it has historically been problematic.

A two-year field monitoring study was performed on six lotic sites and three lentic sites in North Carolina. Observations were conducted every two to four weeks to monitor phenological events, along with seasonal aboveground biomass harvests and annual subterranean turion harvests. Field observations revealed an extended period of subterranean turion formation and sprouting in lotic compared to lentic systems. Lotic site mean aboveground biomass density in August 2022 observed in this study was similar to biomass densities observed in lentic systems in previous studies. Turion densities did not differ between lentic and lotic systems, however a substantial increase in turion density was observed between 2021 and 2022.

With the recent documentation of a new hydrilla biotype in the Connecticut River, there are many gaps in knowledge concerning its growth and phenology. Plants were established in Raleigh and Laurel Springs, NC and periodic harvests were performed. No significant

differences were observed in peak aboveground biomass, relative growth rate or timing of these metrics between hydrilla biotypes, although the predicted peak biomass for dioecious hydrilla did not occur during the study period. Connecticut River hydrilla axillary turion produced 73.8 axillary turions per plant 180 days after planting compared to 32.8 turions produced by monoecious hydrilla and 1.2 turions produced by dioecious hydrilla.

Herbicide concentration exposure time is needed to provide efficacious control of invasive plant species, but maximum exposure time is limited in most lotic systems. To investigate a potential novel application technique for herbicide management in lotic systems, intermittent applications of florpyrauxifen-benzyl, endothall, and fluridone were applied to monoecious hydrilla at three rates and three "rest periods" between intermittent applications. With all three herbicides tested, no decrease in efficacy was observed with the introduction of a rest period between applications.

Vallisneria is a genus of native submersed plants commonly found in lentic and lotic systems in the U.S. and has commonly been used for restoration and revegetation efforts. Recently, it has been determined that there is more variation in this genus than previously thought and the species *Vallisneria neotropicalis* Vict. has been resurrected. This split from *Vallisneria americana* Michx. complicates comparisons to historical studies on *Vallisneria*. Additionally, two invasive *Vallisneria* taxa have recently been documented in the U.S. Six-week mesocosm studies were performed on four *Vallisneria* taxa and hydrilla. No selective treatments were definitively identified between native and invasive *Vallisneria* taxa. Additionally, *V. australis* rapidly gained biomass throughout the duration of the experiment.

Phenology, Growth, and Herbicide Management Strategies for *Hydrilla verticillata* in Lotic Systems

by
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DEDICATION

I would like to dedicate this to all the people that sacrificed countless hours helping me finish my academic endeavors, especially my coworkers and family.

BIOGRAPHY

Jens P. Beets was grew up in Hilo, Hawaii and was exposed to science at a young age by his parents Dr. Jim Beets and Dr. Lisa Muehlstein. His fascination with nature, especially when water was involved led him to pursue a Biology degree at the University of Puget Sound. In his last semester, a botany class drastically increased his interest in plants and when an offer for summer employment at the Center for Aquatic and Invasive Plants at the University of Florida was given to him he jumped at the chance. Jens then began working for Dr. Mike Netherland who quickly encouraged him to consider a MS in aquatic plant management. Dr. Netherland gave Jens many opportunities and a variety of projects to work on to allow him to figure out what he was most interested in scientifically including the declining population of a native grass species and evaluating a newly registered herbicide for use in aquatic plant management. After completing his MS, Jens accepted an offer of a PhD under Dr. Rob Richardson to continue his academic career. Jens has been active in the Aquatic Plant Management Society and several regional chapters. Jens plans to continue invasive plant research and advance our understanding of new invasive species and develop techniques to protect our aquatic ecosystems from nuisance species. In his free time Jens spends as much time outdoors as possible, hiking, backpacking, camping, fishing and kayaking and hopes to get back into judo once he completes his graduate studies.

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There is a near endless list of people who have helped me get to this point, but there are only so many I can fit here. First and foremost, I need to thank Dr. Mike Netherland for giving me a chance as a young scientist and encouraging me to continue on for a PhD. Your mentorship and guidance is dearly missed. I also need to thank my advisor Dr. Rob Richardson for giving the opportunity to start (and finish) my PhD and all of his help and guidance over the past few years. Many thanks to my committee: Dr. Ramon Leon, Dr. Benjamin Sperry, Dr. Greg Cope, and Dr. Wes Everman for their help and guidance. My work would not have been possible without the help of all of my coworkers over the past few years, I am endlessly grateful to you all: Erika Haug, Andrew Howell, Kara Foley, Tyler Harris, Logan Wilson, Delaney Davenport, Doug Fox, Casey Bardier, Michael Punt, Stephanie Nawrocki, and Eric Jones. Without all of your contributions and support I would not have made it across the finish line.

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Introduction

Hydrilla (*Hydrilla verticillata* L.f. Royle) is a submersed, rooted aquatic macrophyte native to Asia. Historically, the continental United States was invaded by two triploid biotypes (monoecious and dioecious). In recent years a genetically distinct third biotype (dioecious) was discovered in the Connecticut River. The monoecious biotype is primarily found between central Georgia and southern Maine on the Atlantic coast, with populations also occurring in California, Washington, and portions of the Midwestern US. Monoecious hydrilla is the dominant biotype found in North Carolina and other mid-Atlantic states, with limited reports of the two biotypes coexisting in lakes (True-Meadows et al. 2016). Although dioecious hydrilla is no longer present, Lake Gaston once contained both biotypes and was the first location where both were confirmed to exist in a single waterbody at the same time (Ryan et al. 1995).

Hydrilla is often incorrectly perceived as desirable for sportfishing and hunting waterfowl; however, infestations of this nuisance species can be extremely problematic. Once established, hydrilla can outcompete desirable native species such as *Vallisneria* (eelgrass), form dense mats that block access to fishing or hunting areas, and result in the long-term decline of sportfish habitat. These dense mats can also impede navigation and recreational uses of a waterbody when they block docks, swimming areas, or boat launches. Populations can increase sedimentation in reservoirs and block irrigation and drainage canals. Hydrilla can also serve as habitat for vectors of diseases to humans and animals, such as mosquitoes and epiphytic cyanobacteria (Wilde et al. 2005). Hydrilla is host to the cyanobacterium *Aetokthonos hydrillicola*, which causes avian vacuolar myelinopathy (AVM), a neurological disease affecting

waterfowl and their predators (Wilde et al. 2005, Wilde et al. 2014). AVM has been documented in North Carolina (Augspurger et al. 2003).

Widespread economic impacts have occurred due to the presence and spread of hydrilla in waterbodies. In the US, over $100 million is spent annually on aquatic macrophyte management (Rockwell 2003). Between 2013 and 2019 over $2.5 million was spent on hydrilla management on Lake Waccamaw alone (GSARP 2019). The indirect economic impacts of hydrilla infestations should also be considered; hydrilla infestations can impede hydroelectric plants, redirect water flow, and cause flooding. As hydrilla begins to affect fish populations, and recreation activities on lakes this would also impact environmental tourism and activities such as sportfishing tournaments.

Spread and Distribution in North Carolina

Monoecious hydrilla was first identified in North Carolina at Umstead Park (Wake County) in 1980 and was found in 10 other locations by 1981(Harlan et al. 1985, Hodson et al. 1984). This infestation coincides with the first documented infestation of monoecious hydrilla in Delaware which may have been due to misidentification as *Elodea Canadensis* Michx. during transplanting efforts. The initial spread was likely unintentional and is often attributed to fragments spread by boaters or waterfowl transporting fragments, seeds, and turions. Intentional spread by individuals believing it to be beneficial to fish and waterfowl habitat have been reported though not confirmed. Currently, 35 of the 100 counties in North Carolina have confirmed hydrilla populations in their waterbodies (Figure 1).

Hydrilla is well established in many North Carolina Piedmont Reservoirs and is the most prevalent aquatic invasive species in some reservoirs. Hydrilla has been present in Lake Gaston since the 1980s and approximately 40% is shallow enough for hydrilla infestation based on

existing water depths and hydrilla recorded at depths up to 5 m (Madsen 2006) . Additionally, 30-40% of Falls Lake and Jordan Lake (reservoirs near Raleigh, NC) are shallow enough for hydrilla infestation. Infestations have been observed on Norman, Badin, Tuckertown and Jordan reservoirs. Native aquatic species are not common in reservoirs due to the lack of a seed bank (Smart et al. 1998). The lack of interspecific competition and high nutrient levels also favor rapid hydrilla growth in reservoirs (Nawrocki 2016). Nutrient loading and sedimentation as a result of land development have resulted in increased eutrophication over time in North Carolina Piedmont reservoirs (Nawrocki 2016). Hydrilla has been shown to allocate more resources to photosynthetic tissue with increased nutrient levels. This leads to surface mat formation and shading out of any native plant growth beneath the hydrilla mat. Sedimentation and increased chlorophyll due in part to an increase in development cause turbid water conditions, decreasing light penetration and creating a disturbed habitat in North Carolina Reservoirs (Nawrocki et al. 2016, Langeland 1996, Steward and Van 1987). Natural adaptations of hydrilla to turbidity and low light conditions provide it with further advantages over other aquatic plant species present in these reservoirs.

North Carolina natural lakes and rivers have the potential for severe hydrilla infestations. Many are shallow enough to support extensive hydrilla populations. These waterbodies can be habitat to many endemic species, including those under federal protection. Hydrilla infestations drastically alter the lake and threaten the habitat these species require. However, preventative action and early treatment can reduce the potential for hydrilla invasion. Hydrilla was discovered in Lake Waccamaw in 2011 and treated in 2013. Subsequent monitoring on Lake Waccamaw found tuber banks in relatively low densities, with no hydrilla tubers found in 2018. Continued monitoring and management efforts are pivotal to ensuring hydrilla does not dominate this rich

natural resource. Additionally, Hydrilla was first observed in the Eno River in 2005 and infested over 20 miles of river in Orange and Durham counties (NCWRC 2019). Hydrilla has also been discovered in the Deep River, this is particularly concerning due to potential damage to the federally endangered Cape Fear Shiner (*Notropis mekistocholas* Snelson), as well as endemic species such as the panhandle pebble snail (*Somatogyrus virginicus* Walker) (NCWRC 2019). Hydrilla infestations have also been noted in the Chowan, Cape Fear, Neuse, and French Broad rivers. Monitoring and preventing the spread of hydrilla in river systems is extremely important due to the additional difficulties of management in dynamic and often fast-flowing river systems such as these.

Agencies and Regulatory Authorities

Hydrilla is a federally listed noxious weed and is classified by the North Carolina Department of Agriculture and Consumer Services as a Class A noxious weed. The North Carolina Wildlife Resources Commission (NCWRC) has hydrilla listed as an aquatic nuisance species and as a result, NCWRC has developed public outreach programs to educate users of the waterways on these species and methods to mitigate the spread of these species. NCWRC implemented a habitat enhancement program to deter the introduction and spread of invasive weedy species in 2013 and has invested heavily in hydrilla management efforts. Two of their most notable projects include Harris Lake and the Eno River. Harris Lake has been stocked with sterile grass carp and herbicide treatments have been performed in high-use areas around boat docks to reduce the risk of spread to other waterbodies. Low-concentration fluridone drip systems have proven effective in managing hydrilla populations in the Eno River. NCWRC often works in conjunction with North Carolina Department of Environmental Quality (NCDEQ) Division of Water Resources on hydrilla management efforts. The Aquatic Weed Control Act of

1991 directed NCDEQ to create the Aquatic Weed Control Program to assist citizens and local governments within North Carolina with aquatic weed infestations. The Aquatic Weed Control Program focuses on early response to limit the spread and mitigate the long-term impacts of aquatic weeds (NCDEQ 2019). These agencies also serve with the NC Division of Parks and Recreation on the Eno River Hydrilla Management Task Force, a partnership between government agencies, academia, and non-profit organizations to prevent the further spread of hydrilla in the Eno River.

Growth and Reproduction

Hydrilla is often confused with the native species *Elodea canadensis* Michx. but can be identified by several key characteristics. Hydrilla leaves grow in whorls of 3-8 (often 5) and the leaves have serrated (toothy) edges (Figure 2). There is also a pronounced midrib on the underside of the leaf. Flowers (pistillate and staminate) are small (less than ¼ in diameter) white and delicate. Perhaps the most defining characteristic of hydrilla, although not always the most obvious, is the formation of axillary and subterranean turions.

Monoecious hydrilla has four primary methods of reproduction: fragmentation, turion production, subterranean turion (tuber) production, and seed (Langeland 1996). Hydrilla can rapidly spread within a waterbody as well as between waterbodies due to fragmentation. Transference of fragments with a single whorl of leaves can result in a new population of hydrilla (Langeland and Sutton 1980). Small amounts of hydrilla can easily spread to new waterbodies in this manner from trailers, bait buckets, aquariums, or intentional spreads for the perceived benefits to fish and waterfowl habitat.

Axillary turions are formed where the leaves meet the stem and appear as compact, green, leaf buds (Figure 3). Subterranean turions, or tubers, are produced at the terminal end of

rhizomes. Turion and tuber production varies, in most North Carolina lakes axillary turions are formed from October through December, while tuber production primarily occurs from June through October, with some production occurring in November and December (Harlan et al. 1985, True-Meadows 2016). In contrast, in North Carolina rivers, tuber formation has been observed from April to December (Jens Beets pers. comm.). Tubers vary in color from white to red, or black, dependent on the sediment composition. Tubers and turions allow monoecious hydrilla to overwinter in cooler climates, where aboveground biomass including fragments and stems generally die back in the winter. There is some evidence that monoecious hydrilla can overwinter in its vegetative form in milder winter climates (Sutton et al. 1992, Steward 1993)

Tubers also serve as an extremely effective method of spread and maintaining populations in waterbodies. Monoecious hydrilla has been shown to produce over 6000 new tubers from a single initial tuber (Sutton et al. 1992). Tubers are typically found in sediment depths up to 8 cm and can occur at densities of over 3000 tubers m^2 (Nawrocki 2011). Monoecious hydrilla tubers exhibit germination rates of 90% in laboratory trials but appear to require a chilling period prior to sprouting which may prevent sprouting in the same year as formation (True-Meadows et al. 2016, Carter et al. 1987). Sprouting typically occurs in March or April, however, during milder winters, sprouting can begin as early as February. Tubers have been observed in undisturbed soil four years after production and 6-year-old tubers can be viable (True-Meadows et al. 2016). These observations may be indicative of an environmentally imposed dormancy that prevents the depletion of tuber populations. Unlike dioecious hydrilla, sprouted monoecious tubers spread shoots laterally, rather than vertically toward the surface (Van et al. 1987). These tubers can also survive ingestion by waterfowl and herbicide applications (Langeland 1996). Seed production has been reported in monoecious hydrilla but

does not appear to be a major method of spread in the United States. The sexual reproduction of hydrilla is of particular concern because it could result in adaptations to a wider range of environments by aiding in dispersal and overwintering. Genetic variability would increase and could result in adaptations that make hydrilla even more difficult to manage.

In addition to robust reproductive capabilities, monoecious hydrilla has several adaptations that help it to displace and outgrow native species including rapid vegetative growth rates, and the ability to grow in lower light and carbon conditions compared to most North Carolina native aquatic plant species. Monoecious hydrilla produces many lateral runners and can elongate towards the water surface at up to 2.5 cm per day where it begins to branch profusely, intercepting sunlight and excluding other plants (Langeland 1996). This combination of characteristics results in a plant where half of the standing crop is in the top half meter of the water column (Haller and Sutton 1975). Hydrilla tissue is approximately 90% water, which allows it to efficiently use available nutrients and prolifically produce plant material from a relatively small supply of essential nutrients. The low light compensation point of hydrilla allows it to photosynthesize earlier in the day and in deeper water than most other aquatic plant species (Langeland 1996). In fact, monoecious hydrilla tubers have been observed to sprout and extend toward the surface in complete darkness (Haug et al. 2019) Reports vary on the salinity tolerance of monoecious hydrilla; however, some researchers have observed sprouting in salinities as high as 13 ppt (True-Meadows et al. 2016). These high tolerances to salinity indicate that brackish water estuaries may also be vulnerable to invasion. Additionally, tubers can sprout in a broad range of pH levels (True-Meadows et al. 2016). Growth is greatest in the summer, with no shoot growth occurring in winter and regrowth in the spring dependent on the sprouting of turions and tubers (Harlan et al. 1985, Sutton et al. 1992, Owens et al. 2012).

Integrated Management Options

There are a variety of management options available for hydrilla control in North Carolina. Management options differ between lakes, with economic and environmental factors greatly influencing the management plan implemented in a specific aquatic system. Integrated management programs are often the most successful at providing long-term hydrilla control; however, these programs require a detailed and well-developed management plan. Integrated management plans can include any combination of prevention, biological, mechanical, physical, and chemical control techniques. Prevention is often overlooked as the most important step in reducing the risk of invasion by aquatic macrophytes. Through education and signage, the risk and spread of invasion can be greatly reduced. Biological control via triploid grass carp can provide long-lasting but non-selective control of aquatic macrophytes. This method is particularly effective for hydrilla control when used in conjunction with herbicides. Mechanical and physical practices are often non-selective, costly, and labor-intensive, but can be useful in localized infestations to augment other methods of control. Lastly, often the most cost effective management strategy is the use of EPA approved herbicides. Several herbicides are available for hydrilla control, with some providing selectivity and minimizing or avoiding non-target damage to desirable native species. Herbicides can be used for localized, spot treatments, or whole-lake control. Additional information on these management options as part of an integrated management plan is outlined below.

Prevention

There are a variety of non-chemical management options for hydrilla control. One basic method of prevention is boat inspections and sanitation. North Carolina Sea Grant has taken steps to educate boaters on proper methods for inspecting and cleaning boats to prevent the

further spread of hydrilla (Figure 4). Hydrilla can easily be trapped in between the boat and trailer bunks, and is also commonly entangled in axles, lights, transducers, and motor propellers (Basiouny et al. 1978, Langeland 1996, Doyle and Smart 2001, Barnes et al. 2013). Self-inspections and signage reminding boaters of potential hydrilla spread are useful for reducing the transport of hydrilla and other aquatic invasive species between lakes (Rothlisberger et al. 2011). However, several states have implemented boat inspection stations and boat ramp monitors to further prevent spread of these invasive species (Haight et al. 2021). Inspection stations are especially beneficial at high-use boat ramps because they are an effective way to inform boaters about invasive species spread, reduce the spread of aquatic invasive plants, and empower boaters to protect the lakes.

The water garden and aquarium trade are likely responsible for some of the early outbreaks of hydrilla in the United States. Hydrilla is listed as a federal noxious weed, making it illegal to import, transport, and sell the plant. Additionally, under NCDA & CS regulation of the Noxious Weed List, the sale and distribution of hydrilla are prohibited in North Carolina. However, it is still possible to purchase invasive species through online vendors. Monitoring these vendors and educating people in the aquarium and water garden trade is a relatively simple preventative measure that can help further prevent the spread of hydrilla in North Carolina.

Biological Control

Sterile triploid grass carp (*Ctenopharyngodon idella* Val.) are the most effective biological control for hydrilla and are often recommended for aquatic macrophyte management in waterbodies. Grass carp grow rapidly with abundant food sources with a mean weight of 20-25 pounds and recorded weights of over 40 pounds. The apparent life span of grass carp is

approximately 10 years (Sutton et al. 2012). Grass carp can tolerate near-freezing temperatures but rarely feed at temperatures below 3 °C. Feeding peaks at 20-26 °C and temperatures above 38 °C are lethal to the fish (Chilton and Muoneke 1992). Grass carp are tolerant of high salinities, with reports of carp withstanding salinities up to 100 g L^{-1} for several days (Liepolt and Weber 1969). Triploid grass carp are non-specific grazers with a preference for the soft tips of tender young plants. (Sutton et al. 2012). Grass carp exhibit a strong preference for hydrilla, however, muskgrass (*Chara spp.*), Southern naiad (*Najas guadalupensis*), and Brazilian elodea (*Egeria densa*) are also among the species highly preferred by grass carp (Sutton et al. 2012).

Stocking rates of grass carp vary between states and water systems but generally range from 10 to 15 fish per vegetated acre. Stocking triploid grass carp in open systems is not recommended due to the possibility of escape to connected systems (Colle 2014). Stocking waterbodies with grass carp is often used in conjunction with herbicide applications (True-Meadows et al. 2016). There are concerns over the direct and indirect impacts the fish have on the waterbodies being stocked. Decreases in water quality, increases in chlorophyll, phosphorus and nitrogen are commonly observed as the herbivory by grass carp shifts the stocked waterbody from a plant-based community to a system dominated by phytoplankton (Colle 2014).

Mechanical Control

Mechanical control, or the use of power-driven equipment is useful in the management of some aquatic plant species, but the utility is limited for submersed species. Most mechanical harvesters are paddle wheel driven machines with hydraulically operated cutterbars that can be lowered to cut plant material which is then conveyed up the machine and offloaded onto a tranport barge. The transport barge is then used to bring the plant material to the shoreline where it can dewater until it is moved to the final disposal site. There are several distinct disadvantages

to mechanical harvest treatments. Mechanical harvesting of hydrilla invariably leads to the production of plant fragments at the cutterbars. These fragments can then float to and infest new areas within the lake. It should also be noted that mechanical harvesting has very low impact on the tuber bank (True-Meadows et al. 2016). Additionally, mechanical harvesting was estimated to result in the loss of 18% of fish biomass in Orange Lake, FL (Haller et al. 1980) and is costly ($455/acre; Haller and Jones 2012). Site-specific management with mechanical harvesting is possible, however this is non-selective and will result in the removal of desirable macrophytes.

Physical Control

There are a variety of other methods for control of hydrilla and other aquatic plant species. Benthic barriers provide localized control of aquatic macrophytes and are most useful on dense pioneer infestations. Barriers are most commonly made of materials such as PVC, fiberglass, and nylon, although fabrics can be used when fastened and anchored properly (Bellaud 2014). Large installations are not practical due to cost and the need to remain in place for one to two months to ensure control. This method is non-selective and impacts fish and benthic organism communities. Hand pulling is a simple and widely used method of control and can be performed from a boat, wading from shore, or with SCUBA in deeper waters. Hand-pulling is highly selective and useful when target species are in extremely low densities. Cost, labor intensity, and time required are major components to consider when implementing hand pulling. Nutrient inactivation and sediment manipulation is another approach for control that provides mixed results but have been largely unsuccessful for monoecious hydrilla (Bellaud 2014, Spencer et al. 1992). Shading via use of EPA-registered dyes limits light penetration and restricts the maximum depth of plant growth. This method is limited to small, highly controlled waterbodies and is non-selective.

Drawdowns, or the lowering of water levels are an effective method of controlling many aquatic invasive macrophytes. This practice is most commonly used in systems with gate valve or flashboard systems in dams (Bellaud 2014). Siphoning or pumping can be used but is time-consuming and costly. Drawdowns are commonly performed in northern states where the plants are exposed to freezing and drying conditions. Drawdown conditions should be maintained for six to eight weeks for exposure. Drawdowns are often most effective in fall to reduce stranding amphibians and benthic organisms. Poovey and Kay (1998) investigated the potential for summer drawdown to control monoecious hydrilla and determined that one-week drawdowns in sandy soils and two-week drawdowns in silt loam were needed to reduce tuber numbers and prevent significant regrowth. In most North Carolina waterbodies a summer drawdown is not feasible due to usage. Additionally, Hodgson et al. (1984) investigated the potential for drawdown treatments on hydrilla in North Carolina and found them to be ineffective due to the presence of an organic detrital layer over the clay substrate most tubers are found in. Drawdowns may be effective when used in conjunction with herbicide treatments to further reduce tuber sprouting (Spencer and Ksander 1999).

Chemical Control

Chemical control of monoecious hydrilla infestations is often the most cost-effective management practice. There are currently nine herbicides registered for hydrilla control that are commonly used in the US: fluridone, endothall, diquat, copper, flumioxazin, florpyrauxifen-benzyl, imazamox, penoxsulam, and bispyribac-sodium.

Fluridone is a bleaching herbicide that targets a plant-specific photosynthetic enzyme responsible for photosynthesis in plants, resulting in bleaching of new growth (Netherland 2014). Fluridone is particularly efficacious on submersed species, with low use rates but long exposure

requirements to provide season-long control (typically 45 days). Selectivity is commonly achieved through the use of low treatment rates (Netherland 2014).

Endothall inhibits respiration and protein synthesis in plants and has traditionally been used for spot treatments of small target areas to non-selectively control submersed plants (Netherland 2014). Endothall should be applied early in the season when hydrilla biomass is low and plants are actively growing. Early treatment will help to reduce impacts on desirable species that grow later in the season (Langeland and Pesacreta 1986, Netherland 2014).

Diquat interferes with photosynthesis and is fast acting on susceptible floating and submersed aquatic macrophytes, with exposure requirements of hours to days depending on water exchange (Netherland 2014). Diquat rapidly kills hydrilla and has similar timing restrictions to endothall and should be applied before hydrilla biomass has peaked.

Copper is commonly used as an algaecide but is also utilized for fast-acting control of aquatic macrophytes with short exposure requirements of hours up to one day. Copper sulfate is the most widely used copper product, however it is corrosive and affected by water alkalinity (Netherland 2014). Concerns over the use of copper for chemical control of aquatic invasive species are commonly related to lack of biodegradation and regular use can increase sediment copper residues, and potential toxicity to fish at high use rates.

Flumioxazin is another fast-acting herbicide that affects a plant enzyme resulting in membrane destruction and tissue necrosis (Netherland 2014, Glomski and Netherland 2013). Flumioxazin has potential for selective hydrilla applications since some native species including *Vallisneria americana,* longleaf pondweed (*Potamogeton nodosus* Poir.), and *Najas guadalupensis* have not exhibited sensitivity (Glomski and Netherland 2013). Water pH can strongly impact treatment efficacy because flumioxazin degrades rapidly at pH of 8 or higher,

altering the concentration and exposure time (Glomski and Netherland 2013, Mudge and Haller 2010, Mudge et al. 2010, Netherland 2014). Diurnal pH fluctuations and plant matter density should be considered when using flumioxazin for hydrilla control.

The recent registration of the auxin-mimic herbicide florpyrauxifen-benzyl provides a new option for aquatic plant management. Florpyrauxifen-benzyl has shown activity on several invasive aquatic macrophytes including hydrilla, crested floating heart (*Nymphoides cristata* [Roxb.] Kuntze), and Eurasian watermilfoil (*Myriophyllum spicatum* L.) (Beets and Netherland 2018, Netherland and Richardson 2016, Richardson et al. 2016). Early symptoms of effective control include unusual growth including twisting of the stems and curling and reddening leaves. There are several other slow-acting systemic herbicides that control hydrilla via inhibition of enzyme activity including topramezone, imazamox, penoxsulam and bispyribac. These herbicides are effective at rates of less than 100 ppb and provide one to two years of control (Netherland 2014). The slow decay of plant matter minimizes oxygen depletion and risk of fish mortality. These herbicides commonly require whole-lake treatments or treatments in areas with low water movement and potential for dilution is low. One critical factor to consider with chemical control of hydrilla is the potential for herbicide resistance to develop in a population. Repetitive treatment of fluridone in Florida led to fluridone-resistant dioecious hydrilla (Michel et al. 2004). The exclusive use of a single herbicide mode of action is not recommended due to the potential for mutations conferring resistance (True-Meadows et al. 2016). Therefore, it is important to integrate a variety of management techniques and alternate modes of action in long-term weed management programs.

Additional information on the chemical control of aquatic weeds can be found at https://content.ces.ncsu.edu/north-carolina-agricultural-chemicals-manual/chemical-weed-

control. As always follow all label instructions for use rates restrictions and personal protective equipment. Remember the label is the law!

Conclusions

Aquatic macrophyte management is of vital importance for the proper maintenance and function of North Carolina water systems. North Carolina water systems, particularly reservoirs, natural lakes, and rivers are at risk of hydrilla infestation. Prevention of the spread of hydrilla within waterbodies and to existing waterbodies can save millions of dollars that would be needed to effectively manage established populations and the impacts these infestations can have on the economic and ecological function of these waterbodies. Existing partnerships between state agencies are important for prevention, education and outreach, and management of hydrilla. Each agency has a role in aquatic macrophyte management. Interagency cooperation and partnership should be maintained to increase the likelihood of success of weed management efforts.

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FIGURES

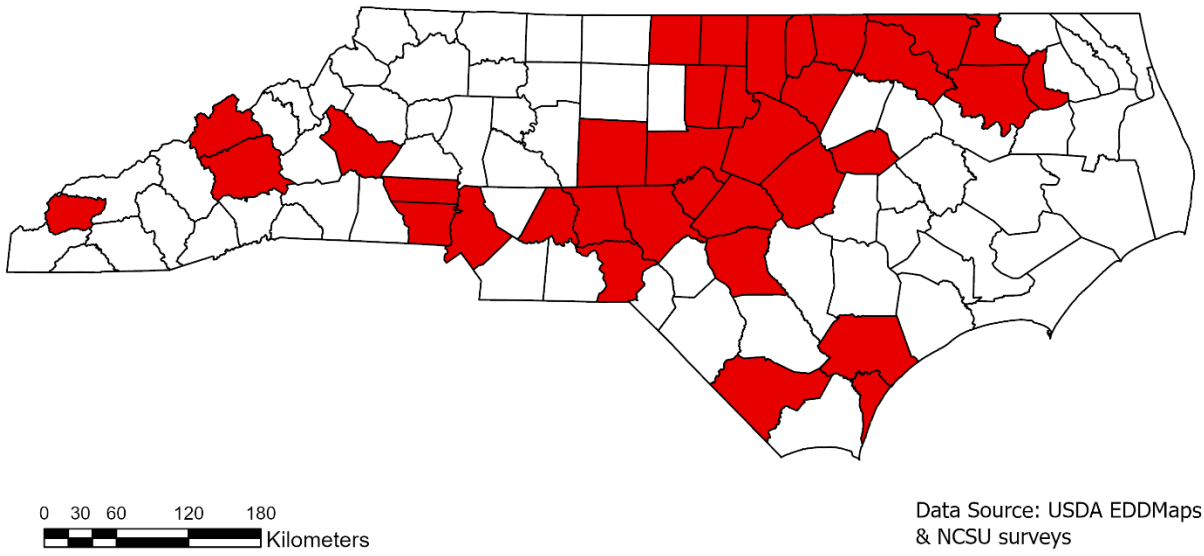

Figure 1-1. Counties in North Carolina with confirmed Hydrilla infestations as of June 2023.

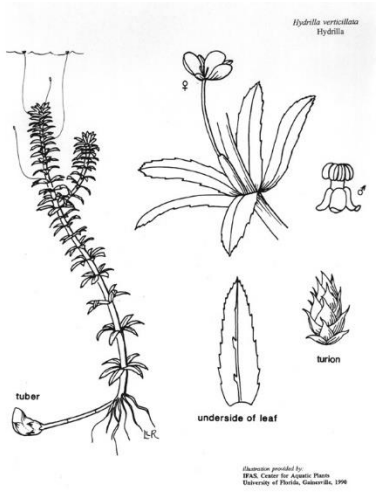

Figure 1-2. Line drawing of *Hydrilla verticillata* (Credit: UF/IFAS).

Figure 1-3. Hydrilla subterranean turions also referred to as tubers (left) and turion (right). Photo Credit: Justin Nawrocki.

Figure 1-4. Example signage from North Carolina Sea Grant to educate boaters on reducing and preventing the spread hydrilla into new water bodies.

CHAPTER 2: Hydrilla Management in North Carolina

Abstract

Hydrilla verticillata (L.f.) Royle is a submersed aquatic macrophyte that has invaded substantial portions of waterbodies throughout the United States. Hydrilla has been previously documented to negatively impact aquatic ecosystems and local economies, these impacts have been closely monitored in lakes and reservoirs where it is historically a problem. However, in recent years hydrilla has invaded lotic systems in North Carolina. While a large body of research has been conducted on hydrilla biology and management in lentic systems, research in lotic systems is lacking, especially concerning monoecious hydrilla. Research was conducted on six lotic sites and three lentic sites containing monoecious hydrilla in North Carolina over a two years period. Phenological observations were conducted every two to four weeks during the study period, revealing an extended period of subterranean turion formation and sprouting in lotic compared to lentic systems. Aboveground biomass density in August 2022 (210 g m^{-2}) at lotic sites observed in this study was similar to biomass densities observed in lentic systems in previous studies. Turion densities did not differ between lentic or lotic systems in 2021 (67.8 and 74.8 turions m^{-2}), however a substantial increase in turion densities was observed in 2022 in lotic systems (138.6 turions m^{-2}) but not lentic systems (65.1 turions m^{-2}) . Variation in timing and duration of sprouting, subterranean turion formation, flowering, and axillary turion formation was observed between 2021 and 2022.

Introduction

Non-indigenous aquatic invasive plants, such as hydrilla [*Hydrilla verticillata* (L.f.) Royle] often have undesirable effects on native aquatic ecology and the associated local

economy within invaded regions (Gordon 1998, Pimentel et al. 2000, Santos et al. 2016). Generally lacking natural herbivores in the invaded areas, these aquatic invasive macrophytes reduce ecosystem biodiversity through resource competition (Madsen and Sand-Jensen 1991, Zhang and Boyle 2010), dominance and displacement (Houlahan and Findlay 2004), and habitat disruption (Thum and Lennon 2009, Schultz and Dibble 2012). The continental United States has three biotypes of hydrilla currently invading its waterbodies. The dioecious biotype was discovered in the 1950s in Florida while the monoecious biotype was first documented in the 1980s in the Potomac River and Lake Umstead in North Carolina (Haller 1982, Schmitz et al. 1991). A third, genetically distinct strain of hydrilla has been identified in the Connecticut River, although it is likely this population has been present for decades (Les et al. 1997, Tippery et al. 2020). The dioecious strain is predominant from Florida to South Carolina and as far west as California. The monoecious strain is generally found from North Carolina to Maine with limited populations further west (True-Meadows et al. 2016). Monoecious and dioecious hydrilla can coexist in the same water body, and the potential for hybridization between these genetically distinct biotypes remains unclear and may be population specific (Steward 1993, Ryan et al. 1995, Williams et al. 2017).

Monoecious hydrilla primarily spreads asexually via fragmentation and turion production (Langeland 1996). Sexual reproduction has also been observed but is not thought to contribute to the spread of this species as significantly as asexual propagation even though it may contribute to genetic variation between populations (Langeland and Smith 1984, Hofstra et al. 2000). Hydrilla produces axillary turions on the leaf axil as well as subterranean turions on rhizome tips. These subterranean turions, also called tubers and both turion types are considered the most important source of hydrilla propagation as well as the process utilized by monoecious hydrilla to

overwinter in cooler climates (Haller et al. 1976, Langeland 1996). Monoecious hydrilla produces subterranean turions at a prodigious rate, with a plant grown from a single subterranean turion producing over 6,000 new subterranean turions after 16 weeks in optimal conditions (Sutton et. al. 1992). These subterranean turions are also persistent in the hydrosoil and able to withstand biotic and abiotic stress (Sutton et. al. 1992, Netherland 1997).

Recently, hydrilla invasions in riverine (hereafter referred to as lotic) systems have become increasingly problematic and are a growing concern among stakeholders as well as state, federal, and international agencies and research groups (Hofstra et al. 2010, True-Meadows et al. 2016). Invasive macrophytes in lotic systems pose a unique problem due to increased management challenges. These difficulties arise from a combination of factors including fast water flow, the presence of desirable and/or rare species, rocky substrate, and limited access. Specific management options may be limited since herbicide applications require adequate concentration exposure times to achieve desired control, and water flow may reduce exposure time requiring increased herbicide concentrations or new application techniques (Netherland et al. 1991, Getsinger and Netherland 1997, Glomski and Netherland 2010, Beets et al. 2019). Continued herbicide applications will require greater volumes of herbicides, making herbicide treatments far more expensive. In many locations, other management options such as mechanical or physical control are not feasible due to access issues. The sterile grass carp [*Ctenopharyngodon idella* (Valenciennes)] is a common biocontrol agent for hydrilla in the Southeastern United States but is typically not permitted for stocking in rivers due to concerns about off-target movement and negative ecological effects (Kirk et al. 2000, Kirk and Henderson 2006, Stich et al. 2013, NCWRC 2017).

Previous studies have examined the establishment of hydrilla in lentic environments (Madsen and Smith 1999, Umetsu et al. 2012, Heidbüchel and Hussner 2019), however, there is limited research focusing on the growth of hydrilla in the presence of water flow. Submersed aquatic vegetation (SAV) distribution in rivers has been found to be influenced by the physical characteristics of streambeds, but differences in plant morphology likely influences propagule drift potential and colonization success (Johansson and Nilsson 1993, Riis and Sand-Jansen 2006). In North Carolina, monoecious hydrilla infestations have become more apparent and of increasing concern in lotic systems, with infestations in the Pee Dee, Neuse, Cape Fear, and Deep Rivers, among others (NCWRC 2017, T Harris and L Wilson pers. comm. 2020)

It is important to study hydrilla behavior in lotic systems due to the increased potential of both short- and long-distance dispersal of this invasive species. For this proposed research we defined a lotic system as a body of water with continuous flow such as (but not limited to) a third order or higher stream. On a large scale, hydrilla infestations in lotic environments pose a much different threat than infestations in lakes and ponds because they are more difficult to identify and manage and impact a broader portion of the landscape. Knowing where hydrilla may, or may not, be able to colonize in a lotic environment will help early detection and rapid response efforts of freshwater resource managers and will also inform future management decisions (Hussner et al. 2017). Thus, our objective was to identify any major phenological differences including timing of major phenological events, aboveground biomass production, and turion density between monoecious hydrilla growing in lentic and lotic systems.

Methods

Site Selection and Environmental Data

Lotic sites on the Deep River, NC, Neuse River, NC, and Cape Fear River, NC, were selected for observation in this study (Figure 1). Deep River sites were chosen based on surveys performed in 2018; Neuse and Cape Fear River sites were based on surveys performed in 2020. Four sites containing hydrilla were identified and selected on the Deep River. Deep River sites 1 and 3 were located in rapids. Deep River sites 2 and 4 were located in runs (slower, consistent flow). Deep River site 4 was flooded in June of 2021 and hydrilla did not persistently reestablish at this site. Monitoring began at Site 2 in July 2021 to compensate for the loss of site 4.

One lotic site was identified and established on the Neuse River (NC) (Figure 1); surveys further downriver revealed no hydrilla present for at least 25 kilometers. One site was identified and established on the Cape Fear River (NC) in a fast-moving side-stream. Due to limited river access no other sites were selected on the Cape Fear River. Three non-flowing (lentic) reference sites of similar depths were identified and established on the shallow edges of Lake Raleigh. This lake was chosen due to the abundance of hydrilla at time of selection, proximity to the laboratory, and previous research conducted on this lake (Meadows 2013). Water depth characteristics for lentic and lotic sites were recorded at time of aboveground biomass sampling (Table 1). A confounding event occurred at the lentic sites when herbicide treatments were unexpectedly performed in Lake Raleigh in September 2021 and 2022. These treatments may confound some findings in our lentic comparison from October 2021 on. However, historical data was used to bolster the comparison of findings in flowing systems to non-flowing systems.

Field data loggers were deployed at all sites except Deep River 2, due to lack of suitable substrate for securing data loggers. Where possible, two loggers were established at each site:

one slightly upstream and one downstream. Data loggers were attached to stabilized t-posts at approximately 5 cm above the sediment level to avoid potential sediment buildup on loggers. Data loggers were set to collect water temperature every six hours. Mean monthly temperature was calculated for each aquatic system, and a one-way ANOVA with a Tukey HSD for comparisons between waterbodies at each month (Table 1). River discharge data were retrieved from the USGS water dashboard using the *dataRetrieval* package from R (Figure 2).

Phenological observations

Phenology data collection began on March 26, 2021 on the Deep River, April 8, 2021 on the Neuse, and April 15, 2021 on Lake Raleigh. The Cape Fear River site was not identified until June 7, 2021. Once the sites were selected, visual assessments were performed every two weeks in 2021 and two to four weeks in 2022. In 2022, intervals were shorter during periods of rapid change or major phenological events were expected, such as turion sprouting and mat formation. Due to the rocky substrate and shallow bedrock in the lotic systems, soil cores were not feasible for subterranean turion collection, and as such the methodology was modified. At each survey site, stratified random sampling was employed to collect hydrilla and sediment from the equivalent of a 1-m^2 quadrat. If hydrilla was not visible from above the water surface at the time of sampling, sediment was collected from stratified random locations at the site to locate subterranean turions. The collected sediment and hydrilla was placed onto a 3-mm mesh sieve and sifted to sort out turions and any other hydrilla biomass. The collected biomass was taken to a laboratory and to confirm life stage (sprouting, subterranean turion formation, flowering, axillary turion formation). Data concerning the life stages was pooled between different sites in lentic and lotic systems.

Turion Density

Subterranean turion density monitoring was conducted in December 2021 on the Neuse and Cape Fear Rivers and Lake Raleigh sites. Due to high water levels, monitoring of the Deep River was in March 2022. Sampling was repeated in December 2022 at all survey sites. Due to substrate and shallow depth, core sampling was not possible at any flowing sites. As an alternative, a spade was used to dig the top 15-20 cm of sediment within three 0.5 m^2 quadrats at each site similar to the methods employed by Regan (2017). At the Lake Raleigh sites, a 10.2 cm diameter core was used with a target depth of 20 cm, and 45 cores were sampled, the equivalent of the quadrat area measured on the flowing sites. Sediment aliquots were sifted through a 3-mm wire screen, and the number of propagules was recorded. Turion densities from cores sampling were calculated from the equation derived by Nawrocki et al. (2016) $T/(C/123.34)$, where T is the number of turions recovered; C is the number of cores sampled, and 123.34 is the number of cores needed to sample 1-m^2.

Density for the sites where a quadrat was employed was calculated by dividing the number of turions collected by the sample area (1.5 m^2). Turion density data were subjected to a two-way ANOVA with year and aquatic system (lotic or lentic) as factors using JMP Pro 17. To meet the assumption of homoscedascity and normal distribution, turion density data were log-transformed and data from lotic and lentic sites were pooled by aquatic system.

Aboveground Biomass Density

Aboveground biomass was collected at quarterly intervals, attempting to capture differential trends in seasonal biomass starting in July of 2021. Weather and logistical constraints prevented biomass sampling earlier in the year. Four 0.5- m^2 quadrats were placed in a stratified random manner, selecting for areas where plants were observed. Water depth was recorded at

each quadrat. Biomass was dried in a forced air dryer for 72 hours and weighed to determine dry weight of collected biomass. Mean water depth was calculated at the time of harvest at each collection site (Table 2). Biomass data were subjected to a two-way ANOVA with harvest time and aquatic system (i.e. lentic and lotic) as factors. To meet the assumption of homoscedascity and normal distribution, biomass data were log-transformed and data pooled by aquatic system.

Results and Discussion

Phenology

As a herbaceous perennial, hydrilla depends on subterranean and axillary turion sprouting for growth in the spring. Based on the two-year monitoring, variation in plant characteristics were observed between lentic and lotic systems as well as between years. Mean monthly temperature was calculated for lentic and lotic systems to provide background as temperature differences may influence timing of phenological events. In 2021, sprouting was first observed in April in lotic sites whereas sprouting had started before the first sampling period in the lentic system (Figure 3). Water temperatures were 3.66°C higher in the lentic site in April 2021 and 2.29-2.91°C higher in January and February in 2022 compared to lotic sites. In 2022, sprouting was observed in February at both the lentic site and lotic sites. Although monoecious hydrilla is typically observed sprouting in April in lentic systems, populations have been observed to sprout in March when water temperatures reached 11-13°C, including the hydrilla population at Lake Raleigh (Harlan et al. 1985, Meadows 2013, True-Meadows et al. 2016). These temperatures were reached by February in the lentic system and by March in the lotic systems. Additionally, in January 2022, the mean monthly water temperature in lotic systems was below the previously established 8°C threshold for sprouting initiation but was above this threshold in lentic systems (Van and Steward 1987). The turion sprouting period may also be extended in lotic systems, in

2021 and 2022 sprouting continued until December. Sprouting in the lentic system was last observed in September in 2021 and October in 2022. The end of the sprouting period in lentic systems was comparable to historical turion sprouting dynamics, although a herbicide treatment was performed both years around this time in the lentic system and may have prevented continued sprouting (Spencer and Ksander 2001, True Meadows et al. 2016).

Water temperatures tended to be higher in lotic systems during the summer months and cooler in the fall, winter, and spring in 2021 but remained warmer from June to December in 2022; this may have been influenced by water depth and discharge (Table 1, Figure 2). Water temperature is important to the initiation of subterranean turion sprouting. Soil temperature and chilling periods also influence sprouting initiation and rates, although results vary on the exact relationship (Basiouny et al. 1978, Carter et al. 1987: Steward and Van 1987, Kojima and Izawa 1989). Additionally, there is no clear delineation on sprouting cessation during a given season. Continued sprouting seemed independent of mean monthly temperature in the lotic systems observed in this study. Future investigations into the potential effects that abiotic factors such as sediment type, turbidity, and dissolved oxygen content have on sprouting, especially in the presence of water flow would be helpful to improve understanding of factors influencing initiation and cessation of subterranean turion sprouting.

Subterranean turion (tuber) formation was first observed in May 2021 in lotic systems and June in the lentic system. The following year formation was observed in April in lotic systems and again in June in the lentic system (Figure 3). Van et al. (1989) reported that monoecious subterranean turion formation was photoperiod and temperature dependent, with increased tuberization in 10-hour photoperiods and in summer conditions. However, differences in photoperiod between study sites are likely negligible due to relatively close geographic

proximity (Figure 1). In 2021 and 2022 lentic temperatures were higher in the period leading to subterranean turion formation indicating factors other than photoperiod and temperature may influence subterranean turion formation (Table 1).

Previous investigations into temperature effects on subterranean turion formation indicate increased water and soil temperatures positively impact sprouting rates (Van et al. 1989, McFarland and Barko 1999, Rybicki and Carter 2002). This is not reflected in the results of this study, the lotic systems were cooler, but formed subterranean turions first. Subterranean turion formation was observed on Lake Raleigh in August in 2010, however, this occurred in a deeper portion of the lake than the sites selected in this study and water temperatures were cooler (Meadows 2013). The timing of subterranean turion formation in our lentic system matches with other historical data. Harlan et al. (1985) observed monoecious hydrilla subterranean turion formation starting in July in three North Carolina lakes. Although it was previously thought subterranean turion formation was tied only to temperature and photoperiod, different populations may have adaptations that alters the timing of subterranean turion formation. Mesocosm studies investigating hydrilla growth and phenology from distinct populations may provide a clearer understanding of the influence of abiotic factors on key phenological events and improve management by targeting management to occur before turion formation, reducing future population potential.

Subterranean turion formation in the lentic system ended in October 2021 and November in 2022. This is likely due to the late season treatments as well as major flood events removing hydrilla biomass from study sites. Formation of subterranean turions has been observed until October in lentic systems of North Carolina (Harlan et al. 1985, Meadows 2013). Subterranean turion formation was still observed in December of both years at all lotic sites in this study,

although aboveground biomass was extremely limited at this time. In 2021, temperatures during this period were higher in the lentic system, but in 2022 late year temperatures were higher in the lotic system (Table 1). Increased temperatures have been shown to increase subterranean turion production, however, these previous studies have mostly focused on the initiation of subterranean turion formation, and less emphasis was placed on the cessation of propagule formation and influence temperature may have (Steward and Van 1987, McFarland and Barko 1990, Rybicki and Carter 2002).

Mat formation, or biomass reaching the water surface and topping out, occurred in lotic systems in June 2021 and July 2022, one month earlier than observed in the lentic system, however decreased water depth and flow rates may have influenced this event. In several locations along the Deep and Cape Fear Rivers, including Deep River site 2, hydrilla was observed to form a mat from bank to bank, especially during periods of low water discharge. Despite the delay in mat formation at the lentic system, flowering initiation did not differ between the lotic and lentic systems in either year, occurring in July. Flowering was not observed past September in the lentic system and was observed until October-November in lotic systems (Figure 3). Floral initiation and presences of flowers appears to vary greatly in monoecious hydrilla, ranging from May to December (Vandiver et al. 1982, Conant et al. 1984, Harlan et al. 1985, Meadows 2013). Sexual reproduction is not thought to be a major form for monoecious hydrilla spread in the US. However, it may contribute to genetic variation and adaptations between populations including those in lentic and lotic systems. The potential for sexual reproduction and increased genetic variation is particularly concerning in hydrilla due to the increased management difficulties of managing genetically diverse macrophytes (Burden and Marshall 1981, Furnier and Mustaphi 1992, Hofstra et al. 2000).

Axillary turion formation initiated in September 2021 and October 2022 at all study sites, continuing until November or December. Future investigations into turion dynamics in lotic systems should investigate the turion production in lotic systems, anecdotally, turion production seemed to be far more prolific in lotic systems than lentic systems, and may have a stress-induced component, with prolific turion production observed following major flood events in lotic systems. Monoecious hydrilla biomass has been observed to detach as rafts containing high numbers of axillary turions, furthering the spread of monoecious hydrilla in aquatic systems (Harlan et al. 1985., Steward and Van 1987, True-Meadows et al. 2016). This method of transport would be particularly effective and problematic in lotic systems, as these rafts can travel rapidly downstream, depositing axillary turions as they are dislodged.

Turion Density

No significant differences in turion density were observed between lotic and lentic sites or years (two-way ANOVA, Figure 4). There was a noticeable large turion density observed for lotic sites in 2022, due to a single quadrat which contained 534 turions m^{-2}. Turion density in this study was considerably lower than the density observed in other North Carolina lakes (Harlan et al. 1985, Nawrocki et al. 2016). This is possibly due to this study being conducted in considerably shallower sections of water bodies (<1 m). The role of water depth in turion density is complicated, several studies indicate that subterranean turion density increases with increasing water depth (Miller et al. 1976, Mitra 1964). However, MacFarland and Barko (1995) reported that monoecious subterranean turion density was greatest at 1.0 m compared to greater and lesser depths. The treatment and subsequent flooding due to a major storm at Lake Raleigh may have also reduced turion density at the sites, as the timing of these events occurred as turion production was increasing and biomass was washed out of the study sites at this location.

Another potential explanation for the low subterranean turion density of lotic systems compared to historical data may be the rocky substrate at lotic sites preventing penetration into the sediment as deep as the coring systems used in other studies. This substrate type may also reduce the subterranean turion density, although it is unclear what effect sediment composition has on subterranean turion density. Sutton and Portier (1985) indicated subterranean turion density was independent of sediment type, while Spencer et al. (1992) reported subterranean turion density was affected by sediment type, especially by increases in organic matter. Given the periodic high flow events in the lotic systems of this study, it is also possible that the subterranean turion density is lower than in lentic systems due to turions being transported downstream with the sediment as a result of the consistent water velocity of lotic systems and flooding events. Although not quantified, high flow events resulted in considerable changes to the sediment composition over time, and subterranean turions were more commonly found in deposits of sediment behind a large rock, where they were less likely to be washed out of the site with the sediment. These were the same areas where hydrilla was commonly first observed prior to mat formation and hydrilla spreading throughout entire sections of river. Future investigation into the effects of flow and sediment characteristics on turion dynamics is warranted and may reveal further insights on the potential for spread in lotic and lentic systems and aid management efforts, particularly in lotic systems with dynamic water flow.

Aboveground Biomass Density

During the initial aboveground biomass sampling time period (July 2021), mean biomass did not significantly differ between lentic and lotic systems (Figure 5). Mean monthly water temperatures were 17% higher in the lentic system during April but did not differ more than 3% until the harvest date (Table 2). Biomass did not differ greatly when sampled again in October

2021. Water temperature did not differ more than 5% between the time of the previous harvest and the October harvest. No biomass was observed at any sites in March 2022, which is consistent with the timing of sprouting and aboveground biomass production of monoecious hydrilla (True-Meadows et al. 2016). In June 2022 biomass did not differ between lentic and lotic systems despite 2.6x more biomass in the lotic sites (Figure 5). Water temperature was 19-27% higher in January – April in the lentic system and less than 2% lower in May and June (Table 2). Water temperature can greatly influence monoecious hydrilla biomass production, with increasing temperature yielding increased biomass, shoot length and number, especially in temperature greater than 16 C, so the increased biomass production in the system with cooler water was surprising (McFarland and Barko 1987, McFarland and Barko 1999, Rybicki and Carter 2002).

In August 2022, biomass in lotic systems was much greater than biomass at the lentic system (Figure 5). Mean water depth was 43.6 cm at the lentic site and 26.3 cm at lotic systems at the time of harvest and water temperatures were 1-6% greater in the lotic sites (Tables 1 & 2). Herbicide treatment the previous year may have negatively impacted biomass in the lentic system as much of the biomass washed out as subterranean turions were being produced, reducing the turion bank. Mean biomass in the lotic systems was comparable to observed aboveground biomass density in Big Lake, NC, despite being in far shallower water depths (Harlan et al. 1985). Biomass in lotic systems decreased in October by 54% which is consistent with monoecious hydrilla aboveground biomass not overwintering and beginning to senesce as temperatures decline (Harlan et al. 1985, True-Meadows et al. 2016). The lack of a major flooding event in the lotic systems in 2022 compared to those observed in 2021 may have contributed to the increased biomass in August and October of 2022 (Figure 2). Although an

exact date was not provided, the lentic site was treated again in September 2022, which likely contributed to aboveground biomass being 5x lower than lotic systems in October. Interestingly, biomass did not decrease significantly between August and October in the lentic site corresponding to pre and post treatment.

Aboveground biomass is not a commonly utilized metric for hydrilla density, so comparisons to historical surveys are limited. Aboveground biomass in the lotic systems studied in 2022 was similar to biomass densities observed in Big Lake at similar harvest time periods. Harlan et al. (1985) reported biomass of 236 g m^{-2} in August compared to the 210 g m^{-2} of this study (Figure 5). However, biomass sampled from our lentic system was considerably less than observed by Harlan et al. (1985) at similar time periods. The mean water depths of our study locations were considerably shallower, (0.29 - 0.6 m) compared to the 1.0 m sites of their study, which likely contributed to this observed difference. Herbicide applications also likely contributed to the decreased biomass especially as the treatment timing in 2021 coincided with a major flood that washed the weakened hydrilla biomass out of the study sites.

Monoecious hydrilla biomass production in lotic systems can match production in lentic systems but may be limited by water depth and be suddenly dislodged from specific areas due to high water discharge events that may be less impactful on aboveground hydrilla biomass in lentic systems. This highlights the importance of treatment timing and early seasonal treatment of monoecious hydrilla in lotic systems to prevent negative impacts on water flow. Dense mats of hydrilla were observed in several locations stretching from bank to bank, impeding paddling between sites. Timing management options before this occurs can greatly reduce necessary herbicide inputs as well as reduce potential flood risks or displacement of native and endangered species.

The highest observed hydrilla biomass in this study was in August 2022 in the lotic systems which may be indicative of increasing biomass in the lotic systems over time (Figure 5). This was further corroborated by the high turion density observed in 2022 and supported by a downstream survey of the Cape Fear and Deep Rivers in 2022 revealing hydrilla present in areas where it had not been previously located in 2017 surveys (Tyler Harris and Logan Wilson pers. comm.). The spread of hydrilla to new lotic systems should be monitored as it may have impacts on recreation, negatively impact habitat and may alter water flow dynamics and increase flood damage. Management strategies are still being adapted for use in lotic systems and priority should be emphasized on developing new use patterns or herbicide combinations for chemical control. Developing new physical, mechanical, or biological control techniques for invasive macrophyte invasions in lotic systems can also provide new tools for integrated plant management.

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TABLES

Table 2-1. Monthly mean temperature at study locations April 2021- December 2022.

Month	Mean Temperature (°C)			
	Lentic		Lotic	
Apr-21	22.44 ± 0.37	*	18.78 ± 0.20	
May-21	23.06 ± 0.19	*	22.39 ± 0.14	
Jun-21	26.17 ± 0.16		26.52 ± 0.07	*
Jul-21	26.86 ± 0.16		28.29 ± 0.07	*
Aug-21	27.46 ± 0.16		28.51 ± 0.05	*
Sep-21	25.01 ± 0.15	ns	25.25 ± 0.08	ns
Oct-21	21.30 ± 0.13	*	20.96 ± 0.10	
Nov-21	13.18 ± 0.13	*	12.44 ± 0.12	
Dec-21	11.62 ± 0.10	*	10.62 ± 0.07	
Jan-22	10.23 ± 0.21	*	7.96 ± 0.11	
Feb-22	12.16 ± 0.19	*	9.25 ± 0.08	
Mar-22	17.42 ± 0.20	*	13.46 ± 0.11	
Apr-22	21.62 ± 0.20	*	17.72 ± 0.11	
May-22	23.60 ± 0.17	*	23.15 ± 0.12	
Jun-22	27.57 ± 0.08	ns	27.76 ± 0.11	ns
Jul-22	28.34 ± 0.10		28.88 ± 0.13	*
Aug-22	27.23 ± 0.12		28.81 ± 0.11	*
Sep-22	24.98 ± 0.12		25.66 ± 0.13	*
Oct-22	18.08 ± 0.08		19.69 ± 0.15	*
Nov-22	15.11 ± 0.18		16.89 ± 0.18	*
Dec-22	9.79 ± 0.13		10.90 ± 0.14	*

[a] Temperature means (±SE) with asterisk are significantly higher than mean at other system type ($\alpha = 0.05$); ns indicates no significant difference between systems.

Table 2-2. Mean water depth at time of aboveground biomass harvest at lentic and lotic systems

Month of Harvest	Mean Depth (cm)	
	Lentic	Lotic
Jul-21	60.0 ± 6.4	32.5 ± 2.9
Oct-21	49.5 ± 2.2	26.7 ± 2.3
Mar-22	43.6 ± 3.1	39.2 ± 2.4
Jun-22	44.7 ± 3.1	40.4 ± 2.9
Aug-22	43.6 ± 3.1	26.3 ± 2.5
Oct-22	44.7 ± 3.1	36.5 ± 3.0

FIGURES

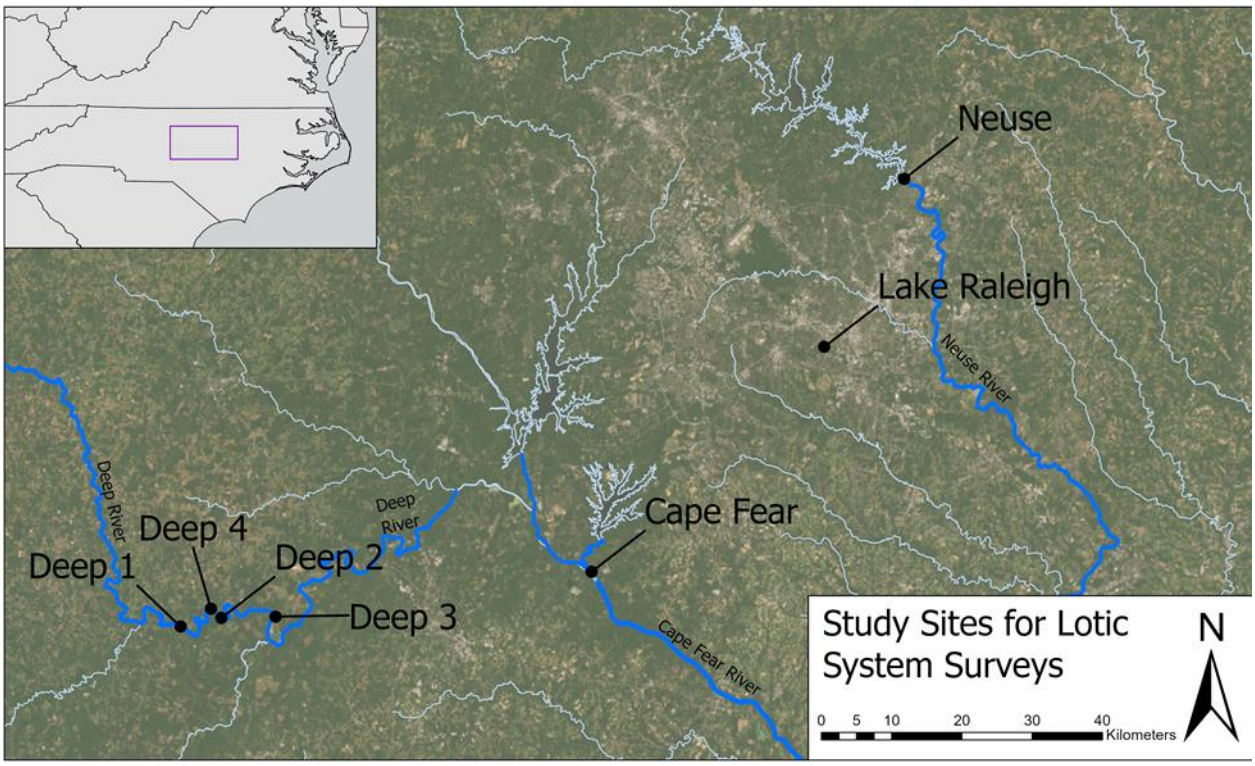

Figure 2-1. Location of NC monoecious hydrilla field sites located on the Deep River, Cape Fear River, Neuse River, and Lake Raleigh.

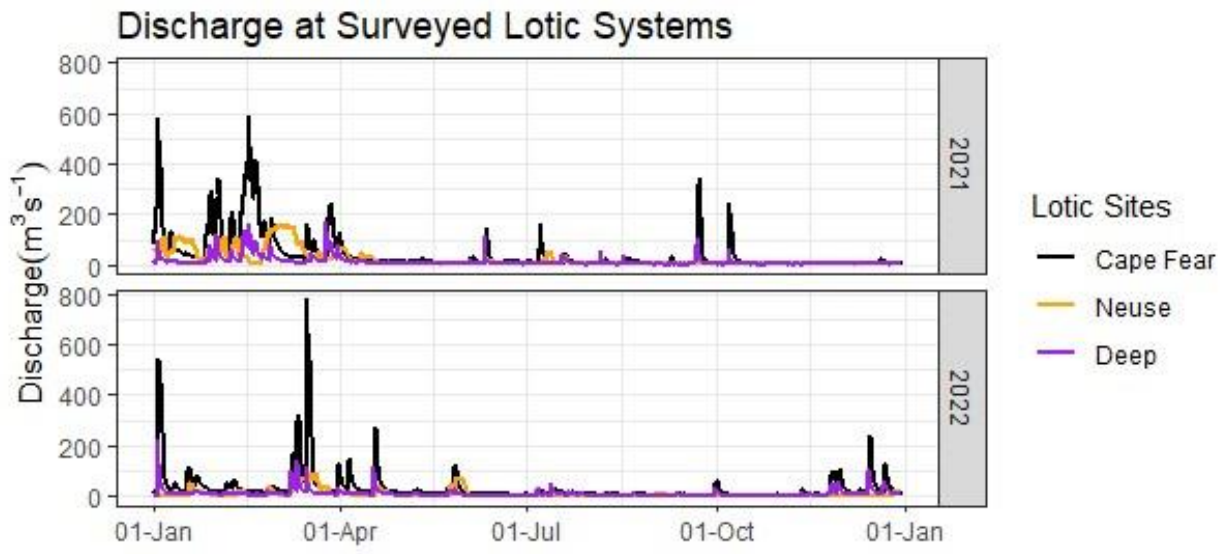

Figure 2-2. Mean daily discharge (cubic feet s^{-1}) at the nearest upstream USGS gauge for the lotic sites for 2021 and 2022.

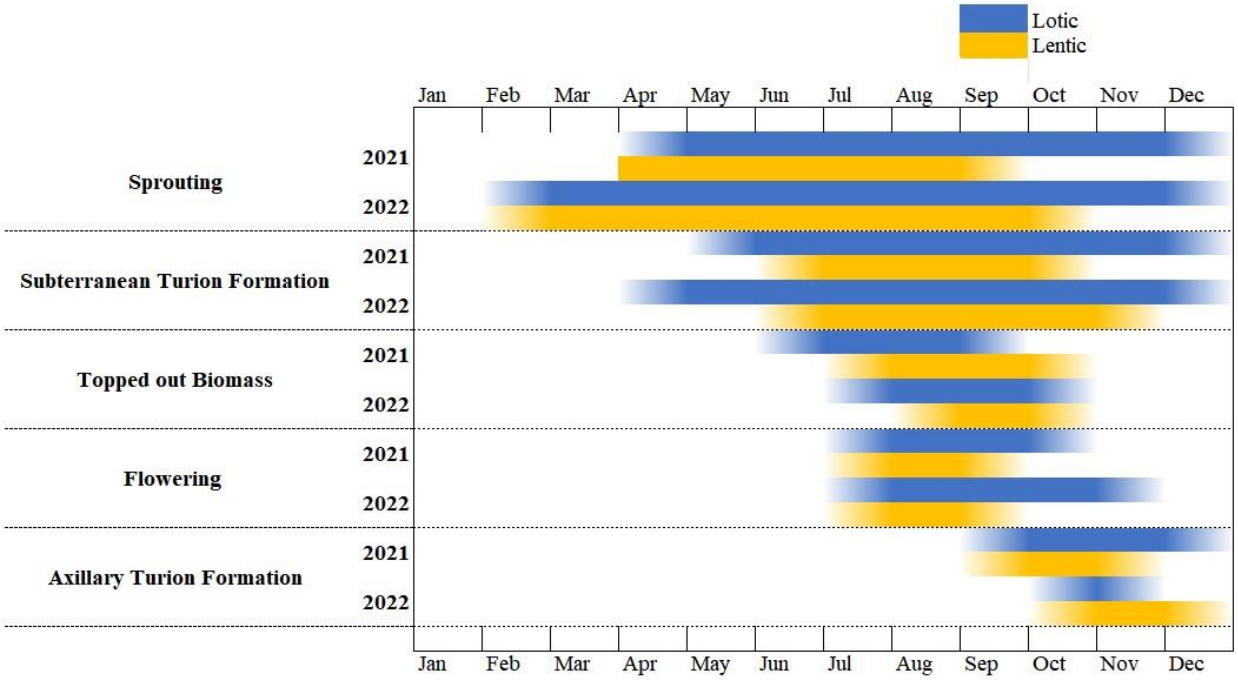

Figure 2-3. Observed patterns in the life cycle of monoecious hydrilla in lentic systems (Lake Raleigh) vs lotic systems (Neuse River, Cape Fear River, Deep River) in North Carolina.

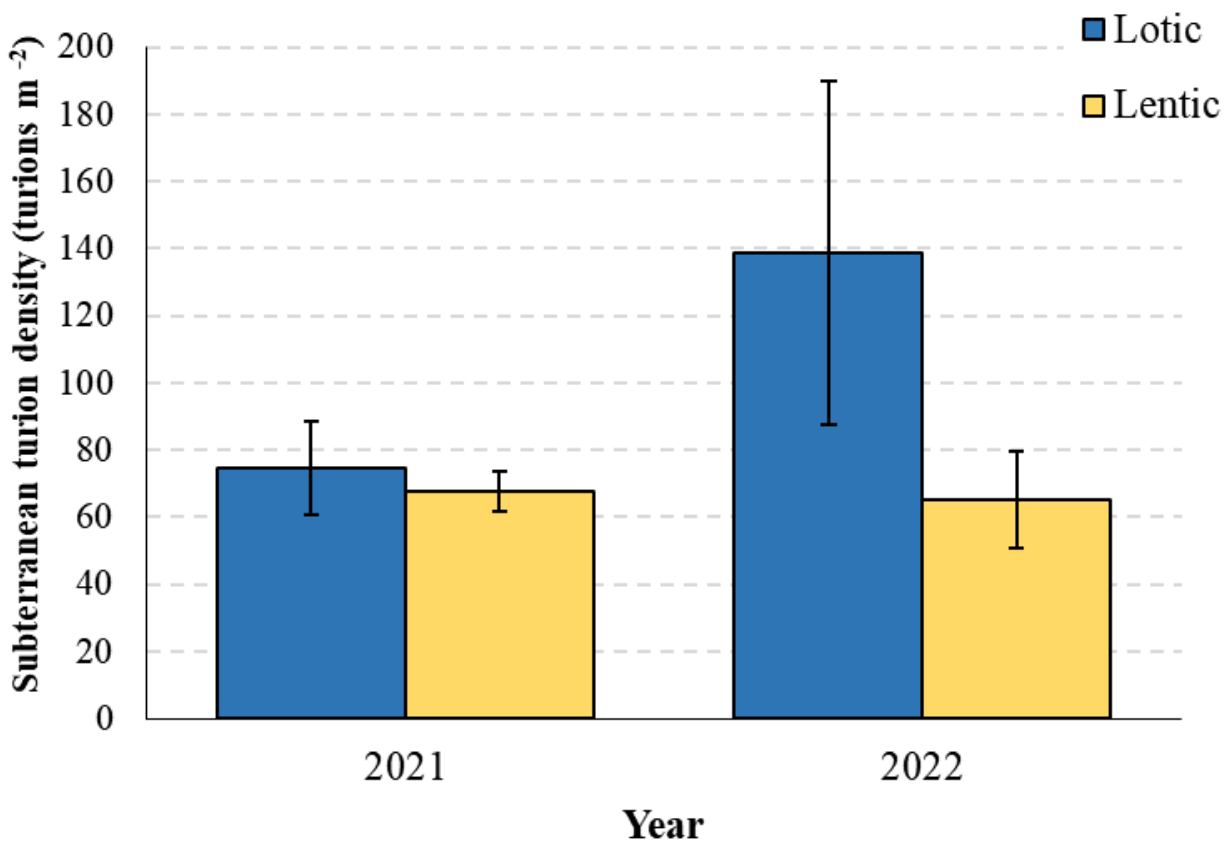

Figure 2-4. Subterranean turion density mean and standard errors for lotic and lentic systems in 2021 and 2022. (n=5 for lotic systems, n=3 for lentic system). No significant difference was observed in a two-way ANOVA between year or system type.

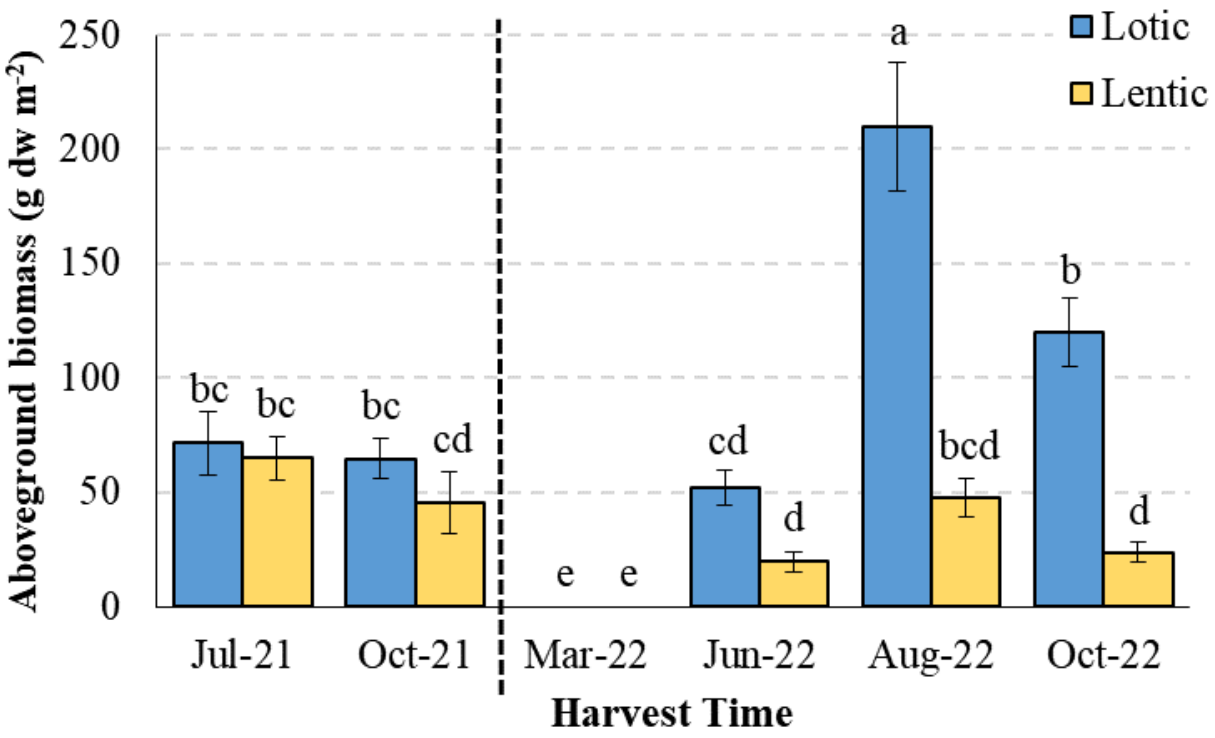

Figure 2-5. Dry aboveground biomass and standard errors for harvests performed once per season in lotic and lentic systems. (n=5 for lotic systems, n=3 for lentic system). Four 0.5-m^2 quadrats were sampled at each site. Sampling could not be performed in June of 2021 due to logistical constraints. Letters over bars indicate difference between harvest periods (ANOVA, Tukey's HSD, $\alpha = 0.05$).

CHAPTER 3: Documenting phenological growth patterns of Connecticut River hydrilla

Abstract

Hydrilla (*Hydrilla verticillata*) is an invasive perennial submersed plant that has been documented in the United States since the 1950s. There have historically been two strains of hydrilla, hereafter referred to as biotypes, recorded in the United States, and have been characterized as 'monoecious' and 'dioecious'. Recently, a third genetically distinct biotype was identified in the Connecticut River, CT. Little is known about growth and phenological differences in this new invader, therefore, we aimed to compare these differences by biotype, including flowering and turion formation in a series of mesocosm experiments. Plants were established in Raleigh and Laurel Springs, NC and periodic harvests were performed. No significant differences were observed in peak aboveground biomass, relative growth rate or timing of these metrics between the three hydrilla biotypes, although the predicted peak biomass for dioecious hydrilla did not occur during the study period. Surprisingly, monoecious hydrilla had lower peak belowground biomass than dioecious and Connecticut River hydrilla despite its abundant subterranean turion production. Monoecious hydrilla had significantly more aboveground biomass and subterranean turions 180 days after planting. Connecticut River hydrilla produced 73.8 axillary turions per plant 180 days after planting compared to 32.8 axillary turions produced by monoecious hydrilla and 1.2 axillary turions produced by dioecious hydrilla. This research suggests the newly documented biotype of hydrilla in the Connecticut River has many similarities to the other two biotypes in the United States but further research is needed to better understand the phenology and ecology of this invasive plant.

Introduction

Hydrilla (*Hydrilla verticillata* (L.f. Royle)) is an extremely problematic herbaceous submersed macrophyte that has successfully invaded waterbodies across the United States. The continental United States has three genetically distinct biotypes of hydrilla currently inhabiting its waterbodies. The dioecious biotype was discovered in the 1950s in Florida and has established in warmer climates of the United States, primarily from Florida to Georgia and westward to Texas, with limited populations in Idaho and California (Steward et al. 1984, Netherland 1997). Monoecious hydrilla was first documented in the United States in the 1980s in Maryland, Delaware, and North Carolina (Haller 1982, Steward et al. 1984, Schmitz et al. 1991). Populations rapidly established in cooler climates and are now primarily found between central Georgia and Connecticut on the Atlantic coast, though populations can also be found in California and Washington. Both of these biotypes of hydrilla primarily spread via fragmentation, stolons, rhizomes, as well as axillary and subterranean turions (Langeland 1996, Netherland 2015).

The third genetically distinct biotype of hydrilla has recently been characterized in the Connecticut River, CT. Its population has been documented to extend from Hartford, CT to Deep River, CT. There is little information about differences in its growth and phenology compared to monoecious and dioecious hydrilla (Tippery et al. 2020). The timing of introduction and sexual reproduction characteristics for the third biotype are also currently unknown. Although it does not yet co-occur with other hydrilla biotypes in the Connecticut River, monoecious and dioecious hydrilla have been historically documented in the state (Les et al. 1997, Madeira et al. 1997, Madeira et al. 2000, Madeira et al. 2004). It was initially believed this novel invasion was similar to “clade C” hydrilla found in Ireland, Japan, Latvia and South Korea (Benoit et al. 2019,

Tippery et al. 2020). Further analysis revealed the hydrilla invasion exactly matched haplotype 17-8, a rare haplotype found only in the Amur-Heilong River Basin and Liao River and Hai River basin in northeast PRC (Harms et al. 2021). In contrast, the monoecious and dioecious hydrilla found elsewhere in the United States are most similar to two haplotypes in "clade B" from the Indian subcontinent and South Korea (Madeira et al. 1997, Benoit et al. 2019).

Although, monoecious and dioecious hydrilla tend to not overlap in the United States, they have been found to coexist in the same water body in some locations, with populations of both previously recorded in Lake Gaston, NC, and several Tennessee Valley Authority reservoirs (Ryan et al. 1995, Netherland and Greer 2014). There is some concern that coexisting populations of genetically distinct hydrilla may hybridize, increasing genetic variation and resulting in adaptations that would complicate management. Test crosses between US monoecious and dioecious hydrilla have produced mixed results, possibly due to both being triploid, and the likelihood that the monoecious biotype in the US arose from a hybridization event between dioecious plants in its native range (Harlan et al. 1985, Langeland 1989, Benoit et al. 2019). Steward (1993) observed 71% of crosses resulted in seed production with 90% seed viability. However, Williams et al. (2017) did not observe seed production in field and greenhouse studies. Differential response of hybrid taxa to management options, hybrid vigor, and increased invasiveness, have been observed and closely monitored in other aquatic invasive species including *Myriophyllum* and *Nymphoides* (Ellstrand and Schierenbeck 2000, Moody and Les 2002, Harms et al. 2021).

Many phenological and growth differences have been observed between monoecious and dioecious hydrilla, as well as differences in response to herbicides. Monoecious hydrilla has been shown to produce subterranean turions at a more rapid rate and broader range of photoperiods

and temperatures than dioecious hydrilla (Spencer and Anderson 1986, McFarland and Barko 1987, Steward and Van 1987, Van 1989). Consequently, monoecious hydrilla typically produces subterranean turions in July - October, while dioecious hydrilla predominantly produces subterranean turions in October - April in their invaded ranges (Bowes et al. 1979, Harlan et al. 1985, True-Meadows et al. 2017). Monoecious hydrilla turions tend to be smaller in size than dioecious turions, allowing more efficient turion production per unit biomass (Steward and Van 1987). Monoecious and dioecious hydrilla also rapidly spread via fragmentation of stems, and rapidly establish in new systems from these fragments (Umetsu et al. 2012). These fragments do not overwinter, and axillary and subterranean turions serve as the primary means of monoecious hydrilla overwintering (Harlan et al. 1985, True-Meadows 2017). Notable differences have also been observed in seasonal biomass production, while peak biomass production occurs in July-October for both biotypes, monoecious hydrilla senesces in the fall and relies on its vegetative propagules for overwintering, while dioecious hydrilla biomass tends to remain uniform throughout the year (Harlan et al. 1985, Madsen and Owens 1998, True-Meadows 2017). There is a current lack of knowledge concerning the turion production and fragmentation dynamics of this “clade C” or Connecticut River hydrilla.

Timing of biomass and turion production are particularly important to understanding the spread of this newly documented hydrilla as this can greatly impact the timing of management efforts. Targeting early growth has been particularly effective in monoecious hydrilla management but is not often an option on well-established stands of dioecious hydrilla (Langeland and Pesacreta 1986). Focusing on easily recognizable indicators of physiological status such as biomass production, relative growth rate, and turion production can help ensure efficacious management timing (Nichols 1991, Wersal and Madsen 2018). Axillary and

subterranean turion production can negatively impact management efforts, since they can allow regrowth in a season following management activities and are often not directly affected by herbicide control (Poovey and Getsinger 2010, Nawrocki et al. 2016). Although some herbicides can suppress turion sprouting, they often do not come into direct contact with quiescent turions due to treatment timing (Steward 1969, MacDonald et al. 1993, Netherland 1997). This necessitates an understanding of the timing and density of turion production of hydrilla for efficacious control, especially with the new hydrilla biotype since its turion dynamics are not yet understood.

The objective of this study was to identify differences in biomass and select phenological events in the new "clade C" hydrilla present in the Connecticut River, (hereafter referred to as Connecticut River hydrilla), compared to monoecious and dioecious hydrilla grown in similar conditions. The capacity for spread and relative growth is currently not understood for this new Connecticut River hydrilla so experiments were performed in two distinct climates. To investigate this, we conducted six-month outdoor mesocosm trials and collected data on biomass production, plant length, axillary and subterranean turion production, and relative growth rate for each of the three biotypes. A basic understanding of the growth and biomass production of the new hydrilla biotype can help inform management strategies as well as provide a basis for comparison in field settings.

Methods

Two distinct research locations (Raleigh, NC and Laurel Springs, NC) were utilized to investigate the differential growth between the three hydrilla biotypes present in the United States under varied climates. The climate in Laurel Springs, NC has been compared to conditions in the Northeast United States, where the Connecticut River hydrilla is currently established

(Henry 2017). Studies were initiated in May of 2022 and repeated in May 2023. Sprouted subterranean turions (monoecious and dioecious) or axillary turions (CT River hydrilla) grown in culture in Raleigh, NC, at North Carolina State University Research Farm #2 (35.810278, -78.721714) were collected and were replanted in 120L mesocosms at Research Farm #2 (2022: n = 16; 2023: n = 24 plants). Additionally, sprouted turions were separately planted (2022: n = 16; 2023: n = 24) at the North Carolina Department of Agriculture & Consumer Service's Upper Mountain Research Station in Laurel Springs, NC (36.39956, -81.30965). This location is at 853 m elevation compared to 96 m of the Raleigh location. Sprouted turions were planted in 6-inch round pots in topsoil amended with Osmocote[1] at 3 g/kg soil and covered with a thin layer of sand to prevent nutrient leaching into the water column. Four pots containing the same biotype were planted in four mesocosms at each location in 2022 and eight mesocosms in 2023.

In year one, destructive harvests were conducted at pre-planting, 30, 60, 90, and 180 days after planting (DAP). In year two, two additional harvests were performed 120 and 150 DAP. At time of harvest, four plants of each biotype were selected and the longest stem length and presence of flowers was recorded. Due to observed differences in turion production in 2022, number of axillary and subterranean turions was recorded in 2023. Plant biomass was clipped at the soil level and belowground biomass was washed. Above and belowground biomass was dried for 72 hours in a forced air dryer at 65 C and dry weight was obtained. Relative growth rate based on total biomass was calculated using the following equation at each time point.

$$RGR = \frac{\ln(Final) - \ln(Initial)}{time\ since\ planting}$$

Aboveground biomass, belowground biomass, and relative growth rate were analyzed over time using nonlinear regression in SigmaPlot 14.0[2] and fit to Gaussian three parameter curves using the following equation, separated by Raleigh and Laurel Springs, NC.

$$f = a \times \exp\left[-0.5\left(\frac{x - x_0^2}{b}\right)\right]$$

Where (a) is the peak value, (x) is the harvest date, (x_0) is the critical date at peak value, (b) is the growth rate, and (f) is hydrilla response. Peak value and date of peak value were derived from this equation and compared between biotypes.

Aboveground biomass, belowground biomass, number of axillary turions, and number of subterranean turions from the 180 DAP harvest, as well as number of axillary turions and subterranean turions, were analyzed using a mixed model two-way ANOVA, with location and biotype as fixed effects, and year included as a random effect. Data was log-transformed to meet assumptions of normality and homogeneity. Significant effects were compared using a Tukey HSD for mean separation using JMP Pro 17.0[3]. Data loggers for temperature were placed in one mesocosm in each location, however, in 2022 the data logger in Raleigh was damaged and no data was recorded. In 2023, the logger in Laurel Springs stopped recording in September 2023. To account for the loss of the water temperature data, air temperature data was retrieved from the North Carolina State Climate Office. A one-way ANOVA was performed with Tukey HSD for mean separation was performed on the air temperature data.

Results and Discussion

Air temperatures were significantly cooler in Laurel Springs, NC than Raleigh, NC, but did not fully simulate the average air temperature for Hartford, CT (11.5 °C) near the Connecticut River hydrilla current infestation (Table 1). Mean air temperature in Raleigh was 5.5 °C warmer than Laurel Springs in 2022 and 5.6 °C warmer in 2023. Temperatures in both locations followed similar trends over time (Figure 1a&b). Due to damaged data loggers, water temperatures could not be compared between locations.

Axillary turion production was first observed in the Connecticut River hydrilla compared to monoecious and dioecious hydrilla, with an axillary turion observed 30 DAP (June) in Laurel Springs. In subsequent harvests, axillary turions were produced in limited quantities until the 120 DAP harvest (September). Axillary turions were not observed on monoecious or dioecious hydrilla until 150 DAP (October).

Flowering also occurred much earlier with Connecticut River hydrilla, male flowers were observed on plants 60 (July) and 90 (August) DAP in Raleigh, but not until 120 DAP (September in Laurel Springs. Monoecious hydrilla did not produce flowers until 120 DAP (September), and 150 DAP (October) for dioecious hydrilla at both locations. Although only male flowers were observed on the Connecticut River hydrilla, there was overlap in timing of flowering between all three biotypes 150 DAP (October), and previous crosses of hydrilla biotypes have resulted in viable seed (Steward 1993). While monoecious hydrilla and Connecticut River hydrilla have not yet been observed in the same waterbody the potential viability of hybrids should be monitored.

Peak aboveground biomass corresponds to the peak value derived by the Gaussian peak equation while timing corresponds to the days after planting this would occur. In Raleigh, there was no significant difference in timing of peak aboveground biomass production or predicted peak value between the three hydrilla biotypes based on the Gaussian peak equation (Figure 2a). However, the predicted peak for dioecious hydrilla aboveground biomass (195 DAP) did not occur during the study period. This is not particularly surprising considering monoecious hydrilla and the Connecticut River hydrilla are observed to senesce in field conditions, whereas, dioecious produces biomass year-round and would be expected to continue accumulating biomass beyond the extent of this study (True-Meadows et al. 2016, Netherland 2017, Tippery 2021, G. Bugbee pers. comm.).

Similar results were observed in Laurel Springs, with the Connecticut River hydrilla peak biomass predicted to occur 144 DAP (Figure 2b). Interestingly, predicted peak biomass did not occur with monoecious or dioecious hydrilla during the study period in Laurel Springs, predicted for 191 DAP and 224 DAP for monoecious and dioecious, respectively. This is surprising considering monoecious and dioecious hydrilla biomass production is typically reduced in cooler climates (Table 1) (McFarland and Barko 1987, Steward and Van 1987). We would have expected a peak in aboveground biomass during the study period, with a decline in biomass occurring towards the end of the growing season as temperatures declined.

Previous mesocosm and field studies have shown monoecious and dioecious hydrilla peak biomass production occurs in July through October (Harlan et al. 1985, Sutton et al. 1992, Madsen and Owens 1998, Shields 2012). This would correspond with our results for monoecious hydrilla which peaked at 167 DAP in Raleigh (October). The lack of significant differences in timing of peak aboveground biomass indicates that the most efficacious timing of management practices for monoecious and dioecious hydrilla can be applied to the new biotype of hydrilla. Although it was not quantified, during this study fragmentation was more prominent in the Connecticut River hydrilla than monoecious or dioecious hydrilla, and further investigation into fragment dynamics, including settling, persistence, and establishment, is needed, as has been investigated with dioecious hydrilla (Madsen and Smith 1991).

Belowground biomass followed a similar trend to aboveground biomass in Raleigh, with no significant differences between peak belowground biomass timing between biotypes (Figure 3a). Interestingly, monoecious biomass did have a significantly smaller peak belowground biomass 0.47 g DW [0.27, 0.66] compared to Connecticut River hydrilla 1.05 g DW [0.72, 1.38] and dioecious hydrilla 0.95 g DW [0.73, 1.18]. This is unexpected considering the prodigious

rate of subterranean turion production in monoecious hydrilla, but may be balanced by a smaller root crown. There were no timing or peak belowground biomass differences between hydrilla biotypes in Laurel Springs (Figure 3b). However, dioecious belowground biomass did not peak during the course of this experiment, with a predicted peak 203 DAP. This again corresponds to the lack of senescence observed in dioecious hydrilla in field settings and is interesting that this difference was observed in the cooler climate of Laurel Springs. This may indicate dioecious hydrilla is more cold tolerant than typically thought, but may not dominate cooler climates monoecious hydrilla currently infests due to a lack of competitive advantage compared to monoecious hydrilla. A previous infestation of dioecious hydrilla in Lake Gaston, NC, was eradicated and only monoecious hydrilla is currently present (Ryan et al. 1995, True-Meadows et al. 2017).

Relative growth rate did not differ in peak or timing of peak growth between hydrilla biotypes in Raleigh or Laurel springs (Figure 4a&b). For all three biotypes, predicted peak relative growth rate was 0.043 - 0.062 g g^{-1} day^{-1} at 81-117 DAP (July-August). Previous investigations into relative growth rates of hydrilla have produced contrasting results. Spencer and Anderson (1986) observed relative growth rates of 0.246 - 0.284 g g^{-1} day^{-1} for monoecious hydrilla and 0.081- 0.264 g g^{-1} day^{-1} for dioecious hydrilla, and Van (1989) observed even greater relative growth rates. Van et al. (1999) observed relative growth rates of 0.266 g g^{-1} day^{-1} for hydrilla in low nutrient conditions and 0.450 g g^{-1} day^{-1} in high nutrient conditions. This is notable, considering that the high and low nutrient conditions tested by Van et al. (1999) were lower than the nutrient amendments used in this study and our relative growth rates were lower than their findings. Puri et al. (2007) observed relative growth rates of 0.001 – 0.014 g g^{-1} day^{-1} in dioecious hydrilla, and Haug et al. (2019) observed relative growth rates of 0.0007 g g^{-1} day^{-1}

for monoecious hydrilla 70 DAP, lower than the findings of this study. Relative growth rate is utilized to measure growth efficiency, and the comparison of the results from this study to previous findings indicates hydrilla growth efficiency can differ greatly between experiments but does not generally differ between biotypes.

Differences in above and belowground biomass production as well as axillary and subterranean turion production were observed between biotypes at 180 DAP. Results from the two-way mixed model analysis revealed no interaction between hydrilla biotype or study location, and no location effect for any of the response variables so only main effects of hydrilla biotype are presented. Monoecious and dioecious hydrilla had similar aboveground biomass production, with average dry weights of 7.71 g and 8.26 g, but were significantly greater than Connecticut River hydrilla (4.41 g) (Figure 5a). However, dioecious hydrilla belowground biomass (1.62 g) was significantly greater than monoecious hydrilla (1.13 g) and Connecticut River hydrilla (0.93 g) (Figure 5b). Previous studies have not found considerable differences in biomass production between monoecious and dioecious hydrilla grown in similar conditions, however, emphasis has been placed on differences in turion ecology due to the complications these propagules can present to management (Spencer and Anderson 1986, McFarland and Barko 1987, Steward and Van 1987, Van 1989). In contrast to the total aboveground biomass, Connecticut River hydrilla produced significantly more axillary turions (73.8) than monoecious hydrilla (32.8) and dioecious hydrilla (1.2) (Figure 5c). However, monoecious hydrilla produced more subterranean turions (16.3) than dioecious hydrilla (2.3) and the Connecticut River hydrilla (3.5) (Figure 5d). Future studies should more clearly distinguish the proportion of total biomass turions comprise compared to above and belowground biomass of the Connecticut River hydrilla.

Previous comparative studies between dioecious and monoecious hydrilla have focused on differences in subterranean turion production due to their typical abundance compared to axillary turions. The findings in this study are corroborated by the 2-7 fold greater axillary and subterranean turion production previously observed in monoecious hydrilla compared to dioecious hydrilla in controlled experiments (Spencer et al. 1987, Steward and Van 1987, Van 1989). Historically, more research emphasis has been placed on hydrilla subterranean turions than axillary turions, however, with the prolific axillary turion production of the new biotype of hydrilla, further investigations into the turion ecology are needed, especially identifying the timing of turion production and turion density in field conditions. This is the first mesocosm experiment investigating growth of the new biotype of hydrilla, so further corroboration concerning growth and phenology will be beneficial to understanding the biology and ecology of this new hydrilla biotype. Improving our understanding of this genetically distinct biotype can help identify proper management strategies to contain the spread of this new invasive macrophyte and determine if management strategies utilized for monoecious and dioecious hydrilla control can be quickly adapted for use on the Connecticut River hydrilla.

The general lack of differences observed in plant response observed between Laurel Springs and Raleigh, particularly with the Connecticut River hydrilla are interesting. This may indicate that while this biotype is currently limited to a cooler Northern climate its growth does not seem to be reduced in a warmer climate, and that it may thrive in warmer waters. Although there is no indication that the Connecticut River hydrilla could outcompete monoecious or dioecious hydrilla, it may be well suited to invade systems where hydrilla is not currently present, similar to is current distribution. Additionally, the prolific turion production of the Connecticut River hydrilla may complicate management. Herbicides are generally ineffective on

quiescent turions, and contact herbicides often do not contact subterranean turions (Van and Haller 1989, Joyce et al. 1992, Netherland 2017). Furthermore, improving our understanding of the timing and density of turion production of Connecticut River hydrilla can greatly aid management strategies as herbicidal management can reduce turion banks in subsequent years (MacDonald et al. 1993, Nawrocki et al. 2016).

This study provides a preliminary understanding of the new hydrilla biotype present in the Connecticut River and identifies the important gaps in our understanding of this new invader that deserve further research. Future studies should increase the scale of mesocosms to provide more realistic conditions, and field studies are needed to corroborate mesocosm findings. Additionally, investigation of herbarium samples may be beneficial to determine if this distinct biotype has been present in the United States longer than currently thought and enhance our understanding of the spread of this new invasive hydrilla biotype.

Sources of Materials

[1] Osmocote Smart Release 15-9-12®, Scotts, 14111 Scottslawn Road, Marysville, OH 43041

[2]SigmaPlot 14.0, Systat Software Inc., 2107 North First Street, Suite 360, San Jose, CA, 95131

[3] JMP Pro (v.17, SAS Institute, Cary, NC 27513)

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TABLES

Table 3-1. Mean (±SE), minimum, and maximum air and water temperatures recorded in Raleigh, NC and Laurel Springs, NC for the duration of the mesocosm study. Air data was retrieved from the North Carolina State Climate Office. Due to loss of data, Tukey HSD was only performed on air data.

2022	Mean	Min	Max	2023	Mean	Min	Max
----------Air----------							
Raleigh	22.2 ± 0.4 *a*	1.3	36.5	Raleigh	21.7 ± 0.4 *a*	-1.9	35.5
Laurel Springs	16.7 ± 0.4 *b*	-2.7	29.3	Laurel Springs	16.1 ± 0.3 *b*	-8.1	28.8
----------Water----------							
Raleigh[a]	-	-	-	Raleigh	22.2 ± 0.3	9.4	31.5
Laurel Springs	19.4 ± 0.4	2.5	31.4	Laurel Springs[b]	20.2 ± 0.3	11.0	29.1

[a]Data logger was flooded and no data was retrieved.
[b]Data logger was damaged and no data after 8/01/23 was recorded.

FIGURES

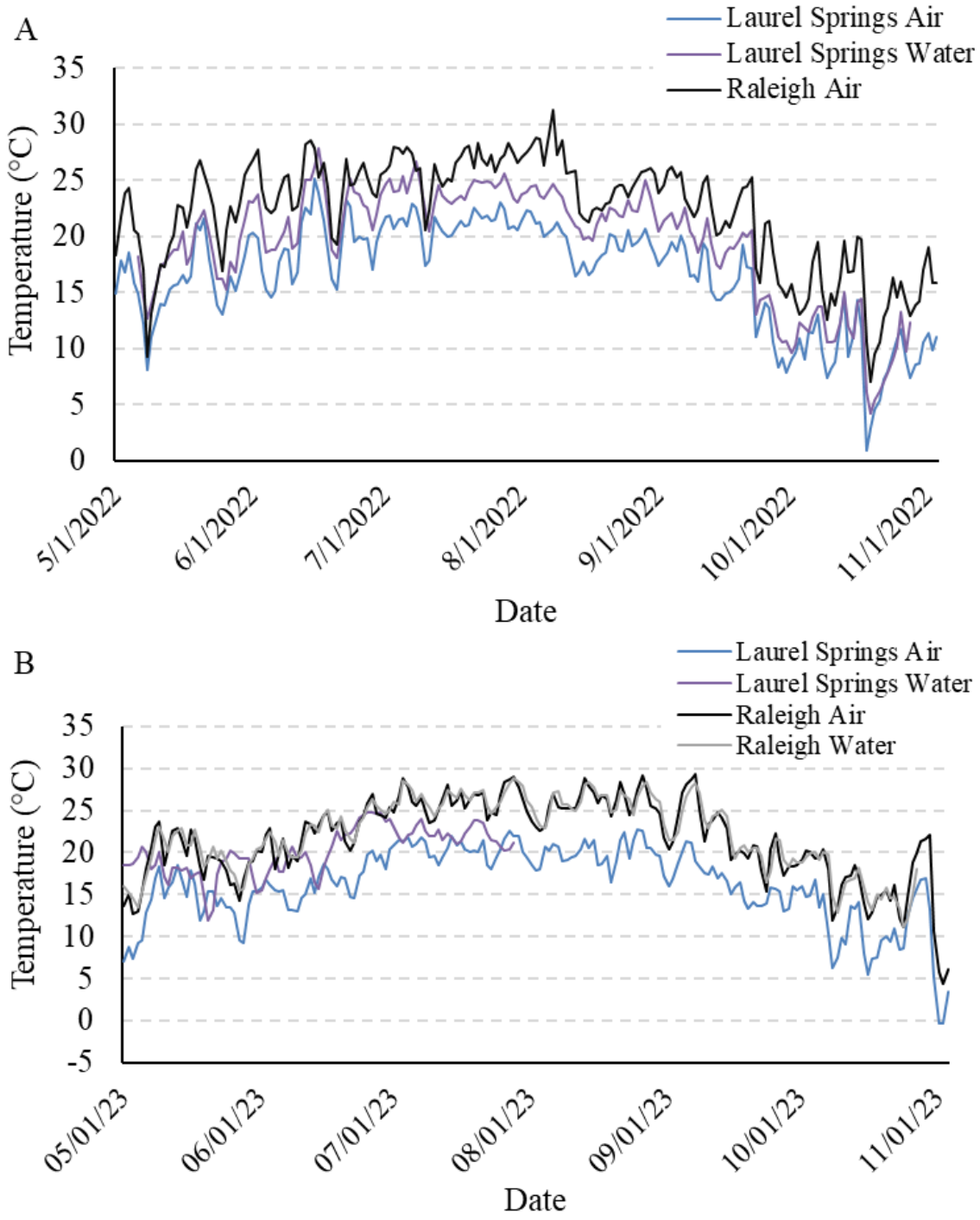

Figure 3-1. Average daily temperatures for (A) 2022 and (B) 2023 during the experiment. Dates correspond to date of planting and approximations of the harvests 30, 60, 90, 120, 150, and 180 days after planting.

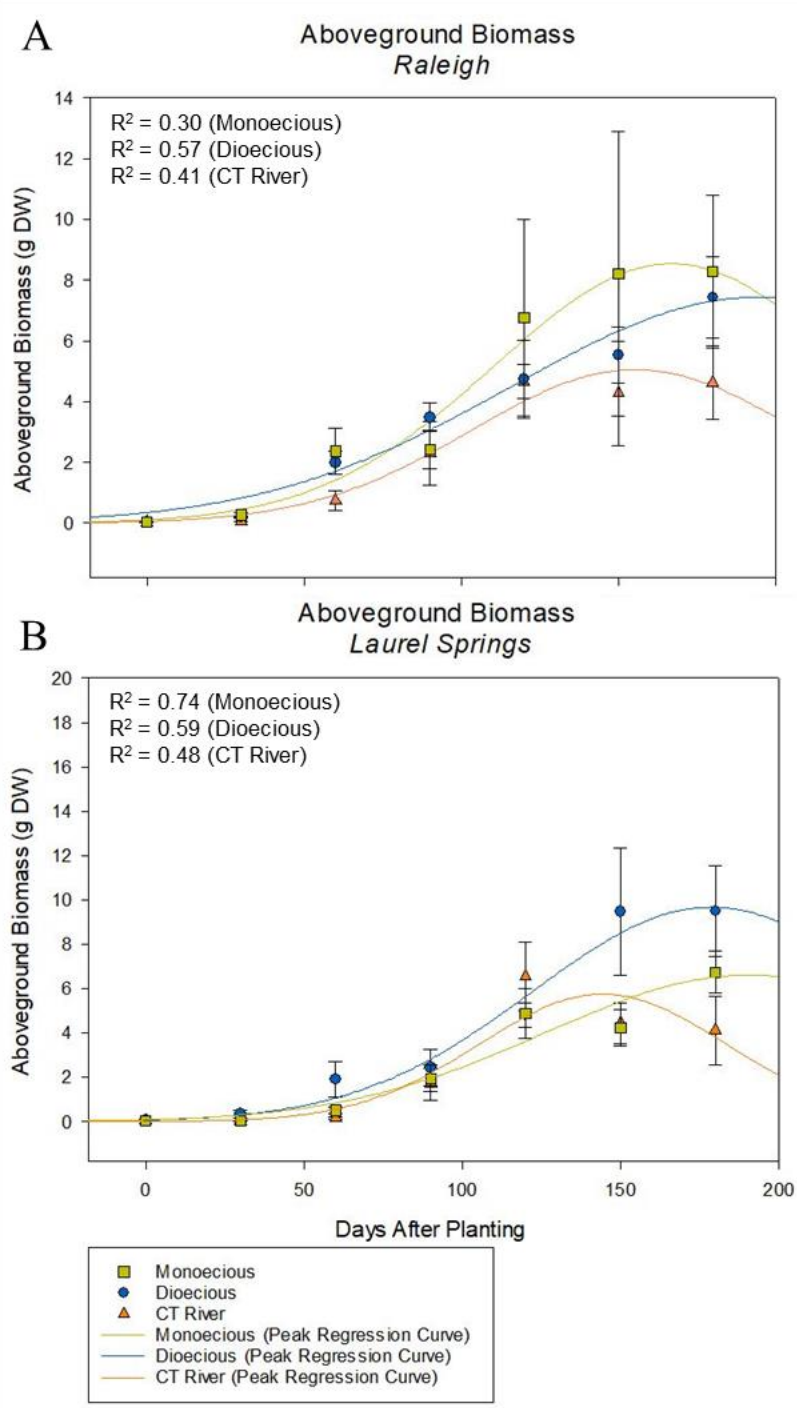

Figure 3-2. Aboveground hydrilla biomass (g DW) over time in (A) Raleigh and (B) Laurel Springs. Points indicate means ± SE. Colored lines are best fit curve for each hydrilla biotype tested. R^2 from non-linear regression analysis is included for each biotype. Results were pooled between years (n=8; except 120 and 150 days after planting where n=4).

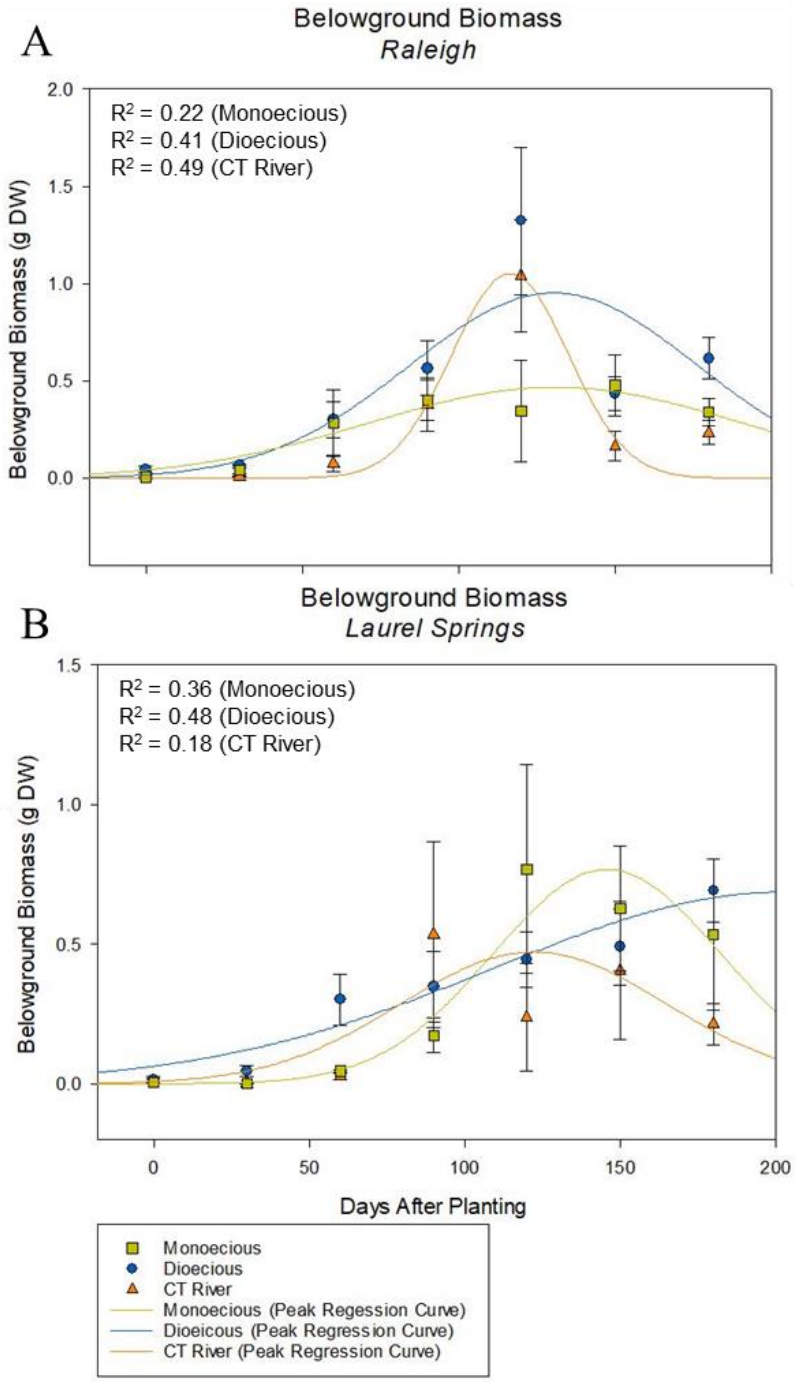

Figure 3-3. Belowground hydrilla biomass (g DW) over time in (A) Raleigh and (B) Laurel Springs. Points indicate means ± SE. Colored lines are best fit curve for each hydrilla biotype tested. R^2 from non-linear regression analysis is included for each biotype. Results were pooled between years (n=8; except 120 and 150 days after planting where n=4).

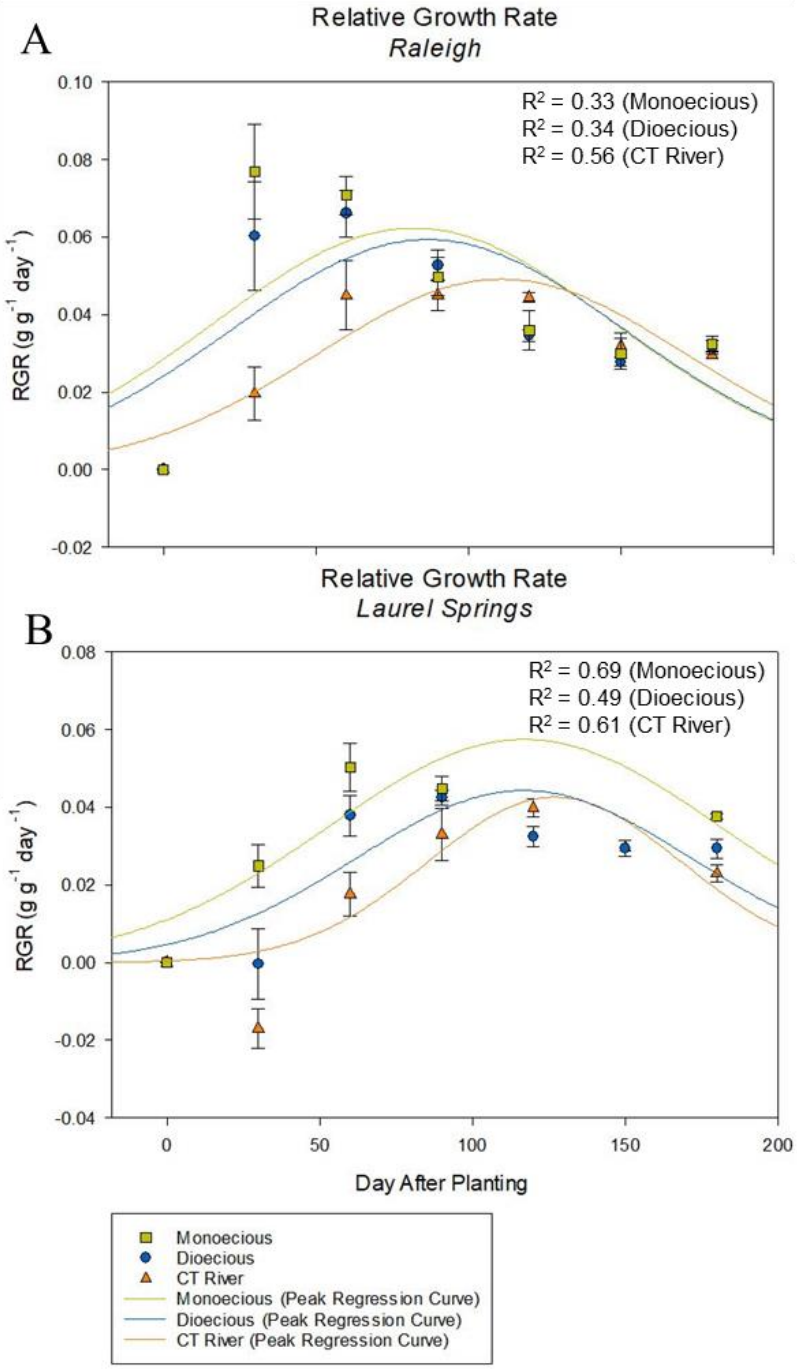

Figure 3-4. Relative growth rate (g g^{-1} day^{-1}) over time in (A) Raleigh and (B) Laurel Springs. Points indicate means ± SE. Colored lines are best fit curve for each hydrilla biotype tested. R^2 from non-linear regression analysis is included for each biotype. Results were pooled between years (n=8; except 120 and 150 days after planting where n=4).

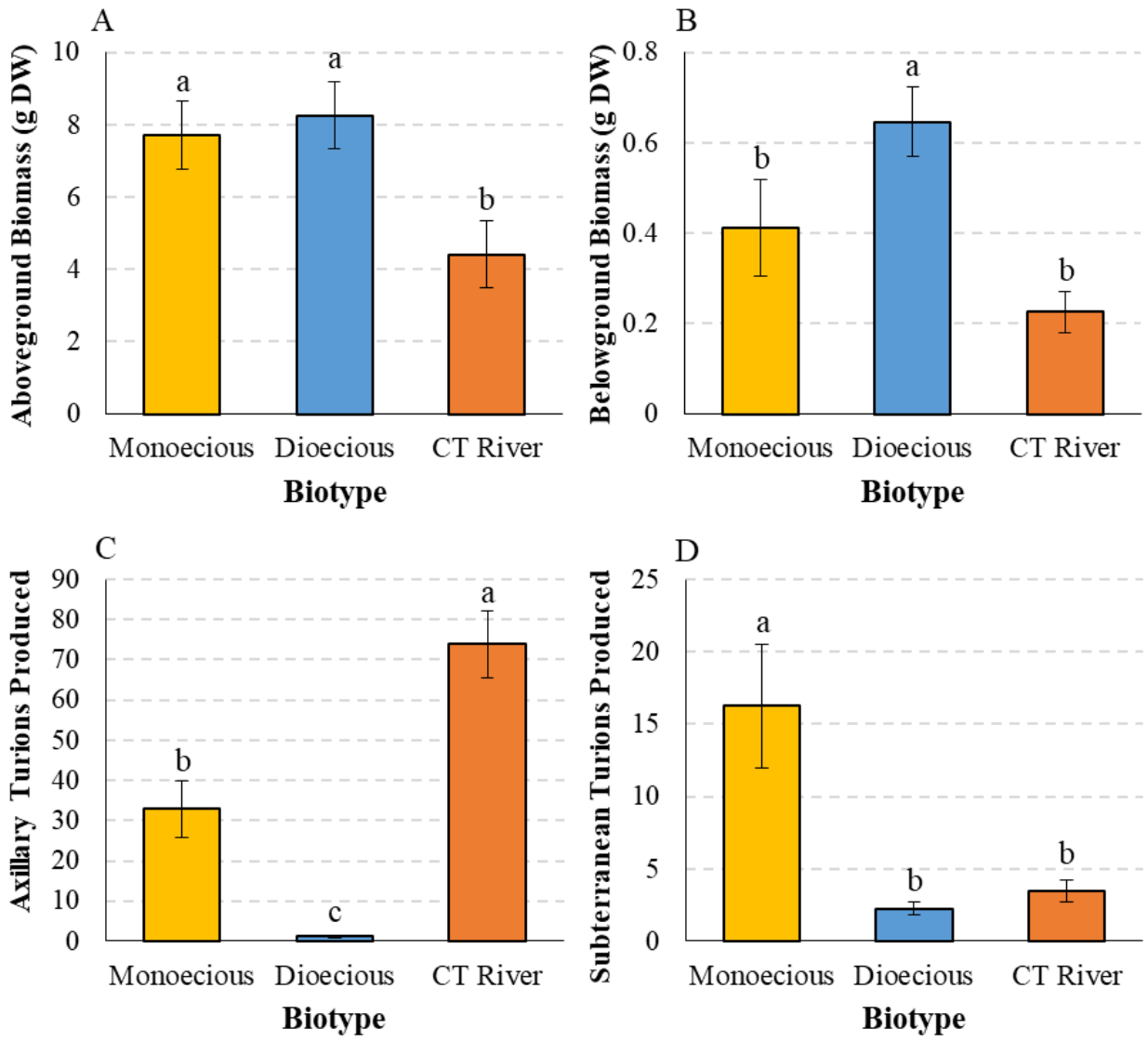

Figure 3-5. Metrics of growth for three hydrilla biotypes 180 days after planting. Mean (±SE): (A) aboveground biomass, (B) belowground biomass, (C) number of axillary turions produced, (D) number of subterranean turions produced. Bars that share a letter are not significantly different (ANOVA, Tukey's HSD, $\alpha = 0.05$). No significant differences were observed between study location or year, therefore, data were pooled (n=16).

CHAPTER 4: Monoecious hydrilla treatments using sequential applications of florpyrauxifen-benzyl, endothall, or fluridone

Abstract

Invasive aquatic macrophytes such as hydrilla (*Hydrilla verticillata*) can have negative impacts in lentic and lotic systems they invade. Hydrilla is a known, persistent issue in many lentic systems across the United States, recently more focus has been placed on the management of invasions in lotic systems. The potential herbicide exposure times are likely to be diminished in lotic environments due to the discharge rates and consistent flow. Previous research has indicated intermittent applications of fluridone, separating single exposures or immediate reapplications with rest periods does not decrease efficacy. We sought to further investigate the efficacy of these intermittent applications with endothall, fluridone, and florpyrauxifen-benzyl utilizing a series of greenhouse mesocosm trials. Three rates and rest periods were compared to sequential or single applications. With all three herbicides tested, no decrease in efficacy was observed with the introduction of rest periods between applications. Injury was apparent in all treatments two weeks after treatment with florpyrauxifen-benzyl and four weeks after treatment with endothall or fluridone. Hydrilla treated with 30 µg L^{-1} florpyrauxifen-benzyl and a 6-day rest period resulted in 90% reduction in aboveground biomass. Endothall treatments of 3 mg L^{-1} with a 16 or 40-hour rest period resulted in similar efficacy to treatments with 4 mg L^{-1}. All treatments with fluridone except for 5 µg L^{-1} with a 12-day rest period resulted in greater than 80% aboveground biomass reduction. Further research into the most efficacious and economical intermittent application is warranted to improve aquatic plant management.

Introduction

Monoecious hydrilla (*Hydrilla verticillata* (L.f Royle)) is an extremely problematic aquatic invasive macrophyte. First documented in the United States in the 1980s, populations rapidly established and spread from Delaware, the Potomac River, and Umstead Lake, NC (Haller 1982, Steward et al. 1984, True-Meadows 2016). Monoecious hydrilla is a herbaceous perennial submersed plant that spreads primarily from fragmentation as well as axillary and subterranean turions. In colder climates, these turions provide monoecious hydrilla with a method for overwintering as the plant senesces during fall months. Monoecious hydrilla produces male and female flowers that bear fruit and may serve as another method of spread as well as genetic variation (Langeland and Smith 1984, Hofstra et al. 2000). Monoecious hydrilla management is more effective following turion sprouting but before biomass has peaked, targeting growing plants (Nawrocki et al. 2016, True-Meadows 2016). Recently, hydrilla invasions in lotic systems have become an increasing concern as they can negatively impact native species, habitat quality, and pose a flood risk (Haller and Sutton 1975, Langeland 1996). Invasions in lotic systems connecting uninvaded reservoirs may also increase the likelihood that hydrilla will establish in previously uninvaded systems.

Concentration exposure time (CET) is pivotal to aquatic plant management and control of invasive aquatic macrophytes. It can provide selectivity of treatment, as well as ensure the control of target weedy species. Increased concentration exposure time improves herbicide efficacy, particularly with herbicides requiring or benefitting from intermediate to long exposure times, such as endothall, fluridone, and florpyrauxifen-benzyl (Netherland et al. 1991, Netherland et al. 1993, Glomski and Netherland 2010, Netherland 2015, Mudge et al. 2015, Netherland 2015, Beets et al. 2019). Standard CET scenarios, in which herbicides are applied a

single time, may not be as applicable for use in lotic systems due to rapid water exchange greatly reducing the potential exposure time. Increasing application rate is often not desirable as this can reduce selectivity and increases costs due to increased volumes of herbicides required. Netherland (2015) observed equivalent efficacy between intermittent and continuous exposures of fluridone in mesocosm experiments. There is lack of knowledge concerning the potential differences in efficacy that intermittent exposures of herbicides have compared to single doses or doses applied sequentially, especially with other aquatic herbicides. This application method of breaking up exposure times may prove beneficial in lentic and lotic environments where longer exposure periods are not feasible due to water discharge.

Florpyrauxifen-benzyl is the newest registered aquatic herbicide, and publications delineating concentration exposure time requirements for monoecious hydrilla are extremely limited. Previous mesocosm research has indicated efficacious dioecious hydrilla control at rates of 24 µg L^{-1} for 24 hours (Beets and Netherland 2018). In 2016, Richardson et al. observed a lowest observable effect concentration (LOEC) for monoecious hydrilla of 3 µg L^{-1} static exposure in a laboratory setting. Few field trials are currently documented with florpyrauxifen-benzyl due to its recent registration. However, Sperry et al. (2021) reported large biovolume reductions of dioecious hydrilla in a small Florida lake with an application of florpyrauxifen-benzyl at 48 µg L^{-1}. Monoecious hydrilla abundance was not reduced when florpyrauxifen-benzyl was applied at the same rate in small plots in a North Carolina reservoir (Sartain et al. 2023). However, dam discharge, rhodamine dye, and herbicide residue analysis revealed rapid water exchange in this system, which likely reduced concentration exposure time below values needed for hydrilla control.

Due to widespread fluridone-resistant biotypes of dioecious hydrilla in Florida, endothall is heavily relied on for management (Albrecht et al. 2004). This should be further evaluated considering that endothall-resistant dioecious biotypes of hydrilla have been recently identified in Florida. Previous research with endothall has found 2.0 ppm for 48 hours or 3-5 ppm for 24 hours provides effective control of dioecious hydrilla (Netherland et al. 1991). Poovey and Getsinger (2010) observed reductions in monoecious and dioecious hydrilla biomass at 1 ppm with at least 96 hours of exposure. Previous research have identified the potential for low-dose metered applications of endothall in rapid flow lotic systems on sago pondweed, but this use pattern has not been widely tested on monoecious hydrilla (Netherland et al. 1998, Sisneros et al. 1998).

Fluridone has been widely adapted for control of monoecious and dioecious hydrilla, although the presence of fluridone-resistant dioecious hydrilla in Florida has necessitated new use patterns for fluridone (Michel et al. 2004, Netherland and Jones 2015, Nawrocki et al. 2016, True-Meadows 2016). Metered drip application of fluridone was effective in suppressing monoecious hydrilla biomass in the Eno River (NC), however hydrilla recovery was observed within one year at the end of the treatment period (Regan 2017). Reapplication of fluridone has also proven effective in control of dioecious hydrilla in the Withlacoochee River (FL) although discharge rates greatly impacted treatment efficacy (Fox et al. 1994). Treatment rates of 12, 24, and 48 $\mu g\ L^{-1}$ have been shown to provide effective control of dioecious hydrilla in growth chambers with 60 to 90 days of exposure (Netherland et al. 1993). While regrowth of dioecious hydrilla was observed with a 30-day recovery period in untreated water, intermittent treatment has yet to be studied for the monoecious biotype (Netherland et al. 1993).

The objective of this study was to determine the potential of intermittent applications of operational rates of florpyrauxifen-benzyl, endothall, and fluridone for efficacious control of monoecious hydrilla biomass. Intermittent exposures were achieved by separating the commonly used exposure periods with different intervals between herbicide reapplication, or "rest periods". Concentration exposure requirements differ between endothall, florpyrauxifen-benzyl, and fluridone, so we tailored reapplication intervals, concentrations, and exposure times to suit each herbicide based on historical data. This study also investigated reapplication intervals shorter than the labeled reapplication period of 14 days for florpyrauxifen-benzyl to investigate the potential that these shorter intervals may have in systems with high water exchange such as lotic systems or lentic systems with periodic high water exchange.

Methods

Three separate experimental trials were performed on monoecious hydrilla in greenhouses at North Carolina State University, Raleigh, NC. Monoecious hydrilla tubers were collected from culture tanks at NCSU, originally sourced from an impoundment in Granville County, NC (36.136993, -78.794979) and sprouted in dechlorinated[1] tap water.

Florpyrauxifen-benzyl

Once the tubers had sprouted and begun to branch, they were placed in 0.09-L pots with topsoil amended with slow-release fertilizer[2] at a rate of 3 g L^{-1} soil and covered with a sand cap to reduce nutrient leaching into the water column. After an establishment period of 3 weeks, a single hydrilla plant was placed in a 1-L glass mesocosm with 900 mL of water. Each mesocosm was considered an experimental unit. Plants were treated with 10, 30, or 50 µg L^{-1} of florpyrauxifen-benzyl[3] for three 24-hour exposure periods. At the end of the 24-hour exposure period, plants were placed in a mesocosm with non-treated water. Plants were given rest periods

of 0, 1, 3, or 6 days between each 24-hour exposure period for a total exposure time of 72 hours. Each application rate and rest period combination had four replications. Herbicide was applied via direct injection to the water column using a serial dilution of florpyrauxifen-benzyl for each 24-hour exposure. Water temperatures averaged 22.1°C and pH 8.0 at time of treatment. A non-treated control was included as a reference in each experimental run.

Mesocosms were placed under 50% shade cloth in a greenhouse to reduce algal proliferation and standardize light and temperature during the trials. During the experiment, observations of plant appearance and visual percent control estimates were taken weekly and compared to non-treated control (0% no control; 100% =no plant biomass remaining). Destructive biomass harvest occurred six weeks after study initiation. Biomass was clipped at the sediment level and aboveground biomass was washed and dried in a forced air dryer for 72 hours at 65°C. Aboveground biomass reduction was calculated by the following equation:

$$\frac{Mean\ Control\ Biomass - Sample\ Biomass}{Mean\ Control\ Biomass} \times 100$$

Values that resulted in negative reduction (sample was larger than mean of the control) were corrected to zero. Biomass reduction data was subjected to a mixed model ANOVA and a Tukey's HSD for mean separations between each treatment in JMP Pro[4]. Biomass data was also subjected to a mixed model ANOVA and Dunnett's test to confirm herbicide treatments resulted in significant biomass reduction compared to the non-treated control. The experimental trial was repeated three times; results from the trials were pooled based on lack of significance when included in a mixed model ANOVA. One replication from the first run was excluded due to poor plant health in this block prior to treatment.

Endothall

Plants were established similar to the florpyrauxifen-benzyl protocol except they were placed in a 0.57-L pot following branching. To account for the larger experimental unit, the establishment period was five weeks to attain sufficient biomass. After the establishment period, a single hydrilla plant was placed in a plastic 15-L mesocosm with 12-L of conditioned water. Plants were treated with 2, 3, or 4 mg L^{-1} of endothall[4] for three 8-hour exposure periods, or a single 24-hour exposure period. At the end of the exposure period, plants were placed in a mesocosm with non-treated water. Plants were given rest periods of 16, 40, or 64 hours between each 8-hour exposure period for a total exposure time of 24 hours. Each application rate and rest period combination had four replications. Herbicide was applied via direct injection to the water column using a serial dilution of for each 8-hour exposure. Water temperatures averaged 22.4°C and pH 7.38 at the time of treatment. A non-treated control was included as reference for each harvest period. During the experiment, observations of plant appearance and visual percent control estimates were taken weekly and compared to non-treated control (0% no control; 100% =no plant biomass remaining) Destructive biomass harvest occurred six weeks after study initiation. Biomass reduction was calculated same as above using the non-treated. Data was analyzed the same as above. This experiment was repeated twice; results from the trials were pooled based on lack of significance when included in a mixed model ANOVA.

Fluridone

Plants were established similar to endothall protocol except they were placed in a 1.8-L pot following branching. After the establishment period, a single hydrilla plant was placed in a plastic 18-L mesocosm with 16-L of water. Plants were treated with 10, 30, or 50 µg L^{-1} of florpyrauxifen-benzyl[3] for three 24-hour exposure periods. At the end of the 24-hour exposure

period, plants were placed in a mesocosm with non-treated water. Plants were given rest periods of 0, 1, 3, or 6 days between each 24-hour exposure period for a total exposure time of 72 hours. Each application rate and rest period combination had four replications. Herbicide was applied via direct injection to the water column using a serial dilution of for each 15-day exposure. Water temperature was 25.3 C and pH 7.6 at time of treatment. A non-treated control was included as reference for each harvest period. During the experiment, observations of plant appearance and visual percent control estimates were taken weekly and compared to non-treated control (0% no control; 100% =no plant biomass remaining). Final harvest occurred six weeks after the final treatment to account for the slow plant response to fluridone (Arnold 1979, Netherland et al. 1993). Biomass reduction was calculated same as above using the non-treated control harvested at the appropriate time point. Data was analyzed the same as above. This experiment was repeated twice; results from the trials were pooled based on lack of significance when included in a mixed model ANOVA.

Results and Discussion

Florpyrauxifen-benzyl

Florpyrauxifen-benzyl applications rapidly resulted in symptoms typically observed in treatments with auxinic herbicides. Visual injury was observed in treated hydrilla plants 24 to 72 hours after treatment at all concentrations. Epinasty was the most common symptom, with fragmentation and shifts in pigmentation at 30 and 50 µg L^{-1}. Minimal visual differences were observed between treatments two and four weeks after treatment (WAT) (Table 1). Some differences were apparent 6 WAT, with highest visual injury in plants treated at 30 µg L^{-1} with a 6-day rest period and 50 µg L^{-1} with a 1 or 3-day rest period. Some treatments were overestimated in visual injury compared to reduction in biomass, whereas injury was

underestimated in other treatments (Figure 1). This is likely due to some heavily injured plants compressing and fragmenting to a high degree, despite biomass remaining in the mesocosm. This discrepancy between visual injury and biomass reduction would probably be lessened if the experiment had continued longer than six weeks. Viable biomass was not differentiated from nonviable biomass due to the subjectivity of this measurement, which highlights the need for further investigation into regrowth potential following treatments from florpyrauxifen-benzyl, especially in plant apical fragments that may serve as a vector for spread of hydrilla invasions (Heidbüchel and Hussner 2019).

All treatments resulted in a significant reduction in aboveground biomass, and mean biomass of the non-treated control increased 4.9 times over the study period compared to pre-harvest. Aboveground biomass reduction of hydrilla was greatest (90%) when hydrilla was exposed to 30 µg L^{-1} with a 6-day rest period (Figure 1). Hydrilla treated at 10 µg L^{-1} with a one- or three-day rest period and hydrilla treated at 30 µg L^{-1} with a three-day rest period resulted in the lowest aboveground biomass reduction. High efficacy of florpyrauxifen-benzyl has been observed in dioecious hydrilla at similar rates with 72 hours of exposure (Beets and Netherland 2018, Mudge et al. 2021). Field trials have further corroborated this with high efficacy in dioecious hydrilla and treatments, as well as providing some selectivity for native desirable species including *Vallisneria sp.* and spatterdock (*Nuphar advena* [Aiton] W. T. Aiton) despite the rapidly declining concentrations of florpyrauxifen-benzyl in treated areas (Sperry et al. 2021).

There is a lack of published data regarding the efficacy of florpyrauxifen-benzyl on monoecious hydrilla. Previous mesocosm data has indicated absorption is lower and EC_{50} values are higher for florpyrauxifen-benzyl in monoecious hydrilla compared to its dioecious

counterpart (Richardson et al. 2016, Haug et al. 2021). Small plot treatments of florpyrauxifen-benzyl on monoecious hydrilla in a high flow reservoir resulted in no reduction in hydrilla abundance despite visual symptomology 72 HAT (Sartain et al. 2023). Residue analysis in Sartain et al. (2023) revealed florpyrauxifen-benzyl rates had dissipated extremely rapidly to 4.2 µg L^{-1} 5 HAT likely tied to dam operation as well as plant absorption and herbicide degradation. The rapid dissipation of florpyrauxifen-benzyl in field studies indicates intermittent applications at rates below the maximum label rate (50 µg L^{-1}) may provide an alternative method for suppression of hydrilla biomass. Our study did not investigate a rest period at the 14-day reapplication interval indicated on the label, so future studies should compare these shorter intervals to the labeled reapplication interval. In rapidly flowing lotic systems, it is possible that most or all water treated with a herbicide application has left the target site and reapplication intervals could be shortened in a special needs situation utilizing a 24(c) label. These short rest periods may also have implications for tidal systems, where treated water may move downstream and backflow.

Endothall

Visual injury was minimal two weeks after treatment, with 40% or greater injury only observed in plants exposed to 4 mg L^{-1} endothall (Table 2). Visual damage had progressed 4 WAT, with all treatments resulting in significant injury. Treatments with 4 mg L^{-1} endothall and the 3 mg L^{-1} endothall 16-hour rest period resulted in the highest visual injury. This trend continued in the 6 WAT rating, with injury similar between plants treated at 4 mg L^{-1} endothall and those treated with 3 mg L^{-1} endothall when a 16 or 40-hour rest period was between intermittent applications.

All plants treated with endothall had reduced biomass compared to the non-treated control. Reduction in aboveground biomass was greater than 80% in monoecious hydrilla exposed to 4 mg L^{-1} endothall as well as plants treated with 3 mg L^{-1} and a 16 or 40-hour rest period between intermittent applications (Figure 2). Hydrilla treated with 2 mg L^{-1} once and with a 16-hour rest period resulted in the lowest observed efficacy. Ortiz et al. (2019) observed accumulation of endothall in monoecious hydrilla greater than treatment solution concentrations and evidence of translocation of endothall to root tissue. Previous studies have also indicated absorption of hydrilla is low when exposed for less than 2-4 days (Haller and Sutton 1973, Ortiz et al. 2022, Van and Conant 1988). However, the results of this study documented intermittent applications of endothall can provide equivalent control when a single 24-hour application is split into three intermittent 8-hour applications, regardless of rest period. Understanding the absorption and physiological dynamics of this can also improve understanding of these management options. There was not a clear relationship between efficacy of endothall concentration and the rest period between intermittent applications. Further studies are warranted to investigate an expanded range of rest periods as well as the efficacy of these treatments on other aquatic macrophyte species. Additionally, the use patterns tested in this study would exceed the 5 ppm per seven day period allowed on endothall labels. Future research should seek to compare these applications to those within label regulations, including decreasing rates of subsequent intermittent applications following initial application.

Fluridone

Visual injury was observed with all treatments except 5 µg L^{-1} with a 12-day rest period 4 weeks after treatment (Table 3). Previous research has also observed some plant response at this time point (Macdonald et al. 1993, Netherland 2015). Differences between treatments decreased

as the study progressed, with minimal observed differences at time of final harvest for each rest period. There were minimal differences in efficacy between treatments, the only treatment that results in less than 80% control was the 5 µg L^{-1} 12-day rest period treatment (Figure 3). This treatment was significantly less efficacious than the 10 µg L^{-1} 3- and 6-day rest period treatments and 15 µg L^{-1} no rest period and 6- and 12-day rest period treatments. Overall, these findings corroborate previous findings that intermittent applications of fluridone does not decrease efficacy (Netherland 2015). The lack of significant differences between most treatments also indicated there is no noticeable increase in efficacy between 5 and 15 µg L^{-1}. This can allow for treatment prescriptions with lower target concentrations, which reduces herbicide costs and can improve selectivity. However, the identification of fluridone resistant dioecious hydrilla supports that caution should be taken when utilizing low doses of fluridone for long-term hydrilla management (Michel et al. 2004, Puri et al. 2009). The utilization of herbicide combinations can help reduce the risk of weed resistance. Further research is warranted into identifying the most efficient combination of herbicide rate, exposure period, and rest period for fluridone applications, considering the large gap between the 15-day exposures tested in this experiment and 3-day exposures tested previously (Netherland 2015). Fluridone can rapidly decrease in concentration depending on discharge values 11-13 days after treatment, emphasizing the need for reapplication for efficacious control, especially in lotic systems (Fox et al. 1994).

Overall, results from these three experiments indicate intermittent applications are a technique that warrants further research, as these small-scale mesocosm trials indicate no loss in efficacy compared to single applications with endothall or repeat applications with florpyrauxifen-benzyl and fluridone. These intermittent applications may provide a particularly

useful management technique for lotic systems where long exposures may not be feasible and an intermittent application approach is more realistic to these field conditions. Additional research in large outdoor mesocosms and field plots to identify the most efficacious intermittent applications is recommended. Additionally, these experiments should be expanded to other herbicides and target and non-target species, as well as herbicide combinations and rotating between herbicides in intermittent applications.

Sources of Materials

[1] API Tap Water Conditioner®, Mars Fishcare North America, 50 E. Hamilton Street, Chalfont, PA 18914

[2] Osmocote Smart Release 15-9-12®, Scotts, 14111 Scottslawn Road, Marysville, OH 43041

[3] ProcellaCOR SC™, SePRO Corporation, Carmel, IN 46032.

[4] JMP Pro (v.17, SAS Institute, Cary, NC 27513)

[5] Aquathol® K, United Phosphorus, Inc., King of Prussia, PA 19406.

[6] Sonar Genesis™, SePRO Corporation, Carmel, IN 46032.

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TABLES

Table 4-1. Visual estimates of hydrilla control following three 24-hour applications of florpyrauxifen-benzyl at 2, 4, and 6 weeks after treatment (WAT). Visual control estimates are expressed as a percentage visual injury (0% = no injury; 100% = complete control; n=11).

Concentration and rest period	2 WAT	4 WAT	6 WAT
Non-treated Control	0.0 ± 0.0 *c*	0.0 ± 0.0 *c*	0.0 ± 0.0 *d*
10 µg L^{-1} No Rest	46.3 ± 6.7 *ab*	65.8 ± 7.7 *ab*	81.9 ± 4.8 *ab*
10 µg L^{-1} 1-day Rest	30.5 ± 5.7 *ab*	46.5 ± 7.7 *ab*	61.0 ± 8.4 *cd*
10 µg L^{-1} 3-day Rest	33.6 ± 7.1 *ab*	49.5 ± 9.7 *ab*	57.5 ± 10.5 *c*
10 µg L^{-1} 6-day Rest	33.6 ± 6.5 *ab*	47.3 ± 6.9 *ab*	59.8 ± 7.8 *cd*
30 µg L^{-1} No Rest	48.8 ± 9.1 *ab*	80.6 ± 6.9 *a*	88.3 ± 6.3 *ab*
30 µg L^{-1} 1-day Rest	54.5 ± 9.8 *ab*	66.4 ± 8.6 *ab*	75.8 ± 7.8 *abc*
30 µg L^{-1} 3-day Rest	45.5 ± 8.9 *ab*	55.0 ± 10.0 *ab*	67.4 ± 8.8 *abc*
30 µg L^{-1} 6-day Rest	48.9 ± 9.9 *ab*	78.7 ± 7.9 *a*	92.6 ± 2.1 *a*
50 µg L^{-1} No Rest	39.6 ± 7.2 *ab*	61.2 ± 9.8 *ab*	69.3 ± 8.9 *abc*
50 µg L^{-1} 1-day Rest	63.9 ± 9.7 *a*	75.0 ± 7.4 *ab*	89.9 ± 2.6 *a*
50 µg L^{-1} 3-day Rest	43.3 ± 8.5 *ab*	77.2 ± 2.9 *a*	89.6 ± 2.3 *a*
50 µg L^{-1} 6-day Rest	38.6 ± 6.2 *ab*	59.5 ± 8.3 *ab*	76.8 ± 6.7 *abc*

Table 4-2. Visual estimates of hydrilla control following a single 24-hour application or three 8-hour applications of endothall. Visual control estimates are expressed as a percentage visual injury (0% = no injury; 100% = complete control; n=8).

Concentration and rest period	2 WAT	4 WAT	6 WAT
Control	0 ± 0.0 d	0.0 ± 0.0 *f*	0.0 ± 0.0 *f*
2 mg L^{-1} Single Exposure	6.3 ± 2.8 *cd*	26.9 ± 5.4 *e*	49.4 ± 6.7 *e*
3 mg L^{-1} Single Exposure	20.6 ± 6.2 *abcd*	50.6 ± 6.7 *cde*	69.4 ± 4.7 *bcde*
4 mg L^{-1} Single Exposure	40.0 ± 8.2 *a*	81.3 ± 3.6 *a*	93.5 ± 2.4 *a*
2 mg L^{-1} 16-hour Rest	11.3 ± 2.8 *bcd*	33.1 ± 5.7 *e*	57.5 ± 5.7 *de*
2 mg L^{-1} 40-hour Rest	10.7 ± 3.0 *bcd*	37.1 ± 7.3 *de*	62.1 ± 5.5 *abcd*
2 mg L^{-1} 64-hour Rest	18.3 ± 3.3 *abcd*	40.8 ± 7.2 *cde*	62.5 ± 8.1 *cde*
3 mg L^{-1} 16-hour Rest	27.5 ± 7.5 *abc*	61.3 ± 8.4 *abcd*	83.8 ± 5.3 *abc*
3 mg L^{-1} 40-hour Rest	23.3 ± 8.9 *abcd*	50.8 ± 10.5 *bcde*	80.8 ± 4.1 *abc*
3 mg L^{-1} 64-hour Rest	17.1 ± 9.2 *abcd*	50.7 ± 8.6 *cde*	71.4 ± 6.8 *bcde*
4 mg L^{-1} 16-hour Rest	40.0 ± 7.8 *a*	80.6 ± 4.1 *a*	92.4 ± 2.4 *a*
4 mg L^{-1} 40-hour Rest	40.0 ± 8.0 *ab*	72.5 ± 6.8 *a*	90.5 ± 5.3 *ab*
4 mg L^{-1} 64-hour Rest	41.3 ± 14.3 *a*	68.1 ± 10.7 *a*	84.8 ± 5.5 *ab*

Table 4-3. Visual estimates of hydrilla control following three 15-day application of fluridone. Visual control estimates are expressed as a percentage visual injury (0% = no injury; 100% = complete control; n=8).

Concentration and rest period	4 WAT	8 WAT	12 WAT	13 WAT	14 WAT	16 WAT
Non-treated Control	0.0 ± 0.0 *d*	0.0 ± 0.0 *c*	0.0 ± 0.0 *d*	0.0 ± 0.0 *d*	0.0 ± 0.0 *c*	0.0 ± 0.0 *c*
5 µg L^{-1} No Rest	27.8 ± 3.8 a*bc*	55.0 ± 3.0 *ab*	81.4 ± 1.4 *a*	-	-	-
5 µg L^{-1} 3-day Rest	33.6 ± 5.0 *abc*	65.0 ± 4.7 *a*	80.7 ± 2.0 *ab*	84.3 ± 2.3 *a*	-	-
5 µg L^{-1} 6-day Rest	38.1 ± 8.8 a*b*	53.3 ± 5.2 *ab*	74.4 ± 4.9 *ab*	77.5 ± 3.5 *ab*	82.5 ± 3.5 *a*	-
5 µg L^{-1} 12-day Rest	13.6 ± 3.4 *cd*	30.0 ± 4.6 *b*	55.0 ± 5.5 *c*	57.9 ± 5.3 *c*	67.9 ± 4.9 *b*	73.6 ± 4.7 *b*
10 µg L^{-1} No Rest	26.3 ± 4.3 *abc*	71.3 ± 3.8 *ab*	87.5 ± 3.2 *a*	-	-	-
10 µg L^{-1} 3-day Rest	52.0 ± 3.7 *a*	73.0 ± 4.1 *a*	83.0 ± 3.4 *ab*	89.0 ± 1.0 *a*	-	-
10 µg L^{-1} 6-day Rest	37.8 ± 6.0 *ab*	59.6 ± 5.3 *ab*	80.0 ± 2.9 *ab*	84.3 ± 1.7 *a*	87.9 ± 2.4 *a*	-
10 µg L^{-1} 12-day Rest	25.6 ± 3.5 *bc*	48.1 ± 3.3 *ab*	64.4 ± 5.6 *bc*	66.3 ± 6.7 *bc*	80.0 ± 4.9 *a*	81.9 ± 3.9 *ab*
15 µg L^{-1} No Rest	34.0 ± 2.4 *ab*	70.0 ± 3.6 *ab*	86.0 ± 1.9 *a*	-	-	-
15 µg L^{-1} 3-day Rest	51.4 ± 2.6 *a*	66.4 ± 3.6 *a*	74.3 ± 4.9 *ab*	84.3 ± 3.7 *a*	-	-
15 µg L^{-1} 6-day Rest	35.0 ± 2.9 *ab*	72.1 ± 2.1 *a*	80.0 ± 2.9 *ab*	87.1 ± 3.4 *a*	90.7 ± 1.7 *a*	-
15 µg L^{-1} 12-day Rest	22.5 ± 2.7 *bc*	45.6 ± 5.1 *ab*	69.4 ± 3.1 *abc*	75.0 ± 3.1 *ab*	81.9 ± 2.1 *a*	86.3 ± 2.1 *a*

FIGURES

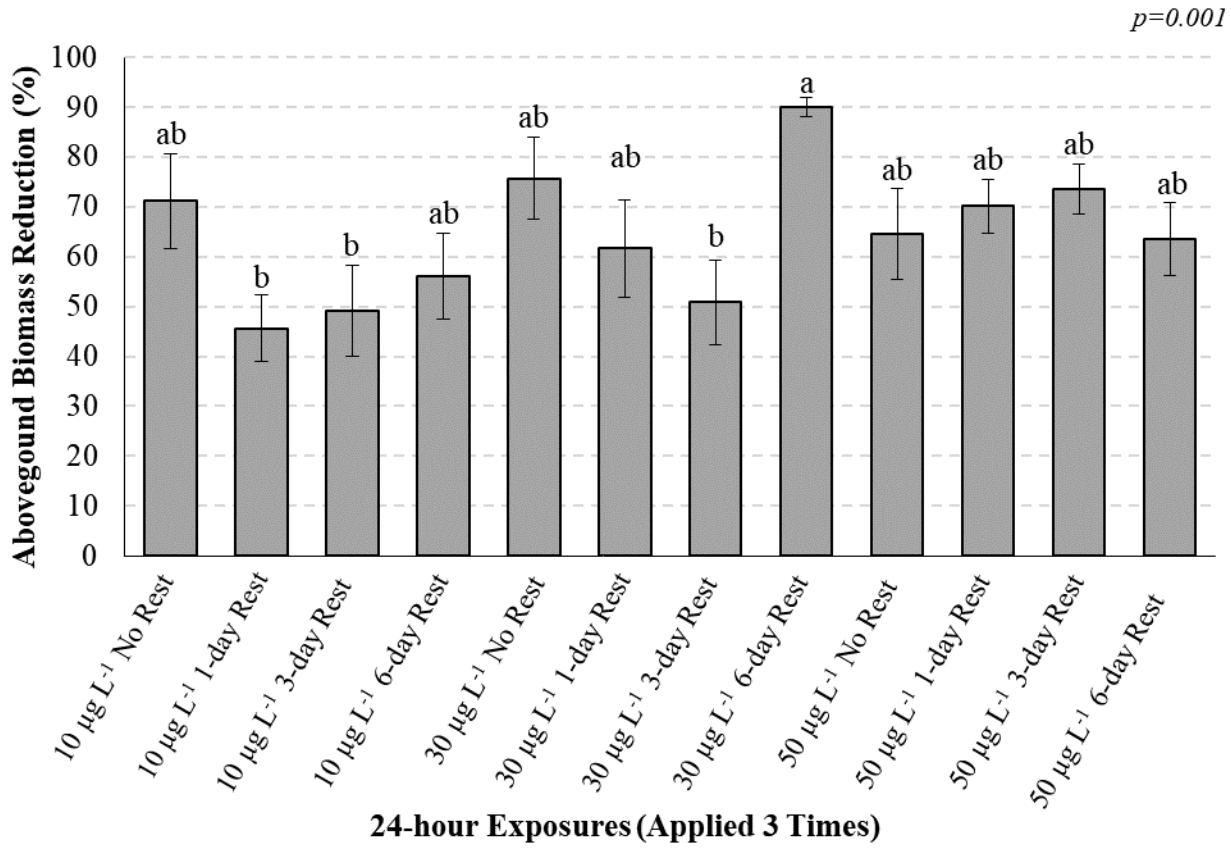

Figure 4-1. Aboveground biomass reduction (mean ± SE) following three 24-hour applications of florpyrauxifen-benzyl with varying times between reapplication (rest periods). Bars that share the same letter are not significantly different (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from three trials were pooled (n=11).

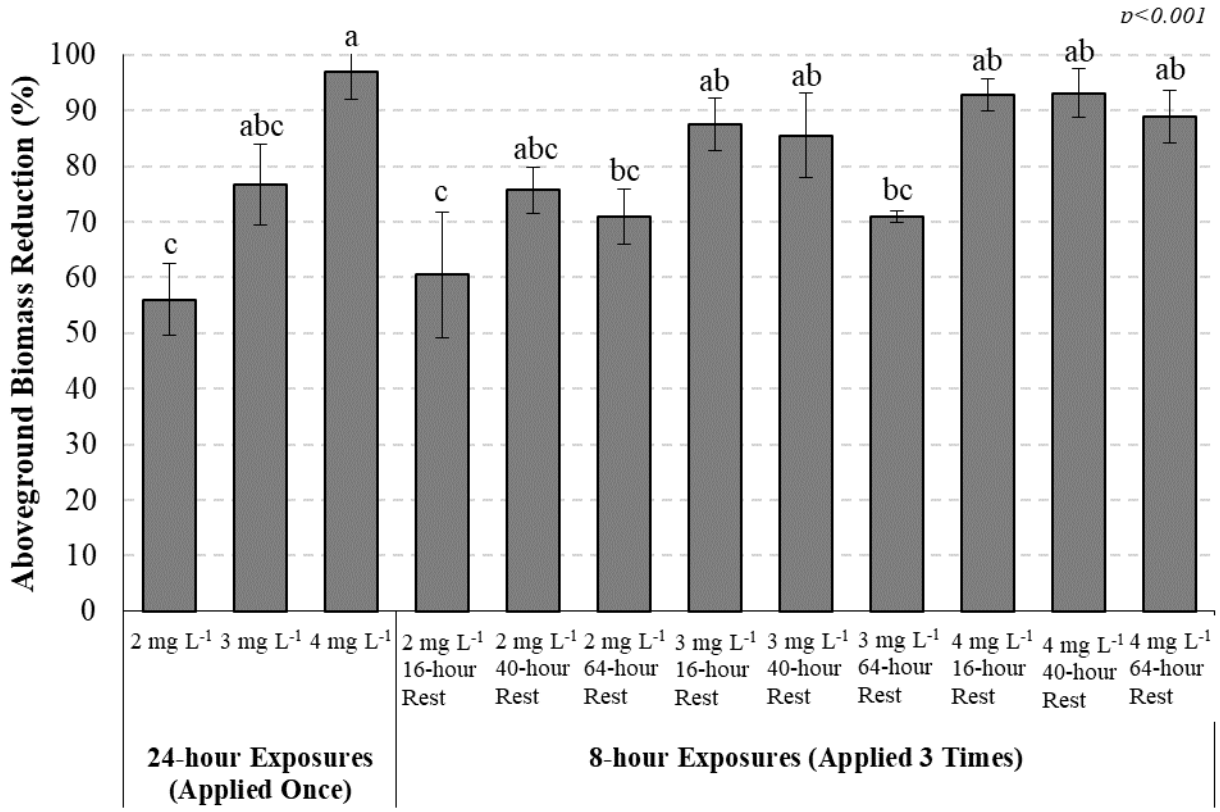

Figure 4-2. Aboveground biomass reduction (mean ± SE) following a single application of endothall with a 24-hour exposure or three 8-hour applications of endothall with varying times between reapplication (rest periods). Bars that share the same letter are not significantly different (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).

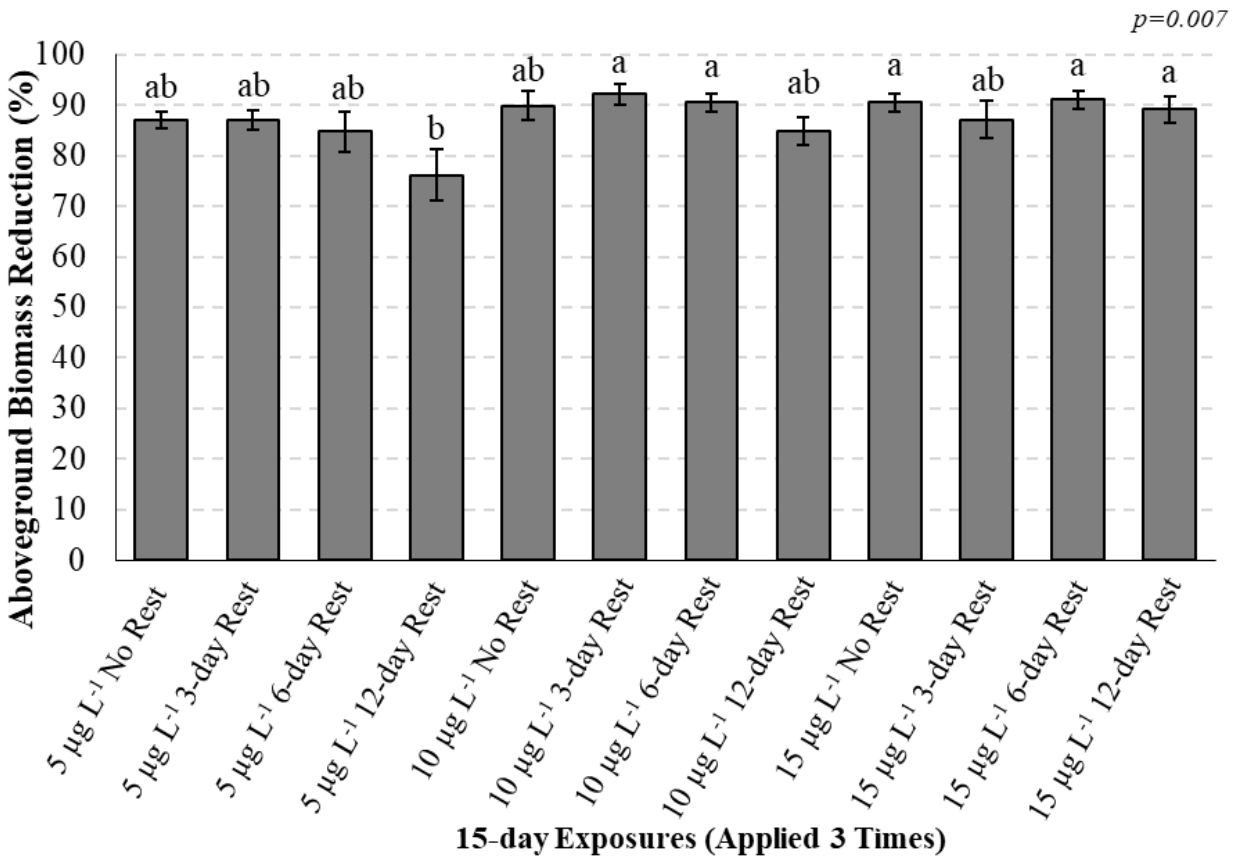

Figure 4-3. Aboveground biomass reduction (mean ± SE) following three 15-day applications of fluridone with varying times between reapplication (rest periods). Bars that share the same letter are not significantly different (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).

CHAPTER 5: Efficacy of herbicide applications on four *Vallisneria* taxa

Abstract

Diverse communities of submersed aquatic vegetation are ecologically beneficial to aquatic ecosystems, however, invasive species can disrupt the ecosystem services that native aquatic plant species often provide. *Vallisneria* is recognized as a desirable species for wildlife resource managers and may outcompete invasive macrophytes such as hydrilla (*Hydrilla verticillata*) in some environmental conditions. Recent documentation of two invasive *Vallisneria* species in the United States has prompted concerns in aquatic systems management. Our primary objective was to provide an initial screening of herbicide treatment regimens used for hydrilla management using two native species (*Vallisneria americana* and *V. neotropicalis*) and two invasive taxa (*V. australis* and hybrid *V. spiralis* × *V. denseserrulata*) in a small-scale mesocosm study. Treatments containing diquat, endothall, florpyrauxifen-benzyl, fluridone, and flumioxazin, or some combination of these herbicides were applied to mesocosms containing one plant of each of these *Vallisneria* taxa and one monoecious hydrilla plant. We observed efficacy on hydrilla with all treatments tested, as well as efficacy in treatments containing endothall or flumioxazin on invasive *Vallisneria* taxa. Treatments with florpyrauxifen-benzyl selectively controlled hydrilla while causing limited damage to both native *Vallisneria* in treatments with florpyrauxifen-benzyl. No selective treatments were definitively identified to control invasive *Vallisneria* taxa while having minimal impact to native species. Additionally, *V. australis* rapidly gained biomass throughout the duration of the experiment having 1.8 – 3x more aboveground biomass 6 weeks after initation and tripling belowground biomass 12 weeks after initation. These insights provide a basis of understanding for differences between these *Vallisneria* for

researchers moving forward with selectively targeting hydrilla in the presence of native *Vallisneria* species and two new aquatic invasive plants.

Introduction

Submersed aquatic vegetation (SAV) are essential communities in most healthy aquatic ecosystems. Diverse native submersed plant assemblages provide a variety of ecosystem services including habitat for fisheries and birds, sediment stabilization, reduce wave action, and improve water quality, among other services (Korschgen et al. 1987, Owens et al. 2008, Moore et al. 2010, Gettys and Haller 2013, Dodd et al. 2021). However, native aquatic plant communities are often sensitive to competition by aquatic invasive species reducing biodiversity and changing habitat quality (Madsen and Sand-Jensen 1991, Smart et al. 1994, Zhang and Boyle 2010). This can reduce the ecosystem services provided by the native species, negatively impact local economies including hydropower, irrigation, and recreation (Langeland 1996, Zhang and Boyle 2010). Herbicide management in conjunction with restoration or revegetation efforts is a common practice to prevent monocultures of aquatic invasive species (Canfield and Hoyer 1992, Gettys and Haller 2013)

These restoration projects in the United States commonly use *V. americana* Michx. (*Vallisneria* or American eelgrass), one species in a genus with global distribution, in restoration efforts. As a herbaceous monocot, *Vallisneria* form rosettes where most of the biomass is located above the hydrosoil (Godfrey and Wooten 1979). *Vallisneria* is dieocious with female flowers in long sessile stalks that are present on the water surface. Male flowers form on short stalks near the basal rosette that detach and float free to the water surface (Les et al. 2008, Martin and Mort 2023). *Vallisneria* also spreads via production of subterranean turions and via stolons of clonal daughter plants. Unlike many other aquatic macrophytes, *Vallisneria* is a meadow-forming plant

with biomass more evenly distributed in the water column (Best et al. 2004, Hauxwell et al. 2007). Many other aquatic macrophytes, especially those that are invasive in the United States such as hydrilla (*Hydrilla verticillata* L.f. Royle), are canopy forming, where biomass is concentrated in the top of the water column (Haller and Sutton 1975).

Vallisneria may also slow or prevent invasions by introduced plant species, especially in low nutrient sediments, where it has been shown to outcompete the aquatic invasive species hydrilla (Van et al. 1999, Owens et al. 2008). Recently, there has been indication that there is divergence between northern and southern populations of *Vallisneria*, with the suggested resurrection of *V. neotropicalis* Vict., previously consolidated with *V. americana*, and the presence of multiple other *Vallisneria* species in the United States (Les et al. 2008, Gorham et al. 2021, Martin and Mort 2023). Previously it was thought that there were only separate ecotypes of *Vallisneria americana,* although differences were observed in phenology, including annual vs perennial habits of overwintering and the lack of turion (overwintering bud) production in southern ecotypes (Godfrey and Wooten 1979, Smart et al. 2005, McFarland and Schafer 2008). The presence of other non-native *Vallisneria* species, cultivars, and hybrids has also been recently documented in the US with the documentation of *V. spiralis* L. × *V. denseserrulata* Makino in the Southeast United States and *V. australis* S.W.L. Jacobs & Les in California (CDFA 2021, Gorham et al. 2021). *V. australis* and *V. neotropicalis* have also been identified as new invasions in Europe and the hybrid *V. spiralis* L. × *V. denseserrulata* has been documented in Japan (Wasekura et al. 2016, Mesterházy et al. 2021). Hybrids between *V. australis* and one of the native species (*V. americana* or *V. neotropicalis* have also been observed being sold in the aquarium trade as *V. americana* (Martin and Mork 2023).

In an effort to combat invasions by other species, managers have been incentivized to identify and cultivate *Vallisneria* populations with growth characteristics favorable for restoration efforts. However, differentiation between cryptic species and hybrids is often difficult with closely related taxa. Hybrids are likely more common than currently recognized and have been observed in several aquatic macrophytes including milfoils, floating hearts, and water chestnut (Tavalire et al. 2012, Parks et al. 2016, Chorak et al. 2019, Harms et al. 2021). Unfortunately, there is the potential that some of these restoration efforts have planted non-native *Vallisneria* species that may become problematic in the waterbodies where that have been introduced. Multiple *Vallisneria* species have been documented to coexist in waterbodies, so selective management actions would be desirable to suppress invasive populations while mitigating adverse effects to native populations (Gorham et al. 2021).

Chemical control is a commonly used tool for selective management of aquatic invasive species. *Vallisneria* has shown sensitivity to fluridone at rates above 20 µg L^{-1} and endothall at rates of 0.5 mg L^{-1}, however, field studies indicate recovery from subterranean propagules can occur at these rates (Netherland et al. 1997, Skogerboe and Getsinger 2002, Mudge 2013). Mudge (2013) also indicated some suppression of *Vallisneria americana* when treated with diquat and high sensitivity to combinations of endothall and flumioxazin. *Vallisneria* has shown limited sensitivity to florpyrauxifen-benzyl although this may be dependent on plant age and size (Beets et al. 2019, Dodd et al. 2022).

Previous studies have largely focused on selectivity for what were thought to be one or two ecotypes of *V. americana*, but there is a lack knowledge concerning differences in herbicide response between multiple *Vallisneria* taxa (Skogerboe and Getsinger 2002, Mudge 2013, Netherland and Glomski 2014, Beets et al. 2019). Additionally, these studies only differentiated

northern and southern V*allisneria*, or narrowleaf and broadleaf, assuming these plants to be *V. americana*, which is likely no longer a valid assumption in future studies without genetic confirmation. Our objective was to provide an improved understanding of the response of three different *Vallisneria* species and a hybrid *Vallisneria* to a variety of concentration exposure time (CET) conditions. This would be valuable to aquatic plant management for both selective control of invasive aquatic weed species as well as future restoration efforts. This investigation was completed using mesocosms containing one plant of each *Vallisneria* taxa, as well as, monoecious hydrilla to partially mimic plants growing together in the same environment, a likely condition in the field. Additionally, we sought to gain a basic understanding of differences in the biomass production of these different *Vallisneria* taxa.

Methods

Experiments were initiated in June and July 2023 in mesocosms at North Carolina State Weed Control Lab greenhouses in Raleigh, NC, to determine sensitivity of *V. americana*, *V. neotropicalis*, *V. australis*, the hybrid *V. spiralis* × *V. denseserrulata*, and monoecious hydrilla. All plants had been kept in culture at North Carolina State University Research Farm #2 (35.810278, -78.721714). Monoecious hydrilla was originally sourced from an impoundment in Granville County, NC (36.136993, -78.794979), *V. americana* from the Connecticut River (41.48333, -72.50656), *V. neotropicalis* from Lake Gaston, NC (36.49792, -77.84371), *V. australis* from Lake Mattamuskeet, NC (35.52013, -76.1088), and *V. spiralis* × *V. denseserrulata* from Wheeler Lake, AL (34.62456, -86.98359). Genetic analyses was performed by Dr. Ryan Thum using ITS sequencing (Les et al. 2008). Single daughter plants of each *Vallisneria* population were planted in 0.52-L pots containing topsoil with slow-release fertilizer[1] at a rate of 3 g L^{-1} soil and covered with a sand cap to reduce nutrient leaching into the water

column. A single sprouted apical stem of hydrilla was planted in a 0.09-L cup with the same amended topsoil. Plants were allowed to establish for four weeks prior to study initiation to allow for recovery following planting.

Following this recovery period one pot of each plant was placed in a 16-L mesocosm containing 12-L of conditioned water[2]. Each mesocosm was considered an experimental unit and was treated with one of the eleven treatments containing diquat[3], endothall[4], florpyrauxifen-benzyl[5], flumioxazin[6], fluridone[7], or a combination of these herbicides (Table 1). These treatments were selected from mesocosm experiments on monoecious and dioecious hydrilla for use in lentic and lotic systems. Four replicates of each plant were harvested for pre-treatment biomass comparison and four mesocosms were left non-treated as experimental controls, with separate controls for plants treated with fluridone to account for the extended study period. Each treatment had four replicate mesocosms, and the experiment was repeated, separated in space and time. Treatments were conducted via injecting the appropriate herbicide in the water column from a serial dilution of herbicide. Following exposure, treated plants were placed in a new mesocosm containing non-treated, conditioned water at the end of their requisite exposure time. Each experimental trial was established using a randomized complete block design. Mesocosms were covered with 50% shade cloth to reduce plant bleaching, minimize algal buildup, and regulate water temperature during the trial period. Mean water temperature was 26.7 C and pH 8.1 at time of treatment. Mesocosms were maintained at a consistent water level via addition of conditioned freshwater.

Six weeks after treatment, a complete destructive harvest was performed of above and belowground biomass. Fluridone treatments were harvested six weeks after the end of their exposure period due to the long exposure requirements of fluridone. Plant dry weights were

obtained after plant biomass was placed in a forced air dryer for 72 hours at 65 C. To standardize data between trials and account for the extended exposure of plants treated with fluridone, biomass reduction was obtained using the following equation:

$$\frac{\boldsymbol{Mean\ Control\ Biomass - Sample\ Biomass}}{\boldsymbol{Mean\ Control\ Biomass}} \times \mathbf{100}$$

Mean control biomass differed for each harvest time point (6, 12, or 14 weeks after treatment). Trial and block factors were determined to not be significant when included in separate mixed model ANOVAs for each plant, so results between trials were combined. Mean separation to determine differences between significantly different treatments was performed using a Tukey honest significant difference (HSD) in JMP Pro 17[8].

Biomass yield of the non-treated controls at each harvest time was compared and subjected to a mixed ANOVA comparing biomass yield differences between plants at each harvest using a Tukey HSD for mean separation. Biomass yield was calculating by subtracting the mean of the pretreatment biomass from the mean of the biomass of the non-treated control harvested at each time period (6 weeks after intitaion (WAI), 12 WAI, 14 WAI).

Results and Discussion

Hydrilla

Rapid symptomology was observed in hydrilla treated with all herbicides except fluridone and the 12-hour exposure of endothall. Six weeks after treatment, hydrilla aboveground biomass was greatly reduced in all treatments except for the 12-hour exposure of endothall (Figure 1). This treatment only resulted in a 7% reduction in aboveground biomass, although belowground biomass was reduced 83% compared to the non-treated controls. The only treatments that did not result in complete control of belowground biomass were the fluridone 45-day exposure,

endothall 2 mg L^{-1} 12 hour exposure, and 3 mg L^{-1} 24 hour exposure (Figure 1). No significant differences between treatments were observed on hydrilla belowground biomass. This is promising for long-term hydrilla control as reductions in belowground biomass can be indicative of low regrowth potential via root crown or subterranean turions. The high efficacy of the intermittent applications of endothall, florpyrauxifen-benzyl, and fluridone also further validates preliminary studies for this treatment method. Hydrilla plants were small at time of treatment, however, rapid biomass accumulation was observed throughout the course of the experiment in the non-treated controls. Monoecious hydrilla overwinters as subterranean turions, so early season management before biomass has peaked is often desirable and has proven highly efficacious (Langeland and Pesacreta 1986, Nawrocki et al. 2016).

Native Vallisneria species

Comparisons of efficacy to historical datasets was complicated due to the likely geographic split between *V. americana* in northern latitudes and *V. neotropicalis* in southern latitudes (Gorham et al. 2021, Martin and Mort 2023). *V. americana* was highly sensitive to treatments with endothall, and the combination of flumioxazin and florpyrauxifen-benzyl, with 98-100% reduction in aboveground biomass with these treatments (Figure 2). Moderate to high efficacy was also observed with diquat and fluridone treatments (63-79%) although treatment efficacy was more variable. Reductions in belowground biomass were also high in these treatments although turions were present in otherwise efficacious treatments. Notably, treatments with endothall for 24 hours at 3 mg L^{-1} resulted in 100% reduction in aboveground biomass but belowground biomass was present in the form of turions (Figure 2). Treatments with florpyrauxifen-benzyl resulted in growth of *V. americana* compared to the non-treated controls.

This is likely explained by the reduction of hydrilla biomass with applications of florpyrauxifen-benzyl and *V. americana* utilizing this space due to a lack of sensitivity to this herbicide.

Herbicide efficacy on the other native *Vallisneria* (*V. neotropicalis*) was similar to the results for *V. americana*. Treatments with endothall resulted in 70 - 100% reduction in aboveground biomass and similar trends in belowground biomass (Figure 3). Interestingly, treatments with diquat alone, florpyrauxifen benzyl alone, and fluridone with a 45-day exposure resulted in lower efficacy than other treatments indicating potential for selective control of hydrilla in the presence of *V. americana* with these treatments. Intermittent applications with fluridone resulted in some suppression of aboveground biomass and reductions in belowground biomass did not differ from other treatments (Figure 3).

Native *Vallisneria* of various previously classified ecotypes has shown sensitivity to endothall at rates of 0.5 mg $^{L-1}$ and higher, validating the findings of this study (Skogerboe and Getsinger 2001, Skogerboe and Getsinger 2002, Mudge 2013). These previous studies have also indicated recovery from viable root crowns and turions, so treatment timing may play an important role in recovery, and further investigation into direct effects on *Vallisneria* turions is warranted to improve strategies for effective selective management of monoecious hydrilla growing within mixed communities with these native species. Similarly, Turnage and Madsen (2015) saw expansion of *Vallisneria* in Minnesota lakes following diquat applications, and Mudge (2013) observed minimal biomass reduction. Previous studies have indicated low efficacy of flumioxazin when applied alone and in combination with endothall or diquat on *Vallisneria* (Mudge and Haller 2010, Mudge 2013). However, combinations with flumioxazin have not been fully investigated at higher rates, especially in combination with florpyrauxifen-benzyl. Our findings of minimal impact on native *Vallisneria* with florpyrauxifen-benzyl are

corroborated with previous mesocosm and field studies (Beets et al. 2019, Sperry et al. 2021, Dodd et al. 2022). *Vallisneria* sensitivity to fluridone has been variable in previous mesocosm and field studies, which may be partially explained by differentiation between *V. americana* and *V. neotropicalis* as seen in this study (Netherland et al. 1997, Nelson et al. 1998, Getsinger et al. 2001, Getsinger et al. 2002, Poovey et al. 2004). With the resurrection of the distinction between *V. americana* and *V. neotropicalis* future studies should take care to confirm which species are being utilized, and new evaluations are likely warranted to properly correlate selectivity with the species present. This study presents some evidence that previous differences in efficacy between *V. americana* population may in fact have been differences in response between these two species, especially when comparing Northern to Southern populations in the US.

Invasive Vallisneria

Herbicide treatments of the invasive hybrid *Vallisneria* and *V. australis* showed some promise for efficacy in this small-scale mesocosm study. Endothall, endothall combined with florpyrauxifen-benzyl or diquat, and the combination of florpyrauxifen-benzyl with flumioxazin resulted in 96-100% reduction in aboveground biomass of the invasive hybrid *V. spiralis* × *V. denseserrulata* (Figure 4). However, no treatment resulted in 100% reduction of belowground biomass, indicating the potential for regrowth in field settings or long-term experimental trials. Minimal biomass reduction was observed in treatments with florpyrauxifen-benzyl, and growth was observed in the 45-day exposure of fluridone (Figure 4). Treatments with diquat alone and the intermittent application of fluridone were moderately effective, resulting in 60 - 63% aboveground biomass reduction. The increased efficacy observed with intermittent applications of fluridone is another indication this management technique may provide increased control of target species. There is a current lack of knowledge about management strategies for this newly

documented invasive hybrid species, and these results can provide an initial understanding of potential efficacious management actions.

Invasive *V. australis* was the only taxon tested that 100% control of aboveground or belowground biomass was not achieved with any treatment (Figure 5). However, treatments with endothall at 3 mg L^{-1} or 2 mg L^{-1} combined with florpyrauxifen-benzyl, and florpyrauxifen-benzyl combined with flumioxazin resulted in 94-98% reduction in aboveground biomass, with similar reductions in belowground biomass. Treatments with florpyrauxifen-benzyl and fluridone resulted in less than 20% reduction in aboveground biomass, and treatments with florpyrauxifen-benzyl resulted in a slight increase in belowground biomass (Figure 5). Chlorosis, a common symptom of fluridone applications was observed in *V. australis* leaves treated with fluridone, however this symptom did not persist, indicating uptake was likely occurring but the plants were recovering.

Our findings with the efficacy of endothall applications are corroborated by previous field and mesocosm studies have indicated that *V. australis* is sensitive to, but not completely controlled by similar concentrations and exposure times (Clements et al. 2018, Dugdale et al. 2019, Dugdale et al. 2022). Dugdale et al. (2019) also observed improved endothall efficacy on *Vallisneria australis* in flowing mesocosms compared to quiescent mesocosms. There is a general lack of knowledge concerning the efficacy of other herbicides on *V. australis* to corroborate our findings. However, our findings with *V. australis*, especially florpyrauxifen-benzyl, flumioxazin, and fluridone are similar to the results we observed with other species. Further investigation into the effects of water flow on herbicide efficacy can greatly improve management strategies, especially in lotic systems such as the San Joaquin River Delta (state acronym) where invasions of *Vallisneria australis* have been noted.

Vallisneria biomass comparison

There were noticeable differences in biomass yield between the non-treated controls of *Vallisneria* by 6 WAI that persisted in the following harvest. *V. australis* had the consistently highest biomass yield in non-treated controls at all three harvest time points (Figure 6a & b). Six weeks after planting, *V. australis* had 1.8 - 3x more biomass yield than other taxa. However, at the 12- and 14-week after planting harvests hydrilla aboveground biomass was similar to *V. australis* (Figure 6a). *V. americana* had considerably lower biomass than other taxa 12 and 14 WAI, although these differences were not significantly different. *V. neotropicalis* and the hybrid *Vallisneria* had comparable aboveground biomass throughout the course of the study. There were fewer differences observed in the belowground biomass, however, six weeks after planting native *V. americana* had 8.2x, and *V. australis* had 6.3x the belowground biomass yield compared to hydrilla (Figure 6b). Twelve weeks after planting, *V. australis* belowground biomass had nearly tripled, interestingly, due to slower growth the below-ground biomass of the hybrid *Vallisneria* was significantly lower than that of *V. australis*. This trend continued fourteen weeks after planting (Figure 6b). These changes in biomass may not be indicative of growth in field conditions due to the plant size at time of planting and environmental factors that influence growth that are more controlled in mesocosm experiments. However, this initial documentation in a mesocosm setting can provide a basis of understanding for future mesocosm research and indicates a growth rate in *V. australis* that may allow it to rapidly outcompete native *Vallisneria* populations. Further investigation into the growth and phenological patterns of these invasive and newly split native species is needed to inform management decisions.

Female flowers were observed in all *Vallisneria* taxa included in this study. However, no male flowers were observed in the hybrid *V. spiralis* × *V. denseserrulata* or *V. australis*. Male

flowers have not been observed in the hybrid *Vallisneria* in other studies, indicating propagation may only be occurring via asexual reproduction (Wasekura et al. 2016, Gorham et al. 2021). Considerable genetic plasticity has been observed in *V. australis* and its documented hybridization with native *Vallisneria* indicates these female flowers are not sterile, or male plants are also present in the United States (Martin and Mort 2023). It may be worth further investigating the sexual reproduction potential of these new invasive taxa, considering the issues that have arisen with hybridization between native and invasive species in the past (Tavalire et al. 2012, Parks et al. 2016, Chorak et al. 2019, Harms et al. 2021).

In conclusion, these small-scale mesocosm experiments provide an initial understanding of herbicide response in two new invasive aquatic species, as well as, two native species that were previously combined. Although no herbicide treatments definitively provided selectivity between invasive and native *Vallisneria* taxa, we were able to selectively manage monoecious hydrilla with minimal damage to native *Vallisneria* with several well-established treatments and further document the efficacy of intermittent applications. Future research should scale up the size of experimental design (i.e. larger mesocosms or small field plots) to provide a more operational understanding of selective management practices. Given the observed resilience of *V. australis* and the hybrid *V. spiralis* × *V. denseserrulata*, rapid endeavours should be made to improve understanding of efficacious management options as well as phenological and growth patterns to improve timing of management efforts.

Sources of Materials

[1] Osmocote Smart Release 15-9-12®, Scotts, 14111 Scottslawn Road, Marysville, OH 43041

[2] API Tap Water Conditioner®, Mars Fishcare North America, 50 E. Hamilton Street, Chalfont, PA 18914

[3] Reward®, Syngenta Crop Protection LLC, Greensboro, NC 27419.

[4] Aquathol® K, United Phosphorus, Inc., King of Prussia, PA 19406.

[5] ProcellaCOR SC™, SePRO Corporation, Carmel, IN 46032.

[6]Clipper™, Valent U.S.A. Corporation, Walnut Creek CA 59639.

[7] Sonar Genesis™, SePRO Corporation, Carmel, IN 46032.

[8] JMP Pro (v.17, SAS Institute, Cary, NC 27513)

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TABLES

Table 5-1. List of herbicides, concentrations, and exposure times tested in this experiment.

Herbicide	Concentration	Exposure
Diquat	0.37 mg L^{-1}	12 hours
Endothall	2 mg L^{-1}	12 hours
Endothall	3 mg L^{-1}	24 hours
Endothall	3 mg L^{-1}	8 hours / 40 hour rest[a]
Florpyrauxifen-benzyl	30 µg L^{-1}	72 hours
Florpyrauxifen-benzyl	30 µg L^{-1}	24 hours / 6 day rest[a]
Fluridone	10 µg L^{-1}	45 days
Fluridone	10 µg L^{-1}	15 days / 6 day rest[a]
Endothall + Diquat	2 mg L^{-1} + 0.37 mg L^{-1}	12 hours
Endothall + Florpyrauxifen-benzyl	2 mg L^{-1} + 30 µg L^{-1}	48 hours
Florpyrauxifen-benzyl + Flumioxazin	30 µg L^{-1} + 300 µg L^{-1}	48 hours

[a] Three applications of the corresponding herbicide were applied to these treatments separated by the rest period

FIGURES

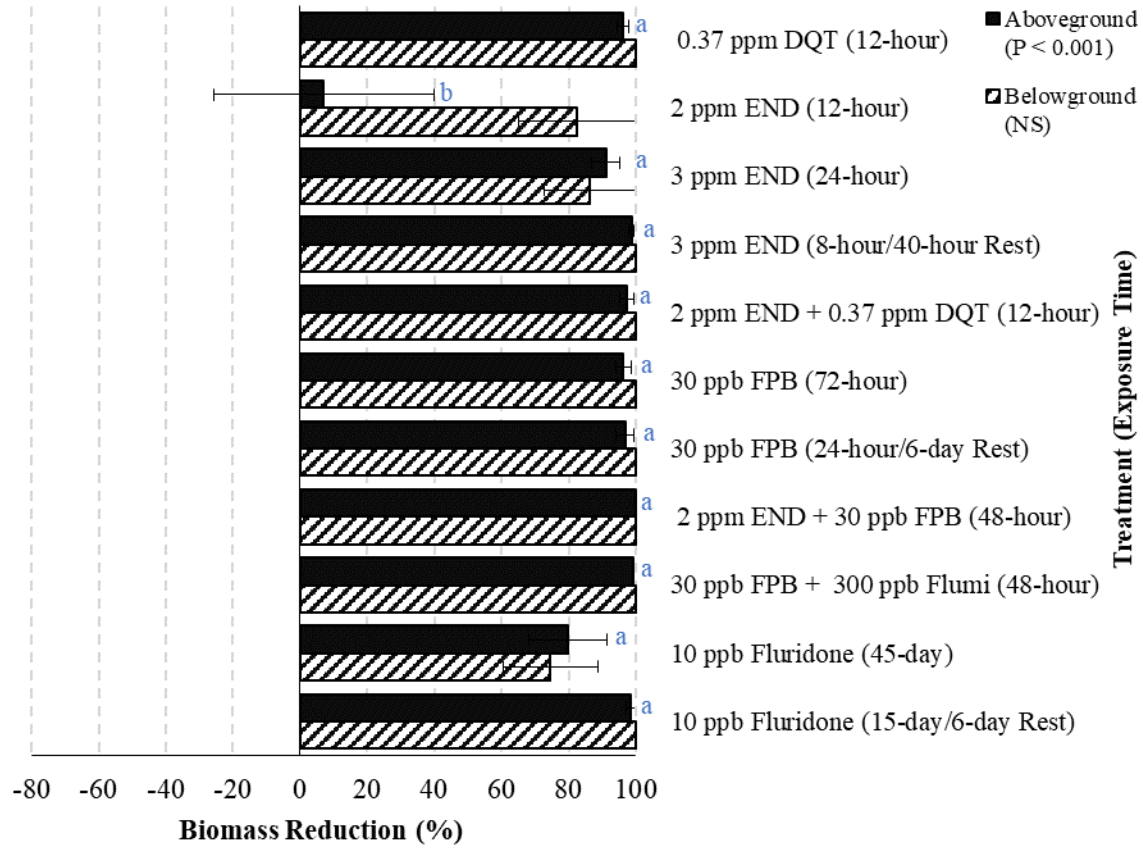

Figure 5-1. Mean (±SE) hydrilla biomass reduction at time of harvest. Herbicides included diquat (DQT), endothall (END), florpyrauxifen-benzyl (FPB), flumioxazin (flumi), and fluridone. Black bars indicate aboveground biomass and bars with diagonal stripes indicate belowground biomass. Treatments that share the same letter are not different (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n = 8).

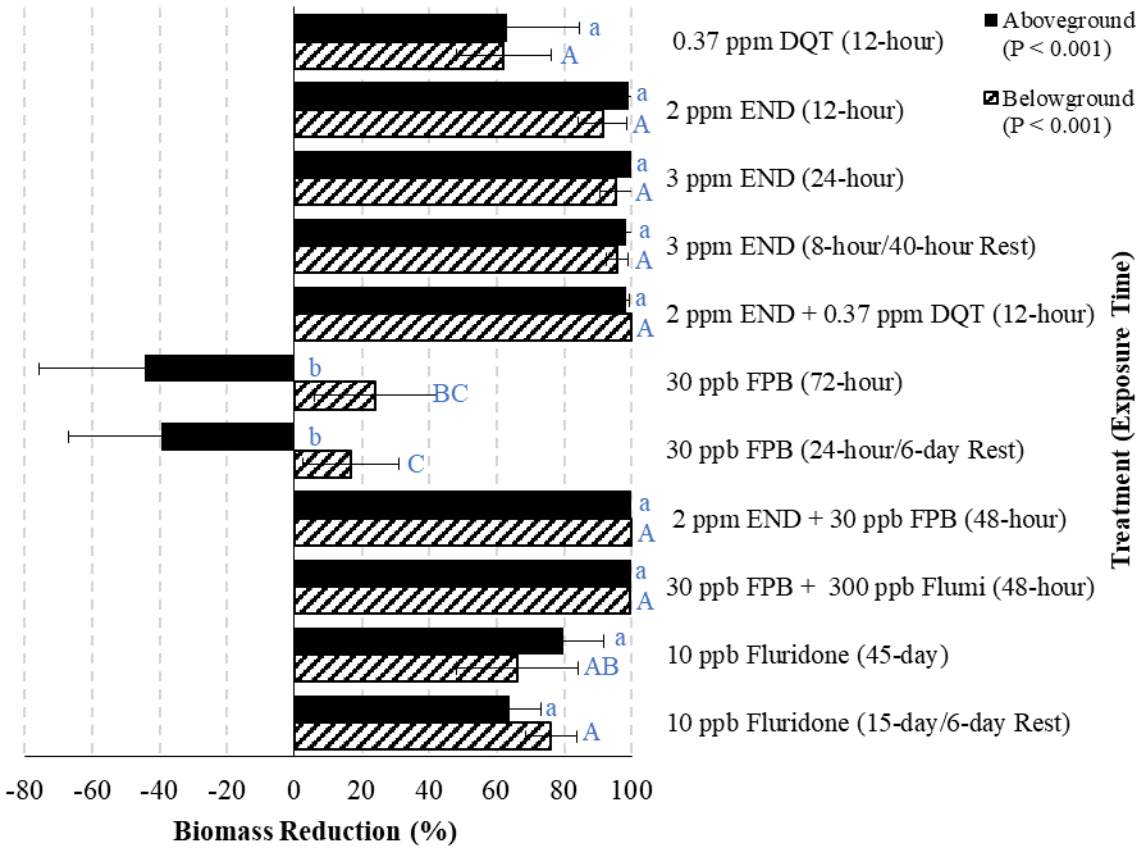

Figure 5-2. Mean (±SE) *Vallisneria americana* biomass reduction at time of harvest. Black bars indicate aboveground biomass and bars with diagonal stripes indicate belowground biomass. Treatments that share the same letter are not different. Lowercase letters correspond to aboveground biomass, uppercase letters correspond to belowground biomass (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).

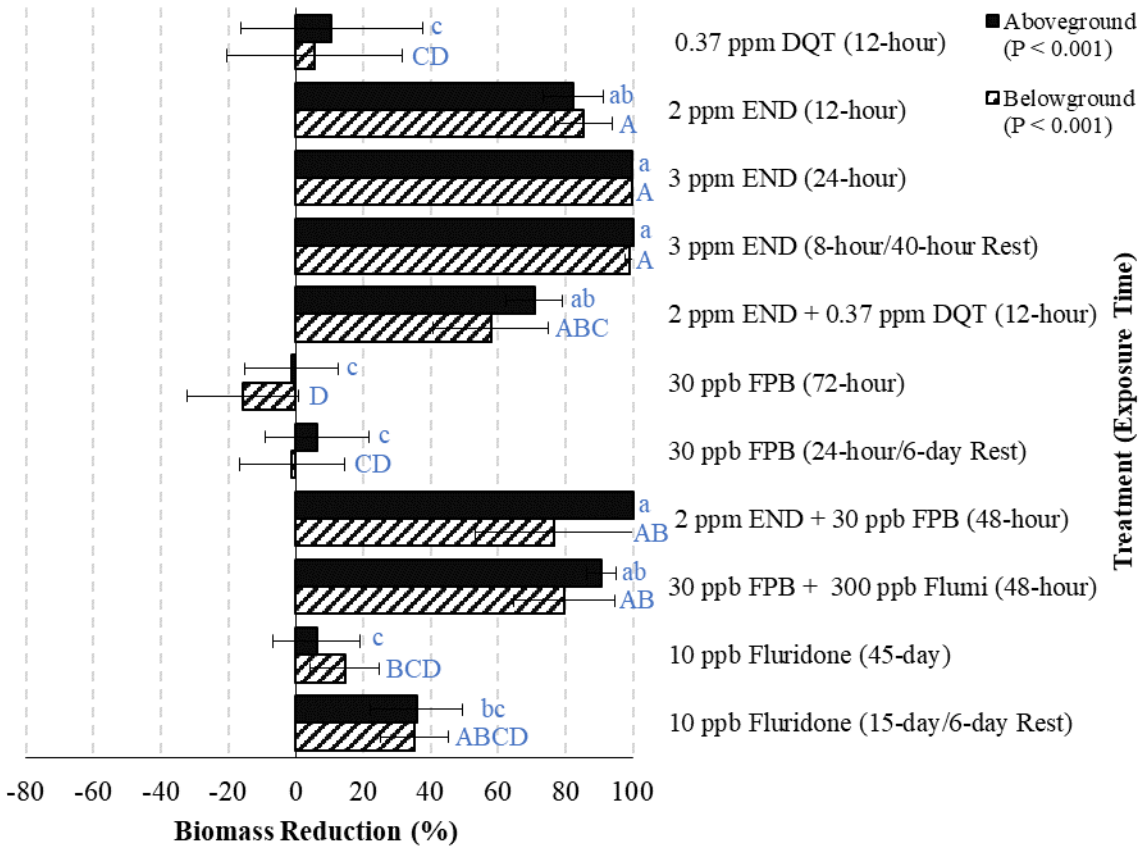

Figure 5-3. Mean (±SE) *Vallisneria neotropicalis* biomass reduction at time of harvest. Black bars indicate aboveground biomass and bars with diagonal stripes indicate belowground biomass. Treatments that share the same letter are not different. Lowercase letters correspond to aboveground biomass, uppercase letters correspond to belowground biomass (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).

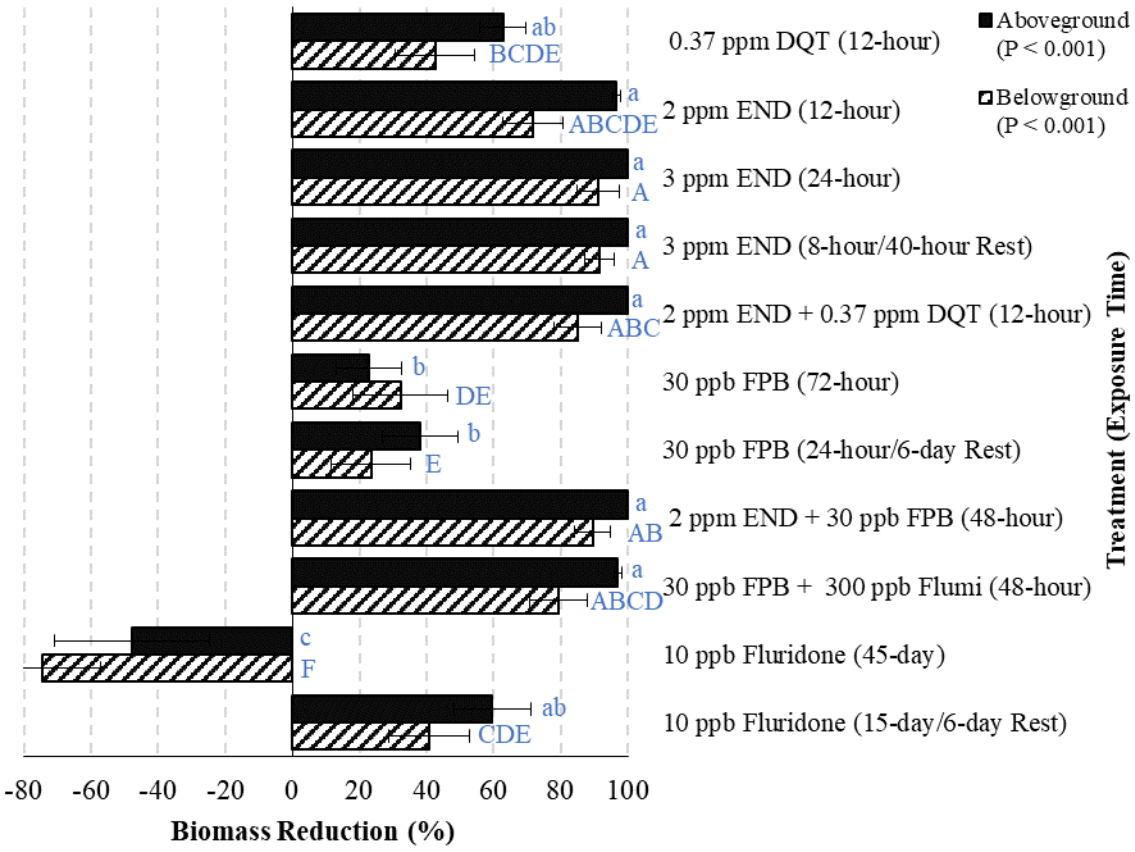

Figure 5-4. Mean (±SE) *Vallisneria spiralis* × *Vallisneria denseserrulata* biomass reduction at time of harvest. Black bars indicate aboveground biomass and bars with diagonal stripes indicate belowground biomass. Treatments that share the same letter are not different. Lowercase letters correspond to aboveground biomass, uppercase letters correspond to belowground biomass (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).

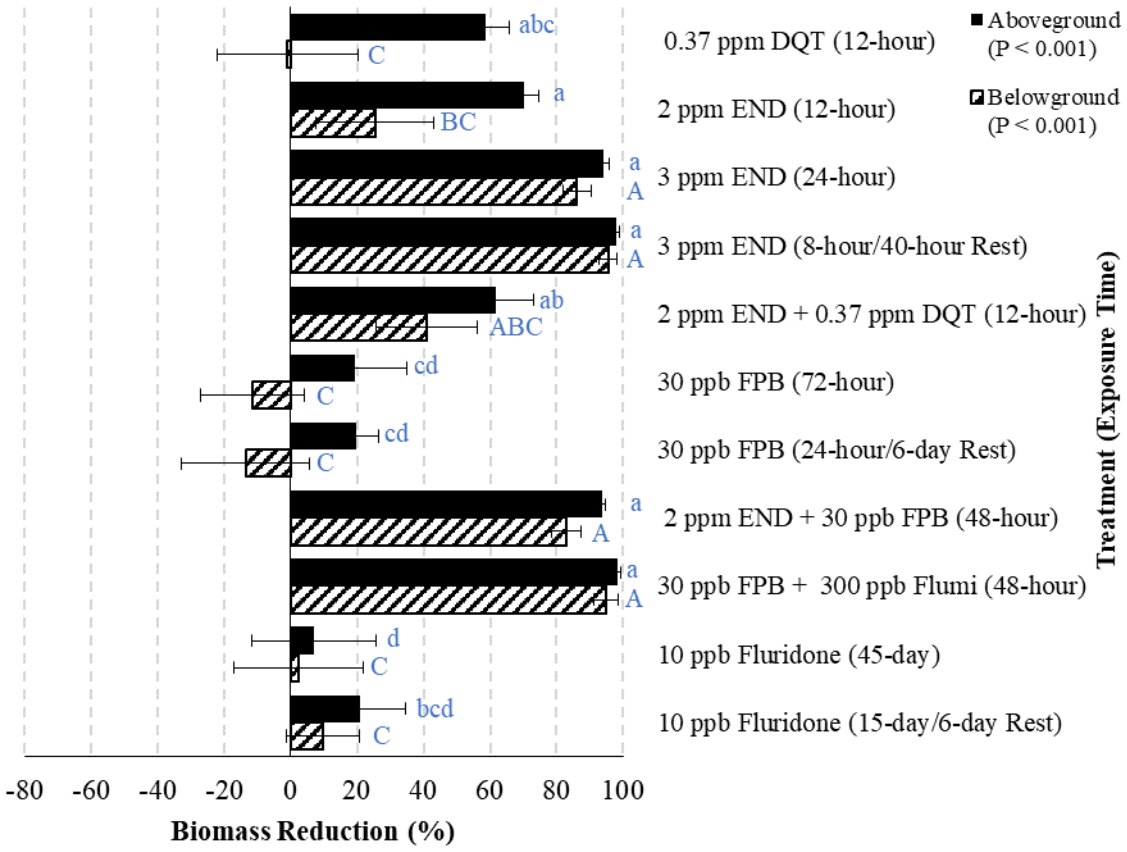

Figure 5-5. Mean (±SE) *Vallisneria australis* biomass reduction at time of harvest. Black bars indicate aboveground biomass and bars with diagonal stripes indicate belowground biomass. Treatments that share the same letter are not different. Lowercase letters correspond to aboveground biomass, uppercase letters correspond to belowground biomass (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).

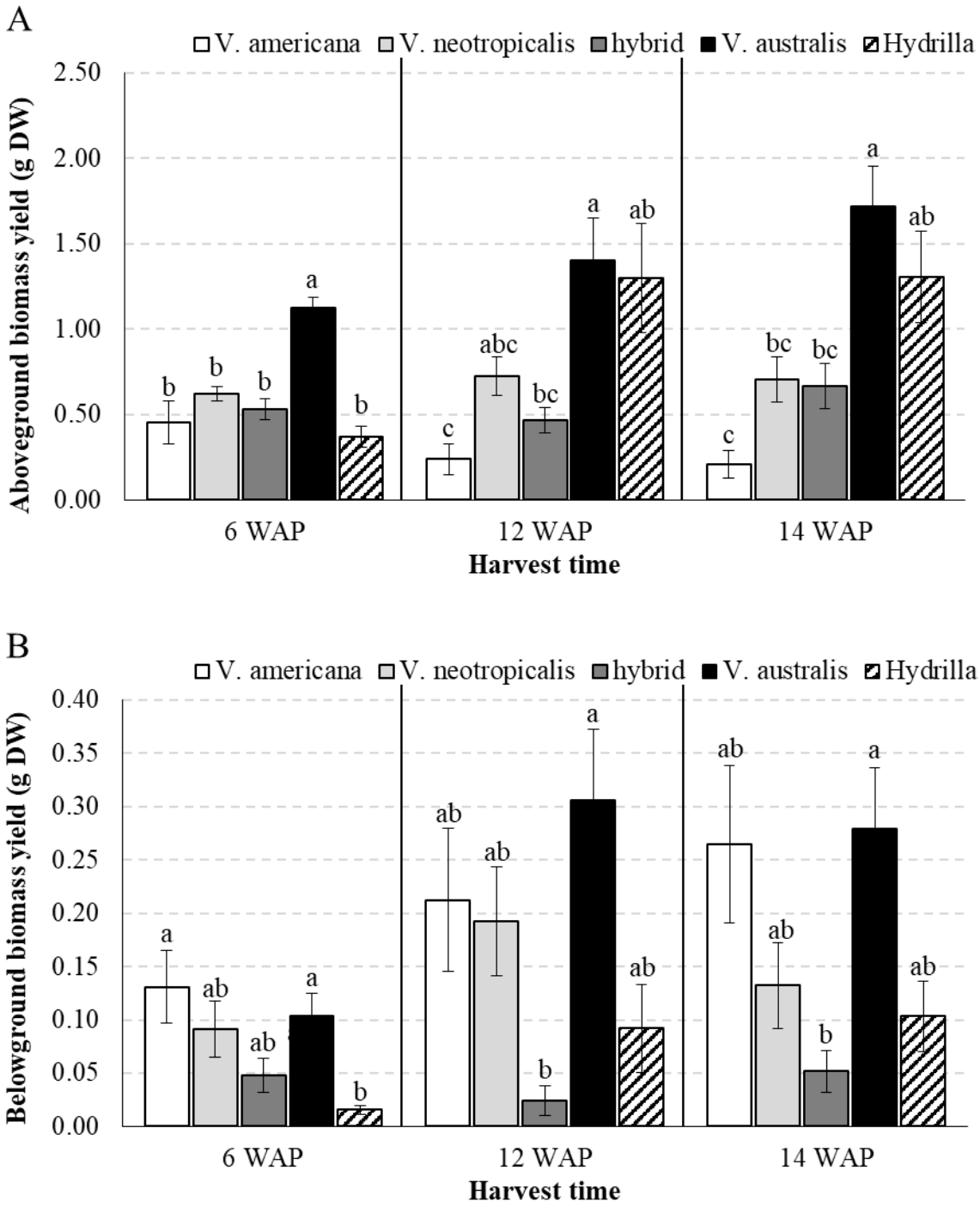

Figure 5-6. Mean (±SE) biomass yield of the non-treated controls at 6, 12, & 14 weeks after planting (WAP) for aboveground biomass yield (a) and belowground biomass yield (b) of four *Vallisneria* taxa. Treatments that share the same letter are not different (ANOVA, Tukey's HSD, $\alpha = 0.05$). Results from two trials were pooled (n=8).