ABSTRACT

HAUG, ERIKA JEANNE. Monoecious Hydrilla and Crested Floating Heart Biology, and the Response of Aquatic Plant Species to Florpyrauxifen-benzyl Herbicide. (Under the direction of Dr. Robert J. Richardson).

Dominance of invasive aquatic plant species can lead to numerous negative impacts to aquatic ecosystems. More research is needed on aspects of the biology of less studied invasive species, such as monoecious hydrilla (*Hydrilla verticillata*) and emerging threats, such as crested floating heart (CFH) (*Nymphoides cristata*). Research on novel active ingredients, such as florpyrauxifen-benzyl, is also needed to respond to new threats and help mitigate the risk of herbicide resistance.

Two studies were conducted to examine aspects of the sprouting, growth, and development of monoecious hydrilla tubers. In the first study, tubers were grown in dark growth chambers for ten weeks to better understand the impacts of light blocking management strategies, and ecosystem driven light reductions. Shoot lengths increased to 32 cm following ten weeks of dark exposure. During this time, the average dry weight of tubers declined from 34.6 mg to 7.6 mg and the average shoot dry weight increased from 0 to 17.4 mg. Starch levels declined significantly and steadily over the course of the 10-week dark growth experiment to 20% of dry weight. The results of this study indicate that monoecious hydrilla is capable of stem extension and development in darkness. In the second study, tuber respiration rate of first year tubers was measured in aerobic conditions for each month from January through July to establish baseline respiration rates under varying conditions. Light exposure and tuber age did not affect respiration rate in the first season. Significantly higher respiration rates were observed for disturbed first season tubers at high (30-33°C) temperatures (-0.159 mgO$_2$ hr$^{-1}$g$^{-1}$) as compared to lower (14 – 20°C) temperatures (-0.084 mgO$_2$ hr$^{-1}$g$^{-1}$). The observed increase in respiration indicates a potential decrease in the longevity of first year disturbed tubers in warmer climates.
The vegetative reproductive capability of CFH was assessed through the monitoring of transversely bisected leaves for plantlet development. Eighty-five percent of the monitored fragments produced independent plantlets within 15 weeks. Leaf size had a strong impact on the production of floating leaves from settled independent plantlets, with 92% of large leaf (26.7-77.3 cm²) plantlets producing floating leaves as compared to 21 and 17% of medium (9.1-22.7 cm²) and small leaves respectively (4.5-7.9 cm²). These results preclude the use of any management technique that would result in the fragmentation of CFH leaf structures.

Two studies were conducted to evaluate efficacy of florpyrauxifen-benzyl on selected aquatic plant species. In the first study, in-water applications of 0 to 81 µgL⁻¹ florpyrauxifen-benzyl and an acid metabolite were maintained for a four-week static exposure on: alligatorweed (*Alternanthera philoxeroides*), bacopa (*Bacopa caroliniana*), fanwort (*Cabomba caroliniana*), monoecious hydrilla, parrotfeather (*Myriophyllum aquaticum*), variable watermilfoil (*Myriophyllum heterophyllum*) and waterwillow (*Justicia americana*). Fanwort was not controlled by florpyrauxifen-benzyl at the rates evaluated. Dry weight EC₅₀ values were <1 µgL⁻¹ for alligatorweed, monoecious hydrilla, parrotfeather, and variable watermilfoil. Bacopa and waterwillow EC₅₀ values were 5.0 and 5.1 µgL⁻¹, respectively. These six species were less sensitive to the acid metabolite (EC₅₀: 1.6 to 77.1 µgL⁻¹). In the second study, the absorption and translocation patterns of florpyrauxifen-benzyl were examined. Ten µg L⁻¹ radiolabeled florpyrauxifen-benzyl was applied to the isolated shoot tissue of Eurasian watermilfoil (*Myriophyllum spicatum*), variable watermilfoil, hybrid watermilfoil (*Myriophyllum spicatum x sibiricum*), dioecious hydrilla, monoecious hydridilla, CFH, tapegrass (*Vallisneria americana*), Brazilian elodea (*Egeria densa*), giant salvinia (*Salvinia molesta*) and water hyacinth (*Eichhornia crassipes*). The highest shoot absorptions were observed for CFH (A₁₉₂=20µg g⁻¹),
dioecious hydrilla (A192=25.3µg g\(^{-1}\)), variable watermilfoil (A192=40.1µg g\(^{-1}\)) and Eurasian watermilfoil (A192=25.3µg g\(^{-1}\)). Evidence of translocation was observed in all rooted species tested. Studies indicate that florpypauxifen-benzyl will provide selective control of several important invasive aquatic plants.
Monoecious Hydrilla and Crested Floating Heart Biology, and the Response of Aquatic Plant Species to Florpyrauxifen-benzyl Herbicide

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Fisheries, Wildlife, and Conservation Biology

Raleigh, North Carolina

2018

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DEDICATION

To those I love and have been loved by,

your support has meant more than words can express.

For now, I will leave you with words to live by:

“Flail forward and hope for the best” ~ Derek Andrew Lyman.
BIOGRAPHY

Erika Haug grew up playing on the shores of a small pond in rural New Hampshire. Her love of lakes led her to study biology with a minor in environmental science at McGill University in Montreal, Canada. During her time at McGill, she interned under the direction of Ms. Amy Smagula at the New Hampshire Department of Environmental Services and developed a passion for invasive aquatic plant management. She continued her studies under the direction of Dr. Sylvie DeBlois, studying aspects of the expansion of *Phragmites australis* haplotype-M on Réserve nationale de faune du Lac-Saint-François. Following graduation with great distinction for a BS from McGill University in 2008, Erika immediately began work as an aquatic plant biologist for Aquatic Control Technology, LLC in Sutton Massachusetts where she worked for close to six years. One evening at a Northeast Aquatic Plant Management Society meeting, Erika and Dr. Richardson began talks about graduate school. Under the direction of Dr. Richardson, she began work on her PhD in Fisheries, Wildlife and Conservation Biology with a focus on invasive aquatic plant biology and management. Erika feels fortunate to have found her passion young in life and looks forward to continuing to study invasive aquatic plant management in the years to come.
ACKNOWLEDGMENTS

The number of people who have helped me to reach this goal are too numerous to count but I will try my best. I must acknowledge my dear friend and colleague Amy P. Smagula, for introducing me to the world of aquatic plant management, for always believing in me, and for encouraging me to go back to school after several years of professional work. I want to thank each of the members of my committee - Dr. Greg Cope, Dr. Randy Wells, Dr. Mike Netherland and Dr. Ramon Leon Gonzalez. I want to thank Dr. Rob Richardson for taking me on as a student, supporting me and my career goals, and giving me the space to explore and try new things. I want to acknowledge Dr. Emily Griffith for all of her statistical help and guidance. I would like to acknowledge Dr. Mark Heilman, Dr. Terri Long and Dr. Jim Burton for their consultation. I would like to thank Dr. Heike Sederoff and all the members of her lab, with particular mention to Mr. Colin Murphree, Mr. Jacob Dums and Dr. Sathya Jali, for lending me lab space and equipment and for teaching me how to conduct a non-structural carbohydrate analyses. Thank you to Mr. Khalied Ahmed and Dr. Travis Gannon for teaching me how to conduct analyses with radio-labeled materials. I thank my family and friends for their never-ending support and love and my partner, Barrett Chambers, for keeping me loved, supported, fed, and sane. Last but certainly not least I would like to thank the members of the aquatics lab past and present - Mr. Steve Hoyle, Mr. Tyler Harris, Dr. Justin Nawrocki, Ms. Stephanie Nawrocki, Mr. Evan Calloway, Mr. Cody Hale, Ms. Shannon Regan, Mr. Andrew Howell, Ms. Amy Henry, Ms. Eryn Molloy, Mr. Logan Wilson and Ms. Kara Foley for their tireless efforts, for working until the wee hours of the morning and for putting up with my insistence on things being just a little closer to perfect.
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CHAPTER 1: Biology and Control of Hydrilla verticillata and Nymphoides Cristata: A Literature Review

Introduction

Dominance of invasive aquatic plant species can lead to numerous negative impacts to aquatic ecosystems. Invasive aquatic plants reduce native plant diversity through competition and displacement and this ultimately results in the formation of dense monotypic stands of vegetation (Gause, 1934; Hardin, 1960; Schultz and Dibble, 2012). These monotypic stands have been shown to reduce native fish and macroinvertebrate populations and diversity through increased hypoxia, changes in habitat complexity, and through decreases in food availability and quality (Schultz and Dibble, 2012). Much less research on the impacts of reduced biodiversity on ecosystem function has been conducted for aquatic ecosystems as compared to terrestrial ecosystems (Covich et al., 2004). However, studies have found that reduced species diversity and changes in species composition in freshwater systems can have far reaching impacts including decreased ecosystem productivity, decreased community respiration, decreased total plant biomass, and decreased abundance of fish and wildlife (Covich et al., 2004; Downing and Leibold, 2002; Engelhardt and Ritchie, 2001). In addition to biodiversity impacts, dense stands of monotypic aquatic plant growth also slow the natural flow of water through aquatic ecosystems leading to a reduction in the utility of drainage canals and hydroelectric power generation and an increase in siltation (Pitlo and Dawson, 1993). These infestations negatively impact the recreational utility of waterbodies, reduce property values and create habitat suitable for disease carrying vectors leading to an increase in the incidence of avian vacuolar myelinopathy (AVM), malaria, yellow fever, encephalitis, and schistosomiasis among others (Gangstad and Cardarelli, 1993; Halstead et al., 2003; Wilde et al., 2005; Zhang and Boyle, 2010).
More research is needed regarding the biology and control of invasive aquatic plant species in the United States. Research on specific aspects of biology and control of the less studied monoecious biotype of hydrilla, which is currently one of the worst aquatic weeds in the US, is needed (True-Meadows et al., 2016). More research is also needed on the biology and control of emerging threats, such as crested floating heart (*Nymphoides cristata*). Lastly, research aiding in the registration of novel active ingredients, such as Florpyrauxifen-benzyl, is needed to respond to potential new threats or novel situations and to help to mitigate the risk of resistance development (Cobb and Reade, 2010; Getsinger et al., 2008).

**Hydrilla verticillata**

To date *Hydrilla verticillata* (L.F.) Royle remains one of the most challenging to manage aquatic weeds invading freshwater systems in the United States (Haller and Sutton, 1975; Langeland, 1996). It has been named the perfect aquatic weed, for its varied adaptations (Langeland, 1996). The negative economic and ecological impacts of this species are immense. Hydrilla has been found to alter water flow, hinder recreational uses, alter fish populations, and provide ideal habitat for the epiphytic cyanobacterium *Aetokthonos hydrillicola*, which has been linked to neurological disorders in several wildlife species (Haller, 2014; Haller and Sutton, 1975; Langeland, 1996; Wilde et al., 2005, 2014; Wiley et al., 2007; Williams et al., 2007).

Although *Hydrilla verticillata* is highly polymorphic, several key characteristics, including whorls of 4 to 8 serrated verticillate leaves with midrib spines, unique floral structures, and the presence of axillary and subterranean turions, allow it to be distinguished from other similar species in a variety of environments (Blackburn et al., 1969; Langeland, 1996). The submersed monocot forms dense underwater stands and often grows to the surface creating a canopy that reduces light attenuation for plants below (Blackburn et al., 1969; Haller and Sutton,
A 10 cm shoot of hydrilla can grow 80 m of shoot tissue in just 35 days (Glomski and Netherland, 2012). Hydrilla has been observed growing in depths of 6 to 7 m in clear water and up to 12 m in pristine spring water (Blackburn et al., 1969; Haller, 2014).

There are two *Hydrilla verticillata* biotypes in the United States, a monoecious biotype with staminate and pistillate flowers on the same plant (Harlan et al., 1985) and a female dioecious biotype with pistillate flowers only (Cook and Lüönd, 1982). These two biotypes have been shown to have large genetic differences, perpetuated by prolonged geographic isolation (Verkleij et al., 1983) and molecular methodologies for distinction of the two biotypes have been improved such that reference samples are no longer required (Madeira et al., 2004). A laboratory study confirmed that these two biotypes can successfully cross pollinate, with 71% of test-crosses producing seed, 90% of which were viable (Steward, 1993). Despite the viability of these test crosses and the cooccurrence of the two biotypes in select waterbodies, field hybridization has yet to be confirmed (Ryan et al., 1995; Netherland and Greer, 2014).

Monoecious hydrilla was first recorded in the Chesapeake Bay area and the Potomac River in the early 1980s and in Umstead Lake in North Carolina in 1980 (Steward et al., 1984; DeMont, 1980). This biotype dominates in the more temperate regions of the mid-Atlantic and as far north as Maine. The dioecious biotype was first recorded in the late 1950’s in Florida and dominates in the warmer climates of the southeastern states (Netherland, 1997). In the more temperate regions, monoecious hydrilla behaves as an herbaceous perennial with standing biomass dying back in the winter (Harlan et al., 1985; Owens et al., 2012). In contrast, dioecious hydrilla can maintain standing biomass throughout the winter in warm southern U.S. climates, such as that found in Florida.
Both biotypes reproduce primarily through vegetative means including through fragmentation. Langeland and Sutton (1980) found that dioecious hydrilla fragments as small as one node were capable of regrowth and vegetative reproduction. Pesacreta (1990) observed an increase in fragmentation with increased temperatures. In laboratory tests, desiccation for longer than 2 hours reduced survival and sprouting of fragments as compared to un-desiccated controls (Baniszewski et al., 2016). Fragment length significantly affected this desiccation effect as well, with longer, 4-whorl fragments showing higher survival rates as compared to shorter, 1-whorl fragments (Baniszewski et al., 2016).

While fragmentation is a means of vegetative reproduction for hydrilla, the prominent means of reproduction for this species is through the prolific production of vegetative propagules including axially turions and subterranean turions (tubers) (Netherland, 1997). Monoecious hydrilla produces tubers that are greater in number but smaller in size than dioecious hydrilla (Owens et al., 2012; Pesacreta, 1990; Spencer et al., 1987; Sutton et al., 1992). In field measurements, monoecious hydrilla tuber densities have been as high as 1,312 to 2,050 tubers m⁻² and mesocosms growth reportedly exceeds these densities by 4,000 to 8,000 tubers m⁻² (Harlan et al., 1985; Hodson et al., 1984; Nawrocki et al., 2016; D. L. Sutton et al., 1992; Van et al., 1987). In contrast, dioecious hydrilla tuber densities only reach a reported maximum of 900 and 9053 tubers m⁻² in the field and mesocosms studies, respectively (Netherland, 1997). In addition to differences in size and numbers, the propagules of these biotypes also vary in their tolerance to environmental gradients. Monoecious hydrilla is capable of producing tubers under shorter photoperiods than dioecious hydrilla, indicating that it may have a competitive advantage over dioecious hydrilla in temperate climates (Spencer and Anderson, 1986). Steward and Van (1987) found that 38 to 68% of monoecious tubers germinated at colder temperatures (15°C) as
compared to only 3% of dioecious tubers germinating at that same temperature. In fact, one study found that a chilling period is a requirement for the sprouting of monoecious hydrilla tubers (Carter et al., 1987). Monoecious hydrilla axillary turions have also been shown to be more cold tolerant than dioecious hydrilla axillary turions (Maki and Galatowitsch, 2004).

In addition to differences among monoecious and dioecious hydrilla vegetative propagules, the axillary and subterranean propagules themselves differ from one another. Physically, monoecious hydrilla tubers are typically more than twice the size of axillary turions (Spencer et al., 1987; Van and Steward, 1990). Monoecious hydrilla plants, which originated from subterranean tubers, produce more propagules, have greater biomass and produce a larger number of root crowns as compared to monoecious hydrilla plants grown from axillary turions (Spencer and Ksander, 1991). Axillary turions will either germinate within a year of production or not at all, whereas subterranean turions may remain viable and quiescent in the sediments at least 4 to 6 years (True-Meadows et al., 2016; Van and Steward, 1990).

**Photosynthesis, Carbon Storage and Respiration:**

In general, submerged macrophytes have adapted to handle the low light conditions underwater. Several anatomical features reflect this, including extremely thin cuticles, increased levels of chloroplasts in epidermal tissue, and thin leaves that are several cells thick at maximum (Wetzel, 2001). Typically, light saturation for leaf photosynthesis ranges from 10 to 50% of full sunlight (ca. 2000 μmol m⁻² s⁻¹) for submerged aquatic plants (Wetzel, 2001). However, the light compensation point for monoecious and dioecious hydrilla is extremely low at 10 μmol m⁻² s⁻¹ (Steward, 1991). Hydrilla can photosynthesize in less than 1% full sunlight (ca. 2000 μmol m⁻² s⁻¹) (Langeland, 1996; Van, Haller, and Bowes, 1976). Spencer and Anderson (1986) observed that chlorophyll and carotenoid content in monoecious hydrilla plants was maximized at longer (14hr
– 16hr) photoperiods and in younger plants. These results are consistent with the observation that monoecious hydrilla shoot biomass was eight times higher when grown in the summer (long photoperiods) as compared to growth in winter conditions (Sutton et al., 1992). Similarly, monoecious hydrilla was observed to produce 20% more biomass when exposed to 10 hours of light and an interrupted dark cycle (9+1hr light/7:7hr dark) as compared to short 10 hours of light and 14 hours of darkness (10hr light:14hr dark) (Macdonald, 1994).

In addition to differences in photosynthetic tissues, production of propagule tissues is also affected by differing light regimes. Steward and Van (1987) observed that monoecious hydrilla only produced tubers under short photoperiod conditions (10hr light) as compared to no tuber production under long photoperiod conditions (16 hr light). Macdonald (1994) observed that monoecious hydrilla produced axillary turions and tubers with both interrupted shortened dark periods (9+1hr light/7:7hr dark) and lengthened uninterrupted dark periods, with the same total hours of light (10hr light: 14hr dark). As such, it seems that the length of photoperiod is the most important aspect of the light regime as opposed to the length of dark-period for production of tubers (Macdonald, 1994; Steward and Van, 1987). Monoecious hydrilla tubers also accumulate starch in response to shorter photoperiods (Pesacreta, 1990).

*Hydrilla verticillata* is one of the best studied freshwater aquatic plants with C4-like metabolism. Research regarding hydrilla photosynthesis began in the 1920’s by Sir J.C. Bose (Raghavendra, 2010). While the results Sir J. C. Bose produced with his photosynthetic recorder showed evidence of inducible C4-like metabolism, the mechanisms behind the varying CO2 compensation points during different seasons were not elucidated until much later (Bowes, 2010; Raghavendra, 2010). Metabolisms in aquatic plants such as *Hydrilla verticillata, Egeria densa* (Planch.) and other related aquatic plants are C4 in nature and occur in single cells (Bowes,
Phosphoenolpyruvate carboxylase (PEPC) is sequestered in the cytosol where the initial carboxylation of Phosphoenolpyruvate (PEP) to malate occurs (Bowes, 2010). Malate is then transported to the chloroplasts, where it is decarboxylated by NADP malic enzyme (NAPME) (Bowes, 2010). This results in a high concentration of CO₂ in the chloroplasts where ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) is located (Bowes, 2010). Hydrilla is also capable of using bicarbonate as an additional carbon source (Holaday and Bowes, 1980; Prins and Elzenga, 1989). Use of bicarbonate likely involves the enzyme carbonic anhydrase (Wetzel, 2001). Laboratory results of carbonic anhydrase utilization in Hydrilla (253 unit/mg chl) were relatively low, though still high enough to support bicarbonate utilization (Van et al., 1976).

Literature on the respiration of monoecious hydrilla is limited. Ryan (1994) conducted a study to examine changes in monoecious hydrilla tuber carbon, protein, and amino acid content per mg dry weight over the course of a winter season. In his study, carbon was lost at an estimated 0.70% per month and nitrogen was lost at an estimated 0.05% per month in overwintering dormant tubers, and he observed no significant linear trends in soluble proteins or free amino acids in overwintering tubers (Ryan, 1994). Respiration rates have been studied in shoot tissues of dioecious hydrilla and in turions of other aquatic plant species. Adamec (2008) noted that aerobic respiration rates of storage organs, such as tubers or turions, are likely to be much lower than respiration rates for shoot tissue. For dioecious hydrilla sub-apical plant segments, respiration rates increased significantly after 16 hours of drying stress (Basiouny, Haller, and Garrard, 2017). Barko and Smart (1981) observed increased dark respiration of hydrilla shoot tissues with increasing temperature above 24°C. Adamec (2008) also observed increased respiration rates with increasing temperatures for turion and tubers of several carnivorous aquatic plants. Stage of quiescence or turion age may also impact respiration.
Adamec (2011) observed higher temperature dependence in respiration rates for older spring turions as compared to fall harvested turions; however, the author did not observe significant differences in the respiration rates at an optimum temperature (Adamec, 2011). The potential change in respiration rates of monoecious hydrilla tubers over time at varying temperatures and lengths of quiescence has yet to be studied. Understanding how the rate of carbon breakdown may change over time and at varying temperatures, may be important in predicting the longevity of monoecious hydrilla tubers as the range of this species expands.

Management:

Physical or Cultural Control:

Physical or cultural management of invasive aquatic plants can take many forms from hand-pulling weeds to the use of reduced water levels to dehydrate and kill invasive plants within exposed areas (drawdowns) (Bellaud, 2014). Hand-pulling is inefficient, expensive, and does not remove fallen axillary or subterranean turions. Hand-dredging is cost prohibitive, time consuming, and re-infestation is rapid for monoecious hydrilla (Killgore, 1987). In North Carolina, fall drawdowns have been determined to be ineffective, where the high clay content in hydrosols prevent full desiccation (Hodson et al., 1984). The addition of a dilute acetic acid (<5%) solution to a drawdown management protocol has been found to reduce tuber sprouting by 80 to 100% (Spencer and Ksander, 1999). A winter drawdown in Florida was observed to stimulate 80% sprouting of dioecious tubers (Haller et al., 1976). While winter drawdown may not control hydrilla on its own, it has been postulated that a combination of winter drawdown to stimulate germination, followed by an effective herbicide treatment or an effective summer drawdown with full dewatering could reduce the tuber bank (Haller et al., 1976). Poovey and Kay (1996) observed highly effective control of monoecious hydrilla in sandy soils with the
application of a short-term summer drawdown. However, when short-term summer drawdown was applied to monoecious hydrilla tubers in slow draining soils, such as clay or loam, researchers observed an increase in the production of tubers and hydrilla biomass (Poovey and Kay, 1996). These results suggest that the efficacy of a summer drawdown for reducing tuber production and biomass is largely dependent on the substrate and length of exposure to desiccation (Poovey and Kay, 1996).

For submersed aquatic plant species, light blocking strategies such as benthic barriers can be a highly effective physical management technique for control of small isolated infestations (Eichler et al., 1995; Helsel et al., 1996; Hofstra and Clayton, 2012; Perkins et al., 1980). Bottom barriers are generally recognized as nonselective for macrophyte control, negatively impacting photosynthesis and growth of native and invasive species alike (Bellaud, 2014). As previously mentioned, hydrilla has a very low light requirement for photosynthetic activity and thus significant light attenuation would be required to inhibit growth (Steward, 1991; Van et al., 1976). Field studies have shown a significant reduction in emergence and biomass of hydrilla using high density light blocking benthic barriers (Hofstra and Clayton, 2012; Wood, 2017). However, laboratory studies have shown that 100% of dioecious tubers will germinate in darkness within 14 days, when maintained between the temperatures of 15°C and 35°C (Haller et al., 1976). Monoecious hydrilla has a large starch reserve allowing growth following germination until a suitable light environment is attained (Miller et al., 1976; Pesacreta, 1990). As such, it is conceivable that monoecious hydrilla may grow in darkness until a seam between sections of benthic fabric can be penetrated, similar to how other submersed aquatic weeds exploit these seams in the fabric (Eichler et al., 1995; Helsel et al., 1996; Killgore, 1987). In addition, recolonization by invasive aquatic macrophytes has been shown to be quite rapid,
following removal of bottom barriers (Eichler et al., 1995). Prolonged use of benthic barriers has been shown to have negative impacts on the diversity and density of macroinvertebrates in underlying sediments (Ussery et al., 1997). Benthic barriers can also negatively impact recreation when the fabric catches on boat propellers in shallow water or becomes clogged with sediment and billows up into swim areas (Killgore, 1987).

**Mechanical:**

Another possible management technique for invasive aquatic plant species is broadly termed mechanical removal of vegetation. Mechanical management can be broken down into mechanical harvesting, hydro-raking, and dredging. Mechanical harvesting involves the use of a floating barge with attached cutting head. The cutting head is hydraulically controlled to sever plant biomass up to 5 feet below the water surface (Bellaud, 2014). This biomass is then conveyed to a transport barge and removed from the system. Multiple studies have found impact to fish populations with mechanical harvesting; however, in large bodies of water these impacts are likely to be minimal, localized, and short lived (Haller et al., 1980; Serafy et al., 1994). While mechanical harvesting does remove biomass from the system, it can also have a pruning effect, resulting in an overall increase in biomass and will invariably lead to lost fragments which can infest previously uninfested areas (Serafy et al., 1994). Hydro-raking is similar to mechanical harvesting; however, this machine uses a York rake attachment to scrape the lake sediments and remove vegetation and attached hydro-soils rather than severing the vegetation with a cutting head. Hydro-raking is also likely to result in the release of vegetative fragments of hydrilla plants. Hydro-raking is not likely to provide long term hydrilla control, as the tines should not consistently remove enough sediment to remove all of the subterranean tubers. While dredging should remove all of the subterranean turions and the overlying vegetative portions of
the hydrilla plant, this method of plant control has many additional non-target ecological impacts that should also be evaluated prior to use.

Biological:

Triploid grass carp (*Ctenopharyngodon Idella* (Val.)) is the primary biological control agent for monoecious hydrilla in the US. One of the major concerns with the use of grass carp for vegetation control is that an unregulated grass carp population could lead to an elimination of all aquatic vegetation (Allen and Wattendorf, 1987). Triploid grass carp have been shown to consume weeds as efficiently as diploid grass carp; however, triploid grass carp are considered functionally sterile and thus population density can be regulated (Allen and Wattendorf, 1987; Sutton, 1985). Additionally, while grass carp will feed on most aquatic vegetation, they reportedly do have preferences and will eat vegetation in order of decreasing palatability and there are genera that they will avoid (Dibble and Kovalenko, 2009). Dibble and Kovalenko (2009) reported 35 studies have confirmed that grass carp do consume hydrilla. Pesacreta (1990) substantiated that regular cutting, such as that produced by grass carp feeding, can suppress tuber formation in monoecious hydrilla. Successful control of monoecious hydrilla has been achieved utilizing lower density stocking rates in North Carolina, than those required in Florida where standing biomass is present year-round (Hodson et al., 1984). An integrated management plan that incorporates the use of selective herbicides in areas of dense hydrilla growth, followed by the lower density stocking of grass carp (30-35 fish ha\(^{-1}\)) to consume any newly emerged growth will likely result in the most cost-effective control of hydrilla, while helping to encourage the growth of native aquatic plant species (Sutton, 1985). However, it should be noted that treatment with diquat or fluridone have been shown to reduce the palatability of hydrilla for triploid carp in laboratory feeding trails (Kracko and Noble, 1993)
Other potential biological control agents for monoecious hydrilla are limited to insects. The host-specific hydrilla leaf-mining fly [Hydrellia pakistanae (Deonier)] could provide control of monoecious hydrilla in climates where hydrilla maintains standing biomass year-round; however in more temperate climates the leaf-mining fly, will not likely survive due to a lack over-wintering habitat for the first and second instars (Harms and Grodowitz, 2011). Additionally, a Chironomid midge [Cricotopus lebetis (Sublette)] larva has been examined for hydrilla control; however, these midges are considered generalists (Stratman et al., 2013). In paired choice tests, larval colonization and female oviposition was greater for the native species Elodea canadensis (Michx.) and Najas guadalupensis (Spreng.) Magnus than for hydrilla, indicating that more data is needed to determine potential non-target impacts (Stratman et al., 2013).

Chemical:

In the United States there are currently eight herbicides registered for hydrilla control. These herbicides include bispyribac sodium, copper, diquat, endothall, fluridone, imazamox, and penoxsulam. Registration of a new product, florpyrauxifen-benzyl, for the control of several invasive aquatic weeds including hydrilla, is expected in the spring of 2018.

Variable and relatively poor control (0 to 60% control) has been observed with diquat in North Carolina waters, likely due, in large part, to the competition for diquat ion by suspended clay particles and dissipation (Hodson et al., 1984; Langeland and Pesacreta, 1985). The use of drop hoses significantly improved control in deep areas; however, multiple applications per season are often necessary for control (Langeland and Pesacreta, 1985). Radiolabeled herbicide uptake studies have shown a positive linear increase in diquat concentration in dioecious hydrilla over time (Van et al., 1987). Only a 6 hour exposure of 2.0 ppm was required for an initial 93%
control of emerging monoecious hydrilla shoot tissue, propagated from tubers, and an 85% reduction in biomass was maintained up to 6 weeks after treatment (Van et al., 1987). In another laboratory study, diquat applied at a concentration of 0.2 mg L\(^{-1}\) provided over 85% control for 10 weeks of both monoecious and dioecious hydrilla (Steward and Van, 1987). When treated with 2 mg L\(^{-1}\) of diquat, total nonstructural carbohydrates of hydrilla were reduced by 40% (Kracko and Noble, 1993).

Fairly good control (70% control) has been achieved in the field with a concentration of 2 mg L\(^{-1}\) of copper, when applied in the spring (Hodson et al., 1984); however, regrowth was rapid following treatment (Hodson et al., 1984). In the laboratory, copper concentrations as low as 1.0 mg L\(^{-1}\) controlled monoecious hydrilla (Steward and Van, 1987). Monoecious hydrilla was observed to be much more susceptible to copper as compared to dioecious hydrilla (Steward and Van, 1987).

Variable levels of control have been observed in field studies using endothall (Aquathol K), from complete control in areas of low dilution to poor control in high dilution treatment areas (Hodson et al., 1984; Langeland and Pesacreta, 1985). In areas of high dilution, granular formulations, and multiple treatments greatly improved control (Langeland and Pesacreta, 1985). Poovey and Getsinger (2010) observed greater than 85% control of monoecious hydrilla shoots, propagated by fragmentation, with 2 mg ai L\(^{-1}\) of endothall (dipotassium salt) for 48 and 72-hour exposures. However, longer exposures or higher concentrations were required to achieve similar levels of control, when plants were propagated by tuber sprouting (Poovey and Getsinger, 2010).

Fluridone is the most commonly used herbicide for the control of monoecious hydrilla. While consistent fluridone use has resulted in the proliferation of resistant strains of dioecious hydrilla, resistance has yet to be observed for monoecious hydrilla (Michel et al., 2004). In field
trials, fluridone (Sonar SRP, Sonar5P, and Sonar 4 AS) provided complete, season-long control of monoecious hydrilla while not impacting native *Chara* (Langeland and Pesacreta, 1985). Treatment with 90 µg L⁻¹ fluridone reduced total non-structural carbohydrates in treated plants by 20% (Kracko and Noble, 1993). Consecutive annual fluridone treatment in North Carolina reservoirs led to consistent reductions in tuber densities (Nawrocki et al., 2016). To reduce tuber densities, consistent management is key (Nawrocki et al., 2016). The loss of one year of management has been observed to result in a fourfold increase in tuber densities in some sites and at other sites no management for one year resulted in a rebound of tuber densities to 85% of the density prior to management (Nawrocki et al., 2016). At concentrations over 3 µg L⁻¹, chlorophyll fluorescence in the apical shoots of newly sprouted tubers was reduced by 85% or more (Netherland, 2015). Established monoecious hydrilla responds much more slowly to fluridone as compared to newly sprouted plants (Netherland, 2015). Netherland (2015) also found that exposure of unsprouted tubers to fluridone concentrations of 6 and 12 µg L⁻¹ completely prevented shoot emergence.

**Crested Floating Heart**

*Origin Introduction and Spread*

The native range of crested floating heart (*Nymphoides cristata* (Roxb.) Kuntze) extends throughout much of southern Asia including confirmed populations in parts of India, Pakistan, Vietnam, southern China, and Taiwan (Burks, 2002; Marwat, Khan, Ahmad, Zafar, & Sultana, 2007; Mason, 1996; Nair, 1973). The first recorded introduction of crested floating heart to the United States occurred in a small residential pond in Naples, Florida in 1996 (Burks, 2002; Willey and Langeland, 2011). Following this introduction, the species spread throughout much of Florida via the canal system and interconnected waterways (Burks, 2002). The species was
first observed in South Carolina in 2005, when an 8 ha population was found in Lake Marion (Willey and Langeland, 2011; pers. comm. Larry McCord). In the Lake Marion system, the initial invasion expanded from 8 ha to covering an estimated 810 ha in just two years (Page, 2010; Willey and Langeland, 2011). To date, the northern-most extent of the species range in the United States, is Burlington, North Carolina and the western-most confirmed populations are in Louisiana and eastern Texas (pers. comm. Dr. Robert J Richardson; Thayer and Pfingsten, 2016). The current extent of the invasion appears to be due to multiple introductions and is likely the result of discarding or intentional planting of contaminated cultivated plants into or near waterways (Burks, 2002; pers. comm. Dr. Robert J Richardson).

Identification Biology and Ecology

Crested floating heart is a rooted, floating leafed dicot aquatic plant species in the Menyanthaceae family. The floating leaf of crested floating heart is cordate at the base, as is characteristic of the Nymphoides genus. In many ways, *N. cristata* resembles its closest relative and sister taxa little floating heart (*Nymphoides cordata* (Elliot) Fernald). However, several features distinguish crested floating heart from the other members of the Nymphoides genus. The margin of this leaf is smooth to slightly wavy and often has a red to brown hue (Gettys et al., 2017; Marwat et al., 2007; personal observation) (Figure 1-1 A, B). The underside of the leaf is light brown in color and covered with brown glands (Marwat et al., 2007) (Figure 1-1 C). The brown glands, however, are far less prominent as compared to the rough texture found on the underside of the leaf of the native big floating heart (*Nymphoides aquatica* (J.F.Gmel)Kuntze) (Willey, 2012). Leaves vary somewhat in their thickness with an average thickness of approximately 290 ± 5 µm (Willey, 2012) In Florida, leaves grow to as long as 25 cm and petioles have been observed to grow to 2 m or longer (Gettys et al., 2017). Sclereids have been
observed in the leaves and stems of crested floating heart and have been postulated to function in the places of lignified tissue to strengthen these vegetative tissues (Malaviya, 1962; Willey, 2012). The flowers form from the petiole in umbellate clusters and have five white petals surrounded by a deeply lobed calyx (Marwat et al., 2007). The feature that most distinguishes crested floating heart from the other members of the Nymphaoides genus is the presence of a characteristic erect, longitudinal fold of petal tissue along the upper side of the midvein of each petal, often referred to as the crest (Burks, 2002) (Figure 1-1B). Robust starchy roots anchor this species in the sediment (Figure 1-1D).

Crested floating heart typically infests the shallower, quieter water of lakes, ponds and canals but has been observed growing in open water depths of 3 m or greater (Burks, 2002; Page, 2010; Willey, 2012). In Lake Marion, the infestation began in a protected cove but spread to infest open water areas and areas with heavy disturbance, where reportedly native species do not grow (Page, 2010). Crested floating heart has also been observed growing with stems and leaves out of the water in saturated soils (personal observation) (Figure 1-2).

_Nymphaoides cristata_ was initially phylogenetically mapped as a sister taxa to _Nymphaoides cordata_ (Tippery et al., 2008). Later it was determined through genetic data that _Nymphaoides cordata_ and big floating heart _Nymphaoides aquatica_ were sister taxa and _N. cristata_, while genetically similar, could not be confirmed as the ancestor of the two taxa (Tippery and Les, 2011). Tippery and Les (2011) also found evidence of hybridization among _Nymphaoides_ species. Crested floating heart hybrids have yet to be documented in the published literature; however, hybrid plants have been observed in the Lake Marion systems (Dr. Rob Richardson, pers. comm.).
Crested floating heart reproduces primarily via vegetative reproduction, including tubers, rhizomes, and ramets (Burks, 2002; Willey and Langeland, 2011). Leaf fragments have been observed to produce bulbils with roots and leaves along insect damaged edges (Nair, 1973). Whether or not these bulbils produce independent plants has yet to be studied. The number of ramets produced is strongly influenced by sediment fertility and planting season and to a lesser degree by substrate composition (Gettys et al., 2017). Mature crested floating heart plants growing in a substrate with moderate nutrient levels are expected to produce approximately 350 ramets per plant in 6 months (Gettys et al., 2017). These ramets can break off naturally; however, fragmentation of the ramets from parent plants can be exacerbated by disturbances, such as boat traffic (Burks, 2002). Once detached from the parent plant, ramets will float for a time, during which they can be transported by wind or water currents to new areas for infestation (Burks, 2002). Ramets will eventually become less buoyant and settle on the sediment surface, where they can remain in what appears to be a quiescent phase (Willey and Netherland, 2015). Ramet sprouting has been shown to be strongly influenced by the level of sediment burial, with surface ramets and partially buried ramets having the highest sprouting numbers (Gettys et al., 2017).

In addition to vegetative reproduction of ramets, there is potential that crested floating heart may spread via sexual reproduction. In India, both female and bisexual flowers have been noted, indicating the plant is gynodioecious (Nair, 1973; Tippery et al., 2008). These bi-sexual flowers have been confirmed as self-compatible through artificial fertilization and successful cross tests have shown that female flowers can be pollinated by bi-sexual flowers (Nair, 1973). Crested floating heart seed viability has also been confirmed in its native range (Mason, 1996). Prominent pollen covered anthers indicate the flowers observed in South Carolina, North Carolina, and Florida are likely to be bi-sexual (Burks, 2002; personal observation) (Figure 1-3).
In India, pollinated fruit typically contain 3-4 seeds (Nair, 1973). Observations of South Carolina populations suggest that the immature fruit will produce approximately 10 ovules and 1 to 2 seeds per fruit (personal observation). The seeds are covered in tiny protrusions (tuberculate) which help them float on the surface of the water (personal observation) (Figure 1-4). Seed viability in the United States has yet to be confirmed.

**Impacts and Invasive Potential**

Nutrients will generally move over time from uphill sites of accumulation in terrestrial systems downslope to lacustrine sites (Likens and Bormann, 1974). It has been suggested that heavy nutrient loading to a waterbody will ultimately result in the displacement of submersed aquatic vegetation with floating leafed vegetation (Portielje and Lijklema, 2008; Scheffer et al., 2003). Floating leafed aquatic plant species have several competitive advantages over submersed species, including superior access to the limited resources, CO₂ and light (Wetzel, 2001). Crested floating heart forms dense mats of overlapping leaves on the water’s surface (Gopal, 1993; Willey and Langeland, 2011). These mats have been shown to attenuate light, disrupt the diffusion of O₂ and CO₂ from the air to the surface waters, and disrupt natural water flow patterns. Clearly infestation by this species will negatively impact recreation and wildlife habitat as well. In its native range, crested floating heart is considered an agricultural weed due to its negative impacts on rice production (Burks, 2002; Gopal, 1993).

Crested floating heart is an example of a highly invasive aquatic plant species being released from the aquarium trade or contaminated water gardens (Burks, 2002; Netherland, 2011). *Nymphoides cristata* has been classified as “High Risk” in a weed risk assessment conducted by the USDA (APHIS, 2012). The USDA predicts that *Nymphoides cristata* will likely invade approximately 11% of the US based on the distribution of the species in its native
range (APHIS, 2012). The predicted area of invasion is concentrated in the southeastern US, ranging as far north as Virginia and as far west as Texas but also includes parts of Puerto Rico and Hawaii as potential habitat (APHIS, 2012) (Figure 1-5). Despite this recognition of elevated risk, crested floating heart can easily be purchased through online retailers, adding to its dispersal potential (Burks, 2002; Maki and Galatowitsch, 2004; pers. comm. Steve Hoyle, NCSU).

**Management:**

Management of crested floating heart has had limited success (Burks, 2002; Page, 2010). Dibble and Kovalenko (2009) found multiple studies in their review of the literature that suggest that grass carp (*Ctenopharyngodon Idella* (Val.)) will avoid the *Nymphoides* genera. Grass carp reportedly do not feed on crested floating heart (Van Dyke et al., 1984). Even if grass carp would feed on crested floating heart, this would still not be an optimal management strategy as the species has been shown to proliferate under grazing conditions (Middleton, 1998; Middleton, 1990). Despite reported herbivorous insect damage in India (Nair, 1973), no insect biocontrol measure has been developed for use in the U.S. (Burks, 2002). The mechanical removal of the plant, using floating harvesters or hydro-rakes, makes little sense given the vegetative reproductive capabilities of the species (Burks, 2002). In addition, a study in its native range found that experimental underwater clipping of the plant did not reduce survivorship (Middleton, 1990). Hand-removal, even by suction assisted divers, is not likely to be a successful management option due to the immense and strong root systems of mature plants. Drawdown and exposure to freezing temperatures, has not provided control (Page, 2010). Pond dyes are generally not effective for control floating-leafed weeds in shallow water; however, some suppression of growth has been observed in deeper water (Kay, 2005). The use of selective herbicides is the most likely viable option for the control of crested floating heart.
Screening herbicides for control of crested floating heart has also had limited success until recent years. Submersed treatments at or near maximum label rates with 2,4-D amine (liquid), 2,4-D ester (granular), bispyribac-sodium, carfentrazone, flumioxazin, and triclopyr (liquid and granular), did not result in significant control (Willey et al., 2014). Similarly, foliar applications with triclopyr, 2,4-D amine, endothall dipotassium salt, penoxsulam and glyphosate did not provide consistent significant reductions in biomass (Willey et al., 2014). Willey et al. (2014) did note that while penoxsulam did not provide significant shoot damage, roots tissues were severely damaged due to treatment, and this damage warrants further exploration of the herbicide for control of crested floating heart (Willey et al., 2014). While the majority of active ingredients screened did not significantly control crested floating heart, submersed applications of endothall (amine salt) or diquat, and foliar applications of imazamox or imazapyr, did provide effective reductions in crested floating heart biomass (Willey et al., 2014). In a mesocosm study, a mixture of flumioxazin (0.43 kg ai ha\(^{-1}\)) and glyphosate (6.05 kg ai ha\(^{-1}\)) provided 100% control of root and shoot tissue for the species at six weeks after treatment (Glomski et al., 2014). In the same study, adding glyphosate to another protox-inhibitor, carfentrazone, increased control; however, the maximum control achieved with this combination was approximately 85% six weeks after treatment (Glomski et al., 2014). Beets and Netherland (2018) found that a 72-hour exposure to 24 µg L\(^{-1}\) florpyrauxifen-benzyl provided a 100% reduction in aboveground biomass and 80% decrease in belowground biomass. Willey and Netherland (2015) compared efficacy of the contact herbicides dipotassium salt of endothall, amine salt of endothall, and diquat on detached quiescent ramets and reported only diquat provided significant control (Willey and Netherland, 2015). These results are in contrast to the previously discussed efficacy screening in which both the amine salt of endothall and the dipotassium salt of endothall also
provided significant reductions in mature crested floating heart plant biomass (Willey et al., 2014). The authors speculate that these differences may indicate that some physiological processes may be reduced in quiescent separated ramets as compared to ramets still attached to the parent plant (Willey and Netherland, 2015).

**Synthetic Auxins**

*Introduction:*

Group 4 herbicides are known as synthetic auxins. The first synthetic auxins were discovered in the early 1940s through four independent and roughly simultaneous discoveries (Troyer, 2001). These herbicides are similar in structure to natural auxins or indole-3-acetic acids (IAA). Auxins are a class of hormones most often associated with cell growth and division, and they function as signaling molecules in most aspects of plant growth and development (Grossmann, 2010; Taiz et al., 2015; Yamada, 1954). Ethylene and auxin interactions have been shown to play a significant role in growth and apical dominance in aquatic plants such as *Regnellidium* spp., in the submersed growth of rice coleoptile and in cell extension and shoot elongation for *Hydrocharis morsus-ranae* (L.), *Regnellidium diphyllum* (Lindm.), and *Ranunculus sceleratus* (L.) (Cookson and Osborne, 1978; Walters and Osborne, 1979; Yamada, 1954). Auxins are also essential for tropism responses in both terrestrial and aquatic plants (Grossmann, 2010). At high concentrations, however, auxin is toxic to plants; therefore plants have evolved sequestration and degradation processes, called homeostatic control mechanisms, that help regulate auxin levels in plant tissues (Taiz et al., 2015). In susceptible plants, synthetic auxins have the same impacts as natural auxin overdose. However, synthetic auxins are more stable within plants and less susceptible to inactivation as compared with naturally produced auxins (Woodward and Bartel, 2005). The historic theory, suggested that synthetic auxins caused
plants to essentially “grow themselves to death” (Gilbert, 1946). Grossmann (2010) argues that it is more accurate to think of the action of synthetic auxin overdosing in three phases; the stimulation phase during which plant metabolic activity is heightened and abnormal growth occurs, such as stem curling and leaf epinasty; the inhibition phase, during which growth is stunted and several responses reducing physiological growth occur, such as stomatal closure and reduced carbon fixation, and finally the decay phase, characterized by cell and plant tissue death. The feedback mechanisms involved in this phase progression is much more complex than that proposed by Gilbert in 1946, and it is because of these complexities that auxin mimics have differential action on monocots versus dicots and among different dicot species (Grossmann, 2010). Other studies have found differential affinity between IAA and 2,4-D for influx and efflux carriers and therefore speculate that differential expression or activity of these carriers may be responsible for the differential herbicide action among species (Delbarre et al., 1996).

Florpyrauxifen-benzyl

Synthetic auxins are easily transported throughout the plant via the same pathways as natural auxins. This herbicide mode of action in aquatic systems has been shown to be selective, with dicots being quite sensitive and monocots and unicellular algae being virtually insensitive (Cedergreen and Streibig, 2005; Grossmann, 2010; Netherland, 2009). The most recently developed synthetic auxin is florpyrauxifen-benzyl (Busi et al., 2017). Florpyrauxifen-benzyl was initially developed by Dow Chemical for use against common weeds in rice including barnyardgrass [Echinochloa crus-galli (L.) P. Beauv.] and some sedges (Cyperus spp.) (Busi et al., 2017; Miller, Norsworthy, and Scott, 2017). Studies have found that simulated drift of florpyrauxifen-benzyl from rice fields was injurious to soybean crops, and as such careful
consideration of drift risk should be considered in this use pattern when soybean fields are nearby (Miller and Norsworthy, 2017; Schwartz-Lazaro et al., 2017).

The carboxylic acid functional group on florpyrauxifen-benzyl and other picolinate auxin herbicides allows for the herbicide to pass through the lipophilic phloem wall and due to the low pKa, the herbicide will ionize into the anionic form inside the phloem effectively concentrating the herbicide in the phloem (Epp et al., 2016). This process is called phloem trapping and allows for the systemic movement of florpyrauxifen-benzyl to growing shoot and root tissues (Epp et al., 2016). In addition to phloem mobility aryl-picolinates are known to have increased efficacy at comparatively low use rates (Busi et al., 2017).

In a small scale efficacy screening, researchers found Eurasian watermilfoil (Myriophyllum spicatum L.) and dioecious hydrilla to be extremely sensitive to florpyrauxifen-benzyl with dry weight effective concentrations (EC\textsubscript{50}) for the parent compound (SX-1552) of 0.11 mg ai L\textsuperscript{-1} and 1.4 mg ai L\textsuperscript{-1}, respectively, and EC\textsubscript{50} values for the acid metabolite (SX-1552A) of 0.23 mg ai L\textsuperscript{-1} and 2.0 mg ai L\textsuperscript{-1}, respectively (Netherland and Richardson, 2016). The high sensitivity noted for Eurasian watermilfoil in these initial small-scale studies carried through to larger mesocosms trials with an EC\textsubscript{50} value of 0.12 mg ai L\textsuperscript{-1} for the parent compound and an EC\textsubscript{50} value of 0.58 mg ai L\textsuperscript{-1} for the acid metabolite. Growth chamber efficacy screenings found crested floating heart (Nymphoides cristata Roxb.) and elodea (Elodea canadensis Michx.) to be less sensitive than Eurasian watermilfoil, although still highly sensitive with EC\textsubscript{50} values for the parent compound of 5.6 mg ai L\textsuperscript{-1} and 6.9 mg ai L\textsuperscript{-1}, respectively, and EC\textsubscript{50} values for the acid metabolite of 23.9 mg ai L\textsuperscript{-1} and 13.1 mg ai L\textsuperscript{-1} respectively (Netherland and Richardson, 2016). This high sensitivity of crested floating heart was confirmed in a later study conducted by Beets and Netherland (2018), in which these researchers observed complete control of
aboveground biomass and 80% reduction in belowground biomass with a 72-hour exposure to 24µg L\(^{-1}\) florpyrauxifen-benzyl. Netherland and Richardson (2016) found the native species megalodonta (\textit{Bidens beckii} Torr. ex Spreng.) was comparatively less sensitive to florpyrauxifen-benzyl than invasive weeds in the screening with EC\(_{50}\) values of 11.3 mg aiL\(^{-1}\) and 14.5 mg ai L\(^{-1}\) for the parent compound and acid metabolite respectively. Research on the target and non-target impacts of florpyrauxifen-benzyl is on-going and registration for aquatics use in the United States occurred on February 27, 2018 (pers. comm. Mark Heilman, Sepro corp.).

\textit{Aquatic Herbicide Absorption and Translocation Research}

Absorption and translocation studies with radiolabeled aquatic herbicides began in the early 1960s (Nissen, 2018). Historically, roots and shoots were separated by inserting the roots through a small hole in a rubber stopper and sealing the edges with a silicone stopcock grease (Funderburk and Lawrence, 1963a, 1963b). Then, the stopper was placed in a small plastic bottle, effectively creating two separate environments such that any radioactivity observed in the section of plant below the stopper could be attributed to translocation (Funderburk and Lawrence, 1963a, 1963b). Authors then used an autoradiograph of the entire plant to note areas where the 14-C labeled herbicide was present (Funderburk and Lawrence, 1963a, 1963b).

Funderburk and Lawrence (1963b) found that simazine was translocated from shoots to roots of waterstar grass [\textit{Heteranthera dubia} (Jacq.) MacMill.] and vise-versa. Funderburk and Lawrence (1963a) found using autoradiographs that 2,4-D acid, ametryne, fenac, and prometryne moved slightly from the shoot to the root of waterstar grass and that 2,4-D butoxyethyl ester moved to a much greater extent in both directions but primarily from the shoot to the root. Typically for emergent weeds, plants are treated on a single leaf and the movement of the herbicide from the leaf to the shoot is measured (Funderburk and Lawrence, 1963a).
More recently, the protocol for the use of radiolabeled aquatic herbicides to study the absorption and translocation in aquatic plants has been improved considerably (Kniss et al., 2011; True-Meadows, 2012; Vassios et al., 2014; Vassios et al., 2017; Nissen, 2018). In most modern absorption and translocation studies of aquatic herbicides in submersed species, small (10-15 cm) plants with established rooted are planted in glass vials filled with washed silica sand or soil (Nissen, 2018). To separate the shoot tissue from the root tissue, modern studies use some form of low melting point wax or agar gel poured over top of the sand around shoot tissues, thereby separating shoot and root tissue without damaging the plant (Kniss et al., 2011; True-Meadows, 2012; Vassios et al., 2014; Vassios et al., 2017). Radiolabeled herbicide is then applied to the water. A total time course and set intervals for measurement are defined with small preliminary studies to capture the near linear increase in absorption until an asymptote is reached (Nissen, 2018). At each of these time intervals, plants are harvested and shoot and root tissue is separated and dried. Dried tissue is then oxidized in a biological sample oxidizer and captured in scintillation cocktail. The radioactivity in each sample is then read on a liquid scintillation counter (LSC) (Kniss et al., 2011; True-Meadows, 2012; Vassios et al., 2014; Vassios et al., 2017). Mean radioactivity per gram dry weight is then plotted for each time point and a regression analysis is run (Vassios et al., 2014). These methods allow for better quantification of herbicide absorption and translocation than the original autoradiography (Nissen, 2018). Use of high performance liquid chromatography (HPLC) can provide even more information than analysis with an LSC (Nissen, 2018). Rather than just quantifying the total radioactivity in a sample, the use of HPLC can also determine the quantity of each of the forms an herbicide is in analyzed tissues (Nissen, 2018).
Literature Cited


Figures and Tables:

Figure 1-1: Photographs of anatomical features of crested floating heart. (A) Floating leaf with cordate base. (B) Characteristic crest along the mid-vein of the flower petal (blue arrow) and a smoother margin with a brown tint (yellow arrow) (C) underside of the leaf with reduced glands. The umbellate flower cluster arises from below the parent leaf and a ramet forms from the petiole of the parent leaf. (D) Large, robust root structure of crested floating heart. A quarter is included in the photo for scale.

Photo credit: Logan Wilson
Figure 1-2: Crested floating heart growing in saturated soils, without standing water.
Figure 1-3: Suspected bi-sexual flower observed in greenhouse-grown plants originating from a population in Lake Marion, South Carolina.
Figure 1-4: Dissected crested floating heart fruit. Two seeds are visible (red arrows). Seeds are covered with tiny protrusions (tuberculated).
Figure 1-5: Figure 1 extracted from “Weed Risk Assessment for Nymphoides cristata (Roxb.) Kuntze (Menyanthaceae) – Crested Floating Heart”. Predicted area of spread in the U.S.A. for Nymphoides Cristata, shown in red. This estimate was based upon three climatic variables and the species native range. Authors note that Alaska, Hawaii and Puerto Rico are not to scale. (APHIS, 2012)
CHAPTER 2: Monoecious *Hydrilla verticillata* Growth in Complete Darkness

Abstract

*Hydrilla verticillata* is one of the most problematic invasive submersed aquatic weeds in the United States. A study was conducted in growth chambers to look at aspects of growth of monoecious *Hydrilla verticillata* in complete darkness. A single tuber was placed in each of forty-eight dark growth chambers. Following differential blackout intervals of zero, two, four, six, eight, and ten weeks, plants in each treatment group were dissected into above ground (shoot) and below ground (tuber) material. Plant sections length, dry weight, and non-structural carbohydrate content were determined. Shoot lengths increased by 32 cm following ten weeks of dark exposure, compared to the zero-darkness exposure. Despite the increase in total shoot length, total dry weight decreased from 34.6 mg (zero-darkness) to 25.1 mg after ten weeks of dark exposure. During this time, tuber dry weight declined from 34.6 mg (zero-darkness) to 7.6 mg and shoot dry weight increased to 17.4 mg. Starch was the most prominent non-structural carbohydrate present in plants throughout the experiment. Starch levels were highest in plants prior to germination (31% of dry weight) and declined significantly and steadily over the course of the 10-week dark growth experiment to an average of 20% of dry weight. The results of this study indicate that monoecious hydrilla has a high elongation and development potential over long periods of time in darkness that could help overcome light blocking measures.

Introduction

Invasion of lentic and lotic freshwater ecosystems by non-native, invasive aquatic plant species leads to detrimental impacts to recreation, water quality, and wildlife habitat, and also disrupts predator-prey interactions (Covich et al., 2004; Engelhardt and Ritchie, 2001; Gettys et al., 2014; Pitlo and Dawson, 1993). *Hydrilla verticillata* (L. f.) Royle is arguably one of the worst invaders in the United States at present. In the United States, there are two biotypes of
*Hydrilla verticillata*, a dioecious biotype, most prevalent in the Southeastern United States and a monoecious biotype, observed more commonly in the Mid-Atlantic and Northeastern United States (True-Meadows et al., 2016). Most of the initial research on the species focused on the dioecious biotype. However, in recent years the body of knowledge surrounding the monoecious biotype has been growing (True-Meadows et al., 2016). The most cost-effective methodology for the control of monoecious hydrilla is the use of selective herbicides in combination with the release of triploid grass carp. However, in many northeastern states the release of triploid grass carp is prohibited and the use of herbicides can be severely limited.

A major invasive aquatic plant management technique and the only remaining alternative to biological and chemical management is physical or cultural management. Physical or cultural management of invasive aquatic plants can take many forms from hand-pulling to altering growing conditions through aeration (Belaud, 2014). For submersed aquatic plant species, light blocking strategies such as benthic barriers can be a highly effective physical management technique for the control of small isolated infestations (Eichler et al., 1995; Helsel et al., 1996; Hofstra and Clayton, 2012; Perkins et al., 1980). Bottom barriers are generally recognized as nonselective for the control of macrophytes, negatively impacting photosynthesis and growth of native and invasive species alike (Belaud, 2014). However, some studies have shown variability in the degree of growth suppression by species (Hofstra and Clayton, 2012), and some studies have found increased shoot length for some aquatic plant species in response to decreased light transmittance to the sediment (Engelhardt, 2006; Steward, 1991). If the duration of the decreased light environment is not long enough to kill the invasive plants, then the longer shoots might constitute a competitive advantage for these species when the light returns.
Field studies have shown a significant reduction in emergence and biomass of hydrilla using certain light blocking benthic barriers, with higher density barrier showing greater control (Hofstra and Clayton, 2012; Wood, 2017). These results are congruent with other studies indicating that hydrilla has a very low light requirement for photosynthetic activity and thus would require high density fabric to inhibit growth (Steward, 1991; Van et al., 1976). One laboratory study confirmed that monoecious hydrilla should not display photomorphogenic growth in less than 1% incident light (Steward, 1991). In preliminary research, an alternate skotomorphogenic development pattern was noted for monoecious hydrilla in complete darkness (Steve Hoyle, pers. comm.). When bottom barriers are used as a management technique, hydrilla only needs to extend shoot tissues skotomorphogenically to a fabric seam to take advantage of higher photosynthetically active radiation (PAR). Observations of submersed aquatic plant growth between seams of benthic fabric are prevalent in the literature (Eichler et al., 1995; Helsel et al., 1996; Killgore, 1987). It should be noted that when dark growth and development or skotomorphogenesis is discussed in this paper, the authors are not referring to the addition of biomass but rather stem extension and the development of structures in darkness, such that upon a change in light availability the plants will be better equipped to begin photosynthesis. This type of dark growth has been observed for other submersed aquatic weeds including sago pondweed \textit{(Stuckenia pectinata} (L.) Borner} (Dr. John Madsen, pers. comm.) Given the large starch reserves in monoecious hydrilla tubers (Miller et al., 1976; Pesacreta, 1990) and the observed dark growth response (Steve Hoyle, unpublished data); it is conceivable that hydrilla would be capable of skotomorphogenesis under a bottom barrier and reach a fabric seam, where it would have access to sufficient light to photosynthesize.
In addition to growth under bottom barriers, skotomorphogenesis would allow hydrilla plants to grow in more turbid water, deeper water, or in dense aquatic plant growth, where light attenuation to the sediment surface is too low for photosynthesis. This competitive advantage would allow hydrilla to develop via skotomorphogenesis until a portion of the water column with higher PAR can be reached. Additionally, upon reaching that portion of the water column, hydrilla would have developed the structures necessary to take advantage of the higher PAR.

Previous studies have evaluated monoecious hydrilla growth, carbohydrate storage, and allocation during a photomorphogenic response (Madsen and Owens, 1998; Pesacreta, 1990). Additionally, Wood (2017) sought to characterize growth of monoecious hydrilla under varying light regimes, including complete darkness. In the aforementioned study, tubers were sprouted in well-lit conditions and grown for 7 days prior to being covered in black plastic bags to simulate complete darkness. While this methodology is akin to the use of light blocking strategies on pre-sprouted tubers, it does not answer questions regarding dark sprouting followed by dark growth, such as that which may occur beneath a complete coverage benthic barrier already in place or at depths within the profundal zone.

Further study of the growth and development of hydrilla in the absence of light is needed. The results of these studies will be useful in informing light blocking management decisions, better informing the delineation of a profundal zone, and increasing the understanding of the competitive advantages of this highly invasive species. The objective of this study was to determine whether monoecious hydrilla could sprout and continue to grow in complete darkness and to characterize that growth and the starch depletion necessary to support it. It is hypothesized that monoecious hydrilla will sprout and undergo high levels of elongation and development over long periods of time in darkness that could help overcome light blocking measures.
Methods

Tuber collection:

Monoecious hydrilla tuber collections were conducted on two dates at Shearon Harris Reservoir in Holly-Springs, North Carolina on September 24, 2015, and January 26, 2016. Tubers were collected after 9 PM on an evening of less than ¼ moon or greater than 85% cloud cover to limit light exposure to the greatest extent feasible. Aliquots of the top six inches of sediment were sifted through a ¼ inch mesh screen until 100 tubers were collected. Green headlamps were used for visualization while sifting sediment in search of tubers. Once collected, tubers were then placed in a ziplock bag with pond-water, and this bag was transported back to the lab in a small cooler. Tubers were stored in pond-water overnight in an open top bucket within a dark room.

Dark growth conditions:

Dark growth conditions were ensured in several ways. The room in which plants were stored during the experiment had no windows and just one door. Light blocking fabric was hung on outside of the door such that light was blocked from entering the room via any gaps between the doorframe and the door. The growth chambers themselves were sprayed with a light-blocking black spray paint as an added precaution against accidental light exposure, which did not occur. A thin strip of clear plastic spanning the length of the growth chamber was left unpainted to allow for viewing the plants during the experiment. This strip was turned away from the door when not in use. All measurements were conducted in the dark room utilizing green light, as this wavelength is the least absorbed by chlorophyll and carotenoid pigments and is not important in triggering responses for phytochromes, cryptochromes or phototropins (Taiz et al., 2015).
Experimental set up:

The morning following collection, a single tuber was placed in each of 48 growth chambers filled with tap water, which was dechlorinated and conditioned using a tap water conditioner product (API Tap Water Conditioner © Mars Fishcare North America Inc. 50E. Hamilton St. Chalfont PA 18914). Half of the water in each chamber was exchanged once every two weeks with fresh dechlorinated and conditioned tap water. Tubers were randomly assigned to one of five experimental groups or to one of the two control groups. Experimental groups were exposed to 2, 4, 6, 8, or 10 weeks of time in complete darkness. One of the control groups was harvested immediately for analysis without exposure to dark growth conditions. This initial control was to determine pre-sprouting conditions. Another control group was planted in the same growth chambers as the experimental groups. However, these chambers were maintained in a greenhouse with natural sunlight for a total of 10 weeks. Due to space and materials constraints only one natural light control harvest was conducted. This secondary control was to determine potential container effects. The second run of the greenhouse container controls died due to a laboratory error in maintenance and as such only one run of these controls (n = 8) is included in the study.

The length and width of all tubers were measured prior to placement in growth chambers. Sprouting of unharvested tubers was monitored once every two weeks. Eight replicate plants were harvested once every two weeks following the assigned darkness exposure. Once harvested, the total shoot length and tuber length and width were measured for each experimental unit. Following these measurements plants were divided into shoot and tuber material and dried at 70°C for 48 hours (Pesacreta, 1990). Once dried to a constant mass, dry biomass measurements
of the shoot and tuber tissue were collected separately. Dry plants were then stored in a freezer at -4 °C until the carbohydrate analysis could be conducted.

**Carbohydrate Analysis:**

In addition to dimension and biomass measurements, a nonstructural carbohydrate profile was conducted following a modified version of the microplate enzymatic assay protocol described in Zhao et al. (2010). Due to the low biomass of hydrrilla tubers and stems, the entire plant was utilized for analysis, rather than a sub-sample of the plant as suggested in Zhao et al. 2010. In order to account for the varying dry weight of samples, the results will be discussed as percent of dry weight (Madsen and Owens, 1998; Pesacreta, 1990). Samples were frozen in liquid nitrogen and ground with silica beads in a Qiagen Tissue Lyser II at a frequency of 30 Hz for 45 second time slots until completely ground and homogenized. Following the Zhao et al. (2010) protocol, the samples were then digested using serial enzymatic digestions to break down starch, sucrose, and fructose into glucose. Fructans were not analyzed in this experiment. Aliquots of these digestions and glucose standards created by serial dilution were pipetted in triplicate into 96-well plates. A glucose assay reagent (Sigma G3285) was then applied. The increase in absorbance at 340nm was read in a microplate reader following each additional digestion and was directly proportional to glucose concentration in each sample at each stage of digestion.

**Statistical Analysis:**

Rotted tubers were removed from analyses, and data from both runs were combined. An ANOVA followed by Tukey-Kramer means separation was run for the means of each metric described above to compare differences among experimental and control groups.
**Results and Discussion:**

**Sprouting:**

After just two weeks of growth in complete darkness, 68% of unharvested tubers had sprouted (Table 2-1). These results are similar to those observed by Miller et al. (1976), in which 58% to 63% of dioecious tubers sprouted following 14 days in complete darkness. In the same experiment, dioecious tubers exposed to continuous light for 14 days showed sprouting rates ranging from 93 to 100% (Miller et al., 1976). In the current study, after 10 weeks of incubation in complete darkness 88% of the remaining unharvested tubers had sprouted (Table 2-1). The percent germination observed in the current study, following 10 weeks of exposure to complete darkness, is similar to monoecious hydrilla germination rates observed in another study under continuous light (94%) (Van and Steward, 1990) or monoecious hydrilla tubers grown under natural light regimes in the current study (100%). It should be noted that removal of tubers from the sediment and the resultant exposure to an aerobic environment has been observed to increase tuber sprouting rates (Van and Steward, 1990).

While 100% of the 8 control plants sprouted, it should be noted that 37% of these plants subsequently rotted and were removed from the study. Perhaps this rotting was a container effect. However, only 4% of tubers grown in the same containers under dark growth conditions rotted and these tubers never sprouted. This demonstrates that it may have been another environmental impact of the greenhouse, such as a buildup of periphytic algae or higher water temperatures, rather than the container leading to the rotting of sprouted tubers.

**Growth:**

Prior to dark exposure, tuber width (p=0.34) and length (p=0.17) did not statistically differ between harvest groups (Figure 2-1). As the trial progressed, stem lengths increased
steadily to an average of 32 cm after 10 weeks of growth in complete darkness (Figure 2-2). This average longest stem length did not statistically differ from control plants grown in the same containers but exposed to full sunlight in a glass greenhouse (Figure 2-2). While lateral branching was not measured quantitatively in this study, far less branching was qualitatively observed in the dark exposures as compared to that observed in greenhouse grown control plants (Figure 2-3). Van et al. (1977) observed that the most branching and the highest decrease in stem elongation occurred under red and blue wavelengths of light for dioecious hydrilla. These wavelengths would be most prevalent nearer to the surface of the water and at higher irradiance levels (Wetzel, 2001). In another study, monoecious hydrilla stem lengths also increased with reduced light availability, and it was suggested that perhaps the absence of red light, which inhibits stem elongation, was responsible for the reduction in the number of lateral shoots (Steward, 1991). The increased stem elongation and reduction in branching observed in the present study is likely indicative of a response to low irradiance. This type of response would allow a plant to grow upward into portions of the water column with higher irradiance and with wavelengths more conducive to photosynthesis (Wetzel, 2001). Such a behavior is particularly advantageous in deep water, waters with high levels of turbidity or areas with dense aquatic plant growth, where irradiance at the sediment water interface is comparably low (Kirk, 1994; Wetzel, 2001).

Overall, dry biomass declined over the course of the experiment from 34.6 mg initially to 25.1 mg after ten weeks of growth in total darkness; however, this difference was not significant (Figure 2-4). There was a significant negative correlation ($\beta = -0.001; p = 0.0004$) between darkness exposure length and total dry biomass; however the strength of this relationship was weak ($r^2 = 0.12$). These limited declines in overall biomass may suggest only minimal losses of
carbohydrates to respiration and heat. The ten-week dark growth dry biomass mean (25.1 mg) was significantly lower than the average dry biomass observed for control plants grown in sunlight for ten weeks (44.4 mg) (Figure 2-4). These results are consistent with a previous study on monoecious hydrilla in which decreasing total biomass correlated with decreasing percent ambient PAR (Steward, 1991) and another study in which significantly lower dry weights were observed for dioecious hydrilla grown under green light as compared to red or blue light (Van et al., 1977). The dry biomass for the tuber tissues significantly declined from the time of planting (34.6 mg) to the end of the experiment (7.6 mg). The final mean tuber biomass after ten weeks in complete darkness did not significantly differ from the tuber biomass in control plants exposed to a natural light regime. This outcome indicates that photosynthesizing plants are also reliant on starch reserves within the tuber for initial growth. The significant decline in tuber biomass observed was mirrored by a significant increase in shoot biomass from the beginning of the experiment (0 mg) to after ten weeks in complete darkness (17.4 mg). After ten weeks of growth, shoot dry biomass was significantly higher in plants grown in the light (36.8 mg) as compared to those grown in the dark (17.4 mg) (Figure 2-4).

Leaves grown in complete darkness appeared yellow. This observation was particularly prominent at the apical meristems (Figure 2-5). While the yellow tissues were not analyzed for pigment content, it is speculated that this yellowing may be indicative of a buildup of carotenoids. Dioecious hydrilla grown under green wavelengths has been shown to have an increase in chlorophyll b, which is more suited to take advantage of the yellow wavelengths found at high depths in freshwater (Van et al., 1977).

During the two runs of this study on two occasions the formation of axillary turions was noted, and on one occasion, a second shoot was noted sprouting from an initial tuber (Figure
2-6). These results, while not common, indicate that vegetative reproduction is possible in the absence of light. The viability of axillary turions was not measured.

Carbohydrate analysis:

Starch was the most prominent non-structural carbohydrate in monoecious hydrilla plants in this experiment. Past studies of dioecious and monoecious hydrilla also found starch to be the most prevalent carbohydrate in tuber tissues as evidenced by the highest percent dry weight (% DW) (Madsen and Owens, 1998; J. L. Miller et al., 1976; Pesacreta, 1990). Starch reserves were highest in plants prior to germination (31% DW) and declined significantly and steadily over the course of the 10-week dark growth experiment to 20% DW (Figure 2-7). This significant decline in starch is indicative of the breakdown of starch molecules for the energy and growth observed in stem elongation metrics (Figure 2-2). Similarly, in past studies starch has been highest in un-germinated monoecious tubers (approximately 50% DW) and declined over an 8-week exposure to natural light regimes to approximately 12% dry weight in monoecious tubers and 30% dry weight in monoecious shoots (Pesacreta, 1990). Madsen and Owens (1998) found that starch reserves in the tubers of sprouted dioecious hydrilla plants remained between 40 to 60% of dry weight in field and container studies over the course of a year in Lewisville, Texas. In the current experiment, starch reserves after ten weeks of growth were 20% DW and 12% DW, for plants grown in complete darkness and those grown under natural light conditions, respectively. This difference was not statistically significant, indicating that light grown plants deplete starch reserves to a similar degree as dark grow plants. Similar to Pesacreta (1990), these results again confirm that monoecious hydrilla does deplete starch reserves following germination but that some starch does still remain even in complete darkness after 10 weeks.
Simple free sugars (glucose, fructose, and sucrose) increased significantly from unsprouted (2.3% DW) tubers to germinated plants grown for two weeks in complete darkness (3.9% DW). Biologically, this increase in free-sugars may be indicative of some low level of breakdown of starch and mobilization of free sugars, but the difference was relatively low from zero to 2 weeks and the pattern of significance did not carry through to future weeks as might be expected. Pesacreta (1990) observed low levels free sugars in tubers (5%) and a statistically and biologically significant increase in free sugar content in tubers during 2 and 4 weeks after planting (15% dry weight). However, the author observed no discernable pattern in free sugars in shoot tissues (Pesacreta, 1990). Perhaps by combining shoot and root tissue in this study some of the differences in tuber simple free-sugar levels were obscured by the lack of pattern in shoot simple free-sugar. Additionally, the higher levels of soluble sugars observed in Pesacreta may be due in part to photosynthate build up following photosynthesis, which did not occur in the current study. It follows that Pesacreta (1990) observed that decreasing photoperiod from 16 to 8 hours of light resulted in a significant decline in free sugars in monoecious hydrilla shoots.

Conclusions

In this experiment, significant increases in shoot elongation, increases in shoot biomass, and decreases in tuber biomass were observed when monoecious hydrilla tubers were sprouted and grown in complete darkness for 10 weeks. Additionally, following this dark growth period there were remaining starch reserves and free sugars, likely indicating that these plants could have continued to survive and grow beyond the 10-week period.

These results clearly have management implications. In particular, the fact that monoecious hydrilla plants can survive and grow for at least 10 weeks in complete darkness and achieve a stem elongation of 32 cm on average, indicates that light blocking strategies may have
limited effect on this plant. It should be noted that a loss of light is not the only management component for bottom barriers. In one study 600g coconut fiber mesh barriers were observed to kill sprouted tubers in a matter of 10 days in Florida mesocosms (Wood, 2017). Wood (2017) speculated that the drop in dissolved oxygen observed below these barriers in combination with low light availability resulted in the more rapid kill of these plants than previously observed. Some additional control has also been achieved with the combination of non-porous bottom barrier and slow release acetic acid for the control of curly-leaf pondweed [Potamogeton crispus (L.)], when porous and non-porous barriers alone did not appear to impact turion sprouting of curly-leaf pondweed (Barr and Ditomaso, 2014). Similar results were observed when instead of acetic acid, water above a temperature of 60°C was applied under barriers (Barr and DiTomaso, 2015). Future studies on the use of light blocking strategies for the control of monoecious hydrilla should focus on the combination of light blocking strategies to deplete starch reserves followed by other control methods, such as acetic acid, warm water temperatures, or contact herbicides.

The utility of light blocking strategies is not the only application of the results of this study. The distribution of submersed aquatic plants at varying depths is most influenced by the available light (Steward, 1991). Steward (1991) argues that knowing the depth at which available light to the sediment surface is less than 1% allows the researcher to predict the potential areas that could support hydrilla growth. We would argue that our results would add an average of 32 cm of depth to that max depth estimate, thereby increasing the littoral zone to be surveyed for monoecious hydrilla growth.


Killgore, K. J. (1987). Aquatic Plant Control Research Program: Evaluation of the diver-operated dredge and bottom-covering material for control of hydrilla in the potomac river. Department of the Army Waterways Experiment Station, Vicksburg MS.


Figures and Tables

Table 2-1: Percent of unharvested tubers that had sprouted at the given darkness exposure in weeks after planting (WAP) or in the control plants.

<table>
<thead>
<tr>
<th>Number of tubers remaining</th>
<th>2 WAP</th>
<th>4 WAP</th>
<th>6 WAP</th>
<th>8 WAP</th>
<th>10 WAP</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent sprouted</td>
<td>68.4</td>
<td>82.5</td>
<td>91.3</td>
<td>93.3</td>
<td>87.5</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 2-1: Initial tuber dimensions collected prior to planting and prior to light or dark exposure. Harvest group indicates the assigned harvest group not the time at which measurements were taken. Points represent the mean tuber dimension for a given harvest group (WAP) and error bars indicate the standard error of this mean. Means sharing a letter did not statistically differ in the ANOVA followed by Tukey-Kramer means separations.
Figure 2-2: Change in the longest shoot length over time. Points represent the average shoot length for a given harvest group in weeks after planting (WAP) and error bars indicate the standard error of this mean. Means sharing a letter did not statistically differ in the ANOVA followed by Tukey-Kramer means separations.
Figure 2-3: Images of Run 1 plants. Letters A, B, C, D, E and F correspond to 2 WAP, 4WAP, 6WAP, 8WAP 10WAP and the Control respectively. The white line on the ruler in each image is for scale and is placed at 20cm on the ruler. Image G is a closer view of the branching observed in the control plant circled in red in image F.
Figure 2-4 Change in the dry biomass of tubers, shoots and plant totals over time. Points represent mean dry biomass for a given harvest group and error bars represent the standard error of that mean. Means sharing a letter did not statistically differ in the ANOVA followed by Tukey-Kramer means separations.
Figure 2-5: Photo of monoecious hydrilla grown in complete darkness. Skotomorphogenic growth is apparent in elongated shoot tissues and lack of chlorophyll pigments. Red circles indicate representative areas suspected of elevated carotenoid content due to coloration.
Figure 2-6: a and b) Axillary turion production in complete darkness c) two shoots sprouted from a single subterranean turion.
Figure 2-7: Percent dry weight for starch and soluble carbohydrates (glucose, fructose and sucrose) over time. Points represent the average percent dry weight for a given harvest group in weeks after planting (WAP) and error bars indicate the standard error of this mean. Means sharing a letter did not statistically differ in the ANOVA followed by Tukey-Kramer means separations.
CHAPTER 3: Monoecious Hydrilla Verticillata Tuber Respiration

Abstract

The primary means of reproduction for monoecious hydrilla is through the prolific production of vegetative propagules, including subterranean and axillary turions. A study was conducted to elucidate changes in respiration rates of subterranean turions over the course of a winter season. Depletion of dissolved oxygen in deionized water, containing 10 tubers, was measured in an airtight biological oxygen demand (BOD) bottle utilizing an optical BOD probe with built-in agitation. Tuber respiration rate was measured in the light and dark and under varying temperatures each month from January through July. The light level that the tubers were exposed to did not affect respiration rate. Respiration rate and Q10 (temperature dependence coefficient) value did not differ from one month to another. Significantly higher respiration rates were observed for tubers maintained in a warm water-bath (-0.159 mg O₂ hr⁻¹ g⁻¹) as compared to those maintained in a cold water-bath (-0.084 mg O₂ hr⁻¹ g⁻¹). These results indicate that temperature may be an important factor in determining the respiration rate of subterranean tubers and thereby may impact the potential longevity of these tubers in the sediment and hydrilla population dynamics.

Introduction

*Hydrilla verticillata* (L. f.) Royle, commonly known as hydrilla, is a highly invasive non-native submersed aquatic weed in the United States. There are two biotypes of hydrilla that have currently invaded the US, a female dioecious biotype, which dominates in the southeastern US and a monoecious biotype, which dominates in the more temperature climates of the mid-Atlantic and northeastern United States. The monoecious biotype is the less studied of the two biotypes (True-Meadows et al., 2016). Where present, monoecious hydrilla negatively impacts recreational uses of the waterbody, hinders natural waterflow, causes changes in water quality,
adversely impacts wildlife habitat, and reduces native plant diversity through displacement (Langeland, 1996; True-Meadows et al., 2016).

Hydrilla has been named the perfect aquatic weed due to its high level of success as an invader (Langeland, 1996). Perhaps the most pronounced reason for this success is the variety and prolific nature of the reproductive capabilities of monoecious hydrilla. Monoecious hydrilla has been reported to produce viable seed (Conant, Van, and Steward, 1984; Lal and Gopal, 1993; Langeland and Smith, 1984; Langeland, 1996). However, hydrilla primarily propagates vegetatively through the prolific production of axillary turions, subterranean turions (tubers), and the fragmentation of stems (Langeland, 1996). While axillary turions must germinate within one year of production, subterranean turions (tubers), have been shown to remain viable for at least 4 years in Florida and at least 6 years in more temperate North Carolina (True-Meadows et al., 2016; Van and Steward, 1990). Tubers can remain viable in the sediment for several years due to high starch reserves and quiescence. This differential ecological longevity results in the build-up of a tuber bank within the sediment, which plays an important role in population dynamics of the species. It is because of this tuber bank that hydrilla can persist within a system even after multiple years of consistent successful management (Nawrocki et al., 2016). While quiescence should slow the rate of depletion of starch, carbohydrate stores have been shown to decline during this quiescence period (Ryan, 1994). Depletion of starch reserves during quiescence is in large part due to cellular respiration.

The range of monoecious hydrilla is expanding, and this expansion is particularly evident in the temperate northeastern US (True-Meadows et al., 2016). Monoecious hydrilla has been shown to produce tubers that are greater in number but smaller in size than dioecious hydrilla (Pesacreta, 1990; Spencer et al., 1987; Sutton et al., 1992; Van and Steward, 1990). However, in
temperate climates monoecious hydriila plants have been shown to produce fewer, yet larger
tubers as compared to monoecious hydriila in more southern climates (Henry, 2017). These
larger tubers produced in more temperate climates, such as the northeastern US, will have larger
carbohydrate stores, and therefore have extended potential longevity, the maximum duration of
viability or capacity to sprout, in the sediment than the current estimates based on southern
climates. However, as previously mentioned, depletion of these starch reserves will be in large
part dependent on the rate of cellular respiration in these tubers.

Respiration rates in aquatic plants have been shown to vary with differing environmental
conditions. For dioecious hydriila sub-apical plant segments, respiration rates increased
significantly after 16 hours of drying stress (Basiouny et al., 2017). Barko and Smart (1981)
observed increased dark respiration of hydriila shoot tissues with increasing temperature above
24°C. Researchers have also observed increased respiration rates with increasing temperatures
for turion and tubers of several carnivorous aquatic plants (Adamec, 2008). Stage of quiescence
or turion age may also impact respiration. The temperature coefficient ($Q_{10}$) is defined as the
increase in biological rate for every 10°C increase in temperature (Taiz et al., 2015). It is a
unitless expression of temperature dependence (Taiz et al., 2015). Adamec (2011) observed
higher respiration rate $Q_{10}$ (temperature coefficient) values in older spring (2.3 -3.4) harvested
turions as compared to the younger fall harvested turions (1.8 – 2.6). These results indicate a
higher temperature dependence in respiration rates for spring turions (Adamec, 2011). The
potential change in respiration rates of quiescent monoecious hydriila tubers over time at varying
temperatures and lengths of quiescence has yet to be studied. Understanding how the rate of
carbohydrate breakdown may change over time and at varying temperatures, will be important in
predicting the potential longevity of monoecious hydriila tubers in the sediment. This would also
be important in predicting the resultant impact on the number of years of consistent management required to control the tuber bank as the range of this species expands.

**Methods**

*Plant Material:*

Monoecious hydrilla tubers were collected from Shearon-Harris Reservoir in Holly Springs, North Carolina. Six-inch pots were filled with topsoil amended with osmocote fertilizer. One sprouted monoecious hydrilla tuber was planted in each pot. Black outdoor mesocosms (278.5 L) were filled with pond water and six planted pots were placed in each. Water was exchanged in mesocosms monthly. Plants were grown for one growing season from April to December. This methodology was employed to ensure that all tubers were of the same age class.

*Tuber harvesting:*

Following natural above ground plant senescence, four pots were randomly selected monthly from January 2017 through July 2017. Pots were removed from mesocosms and submersed in 15 L buckets filled with pond water for transport. In March following data collection, all remaining pots were moved to long-term cold storage in order to effectively extend the winter (Table 3-1). Cold storage was maintained at 4°C. Optimal sprouting for turions occurs between 15°C and 35°C (Netherland, 1997). It has also been shown that monoecious hydrolla tubers maintained at 4°C remain viable longer than colder freezing temperatures (Henry, 2017). Therefore, 4°C is warm enough to hinder freezing of the sediments but not warm enough to promote tuber sprouting. Pots were maintained in dark cold storage with the sediments submersed and undisturbed until harvesting.

To harvest tubers, aliquots of sediment from each pot, either collected at the mesocosms or from cold storage and were rinsed through a quarter inch mesh screen. All tubers were
collected and immediately placed in cold pond water. Preliminary studies indicated that to record respiration rates, a minimum of 8 to 10 tubers were required. If fewer than 10 tubers were collected from a pot, these tubers were disposed of and a new pot was randomly selected for harvest. Pots were sifted one at a time and then immediately processed. While the respiration rates of the tubers from a given pot were measured, the replicate pots remained undisturbed and submersed in the 15 L transport buckets at ambient temperatures or in cold storage. It should be noted that removal from hypoxic sediments and subsequent exposure to aerobic water likely triggers the start of physiological processes associated with tuber sprouting.

*Surface Sterilization:*

Surface sterilization is important to remove microbial respiration as a confounding factor. For the month of January, surface sterilization consisted of triple rinsing harvested tubers with DI water. Following the first analysis of tuber respiration, a marked difference in respiration rates for rinsate water under light and dark conditions was noted. It was hypothesized that this could be due in part to microbial influences and a stronger surface sterilization technique was developed in February for use in March (Table 3-1). Specifically, surface sterilization from March through July consisted of a triple rinse in deionized water, followed by submersion in a 5% household bleach (7% active) solution for 30 minutes with consistent low agitation. Following soaking in the bleach solution, tubers were again triple rinsed in deionized water. Past studies have exposed tubers to a 1.3% solution of pure sodium hypochlorite (bleach) with stirring for 20 minutes to sterilize tubers of bacteria and fungi prior to analysis (Pesacreta, 1990; Sutton, 1986). This 1.3% pure bleach solution is approximately equivalent to an 18% household bleach solution. To ensure tuber tissue integrity only a 5% household bleach solution was used in this study. Due to the possibility that this lower concentration of bleach may not control all bacteria
and fungi in the microsphere surrounding tubers, the measurement of oxygen changes in rinsate water continued for each condition and tuber set despite the surface sterilization.

Respiration Rates:

For each replicate pot, the combined respiration rate of 10 tubers was measured utilizing dissolved oxygen depletion within a 70 ml BOD (biological oxygen demand) bottle (Table 3-1). Oxygen depletion was measured under aerobic conditions using an optical ProBOD meter with built-in agitation and memory storage (Adamec, 2005, 2008, 2011; Van et al., 1976). A rubber stopper was drilled and fitted to create an airtight seal between the bottle and the meter. Prior to measurement, either an opaque or clear BOD bottle was filled with DI water at the appropriate temperature, tubers were placed, and the meter was then twisted into the neck allowing for overflow of excess water, such that air bubbles within the bottle were minimized. Temperature and dissolved oxygen were measured and recorded every 15 seconds for a total of 15 minutes under each condition (Barko and Smart, 1981). The tuber rinsate was measured following exposure to each set of temperature and light combination. During this time the tubers were placed in fresh DI water in the next set of conditions to acclimate for 15 minutes.

In January, respiration rates were only measured at ambient temperatures. Following the collection of results for January harvested tubers, it was posited that a comparative respiration rate at a higher temperature would be useful. Similar to a past study, the higher temperature selected for respiration was 30°C (Barko and Smart, 1981). Changes in dissolved oxygen were measured at ambient temperatures under both light (clear BOD bottle) and dark (opaque BOD bottle) conditions and at 30°C under dark conditions only. Ambient temperatures were determined by water temperature measurements collected from the outdoor mesocosms (Table 3-1). Following movement to cold storage, ambient temperature respiration rates were measured.
at 15°C. Both DI water with the 10 tubers present and the rinsate following measurement were measured under each set of conditions for a total of six data sets collected for each of four replicate samples of ten tubers.

Following respiration rate measurements, a total fresh weight measurement was collected for the ten tubers. Tubers were then dried to a constant mass at 70°C and dry weight measurements were collected.

*Data analysis:*

A simple linear regression model comparing changes in dissolved oxygen levels (mg L\(^{-1}\)) over time (seconds) was run for each dataset to calculate a respiration rate (slope) (Barko and Smart, 1981). All non-significant slopes were set to zero for subsequent calculations. The slope of each rinsate dataset was subtracted from the slope of the corresponding measurement collected with tubers present. This subtraction ensured that the rate of oxygen depletion examined was due to the tubers alone. Utilizing the tuber dry weights and the known volume of water in which measurements were collected, these rates were converted from mg per liter per second to mg of oxygen per hour per gram of dry weight. \(Q_{10}\) (temperature coefficient) values were calculated for each replicate set of tubers using the respiration rate in darkness at ambient temperatures and the respiration rate in darkness at 30°C (Table 3-1). This value is useful for examining the temperature dependence of a reaction rate.

An analysis of variance (ANOVA) followed by Tukey-Kramer means separation was run to elucidate differences by month among mean fresh weights and dry weights. A fixed effects model was run to examine the effects of temperature, light level, and tuber age (month) on the respiration rate. Differences among monthly mean \(Q_{10}\) values were analyzed by ANOVA followed my Tukey-Kramer means separation.
Results and Discussion

Twenty-four measurements of oxygen change over time in pure DI water were collected over the 7 months of the experiment as a control. These measurements were collected under lit conditions at an ambient temperature. Approximately 66% of the measurements resulted in a change in oxygen over-time that significantly differed from zero. 100% of the significant rates were positive, averaging $6.22 \times 10^{-5} \text{ mg O}_2 \text{ L}^{-1} \text{ sec}^{-1}$. These small positive significant results may indicate trace levels of oxygen producing organisms in 66% of the DI water measured. It is for this reason and the potential for the presence of surface microorganisms that may not have been removed during surface sterilization that all significant rates of change in dissolved oxygen over time measured in rinsate waters were subtracted from the corresponding measurement with tubers present.

It should be noted that the initial level of dissolved oxygen in the DI water in which tubers were measured ($8.55 \pm 0.07 \text{ mg L}^{-1}$) would be considered high relative to that which could be expected within the sediment. Dissolved oxygen in water likely only penetrates several centimeters down into the sediment (Wetzel, 2001). Eutrophic stratified lakes can reach oxygen depletion levels at the sediment water interface as low as $<1 \text{ mg L}^{-1}$ (Wetzel, 2001). The oxygen levels in the DI water measured in this experiment would be more akin to what might be expected at the sediment water interface of a well circulated lacustrine or lotic system (Wetzel, 2001). Respiration rates within the hypoxic sediments would likely differ from those measured here and would likely be more dependent on anaerobic (fermentative) metabolism (Taiz et al., 2015). As previously mentioned, the removal of the tubers in this study from sediment may have led to the onset of physiological processes associated with tuber sprouting. In one study, only 2% of monoecious hydrilla tubers germinated in-situ in the first year, however, greater than 90% of
tubers germinated when removed from the sediment (Van and Steward, 1990). As such, the data presented here may be more representative of disturbed tubers in highly circulated waters rather than quiescent tubers in hypoxic sediments.

Over the course of this study, monoecious hydrilla mean tuber fresh weights per replicate ranged from 47.5 mg tuber\(^{-1}\) to 170.4 mg tuber\(^{-1}\) with an overall average for the study of 105.7 mg tuber\(^{-1}\) (SE = 3.8 mg tuber\(^{-1}\)) (Figure 3-1A). The highest mean fresh weights were observed in the months of April (144.3 mg tuber\(^{-1}\)) and July (134.4 mg tuber\(^{-1}\)) (Figure 3-2A). These results are lower and less left skewed than observed mean fresh weights of monoecious hydrilla tubers grown in California (Spencer et al., 1987) but similar to reported mean fresh weights for tubers grown for one season in Raleigh (Henry, 2017). Monoecious hydrilla tuber fresh weight and dry weights were highly correlated, as expected (\(R^2 = 0.987\)). Mean tuber dry weights per replicate ranged from 22.3 mg tuber\(^{-1}\) to 87.9 mg tuber\(^{-1}\) with an overall average of 52.9 mg tuber\(^{-1}\) (SE = 2.04 mg tuber\(^{-1}\)) over the course of the current experiment (Figure 3-1B). These results, again, are lower than dry weights reported from studies in Davis, California (Ryan, 1994) but were similar to results of North Carolina collected tubers grown in Fort Lauderdale, Florida (Van and Steward, 1990). Similar to the fresh weight data for this study, dry weights were significantly higher for the tubers collected in the months of April and July (Figure 3-2B), whereas Ryan et al. (1994) observed no difference in dry weight over the course of the winter. These differences in fresh weight and dry weight of tubers observed in different studies likely points to the morphological plasticity of the species and is the primary reasoning behind analysis on a per gram dry weight basis rather than a per tuber basis.

Changes in month by month respiration rates could indicate the start of physiological processes related to sprouting, if quiescent tubers could be measured in-situ without microbial
influence. However as mentioned, the necessary removal of the tubers from the sediment, subsequent exposure to high levels of oxygen and surface sterilization, likely resulted in the onset of the break of quiescence. Instead, the respiration rate shortly after the break of quiescence is what was measured. The respiration rates of recently germinated seeds have been positively correlated to the vigor of those seeds (Woodstock and Grabe, 1967; Woodstock and Polluck, 1965). Seeds with higher vigor have a longer potential longevity. The month effect was not significant (p = 0.09) in the effects model for respiration rate, indicating a lack of response to tuber age in terms of the respiration rate of disturbed tubers in the first season after production (Figure 3-5). As such, there is not likely to be a link between the age of disturbed tubers within the first season and potential longevity. The authors speculate that if such an effect does exist, a longer study would be required to elucidate it. In contrast, field collected dioecious hydrilla shoot tissues had the highest dark respiration rates and CO₂ compensation points in the winter months and these rates declined by spring (Bowes et al., 1979). Perhaps the respiration of shoot tissue is more sensitive to seasonal changes in daylight due to decreased photosynthesis in winter.

In past studies, turions of some species of carnivorous plants have shown increases in $Q_{10}$ (temperature coefficient values) over the course of the winter, even without significant changes in average respiration rates at a set temperature (Adamec, 2011). In the current study, however, $Q_{10}$ results also did not differ from month to month (p = 0.34) (Figure 3-6). These results indicate that the temperature dependence of disturbed tuber respiration rates for monoecious hydrilla does not change as the first winter season progresses.

When measuring the response of tubers of the same age class (month) to differing temperature and light conditions, tuber vigor is no longer being measured. Instead, the change in
metabolism of disturbed tubers in response to different conditions is being measured. After accounting for potential impacts of rinsate water, light level (dark or light) did not significantly affect the rate of respiration ($p = 0.77$) (Figure 3-3). These results indicate that photosynthesis is likely not occurring in these subterranean tubers, despite having the morphological structure of a compressed preformed axillary buds (True-Meadows et al., 2016). It is possible that after a longer exposure to light these tissues could have produced chlorophyll and begun photosynthesis. However, in practice when subterranean turions are sprouted in full sunlight, the tuber itself does not appear to develop any physical indication of photosynthetic tissue, in terms of the coloration (Erika Haug, personal observation). Temperature, however, did have a significant effect for predicting respiration rates ($p = 0.0003$). Dark respiration rates significantly increased from ambient conditions to hot conditions (Figure 3-4). It must be speculated that these results are an exaggeration of the response of quiescent tubers, as the measurement of respiration rates of quiescent tubers in situ is not currently possible. Plant respiration rates often increase with increasing temperature from $0^\circ C$ to $30^\circ C$ (Taiz et al., 2015). Barko and Smart (1981) observed an increase in dark respiration of dioecious hydrilla shoot tissue with increasing temperature above $24^\circ C$. Aerobic respiration rates of storage organs, such as tubers or turions, are likely to be much lower than respiration rates for shoot tissue (Adamec, 2008). That said, respiration rates of six different carnivorous plant turions were also observed to increase with increased temperature (Adamec, 2008).

Conclusions

The results of this study have implications for the management of monoecious hydrilla tubers. Tuber respiration rates and temperature dependence (Q10) were not significantly impacted by the length of quiescence in the first year, however respiration rates significantly
increased in response to increases in temperature each month. The combination of these results
indicates that temperature, not age, may be a driving factor in respiration rates for disturbed
tubers in the first year after production. Past studies examining the ecological longevity, the
average length of quiescence under natural conditions, of monoecious hydrilla tubers in the field
were conducted in warmer mid-Atlantic to southern climates (True-Meadows et al., 2016; Van
and Steward, 1990). It is possible, given the results of this study, that the potential longevity, the
maximum duration of viability, of monoecious hydrilla tubers may be longer in northern colder
climes due to a reduction in respiration rates with average colder temperatures. An increase in
potential longevity is speculated to impact the ecological longevity. This hypothesis of increased
potential longevity in colder climates is further supported by the fact that monoecious hydrilla
tubers in colder climates tend to be larger and have also been shown to have higher viability than
those grown in southern climates when exposed to long-term laboratory cold storage (Henry,
2017). If the longevity of monoecious hydrilla tubers in northern climates is longer than that in
previous studies of monoecious hydrilla tubers in southern climates, then consistent annual
management would be required for a longer period of time in order to reduce the tuber bank.
This study provides additional support for further study to assess potential differences in the
longevity of monoecious hydrilla tubers in northern climates.
Literature Cited


Figures and Tables

Table 3-1: Surface cleaning methodology 1: Triple rinse with DI water and agitation. Surface cleaning methodology 2: Triple rinse with DI water and agitation, 30 minute soak in 5% household bleach with agitation, Triple rinse with DI water and agitation. Average ambient and hot temperatures provided in Celsius Mean ± Standard Error.

<table>
<thead>
<tr>
<th>Month</th>
<th>Storage</th>
<th>Surface Cleaning Methodology</th>
<th>BOD bottle (ml)</th>
<th>Ambient Temperature (°C)</th>
<th>Hot Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>mesocosms</td>
<td>1</td>
<td>300</td>
<td>17.3 ± 0.02</td>
<td>N/A</td>
</tr>
<tr>
<td>February</td>
<td>mesocosms</td>
<td>1</td>
<td>70</td>
<td>15.0 ± 0.02</td>
<td>30.4 ± 0.07</td>
</tr>
<tr>
<td>March</td>
<td>mesocosms</td>
<td>2</td>
<td>70</td>
<td>20.0 ± 0.01</td>
<td>30.9 ± 0.04</td>
</tr>
<tr>
<td>April</td>
<td>cold storage</td>
<td>2</td>
<td>70</td>
<td>15.2 ± 0.01</td>
<td>30.8 ± 0.04</td>
</tr>
<tr>
<td>May</td>
<td>cold storage</td>
<td>2</td>
<td>70</td>
<td>15.4 ± 0.01</td>
<td>30.5 ± 0.05</td>
</tr>
<tr>
<td>June</td>
<td>cold storage</td>
<td>2</td>
<td>70</td>
<td>14.3 ± 0.02</td>
<td>31.9 ± 0.06</td>
</tr>
<tr>
<td>July</td>
<td>cold storage</td>
<td>2</td>
<td>70</td>
<td>15.5 ± 0.08</td>
<td>32.3 ± 0.09</td>
</tr>
</tbody>
</table>
Figure 3-1: Distribution of per tuber biomass data (grams) collected over the course of the study (A) fresh weight measurements (B) dry weight measurements.
Figure 3-2: Mean per tuber biomass data (grams) per month. Error bars represent one standard error from the mean (A) fresh weight measurements (B) dry weight measurements.
Figure 3-3 Light level effect least squares means plot for the respiration rate effects model. Points represent least squares mean respiration rate at each light level and error bars represent one standard error.
Figure 3-4 Temperature effect least squares means plot for the respiration rate effects model. Points represent least squares mean respiration rate at each temperature and error bars represent one standard error.
Figure 3-5 Harvest month effect least squares means plot for the respiration rate effects model. Points represent least squares mean respiration rate for each harvest month and error bars represent one standard error.
Figure 3-6: Mean Q10 (temperature coefficient) values per month. Error bars represent one standard error from the mean.
CHAPTER 4: Crested Floating Heart Vegetative Reproduction via Leaf Fragmentation

Abstract

Invasive crested floating heart (*Nymphoides cristata*) has been rapidly spreading northward since it was first observed in Naples, Florida in 1996. Despite the apparent threat to waterways, little published data on the growth characteristics of this highly invasive plant are currently available. In 2014, research was initiated at North Carolina State University to determine whether vegetative reproduction could be induced by manual cutting and if so, at which developmental stages could fragmentation result in a new independent plant. Four leaves of three size classes were bisected transversely and monitored weekly for developmental stage and the formation of structures. Eighty-five percent of the monitored fragments produced independent plantlets within 15 weeks. Leaf size had a strong impact on the production of floating leaves from settled independent plantlets, with 92% of large leaf (26.7-77.3 cm²) plantlets producing floating leaves as compared to 21 and 17% of medium (9.1-22.7 cm²) and small leaves respectively (4.5-7.9 cm²). The results of this study serve to elevate awareness of the risks associated with this invader and preclude the use of any management technique or recreational pursuit that would result in the fragmentation of leaf structures.

Introduction

Crested floating heart (*Nymphoides cristata* (Roxb.) Kuntze) is a highly invasive non-native floating leaf aquatic plant species currently inhabiting the southeastern region of the United States. *Nymphoides cristata* has been classified as “High Risk” in a weed risk assessment conducted by the USDA (APHIS, 2012). The native range for the species includes parts of India, Pakistan, Vietnam, southern China, and Taiwan (Burks, 2002; Marwat et al., 2007). The species was first observed in the US in 1996 in Naples, Florida (Burks, 2002; Willey and Langeland, 2011). Following its initial introduction into a small residential pond, crested floating heart
spread throughout much of Florida via interconnected waterways (Burks, 2002). In 2005, the range of the species extended north into South Carolina when a population was found in Lake Marion (Willey and Langeland, 2011; pers. comm. Larry McCord). In the Lake Marion system the initial invasion expanded from covering 8 ha to covering an estimated 810 ha in just two years (Willey and Langeland, 2011). In 2014, the range of crested floating heart expanded further northward when a population was observed in a residential pond Burlington, North Carolina (pers. comm. Dr. Robert J. Richardson). There have also been confirmed populations in Louisiana and eastern Texas (Thayer and Pfingsten, 2016). The current extent of the invasion appears to be due to multiple introductions and is likely the result of discarding of contaminated cultivated plants into or near waterways (Burks, 2002). The USDA predicts that *Nymphoides cristata* will likely invade approximately 11% of the US based on the distribution of the species in its native range (APHIS, 2012). The predicted area of invasion is concentrated in the southeastern US, ranging as far north as Virginia and as far west as Texas (APHIS, 2012).

Species level characteristics as predictors of invasion success are likely to be taxa and site specific (Lake and Leishman, 2004; Sakai et al., 2001). However, multiple meta analyses have shown that both abundance in invaded areas and the ability to reproduce vegetatively are highly correlated with the invasion success for plant species (Hayes and Barry, 2008; Kolar and Lodge, 2001). As such, understanding the reproductive capabilities of invasive plant species is essential for the development of risk assessments and long-term management strategies. Currently the published literature on the population of crested floating heart in the United States is limited (APHIS, 2012; Burks, 2002; Willey and Langeland, 2011).

Little research has been conducted examining the reproductive strategies of the population of *Nymphoides cristata* in the United States. Crested floating heart flowers appear
bisexual (Burks, 2002). While seed has been observed (personal observation, Erika Haug), seed viability has yet to be documented in the published literature (Burks, 2002; Willey and Netherland, 2015). It has been postulated that the primary means of reproduction and spread for this species is through the vegetative production of clonal daughter plants or ramets (Willey and Netherland, 2015). Ramet production has been shown to be strongly influenced by nutrient levels in the substrate and time of year and somewhat influenced by the substrate composition (Gettys et al., 2017). Mature crested floating heart plants growing in a substrate with moderate nutrient levels are expected to produce approximately 350 ramets per plant in 6 months (Gettys et al., 2017). These ramets upon reaching maturity can separate from the mother plant and float unrooted on the water surface for a period of time, allowing for increased dispersal of the plant and establishment of new discrete colonies (Burks, 2002). Many of these ramets will remain quiescent for up to a year after settling (Leif Willey, personal observation in Willey and Netherland, 2015); however, disturbance can increase the probability of sprouting (Willey and Netherland, 2015). In one study, ramets on the sediment surface and partially buried ramets were shown to have higher sprouting rates as compared to fully buried ramets (Gettys et al., 2017). In that same study, approximately 40% of ramets, that settled on the sediment surface, sprouted (Gettys et al., 2017).

In addition to the aforementioned methods of vegetative reproduction, anecdotal evidence of root formation along the leaf edge of crested floating heart plants eaten by insects has been observed (Nair, 1973). While fragmentation induced vegetative reproduction has yet to be studied in crested floating heart leaves, this reproductive strategy is common among stem fragments of many submersed species including those in the genera *Ceratophyllum*, *Elodea*, *Hydrilla*, *Lagrosiphon*, and *Myriophyllum*, and vegetative reproduction from leaf fragmentation
has been observed in several genera within the Cruciferae family (Sculthorpe, 1967). The rapid spread and unabated expansion within invaded systems of crested floating heart over the last 20 years coupled with the limited published literature on the species reproductive capabilities provided the impetus for the research discussed in this paper.

**Objective:**

A study was conducted in order to determine whether the fragmentation response could be induced by manual cutting and to record observations regarding the timing of developmental stages from fragmentation to the formation a new independent plant. It is hypothesized that the fragmentation response can be induced mechanically and that the frequency of formation of independent plants from fragmentation will preclude the use of any management technique or recreational pursuit that would result in the fragmentation of leaf structures.

**Methods**

Stock plants were originally transferred from Lake Marion, South Carolina, to the greenhouses at North Carolina State University in 2013. These populations were planted in sifted lake sediment collected near the Thelma Boating Access on Roanoke Rapids Lake, in Roanoke Rapids, North Carolina. The plants were grown in 120-liter mesocosms with dechlorinated, conditioned tap water (pH = 8.0) using a tap water conditioner product (API Tap Water Conditioner ® Mars Fishcare North America Inc. 50E. Hamilton St. Chalfont PA 18914).

For each run, twelve leaves were selected and excised from stock plants with approximately 0.5 cm of petiole attached to each leaf. Leaves were selected such that three size classes, small (4.5-7.9 cm²), medium (9.1-22.7 cm²), and large (26.7-77.3 cm²), with four leaves per size class, were created. After excision, leaves were bisected transversely such that two sections were formed (Figure 1a). Due to the morphology of crested floating heart leaves, one
fragment per leaf included the petiole. This petiole inclusive fragment type will be referred to as
the proximal fragment (Figure 4-1b). The petiole excluded fragment type will be referred to as
the distal fragment (Figure 4-1b). Fragments were cut at the lengthwise midpoint of each leaf in
an effort to create two fragments of a given leaf that would be approximately equal in leaf area.
Leaf areas for each individual fragment were measured utilizing an LI-3100 area meter (Licor
inc., Lincoln Nebraska) (Table 4-1).

The bases of twelve four-liter black tanks were lined with two centimeters of washed play
sand and filled with 4 liters of tap-water. Black tanks were used in an effort to reduce algal build
up. Water was conditioned with API® tap water conditioner to speed the dechlorination process
and precipitate heavy metals from the water. The distal and proximal fragments of each leaf
were set afloat in individual tanks. Water was refreshed weekly. Tanks were located in glass
greenhouses to allow for a natural sunlight spectrum and day length while prohibiting the
potential overflow of tanks during rain events. Observations during the first run indicated there
was potential for an environmental effect on the response variables. As a result, weekly
temperature and pH readings were collected for run 2 and 3.

Leaf fragments were observed on a weekly basis for 15 weeks. Each week the leaves
were monitored for root development, location and maturation, flower development, parent leaf
health (as assessed by level of chlorotic or necrotic tissue), daughter leaf formation, daughter
plant buoyancy, and other observations. Following parent leaf death (approximately 5 weeks),
the distinction of proximal and distal fragments was no longer clear and as such the data of these
classifications were averaged from this developmental stage forward.

Studies were repeated in space and time for a total of three runs of the experiment. Run
1, 2, and 3 were initiated on August 11th, 2014, May 15th, 2015, and May 21st, 2015, respectively.
Runs 1 and 2 were conducted on different benches of the same greenhouse and Run 3 was conducted in a different greenhouse on the same property.

**Data Analysis:**

Data from all three runs were combined for analysis of trends. While there was some variability within size classes, it should be noted that there was no crossover between size classes (Table 1).

Chi square statistics were run for comparisons of proportions of fragments that reached given developmental stages in each size class ($\alpha=0.05$) and again in each run ($\alpha=0.05$). If an overall chi square was found to be significant, post hoc pairwise chi squares were run utilizing a Bonferroni correction ($\alpha=0.01667$).

A linear effects model was run for each developmental stage observed in the progression from fragmentation to independent plantlet. In the model, the response variable was the time in weeks to reach a given developmental stage and effects included in each model were the fragment area, fragment type (distal or proximal), and run. Following parent leaf senescence, the fragment type was no longer distinguishable. As such in an effort to avoid pseudoreplication the values for development timing for each phase following and including parent leaf senescence reflect an average of the two fragments per leaf and the type effect was removed from these effects models. It should also be noted that effects models were only run on leaf fragments in which a developmental stage occurred (n). After examining residual plots all response variables were log transformed for analysis (untransformed data presented).

**Results and Discussion:**

During the three runs of this experiment, high rates of daughter plant production from artificially severed leaf fragments were observed. There are several differences between these
daughter plants produced by fragmentation and true ramets. Likely the most influential
difference between a true ramet and a daughter plant produced from a leaf fragment is that
during development true ramets remain attached to the entire rooted floating leafed parent plant
via a stolon. This attachment allows for the true ramet to have access to the vast carbohydrate
reserve stored in the root system of the parent plant. In contrast, daughter plants produced by leaf
fragmentation only have access to the nutrients and carbohydrates stored in the section of the leaf
from which they are growing, during development. For clarity, daughter plants produced by
fragmentation will be referred to hereafter as plantlets (Sculthorpe, 1967).

Within 15 weeks of fragmentation 85% of the monitored fragments produced plantlets. Of
fragments that produced plantlets, 85% of those independent plantlets sank to the bottom of
the monitoring tanks. Of the fragments that produced independent plantlets and sank to the
bottom of the tank, 51% produced surface leaves (38% of all leaf fragments). The larger cuttings
produced significantly more plantlets with surface leaves (91%) as compared to the two smaller
size classes (8% and 25%).

For the majority of the developmental stages observed, chi square tests did not provide
significant evidence of a relationship between size class and the proportion of fragments that
reached that developmental stage by the end of the experiment (Table 4-2). However, chi square
statistics did show evidence of dependence between run and occurrence of several developmental
stages (Table 4-3). Linear effects models for the timing of the occurrence of each developmental
stage provided evidence of fragment area and run effects for several of the models (Table 4-4).

*Root Formation and Maturation:*

Roots structures formed at the veins along the cut edge for distal fragments and on the
petiole for proximal fragments (Figure 4-2a-b). *Ceratopteris spp.* will also reproduce
vegetatively through the fragmentation of leaves and for this species new plantlets will form only at the veins of the leaves (Sculthorpe, 1967). It has been suggested that the meristematic cell functions at these sites are strongly influence by diffusible growth substances due to the fact that excised meristematic tissue will only produce new plantlets if it is in contact with a vein or a medium containing adenine or IAA (Sculthorpe, 1967). It is speculated that due to the similar formation of roots at the location of veins, perhaps these diffusible growth substances are responsible for these meristematic cell functions as well.

Root maturation was noted by the formation of ciliate hairs along the roots (Figure 4-2c-d). In addition to forming ciliate hairs, an overall qualitative increase in root biomass was observed. In the distal fragments, some of the roots that formed at separate veins merged together as the roots matured into a root mass (Figure 4-2c).

By the end of the 15-wk trials, 90% of fragments produced roots and 85% of those roots were mature. Chi square tests found no relationship between production or maturation of roots and leaf size class (Table 4-2). It was observed that a significantly lower proportion of run 3 fragments produced roots (71%) and roots that matured (63%), as compared to the other two runs (90-100%) (Table 4-3). On Average, root formation occurred at 1.7 weeks and matured at 2.8 weeks (Figure 4-3). Effects models for these developmental stages provided no evidence of a parent leaf fragment area effect or a leaf type (Proximal or distal) effect (Table 4-4). There was, however, a significant run effect (Table 4-4).

**Daughter Leaf Formation:**

Following root maturation, small daughter leaves developed from the root mass (Figure 4-2e-f). By the end of the 15-week trials, 88% of fragments produced leaves. Two fragments formed leaves without ever forming mature roots. It should be noted that both of these fragments
died before producing independent plantlets. Chi square tests found no relationship between daughter leaf formation and size class (Table 4-2) and a significant relationship between daughter leaf formation and run (Table 4-3).

On average, daughter leaf formation occurred at 3.1 weeks after fragmentation (Figure 4-3). A linear effects model provided evidence of a parent leaf fragment area effect, with larger fragments producing leaves 1 to 2 weeks earlier than small and medium fragments. This earlier formation of photosynthetic tissue allows for earlier photosynthate production through photosynthesis and is likely to increase the survival of plantlets formed from larger fragments. A run effect on the time in weeks for daughter leaves to form was also noted (Table 4-4).

**Flower Formation:**

Flowers only formed on 4 fragments throughout all three runs of this experiment. Flowers emerged from the petioles of daughter leaves. During the 15-week experiments, 4%, 0% and 13% of fragments classified as small, medium, and large, respectively formed flowers. Flower formation occurred only in Run 1.

**Parent Leaf Senescence:**

All parent leaf fragments fully senesced by the end of the 15-week trial (Figure 4-2g). On average, parent leaves began showing signs of senescence including chlorosis, epinasty, and necrosis by 3 weeks after artificial segmentation and were completely senesced by 5 weeks after artificial segmentation (Figure 4-3). As with other phases of development, leaf type (proximal or distal) did not influence the timing of either the onset or completion of parent leaf senescence (Table 4-4). Linear effects models showed a significant impact of run on both the onset and completion of parent leaf senescence (Table 4-4). Fragment area had a significant effect on the time of appearance of the first visible signs of parent leaf senescence, with small leaves showing
visible signs approximately 1 weeks prior to when visible signs were observed with large
fragments. The presence of these visible cues of stress earlier in smaller fragments is likely
indicative of the faster depletion of the more limited supply of carbohydrates in smaller
fragments as compared to larger fragments. Interestingly, fragment size did not have a significant
effect on the time to the death of the parent leaf (Table 4-4).

*Daughter Plant Sinks and Forms Surface Leaves:*

By the end of the 15-week trials, 75% of fragments produced plantlets that sank to the
bottom of the observation tank (Figure 4-2h). Settled true ramets produced by stolons from a
rooted parent plant have been observed remaining quiescent for up to a year (Willey, 2012). In
contrast, 42% of plantlets that settled to the bottom of observation tanks in this study
subsequently produced surface leaves, indicating a lack of a quiescent phase for this type of
vegetative reproduction. This level of sprouting, is comparable to that observed with true ramets,
when the leaves were removed from these ramets and they were placed on the sediment surface
(Gettys et al., 2017). It should be noted that in this study, production of surface leaves largely
resulted from the largest size class of the cuttings. These results suggest that stored
carbohydrates, governed by fragment size, may have a strong influence on the ability of the plant
to become established in deeper waters, where surface leaf production would be necessary.

Chi square tests found no relationship between plantlets sinking and either run (Table
4-3) or size class (Table 4-2). However, chi-square statistics did show significant evidence of a
relationship between the proportion of fragments producing surface leaves and both size class
(Table 4-3) and run (Table 4-2). Pairwise comparisons showed that large leaves produced
significantly more plantlets with surface leaves (91%) as compared to small leaves and medium
sized leaves (Table 4-2).
On average plantlets sank at approximately 7.5 weeks after fragmentation and produced surface leaves at 10.9 weeks after fragmentation (Figure 4-3). Effects models for these developmental stages provided no evidence of a parent leaf fragment area effect, a leaf type effect or a run effect (Table 4-4).

**Mortality:**

The percent of fragments that died without producing plantlets was 15% and of these fragments 36, 45 and 18% belonged to the small, medium and large leaf size classes, respectively. Further, the relationship between size class and mortality was not significant (Table 4-2). The majority (81%) of fragments that died without producing vegetative clones occurred in run 3 (Table 4-3).

**Run effects:**

As previously mentioned, significant run effects were observed in several of the analyses in this experiment (Table 4-3; Table 4-4). Of the eight effects models run for this study, six models were significantly impacted by run, whereas none of the models were impacted by fragment type and only two of the models were impacted by the size of the fragment. While definitive causal conclusions for these run effects cannot be made, the differences are speculated to be due to a difference in environmental conditions. All of the runs followed the same protocol for severing leaves and the maintenance of leaves during observation and all three runs were conducted in glass greenhouses. As such, neither protocol nor greenhouse material is the likely cause of observed differences between runs. Run 1 was begun in the fall and Runs 2 and 3 were conducted in the spring. A day length effect could be argued if in any of the analyses in Run 1 significantly deferred from both run 2 and run 3 and runs 2 and 3 did not significantly differ from each other; however, this did not occur in this study.
Temperature and pH data were collected for runs 2 and 3. Run 1 and run 2 were separated in time and space but were conducted in the same greenhouse, with the same water source. Run 3 was conducted in a separate greenhouse on the opposite side of the property. The water temperature for run 3 was cooler than run 2 throughout the two approximately simultaneous trials (Figure 3). As crested floating heart growth responds favorably to higher temperatures, it is likely that the elevated temperatures in run 2 (compared to run 3) accelerated phenological events. The aqueous pH in run 3 was also consistently slightly more acidic that in run 2 (Figure 4). The increase in pH in run 2 is likely an effect of increased plant and algal growth in run 2 related to the increase in temperature. Since the pH in both run 2 and 3 was generally near neutral, pH is not expected to have been a cause of the run differences observed. Future studies of this fragmentation response should vary the temperature of the water column, while holding other variables constant, to determine if this change in temperature were in fact responsible for the run effect observed.

**Conclusion:**

This study provides clear evidence that fragmentation response can be induced by manual cutting and that this manual cutting can and often does lead to the formation of independent plantlets (Figure 4-2). These findings are in line with both the laboratory observations of Nair (1973), which included reports that crested floating heart can reproduce by fragmentation initiated by insect activity, as well as reported field observations (Willey and Langeland, 2011; pers. comm. Dr. Paul Champion).

This study also confirms that larger leaf cuttings are most successful at producing plantlets with new floating leaves. These results are consistent with results of other studies which have found a greater ability to regrow and greater survivability with larger fragments of
hydrilla *Hydrilla verticillata* (L. f.) Royle (Baniszewski et al., 2016; Langeland and Sutton, 1980). The results are also congruent with studies of submersed macrophytes, which suggested that greater propagule size can be correlated with greater growth, percent emergence, survivability, or longevity (Netherland, 1997; Spencer, 1987; Spencer et al., 1987).

Confirmation of an additional mode of reproduction for crested floating heart is important to elevate the risk level associated with this invader (Hayes and Barry, 2008; Kolar and Lodge, 2001). Clearly, the findings of this study also have management implications. Specifically, the data presented here precludes the use of mechanical harvesting as a management technique for crested floating heart and suggests that limiting motor boat access may be important, due to the risk of exacerbation of the infestation through errant fragments. Future studies should focus on the environmental conditions which increase or decrease the probability of a fragment forming an independent daughter plant.
Literature Cited


Figures and Tables

Table 4-1: Leaf area measurement summary. Data for all three runs were combined and averaged within reclassified size classes.

<table>
<thead>
<tr>
<th>Size Class</th>
<th>Distal Fragment Area (cm(^3))</th>
<th>Proximal Fragment Area (cm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Small</td>
<td>2.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Medium</td>
<td>6.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Large</td>
<td>20.7</td>
<td>8.4</td>
</tr>
</tbody>
</table>
Table 4-2: Chi square results for comparisons of proportion that reached a given developmental stages in each size class (α=0.05). Proportions sharing a letter were not statistically different in pairwise comparisons utilizing and Bonferroni correction (α=0.017).

<table>
<thead>
<tr>
<th>Metric</th>
<th>Percent of Category Developed</th>
<th>Chi p-value</th>
<th>Pairwise Chi results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
</tr>
<tr>
<td>Root formation</td>
<td>88%</td>
<td>83%</td>
<td>100%</td>
</tr>
<tr>
<td>Mature root formation</td>
<td>83%</td>
<td>79%</td>
<td>92%</td>
</tr>
<tr>
<td>Leaf formation</td>
<td>83%</td>
<td>83%</td>
<td>92%</td>
</tr>
<tr>
<td>Parent leaf senescence</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Daughter plant sinks</td>
<td>67%</td>
<td>67%</td>
<td>92%</td>
</tr>
<tr>
<td>Surface leaves form</td>
<td>8% (A)</td>
<td>25% (A)</td>
<td>92% (B)</td>
</tr>
<tr>
<td>Mortality</td>
<td>17%</td>
<td>21%</td>
<td>8%</td>
</tr>
</tbody>
</table>
Table 4-3: Chi square results for comparisons of proportion that reached a given developmental stages in each run ($\alpha=0.05$). Proportions sharing a letter were not statistically different in pairwise comparisons utilizing and Bonferroni correction ($\alpha=0.017$).

<table>
<thead>
<tr>
<th>Metric</th>
<th>Percent Developed</th>
<th>Pairwise Chi results</th>
<th>Chi p-value</th>
<th>Run 1 to Run 2</th>
<th>Run 1 to Run 3</th>
<th>Run 2 to Run 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root formation</td>
<td>100% (A)</td>
<td>100% (A)</td>
<td>70.8% (B)</td>
<td>0.0004</td>
<td>N/A</td>
<td>0.00420</td>
</tr>
<tr>
<td>Mature root formation</td>
<td>92% (A)</td>
<td>100% (A)</td>
<td>63% (B)</td>
<td>0.0008</td>
<td>0.14856</td>
<td>0.01622</td>
</tr>
<tr>
<td>Leaf formation</td>
<td>88% (A, B)</td>
<td>100% (A)</td>
<td>71% (B)</td>
<td>0.0136</td>
<td>0.07364</td>
<td>0.15513</td>
</tr>
<tr>
<td>Parent leaf senescence</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>1.0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Daughter plant sinks</td>
<td>92%</td>
<td>75%</td>
<td>58%</td>
<td>0.16901</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Surface leaves formed</td>
<td>67% (A)</td>
<td>17% (B)</td>
<td>42% (A, B)</td>
<td>0.04570</td>
<td>0.01298</td>
<td>0.21906</td>
</tr>
<tr>
<td>Mortality</td>
<td>8% (A, B)</td>
<td>0% (A)</td>
<td>35% (B)</td>
<td>0.00098</td>
<td>0.14856</td>
<td>0.02241</td>
</tr>
</tbody>
</table>
Table 4-4: Linear effects models for each developmental stage recorded. N = total number of experimental units for that stage. n= number of fragments that reached the developmental stage and are included in the effects model.

<table>
<thead>
<tr>
<th>Response</th>
<th>N</th>
<th>n</th>
<th>Mean (weeks)</th>
<th>Standard deviation</th>
<th>Effect Fragment Area</th>
<th>Effect Type of Fragment</th>
<th>Run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to root formation</td>
<td>72</td>
<td>65</td>
<td>1.69</td>
<td>0.64</td>
<td>p=0.2784</td>
<td>p=0.2935</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Time to mature root formation</td>
<td>72</td>
<td>61</td>
<td>2.77</td>
<td>0.86</td>
<td>p=0.4749</td>
<td>p=0.6155</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Time to leaf formation</td>
<td>72</td>
<td>63</td>
<td>3.13</td>
<td>1.00</td>
<td>p=0.0218</td>
<td>p=0.1429</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Time to visible signs of parent leaf senescence</td>
<td>72</td>
<td>72</td>
<td>2.99</td>
<td>0.66</td>
<td>p&lt;0.0001</td>
<td>p=0.4719</td>
<td>p=0.0138</td>
</tr>
<tr>
<td>Average per leaf (2 fragments) time to death of parent leaf</td>
<td>36</td>
<td>36</td>
<td>5.06</td>
<td>0.83</td>
<td>p=0.9590</td>
<td>N/A</td>
<td>p=0.0025</td>
</tr>
<tr>
<td>Average per leaf (2 fragments) time to Daughter plant sinks</td>
<td>36</td>
<td>27</td>
<td>7.50</td>
<td>1.93</td>
<td>p=0.1141</td>
<td>N/A</td>
<td>p=0.7780</td>
</tr>
<tr>
<td>Surface Leaves</td>
<td>36</td>
<td>15</td>
<td>10.87</td>
<td>2.24</td>
<td>p=0.3626</td>
<td>N/A</td>
<td>p=0.8159</td>
</tr>
</tbody>
</table>
Figure 4-1: Depiction of the fragmentation response. (a) red line shows the location of the transverse cut on the leaf (b) distal (top) and proximal (bottom) fragments and the location of root formation (c) mature roots and daughter leaf production.
Figure 4-2: Developmental stages during the formation of plantlets. (a) Root formation at the veins of a distal fragment, (b) root formation from the petiole of a proximal fragment, (c) root maturation along the cut edge of a distal fragment, (d) root maturation and forming of ciliate hairs from the petiole of a proximal fragment, (e) leaf formation on a distal fragment, (f) leaf formation on a proximal fragment, (g) independent plantlet with senesced parent leaf still attached, and (h) independent plantlet settle on the sediment surface.
Figure 4-3: Progression of the fragmentation response for crested floating heart leaves. Bars represent the mean time after fragmentation for a developmental stage to begin. Error bars depict standard deviation of the mean.
Figure 4-4: Weekly temperature readings for Run 2 and Run 3 are displayed. Individual data points are shown as well as a best fit trendline. Run 1 data not collected.
Figure 4-5: Weekly pH readings for Run 2 and Run 3 are displayed. Individual data points are shown as well as a best fit trendline. Run 1 data not collected.
CHAPTER 5: Response of Seven Aquatic Plants to a new Arylpicolinate Herbicide

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Abstract

The herbicide 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoro-pyridine-2-benzyl ester (SX-1552 or XDE-848 BE; proposed ISO common name in review) is a new arylpicolinate herbicide currently under development for weed management in rice production, aquatic weed management, and other uses. Greenhouse research was conducted to evaluate the effect of SX-1552 and SX-1552A (an acid metabolite) on seven aquatic plants: alligatorweed, bacopa, fanwort, monoecious hydrilla, parrotfeather, variable watermilfoil, and waterwillow. SX-1552 and SX-1552A were applied to these species as an in-water, four week static exposure at rates of 0 to 81 µg/L. Fanwort was not controlled by SX-1552 at the rates evaluated, in contrast to the other species tested. Dry weight EC50 values were <1 µg/L SX-1552 for alligatorweed, monoecious hydrilla, parrotfeather, and variable watermilfoil. Bacopa and waterwillow SX-1552 EC50 values were 5.0 and 5.1 µg/L, respectively. These six species were less sensitive to SX-1552A with dry weight EC50 values of 1.6 to 77.1 µg/L. Plant control ratings also indicated that response of the six sensitive species increased from two to four weeks after treatment. Further research is needed on additional species as well as concentration exposure time determination for the species evaluated here.

Key words: herbicidal control, synthetic auxin
Introduction

Despite an increased number of US aquatic registrations in the last decade, additional technologies are still needed for successful management of aquatic weeds. While 244 herbicide actives are currently registered in the US, only 14 are registered as aquatic herbicides (NPIRS, 2015). Additional herbicides can improve control of weed species not optimally addressed by current product registrations, enhance selectivity to desirable native aquatic vegetation, reduce use rates, and mitigate risk of potential herbicide resistance development (Getsinger et al., 2008; APMS, 2014). Selectivity to native aquatic vegetation and longevity of control are key criteria in the management of invasive aquatic plants. Effects of a specific herbicide chemistry on a given target weed and co-occurring native plants, general characteristics of its mode of action, and herbicide concentration and exposure time (CET) achieved with in-water treatments dictate the selectivity and duration of control of aquatic herbicide treatments (Getsinger et al., 1993; Netherland and Getsinger, 1992; Netherland et al., 1997). Research and development of new aquatic herbicides is generally focused on finding new selective, systemic chemistries that have short exposure time requirements for in-water, partial-site treatment of major target aquatic weeds such as hydrilla (Hydrilla verticillata L.) and Eurasian watermilfoil (EWM).

Auxin-mimic herbicides (2,4-D and triclopyr) are well-documented for their selective, systemic control of problem weeds such as EWM and water hyacinth. Auxins are a group of plant growth hormones that affect many plant processes, such as root initiation, tropism, shoot growth, plant development and apical dominance, among other essential plant growth processes (Grossman, 2010; Yamada, 1954). In susceptible plants, synthetic auxins have the same impacts as natural auxin overdose. However, synthetic auxins are more stable within plants and less susceptible to the plant’s methods of inactivation as compared to the naturally produced auxins.
(Woodward and Bartel, 2005). The prevailing theory until recently has suggested that synthetic auxins caused plants to essentially “grow themselves to death” (Gilbert 1946). The action of synthetic auxin overdosing can be summarized in three phases; the stimulation phase during which the plants metabolic activity is heightened and abnormal growth occurs, such as stem curling and leaf epinasty; the inhibition phase, during which growth is stunted and several growth reducing physiological responses, such as stomatal closure and reduced carbon fixation, occur, and finally the decay phase, characterized by cell and plant tissue death (Grossman, 2010). The feedback mechanisms involved in this phased progression is much more complex than that proposed by Gilbert (1946) and it is because of these complexities that auxin mimics have differential action on monocots versus dicots and among different dicot species (Grossman, 2010).

Synthetic IAA (auxin) derivatives were developed for use in plant management as early as 1940 (Cobb 1992). Synthetic auxins are translocated throughout the plant due to their similarity to natural auxins (Grossman 2010). Generally, dicotyledonous plants are more susceptible to synthetic auxins than monocots, while unicellular algae in the water column are not impacted (Cedergreen and Streibig 2005). As such, synthetic auxins are often used to selectively control aquatic weeds, while limiting the impact to non-target native plant and algal species (Madsen and Wersal 2009, Glomski and Netherland 2010, Wersal et al. 2010). Although currently registered auxin-mimic herbicides fit a number of needs for selective aquatic weed control, a systemic herbicide with this selective mode of action has not been previously identified with sufficient activity on hydrilla (Hydrilla verticillata). Hydrilla may be considered the most problematic US aquatic weed and despite efforts to register several new herbicides for hydrilla control, the species continues to have the most urgent need for additional herbicide
options (Hoyer et al., 2005; Richardson, 2008; APMS, 2014). Several other aquatic weeds, such as crested floating heart and certain biotypes of hybrid watermilfoils, show insufficient response to current auxin-mimic herbicides to be optimally controlled with typical use rates (LaRue et al., 2013; Willey et al., 2014).

The herbicide SX-1552, 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoro-pyridine-2-benzyl ester is also under development by Dow AgroSciences for rice production (XDE-848 BE; proposed ISO common name in review; active tradename Rinskor™) and other agricultural crops and is also in development in partnership with SePRO Corporation as an aquatic herbicide (SX-1552; Procellacor™ Aquatic Herbicide Technology System). SX-1552 is a member of a new class of synthetic auxins in the arylpicolinate herbicide family. Studies of Arabidopsis thaliana with mutations in select auxin-binding receptor proteins, along with direct molecule-protein interaction testing of these same receptor proteins, support that arylpicolinate chemistry including SX-1552 has a different binding affinity versus 2,4-D and other currently registered synthetic auxin herbicides (Walsh et al., 2006; Villalobos et al., 2012; Lee et al., 2013; Bell et al., 2015). In preliminary screening, SX-1552 exhibited strong activity on several problematic US aquatic plants including the submerged weeds hydrilla and EWM, the free-floating weed water hyacinth (Eichhornia crassipes), and floating leaf weed crested floating heart [Nymphoides cristata (Roxb.) Kuntze] (Netherland et al. in review; SePRO Corporation, unpublished data). SX-1552 would represent a new mode of action for hydrilla control and a number of other important aquatic weed management uses. The objective of this study was to evaluate the activity of SX-1552 and SX-1552A—a less active acid metabolite—against seven aquatic plant species using a small-scale screening method under greenhouse conditions to confirm activity and potential utility.
Methods

Propagation

Seven species were propagated for this evaluation: alligatorweed [Alternanthera philoxeroides (Mart.) Griseb.], fanwort (Cabomba caroliniana A. Gray), bacopa [Bacopa caroliniana (Walter) B.L. Rob.], monoecious hydrilla, parrotfeather [Myriophyllum aquaticum (Vell.) Verdc.], variable watermilfoil (Myriophyllum heterophyllum Michx.) and water willow (Justicia americana (L.) Vahl.). Laboratory stock plants were used for the propagation of alligatorweed and parrotfeather. Variable watermilfoil shoot tissue, monoecious hydrilla subterranean turions, and water willow stems were field collected from local NC sources. Lemon bacopa1 and fanwort2 were purchased from commercial sources. Alligatorweed, parrotfeather, and water willow shoot tips were cut to approximately 15cm in length. These tips were first stored upright in de-chlorinated tap water. Following the production of viable root tissue, tips were planted in soil and submersed in de-chlorinated tap water for establishment. Approximately 10-cm sections of variable watermilfoil and fanwort shoot tissue were cut and immediately planted in soil and submersed in de-chlorinated tap water for establishment. Lemon bacopa purchased from an aquarium plant dealer was first submersed in de-chlorinated water with the roots in the nutrient gel provided by the dealer. The nutrient gel was removed after one week and shoots were then planted in soil and submersed in de-chlorinated tap water for establishment. Monoecious hydrilla subterranean turions were collected at Lake Gaston and stored at 4°C prior to sprouting in de-chlorinated tap water. Sprouted turions were planted in soil and submersed in de-chlorinated tap water for establishment. All propagules were planted in 3 oz (89 ml) pots, filled with lake sediment collected from Roanoke Rapids Lake, NC. Collected soil was sifted to remove debris and propagules and homogenized before filling pots. After propagules of test
species were planted, a thin layer of fine sand was placed over the lake sediment. Plants were allowed to establish for one week following planting in soil. Experimental mesocosm size was 15L with plastic liner in each container. All mesocosms were maintained in a temperature controlled poly-covered greenhouse with minimum temperature of 26 C.

Treatment

Each species was exposed to a four week static exposure of 0, 0.3, 1, 3, 9, 27, or 81 µg/L of SX-15523 or of SX-1552A, the acid metabolite. Due to the limited maturity of tested plants, competition between plants did not appear to impact the growth of plants. Treatments were arranged into a randomized complete block design with four replicates. The experiment was conducted twice, non-concurrently, to confirm consistent results.

Data collection and analysis

Percent control of treated plants as compared to untreated controls was assessed visually at two weeks and four weeks after treatment. Plants were rated on a scale of 0 (no signs of impact) to 100% control (no living shoot tissue remaining). Intermediate symptomology of treatment varied by species and included evaluations of shoot swelling, stem twisting, leaflet curling, chlorosis, and tissue death. Visual observations are described, but data is not presented. The total length of all living shoot tissue was measured in millimeters prior to treatment and again after four weeks of exposure. Due to tissue damage following herbicide treatment, intermediate measurements of living shoot tissue were determined to be too destructive to the remaining live tissue and as such only pretreatment and post-treatment measures were collected. Four weeks after treatment, above sediment shoot biomass was harvested for both fresh-weight and dry-weight determination. The fresh biomass of all tissue harvested for each plant was measured within 2 hours of harvesting using a laboratory balance with 0.001 gram accuracy. A
short period of time after harvest was allowed for excess moisture to drain from plant biomass. Following fresh-weight measurement, plant samples were placed in labeled paper bags for drying. Plant samples were dried to a constant mass at 60°C. The biomass of the dried plant tissue was again measured on a laboratory balance with 0.001 gram accuracy.

Water samples were collected using glass instrumentation and stored in amber color glass vials. Methanol (1.5 ml) was placed in each vial prior to collection of 29 ml sample water. Formic acid (1.2 ml) was titrated into the vial after collection to prevent potential hydrolytic degradation of SX-1552 by achieving approximate pH 3. Following collection and acidification, samples were stored in a laboratory grade freezer at -5°C. Frozen samples were then shipped overnight on ice to EPL Bio Analytical Services, Ninantic, IL, for analysis via LC-MS (Liquid Chromatography with Mass Spectroscopy) in a dedicated method developed for analysis of SX-1552 and its major metabolites in water in support of registration studies (EPL Method Number 477G696A-1, unpublished). Samples were collected from the first replicate of 3 µg/L, 9 µg/L and 81 µg/L concentrations for SX-1552 immediately after treatment to verify target concentrations. Mean starting concentrations were within 10% of target rates.

Water Temperature and pH measurements were collected utilizing a YSI field probe (Model 556). Measurements were made prior to treatment and weekly thereafter. Measurements were collected from all replicates of the untreated control, 9 µg/L and 81 µg/L treatment chambers prior to treatment and during the final percent control evaluation. Interim temperature and pH measurements were collected only from the replicates of the untreated control chambers.

All data were subjected to ANOVA in SAS v. 9.3. No significant treatment by trial interactions were observed, therefore, data was pooled over experiments. Shoot length, fresh weight, and dry weight were converted to percent inhibition of the untreated control and then
subjected to regression analysis along with visual control. The non-linear equation \( y = a (1 - e^{-b\cdot x}) \) was used for all models in SigmaPlot v.12.0. This model was utilized because it converged across all data sets while the three and four parameter logistic equations evaluated did not. EC50 concentrations were then determined for each regression model. In addition, a Dunnet’s test \((\alpha=0.05)\) comparing biomass of treated plants to the non-treated control was utilized to determine the lowest observed effect concentration (LOEC).

**Results and Discussion**

All seven weed species displayed some level of sensitivity to both herbicides (Figure 5-1). Alligatorweed was sensitive to both SX-1552 and SX-1552A (Figure 5-1). Treatment symptomology on alligatorweed included increased stem growth, limited chlorosis, and stem swelling at and below the surface of the water and progressed to tissue necrosis and plant death. Visual symptoms were observed at two weeks after treatment with SX-1552, while response to the acid form occurred more slowly (data not presented). At four weeks after treatment, SX-1552 EC50 values ranged 0.96 to 1.8 µg/L, while SX-1552A EC50 values ranged 9.7 to 17.8 µg/L, indicating less sensitivity to the acid form (Table 5-1). Dry weight LOEC values were 1 and 9 for SX-1552 and SX-1552A, respectively.

Previous research has indicated that triclopyr may reduce the biomass of young alligatorweed plants (Hofstra and Champion 2010) and that quinclorac may provide moderate control in a greenhouse setting (Kay 1992). Alligatorweed is generally not controlled by 2,4-D and this has been attributed to poor basipetal translocation (Earle et al. 1951). The control observed with SX-1552 is greater than would have been expected from either triclopyr or 2,4-D.

Bacopa response was generally similar to alligatorweed with the plant being distinctly more sensitive to SX-1552 than SX-1552A (Figure 5-1). Symptomology associated with SX-
SX-1552A was minor at two weeks after treatment, but more pronounced by four weeks after treatment (data not presented). SX-1552 EC50 values ranged from 3.2 to 5.0 µg/L (Table 5-1). SX-1552A EC50 values ranged from 9.7 to 17.8 µg/L. At SX-1552 rates of 9.7 µg/L and greater, bacopa response progressed to eventual tissue and plant death. However, at rates lower than 3 µg/L leaves were initially abscised but some leaf tissue regrowth had occurred by trial conclusion. Conversely, bacopa plants exposed to low <3 µg/L SX-1552A rates did not lose foliage. This plant response likely explains the disparity between shoot and weight inhibition EC50 values for SX-1552A. LOEC values were 9 µg/L for SX-1552 and 27 µg/L for SX-1552A again supporting better activity from the SX-1552 molecule (Table 5-1).

Unlike the other species evaluated, fanwort was not as sensitive even with the static four-week exposure (Figure 5-1). Symptomology observed at the highest exposure rates included curling of young leaves and progressed to limited stem epinasty. Our evaluated rates were not sufficient to generate EC50 or LOEC values and this is consistent with previous research on fanwort sensitivity to auxin mimics. Bultemeir et al. (2009) reported that 2,4-D, quinclorac, and triclopyr (maximum test rates of 4,400, 400, and 4,900 µg/L, respectively) did not reduce fanwort photosynthesis by 50%. Due to the relative tolerance of cabomba to synthetic auxins, there is no need to evaluate a broader rate range of SX-1552 in order to generate an EC50 value unless registered use rates will exceed 81 µg/L.

Monoecious hydrilla was sensitive to both SX-1552 and SX-1552A (Figure 5-1). EC50 values for all data at 4 WAT ranged 0.71 to 1.6 µg/L, while LOEC was 3 µg/L (Table 5-1). Visual symptoms did progress from 2 to 4 WAT with both SX-1552 and SX-1552A (data not presented). Symptomology consisted of leaf pigmentation changes (purpling) and stunted growth, progressing to leaf curling, chlorotic/necrotic tissue and eventual plant death. Hydrilla
stem tissue also became fragile to touch and broke easily at nodes as symptomology progressed. While hydrilla (like many other monocots) is commonly known to be tolerant of the synthetic auxins 2,4-D and triclopyr, quinclorac has been reported to provide significant control of hydrilla (Zawierucha et al. 2006). Our results are also consistent with those of Netherland et al. (in review), who reported dioecious hydrilla EC50 values of 1.7 to 6.8 µg/L with both SX-1552 and SX-1552A, respectively. This new mode of action provided by SX-1552 could provide a needed herbicide for resistance management in control efforts of dioecious hydrilla since fluridone and endothall-resistant dioecious biotypes have been detected in Florida (Michel et al 2004, APMS 2014, MD Netherland – unpublished data). It may also provide a new pattern of selectivity for removing hydrilla from mixed aquatic plant communities. Future research should be conducted to determine this pattern of selectivity as well as the necessary concentration exposure time for both hydrilla biotypes.

The two milfoil species, parrotfeather and variable watermilfoil, were the most sensitive species evaluated to SX-1552 (Figure 5-1). Symptomology occurred within one week of treatment, particularly in plants treated with SX-1552, and rapidly increased. Increased stem growth and epinasty were the first observed symptoms, but this quickly progressed to tissue necrosis and plant death. Our rate range was generally not low enough to calculate SX-1552 EC50 values for most parameters, although dry weight inhibition of parrotfeather was 0.68 µg/L (Table 1). Both plants were more tolerant to SX-1552A as parrotfeather had EC50 values of 6.0 to 10.5 µg/L while variable watermilfoil had EC50 values of 21.3 to 35.1 µg/L across plant growth data. LOEC values for SX-1552 was 0.3 µg/L on both species and 9 and 21 for SX-1552A on parrotfeather and variable milfoil, respectively. Progression of visual symptoms was also observed with both species from two to four weeks after treatment (data not presented).
The sensitivity of milfoil species to synthetic auxins is well documented. Netherland et al. (in review) reported Eurasian watermilfoil EC50 values of 0.17 to 1.4 µg/L for SX-1552 and SX-1552A. Numerous other researchers have previously described sensitivity of Eurasian watermilfoil, parrotfeather, and variable watermilfoil to the synthetic auxins 2,4-D and triclopyr (Getsinger et al. 2003; Haug and Bellaud 2013; Hofstra et al. 2006; Netherland and Getsinger 1992; Parsons et al. 2001; Poovey et al. 2007; Sutton and Bingham 1970). Thus, Myriophyllum species are likely to be among the most sensitive to SX-1552 and these species may be significantly injured in SX-1552 treatment areas.

Water willow was more sensitive to SX-1552 than SX-1552A (Figure 1). EC50 values ranged 1.4 to 9.3 µg/L for SX-1552 and 59.1 to 77.7 µg/L for SX-1552A, which was the largest difference in response among species evaluated (Table 5-1). Likewise, LOEC values were 9 and 81 µg/L for SX-1552 and SX-1552A, respectively. In piedmont reservoirs, water willow is one of the most important native species and hydrilla one of the most significant invaders. The difference in plant response between these species makes it likely that SX-1552 could selectively remove hydrilla from water willow beds, a necessity for this use pattern.

Our results indicate that SX-1552 has the potential to control several important North American weed species. The strong activity of this new mode of action herbicide observed for monoecious hydrilla supports its development for selective hydrilla control. Further, high activity on invasive/nuisance milfoils such as parrotfeather and variable watermilfoil also support potential future fit in selective control of these species. The four-week static exposure used in these small-scale trials may overestimate control that could be obtained in field situations where plant establishment and degradation/dilution in typical partial treatment designs will reduce achieved exposure and can reduce efficacy. However, Netherland et al. (in review)
showed that static greenhouse treatments of well-established Eurasian watermilfoil with SX-1552 provided control at similar ≤1 ppb rates as observed in small-scale testing similar to that presented here. Current results provide a good baseline for the establishment of CET protocols on more established plants necessary to fully develop field use patterns. Similar to use of currently registered auxin-mimic herbicides, focus should concentrate on partial treatment designs as these are expected to be the primary approach for potential use of SX-1552. The four week exposure also provided an important detail on the acid form; control of all species except cabomba increased from two to four weeks. In addition to CET trials, future research should also evaluate the sensitivity of additional target and non-target submersed plants so that complete use pattern guidelines can be developed.

**Sources of Materials**

1Bacopa (Bacopa caroliniana), The Fish Room Cary, NC for experiment 1 and PetSmart, Cary, NC for experiment 2.

2Fanwort, LiveAquaria.com. 2253 Air Park Road, P.O. Box 100 Rhinelander, Wisconsin 54501.

3SX-1552 and SX-1552A, SePRO Corporation, Carmel, IN 46032.

**Acknowledgements**

The authors would like to acknowledge the following members of the NCSU Aquatic Weed Science Lab for their efforts with data collection: Shannon Auell, Evan Calloway, Tyler Harris, Andrew Howell, Steven Hoyle, Amy Miller, and Stephanie Nawrocki.
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Figures and Tables

Table 5-1: Calculated EC50 values for seven aquatic plants treated with SX-1552 and SX-1552A at concentrations ranging from 0.3 to 81 ppb. Values derived from non-linear regression analysis of shoot length, fresh weight, and dry weight converted to percent inhibition of untreated plants using the equation $y = a \cdot (1 - e^{-b \times})$. Lowest observed effect concentration (LOEC) derived via Dunnett’s test ($\alpha=0.05$).

<table>
<thead>
<tr>
<th>Species</th>
<th>Shoot inhibition</th>
<th>Fresh weight inhibition</th>
<th>Dry weight inhibition</th>
<th>Dry weight LOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 values (µg/L) – SX1552</td>
<td>SX-1552 (µg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alligatorweed</td>
<td>1.37</td>
<td>1.8</td>
<td>0.96</td>
<td>1</td>
</tr>
<tr>
<td>Bacopa</td>
<td>3.2</td>
<td>3.7</td>
<td>5.0</td>
<td>9</td>
</tr>
<tr>
<td>Cabomba</td>
<td>&gt;81</td>
<td>&gt;81</td>
<td>&gt;81</td>
<td>&gt;81</td>
</tr>
<tr>
<td>Monoecious hydrilla</td>
<td>1.32</td>
<td>0.94</td>
<td>0.71</td>
<td>3</td>
</tr>
<tr>
<td>Parrotfeather</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>0.68</td>
<td>0.3</td>
</tr>
<tr>
<td>Variable watermilfoil</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Water willow</td>
<td>1.4</td>
<td>9.3</td>
<td>5.1</td>
<td>9</td>
</tr>
</tbody>
</table>

|                     | EC50 values (µg/L) – SX1552A | SX-1552A (µg/L)          |
| Alligatorweed       | 15.8             | 17.8                    | 9.7                   | 9               |
| Bacopa              | 2.5              | 36.1                    | 12.2                  | 27              |
| Cabomba             | >81              | >81                     | >81                   | >81             |
| Monoecious hydrilla | 1.2              | 1.4                     | 1.6                   | 3               |
| Parrotfeather       | 10.5             | 6.0                     | 6.9                   | 9               |
| Variable watermilfoil | 21.3            | 33.5                    | 35.1                  | 27              |
| Water willow        | 74.8             | 59.1                    | 77.7                  | 81              |
Figure 5-1 Plant dry weights at 4 weeks after static exposure of SX1552 and SX-1552A at 0, 0.3, 1, 3, 9, 27, and 81 µg/L expressed as percent inhibition of the untreated control. Regression analysis performed using the non-linear equation $y = a (1 - e^{-b \times})$. 
CHAPTER 6: Absorption and Translocation of Florpyrauxifen-benzyl (Procellacor™) in Ten Aquatic Plant Species

Abstract

There is a need for the registration of more active ingredients for use in aquatic systems in order to respond to new threats or treatment scenarios, enhance selectivity, reduce use rates, and to mitigate the risk of potential herbicide-resistance. Florpyrauxifen-benzyl is a new synthetic auxin under development for use as an aquatic herbicide. In 2017, a study was conducted at North Carolina State University, in which 10 µg L⁻¹ radiolabeled florpyrauxifen-benzyl was applied to the isolated shoot tissue of ten different aquatic plant species in order to elucidate absorption and translocation patterns in these species and thus infer the potential for systemic control. Extremely high levels of shoot absorption were observed for all species tested and the uptake observed was rapid. Highest shoot absorptions were observed for crested floating heart (A₁₉₂ =20 µg g⁻¹), dioecious hydrilla (A₁₉₂ =25.3 µg g⁻¹), variable watermilfoil (A₁₉₂ =40.1 µg g⁻¹) and Eurasian watermilfoil (A₁₉₂ =25.3 µg g⁻¹). Evidence of translocation was observed in all rooted species tested. The highest amount of herbicide translocated was observed for crested floating heart with a predicted translocation of 1.28 µg g⁻¹ at 192 hours after treatments. Given the high level of relative activity of this herbicide, the herbicide quantity translocated to the roots should be sufficient to provide control of root tissues.

Introduction

Dominance of invasive aquatic plant species can lead to numerous negative impacts to aquatic ecosystems. Through competition and displacement, invasive aquatic plants reduce native plant diversity, and ultimately infestation by non-native invasive plants results in the formation of dense monotypic stands of vegetation (Gause, 1934; Hardin, 1960; Schultz and Dibble, 2012). These dense monotypic stands can reduce native fish and macroinvertebrate
populations and diversity (Covich et al., 2004; Downing and Leibold, 2002). They can also reduce overall ecosystem productivity and impede the natural flow of water through an ecosystem (Engelhardt and Ritchie, 2002; Pitlo and Dawson, 1993; Schultz and Dibble, 2012). Additionally, these infestations negatively impact the recreational utility of waterbodies, reduce property values, and create habitat suitable for disease carrying vectors (Gangstad and Cardarelli, 1993; Halstead et al., 2003; Wilde et al., 2005; Zhang and Boyle, 2010).

One of the most commonly used and cost-effective methodologies for the selective management of invasive aquatic plants is the application of aquatic herbicides. However, there is a need for the registration of more active ingredients for use in aquatic systems in order to respond to new threats or treatment scenarios, enhance selectivity, reduce use rates, and to mitigate the risk of potential herbicide-resistance (Cobb and Reade, 2010; Getsinger et al., 2008).

Synthetic auxin herbicides have favorable properties for invasive plant control as they 1) impact plant specific processes and thus pose limited risk to wildlife, 2) are easily absorbed and translocated throughout sensitive plants, and 3) are generally selective to control dicots, with minimal impact to monocots (Epp et al., 2016; Grossmann, 2010; Netherland, 2009). Only 14 herbicides are currently registered for aquatic use, two of which, triclopyr and 2,4-D can be considered auxin mimics (Netherland, 2009). Recently, a new herbicide, florpyrauxifen-benzyl, was developed for use in aquatics. Florpyrauxifen-benzyl is a synthetic auxin with a highly favorable toxicology profile (aquatic tradename – Procellacor™) (Miller and Norsworthy, 2015; Wells and Taylor, 2016). This herbicide has been shown to be highly effective in controlling some of the most invasive aquatic plants species in the US, including hydrilla [*Hydrilla verticillata* (L. f.) Royle] and invasive watermilfoils (*Myriophyllum spp.*) (Netherland and Richardson, 2016; Richardson et al., 2016).
In order to better understand the required in-water exposure times for the product to be efficacious on different species and to estimate the longevity of control, it is important to understand the rates at which a new product is absorbed and translocated. Theoretically, the carboxylic acid functional group on florpyrauxifen-benzyl (lipid-soluble) and other picolinate auxin herbicides allows for the herbicide to pass through the lipophilic phloem wall and with a low pKa, the herbicide should ionize into the anionic form inside the phloem effectively concentrating the herbicide in the phloem (Bromilow et al., 1990; Epp et al., 2016). This process is called phloem trapping and would allow for systemic movement of the ionized herbicide to growing shoot and root tissues (Epp et al., 2016). However, the response of plants to synthetic auxins is often species-specific and may vary in an aquatic environment as compared to a terrestrial environment (Cobb and Reade, 2010; Delbarre et al., 1996). Therefore, absorption and translocation of florpyrauxifen-benzyl needs to be evaluated for species-specific responses in order to confirm theoretical hypotheses regarding the behavior of this herbicide within aquatic plants. In the last several years, protocols have been utilized with radiolabeled aquatic herbicides to study the absorption and translocation in aquatic plants (Kniss et al., 2011; True-Meadows, 2012; Vassios et al., 2014; Vassios et al, 2017). The objective of this study was to elucidate absorption and translocation patterns of florpyrauxifen-benzyl in these species. It was hypothesized that as compared to other herbicides, florpyrauxifen-benzyl absorption would be high, uptake would be fast, and translocation would be evident in sensitive species.
Methods

[Methodology adapted from Vassios, Nissen, Koschnick, and Heilman, 2014]

Plant Propagation and Preparation

The ten aquatic plant species utilized in this experiment were Eurasian watermilfoil (Myriophyllum spicatum L.), hybrid Eurasian watermilfoil (Myriophyllum spicatum L. x Myriophyllum heterophyllum Michx.), variable milfoil (Myriophyllum heterophyllum Michx.), dioecious hydrilla [Hydrilla verticillata (L.F.) Royle], monoecious hydrilla [Hydrilla verticillata (L.F.) Royle], Brazilian elodea (Egeria densa Planch.), tape-grass (Vallisneria americana Michx.), crested floating heart [Nymphoides cristata (Roxb.) Kuntze], giant salvinia (Salvinia molesta Mitchell) and water hyacinth [Eichhornia crassipes (Mart.) Solms]. Plant source locations are listed in Table 6-1. Submersed species and crested floating heart were planted in flat-bottom 25 mm diameter glass test tubes. The test tubes were filled with sterilized topsoil amended with slow release fertilizer (Osmocote Smart Release 15-9-12 ® The Scotts Company 14111 Scottslawn Road Marysville OH 43041) at a rate of 3 grams per liter of soil. 15 cm apical segments were cut from stock plants and planted in the fertilizer amended topsoil, and a thin layer of sand was placed on top. Once planted, submersed plants were established in dechlorinated tap water in a glass greenhouse with 30% shade-cloth for 8 weeks, with the exception of hybrid Eurasian watermilfoil (HWM). Due to poor growth following initial planting, new 15 cm apical segments of HWM were planted two weeks prior to treatment. At the same time, submersed species that had been growing for 6 weeks were trimmed to approximately 15 cm of shoot tissue. One week prior to treatment, plants were moved to the treatment room to acclimate.
One to two days prior to treatment, plants were briefly removed from the growth chambers and placed in test-tube racks with the majority of shoot tissue submersed in adjacent beakers of dechlorinated tap water. Water was dried from the surface of the sand and the walls of the test tube using paper towels to provide a tighter seal between the gel and the wall of the test tube. Once the surfaces were dried, a layer of agarose gel (1.5% v/v) (Phytagar, Invitrogen Corp., Grand Island, NY) was placed on the sand surface. The gel was maintained in a liquid state at a temperature of 30°C to 38°C.

Two floating species, giant salvinia and water hyacinth were also included in this experiment. Small (5 to 7 cm diameter) daughter plants of water hyacinth and small (3-5 node) giant salvinia plants were selected from established stock plants. Because roots were already established on these plants, they were immediately transferred to the treatment room to acclimate without an establishment period.

**Shoot Absorption and Translocation**

Treatments were carried out in a small temperature and light controlled room in the NCSU weed science laboratory. Treatment vessels were maintained under full spectrum lights (21µmol s\(^{-1}\) m\(^{-2}\) µA) with a 14hr light: 10hr dark light regime for the duration of the experiment. Natural sunlight was blocked from entering the room, just prior to and throughout the treatment.

Glass treatment vessels (7.6 L) were filled with 7 L of tap water treated with water conditioner (API Tap Water Conditioner ® Mars Fishcare North America Inc. 50E. Hamilton St. Chalfont PA 18914). Each treatment vessel was equipped with a custom built, 24-slot, stainless steel test tube rack. In order to contain the ten species slated for treatment, two tank types labeled tank A and tank B were established, each with 4 submersed species and one floating species. Each tank type was replicated 3 times for a total of 6 treatment tanks. Six experimental units of
five species were placed in Tank A and Tank B for a total of 10 different species. Tank A species included dioecious hydrilla, Eurasian watermilfoil, variable watermilfoil, crested floating heart, and giant salvinia. Tank B species included monoecious hydrilla, hybrid Eurasian watermilfoil, Brazilian elodea, valisneria, and water hyacinth. Plants were placed in test tube racks within holding buckets prior to treatment and all plants were lowered into the treatment vessels following application of the 14-C labeled herbicide and formulation blank.

All six treatment vessels were treated at a rate of 10 µg a.i. L\(^{-1}\) (or ppb). A mixture of 75% non-radiolabeled technical material, 25% radiolabeled technical material, and sufficient formulation blank equivalent to the 300 g a.i. L\(^{-1}\) suspension concentrate commercial aquatic formulation (Procellacor SC) was used to reach this target treatment rate. Radiolabeled and non-radiolabeled technical material were dissolved in acetone separately and then mixed. The mixed solution was tested using liquid scintillation spectroscopy (LSS). The formulation blank was diluted in water prior to application. Dissolved technical material was applied to the treatment vessels immediately followed by the application of diluted formulation blank. The vessel was then stirred with a glass stir rod to ensure uniform distribution of active ingredients and formulation. Following application, the test tube racks containing the plants to be treated were placed in each of the treatment vessels. This placement marked time zero for treatment and three replicate plants of each species were harvested without exposure to treatment vessels.

Plants were harvested at 0, 0.5, 1, 6, 12, 36, and 192 hours following treatment. Preliminary studies had shown that florpyrauxifen-benzyl is absorbed very quickly. As such, these harvest times were selected to be more heavily weighted toward earlier times after treatment, as compared to other studies, in order to capture the initial hyperbolic increase in absorption. At each designated harvest time, three replicate plants of each species were removed.
from the treatment vessels. Above and below ground material was removed from the test tube and separated at the agar line. Each tissue type was triple rinsed separately and then allowed to air dry. One milliliter of rinsate from the third rinse of each plant portion was analyzed by LSS to ensure that all surface radiation was removed and only absorbed radiolabeled material remained. Following approximately 1 to 2 hours of air drying plant tissue was placed in a paper bag. Paper bags with plant tissue were dried at 70°C for 48 hours. Dry biomass data was collected for each plant tissue type using an analytical balance. The plant tissue was then rolled in ash-free filter paper, labeled and stored at -4°C. Plant tissue and filter paper were combusted in a biological oxidizer (OX500® RJ Harvey Instrument Corporation 11 Jane St, Tappan, NY 10983) for 2 minutes and collected in 10mL of 14-C trapping oxidizing cocktail (OX161® RJ Harvey Instrument Corporation 11 Jane St, Tappan, NY 10983). The efficiency of the oxidizer was tested daily to meet EPA standards of 80 to 120%. Following combustion, radioactivity of samples was quantified by LSS (Tri-carb 2100TR Liquid Scintillation Counter ® Spectrofuge Corporation of North Carolina, Inc 4915 Prospectus Drive Suite A Durham, NC 27713) for 4 minutes per sample.

Statistical Analysis:

Analysis follows that of Vassios et al., 2014 and Vassios et al., 2017. Herbicide concentration in micrograms of herbicide per gram of dry weight was calculated in Microsoft Excel (2010) utilizing measured DPM of each sample, the measured dry biomass of each sample analyzed, and the specific activity of the radiolabeled florpypyauxifen-benzyl received. Levene’s Test for Homogeneity of Variance was run in MS Excel for each species prior to combining the two runs of concentration data for analysis. Following Levene’s Test, means and standard errors were calculated using the JMP Pro (ver. 13) extension platform for SAS software. These
tabulated values were then exported back to MS Excel and finally imported into SigmaPlot (ver. 13) for non-linear regression analysis. The regression analysis fit the hyperbolic model adapted from Kniss et al. (2011) by Vassios et al (2014; 2017) (Equation 1), where y is the predicted absorption at time x, and a and b are constants.

(Equation 1)

\[ y = \frac{ax}{1 + bx} \]

The Plant Concentration Factor (PCF) metric is often utilized to compare absorption across different herbicides with varying properties and applied at varying rates. While this experiment focused on the absorption and translocation of only one herbicide at one rate, we felt the inclusion of the PCF metric would be beneficial for future comparisons across studies. PCF was calculated following the equation presented in Vassios et al. (2017) as adapted from de Carvalho et al. (2007) (Equation 2), where plant concentration is the concentration of the herbicide (ng) in plant tissues divided by the fresh biomass (g) and water concentration is the concentration of the herbicide in the treatment water at the time of treatment (ng ml\(^{-1}\)).

(Equation 2)

\[ PCF = \frac{\text{plant concentration}}{\text{water concentration}} \]

The non-linear regression equations resulting from these analyses were used to calculate the predicted absorption at 192 hours after treatment \( (A_{192}) \) and the predicted time it would take to reach 90% of that absorption \( (T_{90}) \). These two metrics were recommended for comparisons of absorption in different species and plant portions in Vassios et al. (2017). The \( A_{192} \) value was used to compare the theoretical max absorption of different species and \( T_{90} \) will be was used to compare the rate of absorption or how quickly the plant absorbed to its maximum.
It is important to note that fresh weights were only collected during run two. Linear regression equations of second run dry weight to fresh weight comparisons were conducted for each species with separate equations for shoot ($r^2 = 0.48-0.97; p <0.001$) and root tissue ($r^2 = 0.49-0.95; p <0.001$). The equations were used to estimate the fresh weights for run one samples.

Translocation of herbicide from aboveground to belowground tissue was calculated as the percent of total radioactivity in the plant that was observed in the belowground portion of the plant. The mean and standard error of this value for each time interval (hours after treatment) were plotted using JMP Pro (ver. 13) graph builder.

**Results and Discussion**

The non-linear hyperbolic regression model fit Eurasian watermilfoil (pure EWM) aboveground florpyrauxifen-benzyl accumulation over time ($r^2 = 0.98$; Table 6-2;Figure 6-2). Our results showed a rapid increase in product concentration in the plant within the first 24 hours, and this is reflected by the predicted $T_{90}$ value of 24.21 hours (Table 6-2). Results from PCF also show this maximum concentration occurring between the 12 hr and 36 hr harvests (Table 6-3). The $PCF_{192}$ (120) and $A_{192}$ (18.14) indicate very high levels of absorption for Eurasian watermilfoil (Table 6-2; Table 6-3). The Eurasian watermilfoil $PCF_{192}$ (120 ± 34) for florpyrauxifen-benzyl was more than twice times the Eurasian watermilfoil $PCF_{192}$ (35±6) reported for triclopyr, another synthetic auxin (Vassios et al. 2017). This discrepancy indicates that florpyrauxifen-benzyl bioconcentrates in Eurasian watermilfoil tissues to a greater extent than trichlopyr. The results are not unexpected given the high level of sensitivity of EWM to florpyrauxifen-benzyl observed in other studies (Netherland and Richardson, 2016).
Results of belowground tissue analysis indicate only limited translocation to root tissue with an A$_{192}$ of 0.19 µg g$^{-1}$ (Table 6-2) and an average translocation of 0.48 ± 0.28% at 192 hours after treatment (data not shown). Vassios (2014) found high accumulation of triclopyr, an auxin mimic herbicide, with both granular and liquid formulation, however the authors indicate that this may have been due in part to pore water movement as the shoots and roots were not separated. In another study, Vassios et al. (2017) observed only 2.6±0.3% of the absorbed triclopyr herbicide was translocated to belowground tissue. The high herbicidal activity of florpyrauxifen-benzyl compared to triclopyr may be relevant in interpreting percent translocation results. Small-scale screening has documented that the new herbicide is as much as 370 times more active on EWM than triclopyr (Beets and Netherland, 2018). Vassios et al. (2017) observed a belowground A$_{192}$ of 71.42 µg a.i. g$^{-1}$ with triclopyr in EWM. When corrected for relative activity of the herbicides, the triclopyr EWM belowground A$_{192}$ would project to a belowground A$_{192}$ value for florpyrauxifen-benzyl of 0.19 µg a.i. g$^{-1}$. This value is identical average belowground A$_{192}$ value reported here for florpyrauxifen, thereby indicating similar translocation patterns for florpyrauxifen-benzyl as compared to triclopyr.

Absorption values for hybrid Eurasian watermilfoil (HWM) (A$_{192}$ = 10.43 µg g$^{-1}$; PCF$_{192}$ = 62 ±11) were lower than that of the genetically pure EWM (A$_{192}$ = 18.14 µg g$^{-1}$; PCF$_{192}$=120 ±34) (Table 6-2; Table 6-3). However, HWM reached 90% of max absorption faster ($t_{90}$ = 5.91 hr) than EWM ($t_{90}$ =24.21 hr) (Table 6-2). The lower absorption and lower PCF$_{192}$ observed in this study explains the reported reduced sensitivity to florpyrauxifen-benzyl of the Hayden Lake HWM as compared to pure EWM (Beets and Netherland, 2018). Similar to pure EWM, translocation in the Hayden Lake hybrid (HWM) appears low as a fraction of total absorbed herbicide with an average of 1.11±0.28 % of the total amount of herbicide absorbed (data not
shown). Regression equations for belowground tissue for HWM could not be calculated in Sigmaplot using the non-hyperbolic regression equation. However, raw data did indicate radioactivity in root tissue and thereby indicated some translocation did occur.

Variable watermilfoil florpyrauxifen-benzyl absorption ($A_{192} = 40.06 \mu g \text{ g}^{-1}$) was more than twice that of pure EWM and almost four times that of HWM (Figure 6-2). However, the fit curve had a much slower rise to maximum as evidenced by the $t_{90}$ of 92.7 hours (Table 6-2). This high level of absorption might be an important factor favoring previously reported extremely high sensitivity of variable milfoil to florpyrauxifen-benzyl with an EC$_{50}$ in small-scale, static exposure studies of less than 0.3 ppb (Richardson et al., 2016).

A hyperbolic non-linear regression model fit dioecious hydrilla shoot data very well ($r^2 = 0.97$). Vassios et al. (2017) observed an $A_{192}$ for dioecious hydrilla that was approximately 25% of that observed for EWM, when studying another auxin mimic herbicide, triclopyr. In contrast the results of this florpyrauxifen-benzyl study indicate that dioecious hydrilla shoots ($A_{192} = 25.31 \mu g \text{ g}^{-1}$) absorbed approximately 40% more herbicide than EWM shoot tissue. Higher absorption in dioecious hydrilla as compared to EWM is particularly interesting in light of the fact that efficacy studies found the EC$_{50}$ for EWM (EC$_{50} = 0.11 \pm 0.11 \mu g \text{ ai L}^{-1}$) to be lower than that of dioecious hydrilla (EC$_{50} = 1.4 \pm 0.1 \mu g \text{ ai L}^{-1}$) indicating higher sensitivity (Netherland and Richardson, 2016). EWM is a dicot and hydrilla is a monocot. It is plausible that there is a difference in the way the herbicide is metabolized in these two plants, which could explain these seemingly contrasting results.

Several studies have indicated that experimental results utilizing dioecious hydrilla cannot be universally applied to monoecious hydrilla (True-Meadows et al., 2016). The aboveground absorption results of this study are yet another example of such. The predicted
absorption in monoecious hydriilla based on our results (A_{192} = 4.83 \text{ cg g}^{-1}) is approximately 19% of the absorption predicted for dioecious hydriilla (A_{192} = 25.31 \text{ µg g}^{-1}) (Table 6-2; Figure 6-1; Figure 6-2). It should be noted that separate sensitivity studies have found florpyrauxifen-benzyl to be highly efficacious for both dioecious hydriilla and monoecious hydriilla (Netherland and Richardson, 2016; Richardson et al., 2016). Beets and Netherland (2018) found that doubling the rate of florpyrauxifen-benzyl from 24 µg L^{-1} to 48 µg L^{-1} did not increase observed efficacy nor did increasing the exposure from 24 to 72 hours. In this same study, the authors observed a significant decrease in belowground biomass of dioecious hydriilla (Beets and Netherland, 2018).

Regression equations for belowground tissue for monoecious and dioecious hydriilla could not be calculated in Sigmaplot using the non-hyperbolic regression equation. However, raw data did indicate radioactivity in root tissue and thereby indicated some translocation did occur.

Results for Brazilian elodea (A_{192} = 6.49 \text{ µg g}^{-1}), another submersed monocot, indicate a shoot absorption pattern somewhere between that of monoecious hydriilla and hybrid EWM (Table 6-2). Whereas results for tape-grass, a native submersed monocot, indicate a shoot absorption pattern that is the lowest of all plant species tested in this experiment (A_{192} = 2.57 \text{ µg g}^{-1}; PCF_{192} = 8.89) (Table 6-2; Table 6-3; Figure 6-1). Translocation was relatively low for both tape-grass and Brazilian elodea.

The hyperbolic non-linear regression model fit the shoot absorption data for crested floating heart, the only floating leaved dicot in this experiment, very well with an r^2 of 0.97 (Table 6-2; Figure 6-2). Predicted shoot absorption for crested floating heart at 192 hours after treatment (A_{192} = 20.06 \text{ µg g}^{-1}) was similar to that of EWM and dioecious hydriilla (Table 6-2). Again this high level of absorption is congruent with the high level of sensitivity to florpyrauxifen-benzyl observed for crested floating heart (Netherland and Richardson, 2016).
The highest level of belowground concentration of herbicide observed in this experiment was observed in crested floating heart, with $A_{192}$ of 1.28 µg g$^{-1}$ (Table 6-2; Figure 6-2). Observations of high belowground concentrations of florpyrauxifen-benzyl are consistent with a mesocosm study, in which treatment with 24 µg L$^{-1}$ of the herbicide for 24 to 72 hours resulted in significant reductions in belowground biomass (Beets and Netherland, 2018). This result is certainly encouraging given the starchy overwintering root structures for crested floating heart and may indicate true systemic control, rather than starch reduction from foliar control.

Water hyacinth and giant salvinia both showed moderate to high levels of absorption with predicted absorption levels of 8.66 µg g$^{-1}$ and 7.66 µg g$^{-1}$ respectively (Table 6-2; Figure 6-1). PCF values were also moderate to high at 61 for giant salvinia and 55 for water hyacinth (Table 6-3). As this was an in-water treatment, we were not able to physically separate the shoots and roots to quantify translocation in these floating species.

While the predicted shoot absorption values for monocots may appear to be on average lower than that of dicots in this experiment, it should be noted that this apparent difference was not significant in a one-way ANOVA ($p = 0.24$).

The percent of herbicide translocated from shoot to root tissue has been low, less than 5-10%, in other experiments examining radiolabeled herbicide translocation in submersed aquatic plant species (Vassios et al., 2017). Evidence of florpyrauxifen-benzyl translocation was observed in all rooted plant species tested, as indicated by the radioactivity measured in isolated root tissues. However, more florpyrauxifen-benzyl translocation was expected than was observed based on the classification of this herbicide as an aryl-picolinate. Aryl-picolinates as a class of herbicides have been shown to translocate well in terrestrial plant tissues (Bromilow et al., 1990; Epp et al., 2016). One possible explanation for the reduced translocation observed could be due
to application of the formulation blank separate from the application of the active ingredient. This methodology was recommended by the manufacturer; however, it is hard to predict how the chemicals will behave when applied separately in a novel (aqueous) environment. Alternatively, it is possible that the vascular tissue alteration was fast enough to reduce movement to belowground tissues, due to the small scale at which this experiment took place and the moderate florpypaauxifen-benzyl application rate utilized.

Conclusions:

While synthetic auxin herbicides generally affect dicots more than monocots, sensitivity analyses for florpypaauxifen-benzyl have shown high activity on a select number of monocot invasive aquatic plants. Evidence of translocation was observed in all rooted species tested and moderate to high translocation was observed in crested floating heart. High levels of shoot absorption and high plant concentration factors were observed for susceptible species and reduced absorption levels were absorbed for insensitive species. Overall, this study provides additional evidence that florpypaauxifen-benzyl is a good candidate for the selective control of invasive aquatic plant species.
Literature Cited


Figures and Tables:

Table 6-1: A list of the plant species utilized in this experiment and the origin of the plant collections.

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eurasian watermilfoil</td>
<td><em>Myriophyllum spicatum</em></td>
<td>Roanoke Rapids Lake, NC</td>
</tr>
<tr>
<td>Hybrid Eurasian watermilfoil</td>
<td><em>Myriophyllum spicatum</em></td>
<td>Hayden Lake, ID</td>
</tr>
<tr>
<td>Variable milfoil</td>
<td><em>Myriophyllum heterophyllum</em></td>
<td>farm pond, Sanford, NC</td>
</tr>
<tr>
<td>Dioecious Hydrilla</td>
<td><em>Hydrilla verticillata</em></td>
<td>Gainesville, FL</td>
</tr>
<tr>
<td>Monoecious Hydrilla</td>
<td><em>Hydrilla verticillata</em></td>
<td>Shearon Harris Reservoir, NC</td>
</tr>
<tr>
<td>Brazilian Elodea</td>
<td><em>Egeria densa</em></td>
<td>small pond, Raleigh, NC</td>
</tr>
<tr>
<td>Tape grass</td>
<td><em>Vallisneria americana</em></td>
<td>Lake Mattamuskeet, NC</td>
</tr>
<tr>
<td>Crested Floating Heart</td>
<td><em>Nymphoides cristata</em></td>
<td>Lake Marion, SC</td>
</tr>
<tr>
<td>Giant Salvinia</td>
<td><em>Salvinia molesta</em></td>
<td>stock plants from controlled infestation in a swamp in Wilmington, NC</td>
</tr>
<tr>
<td>Water Hyacinth</td>
<td><em>Eichhornia crassipes</em></td>
<td>small pond, Johnston County, NC</td>
</tr>
</tbody>
</table>
Figure 6-1: Hyperbolic non-linear regression plots for Eurasian watermilfoil hybrid, monococious hydrilla, Brazilian elodea, giant salvinia, water hyacinth and tapegrass. Aboveground data is symbolized with a dashed line for the regression and open triangles for the mean concentration. Belowground data is symbolized with a solid line for the regression and closed circles for the mean concentration. Error bars represent the standard error of the mean.
Figure 6-2: Hyperbolic non-linear regression plots for Eurasian watermilfoil, variable watermilfoil, dioecious hydrilla and crested floating heart. Aboveground data is symbolized with a dashed line for the regression and open triangles for the mean concentration. Belowground data is symbolized with a solid line for the regression and closed circles for the mean concentration. Error bars represent the standard error of the mean.
Table 6-2: Results of hyperbolic regression analysis and predicted values for the absorption at 192 hours ($A_{192}$) and the time to reach 90% of $A_{192}$ ($t_{90}$)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Scientific Name</th>
<th>Dicot or Monocot</th>
<th>Portion</th>
<th>a ± SE</th>
<th>b ± SE</th>
<th>$r^2$</th>
<th>$A_{192}$</th>
<th>$t_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eurasian watermilfoil</td>
<td><em>Myriophyllum spicatum</em></td>
<td>Dicot</td>
<td>Aboveground</td>
<td>5.89 ± 1.01</td>
<td>0.32 ± 0.06</td>
<td>0.98</td>
<td>18.14</td>
<td>24.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Belowground</td>
<td>4.70 ± 46.69</td>
<td>24.59 ± 248.92</td>
<td>-0.41</td>
<td>0.19</td>
<td>0.37</td>
</tr>
<tr>
<td>Hybrid Eurasian watermilfoil</td>
<td><em>Myriophyllum spicatum x sibericum</em></td>
<td>Dicot</td>
<td>Aboveground</td>
<td>15.38 ± 8.15</td>
<td>1.47 ± 0.85</td>
<td>0.85</td>
<td>10.43</td>
<td>5.91</td>
</tr>
<tr>
<td>Variable milfoil</td>
<td><em>Myriophyllum heterophyllum</em></td>
<td>Dicot</td>
<td>Aboveground</td>
<td>2.01 ± 0.79</td>
<td>0.05 ± 0.02</td>
<td>0.89</td>
<td>40.06</td>
<td>92.7</td>
</tr>
<tr>
<td>Dioecious Hydrilla</td>
<td><em>Hydrilla verticillata</em></td>
<td>Monocot</td>
<td>Aboveground</td>
<td>1.51 ± 0.29</td>
<td>0.05 ± 0.01</td>
<td>0.97</td>
<td>25.31</td>
<td>84.52</td>
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<tr>
<td>Dioecious Hydrilla</td>
<td><em>Hydrilla verticillata</em></td>
<td>Monocot</td>
<td>Aboveground</td>
<td>17.44 ± 10.05</td>
<td>3.61 ± 2.22</td>
<td>0.87</td>
<td>4.83</td>
<td>2.46</td>
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<tr>
<td>Brazilian Elodea</td>
<td><em>Egeria densa</em></td>
<td>Monocot</td>
<td>Aboveground</td>
<td>12.92 ± 6.82</td>
<td>1.98 ± 1.14</td>
<td>0.93</td>
<td>6.49</td>
<td>4.42</td>
</tr>
<tr>
<td>Tape grass</td>
<td><em>Vallisneria americana</em></td>
<td>Monocot</td>
<td>Aboveground</td>
<td>1.43 ± 0.22</td>
<td>0.55 ± 0.10</td>
<td>0.99</td>
<td>2.57</td>
<td>14.87</td>
</tr>
<tr>
<td>Crested Floating Heart</td>
<td><em>Nymphoides cristata</em></td>
<td>Dicot</td>
<td>Aboveground</td>
<td>1.59 ± 0.27</td>
<td>0.07 ± 0.01</td>
<td>0.97</td>
<td>20.06</td>
<td>71.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Belowground</td>
<td>0.004 ± 0.001</td>
<td>-0.002 ± 0.001</td>
<td>0.99</td>
<td>1.28</td>
<td>181.15</td>
</tr>
<tr>
<td>Giant Salvinia</td>
<td><em>Salvinia molesta</em></td>
<td>N/A</td>
<td>Aboveground</td>
<td>5.07 ± 0.94</td>
<td>0.65 ± 0.13</td>
<td>0.98</td>
<td>7.76</td>
<td>12.85</td>
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<td>Water Hyacinth</td>
<td><em>Eichhornia crassipes</em></td>
<td>Monocot</td>
<td>Aboveground</td>
<td>2.34 ± 0.35</td>
<td>0.26 ± 0.05</td>
<td>0.99</td>
<td>8.66</td>
<td>28.41</td>
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</table>
Table 6-3: Plant Concentration Factor (PCF) results for each species at each time interval after treatment expressed as mean ± standard error

<table>
<thead>
<tr>
<th>Species</th>
<th>0.5</th>
<th>1</th>
<th>6</th>
<th>12</th>
<th>36</th>
<th>192</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eurasian Watermilfoil</td>
<td>23 ± 3</td>
<td>37 ± 2</td>
<td>75 ± 11</td>
<td>82 ± 11</td>
<td>125 ± 31</td>
<td>120 ± 34</td>
</tr>
<tr>
<td>Hybrid Eurasian Watermilfoil</td>
<td>30 ± 5</td>
<td>37 ± 6</td>
<td>53 ± 9</td>
<td>98 ± 30</td>
<td>50 ± 6</td>
<td>62 ± 11</td>
</tr>
<tr>
<td>Variable Watermilfoil</td>
<td>18 ± 4</td>
<td>25 ± 5</td>
<td>63 ± 11</td>
<td>7 ± 10</td>
<td>145 ± 40</td>
<td>217 ± 64</td>
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<tr>
<td>Dioecious Hydrilla</td>
<td>14 ± 4</td>
<td>18 ± 4</td>
<td>32 ± 7</td>
<td>47 ± 6</td>
<td>93 ± 22</td>
<td>90 ± 20</td>
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<tr>
<td>Monoeocious Hydrilla</td>
<td>14 ± 4</td>
<td>18 ± 5</td>
<td>26 ± 6</td>
<td>24 ± 4</td>
<td>26 ± 4</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Brazilian Elodea</td>
<td>23 ± 3</td>
<td>35 ± 4</td>
<td>51 ± 8</td>
<td>48 ± 11</td>
<td>64 ± 14</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>Tapegrass</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
<td>4 ± 1</td>
<td>6 ± 1</td>
<td>7 ± 2</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Crested Floating Heart</td>
<td>5 ± 1</td>
<td>14 ± 7</td>
<td>13 ± 4</td>
<td>20 ± 4</td>
<td>34 ± 5</td>
<td>47 ± 11</td>
</tr>
<tr>
<td>Giant Salvinia</td>
<td>14 ± 2</td>
<td>27 ± 2</td>
<td>47 ± 7</td>
<td>63 ± 3</td>
<td>82 ± 8</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>Water hyacinth</td>
<td>10 ± 1</td>
<td>15 ± 2</td>
<td>34 ± 6</td>
<td>43 ± 5</td>
<td>49 ± 9</td>
<td>55 ± 9</td>
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