EVALUATION OF EURASIAN AND HYBRID WATERMILFOIL ACCESSIONS FOLLOWING EXPOSURE TO DIFFERENT ENVIRONMENTAL CONDITIONS AND HERBICIDES

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A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2018
To the memory of Dr. Mike Netherland
ACKNOWLEDGMENTS

I would like to thank my committee for their support: Dr. Mike Netherland, Dr. Stephen Enloe, Dr. Bill Haller, and Dr. Dail Laughinghouse. I would especially like to thank Dr. Netherland for all of his support and guidance. He gave me the opportunity to work for him and be his student, and he helped me tremendously since I started working for him both professionally and personally. Dr. Netherland dedicated so much time and energy into my work and personal development, and for that I will be forever grateful. His influence and support kept me in the aquatic plant management field and helped guide me to pursue a doctoral program. Dr. Jay Ferrell has also been invaluable in his support and guidance. Conversations with Dr. Ferrell have allowed me to look for new approaches and justifications in my work.

Thank you to everyone else that has helped and guided me in my work at UF especially Carl Della Torre, Chetta Owens, Dean Jones, Sherry Bostick, Cody Lastinger, and Joshua Wood.

I am also thankful for the organizations that helped fund my research: Florida Fish and Wildlife Conservation Commission, Aquatic Ecosystem Research Foundation, US Army Corps of Engineers Research and Development Center, and SePRO, especially Dr. Mark Heilman who has provided support and feedback in several studies.

Finally, I would like to thank my friends and family for their support and guidance. My parents and my sister, Kalmia, have been extremely supportive, helpful and are a continuing inspiration. My cousin, Jeremy, convinced me to make the move to Florida and get started in the field of aquatics, that I have grown to enjoy and appreciate.
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LIST OF ABBREVIATIONS

2,4-D 2,4-D [2,4-dichlorophenoxy] acetic acid
AI Active Ingredient
CAIP Center for Aquatic and Invasive Plants
CET Concentration exposure time
DAT Days after treatment
DW Dry Weight
HAT Hours after treatment
LAERF Lewisville Aquatic Ecosystem Research Facility
SE Standard Error
Eurasian watermilfoil (EWM) and Hybrid watermilfoil (HWM) are problematic submersed aquatic invasive plants in many waterways of the northern United States. Auxin-mimic herbicides, such as 2,4-D and triclopyr, are commonly used herbicides to manage to control invasive populations of EWM and HWM. The arylpicolinate herbicide florpyrauxifen-benzyl provides a new tool to augment control options of problematic aquatic weedy species including EWM and HWM. Experiments were conducted in growth chambers and large-scale mesocosms to better understand the efficacy of florpyrauxifen-benzyl on EWM and HWM and differences between HWM accessions. The aims of these studies were to: 1) evaluate HWM and EWM response to several auxin-mimic herbicides under static, environmentally controlled conditions, 2) evaluate the growth of two submersed plant species in OECD-recommended sediment, 3) investigate the potential for increased herbicide tolerance of HWM, 4) evaluate a wide range of CET conditions to determine the effect of florpyrauxifen-benzyl on well-established EWM, HWM, and several native aquatic plant species under large-scale mesocosm conditions, and 5) document differences in biomass and response to
herbicides between populations of NWM, EWM and HWM. Growth chamber results indicate strong response to florpyrauxifen-benzyl in both EWM and HWM, with differences in response between EWM and HWM for the auxin-mimic herbicides tested. Similar to growth chamber results large-scale studies demonstrated significant reduction in EWM and HWM in several CET scenarios. In addition to growth differences between HWM accessions, there were differences in herbicide response between accessions.
CHAPTER 1
LITERATURE REVIEW

Invasive species are a recognized threat to biodiversity and economic functions of many ecosystems worldwide. Invasive species are often non-native, and invasive plants have several key life history traits that make them better competitors compared to native species of the ecosystem they invade. Some of these traits include sexual and asexual reproduction, rapid growth, phenotypic plasticity, and tolerance to environmental heterogeneity (Sakai et al. 2001). Invasive species often lack the predators or pathogens that control the populations in their native habitats, increasing resource availability for these invasive species and their ability to invade. There are an estimated 25,000 introduced plant species in the United States, costing nearly $35 billion between losses, damages, and control. Of this, aquatic weeds cost $110 million (Pimentel et al. 2005). Similar to terrestrial systems, there many native aquatic plants desirable to ecosystems because of their benefit to biotic and abiotic factors within a lake. Aquatic plant species serve not only as food and habitat to fish and other aquatic organisms, but also improve diversity, stabilize sediment, and improve water clarity (Madsen 2014). Invasive aquatic species such as Eurasian watermilfoil (*Myriophyllum spicatum* L.) can destabilize the biodiversity as well the as economic and ecologic function of water bodies.

There are vascular plant species that dominate aquatic ecosystems and the majority or entirety of their life cycle occurs while submersed in water. These plants display a wide range of adaptations to submersed life, dependent on the conditions where they evolved. Most of these macrophytes have terrestrial ancestors, evidenced
Haloragaceae, the watermilfoil family, primarily includes marsh or water herbaceous eudicots distributed worldwide, with the one shrubby genus *Haloragodendron* endemic to Australia. Haloragaceae is comprised of eight genera with 145 recorded species. Defining characteristics include flowers that are often unisexual, although some species have bisexual flowers. The genus of most interest in the family is the aquatic *Myriophyllum*, with approximately 68 species (Moody and Les 2010). There are fourteen milfoil species present in the United States, including both native and invasive species. There is confirmed hybridization between native and non-native species, with reported hybrids of *M. heterophyllum* Michx. × *M. pinnatum* (Walter) Britton et al. and *M. spicatum* L. × *M. sibiricum* Kom. confirmed (Moody and Les 2002).

**Eurasian Watermilfoil**

Eurasian Watermilfoil, hereafter referred to as EWM is a submersed eudicot native to Eurasia first reported in the United States in the 1940s. Since then EWM has spread throughout aquatic ecosystems the Northern third of the country. There are at least two distinct genetic lineages of EWM present in North America (Zuellig and Thum 2012). EWM is listed as a noxious weed in several southern states including Florida, North Carolina, South Carolina, and Alabama. Despite a variety of management options, EWM has proven to be a persistent problem in lakes and rivers (Smith and Barko 1990, Madsen et al. 1991).

EWM is a rooted perennial plant with slender, heavily branched stems and grayish-green, whorled leaves with a feathery appearance and 24 or more leaflets (Figure 1-1; Godfrey and Wooten 1981). EWM rapidly establishes in a variety of
habitats, including impounded and natural freshwater, as well as both brackish and spring waters. One factor that contributes to the invasive potential of EWM in displacing native vegetation is its ability to spread via vegetative growth as well as fragmentation. Milfoil species have a high propensity to autofragment after peak biomass is attained. Autofragmentation is a self-induced abscission of apical stems that have formed roots (Madsen and Smith 1997). This allows for a more rapid rate of long-distance spread than stolon expansion. Autofragmentation accounts for up to 26% of EWM expansion, with stolon growth remaining as the primary means of local spread (Madsen and Smith 1997). EWM can also use allofragmentation, or mechanical breakage, as a means of fragment spread (Madsen and Smith 1997).

Invasive milfoils have a longer growing period and photosynthetic tissue configuration that allows for growth a wider range of light conditions than many common native species such as Northern watermilfoil \((Myriophyllum sibiricum)\) Kom., coontail \((Ceratophyllum demersum)\) L., and \(Vallisneria\) spp. (Grace and Wetzel 1978). Peak biomass \((200-2000 \, g \, m^{-2} \, DW)\) occurs from June to September (Adams and McCracken 1974, Stanley et al. 1975), with biomass remaining unchanged during early winter. Flowering and seed production coincide with peak biomass production during the warmer summer months, with fluctuations in growth occurring after flowering due to fragmentation (Stanley et al. 1975, Adams and McCracken 1974, Smith and Barko 1990). Overwintering generally occurs in an evergreen form, however, new unexpanded shoots attached to rootstocks also aid overwintering (Stanley et al. 1975). Dormancy may also play a role in preventing localized extinction events (Grace and Wetzel 1978).
EWM is typically found in water 1 to 10 m deep, with growth to the water surface commonly observed in water 3 to 5 m deep (Aiken et al. 1979). This is due to a low light compensation point, which allows it to inhabit deeper regions of water bodies depending on water clarity (Grace and Wetzel 1978). As milfoils reach the water surface the stems tend to branch profusely, while the lower leaves slough off, thus forming a dense surface canopy that has the potential to shade out native vegetation and subsequently reduce species diversity (Grace and Wetzel 1978, Madsen et al. 1991). Buoyancy is provided via vascular lacunae that oxygenate the roots (Moody and Les 2010). Like many submersed macrophytes, EWM shows a preference for nutrient-rich sediments and tends to grow better on fine-textured inorganic sediments (Li et al. 2015, Smith and Barko 1990).

Despite having some aspects of Krantz anatomy that are typical of C₄ plants, milfoil is a C₃ plant (Stanley and Naylor 1972). Like several other aquatic species, EWM photorespiration is much lower than that of terrestrial C₃ plants, however, the ratio of photosynthesis to photorespiration is more similar to terrestrial C₃ plants (Van et al. 1976).

**Northern Watermilfoil**

Northern watermilfoil, hereafter referred to as NWM is a native species with a range spanning from Alaska throughout the continental United States excluding the southeastern United States (USDA 2011). Much like EWM, NWM is a rooted perennial with leaves in whorls around the stem with six to eleven leaflets per leaf. While it can produce high plant biomass, it is not generally recognized as a canopy forming plant. In addition to reproducing vegetatively and via seed, NWM produces turions at the terminal node (Berger 2011). NWM provides a high-quality habitat for aquatic wildlife,
serving as both cover and a food source, making it integral to proper ecosystem function.

**Hybrid Watermilfoils**

Hybridization between species is recognized as important and frequent in the evolution and speciation of plants. Hybridization can contribute to genetic diversity, adaptations, and the formation of ecotypes and species (Rieseberg et al. 1993). Hybridization has also been implicated in contributing to and promoting invasions (Rieseberg et al. Ellstrand 1993). Plant Communities where hybridization can occur between invasive and native species may be more susceptible to invasion and extinction of a parental species may occur (Sakai et al. 2001). From a management standpoint, hybridization can further complicate identification due to the ability to present characteristics similar to either parent or even novel morphologies.

Hybridization in invasive plant management creates two major concerns: 1) gene contamination via outbreeding depression or genetic assimilation that can drive the native parent species extinct and 2) hybridization that leads to heterosis or hybrid vigor (Moody and Les 2002). Thompson (1991) reported that the hybridization of a native and invasive *Spartina* sp. has the potential to create recombinant genomes with genotypes superior in fitness to parental species. Heterosis is of particular concern in species that spread through vegetative means as the dissolution of heterosis is less likely and can spread indefinitely (Moody and Les 2002).

Human disturbance may lead to the creation of novel niches that hybrids are better suited to fill compared to their parent species (Ellstrand and Schierenbeck 2000). One example of this is chemical applications to control invasive species in water bodies, where an herbicide treatment may control more susceptible parent plants and create the
opportunity for hybrids to spread within a lake. There is even the possibility for hybridization to stimulate the evolution of invasive potential (Ellstrand and Schierenbeck 2000).

Hybrid Watermilfoil (Myriophyllum spicatum L. x Myriophyllum sibiricum Kom.), hereafter called HWM, was first documented by Moody and Les (2002). Due to morphological similarities between HWM and its parental species it is suspected that hybrids went unnoticed for several years or possibly decades and were only definitively identified as hybrids based on ribosomal DNA analysis (Moody and Les 2002). HWM may pose a greater threat due to higher invasive potential and lower sensitivity to herbicides than EWM (Larue et al. 2013, Berger et al. 2015). The genetic mechanisms for these concerns are not well understood but could be due to heterosis or increased genetic variation (Larue et al. 2013).

There may be significant differences between HWM in different lakes due to the hybrid populations arising independently (Sturtevant et al. 2009). Several studies have indicated there are populations of HWM that show differences in response to several herbicides compared to EWM. The HWM population in Townline Lake (Big Rapids, MI) has shown reduced and variable response to the herbicides fluridone, 2,4-D, and triclopyr (Berger et al. 2012, Berger et al. 2015, Glomski and Netherland 2010, LaRue et al. 2013, Thum et al. 2012). HWM can be more abundant in lakes historically treated with 2,4-D than either parental species (LaRue et al. 2013). Resource managers in northern states have reported differential herbicide response in several northern lakes following treatment. While significant efforts have been placed on identifying hybrid
watermilfoils, there has been much less information generated regarding the potential for hybrid vigor, invasive potential, and response to different herbicides.

**Management**

Aquatic weeds that form surface canopies, or mats, are known to negatively affect water quality by reducing dissolved oxygen in the water column below the mat and leading to high variation in temperatures and pH (Bowes et al. 1979). In addition to creating severe daily fluctuations in environmental conditions, mat formation by invasive species such as EWM also shades out other, often lower growing submersed species present, which reduces plant species diversity and promotes a monoculture. Large-scale aquatic invasive species infestations have multiple economic and ecological impacts, such as interrupting navigation, providing habitat for disease vectors, impeding water flow, altering macroinvertebrate diversity, and nitrogen and phosphorus loading from plant degradation (Madsen et al. 1991). Control measures to mitigate impacts on recreation, fisheries, and wildlife diversity in water bodies is often necessary due to the combination of negative water quality impacts, water flow reduction/blockage, and native species displacement.

There are several non-chemical methods of control used to manage aquatic invasive submersed plant species. Mechanical harvesting can provide large-scale control of invasive aquatic species, though it is usually non-selective and results are often temporary. Mechanical control has the potential to spread fragments and may create mats of decomposing fragmented plant matter in the water that pose health risks such as mosquito breeding habitat (Madsen 2000, Grace and Wetzel 1978). Costs of submersed weed harvesting vary and are dependent on location and the species of submersed plants. Harvesting usually requires the harvested plant biomass to be
transported off site which further increases the cost of mechanical control. Milfoil harvest costs have ranged from $300 to $600/acre and hydridlla (*Hydrilla verticillata* [L.f.] Royle) approximately $455/acre (University of Minnesota 2018, Haller and Jones 2012).

Drawdowns are a common method used to desiccate milfoil and other evergreen perennial aquatic species (Tarver 1980). Drawdowns can be particularly effective in winter, and can provide long-term control, but require six to eight weeks for drying and can interfere with the use of the water body as well as increase the potential for annual plants to spread (Haller 2014). While drawdowns are generally feasible on reservoirs, they are much less likely to be an option for control in natural lakes.

Biological control has been used with varying levels of success. Grass carp (*Ctenopharyngodon idella* Valenciennes) can provide a long-term and cost-effective means of invasive plant management, especially when combined with chemical management practices (Eggeman 1994). They are a popular control method in isolated water bodies and grass carp preferentially feed on specific aquatic plant species such as hydridlla (Madsen 2000). However, grass carp have a lower preference for EWM than other invasive plant species and may in fact release EWM infestations from hydridlla and other targeted species (Richardson 2008). For this reason, grass carp are rarely used in public waters of northern states for EWM control. The milfoil weevil (*Euhrychiopsis lecontei* D.) is native to North American with the ability to reduce milfoil growth at insect densities above 25 m$^{-2}$ (Newman and Inglis 2009). *Euhrychiopsis lecontei* has the ability to reduce root biomass as well as aboveground biomass (Newman et al. 1996). Detached biomass increases with weevil density, while the total above ground biomass
does not significantly differ from populations not affected by the weevil, which indicates insect damage is often too low to provide effective control (Newman et al. 1996).

**Response of EWM to Herbicides**

EWM has a long history of control with herbicide treatments, such as 2,4-D which has been used since the 1950s (Gallagher and Haller 1990). Understanding concentration and exposure time (CET) is critical to proper control of EWM and other aquatic plants (Netherland and Getsinger 1992). Laboratory and field studies have proven invaluable in developing effective herbicide use patterns for control of EWM (Berger et al. 2012, Poovey et al. 2007). Poovey et al. (2007) suggested that the effectiveness of herbicide applications is dependent on several factors, including plant growth, age, and density. Seasonal timing of triclopyr treatments can provide selective control of EWM (Netherland and Glomski 2014). Triclopyr and 2,4-D have been used to control EWM in both small-scale spot treatments and whole-lake treatments at lower dosages (Glomski and Netherland 2010, Green and Westerdahl 1990, Nault et al. 2014, Wersal et al. 2010). Low dose long-term exposure treatments across an entire water body, while initially highly effective, have been suggested to provide selection pressure that could result in displacement of more sensitive parental genotypes and selection for a more tolerant hybrid.

Several other herbicides and herbicide combinations have been used to control EWM. Fluridone has historically provided selective control with large-scale, low concentration applications (Madsen et al. 2002) and have the added benefit of multiple year control and cost effectiveness (Berger et al. 2012, Madsen et al. 2002). Carfentrazone has also been shown to reduce EWM biomass, however, more effective control is attained when used in combination with 2,4-D (Gray et al. 2007). Diquat can
provide control of EWM even with relatively short half-lives (Skogerboe et al. 2006). Similarly, endothall provides selective control of EWM and is dependent on rate and timing (Skogerboe and Getsinger 2002). The contact herbicides diquat and endothall tend to provide only short-term control, causing applicators to favor the use of triclopyr, 2,4-D, and fluridone for long term control of EWM (Berger et al. 2012). Due to reduced efficacy of herbicide treatments on HWM and repeat applications of fluridone leading to resistance in hydrilla (Netherland and Jones 2015), there is a need for the development of new herbicide chemistry and a better understanding of differences between HWM and EWM.

Auxin Mimic Herbicides

Auxin-mimic herbicides, such as 2,4-D and triclopyr, are commonly used management tools to control invasive populations of EWM and HWM (Netherland and Getsinger 1992; Poovey et al. 2007; Wersal et al. 2010). These herbicides provide selective and systemic control of many aquatic invasive species. Auxin hormones are involved in root initiation, shoot growth, and development, among other plant growth processes (Grossman 2010). Auxin-mimic herbicides simulate auxin overdose in plants; however, synthetic auxins are more stable than natural auxins, making the synthetic auxins more resistant to inactivation by the plant (Grossman 2010, Richardson et al. 2016). Auxin-mimic herbicide damage acts in three successive phases: 1) stimulation, where abnormal plant growth is due to uncontrollable cell division, 2) inhibition, where plant growth is stunted, and physiological responses are suppressed, and 3) decay due to cell wall degradation (Richardson et al. 2016). Auxin herbicides are perceived by the TIR1/AFB auxin receptors, inactivating Aux/IAA repressors and depressing auxin response factors (Grossman 2010). This causes an overexpression of the genes
responsible for ethylene and abscisic acid (ABA) biosynthesis. Excess ethylene in shoots leads to epinasty and permanent over expression of ABA. ABA distribution throughout the plant mediates stomatal closure and reactive oxygen species (ROS) are overproduced along with limited transpiration and carbon assimilation. ABA also limits cell division and combined with ethylene cause chloroplast damage and destroy cell membranes. Ultimately, this leads to growth inhibition, tissue desiccation, and plant death (Grossmann 2010).

**Florpyrauxifen-benzyl (ProcellaCOR)**

Florpyrauxifen-benzyl is a new herbicide chemistry developed by SePRO Corporation (Carmel, Indiana, USA) in partnership with Dow Agrosciences (Indianapolis, Indiana, USA). The herbicide is part of a new class of synthetic auxins, the arylpicolinates, that differ in binding affinity compared to other auxins registered for aquatic use such as 2,4-D and triclopyr (Bell et al. 2015, Lee et al. 2013). Florpyrauxifen-benzyl has a binding affinity more similar to the terrestrially registered herbicide, aminopyralid (Epp et al. 2016). Florpyrauxifen-benzyl is translocated to growing points within affected plants, however previous studies show little translocation to the roots (Miller and Norsworthy 2018).

Dow AgroSciences has developed this chemistry for use in rice to control multiple herbicide resistant barnyard grass (*Echinochloa crus-galli* [L.] P Beauv.). Florpyrauxifen-benzyl shows high efficacy on barnyard grass at label rates, with no difference between plants resistant or susceptible to other herbicides (Duy et al. 2018; Miller et al. 2017). In small-scale laboratory screening, florpyrauxifen-benzyl is also shown to be active on several aquatic weed species including crested floating heart (*Nymphoides cristata* [Roxb.] Kuntze), hydrilla – (both dioecious and monoecious
biotypes), and Eurasian watermilfoil (Beets and Netherland 2018a, Netherland and Richardson 2016, Richardson et al. 2016). These studies suggest rapid uptake and activity under static conditions at low concentrations from 1 to 27 µg L⁻¹ as well as selectivity against several desirable native species.

**Growth Chambers and OECD**

Growth chambers provide highly controlled, uniform conditions allowing for increased repetition and elimination of external variables such as shading, herbivory, and temperature fluctuations. While growth chambers have their own set of limitations, such as algal interference, they have small space requirements and provide a controlled environment for testing (Netherland and Getsinger 2018). Protocols developed by the European Organisation for Economic Co-operation and Development (OECD) have been adopted to further standardize studies in growth chambers and allow for direct comparisons between trials performed at different times and by different laboratories.

The OECD developed several protocols for testing non-target damage by chemicals, especially herbicides, on aquatic species. The protocol was historically used on *Lemna* spp. and algae, but more recently was adopted for *Myriophyllum* spp. to allow for meaningful testing of different modes of action (OECD 2014). The protocol provides specific guidelines for light and temperature conditions, water quality, sediment, and experimental design to ensure consistency between replicates. OECD protocol allows for greater replication in small-scale studies than commonly used methods such as aquaria or small mesocosms may allow. Although the protocol was designed for risk assessment of chemicals on rooted dicotyledons, it has been shown to be effective for testing new herbicides (Netherland and Richardson 2016), as well as bioassays of contaminated sediments (Feiler et al. 2004). Consistency of purchased sediments can
often be problematic in comparisons between trials, creating a need for a standardized alternative, which OECD protocols provide. The OECD-recommended substrate requires mixing of components, which can be time intensive and costly to have the components shipped.

The overall goal of this research is to evaluate differences in growth and herbicide response between HWM and its parental genotypes (EWM and NWM), as well as possible variation between HWM populations. The first objective was to evaluate auxin herbicide response in EWM and HWM in a series of small-scale experiments. The second objective was to evaluate differences in submersed species growth in various substrate types and fertilization levels, to compare the OECD-recommended growth media to other growth media in small-scale studies. The third objective was to evaluate florpyrauxifen-benzyl activity on well-established milfoils and several native submersed species in large-scale mesocosm evaluations. This was based on high activity of florpyrauxifen-benzyl seen in small-scale studies. The final objective was to better understand growth and herbicide efficacy differences between HWM populations, as well as populations of its parental species. This was done by microsatellite analysis of several HWM accessions, as well as small and large-scale evaluations of herbicide response. This research can provide a better understanding of how results from small-scale studies translate to studies performed in larger, more realistic systems. These results can inform management practices and improve the knowledge of invasive milfoils.
Figure 1-1. Line drawing of Eurasian watermilfoil (EWM; *Myriophyllum. spicatum* L.).
CHAPTER 2
GROWTH CHAMBER EVALUATION OF FIVE AUXIN-MIMIC HERBICIDES AGAINST EURASIAN AND HYBRID WATERMILFOIL

There is increasing concern by aquatic plant managers regarding apparent decreased herbicide efficacy on hybrid watermilfoil (*M. spicatum x M. sibiricum* Kom.; HWM) compared to Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) control. For example, 2,4-D tolerance has been observed in the HWM population from Hayden Lake, Idaho (Tom Woolf, personal communication, Taylor et al. 2017). These anecdotal observations by plant managers on the apparent differential responses of EWM and HWM require additional studies. Studies have compared auxin efficacy on EWM and HWM under different conditions, but there is a lack of direct comparisons between these two milfoils to auxin mimics tested under uniform laboratory conditions (Glomski and Netherland 2010, Netherland and Willey 2017, Poovey et al. 2007).

EWM and HWM submersed aquatic invasive plants that have spread across the northern United States and are problematic in several water bodies such as Hayden Lake, ID and Lake Minnetonka, MN. Both genotypes quickly displace native vegetation by often forming dense surface mats, which outcompete other submersed species, impede water flow, alter macroinvertebrate diversity, and can lead to nitrogen and phosphorus loading from plant degradation (Madsen et al. 1991). This can alter water quality parameters such as DO, temperature, and pH, by stratification similar to that observed for invasive hydrilla (Bowes et al. 1979). The combination of negative water quality impacts, water flow reduction/blockage, and native species displacement often necessitates control to mitigate negative impacts on recreation, fisheries, aesthetics, and wildlife diversity in water bodies.
Auxin mimic herbicides, such as 2,4-D and triclopyr, are commonly used management tools to control invasive populations of EWM and HWM (Netherland and Getsinger 1992; Poovey et al. 2007; Wersal et al. 2010). These herbicides provide selective and systemic control of many aquatic invasive dicotyledons, including EWM and HWM. Auxin-mimic herbicides simulate auxin overdose in plants, however, these are more stable than natural auxins, making the synthetic auxins more resistant to inactivation by the plant. Auxin hormones are involved in root initiation, shoot growth, and development, among other processes (Grossman 2010). Auxin-mimic herbicide damage occurs in three successive phases: 1) stimulation, where abnormal plant growth occurs due to uncontrollable cell division; 2) inhibition, where plant growth is stunted, and physiological responses are suppressed; and 3) decay due to cell wall degradation (Grossman 2010, Sterling and Hall 1997).

The development of the arylpicolinate herbicide, florpyrauxifen-benzyl, provides a potential new product to augment control options of problematic aquatic weed species. Florpyrauxifen-benzyl is part of a new class of synthetic auxins, the arylpicolinates, that differ in binding affinity compared to currently registered auxins such as 2,4-D and triclopyr (Bell et al. 2015, Lee et al. 2013). In small-scale laboratory evaluations, florpyrauxifen-benzyl has shown high activity on EWM (Netherland and Richardson 2016, Richardson et al. 2016). Aminocyclopyrachlor and aminopyralid have also shown high activity on many invasive species. Bukun et al. (2010) reported that aminocyclopyrachlor has the potential for greater biological activity than other auxin herbicides due to higher absorption. Aminopyralid has shown comparable or improved efficacy for control of Canada thistle (*Cirsium arvense*) (Enloe et al. 2007). It is unknown
if this high level of terrestrial activity translates into aquatic systems on a plant with
known auxin mimic susceptibility, but studies have shown aminocyclopyrachlor efficacy
on several aquatic floating plants, including water hyacinth (*Eichhornia crassipes* [Mart.]
Solms) (Israel 2011).

The objectives of these experiments were to 1) evaluate HWM and EWM
response to five auxin-mimic herbicides in static, environmentally controlled conditions,
using EC values derived from length and biomass of treated plants, and 2) investigate
decreased herbicide efficacy on HWM and provide insight concerning tolerance to a
specific auxin-mimic herbicide or tolerance to the family of herbicides. This study will
focus on a confirmed HWM from a single population that has been reported to be
tolerant to 2,4-D (Tom Woolf, personal communication), however, it is important to
consider that hybrid populations arise independently, and herbicide response may vary
greatly between populations due to inherited traits.

**Materials and Methods**

The efficacy of five auxin-mimic herbicides was evaluated on EWM and HWM in
growth chambers at the University of Florida Center for Aquatic and Invasive Plants
(CAIP), Gainesville, FL. All chambers were kept at 25 C on a 16-hour light cycle. Trial 1
was initiated on 7/21/15 for EWM and 5/11/16 for HWM, and repeated (Trial 2) on
10/11/16 for HWM and 2/26/18 for EWM. Five auxin herbicides were tested on EWM
and HWM, with an untreated control in each trial. All plant material was taken from
culture tanks at CAIP (EWM was originally collected from Crystal River, FL and HWM
originally from Hayden Lake, ID). Two 10 cm apical stems were collected from the EWM
and HWM stock tanks and planted separately in soil in 250 mL beakers hen placed in
2L beakers containing nutrient solution in the growth chambers. The plants were
allowed to grow for seven days prior to treatment to allow root development. Each experimental unit consisted of a 2L beaker containing a 250 mL beaker with 200 mL of Organisation for Economic Co-operation and Development (OECD) sediment with two apical stems of EWM or HWM in growth solution as described in Smart and Barko (1985) (OECD 2014).

Each of the five auxin herbicides was tested at eight concentrations with three replications in trial 1 and five replications in trial 2 (Table 2-1). Each beaker was randomly assigned an herbicide concentration. In addition, five beakers were randomly selected for pretreatment harvest. Due to space constraints in growth chambers, treatments were tested sequentially, with 2,4-D¹, aminocyclopyrachlor², and aminopyralid³ tested first on HWM followed by triclopyr⁴ and florpyrauxifen-benzyl⁵. This treatment plan was then repeated on EWM. Two weeks after each trial was initiated, all plant root and shoots were harvested and combined then sprayed with water to remove necrotic material, and total length of each plant (sum of main stem and all branching stems) measured to the nearest 0.1 cm. Treatment placement was randomized on each shelf for all trials. Total plant length and plant dry biomass were used to determine half maximal effective concentration (EC₅₀) values. Samples were placed in labeled bags in a forced air-drying oven at 60 C for 72 hours to determine dry biomass to nearest 0.01 g.

**Statistical analysis:** To account for temporal differences between treatments as well as variation in growth chamber temperature and light source fluctuations, each shelf contained one replication of each treatment in a Randomized Complete Block Design (RCBD). Each shelf was be treated as a separate block. Data between the repeated trials were not statistically different at the 5% level and therefore combined for
analysis. Analysis was performed in R using the drc package based on dry viable biomass and total length (Knezevic et. al 2007). The drc package was also used to create the dose-response figures.

**Results and Discussion**

Symptoms typical of auxin herbicides were observed within two days of treatment with epinasty and necrosis being the most prevalent. Florpyrauxifen-benzyl was extremely active on EWM based upon dry biomass reduction with an EC$_{50}$ value of 0.001 µg L$^{-1}$ ai, 5600x less than the next lowest EC$_{50}$ value, 5.6 µg L$^{-1}$ ai for aminocyclopyrachlor (Table 2-2). EWM sensitivity to triclopyr, aminocyclopyrachlor, and 2,4-D did not significantly differ based on EC$_{50}$ values for dry biomass (Table 2-2).

Similar trends were observed for EC$_{50}$ values based on length, although the drc package was not able to determine an EC$_{50}$ value for florpyrauxifen-benzyl due to high mortality (Table 2-3). Aminopyralid did not differ in EC$_{50}$ values from the other auxin mimics tested, despite its EC$_{50}$ value being noticeably greater than the other EC$_{50}$ values. The high variation in aminopyralid EC$_{50}$ values was likely due to two replicates being observably less robust at the 1 and 27 µg L$^{-1}$ ai treatments than other plants at the same rate. The results from these trials indicate that florpyrauxifen-benzyl is highly active on EWM, and at concentrations up to four orders of magnitude lower than other auxin-mimic herbicides.

Florpyrauxifen-benzyl was also highly active on HWM with a biomass EC$_{50}$ value of 0.38 µg L$^{-1}$ ai, 38x lower than the triclopyr EC$_{50}$ value of 14.7 µg L$^{-1}$ ai. Triclopyr had a significantly lower biomass EC$_{50}$ value than 2,4-D, aminocyclopyrachlor, and aminopyralid. Aminocyclopyrachlor, 2,4-D, and aminopyralid did not have different EC$_{50}$ values for HWM based on biomass (Table 2-2). Herbicides fell into three groups in
terms of efficacy on HWM biomass, with florpyrauxifen-benzyl exhibiting the highest efficacy followed by triclopyr, with efficacy not differing between 2,4-D, aminocyclopyrachlor, and aminopyralid. A similar trend was observed in EC$_{50}$ values based on length, with variation in the magnitudinal differences between compounds compared to biomass values (Table 2-3).

EC values for florpyrauxifen benzyl increased from 0.001 for EWM to 0.38 µg L$^{-1}$ ai for HWM, indicating HWM is less sensitive to florpyrauxifen-benzyl (Figure 2-1a; Table 2-2). HWM response to triclopyr did not significantly differ from EWM, while HWM was 5.8x less sensitive to 2,4-D than EWM (Figure 2-1c). EWM was 17x more sensitive to aminocyclopyrachlor than HWM (Figure 2-1d) but sensitivity to aminopyralid did not significantly differ between EWM and HWM (Figure 2-1e). These differences indicate that Hayden HWM is not only 2,4-D tolerant but also exhibits differences in herbicide sensitivity across auxin-mimic herbicides in short-term static conditions. Florpyrauxifen-benzyl shows a high degree of activity on both EWM and HWM at concentrations significantly lower than the other herbicides tested, suggesting it has promise as an effective tool for managers with EWM and HWM infestations.

While aminocyclopyrachlor and aminopyralid did provide control at use rates similar to 2,4-D and triclopyr, they were not as effective as florpyrauxifen-benzyl. Due to high herbicidal activity noted in terrestrial systems, it is interesting that this is not observed in aquatic systems, or at least with milfoils. There may be a bias issue in the protocol used. Based on the data from these trials, it could be predicted that florpyrauxifen-benzyl would have a strong effect in terrestrial systems, while aminocyclopyrachlor and aminopyralid would have less of an effect, which is not what
has been observed with aminocyclopyrachlor (Enloe et al. 2007, Minogue et al. 2011). It is apparent that florpyrauxifen-benzyl is more toxic to milfoils based on activity at much lower concentrations than the other auxin-mimic herbicides tested. However, there are also efficacy differences between EWM and this accession of HWM with some of the other auxin-mimic herbicides.
Table 2-1. Overview of five auxin-mimic herbicides tested at eight concentrations on EWM and HWM in each growth chamber trial (n=3 Trial 1, n=5 Trial 2).

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Concentrations (µg a.i. L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>0, 0.1, 0.3, 1, 3, 9, 27, 81</td>
</tr>
<tr>
<td>Triclopyr</td>
<td>0, 1, 3, 9, 27, 81, 243, 729</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0, 1, 3, 9, 27, 81, 243, 729</td>
</tr>
<tr>
<td>Aminocyclopyrachlor</td>
<td>0, 1, 3, 9, 27, 81, 243, 729</td>
</tr>
<tr>
<td>Aminopyralid</td>
<td>0, 1, 3, 9, 27, 81, 243, 729</td>
</tr>
</tbody>
</table>
Table 2-2. Average EC$_{50}$ (µg a.i. L$^{-1}$) values based on dry biomass with 95% confidence intervals of EWM and HWM for the five auxins tested at eight concentrations (pooled; n=8).

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>EWM EC$_{50}$</th>
<th>HWM EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>0.001 [0, 0.006]</td>
<td>0.38 [0.10, 0.65]</td>
</tr>
<tr>
<td>Triclopyr</td>
<td>9.9 [6.2, 13.5]</td>
<td>14.7 [7.4, 22.1]</td>
</tr>
<tr>
<td>2,4-D</td>
<td>10.4 [4.9, 15.8]</td>
<td>60.3 [25.0, 95.7]</td>
</tr>
<tr>
<td>Aminocyclopyrachlor</td>
<td>5.6 [2.0, 9.2]</td>
<td>94.1 [38.0, 150]</td>
</tr>
<tr>
<td>Aminopyralid</td>
<td>66.3 [0, 149]</td>
<td>73.7 [34.7, 113]</td>
</tr>
</tbody>
</table>
Table 2-3. Average EC\textsubscript{50} (µg a.i L\textsuperscript{-1}) values based on total plant length with 95% confidence intervals of EWM and HWM for the five auxins tested at eight concentrations (pooled; n=8).

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>EWM EC\textsubscript{50}</th>
<th>HWM EC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>N/D*</td>
<td>0.19 [0.01, 0.37]</td>
</tr>
<tr>
<td>Triclopyr</td>
<td>5.5 [1.48, 9.6]</td>
<td>14.2 [2.0, 26.4]</td>
</tr>
<tr>
<td>2,4-D</td>
<td>8.3 [2.80, 13.7]</td>
<td>45.7 [22.3, 69.0]</td>
</tr>
<tr>
<td>Aminocyclopyrachlor</td>
<td>6.4 [0.50, 12.2]</td>
<td>111 [44.8, 176]</td>
</tr>
<tr>
<td>Aminopyralid</td>
<td>25.4 [0, 68.2]</td>
<td>92.9 [42.3, 143]</td>
</tr>
</tbody>
</table>

* The drc package was not able to determine an EC\textsubscript{50} based on length due to high mortality with florpyrauxifen-benzyl.
Figure 2-1. Dose response curves for mean dry biomass for five auxin-mimic herbicides tested on EWM and HWM: a) florpyrauxifen-benzyl, b) triclopyr, c) 2,4-D, d) aminocyclopyrachlor, and e) aminopyralid. Each symbol represents mean values (± standard error, n=8). Solid lines are fits for Eurasian Watermilfoil (EWM) and dashed lines are fits for Hybrid Watermilfoil (HWM).
CHAPTER 3
GROWTH CHAMBER EVALUATION OF SUBSTRATE TYPE AND FERTILIZATION ON GROWTH OF EURASIAN WATERMILFOIL AND HYDRILLA

Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) and monoecious hydrilla (*Hydrilla verticillata* [L.f.] Royle) are problematic, submersed aquatic invasive species that have invaded water bodies across the northern region of the United States. Like many submersed macrophytes, watermilfoil shows a preference for nutrient-rich, fine textured inorganic sediments (Smith and Barko 1990, Li et al. 2015). Monoecious hydrilla has been shown to prefer sediments low in organic matter (MCFarland and Barko 1987). Both species grow as rooted, submersed plants, and detached fragments may spread to form new rooted populations within a water body (True-Meadows et al. 2016; Smith and Barko 1990).

The Organisation for Economic Cooperation and Development (OECD) developed a protocol for testing non-target damage by chemicals, especially herbicides, on aquatic species (OECD 2014). The protocol provides specific guidelines for light and temperature, water quality, substrate, and experimental design to ensure consistency between replicates and allow for higher replication in small-scale studies than common research methods conducted in aquaria or small mesocosms. Although the test was designed for risk assessment of chemicals on rooted dicotyledons, it has been shown to effective for testing new herbicides (Netherland and Richardson 2016) as well as bioassays of contaminated sediments (Feiler et al. 2004).

A modified version of substrate recommended by the OECD for herbicide testing in growth chambers has been used in herbicide testing on EWM and hydrilla to standardize small-scale studies. Although it allows for consistency in substrate parameters such as pH and soil composition, the substrate requires mixing of specific
components according to strict guidelines. This is costly and more time-consuming to prepare than using commercially available potting soils or sand.

Nutrients, especially nitrogen and phosphorus, are necessary for plant growth, but in aquatic systems can also lead to increased algal populations. A higher fertilizer rate than that recommended by the OECD protocol could increase short-term growth or allow longer term studies, or higher fertilizer rates may create prohibitive algal blooms or inhibit growth due to root burn. It would save significant time and work in study preparation for evaluating response of hybrid watermilfoils, hydrilla, and other submersed species to various herbicide treatments, if a commercial potting soil provides similar or improved growth compared to the OECD substrate. Therefore, the objectives of this study were: 1) determine if the OECD recommended substrate increases short-term growth compared to commercially available potting soils or builders sand amended with nutrients and 2) determine if the OECD recommended fertilizer rate and substrate promotes the greatest growth.

**Materials and Methods**

The experiment to evaluate the efficacy of OECD on aquatic plant growth was conducted using two trials carried out in four growth chambers at the University of Florida Center for Aquatic and Invasive Plants (CAIP). This experiment had 2 factors: substrate type, and fertilizer rate. Hydrilla and EWM were planted in either OECD substrate (OECD 2014), commercial potting soil historically used at the Lewisville Aquatic Ecosystem Research Facility (LAERF) in Lewisville, TX (Hapi-gro Hope Agri Products, INC. top soil); commercial potting soil used at CAIP (Margo Garden Products professional top soil), or pure sand (Table 1). All four soil media were then amended with either 200 mg kg⁻¹ (1x), 400 mg kg⁻¹ (2x), 1000 mg kg⁻¹ (5x) ammonium chloride.
and sodium phosphate mixed with deionized water, or 3g kg\(^{-1}\) 15-9-12 Osmocote\(^6\). The 3g kg\(^{-1}\) 15-9-12 Osmocote is commonly used in fertilization of submersed aquatic species for stock cultivation (Mudge 2018). This is equivalent to 2.25x more phosphate and 7.5x more nitrogen than the OECD protocol requires (Table 3-2). The experiment was conducted as a randomized complete block design with treatments arranged as a 4x4 factorial comparing 4 substrate types and 3 rates of ammonium chloride and sodium phosphate and the Osmocote.

The experiment consisted of two identical trials, trial 1 was initiated on 2/8/17, and trial 2 was initiated 6/28/18. All chambers were kept at 25°C on a 16-hour light cycle. All of the plant material was collected from culture tanks at the CAIP, the EWM originated from Crystal River, FL while the monoecious hydrilla originated from Lake Harding, GA. Establishment in the growth chamber began by collecting 10 cm apical stems from the stock tanks. Each experimental unit consisted of a 250 mL beaker with 200 mL of substrate with two 10 cm apical stems of EWM or one sprouted tuber of monoecious hydrilla. The 250 mL beaker was then lowered into a 2 L beaker containing Smart and Barko (1985) culture solution. Each treatment contained four replicates and the trial was repeated. Due to algal growth, all beakers were flushed and replaced with new Smart and Barko solution 15 days after planting. A destructive harvest was conducted following the 30-day growth period. All plants were rinsed, sorted and placed in a forced-air drying oven to obtain dry biomass. Two-way ANOVA tests were performed in R on dry biomass data with Tukey HSD test for multiple comparisons.

**Results and Discussion**

No substrate type or fertilizer rate indicated a clearly advantageous medium for short-term growth of hydrilla or EWM in these growth chamber trials. There was a
significant interaction between substrate type and fertilizer treatment on biomass accumulation of monoecious hydrilla (p = 0.04; Figure 3-1). Few differences were observed between substrate and fertilizer combinations, however there were some notable trends. Monoecious hydrilla biomass planted in OECD substrate was never larger than hydrilla planted in other substrate types. Also, monoecious hydrilla planted in Margo potting soil had increased biomass compared to initial biomass with all fertilizer treatments (p<0.05). At the 5x rate of OECD fertilizer, hydrilla grown in sand did not significantly increase in biomass compared to initial biomass and was significantly smaller than hydrilla planted in potting soil at the same fertilizer rate. This may be indicative of root burning by the fertilizer in sand, while organic matter content in the Margo potting soil prevented root burn via redox reactions (Reddy and DeLaune 2008).

There was no significant interaction between substrate type and fertilizer treatment on EWM biomass, therefore, one-way ANOVAs were run on the main effects (Figure 3-2). Similar to monoecious hydrilla, no fertilizer rate or substrate type provided a clear advantage to EWM biomass production. No fertilizer rate resulted in significantly different biomass from the 1x fertilizer rate, indicating increased fertilizer does not provide an advantage to EWM biomass production in small-scale growth chamber studies (Figure 3-2a). EWM biomass accumulation was 34% lower in sand than plants in the OECD substrate but biomass did not differ between this treatment and that of the two potting soils (Figure 3-2b).

These data suggest that EWM and monoecious hydrilla can grow just as well, if not better, in commercially available potting soil compared to the substrate required by the OECD protocol. Although monoecious hydrilla growth does differ in substrates
depending on fertilizer, biomass of the plants grown in Margo potting soil was not
different from biomass of plants grown in OECD substrate at every fertilizer treatment.
Plants grown in Margo potting soil also resulted in significantly more biomass than the
initial biomass even at fertilizer rates in which the OECD substrate did not increase.
Monoecious hydrilla and EWM grown in pure sand had reduced biomass in several
treatments and did not produce increases in biomass. Which may be a result of burn
from the fertilizer at increased rates, or competition due to the higher algal density
observed in some trials. Both root damage and algae growth are common problems in
propagation of submersed aquatic vegetation (Mudge 2018). Although it was not
quantified, algae were observably more abundant in the 5x fertilizer rate and sand
treatments. Reduced growth in these treatments may be attributed to reduced light,
nutrient competition, or root burn, or a combination of all three factors.

It is surprising that EWM and hydrilla did not grow significantly more in OECD-
required substrate or Hapi-gro potting soil, due to their reported preference for inorganic
substrates (McFarland and Barko 1987; Smith and Barko 1990). Both species produced
equal or greater biomass in the Margo top soil compared to OECD substrate which was
not expected, given the greater organic matter content of the Margo top soil (Table 3-1).
Increased fertilizer rates do not seem to have a consistently significant positive effect on
plant biomass, therefore the rate recommended by the OECD protocol appeared
sufficient and may help prevent algal competition from the lower nitrogen and
phosphorus concentrations in the OECD sediment. Similar plant biomass growth in
potting soil with higher organic matter compared to the OECD-required substrate
indicates that these potting soil mixes can be used in place of the OECD-required
substrate for small-scale growth studies potentially reducing costs and study setup times. It should be noted that there is variation in commercially purchased soils, and composition varies. OECD-required substrate may be beneficial when performing studies requiring standardized substrate conditions, such as studies with new herbicide chemistries, but the OECD-required substrate does not provide better growth compared to that in commercial potting soils.
Table 3-1. Soil analysis of substrates used in growth assay prior to addition of fertilizer.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total P (mg kg(^{-1}))</th>
<th>pH</th>
<th>Total N (mg kg(^{-1}))</th>
<th>Organic Matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Margo</td>
<td>189.9</td>
<td>7.67</td>
<td>390.6</td>
<td>35.09</td>
</tr>
<tr>
<td>Hapi-gro</td>
<td>49.91</td>
<td>8.28</td>
<td>391.3</td>
<td>5.31</td>
</tr>
<tr>
<td>Sand</td>
<td>1.02</td>
<td>8.28</td>
<td>10.58</td>
<td>0.13</td>
</tr>
<tr>
<td>OECD</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>4.5</td>
</tr>
</tbody>
</table>
Table 3-2. Available Nitrogen and Phosphate of fertilizer additions used in growth chamber study.

<table>
<thead>
<tr>
<th>Fertilizer</th>
<th>Total N (g kg(^{-1}))</th>
<th>Phosphate (g kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OECD (1X)</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>OECD (2X)</td>
<td>0.12</td>
<td>0.24</td>
</tr>
<tr>
<td>OECD (5X)</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Osmocote (3g kg(^{-1}))</td>
<td>0.45</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Figure 3-1. Influence of growth media and fertilizer on mean (±SE) biomass accumulation of monoecious hydrilla among fertilizer treatment levels (pooled; n= 8) 30 days after planting. Horizontal black line indicates mean initial biomass. Asterisks indicate biomass significantly higher than average initial growth. Means with the same letter are not significantly different at (p=0.05).
Figure 3-2. Influence of a) fertilizer and b) substrate on mean (±SE) biomass accumulation of EWM (pooled; n= 8) 30 days after planting. Horizontal black line indicates average initial biomass. Significant increase in biomass was observed across all fertilizer rates and sediment types compared to initial. Means with the same letter are not significantly different at (p=0.05).
Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) and Hybrid Eurasian watermilfoil (*M. spicatum* L. *x M. sibiricum* Kom.; HWM) are problematic submersed aquatic invasive plants in many North American waterways. Auxin-mimic herbicides, such as 2,4-D and triclopyr, are commonly used for selective control of invasive populations of EWM, HWM, and other dicotyledonous species by stimulating auxin overdose (Netherland and Getsinger 1992; Poovey et al. 2007; Wersal et al. 2010). Differences in response to 2,4-D between EWM and HWM has led to discussion if this response is specific to 2,4-D or auxin mimics in general. These synthetic auxins are more stable in their binding to auxin receptors than natural hormones making the synthetic auxins more resistant to inactivation by the plant (Grossman 2010).

Moody and Les (2002) documented hybrid populations of watermilfoil, previously thought to be EWM, using nuclear ribosomal DNA analysis. Due to their highly similar morphology, DNA analysis is the most accurate method for discerning between EWM and HWM. The potential for inherited traits in HWM, such as increased invasiveness, hybrid vigor, or increased tolerance to herbicides presents additional concerns for aquatic weed control programs (Ellstrand and Schierenbeck 2000, Moody and Les 2002, Thompson 1991). Chemical applications have the potential to create niche habitats for HWM if herbicides have reduced efficacy (LaRue et al. 2013). In this situation, EWM could be drastically reduced or eliminated by exposure to auxin herbicides, while HWM survives to spread and repopulate treated sites (Ellstrand and Schierenbeck 2000). However, it is important to consider that hybrid populations can
arise independently, and herbicide response may vary greatly between hybrid populations due to different inherited traits.

Development of a new class of synthetic auxins, the arylpicolinates, has resulted in production of a new herbicide called florpyrauxifen-benzyl, and it may herbicide provide a tool to augment control options of problematic aquatic weedy species. The arylpicolinates differ in binding affinity compared to currently registered auxins such as 2,4-D and triclopyr (Bell et al. 2015, Lee et al. 2013). In small-scale laboratory studies florpyrauxifen-benzyl has been shown to be active on several aquatic weeds, including crested floating heart (Nymphoides cristata [Roxb.] Kuntze), hydrlilla (Hydrilla verticillata [L.f] Royle – both dioecious and monoecious biotypes) and EWM (Netherland and Richardson 2016, Richardson et al. 2016). Results from these studies suggested that concentrations of florpyrauxifen-benzyl had activity on EWM well below typical use rates for 2,4-D and triclopyr.

Concentration and exposure time (CET) requirements are key factors in evaluation of a new herbicide to determine use patterns. CET represents the amount of time that various herbicide concentrations are in contact with a plant and describes how an aquatic herbicide should affect a given plant species (Getsinger and Netherland 1997, Getsinger and Netherland 2018). Under operational herbicide use, a wide range of potential CET scenarios may occur due to various factors such as treatment scale, water flow or exchange, application rate, adsorption, degradation, and diffusion (Nault et al. 2014, Netherland and Jones 2015, Green and Westerdahl 1990, Netherland and Glomski 2014, Glomski and Netherland 2010, Glomski and Netherland 2014, Glomski et al. 2009, Skogerboe et al. 2006). CET is species dependent and can play an important
role in herbicide selectivity. There has been considerable research conducted to define the CET requirements for control of EWM with the herbicides 2,4-D (Green and Westerdahl 1990, Nault et al. 2014) and triclopyr (Netherland and Getsinger 1992, Netherland and Glomski 2014, Netherland and Jones 2015). Further investigation of CET requirements is needed to evaluate the efficacy and use patterns of the new compound, florpyrauxifen-benzyl.

Large mesocosms allow for the inclusion of more plant species in a single unit and are less prone to the plants rapidly reaching carrying capacity compared to small-scale studies. When large mesocosms are planted in the late summer or fall and treated the following spring, they better represent plant phenology and field conditions and provide a more realistic environment than newly planted small mesocosms or growth chambers (Netherland and Glomski 2014). The large-scale mesocosms using more robust plants are generally used to confirm results from small-scale studies (Netherland and Richardson 2016).

The goal of this research was to evaluate a wide range of CET conditions to determine the effect of florpyrauxifen-benzyl on well-established EWM, HWM, and several native submersed species. Our objectives with this experiment were to determine the most effective CET combinations for EWM and HWM control and to observe the effect of these CET scenarios on native species. Native submersed species from North America included: American pondweed (Potamogeton nodosus Poir.), elodea (Elodea canadensis Michx.), water stargrass (Heteranthera dubia [Jacq.] MacMill.), Illinois pondweed (Potamogeton illinoensis Morong), as well as vallisneria
(Vallisneria americana Michx.) from southern (Gainesville, FL) and northern (NY) locations. These species are considered desirable and less problematic than EWM.

**Materials and Methods**

Plants were established on 9/15/2015 from apical stems or root nodes (Vallisneria) at the U.S. Corps of Engineers Lewisville Aquatic Ecosystem Research Facility (LAERF) in Lewisville, TX. Each 6,700L mesocosm was planted with two 3-L pots for each species of American pondweed, Illinois pondweed, elodea, water stargrass, EWM, HWM, and two populations of vallisneria from southern and northern locations. Specimens of HWM with reported tolerance to 2,4-D were used from a single population (Hayden Lake, Idaho), (Beets and Netherland 2018b, Taylor et al. 2017). All plants were established in topsoil amended with Forestry Supply® 20-10-5 fertilizer tablets (4.5 g kg⁻¹). Plants were allowed to establish from 9/2015 to 4/2016, and then were treated with herbicide as noted below. One treated and one control tank contained HOBO¹⁰ data loggers to observe daily temperature fluctuations during the study period.

Florpyrauxifen-benzyl treatments were applied at concentrations of (0, 3, 9 and 27 µg a.i. L⁻¹) for 6 and 24-hour water-exchange half-lives as well as two concentrations (3 and 9 µg L⁻¹) as static treatments with no water exchange. Untreated water was circulated through the mesocosms at appropriate times to provide nominal target water exchange half-lives (Netherland and Glomski 2014). Each of the nine treatments had three replications randomly assigned to mesocosms. Water samples were collected from representative treatments and analyzed via liquid chromatography and tandem mass spectroscopy to determine actual herbicide concentrations (EPA 2015). Harvests were conducted at 30 and 60 days after treatment by collecting aboveground standing crop of plants. Samples were dried in a forced air dryer at 70 C for two weeks and then
weighed to the nearest 0.1 g. Results were analyzed using separate one-way ANOVA and Tukey’s HSD to determine statistical differences in aboveground biomass (p=0.05) among treatments at each harvest period. Heteroscedascity (unequal variance in predicted vs residual data) was an issue, and data for EWM and HWM were square root transformed to meet assumptions of normality and equal variance. Nontransformed data are presented.

**Results and Discussion**

Temperature in the mesocosms ranged from 16.6 to 26.9 C with a mean temperature of 21.7 C during the study period. Herbicide analysis determined florpyrauxifen-benzyl degradation was within expectations based on dilution scenarios and the herbicide’s physical chemistry and relatively fast photolytically-driven breakdown (Table 4-1; WA Dept. of Ecology 2017). Sample concentration fluctuations are likely due to a combination of herbicide photolytic degradation (0.6 day half-life), plant uptake, and limitations in analysis due to herbicide solubility in water (10 to 15 µg L⁻¹).

**Milfoil Efficacy**

Florpyrauxifen-benzyl provided near complete reduction of EWM and HWM biomass for up to 60 days following treatment even at the lowest concentrations and exposure times evaluated (Figure 4-1a and b). EWM biomass was significantly reduced by all CET scenarios, whereas, untreated control biomass showed an increase between harvest periods (Figure 4-1a). All exposure scenarios resulted in large reductions in HWM biomass, thirty and sixty days after treatment compared to the untreated control. However, 30 days after treatment HWM biomass in the 3 µg L⁻¹ 6 hour treatment (the lowest scenario) was greater than HWM biomass in the other CET treatments (Figure 4-
2b). Differences in herbicide sensitivity between EWM and HWM have been anecdotally observed in the field and seen in small-scale studies (Beets and Netherland 2018b, Taylor et al. 2017). These use rates were also two orders of magnitude below the use rates for currently registered herbicides such as triclopyr and 2,4-D (Green and Westerdahl 1990, Nault et al. 2014, Netherland and Getsinger 1992) and suggest the potential use of florpyrauxifen-benzyl for milfoil control programs.

**Native Species**

Overall, florpyrauxifen-benzyl had minimal effect on the native species evaluated in this study. It had no significant effect on American pondweed or Illinois pondweed biomass (Figure 4-2a and b) and some treatments of Illinois pondweed had greater biomass than the untreated control at 30 days. Increases in growth in treated mesocosms compared to untreated controls may be indicative of a lack of competition from the controlled milfoil. Elodea was not significantly affected by time or treatment (Figure 4-3a) and *Heteranthera* showed the most treatment related variability, with one treatment (3 µg L\(^{-1}\)/6 hr) showing a large increase in biomass and another (9 µg L\(^{-1}\) static) showing injury symptoms (Figure 4-3b). Given its sensitivity to 2,4-D, *Heteranthera* may be a plant that requires further refinement of CET for selective milfoil treatments and did not grow well in this study. No treatment scenario resulted in a significant reduction in southern vallisneria (Figure 4-4a). Northern vallisneria growth was minimal, however, northern vallisneria biomass in the 9 µg L\(^{-1}\)/24 hr and 27 µg L\(^{-1}\)/24 hr scenarios after 60 days was greater than the untreated control after 30 days (p < 0.001; Figure 4-4b).

Overall, this study confirms preliminary studies indicating a high level of activity on EWM and HWM by florpyrauxifen-benzyl. In addition, exposure requirements were
much shorter than expected, as evidenced by the strong control of EWM and HWM at the 3 µg L\(^{-1}\)/6 hr water-exchange scenario. This information is promising for selective control of target milfoil populations when compared to the lack of response by native plants in the majority of CET scenarios. EWM and HWM were completely controlled in the 3 µg L\(^{-1}\) static treatments and also scenarios with higher herbicide concentrations, whereas, native species exhibited variable but largely insignificant responses to higher concentration as well as in both static treatments. While low-rate, static treatments are often used in targeting invasive aquatic species, hydrodynamic processes can greatly alter CET and therefore herbicide treatment efficacy. Static applications such as whole pond treatments have the potential to lack selectivity depending on the initial application rate. However, based on these results florpyrauxifen-benzyl provides selective control of EWM and HWM under multiple CET scenarios.

In species rich areas, the ability to use low use rates to control milfoil invasions and allow the spread of native species via post-treatment regrowth and sustained control of EWM and HWM is vital to management. This study also indicated that prior small-scale trials were useful predictors of use patterns for larger-scale studies. Given the level of sensitivity of both EWM and HWM to the rates and exposures evaluated, the question of potential treatment related differences between EWM and HWM was not adequately addressed. Although there is some evidence of increased tolerance by HWM, further trials (with this and additional strains of HWM) to determine if there are real differences in response to florpyrauxifen-benzyl are warranted.
Table 4-1. Mean (SE) florypyrauxifen-benzyl concentration (µg L\(^{-1}\)) collected at hours after treatment (HAT) and days after treatment (DAT) intervals following treatment (n=3). Dashes indicate time periods where no sample was collected.

<table>
<thead>
<tr>
<th>CET scenario</th>
<th>1 HAT</th>
<th>6 HAT</th>
<th>24 HAT</th>
<th>48 HAT</th>
<th>72 HAT</th>
<th>7 DAT</th>
<th>10 DAT</th>
<th>14 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 µg L(^{-1}) 6 hr</td>
<td>16.2 (2.8)</td>
<td>8.1 (0.66)</td>
<td>3.9 (2.9)</td>
<td>1.3 (0.21)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27 µg L(^{-1}) 24 hr</td>
<td>14.3 (2.1)</td>
<td>8.9 (0.72)</td>
<td>9.0 (0.31)</td>
<td>8.6 (2.48)</td>
<td>2.0 (0.42)</td>
<td>0.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 µg L(^{-1}) static</td>
<td>2.2 (0.2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.6 (0.67)</td>
<td>0.77 (0.43)</td>
<td>0.10 (0.03)</td>
<td>0.07 (0.03)</td>
</tr>
<tr>
<td>9 µg L(^{-1}) static</td>
<td>6.9 (0.5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.6 (0.04)</td>
<td>1.2 (0.27)</td>
<td>0.25 (0.04)</td>
<td>0.08 (0.04)</td>
</tr>
</tbody>
</table>
Figure 4-1. Mean (± SE) dry aboveground biomass at 30 and 60 days after treatment (DAT) with flropyrauxifen-benzyl at 3 µg L⁻¹ for 6 hr, 24 hr and static water-exchange half-lives, 9 µg L⁻¹ for 6 hr, 24 hr and static water-exchange half-lives, and 27 µg L⁻¹ for 6 and 24 hr water-exchange half-lives on (a) EWM and (b) HWM (n=3). Letters above bars represent differences between treatments according to Tukey’s test (α=0.05). Uppercase letters indicate 60 day harvest dates that were analyzed separately.
Figure 4-2. Mean (± SE) dry aboveground biomass at 30 and 60 days after treatment (DAT) with florpypaixifen-benzyl at 3 µg L\(^{-1}\) for 6 hr, 24 hr and static water-exchange half-lives, 9 µg L\(^{-1}\) for 6 hr, 24 hr and static water-exchange half-lives, and 27 µg L\(^{-1}\) for 6 and 24 hr water-exchange half-lives on (a) American pondweed and (b) Illinois Pondweed (n=3). Letters above bars represent differences between treatments according to Tukey’s test (α=0.05). Differences in mean biomass between 60 day treatments were not observed.
Figure 4-3. Mean (± SE) dry aboveground biomass at 30 and 60 days after treatment (DAT) with florpyrauxifen-benzyl at 3 µg L\(^{-1}\) for 6 hr, 24 hr and static water-exchange half-lives, 9 µg L\(^{-1}\) for 6 hr, 24 hr and static water-exchange half-lives, and 27 µg L\(^{-1}\) for 6 and 24 hr water-exchange half-lives on (a) elodea and (b) Heteranthera (n=3). Differences in mean biomass were not observed between treatments at 30 and 60 DAT.
Figure 4-4. Mean (± SE) dry aboveground biomass at 30 and 60 days after treatment (DAT) with florypyrauxifen-benzyl at 3 µg L\(^{-1}\) for 6 hr, 24 hr and static water-exchange half-lives, 9 µg L\(^{-1}\) for 6 hr, 24 hr and static water-exchange half-lives, and 27 µg L\(^{-1}\) for 6 and 24 hr water-exchange half-lives on (a) Southern vallisneria and (b) Northern vallisneria (n=3). Differences in mean biomass were not observed between treatments at 30 and 60 DAT for S. vallisneria or 60 DAT for N. vallisneria.
Hybridization in plants can be problematic to invasive plant management. Hybridization between native and invasive species is of particular interest due to two main issues: 1) communities where hybridization between native and invasive species occurs may be more susceptible to invasion leading to extinction of parental species (Sakai et al. 2001), and 2) hybridization can complicate identification due to the ability to present characteristics similar to either parent or novel morphologies. A primary example of this in aquatic plant management is hybridization between invasive Eurasian watermilfoil (Myriophyllum spicatum L.; EWM) and native Northern watermilfoil (Myriophyllum sibiricum Kom.; NWM).

Concerns of hybrid watermilfoil (M. spicatum L. x M. sibiricum Kom.; HWM) have existed for decades, however, the highly similar morphology of EWM and suspected HWM complicated identification. Techniques for molecular confirmation advanced and the use nuclear ribosomal DNA (nrDNA) analysis proved to be an accurate method of identification, and populations of HWM previously thought to be EWM were documented (Moody and Les 2002). New or inherited characteristics that are problematic for management, such as hybrid vigor, increased invasiveness, or increased herbicide tolerance could potentially occur in hybrid populations. If HWM populations have increased herbicide tolerance compared to parental species, herbicide treatments for control of EWM may create niche habitats for HWM as described by Ellstrand and Schierenbeck (2000). EWM was drastically reduced or eliminated by exposure to auxin-mimic herbicides, while HWM survived to spread and repopulate former sites of EWM. Significant differences between hybrid populations in different lakes, or even within
lakes may occur due to hybrid populations likely arising independently (Sturtevant et al. 2009).

A few studies have indicated there are populations of HWM that exhibit differential responses to several herbicides compared to EWM. The HWM population from Townline Lake, MI has shown reduced and variable response to fluridone, 2,4-D, and triclopyr (Berger et al. 2012, Berger et al. 2015, Glomski and Netherland 2010, LaRue et al. 2012, Thum et al. 2012). HWM is often more abundant in lakes historically treated with 2,4-D than either parental species (LaRue 2012, Moody and Les 2007). Differential herbicide response in HWM has been reported by resource managers following treatment of several lakes in Northern states (Nault et al. 2014). HWM populations are genetically diverse and distinct populations can occupy the same water body (Taylor et al. 2017). This creates a need for identification of problematic populations as well as molecular identification of milfoils when studies are performed. Techniques have been developed to identify genetically distinct hybrid watermilfoil populations using several molecular techniques, including microsatellite analysis (Thum et al. 2006, Wu et al. 2013). Microsatellite analysis is particularly effective in comparing genetic diversity among populations with more recent ancestry, such as independently arising HWM populations (Thum 2018). While significant efforts have been invested in identifying hybrid watermilfoils, there is much less information generated regarding the potential for hybrid vigor, invasive potential, and response to herbicides. There are also relatively few comparisons between different hybrid watermilfoil populations.

Florpyrauxifen-benzyl has shown high activity on milfoil and several other invasive plant species, however, studies with florpyrauxifen-benzyl have been limited to
small-scale systems (Beets and Netherland 2018a, Netherland and Richardson 2016, Richardson et al. 2016). Small-scale studies can provide rapid evaluation of herbicide activity as well as dose-response measurements. However, they control abiotic factors and do not allow a realistic analysis of exposure requirements in field conditions.

Concentration exposure time (CET), the amount of time that a concentration of herbicide is in contact with a plant, is an extremely critical factor in aquatic herbicide treatment efficacy (Getsinger and Netherland 1997). Exposure time and concentration of herbicide treatments are essential factors to aquatic plant management and allow better understanding for potential use rates, which may differ between genetically distinct HWM populations.

The goal of this research was to evaluate potential differences in herbicide tolerance, growth of hybrids and parental genotypes, and variation among hybrid accessions. Our objectives with this study were to: 1) use molecular techniques to confirm that the accessions of Northern watermilfoil (*Myriophyllum sibiricum* Kom.; NWM), EWM, and HWM were genetically distinct, 2) obtain herbicide dose response data for four HWM accessions and compare concentration and exposure time combinations of florpyrauxifen-benzyl on EWM, NWM, and HWM populations, 3) observe seasonal growth (biomass allocation, surface matting, etc.) in four HWM populations and its parental species (NWM and EWM), and 4) compare the CET scenarios for florpyrauxifen-benzyl to similar rates of 2,4-D and 2,4-D + endothall.

**Materials and Methods**

**Molecular Confirmation of Hybrids**

Plant samples were collected from apical shoots of milfoil plants in the field as well as stock culture tanks at the Center for Aquatic and Invasive Plants (CAIP),
Gainesville, FL. Field sites were sampled at Ham Lake, MN, Minnetonka Lake, MN, and Big Cornelian Lake, MN. Samples were collected from the stock tanks at CAIP originated from Crystal River, FL, Ham Lake, MN, Alpine Lake, WI, Lake Minnetonka, MN, and Hayden Lake, ID. Tissue from 2-3 apical meristems was cut from separate stems and flash frozen with liquid nitrogen before storing in a deep freezer at -80°C with five replications from each site. Additional apical meristems were planted in mesocosms at Montana State University (MSU) for future reference and redundancy.

**DNA Analysis**

Plant processing and analysis was completed at Montana State University in Dr. Ryan Thum’s genetics lab in August 2017. DNA was extracted from the frozen samples using DNEasy Plant Mini Kits (Qiagen). The internal transcribed spacers (ITS) were amplified using universal primers ITS1 and ITS4 before subjecting to PCR reactions (Thum et al. 2006). Thermal cycling was completed at 94°C for 2 minutes followed by 25 cycles of: 94°C 1 minute, 56°C 30 seconds, 72°C 1 minute, with a final extension at 72°C for 8 minutes before holding at 4°C. The PCR product was then run on a 1% agarose gel to check for correct size and purity. Seven microsatellite markers were used to genotype the samples: Myrsp1, Myrsp 5, Mysrp 9, Myrsp 12, Myrsp 13, Myrsp 15, and Myrsp 16 (Thum et al. 2017, Wu et al. 2013). Microsatellite data were scored using GeneMapper and POLYSAT was used to distinguish clones based on microsatellite loci. Unique clones were identified using a Principal Coordinates Analysis.

**Small-scale Growth Chamber Study**

Experiments were carried out in four growth chambers at the University of Florida Center for Aquatic and Invasive Plants (CAIP). All chambers were kept at 25°C on a 16-hour light cycle. This experiment was conducted twice, with trial one initiated on 5/22/17.
and harvested 6/9/17, and trial two began on 8/13/18 and harvested 9/4/18.

Florpyrauxifen-benzyl was tested on four accessions of HWM (Hayden Lake, ID; Ham Lake, MN; Minnetonka Lake, MN; Alpine Lake, WI). All plant material was collected from stock culture tanks at the CAIP. Ten cm apical stems were harvested and planted in 250 mL beakers placed in 2 L beakers containing nutrient solution in the growth chambers. Each experimental unit consisted of a 250 mL beaker with 200 mL of Organisation for Economic Co-operation and Development (OECD) substrate (OECD 2014) with two apical stems of EWM (Crystal River, FL) or HWM (Hayden Lake, ID) in a 2 L beaker containing growth solution as described in Smart and Barko (1985).

Five herbicide concentrations were randomly tested on each accession with six replications for each treatment. Each beaker was randomly assigned an herbicide concentration. Prior to treatment, four beakers of each accession were harvested to collect pretreatment data. All plants were harvested seventeen days after trial initiation, and shoots and roots were washed with water to remove necrotic material and residual sediment and then combined. Samples were sorted based on treatment and accession, then placed in labeled bags in a forced air-drying oven at 70 C for 72 hours to determine dry biomass. Data were analyzed using the drc package in R to determine EC50 and EC90 values as well as derive dose-response curves (Knezevic et. al 2007). Plant dry biomass (roots and shoots combined) was used to determine EC50 and EC90 values for each accession. Trials were not statistically different at the 5% level and were pooled to improve statistical analysis.

**Mesocosm Growth Study**

Apical stems were planted on 9/28/16 and allowed to establish for 7 ½ months until 4/12/17 at the Lewisville Aquatic Ecosystem Research Facility (LAERF) in
Lewisville, TX. All plants were grown in commercial top soil amended with Forestry Supply\textsuperscript{9} 20-10-5 fertilizer tablets (4.5 g kg\textsuperscript{-1}). Three 6,700 L mesocosms were planted with nine 3 L pots containing 10 cm apical stems of each biotype of NWM (Minnetonka, MN) EWM (Crystal River, FL and Lake Minnetonka, MN) and HWM (Hayden Lake, ID; Ham Lake, MN; Minnetonka MN; Alpine Lake WI) and harvested at study initiation on 4/12/17. Three additional mesocosms were planted with 18 3 L pots containing 10 cm apical stems of each biotype of NWM, EWM, and HWM biotypes on 9/28/16. Each pot contained plants from a single location. Harvests were performed 30 and 60 days after initiation by harvesting one of the two pots of each plants in the three mesocosms at each harvest time. Above and belowground samples were collected from plants at each harvest date and washed to remove sediment then dried in a forced air dryer at 70 C until desiccated and weighed. Data were analyzed using two one-way ANOVAs and Tukey’s HSD to determine statistical differences in aboveground biomass (p=0.05) between harvest dates.

**Mesocosm CET Comparison**

Twenty-one 6,700 L mesocosms were set up at LAERF similar manner to those described above on 9/28/16 with each the nine milfoil accessions or biotypes planted in a single 3 L pot. Plants were allowed to establish for 5 ½ months and herbicide treatments were applied on 4/12/2017, when they were treated. Mesocosm treatments were applied as follows: florpypyrauxifen-benzyl\textsuperscript{5} at 3 and 6 µg L\textsuperscript{-1} six hour water-exchange half-life; and 12 µg L\textsuperscript{-1} six hour water-exchange half-life; 1.5 µg L\textsuperscript{-1} for seven day water-exchange half-life; 0.3 mg L\textsuperscript{-1} 2,4-D\textsuperscript{11} seven day water-exchange half-life, 1.2 mg L\textsuperscript{-1} 2,4-D + 3.0 mg L\textsuperscript{-1} endothall\textsuperscript{12} six hour water-exchange half-life, and 0.3 mg L\textsuperscript{-1} 2,4-D + 0.75 mg L\textsuperscript{-1} endothall water-exchange half-life (Table 1). Exposure times were
achieved by circulating untreated water through the mesocosm to provide nominal water exchange at the target retention time (Netherland and Glomski 2014). Each treatment was randomly applied to three mesocosms (replicates). A destructive harvest was performed 60 days after treatment when aboveground biomass was collected, and samples were dried in a forced air dryer at 70 C until desiccated and weighed. Data were analyzed using one-way ANOVA and Tukey’s HSD to determine statistical differences in aboveground biomass between treated and untreated milfoil 60 DAT (p = 0.05). One treated and one control tank contained HOBO\textsuperscript{10} data loggers to observe temperature fluctuations during the study period.

**Results and Discussion**

**Molecular Confirmation of Milfoil Populations**

The HWM accessions and EWM and NWM biotypes used in this study were confirmed to be genetically distinct based on the seven microsatellite loci tested. Principal coordinates analysis distinguished the different populations according to variant (Figure 5-1). The reference samples provided by the Thum database were used to validate the identification. Large symbols in Figure 5-1 indicate samples collected and processed in this study, and samples with the same x and y-coordinates are considered genetically identical (Thum et al. 2017). Fresh field samples from Ham Lake and Lake Minnetonka were identical to and CAIP culture stock. with one exception; one sample from Minnetonka CAIP stock was identical to that from Crystal River. This is likely due to sample contamination during preparation or tip collection from stock tanks. It also highlights the importance of verifying the accession on which herbicide assays are performed. This also implies that there is no genetic drift in tanks, despite cultures being maintained for several years.
The two samples from Big Cornelian (squares) were identified as NWM based on location within cluster of NWM samples (Figure 5-1). Samples from Ham, Alpine, Minnetonka, and Hayden lakes clustered together in the middle, and were classified as HWM. Hayden HWM has a different EWM parental lineage than the other HWM accessions tested, as indicated by its documented genetic distance from other lineages (Figure 5-1). This parental lineage is indicated by the grouping of EWM from the Thum reference samples. The samples from Crystal River CAIP stock were distinguished as EWM (Figure 5-1).

**Small-scale Growth Chamber Study**

Florpyrauxifen-benzyl was highly active on all HWM accessions tested with EC$_{50}$ values from 0.11 to 0.57 µg L$^{-1}$ ai (Table 5-2). The HWM accession from Hayden, ID had an EC$_{50}$ value of 0.11 µg L$^{-1}$ ai, 3 to 5x lower than the other HWM accessions tested. HWM from Minnetonka, Alpine, and Ham lakes did not have significantly different EC$_{50}$ values (Table 5-2; Figure 5-2). EC$_{90}$ values were not significantly different between any HWM accession tested. While the Hayden accession is more sensitive to florpyrauxifen-benzyl than the other accessions tested based on EC$_{50}$ values, control of all accessions may be achieved at similar EC$_{90}$ concentrations. These results are inconsistent with field operations where plant managers have suspected differential response of HWM to other auxin herbicides. Further research should be conducted to identify problematic populations and determine proper management strategies with florpyrauxifen-benzyl.

**Mesocosm Growth Study**

No significant interaction was observed between harvest date and species among the untreated controls, and there was no significant difference in aboveground
biomass between harvest dates (p>0.05; Figure 5-3a). There was a 39% increase in belowground biomass between the 7-month and 9-month harvests (Figure 5-3b). The increase in belowground biomass likely due to plants being well established at the time of initial harvest and having little room for continued aboveground growth within the tanks, but still having space within pots for root growth. There were significant differences in above and belowground biomass between milfoil biotypes (Figure 5-4). Both NWM populations, the Florida EWM, and Hayden HWM had statistically similar aboveground biomass. These milfoil biotypes had significantly less aboveground biomass than the other HWM accessions as well as the Minnetonka EWM populations. Belowground biomass production followed similar trends of growth between biotypes. The differences observed between accessions indicated HWM accessions have variation not only in morphology (Moody and Les 2002; LaRue et al. 2013) but also in biomass production. HWM demonstrated a capacity to attain higher biomass than its native parent, NWM, although this does appear limited to certain accessions. HWM accessions also produced higher biomass than EWM, dependent on the HWM accession and EWM population being compared.

**Mesocosm CET Comparison**

Temperature in the mesocosms ranged from 15.4 to 29.6 C with a mean temperature of 23 C during the 60 day study period. The CET experiment indicated that there are differences in response between different accessions of HWM in certain concentration exposure scenarios. The biomass in untreated controls of both NWM populations was extremely variable and resulted in non-significant reductions in all treatment scenarios despite 100% reduction in biomass
The 300 µg L⁻¹ 7 day 2,4-D CET scenario resulted in 30-87% reduction of milfoil biomass, however, only EWM biotypes were significantly reduced (Figure 5-5a). This is comparable to whole-lake treatments with 2,4-D, which result in 40-88% control, dependent on hydrologic conditions (Nault et al. 2014, Wersal et al. 2010). When endothall was added to the 2,4-D CET scenario, biomass reduction increased, ranging from 58-99%, with significant reductions of EWM biotypes as well as HWM from Alpine and Ham Lakes (Figure 5-5b). When rates of 2,4-D were increased, but endothall concentration and retention time was decreased, efficacy was reduced on HWM, with no significant reductions in biomass (Figure 5-5c). This is indicative of decreased 2,4-D efficacy on HWM compared to EWM in short exposure scenarios, and either combinations with other fast-acting herbicides or longer herbicide exposure times may be required spot treatments and treatments in hydrologically fluctuating systems. A 65-99% reduction in biomass was observed on both NWM and EWM (Figure 5-5c).

Biomass control on HWM ranged from 0% on Alpine HWM to 73% on Hayden HWM (Figure 5-5c). These data clearly show that HWM is more tolerant to 2,4-D treatments as has been noted in the field by aquatic applicators.

Milfoil biomass reduction due to florpyrauxifen-benzyl treatment varied between CET scenarios. Reduction in biomass was only achieved in the 1.5 µg L⁻¹ 7-day florpyrauxifen-benzyl treatment with Florida EWM, one population of Minnetonka EWM, and Alpine HWM (Figure 5-6a). When concentration was increased to 3 µg L⁻¹ and exposure time was decreased to 6 hours, significant biomass reduction was only achieved with the Florida EWM population (Figure 5-6b). The 6 µg L⁻¹ 6-hour and 12 µg L⁻¹ 3-hour treatments resulted in significant reductions of biomass for all EWM and
HWM (Figure 5-6c and d). NWM was also completely controlled in these CET scenarios but were not significantly different from controls. These results further indicate differences in herbicide sensitivity between HWM accessions as well as differences between EWM populations. Efficacy differences between biotypes were not observed at high CET’s, and total control was achieved for all HWM accessions tested. This has promising management implications since florpyrauxifen-benzyl has shown selective control for milfoil in the presence of native species and further corroborates that milfoil control can be achieved with florpyrauxifen-benzyl at relatively low use rates with sufficient concentration exposure time. However, low dose treatments should be approached with caution as they may select for more herbicide tolerant HWM accessions. This highlights that CET for the entire water body must be taken into consideration even in spot treatments.
Table 5-1. Treatment rates for mesocosm trial at four exposure times with herbicides applied in mesocosm trial at LAERF on 4/12/2017 (n=3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate</th>
<th>Exposure Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>300 µg L(^{-1})</td>
<td>7 days</td>
</tr>
<tr>
<td>2,4-D + Endothall</td>
<td>300 µg L(^{-1}) (as acid) +</td>
<td>7 days</td>
</tr>
<tr>
<td></td>
<td>750 µg L(^{-1}) (as salt)</td>
<td></td>
</tr>
<tr>
<td>2,4-D + Endothall</td>
<td>1200 µg L(^{-1}) (as acid) +</td>
<td>6 hours</td>
</tr>
<tr>
<td></td>
<td>300 µg L(^{-1}) (as salt)</td>
<td></td>
</tr>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>1.5 µg L(^{-1})</td>
<td>7 days</td>
</tr>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>3 µg L(^{-1})</td>
<td>6 hours</td>
</tr>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>6 µg L(^{-1})</td>
<td>6 hours</td>
</tr>
<tr>
<td>Florpyrauxifen-benzyl</td>
<td>12 µg L(^{-1})</td>
<td>6 hours</td>
</tr>
</tbody>
</table>
Table 5-2. Dry biomass effective concentration of florpypyrauxifen-benzyl (µg L-1) for four accessions of hybrid watermilfoil (n=10; pooled) in growth chamber study 17 DAT. Values in brackets indicate 95% confidence intervals. Values that share the same letter within an EC are not significantly different at the 5% level.

<table>
<thead>
<tr>
<th>Accession</th>
<th>EC50 (µg L⁻¹) [EC90 (µg L⁻¹)]</th>
<th>EC90 (µg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hayden</td>
<td>0.11 [0.04, 0.17] a</td>
<td>1.1 [0.37, 1.7] b</td>
</tr>
<tr>
<td>Alpine</td>
<td>0.57 [0.30, 0.84] b</td>
<td>1.4 [0.46, 2.4] b</td>
</tr>
<tr>
<td>Minnetonka</td>
<td>0.37 [0.22, 0.53] b</td>
<td>0.83 [0.25, 1.4] b</td>
</tr>
<tr>
<td>Ham</td>
<td>0.32 [0.14, 0.49] ab</td>
<td>1.1 [0.36, 1.9] b</td>
</tr>
</tbody>
</table>
Figure 5-1. Results of Principal Coordinate Analysis of milfoil DNA samples combined with reference samples from Thum database. Squares indicate NWM, circles HWM, and triangles EWM. Small symbols indicate reference samples from Thum database, large symbols are samples from this study. Symbols are based on replications of each sample (n = 5). Genetically identical samples have overlaid symbols.
Figure 5-2. Dose-response curves of average biomass for four accessions of HWM after exposure to florpyrauxifen-benzyl in growth chambers. Each symbol represents mean values (± standard error, n = 10).
Figure 5-3. Mean plant dry biomass (±SE) (a) aboveground and (b) belowground at each harvest period (7, 8, and 9 months after planting) for all HWM accessions in the 6,700 L mesocosm growth study at Lewisville, TX (n=27). Means with the same letter are not significantly different at (p = 0.05).
Figure 5-4. Mean plant dry biomass (±SE) (a) aboveground and (b) belowground at for each milfoil biotype across all harvest periods in 6,700 L mesocosm growth study at Lewisville, TX (n=27). Means with the same letter are not significantly different at (p = 0.05).
Figure 5. Mean reduction in dry biomass (±SE) for (a) 300 µg L\(^{-1}\) 2,4-D 7-day, (b) 300 µg L\(^{-1}\) 2,4-D+750 µg L\(^{-1}\) endothall 7 day, and (c) 1200 µg L\(^{-1}\) 2,4-D+300 µg L\(^{-1}\) endothall 6 hr CET scenarios 60 days after treatment in mesocosms. Green bars represent NWM biotypes, orange bars represent EWM biotypes, and black bars represent HWM accessions. Asterisks indicate significant reduction in biomass compared to untreated controls (n = 3).
Figure 5-6. Mean reduction in dry biomass (±SE) for (a) 1.5 µg L⁻¹ florpyrauxifen-benzyl 7 day, (b) 3 µg L⁻¹ florpyrauxifen-benzyl 6 hr, (c) 6 µg L⁻¹ florpyrauxifen-benzyl 6 hr, and (d) 12 µg L⁻¹ florpyrauxifen-benzyl 3 hr CET scenario 60 days after treatment in mesocosms. Green bars represent NWM biotypes, orange bars represent EWM biotypes, and black bars represent HWM accessions. Asterisks indicate significant reduction in biomass compared to untreated controls (n = 3 except (c) where n=2). Several treatments resulted in 100% biomass reduction in all replicates, as indicated by no error bars.
CHAPTER 6
CONCLUSIONS

Hybrid milfoil populations are likely to arise independently through sexual reproduction (Thum and McNair 2018) and can have genetic variation (i.e. different accessions) within a lake (Taylor et al. 2017). This is problematic for management efforts, as HWM have a higher invasive potential and show lower sensitivity to several currently used herbicides (LaRue et al. 2013, Berger 2011). It is important to properly identify problematic HWM accessions and distinguish those with herbicide sensitivity differences to make better informed management decisions. It appears that culture tanks of milfoil can be maintained without concern for genetic drift compared to field samples. Management of problematic milfoil populations is imperative to prevent spread within a water body as well as to other, nearby water bodies. Invasive watermilfoils can displace desirable native species, as well as impact economic functions of lakes such as recreation, fishing, and transport. Impacts on ecologic functions such as species richness, abundance and diversity can also be observed in milfoil monocultures (Madsen et al. 1991).

Both small-scale growth chamber studies, and large-scale mesocosm studies are effective methods for evaluation of herbicide sensitivity. Small-scale studies provide data on sensitivity differences, while large-scale mesocosm studies allow for more complex manipulations of potential hydrologic conditions in a more realistic system. As expected, testing the efficacy of florpyrauxifen-benzyl, 2,4-D, and endothall showed variation in efficacy among HWM accessions confirming anecdotal reports of aquatic plant managers. Concentration exposure time also proved to be instrumental, with accessions showing variation in control dependent on the CET scenario. Care should
be taken in determining necessary CET, as low dose treatments could result in selecting for herbicide tolerant HWM accessions. From these studies, florpyrauxifen-benzyl as well as 2,4-D/endothall combinations were effective for milfoil control with appropriate CET.
APPENDIX:

SOURCES OF MATERIALS

1DMA® 4 IVM, Dow AgroSciences LLC. 9330 Zionsville Road Indianapolis, IN 46268. http://www.cdms.net/ldat/id4JS003.pdf


5SX-1552 SePRO Corporation. 11550 North Meridian Street, Suite 600 Carmel, IN 46032.

6Hapi-gro Top Soil, Hope Agrip­roducts, Inc. 2400 Old Lewisville Road Hope, AR 71801.

7Professional Topsoil, Margo™ Garden Products. 50 N Laura Street Suite 2550, Jacksonville, FL 32202.

8Osmocote® Plus Smart-release® Plant Food 15-9-12. Marysville, OH

920-5-10 Planting Tablets, Forestry Suppliers, Inc. 205 West Rankin Street Jackson, MS 39201.


LIST OF REFERENCES


LaRue EA, Zuellig MP, Netherland MD, Heilman MA, Thum RA. 2013. Hybrid watermilfoil lineages are more invasive and less sensitive to a commonly used herbicide than their exotic parent (Eurasian watermilfoil). Evol. Appl. 6:462-471.


Jens Beets was born in Richmond, Virginia in 1992 to Dr. Lisa Muehlstein and Dr. Jim Beets, both professors at University of Richmond at the time. After graduating from Hilo High School, he attended University of Puget Sound and graduated with a Bachelor of Science degree, majoring in biology. After completing his degree, Jens moved to Gainesville to work as an OPS employee for Dr. Mike Netherland. This developed his interest in aquatic invasive plant management and led to pursuing a Master of Science degree under Dr. Netherland. In addition to his thesis studies, Jens has performed several studies involving the native grass *Paspalidium geminatum* (Firssk.) Stapf. With Florida Fish and Wildlife Conservation Commission funding. After completion of his Master of Science, Jens intends to enroll at North Carolina State University and pursue a Doctor of Philosophy with Dr. Rob Richardson in Fish and Wildlife.