

CHARACTERIZATION OF A SUSPECTED HERBICIDE TOLERANT HYBRID
WATERMILFOIL (*Myriophyllum spicatum* x *M. sibiricum*)

By

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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

2011

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To my friends and family

ACKNOWLEDGMENTS

For their support and input throughout my time at the University of Florida I would like to thank my committee: Dr. Greg MacDonald, Dr. Mike Netherland, Dr. Bill Haller, and Dr. Michael Kane. Specifically I would like to extend my appreciation to Dr. Mike Netherland for additional assistance and guidance he offered. I appreciate Dr. MacDonald for his advice and encouragement from the beginning. In addition, I would like to thank Dr. Ferrell for sharing his thoughts and guidance throughout my tenure as a Master's student. Fellow Weed Science graduate students have made this experience enjoyable while also providing invaluable help.

I am grateful for the funding for this research provided by Florida Fish and Wildlife Conservation Commission and support from the Center for Aquatic and Invasive Plants.

I appreciate my parents' support and encouragement to further my education and appreciate all they have done to encourage me throughout the years. Finally, I would like to thank Brandon Theisen for his support.

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Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

CHARACTERIZATION OF A SUSPECTED HERBICIDE TOLERANT HYBRID
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By

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December 2011

Chair: Gregory MacDonald

Major: Agronomy

An invasive watermilfoil population from a lake in western Michigan was documented as a hybrid between the native northern watermilfoil and the exotic Eurasian watermilfoil (*Myriophyllum sibiricum* x *M. spicatum*). This population survived normally lethal concentrations of fluridone herbicide; therefore studies were conducted to elucidate the possibility and level of tolerance. Mesocosm studies showed a differential response in biomass and fluorescence yield, measure by Pulse Amplitude Modulated (PAM) fluorometry, to fluridone and this population was tolerant of the maximum permitted concentration of fluridone in Michigan ($6 \mu\text{g L}^{-1}$). Further laboratory studies refined the use of the PAM fluorometer to document tolerance to fluridone while comparing the tolerant population to 12 invasive watermilfoils from across the United States. Simultaneous comparative pigment analysis confirmed the accuracy of the PAM fluorometer in detecting the responses of fluridone to watermilfoil at concentrations up to $48 \mu\text{g L}^{-1}$. The tolerant population was also found to respond similarly to norflurazon and topamezone herbicides when compared to susceptible populations. This common response to herbicides with differing mechanisms of action suggests the mechanism of tolerance is fluridone in watermilfoil is not at the active enzymatic site of fluridone.

Collectively these studies confirmed herbicide tolerance and cross-tolerance in this population of hybrid watermilfoil and also demonstrated the utility of PAM fluorometry as a quick and reliable method to document fluridone plant responses.

CHAPTER 1 INTRODUCTION

Submersed aquatic vascular macrophytes are a unique group of plants that display a wide range of physiological adaptations to life under water. These plants comprise less than 1% of angiosperms, and most trace lineage to terrestrial ancestors. Many have vestigial features such as a thin cuticle, poorly developed stomata and xylem elements which are essentially functionless (Sculthorpe 1967). Submersed plants are often desirable and beneficial in waterbodies for a number of reasons. Increased diversity and abundance of aquatic flora directly correlates to an increase of aquatic fauna (Rosine 1955) and beds of submersed aquatic plants have been found to provide a rich foraging habitat for fish and greater protection from predators (Rozas and Odum 1988). In addition to biotic benefits, aquatic vegetation can also benefit abiotic factors such as stabilization of sediment and improved water clarity (Madsen 2009a).

Although native submersed aquatic plants are desirable in aquatic ecosystems, invasive submersed species have the potential to overtake native plant communities and produce detrimental ecological effects. In Lake George, New York, an invasion of Eurasian watermilfoil (*Myriophyllum spicatum* L.) caused a rapid decline in the number of native aquatic plant species over a three year period (Madsen et al. 1988). Schmitz and Osborne (1984) found that beneficial zooplankton abundance decreased with increased hydrilla (*Hydrilla verticillata* [L.f] Royle) growth. Anthropogenic uses of waterbodies are also impacted by invasive aquatic plants. Dense, often monotypic infestations with surface canopies can impair commercial and recreational navigation, disrupt flood control, and provide a habitat for vectors of disease. Rapidly growing

invasive aquatic plants are often managed to maintain the ecological balance and recreational use of infested waters.

Management of aquatic plants differs from traditional weed control. Non-target organisms such as native plants and wildlife will likely be exposed to submersed herbicide applications and their response must be taken into consideration when herbicide applications are made to water bodies. Whereas chemical terrestrial weed control is focused on defined areas of land, water dynamics such as flow rate and dilution complicate aquatic plant management efforts. Furthermore, large water bodies that contain invasive plants requiring management are often public waters. Public waters have many different stakeholders including resource managers, federal and state agencies, residents with high value real estate, recreational end users, and other special interest groups. The vast and varying opinions of these stakeholders must be considered when formulating treatment plans for aquatic weeds in these types of water bodies.

***Myriophyllum* Biology**

The watermilfoils, contained in the genus *Myriophyllum*, are dicotyledonous aquatic plants and members of the Haloragaceae family. Approximately 68 species make up this genus, which are present on all continents excluding Antarctica with at least seven species common between continents (Moody and Les 2010). Fourteen watermilfoils are present in North America and include both native and invasive species. Eurasian watermilfoil and parrotfeather (*M. aquaticum* [Vell.] Verd.) are invasive and listed as noxious weeds in several states. Variable-leaf watermilfoil (*M. heterophyllum* Michx.) is considered invasive outside of its original range of the Southeast and has also invaded Europe and Asia (Les and Mehrhoff 1999; Yu et al. 2002). Two

hybridizations are known between species of this family. *M. spicatum* x *M. sibiricum* and *M. heterophyllum* x *M. laxum*(Shuttlw. ex Chapm.) hybridization were first documented by Moody and Les (2002). Species of interest in this document are Eurasian watermilfoil, northern watermilfoil (*M. sibiricum* Kom.), and the hybrid from the genetic cross of Eurasian and northern watermilfoil.

Northern watermilfoil is a native plant whose range stretches from California north to the northern Canadian provinces and east to Maine and the eastern seaboard. It is not found in the southeastern United States (USDA 2011). Eurasian watermilfoil has been documented in every state in the U.S. and several Canadian provinces (Jacono and Richerson 2011). This species is the most widely managed aquatic weed in the country (Smith and Barko 1990). It was first documented in North America in the Chesapeake river area in 1890 (Reed 1977) and introduction is speculated to be the result of the aquarium trade or the shipping industry via ship ballasts (Madsen 2009b). The hybrid of Eurasian x northern watermilfoil (hereafter referred to as 'hybrid') has been documented from Michigan to Washington in the northern part of the country (Moody and Les 2002, 2007).

Northern watermilfoil, along with most *Myriophyllum* species, is a rooted submersed aquatic plant that has pectinate leaves in whorls around a white or pink-colored stem. Leaves consist of 6 to 11 pairs of leaflets (Aiken 1980). This species reproduces through a variety of ways such as stem fragmentation, runners from root crowns, seed, and axial turions, or winter buds, provide a dormancy mechanism in the winter months (Aiken 1980). Northern watermilfoil is not considered to be a nuisance species as it does not form dense canopies at the water surface (Aiken 1980). This

native species is considered excellent habitat for aquatic wildlife, specifically providing necessary aquatic plant architecture for fisheries success (Valley et al. 2004).

Eurasian watermilfoil is a rooted submersed aquatic plant with pectinate leaves in whorls of four around a submersed stem. Leaves consist of 14 to 21 pairs of leaflets. It is closely related to the native northern watermilfoil although the two species rarely coexist for extended durations due to the rapid growth rate and subsequent displacement by the Eurasian species (Aiken 1980; Nichols 1992).

The plant also reproduces in a variety of ways including runners from root crowns, stem fragmentation, and seed (Madsen 2009b). Vegetative means are considered the major mechanism of wide-spread distribution (Madsen et al. 1988). Eurasian watermilfoil has been shown to autofragment primarily after flowering and at the end of the growing season (Madsen et al. 1988). This species, when compared with other invasive aquatic plants, does not produce high levels of biomass (Grace and Wetzel 1978). While biomass production is not excessive, Eurasian watermilfoil has been documented to form dense canopies (>80% cover) that shade out native vegetation, reducing species diversity in as little as 2 years (Madsen et al. 1991).

Eurasian watermilfoil is photosynthetically a C₃ plant. Van et al. (1976) found that the ratio of Rubisco to Phosphoenolpyruvate (PEP) carboxylase was similar to that found in spinach (*Spinacia oleracea* L.), a C₃ plant. Another determinant of C₃-photosynthesis is the photorespiration pathway, documented by glycolate levels in the plant. Eurasian watermilfoil was found to have glycolate levels lower than most C₃ plants, but higher than observed in C₄ plants (Stanley and Naylor 1972). Interestingly, this plant does exhibit some form of Kranz-anatomy that is usually associated with C₄

plants (Stanley and Naylor 1972). Several authors detail the preference of free CO₂ as a carbon source, but the plant has the ability to use bicarbonate at high pH levels (Steeman Nielsen 1947; Stanley 1970; and Van et al. 1976). The ability of some aquatic plants to use bicarbonate as a carbon source is advantageous in that free carbon dioxide is usually limited in the freshwater environment. With an alternative carbon source, such as bicarbonate, this species can continue to photosynthesize under low carbon dioxide conditions (Prins and Elzenga 1987).

While present in Florida, Eurasian watermilfoil has never grown to problematic levels of infestation. An article published in 1967- "Eurasian Watermilfoil-Florida's New Underwater Menace" by Blackburn and Weldon (1967) described the potential invasiveness of watermilfoil as the plant had invaded thousands of acres in the Chesapeake Bay and Tennessee Valley Authority reservoirs. Although the plant remains present in Florida, it has never reached nuisance levels requiring management.

Hybrid watermilfoil was first documented in 2002 by Moody and Les. Populations previously thought to be the invasive Eurasian watermilfoil were found, through nuclear ribosomal DNA analysis, to be hybrid populations from the parental species Eurasian watermilfoil and northern watermilfoil (Moody and Les 2002). It is suspected that hybrids went unnoticed for some time due to the morphological similarities between parental species and the hybrid. The two parental species are visibly distinguished by number of leaflet pairs (northern 6 to 11 pairs, Eurasian 14 to 21 pairs), stem diameter, and presence of axial turions (Coffey and McNabb 1974; Crow and Helquist 2002). However, the hybrid plant can exhibit a range of leaflet pairs similar to either parent and have other characteristics similar to each parent. Hybrid watermilfoils present unique

challenges for management due to inherited traits such as invasiveness and rapid growth rate from the Eurasian parent and the potential for turion formation in some hybrid populations from the northern parent. There are differing reports as to the dominance of a hybrid population compared to the Eurasian parent. Moody and Les (2007) found that hybrids tend to overtake lakes so that the parental species are no longer found. However, a more recent study by Sturtevant et al. (2009) found that hybrids and Eurasian watermilfoil parents often co-existed in the same bodies of water.

It is important to note that numerous hybrid watermilfoil populations have arisen independently and therefore traits associated with hybrids from one lake may be quite different when compared with those from another lake (Sturtevant et al. 2009). These genotypic and phenotypic differences that exist between populations of the hybrid plants preclude the use of generalities regarding specific growth or management traits. Repeated hybridization as well as back-crossing has also been documented in the field (Moody and Les 2002). Therefore, it is important to refer to each hybrid population independently.

Impacts and Management of Invasive Watermilfoil Species

Invasive watermilfoils (Eurasian and hybrid) are problematic weeds in many water bodies. Plants grow to form dense canopies displacing native vegetation, inhibiting flood control and obstructing recreational uses of waterways. Madsen et al. (1991) found that Eurasian watermilfoil formed dense canopies on the water surface that shade out desirable native plants. Dense canopy formation has been shown to negatively impact water quality by reducing dissolved oxygen in water below the mat and increasing surface temperatures and pH (Bowes et al. 1979). Submersed aquatic weeds have also been shown to harbor algal species harmful to both wildlife and human health (Wilde et

al. 2005). When watermilfoil densities result in unfavorable conditions for wildlife and fisheries, displace desirable native vegetation, and impact recreational access or aesthetics, management of these plants is often required.

Preventative management is necessary to limit spread of invasive plants to uninfested waterbodies. Practices such as removing fragments from boat trailers are helpful in reducing human-vectored spread of the plants. Several states have highly visible public education and outreach programs to educate the public on preventative methods (UF/CAIP 2011, Cal-IPC 2011, ISDA 2011).

Mechanical control of invasive watermilfoils, like many submersed plants, is not always an effective choice for management. Mechanical harvesters segment the plants, increasing fragmentation and possibly assisting in the spread and intensity of the invasion. This method is also non-selective and has the potential to also damage native plant communities. Hand harvesting does limit fragmentation but requires a large financial investment (Kelting and Laxson 2010). Despite the problems associated with mechanical harvesting, there are numerous operational programs that continue to rely on mechanical harvesters as a primary means of control.

Several biological controls are available for submersed Eurasian and hybrid watermilfoils. Triploid grasscarp (*Ctenopharyngodon idella* Val.) are a common choice to manage many submersed plant infestations. Although grasscarp are an attractive choice in some situations, invasive watermilfoil management is not recommended because the fish prefer native plants over the invasive watermilfoils (Stroganov 1963). A native weevil, *Euhrychiopsis lecontei* (Dietz) does prefer the invasive watermilfoils to native submersed plants and is sold commercially (Alwin et al. 2010). The use of this

biocontrol has been associated with seasonal declines but has not been shown to provide sufficient control to eliminate an invasion (Newman et al. 2001). While there is a large body of literature on *Eurychiopsis* and watermilfoil control, predictable and consistent control remains a problem (Alwin et al. 2010).

Chemical control methods are also available for invasive watermilfoils. Herbicides effective for control compromise several modes of action including plant growth regulators such as 2,4-D ([2,4-dichlorophenoxy] acetic acid) and triclopyr ([3,5,6-trichloro-2-pyridinyl]oxy acetic acid), the carotenoid biosynthesis inhibitor fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone), and cell membrane disrupters such as diquat (6,7-dihydrodipyrido[1,2- α :2',1'-*c*] pyrazinediium ion) and endothall (7-oxabicyclo[2.2.1] heptanes-2,3-dicarboxylic acid). Diquat and endothall are contact materials that do not translocate within the plant and typically provide short term control of infestations. The systemic herbicides generally provide long term, season-long or even multiple season control of invasive watermilfoils. Triclopyr and 2,4-D have been used and are documented to provide control as long as exposure times are sufficient (Netherland and Getsinger 1992; Green and Westerdahl 1990). Fluridone is an attractive choice for water managers due to low use concentrations (<10 μgL^{-1}), native plant selectivity, ability to target watermilfoil in the entire lake, cost effectiveness, and potential for multiple years of control from a single treatment. This herbicide is used frequently by water managers to control Eurasian and hybrid watermilfoils on a whole-lake or whole-system basis. There have been numerous claims of reduced herbicide response by hybrid watermilfoils, however there is limited published information on this topic. Triclopyr and 2,4-D amine were found to inhibit growth of both Eurasian and

hybrid watermilfoil accessions in a similar manner following exposure to labeled use concentrations (Poovey et al. 2007). Recently, differences between Eurasian and hybrid watermilfoil populations were noted following exposure to low continuous concentrations of 2,4-D (Glomski and Netherland 2010). This shows the potential for increased tolerance to fluridone.

Fluridone Herbicide

Fluridone was first described as having herbicidal effects in 1976. The compound was discovered and evaluated by Eli Lilly & Company during greenhouse screening and investigated as a selective herbicide for pre-emergence use in cotton (Waldrep and Taylor 1976). It was found to have activity against both monocotyledonous and dicotyledonous weeds such as redroot pigweed (*Amaranthus retroflexus* L.), sicklepod (*Cassia obtusifolia* L.), johnsongrass (*Sorghum halepense* Pers.), and large crabgrass (*Digitaria sanguinalis* [L.] Scop.), among others (Waldrep and Taylor 1976). Fluridone was then evaluated for use on aquatic vascular plants and was found to provide excellent control at low use concentrations on hydrilla, Eurasian watermilfoil, duckweed (*Lemna* spp.), cabomba (*Cabomba caroliniana* Gray), and several other problematic aquatic species (McCowen et al. 1979).

Fluridone was registered in the United States in 1986 for use in aquatic environments by the U.S. Environmental Protection Agency. Several liquid and granular formulations are available for use in both lotic and lentic waters to control a variety of submersed and emergent aquatic vegetation.

Fluridone belongs to the substituted-tetrahydropyrimidinone class of herbicides which are commonly referred to as the bleacher herbicides. Fluridone is an inhibitor of the carotenoid biosynthesis pathway. Specifically, it is a noncompetitive inhibitor of the

phytoene desaturase (PDS) enzyme. The PDS enzyme catalyzes the desaturation of phytoene in the rate-limiting step of this pathway (Chamovitz et al. 1993). When PDS is inhibited, phytoene levels increase and carotenoid production in the cell is limited. Carotenoids function to shield chlorophyll from excess light and help dissipate the oxidative energy of singlet oxygen. In fluridone treated plants, carotenoids are not present to quench the energy of oxygen radicals, allowing for the formation of lipid radicals in chlorophyll molecules (Senseman 2007). This results in degradation of chlorophyll, bleaching of new tissue and subsequent necrosis and plant death.

Fluridone is an attractive management tool in aquatic ecosystems for several reasons. Use rates of fluridone in lentic waters are 5-20 $\mu\text{g L}^{-1}$ and 10-40 $\mu\text{g L}^{-1}$ in lotic waters, which is significantly lower than the mg L^{-1} range typical of several other aquatic herbicides. Large scale treatments of fluridone have the potential to provide multiple years of control of submersed plants. Fluridone is also a fairly selective herbicide that does not adversely impact many desirable native plants. At low use rates of 5 $\mu\text{g L}^{-1}$, fluridone was found to reduce the biomass of Eurasian watermilfoil while increasing the biomass of native species such as vallisneria (*Vallisneria Americana* Michx.) and two pondweed species (*Potamogeton nodosus* Poir. and *P. pectinatus* L.) over untreated controls (Netherland et al. 1997). Field studies have shown that low use rates (5-6 $\mu\text{g L}^{-1}$) of fluridone applied on a whole-lake scale have controlled invasive watermilfoil populations while leaving desirable native plant communities intact (Getsinger et al. 2001, Getsinger et al. 2002a, Getsinger et al. 2002b). For these reasons, resource managers often use this herbicide to control undesirable aquatic vegetation while preserving native vegetation in a wide range of aquatic ecosystems.

In addition to control of Eurasian watermilfoil, fluridone has been used to control hydrilla since the late 1980s. Schmitz et al. (1987) describe using fluridone to control hydrilla in a central Florida lake such that the plant could no longer be found in the water body. Haller et al. (1990) successfully used fluridone to control hydrilla for one year in the St. Johns River. As such, fluridone was heavily used for hydrilla control in public waters of Florida from the late 1980's until the early 2000s. Between 1999 and 2001, formerly susceptible hydrilla populations in several major lakes were not exhibiting the level of control previously associated with fluridone use. Subsequent laboratory testing documented that several populations of hydrilla had developed resistance to fluridone (Michel et al. 2004, Arias et al. 2005, Puri et al. 2006). Specifically, an amino acid substitution in the phytoene desaturase enzyme conferred 2 to 5 fold resistance to fluridone in hydrilla (Michel et al. 2004).

Previous to this discovery, herbicide resistance in hydrilla was thought to be unlikely due to the strictly vegetative reproduction exhibited by dioecious female hydrilla in Florida. However, low use rates, extended exposure times, and repeated use of fluridone led to tremendous selection pressure in these water bodies. Repeated fluridone applications selected for plants that were resistant to typical use rates of the herbicide. Since this time, resistance has developed in many water bodies throughout the state and fluridone is no longer a widespread tool for hydrilla management in Florida's public waters. To date, hydrilla is the only plant that has been confirmed to have developed resistance to fluridone.

Herbicide resistance is defined as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type"

(WSSA 1998). Herbicide tolerance “implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant” (WSSA 1998). Some discussion has occurred debating whether the fluridone resistance in hydrilla is actually resistance or tolerance. Since resistant plants were selected following low use rates of the herbicide, fluridone resistance is the proper term.

Fluridone has been used for the past 20 years for invasive watermilfoil management. Watermilfoil control requires similar use patterns to that of hydrilla control. Use rates ranging from 4 to 25 $\mu\text{g L}^{-1}$ have been shown to provide watermilfoil control given that sufficient exposure time is also met (Netherland and Getsinger 1995). Using fluridone as a chemical tool to control Eurasian watermilfoil did meet some opposition as concerns arose as to the effect of the herbicide on native plants and wildlife habitat at a whole lake level. Getsinger et al. (2001) documented that after whole lake fluridone treatments in Michigan, species diversity increased 1.5 to 2.3 fold from pre-treatment measurements. Native plant cover was also not negatively impacted. Concerns over native plant selectivity and economic factors continued to drive the use of lower rates. Currently, use patterns in several states consist of an initial fluridone treatment between 5 to 10 $\mu\text{g L}^{-1}$ followed by a subsequent application to return the fluridone concentration to these levels. Although low use rates may be cost-effective and increase native plant selectivity, these practices could have implications for resistance as well. In terrestrial systems, low use rates of selective herbicides have been shown to cause rapid development of resistance to those herbicides, as well as cross resistance, in annual ryegrass (*Lolium rigidum* L.) (Neve and Powles 2005).

In May 2010, a 220 acre lake in western Michigan (Townline Lake) was treated with $6 \mu\text{g L}^{-1}$ of fluridone, followed by a subsequent treatment to return the concentration to the original level, which was permitted by the Michigan Department of Natural Resources. The population of documented hybrid watermilfoil in the water body was not controlled with this rate of the herbicide. Initial mesocosm studies found that the Townline population of watermilfoil did not experience declines in biomass like that of susceptible watermilfoil populations when exposed to several rates of fluridone (Thum et al. submitted). The observation that a hybrid watermilfoil may show increased tolerance to fluridone has fueled more speculation on the nature of watermilfoil hybrids and their response to herbicides.

Pigment analysis has been used traditionally to document the biochemical response of a plant to fluridone. Fluridone inhibits the phytoene desaturase enzyme of the carotenoid biosynthesis pathway and by analyzing pigment levels in this pathway the plant's response to fluridone can be studied. Sprecher et al. (1998) developed an extraction method that measures absorbance spectrophotometrically to quantify phytoene and β -carotene levels. In fluridone susceptible plants, phytoene levels increase after fluridone treatments and β -carotene levels would be lower than control plants. In fluridone resistant plants, phytoene and β -carotene levels are similar to those of untreated control plants. Puri et al. (2006) used this technique to demonstrate fluridone resistance in several hydrilla populations collected throughout Florida.

Chlorophyll analysis is an indirect method of determining fluridone resistance. Although fluridone does not directly affect chlorophyll biosynthesis, the absence or reduction of β -carotene leads to the destruction of chlorophyll molecules. Therefore, in a

susceptible plant exposed to fluridone, chlorophyll levels in new tissue will be lower than those in control plants. Several extraction methods have been evaluated and utilized for pigment analysis using various solvents (Iriyama et al. 1974, Hiscox and Isrealstam 1979, Moran and Porath 1979) but the non-macerated method of Wellborn (1994) using dimethyl sulfoxide (DMSO) proved to be the most useful in these studies. This method provides pigment analysis that is comprehensive in the study of fluridone response in plants in that two pigments affected by fluridone can be quantified. Although pigment analysis provides a method for determining herbicide activity in the plant, the methods employ a destructive harvest of plant tissue. There is a need for non-destructive and repeatable methods of analysis.

PAM Fluorometry

In functioning plants, energy from light, in the form of photons, comes into contact with the various pigments of the plants in the light harvesting complexes. These pigments, such as chlorophyll *a*, chlorophyll *b*, and the carotenoids, have many double bonds which are capable of absorbing this energy and performing photochemistry to pass an excited electron to the electron transport chain of photosynthesis. The carotenoids function to absorb excess light energy in the light harvesting complexes thereby protecting chlorophyll against this energy and conversion to radical oxygen.

When excess light is absorbed by chlorophyll in the light harvesting complex, several events can occur. Energy can be reradiated as heat, energy can be transferred to adjacent molecules via inductive resonance, photochemistry or charge separation can pass an excited electron to the electron transport chain, or lastly energy can be reradiated as fluorescence. Chlorophyll *a* and *b* molecules drive photosynthesis by absorbing light energy, which causes excitation of electrons in those molecules, and

transfer this energy to adjacent molecules via inductive resonance. When chlorophyll is damaged or not functioning properly due to a variety of reasons such as stress or herbicidal effects, the chlorophyll will often emit the excess energy as reradiated light. Measuring this reradiated light can give insight as to the efficiency and functionality of chlorophyll and photosynthesis in the plant (Papageorgiou 1975).

Pulse-amplitude modulated (PAM) fluorometry is used to measure chlorophyll fluorescence and works by focusing a saturating beam of light on the desired region of the plant. Yield ratio is calculated by the instrument. Higher fluorescence yield ratio indicates highly functioning chlorophyll whereas lower yield ratios indicate damaged or non-functioning chlorophyll. Yield is a ratio of F_v/F_{max} . F_{max} is equal to the fluorescence when the saturating pulse is applied to the tissue. F_v is equal to $F_{max}-F$ where F is the fluorescence of the tissue with no light pulse applied. The plant fluoresces more when chlorophyll is damaged, which indicates a higher F_{max} value. However, the yield output ratio is lower because damaged chlorophyll fluoresces more under ambient light conditions (Figure 1-1). Therefore, a higher Y ratio value indicates chlorophyll that is functioning normally where a lower Y value indicates damaged chlorophyll (Bolhar-Nordenkamp et al. 1989).

PAM fluorometry has been used to study irradiance stress (Ralph et al. 1998), salinity stress (Kamermans et al. 1999), and shoot-to-landscape differences in photosynthesis in sea grasses (Durako and Kunzelman 2002). *In situ* measurement of photosynthetic activity of Red Sea faviid corals has also been measured (Beer et al. 1998). This technique is useful because it is a non-destructive method of evaluating the activity of chlorophyll and has also been used to evaluate herbicidal effects on plants.

Ireland et al. (1986) used fluorometry to document decreased fluorescence in wheat (*Triticum* spp.) 30 minutes after exposure to glyphosate (*N*-[phosphonomethyl] glycine) herbicide. The herbicide diuron (*N*-[3,4-dichlorophenyl]-*N,N*-dimethylurea), a photosystem II inhibitor, was shown to reduce fluorescence yield ratio in sea grasses two hours after exposure as measured with a diving-PAM (Haynes et al. 2000). Junea et al. (2001) evaluated the effects of mercury and metolachlor (2-chloro-*N*-[2-ethyl-6-methylphenyl]-*N*-[2-methoxy-1-methylethyl] acetamide), a mitosis inhibiting herbicide, on six algal species.

This research investigates the suspected fluridone tolerance in a hybrid watermilfoil population in Michigan and methods used to document response to fluridone. Chapter 2 focuses on mesocosm-scale studies used to evaluate the response several populations of invasive watermilfoil through PAM fluorometry and biomass evaluations. While these studies are informative, they are limited by the significant inputs of time and space required. Laboratory methods are needed to circumvent these limitations, but specific methods to evaluate submersed plants' response to fluridone have not been documented. Refinement of methods including pigment extraction incubation time, using PAM fluorometry to evaluate fluridone response, and determining optimal shoot length for these studies are discussed in Chapter 3. Chapter 4 employs these methods in small-scale laboratory evaluations of a number of invasive watermilfoil populations from several states. Chapter 5 evaluates the potential cross-tolerance of the fluridone tolerant Townline population to pigment synthesis inhibiting herbicides with differing modes of action.

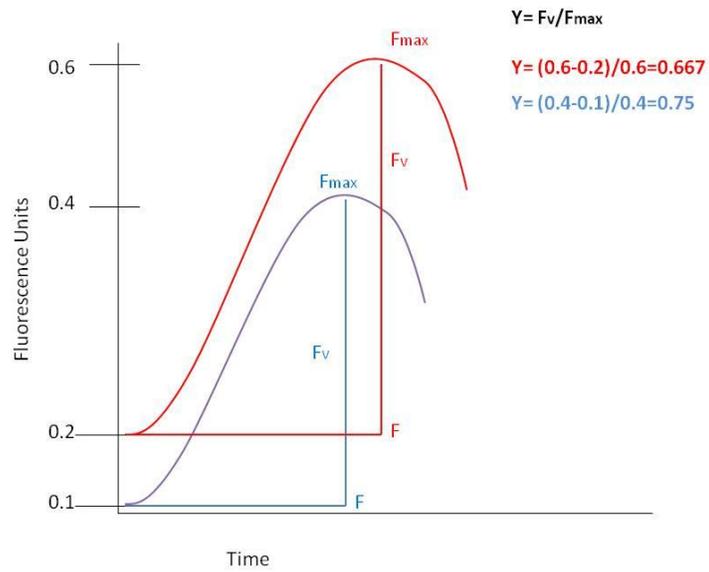


Figure 1-1. An example of two fluorescence induction curves measured by PAM fluorometer. The upper curve indicates damaged chlorophyll and the lower curve indicates functioning chlorophyll. Equations for Y ratio depicted in figure.

CHAPTER 2 EVALUATION OF SUSPECTED FLURIDONE TOLERANT HYBRID WATERMILFOIL UNDER STATIC MESOCOSM CONDITIONS

Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hybrid watermilfoil (*M. spicatum* x *M. sibiricum*), are problematic invasive weeds in many water bodies throughout the northern tier of the United States. Plants grow to form dense surface canopies displacing native vegetation, altering water quality, and obstructing recreational uses of waterways. Madsen et al. (1991) found that Eurasian watermilfoil formed dense which can shade out desirable native plants in the ecosystem. Dense canopy formation has been shown to negatively impact water quality by reducing dissolved oxygen in water below the mat and increasing surface temperatures and pH (Bowes et al. 1979). Submersed aquatic weeds have also been shown to harbor algae species harmful to both wildlife and human health (Wilde et al. 2005). When watermilfoils spread within waterbodies and result in unfavorable conditions for wildlife and fisheries, displacement of desirable native vegetation, and impacts on recreational access or aesthetics, management of these plants is often required.

Hybrid populations of watermilfoil were first documented in 2002 by Moody and Les. Populations previously thought to be the invasive Eurasian watermilfoil were found, through nuclear ribosomal DNA analysis, to be hybrids from the parental species Eurasian watermilfoil and northern watermilfoil (Moody and Les 2002). Hybrid watermilfoils may present unique challenges for management due to inherited traits such as increased invasiveness, or hybrid vigor, and the potential to acquire a trait such as turion formation from the northern parent that could confound management efforts. It is important to note that numerous hybrid watermilfoil populations have arisen independently and therefore traits associated with hybrids from one lake may be quite

different when compared with those from another lake (Sturtevant et al. 2009). The extent of hybridization in natural lakes is unclear and currently being investigated. Genotypic and phenotypic differences exist between populations of the hybrid plants and generalities cannot be made regarding specific growth or management traits. Repeated hybridization as well as back-crossing has been documented in the field (Moody and Les 2002). Therefore, it is important to refer to each hybrid population independently.

Chemical control methods are commonly used to manage invasive watermilfoils. Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) is an attractive choice for water managers due to its low use rates, native plant selectivity, ability to target watermilfoil in the entire lake, and potential for multiple years of control from a single treatment. This herbicide is used frequently by water managers to control Eurasian and hybrid watermilfoils. There have been numerous claims of reduced herbicide response by hybrid watermilfoils, however there is limited published information on this topic. Eurasian and northern parents are both highly susceptible to low use rates typical of fluridone treatments (Crowell et al. 2006). Triclopyr ([3,5,6-trichloro-2-pyridinyl]oxy acetic acid) and 2,4-D amine ([2,4-dichlorophenoxy] acetic acid) were found to inhibit growth of both Eurasian and hybrid watermilfoil accessions in a similar manner following exposure to labeled use rates (Poovey et al. 2007). Recently, differences between Eurasian and hybrid watermilfoil biotypes were noted following exposure to low continuous concentrations of 2,4-D (Glomski and Netherland 2010).

In May 2010, a 220 acre lake in western Michigan (Townline Lake) was treated with a concentration of fluridone permitted by the Michigan Department of Natural

Resources. The population of documented hybrid watermilfoil in the water body was not controlled with the legal rate of the herbicide. The observation that a hybrid watermilfoil may show increased tolerance to fluridone has fueled more speculation on the nature of hybrids and their response to herbicides. Mesocosm studies are needed to compare the Townline population of watermilfoil to other watermilfoil populations when exposed to fluridone. As fluridone is typically used to treat an entire lake, a failure to perform is particularly notable due to costs, exposure of native plants (some quite sensitive to fluridone) through the entire system, and subsequent requests to provide additional herbicide treatments for summer relief from the watermilfoil infestation.

In order to determine if the hybrid watermilfoil population from Townline Lake shows a unique response to fluridone compared to other watermilfoil accessions, a series of mesocosm studies was conducted. The objective of these studies was to evaluate the response of several different watermilfoil populations to a range of fluridone concentrations to determine variation across a spectrum of watermilfoils (both Eurasian and hybrid) against Townline, which is suspected to have increased tolerance to fluridone.

Materials and Methods

Experiment 1

An initial screening study was conducted in a greenhouse at the Center for Aquatic and Invasive Plants in Gainesville, Florida during the summer of 2010. The suspected susceptible hybrid watermilfoil originating from Otter Lake, MN (hereafter referred to as 'Susceptible') was kept in outdoor culture and exposed to ambient light and temperature. This population was confirmed as a hybrid watermilfoil through ITS analysis (Thum et al. submitted). Shoot tips were harvested from this stock culture for

the study. Apical shoot tips of the suspected tolerant hybrid watermilfoil, a population from Townline Lake, were obtained from Michigan for the study. Apical shoot tips 10-15 cm in length of Townline hybrid watermilfoil or Susceptible hybrid watermilfoil were planted in 4.25 inch square pots containing top soil amended with osmocote (15-9-12) fertilizer at a rate of 1g kg⁻¹ (soil). Pots were capped with approximately 1 cm of sand prior to planting. Each 95-L container contained one pot of Townline watermilfoil and one pot of susceptible watermilfoil. Artificial light in the greenhouse provided a photoperiod of 14 hour light: 10 hour dark. Plants were allowed to establish and grow for 2 weeks prior to treatment. After this initial time, each 95-L tank was treated with 0, 2, 4, 6, 8, 12, or 24 µg L⁻¹ of fluridone (Sonar AS®, SePro Corporation, Carmel, IN). Each treatment was replicated 5 times (5 95-L tanks per fluridone concentration) using a completely randomized design. Plants were allowed to grow and respond to treatment for 7 weeks until a destructive harvest of all shoot biomass was conducted. Harvested biomass was dried for 3 days in a 70°C drying oven. Samples were weighed and results analyzed using analysis of variance (ANOVA) and Fisher's Protected LSD ($\alpha=0.05$) to determine differences between populations at each concentration.

Experiment 2

Experiment 2 was conducted to compare the tolerant Townline population to a greater geographical range of invasive watermilfoils that were collected from several states. This study was conducted in an enclosed greenhouse at the Center for Aquatic and Invasive Plants in Gainesville, Florida during the winter of 2010 and 2011. Townline plants were harvested from outdoor stock cultures for the study. Plant material collected from 3 populations of suspected fluridone susceptible watermilfoils was obtained for the study (Table 2-1). One susceptible population was collected at Frog Lake in Wisconsin

and is a hybrid watermilfoil. The second population was obtained from Auburn Lake, Minnesota and is Eurasian watermilfoil (EWM). The final population is an EWM population from Texas. All populations' genotypes were confirmed as hybrid or EWM through ITS analysis (Thum et al. submitted). As in the previous study, 10-15 cm apical shoot tips were used for planting. A single apical shoot tip was planted in a 164 mL cone-tainer containing topsoil amended with Osmocote and capped with sand. One cone-tainer of each population was placed in a 4.5 inch square pot containing topsoil amended with slow release fertilizer and again capped with sand prior to planting. Each pot contained one container from each population. Two square pots were placed in each 95-L tank so that biomass could be sampled at each of two harvest intervals by removing a single pot. Plants were allowed to establish and grow for 2 weeks prior to treatment. At this time, 6 replications of each treatment of 0, 5, 10, and 20 $\mu\text{g L}^{-1}$ of fluridone were added to the appropriate tanks. This study was conducted using a completely randomized design. The first set of plants was harvested at 7 weeks after treatment (WAT). The fluorescence of shoot tips were measured with a PAM fluorometer and above ground biomass of each population was destructively harvested. Biomass samples were dried for 3 days at 70°C. The second harvest occurred 11 WAT and the same parameters were measured. Both fluorescence yield data and biomass data were combined across all susceptible populations at each harvest since no significant differences were found between these populations when data were subjected to ANOVA ($\alpha=0.05$). Non-linear regression was fitted to the data. Data from each harvest were analyzed with ANOVA and Fisher's Protected LSD ($\alpha=0.05$) to determine

significant differences between the combined susceptible populations and the Townline population.

Experiment 3

Experiment 3 was designed to examine the response over time to fluridone by several invasive watermilfoils as compared to Townline. This study was conducted in outdoor mesocosms at the Center for Aquatic and Invasive Plants in Gainesville, Florida during the spring and summer of 2011. Four populations of watermilfoils were harvested from outdoor stock cultures of plants on site (Table 2-2). These populations were Townline, Indian, Auburn and Texas. Townline, Auburn, and Texas populations are identical to those in Experiment 2. The Indian population is from Indian Lake in Michigan and has geographical and genetic similarities to Townline Lake. It is also a hybrid and suspected to be tolerant to fluridone. One additional population of known susceptible EWM was obtained from North Carolina. Populations were again confirmed genetically as hybrid or EWM through ITS analysis (Thum, pers. comm.¹). Apical shoots were planted in an identical manner to that of Experiment 2. The study was designed to allow for 3 harvesting dates, so 3 pots, each containing all populations, were added to each mesocosm. Plants were allowed to grow for 2 weeks prior to treatment. Treatments for this study included an untreated control, 3, 6, 9, 12, 18, and 36 $\mu\text{g L}^{-1}$ with 4 replications each in a completely randomized design. Due to the potential photodegradation of fluridone herbicide in outdoor mesocosms, water samples were collected every 2 days to determine the half life of the herbicide in the mesocosms. Samples were analyzed using ELISA Quanti-Plate kits (QuantiPlate Kit for Fluridone,

¹ R. Thum, Grand Valley State University, Annis Water Resources Institute Muskegon, MI 49441.

Envirologix, Portland, ME) and half-life was determined to be 10 days. Therefore, every ten days for the duration of the study, each mesocosm was treated with a half concentration of the appropriate initial treatment. The first and second harvests occurred at 3 and 6 weeks after treatment, respectively. PAM yield and above ground biomass data were collected at each harvest. The final harvest occurred 8 weeks after treatment with only above ground biomass data being collected. All biomass data from this experiment was dried and weighed in the same manner as previous experiments. All susceptible populations were again combined due to no treatment by experiment significance as found with ANOVA. Non-linear regression was fitted to the data. Fluorescence yield data and biomass data from each harvest were analyzed using ANOVA and Fisher's Protected LSD ($\alpha=0.05$) to detect differences between Townline and the combined susceptible populations within each concentration.

Results and Discussion

Experiment 1

The initial study determined that the Townline hybrid watermilfoil had a different response to fluridone herbicide at all concentrations used in the experiment (Figure 2-1). The susceptible population had biomass reduced to less than 40% of untreated control at $2 \mu\text{g L}^{-1}$ fluridone while Townline did not exhibit that level of reduction at even the highest concentration. For reference, the maximum permitted concentration to treat using fluridone for control of invasive watermilfoils in the state of Michigan is $6 \mu\text{g L}^{-1}$. There was very limited reduction in biomass of Townline plants at the essentially highest treatment it could be exposed to in the field. This initial screening study indicated that Townline exhibits an increased tolerance or possibly resistance to the herbicide fluridone and warrants further investigation.

Experiment 2

The second experiment resulted in differences in biomass and fluorescence yield at 5 and 10 $\mu\text{g L}^{-1}$ between Townline and the combined susceptible populations (Tables 2-3 and 2-4). At the first harvest fluorescence yield decreased by more than half in the susceptible populations at 5 $\mu\text{g L}^{-1}$ while Townline showed essentially no decrease in fluorescence (Table 2-3). At 11 WAT, fluorescence yield was decreased to less than 20% of untreated plants at 5 $\mu\text{g L}^{-1}$ in susceptible populations while Townline actually showed increased fluorescence from untreated controls (Table 2-3). Dry biomass of susceptible populations had decreased to less than 10% by 7 WAT at the lowest concentration and decreased further to less than 5% at 11 WAT (Table 2-4). Townline did not exhibit a decline lower than 80% of untreated control at either 5 or 10 $\mu\text{g L}^{-1}$ at either harvest. This experiment confirmed that the Townline population does not respond to fluridone herbicide at the concentrations that would be legally permitted for control in Michigan. Plants originating from locations throughout the country showed little variation and results were combined. This speaks to the success and applicability of fluridone as a tool to treat invasive watermilfoils at low use rates.

Experiment 3

The outdoor mesocosm experiment again demonstrated the response to fluridone by the different populations of watermilfoils. Due to the slow acting nature of fluridone, significant differences in fluorescence yield and biomass between susceptible and Townline populations were limited at 3 WAT (data not shown).

The second harvest was conducted at 6 WAT and data from susceptible populations was combined since no significant differences in response to fluridone were found between populations. By this time, fluorescence yield differences were found at

all concentrations $6 \mu\text{g L}^{-1}$ and above (Figure 2-2a). Biomass differences existed at 3, 6, 9, and $12 \mu\text{g L}^{-1}$ for the susceptible populations when compared to Townline (Figure 2-2b). Of note is the 200% increase in biomass of Townline plants from control to even the lowest concentration of fluridone herbicide. It appears from this data that Townline did not successfully compete with the other populations when no herbicide was present, however when fluridone decreased biomass of other populations the Townline population biomass increased dramatically.

By 8 WAT apical shoot tips were not present in many of the mesocosms so PAM yield data was not collected. Biomass data from susceptible populations was combined since no significant differences were found between populations. Townline plant biomass again continued to increase to 400% of untreated mesocosms at the lowest concentrations of fluridone (Figure 2-3). The combined susceptible populations showed significant differences from Townline at all concentrations (Figure 2-3).

This study verified that Townline does not respond to fluridone herbicide in a similar manner to any of the other analyzed populations. While the hybrid Indian Lake plants are genetically similar to Townline, their response was significantly different by 8 WAT. Even though hybrid watermilfoils were geographically and genetically similar, as with Townline and Indian Lake populations, herbicide response was distinct. Little variation was found between all susceptible populations used in this study, which allowed results to be combined. This limited variation in response and sensitivity to fluridone is important to the success of fluridone as a tool to treat invasive watermilfoils across the country.

Herbicide resistance is defined as “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type” (WSSA 1998). Herbicide tolerance “implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant” (WSSA 1998). Since there is no ‘wild type’ hybrid watermilfoil, the differing response to fluridone of hybrid populations, specifically Townline population, cannot be referred to as herbicide resistance. These populations of plants do, however, exhibit an increased tolerance to fluridone. It is unknown if the Townline population was selected for by previous use of fluridone herbicide or if this population developed tolerance for the herbicide during hybridization.

Now that a fluridone tolerant population of hybrid watermilfoil has been confirmed, it is important that resource managers take steps to prevent the spread of this unique population to neighboring lakes. Townline Lake is located in central Michigan in the near vicinity of numerous other bodies of water. The potential for spread to other water bodies is highest in close proximity to the originally infested lake (Roley and Newman 2008). Resource managers should consider limiting public access to Townline Lake until the population of plants is controlled and also monitor neighboring lakes to detect any possible invasions of this unique hybrid watermilfoil.

Table 2-1. Invasive watermilfoil (*Myriophyllum* spp.) populations used in Experiment 2.

Population	Species	Location	Tolerance
Townline	Hybrid	Michigan	probable
Frog	hybrid	Wisconsin	susceptible
Auburn	EWM	Minnesota	susceptible
Texas	EWM	Texas	susceptible

Note: Hybrid watermilfoil is a cross between northern watermilfoil (*M. sibiricum*) and Eurasian watermilfoil (EWM) (*M. spicatum*)

Table 2-2. Invasive watermilfoil (*Myriophyllum* spp.) populations used in Experiment 3.

Population	Species	Location	Tolerance
Townline	hybrid	Michigan	probable
Indian	hybrid	Michigan	unknown
Auburn	EWM	Minnesota	susceptible
Texas	EWM	Texas	susceptible
North Carolina	EWM	North Carolina	susceptible

Note: Hybrid watermilfoil is a cross between northern watermilfoil (*M. sibiricum*) and Eurasian watermilfoil (EWM) (*M. spicatum*).

Table 2-3. Fluorescence yield (F_v/F_m), represented as percent of the untreated control, in susceptible and Townline populations of invasive watermilfoils as a function of fluridone concentration 7 and 11 weeks after treatment (WAT).

fluridone concentration ($\mu\text{g L}^{-1}$)	7 WAT		11 WAT	
	Susceptible	Townline	Susceptible	Townline
5	44.9±10.0 *	98.1±2.2	15.4±5.8 *	124.6±9.1
10	13.9 ± 6.9 *	98.4±4.0	2.7 ± 2.7 *	119.2±13.7
20	5.3 ±3.9	23.5±14.9	0	0

Note: Values indicate means with standard error (n=6). Concentrations with significant differences between populations found using Fisher's Protected LSD ($\alpha=0.05$) are marked with an asterisk.

Table 2-4. Biomass, represented as percent of the untreated control, in susceptible and Townline populations of invasive watermilfoils as a function of fluridone concentration 7 and 11 weeks after treatment (WAT).

fluridone concentration ($\mu\text{g L}^{-1}$)	7 WAT		11 WAT	
	Susceptible	Townline	Susceptible	Townline
5	9.3 ± 3.3 *	111.2±11.3	2.3 ± 0.9 *	91.1±4.4
10	2.0 ± 1.6 *	92.3±16.1	0.08±0.07 *	81.4±5.9
20	2.7 ±2.6	10.3±7.3	0	0

Note: Values indicate means with standard error (n=6). Concentrations with significant differences between populations found using Fisher's Protected LSD ($\alpha=0.05$) are marked with an asterisk.

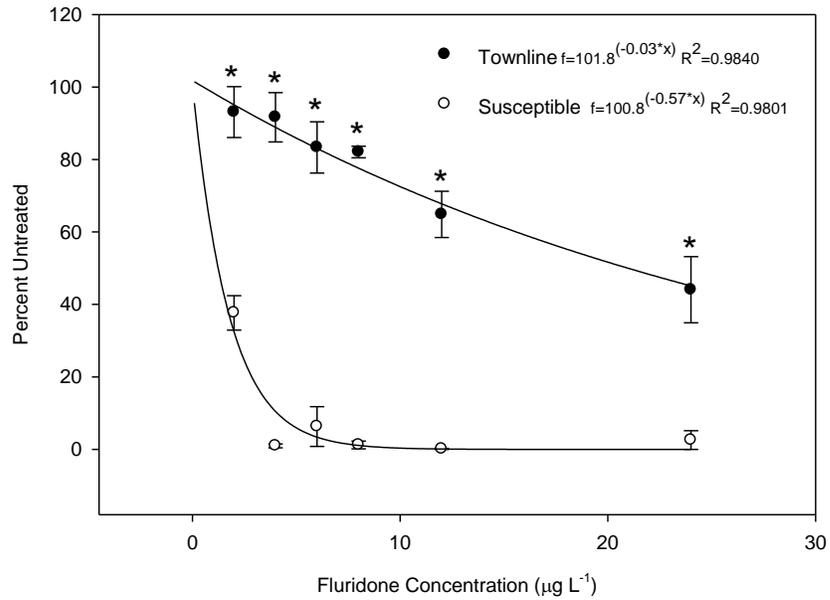


Figure 2-1. Above ground biomass, represented as percent of the untreated control, in susceptible and Townline invasive watermilfoil plants as a function of fluridone concentration 7 weeks after treatment. Symbols represent means and error bars indicate standard error (n=5). Curves represent nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk.

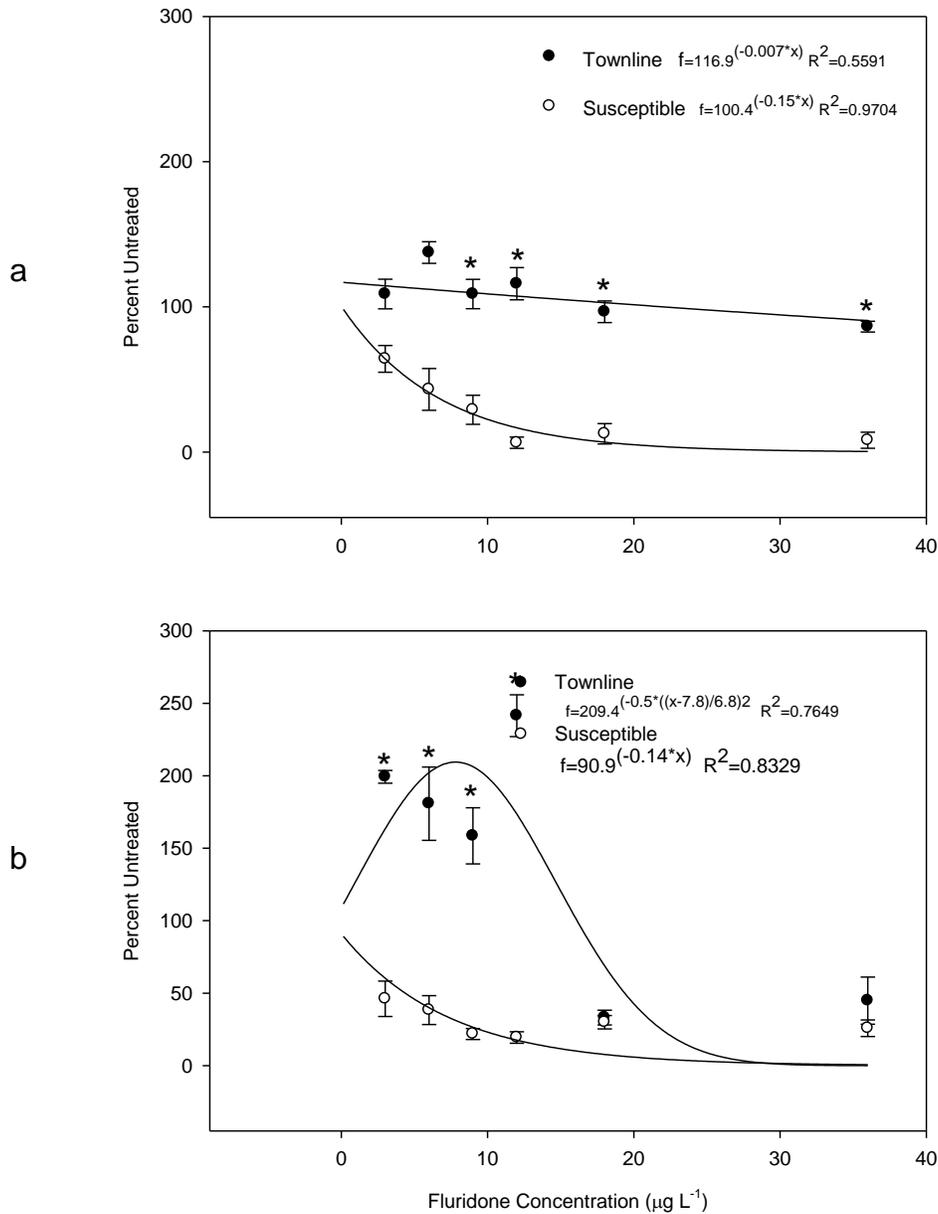


Figure 2-2. Fluorescence yield (F_v/F_m) (a) and dry biomass (b) of suspected fluridone tolerant Towline hybrid watermilfoil and 4 combined susceptible populations of invasive watermilfoils 6 weeks after treatment with fluridone, represented as percent of the untreated control. Symbols represent means and error bars indicate standard error ($n=4$). Curves indicate nonlinear regression. Significant differences within each concentration between Towline and susceptible populations, as found using Fisher's Protected LSD ($\alpha=0.05$), are marked with an asterisk.

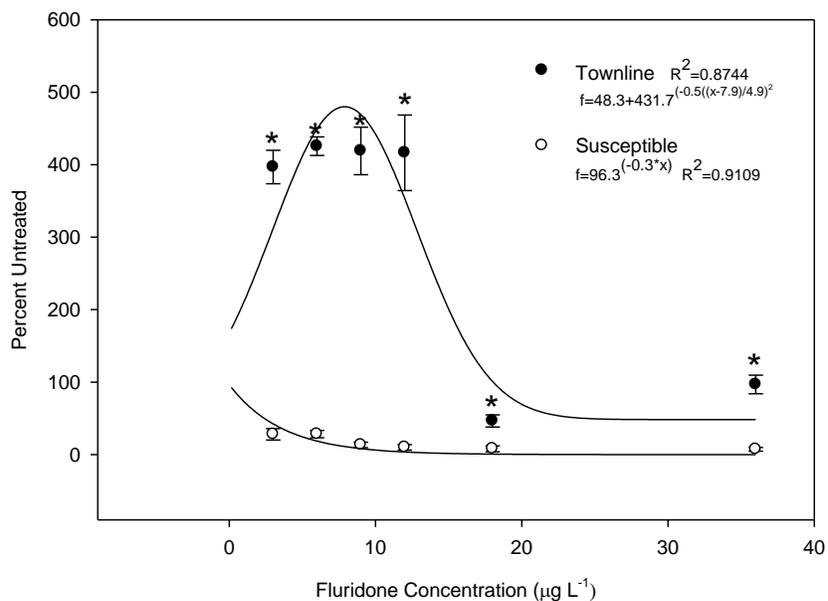


Figure 2-3. Dry biomass of suspected fluridone tolerant Townline hybrid watermilfoil and 4 combined populations of susceptible invasive watermilfoils 8 weeks after treatment with fluridone, represented as percent of the untreated control. Symbols represent means and error bars indicate standard error (n=6). Curves represent nonlinear regression. Significant differences within each concentration between Townline and the combined susceptible populations, as found using Fisher's Protected LSD ($\alpha=0.05$), are marked with an asterisk.

CHAPTER 3

A COMPARISON OF METHODS FOR CHARACTERIZING HERBICIDAL EFFECTS ON SUBMERSED AQUATIC VASCULAR PLANTS

Although native submersed aquatic plants are often desirable in certain ecosystems, invasive plants have the potential to overtake desirable plant communities and have many other detrimental effects. In Lake George, New York, an invasion of Eurasian watermilfoil (*Myriophyllum spicatum* L.) caused a rapid decline in the number of aquatic plant species over a three year period (Madsen et al. 1988). Anthropogenic uses of waterbodies are also negatively impacted by invasive aquatic plants. Heavy infestations and dense surface canopies can impair commercial and recreational navigation, disrupt flood control, and become a habitat for disease vectors. For this reason, invasive aquatic plants are often managed to maintain a non-harmful level or eradicated from a water body.

Many submersed aquatic plants have vestigial features from their terrestrial heritage such as a thin cuticle, poorly developed stomata and xylem elements which are essentially functionless (Sculthorpe 1967). Since submersed plants greatly differ from their terrestrial counterparts, research methods to characterize these plants are often vastly different than those developed for terrestrial plants. Research concerning invasive submersed aquatic macrophytes has been ongoing for several decades both in the field and in the lab (Haller and Sutton, 1973, Van et al. 1976, Bowes et al. 1977). Laboratory techniques have been used to evaluate many aspects of these plants including physiological characteristics, herbicide response, and metabolic activity (Holladay and Bowes 1980, Kane and Gilman 1991, MacDonald et al. 1993). These evaluations are advantageous due to the controlled nature of the laboratory

environment, smaller area needs compared to field or mesocosm studies, and speed of determining outcomes.

Pigment analysis has been used traditionally to characterize the biochemical response of a plant to fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone). Fluridone inhibits the phytoene desaturase enzyme of the carotenoid biosynthesis pathway and by analyzing these pigment levels the plant's response to fluridone can be elucidated. Sprecher et al. (1998) developed an extraction method that measures absorbance spectrophotometrically to quantify phytoene and β -carotene levels. In fluridone susceptible plants, phytoene levels increase after fluridone treatments and β -carotene levels would be lower than control plants. In fluridone resistant plants, phytoene and β -carotene levels are similar to those of untreated control plants. Puri et al. (2006) used this technique to determine and document fluridone resistance in several hydrilla (*Hydrilla verticillata* [L.f.] Royle) populations in Florida.

Chlorophyll analysis is an indirect method of determining fluridone resistance. Although fluridone does not directly affect chlorophyll biosynthesis, the absence or reduction of β -carotene leads to the destruction of chlorophyll. Therefore, in a susceptible plant exposed to fluridone, chlorophyll levels in new tissue will be lower than those in control plants. Hiscox and Israelstam (1979) developed a method of chlorophyll extraction without maceration using dimethyl sulfoxide (DMSO). This study documented that chlorophyll extracted in DMSO is a simple process that produces stable chlorophyll extracts. A more recent study determined that chlorophyll *a*, chlorophyll *b*, and total carotenoids could all be quantified by the same extract in DMSO and analyzed with a spectrophotometer at different absorbances (Wellburn 1994). This method provides

pigment analysis that is comprehensive in the study of fluridone resistance or tolerance and less complex than the previously described Sprecher method.

Hiscox and Israelstam (1979) used an incubation period of 6 hours in DMSO and a 65°C water bath for chlorophyll extraction in terrestrial plants with a developed cuticle. This method has also been applied to submersed aquatic plants by several authors (Netherland et al 1993, Netherland and Getsinger 1995, Bultemeier 2008). However, since the thickness of the cuticle in the plant sample is determinant of the incubation time, it is thought that incubation time for submersed plants could be significantly less than 6 hours due to the poorly developed cuticle.

Although pigment analysis provides a method for determining herbicide activity in the plant, the methods require a destructive harvest of plant tissue. There is a need for non-destructive and repeatable methods of analysis on the same tissue source. Pulse-amplitude modulated (PAM) fluorometry can give information as to chlorophyll functionality by measuring chlorophyll fluorescence. A PAM fluorometer works by focusing a saturating beam of light on the desired region of the plant. By measuring the re-radiation, or fluorescence, a yield ratio is calculated by the instrument. Higher fluorescence yield ratio indicates highly functioning chlorophyll whereas lower yield ratios indicate damaged or non-functioning chlorophyll. Yield is a ratio of F_v/F_{max} , where F_{max} is equal to the fluorescence when the saturating pulse is applied to the tissue. F_v is equal to $F_{max}-F$ where F is the fluorescence of the tissue with no light pulse applied. The plant fluoresces more when chlorophyll is damaged, which indicates a higher F_{max} value. However, the yield output ratio is lower because damaged chlorophyll also fluoresces more under ambient light conditions. Therefore, a higher Y ratio value

indicates chlorophyll that is functioning normally where a lower Y value indicates damaged chlorophyll (Bolhar-Nordenkampf et al. 1989).

PAM fluorometry has been used to study irradiance stress (Ralph et al. 1998), salinity stress (Kamermans et al. 1999), and shoot-to-landscape differences in photosynthesis in sea grasses (Durako and Kunzelman 2002). *In situ* measurement of photosynthetic activity of Red Sea faviid corals has also been measured (Beer et al. 1998). This technique is useful because it is a non-destructive method of evaluating the activity of chlorophyll and has also been used to evaluate herbicidal effects on plants. Ireland et al. (1986) used fluorometry to document decreased fluorescence in wheat (*Triticum* spp.) 30 minutes after exposure to glyphosate (*N*-[phosphonomethyl] glycine) herbicide. The herbicide diuron (*N*-[3,4-dichlorophenyl]-*N,N*-dimethylurea), a photosystem II inhibitor, was shown to reduce fluorescence yield ratio in sea grasses two hours after exposure as measured with a diving-PAM (Haynes et al. 2000). Junea et al. (2001) evaluated the effects of mercury and metolachlor (2-chloro-*N*-[2-ethyl-6-methylphenyl]-*N*-[2-methoxy-1-methylethyl] acetamide), a mitosis inhibiting herbicide, on six algal species. Using a PAM fluorometer to detect the effects of pigment synthesis inhibiting herbicides such as fluridone has not been documented in aquatic plants, but would potentially be a non-destructive method for evaluating fluridone activity in the plant.

Several authors have studied invasive watermilfoils in the laboratory, but different methods were used to evaluate herbicide response. Netherland (1995) used apical shoot tips 10-15cm in length, while Forney and Davis (1981) documented using apical shoot tips 7 cm in length. Shorter shoot segments are not expected to have the

carbohydrate reserves necessary to thrive for long periods in culture. However, these shoot segments are thought to respond more quickly to herbicide application for this same reason. On the other hand, longer shoots have the carbohydrate reserves to thrive in culture, but it is speculated that they also can use these same reserves to initially withstand the herbicide application.

A multitude of methods have been well published using small-scale laboratory experiments to study aquatic plants, however, no studies report on pigment analysis incubation periods, optimal shoot length for determining herbicide response in invasive watermilfoils, or using pulse-amplitude modulated (PAM) fluorometry to evaluate herbicide resistance or tolerance in aquatic plants.

Materials and Methods

Pigment Extraction Incubation Time

Stock plants of hydrilla and Eurasian watermilfoil (EWM) were maintained in 950-L outdoor mesocosms at the Center for Aquatic and Invasive Plants in Gainesville, Florida throughout the study period. These plants were exposed to ambient temperature and photoperiod. Actively growing shoots were harvested for use in the study and washed thoroughly with tap water to remove algae and microorganisms. Apical shoot tips weighing 0.1 g were placed into 5 mL dimethyl sulfoxide (DMSO) in HDPE centrifuge tubes and incubated in a 65°C water bath for 1 hour, 3 hours, or 6 hours. A second experiment utilized shorter time intervals of 15 minutes, 30 minutes, and 1 hour. Both experiments were conducted using a completely randomized design in a factorial arrangement where each plant species received each incubation interval with 4 replications. At the designated time interval, a 2 mL aliquot of extract was analyzed using a BioMate spectrophotometer (Thermo Scientific, Pittsburgh, PA) at 480, 649, and

665 nm to determine total carotenoids, chlorophyll *a* and chlorophyll *b*, respectively. DMSO was used as a blank. Total chlorophyll was calculated with the following equation: $(12.19 \times \text{absorbance at } 665\text{nm} - 3.45 \times \text{absorbance at } 649\text{nm}) + (21.99 \times \text{absorbance at } 649\text{nm} - 5.32 \times \text{absorbance at } 665\text{nm})$. Total carotenoids were calculated as $(1000 \times \text{absorbance at } 480\text{nm} - 2.14 \times \text{chlorophyll } a - 70.12 \times \text{chlorophyll } b) / 200$. Experiments were repeated. Data were converted to percent of longest incubation time and subjected to analysis of variance (ANOVA). There was not a significant treatment by run interaction for either experiment, therefore results were combined. Means were separated using Fisher's Protected LSD ($\alpha=0.05$).

PAM Fluorometry

Stock plants of EWM were maintained in 950-L outdoor mesocosms at the Center for Aquatic and Invasive Plants in Gainesville, Florida throughout the study period. These plants were exposed to ambient temperature and photoperiod. The first population used in this study, a hybrid watermilfoil, originated from Townline Lake in western Michigan, which is thought to have increased tolerance to the herbicide fluridone. The second population used originated from Texas and is confirmed EWM suspected to be susceptible to fluridone. This susceptible population is hereafter referred to as 'Susceptible'. Actively growing shoots of each population were harvested for use in the study and washed thoroughly with tap water to remove algae and microorganisms. Apical shoots 6 cm in length were placed in glass culture tubes containing 100mL Andrew's media, as described by Selim et al. (1989), supplemented with 4 g L^{-1} sodium bicarbonate. Plants were treated with 0, 2.5, 5, 10, or $20 \mu\text{g L}^{-1}$ fluridone herbicide (Sonar AS®, SePRO Corporation, Carmel, IN), with 4 replications per treatment in a completely randomized design. Plants were kept in a climate

controlled growth chamber for the duration of the experiment (23°C: 21°C day:night temperature, 14 light:8 dark photoperiod, and 350 $\mu\text{mol}/\text{m}^2$ light). Fluorescence was measured at the growing point 7 days after treatment (DAT) using a PAM fluorometer (Mini-PAM, Walz, Effetrich, Germany). New apical tissue was harvested for pigment analysis as described previously. The experiment was repeated. There was not a significant treatment by run interaction for either experiment, therefore results were combined. Data were converted to percent of the untreated control and subjected to nonlinear regression. Confidence intervals ($\alpha=0.05$) of the means were used to compare the effective concentration to reduce pigments by 50% (EC_{50}) for fluorescence yield, total chlorophyll, and total carotenoids for each population.

Shoot Length Response

Stock plants of fluridone-susceptible EWM were maintained in 950-L outdoor mesocosms at the Center for Aquatic and Invasive Plants in Gainesville, Florida throughout the study period. Established and thriving plants were harvested for use in the study and washed thoroughly with tap water to remove algae and microorganisms. The study was repeated using EWM plants harvested from the Chassahowitzka River in Central Florida. Apical shoot tips were excised into 2, 4, 6, or 8 cm segments and washed thoroughly in tap water to remove algae and microorganisms. These segments were placed in glass culture tubes containing 150 mL of Andrew's media supplemented with 4 g L^{-1} sodium bicarbonate. Each shoot tip size was then treated with 5, 10, or 20 $\mu\text{g L}^{-1}$ fluridone herbicide. An untreated control of each shoot length was also included. Plants were kept in a climate controlled growth chamber for the duration of the experiment (23°C: 21°C day:night temperature, 14 light:8 dark photoperiod, and 350 $\mu\text{mol}/\text{m}^2$ light). Fluorescence was measured at the growing point 2, 6, and 10 DAT using

a PAM fluorometer. At 10 DAT, 0.1 g of new growth from each shoot was harvested for pigment analysis. The previously described method of total carotenoid and total chlorophyll quantification was used. The study was conducted using a completely randomized design with 4 replications. Data were converted to percent of the untreated control, combined and subjected to ANOVA. There was not a significant treatment by run interaction for either experiment, therefore results were combined. Means were separated using Fisher's Protected LSD ($\alpha=0.05$) to detect significant differences between treatments within each shoot length.

Results and Discussion

Pigment Extraction Incubation Time

In the first experiment there were no significant differences in the levels of total chlorophyll or carotenoids for either species, with the exception of slightly lower levels at the 6 hour incubation time (Table 3-1). In fact, lower levels were measured at the longer incubation times of 3 and 6 hours. Chlorophyll and carotenoids are known to degrade when exposed to light (MacKinney 1941), and it is speculated that the longer incubation times showed a loss of these pigments due to photodegradation. In the second experiment, the total incubation time was 1 hour since Experiment 1 had previously shown that this interval was sufficient incubation time. However, in this experiment a significantly lower percentage of total chlorophyll was extracted for both hydrilla and EWM at 15 and 30 minutes (Table 3-2). For total carotenoids, no significant differences were shown for hydrilla at 30 minutes and 1 hour incubation time, however 15 minutes was significantly lower. EWM exhibited significantly decreased total carotenoids at both 15 and 30 minutes compared to 1 hour.

These results indicated that the 6 hour incubation time period as suggested by Hiscox and Israelstam and employed in aquatic plant research is not necessary. A 1 hour incubation time is sufficient, but shorter extraction periods are not long enough to obtain complete pigment extraction. These results do not correspond to those of Spencer and Ksander (1987) who found that a 20 minute incubation time at 65°C was sufficient for chlorophyll analysis in hydrilla and EWM. However, due to the lag period after extraction and spectrophotometric analysis some pigment degradation could have occurred, thus compromising the actual pigment content. It is also possible that the pigment concentration in these shoots was significantly lower, thus requiring a shorter incubation time.

PAM Fluorometry

This study was conducted to evaluate the accuracy of three different methods of determining fluridone response in watermilfoil. More specifically, PAM fluorometry was compared to more traditional chemical analysis of pigment content, which is laborious, expensive, time consuming, and requires destructive harvest of the tissue. Regression analysis was used to interpret the response of the plants to fluridone as a function of concentration. From this analysis, the effective concentration that would cause a predicted reduction of 50% was calculated (EC_{50}). To statistically compare each method, 95% confidence intervals of the mean EC_{50} values were generated. Fluorescence yield EC_{50} values for both the tolerant and susceptible populations overlapped with EC_{50} values of total carotenoids and total chlorophyll (Table 3-3). These results indicate that measuring fluorescence yield gives similar and statistically equivalent results as that of the more complex and destructive pigment analysis. As expected, fluorescence yield, total carotenoids, and total chlorophyll EC_{50} values of the

tolerant population were all significantly higher than those of the susceptible population of Eurasian watermilfoil (Table 3-3).

Within each population, the EC₅₀ values of fluorescence yield, chlorophyll, or carotenoid content were not significantly different. This indicates that each method provides a similar measure of fluridone response, and the ability to elucidate tolerance or resistance, as shown by the differences between populations, is reliable. This finding will greatly aid resource managers because PAM fluorometry determines results in a much more time sensitive fashion without a requirement for destructive harvest of plant tissue.

Shoot Length Response

One of the most challenging aspects of laboratory experimentation with aquatic plants, especially submersed aquatic macrophytes, is the selection of uniform plant material. Herbicide response studies further complicate this situation, because many herbicides work only on new growth. Therefore, the growth status of the plant tissue and the ability to have fairly uniform growth potential from plant material is critical. A key factor for growth in these types of plants is thought to be initial shoot length. Therefore, these studies were conducted to determine the optimal shoot length to provide accurate results of fluridone response. PAM fluorometry was shown to provide an accurate assessment of fluridone in the previous experiment and was thus used in this study to measure response over time as a function of shoot length.

At 2 DAT, differences were observed at the lowest concentration (5 µg L⁻¹) for shoot lengths ≥ 4 cm (Table 3-4). At concentrations of 10 µg L⁻¹ or higher, differences were observed in all segment lengths. By 6 DAT, differences between the control and all concentrations were observed regardless of shoot length (Table 3-5). In 2 cm and 4

cm shoots, differences were also observed between concentrations $\leq 5 \mu\text{g L}^{-1}$ and concentrations $\geq 10 \mu\text{g L}^{-1}$. Differences were also observed at the lowest concentration in all shoot lengths by 10 DAT (Table 3-6). This demonstrates the ability to determine different responses to $\geq 10 \mu\text{g L}^{-1}$ of fluridone within 2 DAT if 4 cm or longer shoots are utilized. To determine differences between lower concentrations over a longer period of time, 2 cm shoot lengths provide the most consistent results of lengths studied. Resource managers concerned with potentially fluridone tolerant or resistant EWM populations would benefit from obtaining this knowledge as quickly as possible, therefore 4 cm or 6 cm shoot segments lengths would be recommended in such studies.

Total chlorophyll content was significantly lower at the highest concentration of fluridone ($20 \mu\text{g L}^{-1}$) in all shoot lengths while shoots 2 cm in length displayed no difference in total chlorophyll at 0, 5, or $10 \mu\text{g L}^{-1}$ fluridone (Table 3-7). Shoots 4 cm, 6 cm, and 8 cm in length showed differences between the control and each concentration of fluridone. Total carotenoids were also significantly lower at the highest concentration in all shoot lengths (Table 3-8). Shoots 6 cm in length produced the most logical differences between concentrations, and would likely be the best choice of shoot length to determine pigment responses to fluridone in EWM.

Taking into consideration fluorescence yield data and pigment data, 6 cm shoot length segments would provide the most accurate determination of fluridone response in EWM. At all data collection intervals after treatment, 6 cm shoot tips produced significant responses between the control and different concentrations. Pigment analysis was also most informative with 6 cm shoot tips in that differences were

observed between the control and each concentration, and differences were also observed between some concentrations. Based on visual observations from the study, 2 cm and 4 cm shoot lengths do not have enough carbohydrate reserves to produce the biomass needed for effective pigment analysis. Shoots 8 cm in length did not exhibit logical differences between rates in fluorescence yield or pigment analysis. This is likely due to the excess reserves in the 8 cm shoot that allow it to initially withstand herbicide treatment.

Refining and developing these methods are integral for herbicide response research with submersed aquatic plants, specifically Eurasian and hybrid watermilfoils. Decreasing incubation time during chlorophyll and carotenoid extraction not only is a time saving measure, but reduces the likelihood of pigment degradation during extended incubation times. Utilizing PAM fluorometry to document herbicide response is also more efficient. This method is an improvement from destructive pigment analysis in that plants remain intact. In turn, the analysis is able to be repeated over time to provide more insight on specific populations' response to pigment synthesis inhibiting herbicides. Choosing the optimal shoot length for laboratory experiments is essential to obtaining accurate and rapid results. These methods will help resource managers to efficiently evaluate potentially tolerant populations of invasive watermilfoils and allow for more rapid decision making to occur.

Table 3- 1. Total chlorophyll and total carotenoids extracted from hydrilla and Eurasian watermilfoil (EWM) tissue in dimethyl sulfoxide as a function of 6 hour incubation time.

Incubation Time	Total Chlorophyll		Total Carotenoids	
	Hydrilla	EWM	Hydrilla	EWM
1 hr	109.8ab	89.4a	113.5a	98.7a
3hr	115.0a	98.8a	110.3a	102.3a
6hr	100.0b	100.0a	100.0a	100.0b
LSD	12.3	11.4	9.2	6.3

Note: Values represent percentage of total (6 hour incubation) pigment extracted at each time interval (n=8). Letters indicate significant differences between incubation times within a column as found using Fisher's Protected LSD ($\alpha=0.05$).

Table 3 2. Total chlorophyll and total carotenoids extracted from hydrilla and Eurasian watermilfoil (EWM) tissue in dimethyl sulfoxide as a function of 1 hour incubation time.

Incubation Time	Total Chlorophyll		Total Carotenoids	
	Hydrilla	EWM	Hydrilla	EWM
15 min	62.7c	48.7c	85.5b	56.7c
30 min	85.8b	78.6b	96.3a	83.1b
60 min	100.0a	100.0a	100.0a	100.0a
LSD	8.3	17.3	5.8	15.0

Note: Values represent percentage of total (1 hour incubation) pigment extracted at each time interval (n=8). Letters indicate significant differences between incubation times within each column as found using Fisher's Protected LSD ($\alpha=0.05$).

Table 3-3. EC_{50} values (\pm confidence intervals, $\alpha=0.05$) for fluorescence yield, total chlorophyll, and total carotenoids for suspected fluridone-tolerant and fluridone-susceptible invasive watermilfoil populations.

	Tolerant Population		Susceptible Population	
	EC_{50} 95% Confidence Interval	R^2 value	EC_{50} 95% Confidence Interval	R^2 value
Fluorescence Yield	21.3 \pm 6.3	0.7376	3.7 \pm 0.5	0.8924
Carotenoids	25.4 \pm 10.7	0.6064	7.4 \pm 3.2	0.5531
Chlorophyll	24.9 \pm 11.8	0.5648	7.4 \pm 2.7	0.6772

Note: R^2 values indicate the fit of the exponential-decay model ($y=a*\exp(-b*\text{fluridone concentration})$) with n=8.

Table 3-4. Fluorescence Yield (F_v/F_m), as percent of the untreated control, in Eurasian watermilfoil shoot lengths as a function of fluridone concentration 2 days after treatment.

Fluridone Concentration ($\mu\text{g L}^{-1}$)	Shoot Length			
	2 cm	4 cm	6 cm	8 cm
0	100.0 a	100.0 a	100.0 a	100.0 a
5	98.1 a	89.2 b	92.9 bc	94.2 b
10	89.3 b	88.2 bc	95.9 ab	87.1 c
20	91.1 b	80.4 c	87.9 b	90.0 bc
LSD	5.9	7.8	5.3	4.8

Note: Letters following percent of the untreated control values represent significant differences between concentrations in each shoot length according to Fisher's Protected LSD ($\alpha=0.05$) with $n=8$.

Table 3-5. Fluorescence Yield (F_v/F_m), as percent of the untreated control, in Eurasian watermilfoil shoot lengths as a function of fluridone concentration 6 days after treatment.

Fluridone Concentration ($\mu\text{g L}^{-1}$)	Shoot Length			
	2 cm	4 cm	6 cm	8 cm
0	100 a	100 a	100 a	100 a
5	80.9 b	66.7 b	68.0 b	72.8 bc
10	62.1 c	70.1 b	73.6 b	86.4 ab
20	53.4 c	55.2 c	69.4 b	62.2 c
LSD	14.1	10.1	12.2	19.3

Note: Letters following percent of the untreated control values represent significant differences between concentrations in each shoot length according to Fisher's Protected LSD ($\alpha=0.05$) with $n=8$.

Table 3-6. Fluorescence Yield (F_v/F_m), as percent of the untreated control, in Eurasian watermilfoil shoot lengths as a function of fluridone concentration 10 days after treatment.

Fluridone Concentration ($\mu\text{g L}^{-1}$)	Shoot Length			
	2 cm	4 cm	6 cm	8 cm
0	100 a	100 a	100 a	100 a
5	72.9 b	57.9 b	57.1 b	65.5 bc
10	60.9 c	51.6 b	59.0 b	75.5 b
20	53.7 c	53.1 b	53.2 b	47.1 c
LSD	11.3	14.5	12.1	8.2

Note: Letters following percent of the untreated control values represent significant differences between concentrations in each shoot length according to Fisher's Protected LSD ($\alpha=0.05$) with $n=8$.

Table 3-7. Total chlorophyll, represented as percent of the untreated control, in Eurasian watermilfoil shoot lengths as a function of fluridone concentration 10 days after treatment.

Fluridone Concentration ($\mu\text{g L}^{-1}$)	Shoot Length			
	2 cm	4 cm	6 cm	8 cm
0	100 a	100 a	100 a	100 a
5	98.8a	70.6 b	79.1 b	52.8 bc
10	88.1 ab	76.0 b	63.1 bc	64.8 b
20	76.5 b	57.8 b	50.2 c	37.2 c
LSD	19.9	21.0	17.2	18.3

Note: Letters following percent of the untreated control values represent significant differences between concentrations in each shoot segment length according to Fisher's Protected LSD ($\alpha=0.05$) with $n=8$.

Table 3-8. Total carotenoids, represented as percent of the untreated control, in Eurasian watermilfoil shoot lengths as a function of fluridone concentration 10 days after treatment.

Fluridone Concentration ($\mu\text{g L}^{-1}$)	Shoot Length			
	2 cm	4 cm	6 cm	8 cm
0	100 a	100 a	100 a	100 a
5	78.1 b	72.6 b	70.5 b	57.8 c
10	78.2 b	73.1 b	58.1 bc	73.2 b
20	66.3 b	61.9 b	50.1 c	41.4 d
LSD	13.4	16.8	13.9	15.0

Note: Letters following percent of the untreated control values represent significant differences between concentrations in each shoot segment length according to Fisher's Protected LSD ($\alpha=0.05$) with $n=8$.

CHAPTER 4

CHARACTERIZATION OF FLURIDONE TOLERANT HYBRID WATERMILFOIL UTILIZING PIGMENT ANALYSIS AND PAM FLUOROMETRY

Invasive watermilfoils, Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hybrid watermilfoil (*M. spicatum* x *M. sibiricum*), are problematic weeds in many water bodies they inhabit. Plants grow to form dense canopies displacing native vegetation, altering water quality, and obstructing recreational uses of waterways. Madsen et al. (1991) found that Eurasian watermilfoil formed dense canopies which can shade out desirable native plants in the ecosystem. Dense canopy formation has been shown to negatively impact water quality by reducing dissolved oxygen in water below the mat and increasing surface temperatures and pH (Bowes et al. 1979). Submersed aquatic weeds have also been shown to harbor algae species harmful to both wildlife and human health (Wilde et al. 2005). When watermilfoils spread within waterbodies and result in unfavorable conditions for wildlife and fisheries, displacement of desirable native vegetation, and impacts on recreational access or aesthetics, management of these plants is often required.

Hybrid watermilfoil was first documented in 2002 by Moody and Les. Populations previously thought to be the invasive Eurasian watermilfoil were found, through nuclear ribosomal DNA analysis, to be hybrid populations from the parental species Eurasian watermilfoil and northern watermilfoil (Moody and Les 2002). Hybrid watermilfoils present unique challenges for management due to inherited traits such as invasiveness and rapid growth rate from the Eurasian parent and the potential for turion formation in some hybrid populations from the northern parent. Once a hybrid population is established in a water body, the gene combinations gained from parental species tend to become incorporated in the hybrid population (Sturtevant et al. 2009). It is important

to note that numerous hybrid watermilfoil populations have arisen independently and therefore traits associated with hybrids from one lake may be quite different when compared with those from another lake. Genotypic and phenotypic differences exist between populations of the hybrid plants and generalities cannot be made regarding specific growth or management traits. Repeated hybridization as well as back-crossing has been documented (Moody and Les 2002). Therefore, it is important to refer to each hybrid population independently.

Chemical control methods are commonly used to manage invasive watermilfoils. Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) is an attractive choice for water managers due to its low use rates, native plant selectivity, ability to target watermilfoil in the entire lake, and potential for multiple years of control from a single treatment. This herbicide is used frequently by water managers to control Eurasian and hybrid watermilfoils. There have been numerous claims of reduced herbicide response by hybrid watermilfoils; however there is limited published information on this topic. Triclopyr ([3,5,6-trichloro-2-pyridinyl]oxy acetic acid) and 2,4-D amine ([2,4-dichlorophenoxy] acetic acid) were found to inhibit growth of both Eurasian and hybrid watermilfoil accessions in a similar manner following exposure to labeled use rates (Poovey et al. 2007). Recently, differences between Eurasian and hybrid watermilfoil biotypes were noted following exposure to low continuous concentrations of 2,4-D (Glomski and Netherland 2010).

Pigment Analysis

Fluridone is an inhibitor of the carotenoid biosynthesis pathway; specifically a noncompetitive inhibitor of the phytoene desaturase (PDS) enzyme. The PDS enzyme

catalyzes the desaturation of phytoene in the rate-limiting step of this pathway (Chamovitz et al. 1993). When PDS is inhibited, phytoene levels increase and carotenoid production in the cell is limited. Carotenoids function to shield chlorophyll from excess light and help dissipate the oxidative energy of singlet oxygen. In fluridone treated plants, carotenoids are not synthesized in new tissue. Therefore, they are not present to quench the energy of oxygen radicals, allowing for the formation of lipid radicals in chlorophyll molecules (Senseman 2007). This results in degradation of chlorophyll, bleaching of new tissue and subsequent necrosis and plant death. By analyzing pigment levels in this pathway the plant's response to fluridone can be studied. Sprecher et al. (1998) developed an extraction method that utilizes absorbance spectroscopy to quantify phytoene and β -carotene levels. In fluridone susceptible plants, phytoene levels increase after fluridone treatments and β -carotene levels are less than control plants. In fluridone resistant plants, phytoene and β -carotene levels are similar to those of untreated control plants. Puri et al. (2006) used this technique to demonstrate resistance in several hydrilla (*Hydrilla verticillata* [L.f.] Royle) populations collected throughout Florida.

Chlorophyll analysis is an indirect method of determining fluridone resistance. Although fluridone does not directly affect chlorophyll biosynthesis, the absence or reduction of β -carotene leads to the destruction of chlorophyll molecules. Therefore, in a susceptible plant exposed to fluridone, chlorophyll levels in new tissue will be lower than those in control plants. Several extraction methods have been evaluated and utilized for pigment analysis using various solvents (Iriyama et al. 1974, Hiscox and Isrealstam

1979, Moran and Porath 1979) but the non-macerated method of Wellborn (1994) using dimethyl sulfoxide (DMSO) proved to be the most useful in these studies.

Although pigment analysis provides a method for determining herbicide activity in the plant, the methods employ a destructive harvest of plant tissue. Due to the transitional nature of fluridone response, there is a need for non-destructive and repeatable methods of analysis. Pulse-amplitude modulated (PAM) fluorometry can give information as to chlorophyll functionality as a measure of chlorophyll fluorescence.

PAM fluorometry has been used to study irradiance stress (Ralph et al. 1998), salinity stress (Kamermans et al. 1999), and shoot-to-landscape differences in photosynthesis in sea grasses (Durako and Kunzelman 2002). *In situ* measurement of photosynthetic activity of Red Sea faviid corals has also been measured (Beer et al. 1998). This technique is useful because it is a non-destructive method of evaluating the activity of chlorophyll and has also been used to evaluate herbicidal effects on plants. Ireland et al. (1986) used fluorometry to document decreased fluorescence in wheat (*Triticum* spp.) 30 minutes after exposure to glyphosate (*N*-[phosphonomethyl] glycine) herbicide. The herbicide diuron (*N*-[3,4-dichlorophenyl]-*N,N*-dimethylurea), a photosystem II inhibitor, was shown to reduce fluorescence yield ratio in sea grasses two hours after exposure as measured with a diving-PAM (Haynes et al. 2000). Junea et al. (2001) evaluated the effects of mercury and metolachlor (2-chloro-*N*-[2-ethyl-6-methylphenyl]-*N*-[2-methoxy-1-methylethyl] acetamide), a mitosis inhibiting herbicide, on six algal species.

A PAM fluorometer works by focusing a saturating beam of light on the desired region of the plant. Yield ratio is calculated by the instrument. Higher fluorescence yield

ratio indicates highly functioning chlorophyll whereas lower yield ratios indicate damaged or non-functioning chlorophyll. Yield is a ratio of F_v/F_{max} . F_{max} is equal to the fluorescence when the saturating pulse is applied to the tissue. F_v is equal to $F_{max}-F$ where F is the fluorescence of the tissue with no light pulse applied. The plant fluoresces more when chlorophyll is damaged, which indicates a higher F_{max} value. However, the yield output ratio is lower because damaged chlorophyll fluoresces more under ambient light conditions which is noted in a higher F value. Therefore, a higher Y ratio value indicates chlorophyll that is functioning normally where a lower Y value indicates damaged chlorophyll (Bolhar-Nordenkamp et al. 1989).

In May 2010, a 220 acre lake in western Michigan (Townline Lake) was treated with a rate of fluridone permitted by the Michigan Department of Natural Resources. The population of documented hybrid watermilfoil in the water body was not controlled with the legal rate of the herbicide. The observation that a hybrid watermilfoil may show increased tolerance to fluridone has fueled more speculation on the nature of hybrids and their response to herbicides.

The use of a PAM fluorometer to detect the effects of pigment synthesis inhibiting herbicides such as fluridone has been documented in aquatic plants, but with limited results (unpublished data). Fluorometry would potentially be a non-destructive method for evaluating fluridone activity in specific plant tissues over a period of time. This method will be compared to traditional pigment analysis when determining differential responses of Townline and several susceptible invasive watermilfoil populations in laboratory experiments. Although mesocosm studies have been used previously to evaluate invasive watermilfoils' response to fluridone, these studies are labor intensive

and time consuming. Laboratory methods could potentially reduce the time needed to document response from several weeks to several days. This reduction in lag time could quickly provide resource managers with valuable information to efficiently treat watermilfoil infestations in the field. This study focuses on further evaluating Townline hybrid watermilfoil and implementing laboratory methods when making comparisons with watermilfoil populations obtained from several states.

Materials and Methods

Experiment 1

Apical shoot tips 6 cm in length were harvested from outdoor stock cultures of watermilfoil populations at the Center for Aquatic and Invasive Plants in Gainesville, Florida. Populations used in this study included Townline, Indian, Auburn, and North Carolina (Table 4-1). Townline is a hybrid watermilfoil suspected to be tolerant to fluridone. The Indian population is also a hybrid and is closely related to Townline due to similar geography and genetics (Thum, pers. comm.¹). Both Auburn and North Carolina populations are confirmed EWM populations.

Apical shoot tips were thoroughly washed with tap water and a single tip was placed in a glass culture tube containing Andrew's media, as described by Selim et al. (1989), supplemented with 4 g L⁻¹ of sodium bicarbonate as an additional carbon source. Plants were immediately treated with 0, 3, 6, 9, 12, 24, or 48 µg L⁻¹ of fluridone (Sonar AS, SePro Corporation, Carmel, IN). Culture tubes were maintained in a climate controlled growth chamber for the duration of the experiment (23°C: 21°C day:night temperature, 14 light:8 dark photoperiod, and 350 µmol/m² light).

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Shoot tips were removed 3, 5, and 7 days after treatment (DAT) and new apical growth was measured with a PAM fluorometer (Mini-PAM, Walz, Effetrich, Germany) to determine fluorescence yield. After measurement, shoot tips were returned to their respective culture tubes. At 7 DAT, 0.2 g of new apical shoot growth was harvested from each shoot for pigment analysis. Total chlorophyll and total carotenoids were quantified using methods described by Wellburn et al (1994). Harvested samples were placed in plastic tubes with 5mL of dimethyl sulfoxide (DMSO) and incubated for 1 hour in a 65°C water bath. Extract from each sample was analyzed spectrophotometrically (BioMate 5, Thermo Scientific, Pittsburgh, PA) at 480nm for total carotenoids, 649 nm for chlorophyll *b* and 665 nm for chlorophyll *a*. DMSO was used as a blank. Total chlorophyll was calculated with the following equation: $(12.19 \times \text{absorbance at 665nm} - 3.45 \times \text{absorbance at 649nm}) + (21.99 \times \text{absorbance at 649nm} - 5.32 \times \text{absorbance at 665nm})$. Total carotenoids were calculated as $(1000 \times \text{absorbance at 480nm} - 2.14 \times \text{chlorophyll } a - 70.12 \times \text{chlorophyll } b) / 200$. Each treatment was replicated four times and the entire study was repeated. Analysis of variance (ANOVA) was used to determine mean effects and possible interactions. There was no experiment by treatment interaction so the results from both experiments were combined. Fisher's Protected LSD ($\alpha=0.05$) was used to detect significant differences in response between each individual population and Townline population.

Experiment 2

Plants with an unknown response to fluridone were obtained from 5 lakes in Michigan and from 3 bays (Phelps, Gideon's, and Carman's) on 5800 ha Lake Minnetonka, Minnesota to be compared to the response of Townline population. The Michigan lakes tested were Missaukee, Cadillac, Shamrock, Ryerson, and Round

Lakes. All of these populations were confirmed as EWM. The study was identical to Experiment 1 in methodology except that PAM yield was only measured at harvest (7 DAT). No significant differences were found between the 8 populations tested. Data was combined and compared to Townline using ANOVA and Fisher's Protected LSD ($\alpha=0.05$) to detect significant differences within each concentration. Nonlinear regression was fitted to the data.

Results and Discussion

Experiment 1

Chlorophyll fluorescence data showed limited differences at 3 DAT (Table 4-2). However, by 5 DAT differences were detected between Townline and each population at each concentration (Table 4-3). By 7 DAT, significant differences were still apparent at most concentrations (Table 4-4). Total chlorophyll data did not exhibit the same level of differences, while total carotenoids also showed fewer differences than fluorescence data as well (Tables 4-5 and 4-6). The lack of differences found using pigment analysis is most likely due to possible variation when using these methods. The PAM fluorometer resulted in less variation in results at 5 and 7 DAT. These results suggest that using a PAM fluorometer to measure fluorescence provides more distinguishable results than typical pigment analysis.

Indian Lake is suspected to have a similar response to fluridone as that of Townline Lake due to geographical proximity and genetic similarities between populations. However, the fluorescence yield response differed at several concentrations 3 DAT, all concentrations at 5 DAT, and all concentrations but the highest concentration at 7 DAT (Tables 4-2, 4-3, and 4-4). Chlorophyll analysis showed differences between the two populations at 9 and 12 $\mu\text{g L}^{-1}$ while total carotenoids were

significantly different at all concentrations but the highest (Tables 4-5 and 4-6). Although reported as being genetically similar to Townline Lake, the response of the hybrid Indian Lake population to fluridone was different to that of Townline.

Fluorescence yield of Auburn differed from Townline at only the lowest and highest concentrations 3 DAT, but differences were found at all concentrations at 5 and 7 DAT (Tables 4-2, 4-3, and 4-4). This suggests that the PAM fluorometer does not detect a quantifiable response to fluridone until at least 5 DAT. Chlorophyll and carotenoid data did not exhibit the same distinguishable trends as fluorescence yield. Pigment data was only significantly different from Townline at $9 \mu\text{g L}^{-1}$ for chlorophyll and 6 and $9 \mu\text{g L}^{-1}$ for carotenoids (Tables 4-5 and 4-6). This is again evidence that the PAM fluorometer provides more precise measurement when compared to pigment analysis. As evidenced by the fluorescence yield results, Auburn and Townline plants had a different response to fluridone, with Townline being less affected at each concentration.

The population of watermilfoil from North Carolina began to show significant differences at several concentrations beginning at 3 DAT. At this time, differences were found at 3, 6, 12 and $48 \mu\text{g L}^{-1}$ (Table 4-2). By 5 DAT, differences were found at each concentration and this trend continued at 7 DAT (Tables 4-3 and 4-4). Differences were found in total chlorophyll at all concentrations but the highest (Table 4-5). Differences were found in total carotenoids at all concentrations tested (Table 4-6). North Carolina plants also had a different response than Townline plants.

These results show the different responses of invasive watermilfoils to fluridone. Although the product is labeled for identical use concentrations for all invasive watermilfoils, differences in response could account for the varying levels of treatment

success observed in the field. Townline population has an increased tolerance for the herbicide fluridone when compared to other known susceptible populations.

Experiment 2

Plants collected from 5 Michigan lakes and 3 bays in Lake Minnetonka, Minnesota showed varying responses to fluridone when compared to Townline Lake. These populations differ in history of fluridone exposure. Fisher's Protected LSD also confirmed that the combined susceptible populations' response, as measured as fluorescence yield, was different to that of Townline at all concentrations tested (Figure 4-1). Both total chlorophyll and total carotenoids were different between Townline and susceptible at all concentrations but the highest (Figures 4-2 and 4-3).

This study demonstrates that Townline plants have an increased tolerance to fluridone when compared to other invasive watermilfoils from several states. Use of a PAM fluorometer was less variable than traditional pigment analysis and is a less complex method to utilize. The limited variability between susceptible watermilfoils collected across the country demonstrates the high level of sensitivity these species often exhibit to fluridone. Fluridone has traditionally been a successful tool in controlling watermilfoil invasions due in part to the widespread response of these plants. Townline Lake is the first documented population of invasive watermilfoils that does not show susceptibility to fluridone. This population should be carefully monitored by resource managers to prevent spread to neighboring lakes. Lakes in close proximity need to be monitored as well in order to rapidly detect any invasion of fluridone tolerant plants. The use of PAM fluorometry in lab-scale studies is a promising tool for quickly evaluating the response to fluridone of other populations of invasive watermilfoils.

Table 4-1. Populations of invasive watermilfoils used in Experiment 1.

Population	Species	Location	Tolerance
Townline	hybrid	Michigan	probable
Indian	hybrid	Michigan	suspected
Auburn	EWM	Minnesota	susceptible
North Carolina	EWM	North Carolina	susceptible

Note: Hybrid watermilfoil is a cross between northern watermilfoil (*Myriophyllum sibiricum*) and Eurasian watermilfoil (EWM) (*M. spicatum*).

Table 4-2. Fluorescence yield (F_v/F_m), represented as percent untreated, in Townline plants and 3 different populations of invasive watermilfoils collected from three states after exposure to fluridone 3 days after treatment.

fluridone concentration ($\mu\text{g L}^{-1}$)	Townline	Indian	Auburn	North Carolina
3	102.1	98.3*	99.8*	95.5*
6	97.9	91.7	97.4	94.3*
9	96.6	91.9*	90.6	95.4*
12	93.4	91.2	88.8	85.2
24	87.7	88.3	87.2	86.0
48	91.1	84.3*	83.7*	81.2*

Note: Differences between each population compared to Townline within each concentration, found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk with n=8.

Table 4-3. Fluorescence yield (F_v/F_m), represented as percent untreated, in Townline plants and 3 different populations of invasive watermilfoils collected from three states after exposure to fluridone 5 days after treatment.

fluridone concentration ($\mu\text{g L}^{-1}$)	Townline	Indian	Auburn	North Carolina
3	104.4	96.5*	101.1*	96.9*
6	102.4	85.7*	93.8*	88.7*
9	101.8	84.7*	81.4*	89.7*
12	97.7	88.8*	82.3*	83.8*
24	91.2	75.9*	76.0*	73.9*
48	86.7	69.4*	72.1*	63.5*

Note: Differences between each population compared to Townline within each concentration, found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk with n=8.

Table 4-4. Fluorescence yield (F_v/F_m), represented as percent untreated, in Townline plants and 3 different populations of invasive watermilfoils collected from three states after exposure to fluridone 7 days after treatment.

fluridone concentration ($\mu\text{g L}^{-1}$)	Townline	Indian	Auburn	North Carolina
3	101.6	100.6	95.7*	98.2
6	99.2	85.8*	87.7*	82.4*
9	97.0	80.6*	67.7*	86.5*
12	97.5	80.3*	78.7*	77.2*
24	84.0	62.3*	69.5*	65.7*
48	70.0	59.1*	59.1*	41.3*

Note: Differences between each population compared to Townline within each concentration, found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk with $n=8$.

Table 4-5. Total chlorophyll, represented as percent untreated, in Townline plants and 3 different populations of invasive watermilfoils collected from three states after exposure to fluridone 7 days after treatment.

fluridone concentration ($\mu\text{g L}^{-1}$)	Townline	Indian	Auburn	North Carolina
3	112.9	101.3	96.8	83.3*
6	106.5	98.0	85.7	75.3*
9	90.7	62.4*	78.2*	67.1*
12	93.1	72.0*	77.6	57.7*
24	79.1	60.1	64.1	48.5*
48	54.4	63.7	55.3	41.2

Note: Differences between each population compared to Townline within each concentration, found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk with $n=8$.

Table 4-6. Total carotenoids, represented as percent untreated, in Townline plants and 3 different populations of invasive watermilfoils collected from three states after exposure to fluridone 7 days after treatment.

fluridone concentration ($\mu\text{g L}^{-1}$)	Townline	Indian	Auburn	North Carolina
3	107.3	93.2*	89.1	74.2*
6	98.4	76.7*	72.1*	65.2*
9	80.4	54.0*	68.9*	53.4*
12	81.0	62.0*	79.2	47.7*
24	69.6	51.3*	56.1	39.2*
48	51.2	62.9	56.8	39.4*

Note: Differences between each population compared to Townline within each concentration, found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk with $n=8$.

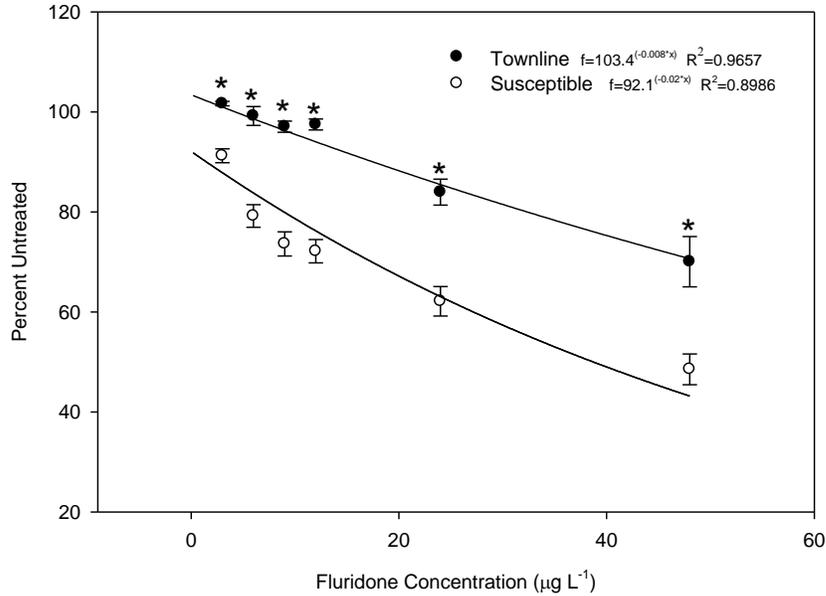


Figure 4-1. Fluorescence Yield (F_v/F_m), represented as percent of the untreated control, in Townline plants and nine different populations of susceptible invasive watermilfoils collected from two states 7 days after treatment with fluridone. Symbols represent means at each concentration and error bars represent standard error (Townline $n=8$, Susceptible $n=32$). Curves indicate nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are marked with an asterisk.

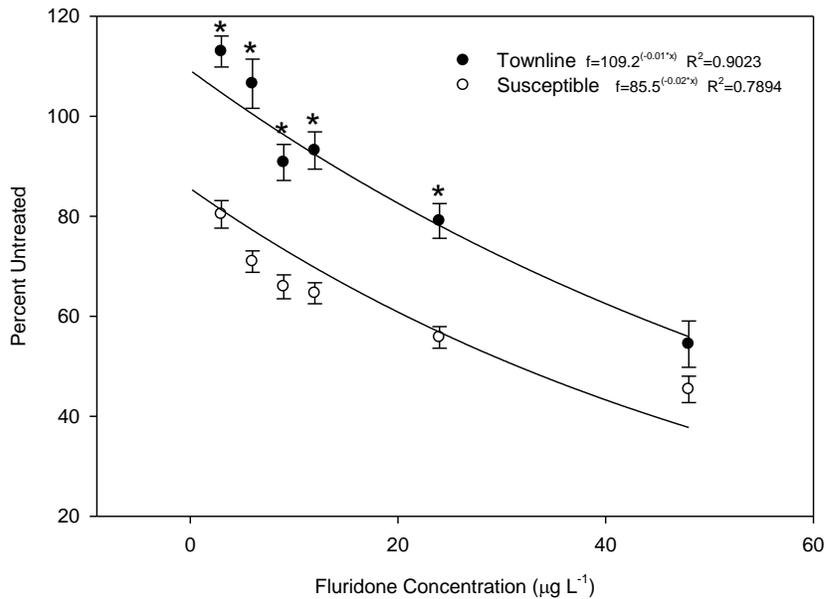


Figure 4-2. Total chlorophyll, represented as percent of the untreated control, in Townline plants and nine different populations of susceptible invasive watermilfoils collected from two states 7 days after treatment with fluridone. Symbols represent means at each concentration and error bars represent standard error (Townline n=8, Susceptible n=32). Curves indicate nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are marked with an asterisk.

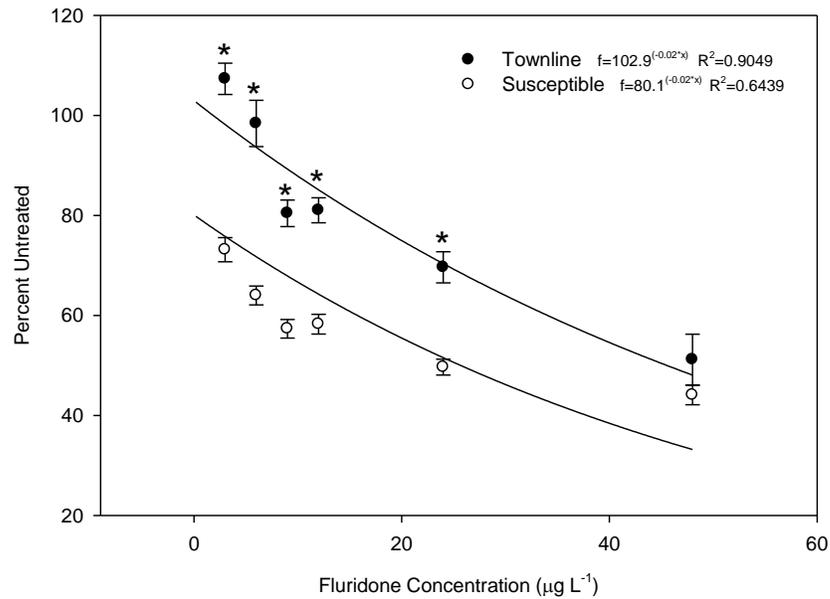


Figure 4-3. Total carotenoids, represented as percent of the untreated control, in Townline plants and nine different populations of susceptible invasive watermilfoils collected from two states 7 days after treatment with fluridone. Symbols represent means at each Concentration and error bars represent standard error (Townline n=8, Susceptible n=32). Curves indicate nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are marked with an asterisk.

CHAPTER 5

EVALUATION OF A HYBRID WATERMILFOIL RESPONSE TO PIGMENT SYNTHESIS INHIBITING HERBICIDES

Invasive watermilfoils, Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hybrid watermilfoil (*M. spicatum* x *M. sibiricum*), are problematic weeds in many water bodies they inhabit. Plants grow to form dense canopies displacing native vegetation, inhibiting flood control and obstructing recreational uses of waterways. Madsen et al. (1991) found that Eurasian watermilfoil formed dense canopies on the water surface to shade out desirable native plants in the ecosystem. Dense canopy formation has been shown to negatively impact water quality by reducing dissolved oxygen in water below the mat and increasing surface temperatures and pH (Bowes et al. 1979). Submersed aquatic weeds have also been shown to harbor algae species harmful to both wildlife and human health (Wilde et al. 2005). When watermilfoils spread within waterbodies and result in unfavorable conditions, management of these plants is often required.

Fluridone herbicide (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) has been used to control Eurasian watermilfoil and hydrilla (*Hydrilla verticillata* [L.f.] Royle) since the late 1980s. Schmitz et al. (1987) describe using fluridone to control hydrilla in a central Florida lake such that the plant could no longer be found in the water body. Haller et al. (1990) successfully used fluridone to control hydrilla for one year in the St. Johns River. As such, fluridone was heavily used for hydrilla control in public waters of Florida from the late 1980's until the early 2000s. Between 1999 and 2001, formerly susceptible hydrilla populations in several Florida lakes were not exhibiting the level of control previously associated with fluridone use. Subsequent laboratory testing documented that several populations of hydrilla had developed resistance to fluridone (Michel et al. 2004, Arias et al. 2005, Puri et al. 2006).

Specifically, an amino acid substitution in the phytoene desaturase enzyme conferred 2 to 5 fold resistance to the herbicide fluridone in hydrilla (Michel et al. 2004).

Previous to this discovery, herbicide resistance in hydrilla was thought to be unlikely due to the strictly vegetative reproduction exhibited by dioecious hydrilla in Florida. However, low use rates, extended exposure times, and repeated use of fluridone led to tremendous selection pressure on these waterbodies. Repeated fluridone applications selected for plants that were resistant to typical use rates of the herbicide fluridone. Since this time, resistance has developed in many water bodies throughout the state and fluridone is no longer a widespread tool for hydrilla management in Florida's public waters. To date, hydrilla is the only plant that has been confirmed to show resistance to fluridone. Since the fluridone use patterns are almost identical for hydrilla and invasive watermilfoils, the potential for resistance in watermilfoils is expected.

In 2010, a population of hybrid watermilfoil in Townline Lake, Michigan was treated with standard rates of fluridone herbicide. After a lack of response, the plants were evaluated in controlled studies and found to be tolerant of fluridone rates exceeding the maximum rate permitted by the Michigan Department of Natural Resources. Hydrilla resistance to fluridone was found to be a conformational change in the target enzyme, phytoene desaturase. Due to the specificity of this resistance, other pigment synthesis inhibitors that target alternative enzymatic reactions are effective on both susceptible and resistant populations. Moreover, studying the response of hydrilla to multiple herbicide mechanisms of action allowed researchers to elucidate the primary mechanism of resistance. Therefore, the objective of this research was to determine the

response of the tolerant Townline hybrid watermilfoil to phytoene desaturase inhibiting herbicides, specifically norflurazon (4-chloro-5-[methylamino]-2-[3-(trifluoromethyl)phenyl]-3[2*H*]-pyridazinone), and a pigment synthesis inhibiting herbicide with a different mechanism of action, topramezone ([3-4,5-dihydro-isoxazol-3-yl)-4-methylsulfonyl-2-methylphenyl](5-hydroxy-1-methyl-1*H*-pyrazole-4-yl)methanone), in an effort to elucidate the possible mechanism of resistance and determine if alternative herbicides would be effective in controlling this population.

Materials and Methods

Populations of hybrid and Eurasian watermilfoil were obtained and put into culture at the Center for Aquatic and Invasive Plants in Gainesville, Florida outdoor mesocosm tanks. Stock cultures of plants were exposed to ambient water and air temperature throughout the culture period. For the first experiment, plants originating from Townline Lake in central Michigan were used and compared to plants with a known susceptibility to fluridone from a Texas population of watermilfoil. In the second study, the Townline population of plants was compared to a population of fluridone susceptible Eurasian watermilfoil originating from Auburn Lake in Minnesota.

Apical shoot tips 6 cm in length were harvested from stock cultures, cleaned thoroughly with flowing tap water, and placed in 100mL of Andrew's culture medium (Selim et al. 1989) supplemented with 4 g L⁻¹ sodium bicarbonate. Plants were treated with fluridone (Sonar AS®, SePRO Corporation, Carmel, IN), topramezone (BAS 670, BASF, Raleigh, NC), or norflurazon (Solicam 80DF, Syngenta Crop Protection Inc., Greensboro, NC) at 0, 5, 10, 20, 40, or 80 µg L⁻¹. Culture tubes were maintained in a climate controlled growth chamber for the duration of the experiment (23°C: 21°C day:night temperature, 14 light:8 dark photoperiod, and 350 µmol/m² light).

Chlorophyll fluorescence was measured at 10 days after treatment (DAT) with a PAM fluorometer (Mini-PAM, Walz, Effetrich, Germany). At this time, 0.1g of new apical growth was harvested for total chlorophyll and total carotenoid analysis with methods similar to those described by Wellburn (1994). Pigment extract in dimethyl sulfoxide (DMSO) was analyzed at 480 nm, 649 nm, and 665 nm for quantification of total carotenoids, chlorophyll *b* and chlorophyll *a*, respectively. DMSO was used as a blank. Total chlorophyll was calculated with the following equation: $(12.19 \times \text{absorbance at } 665\text{nm} - 3.45 \times \text{absorbance at } 649\text{nm}) + (21.99 \times \text{absorbance at } 649\text{nm} - 5.32 \times \text{absorbance at } 665\text{nm})$. Total carotenoids were calculated as $(1000 \times \text{absorbance at } 480\text{nm} - 2.14 \times \text{chlorophyll } a - 70.12 \times \text{chlorophyll } b) / 200$. Extracts were analyzed using a spectrophotometer (Biomate 5, Thermo Scientific, Pittsburgh, PA).

Each treatment was replicated four times and the entire study was repeated. Analysis of variance (ANOVA) was used to determine mean effects and possible interactions. There was no experiment by treatment interaction so the results from both experiments were combined. Fisher's Protected LSD ($\alpha=0.05$) was used to detect significant differences in response between the susceptible populations and Townline population within each concentration.

Results and Discussion

Fluridone

Fluorescence yield showed differences in response to fluridone between Townline and the susceptible population at 5, 10, and 20 $\mu\text{g L}^{-1}$ (Figure 5-1a). Total chlorophyll had differences at 5, 10, and 40 $\mu\text{g L}^{-1}$ while total carotenoids showed differences at 5, 20, and 40 $\mu\text{g L}^{-1}$ (Figures 5-1b and 5-1c). Differences were not expected at the two highest concentrations since fluridone was previously documented to be lethal to

Townline plants between 20 and 30 $\mu\text{g L}^{-1}$. The legally permitted rate in Michigan, where Townline is located, is 6 $\mu\text{g L}^{-1}$. Considering the differences between the two populations at 5 $\mu\text{g L}^{-1}$ in both at harvest fluorescence yield and pigment analysis, it is logical to surmise that Townline would not be controlled at the legally permitted rate, while the known susceptible population would be controlled.

Norflurazon

Differences in fluorescence yield were only found at 40 $\mu\text{g L}^{-1}$ of norflurazon 10 DAT (Figure 5-2). The typically lethal concentration for norflurazon on watermilfoil is unknown, hence the wide range of concentrations in this study. Total chlorophyll varied in response at all concentrations but the highest and total carotenoids were different at all concentrations (Figures 5-2b and 5-2c). It is possible that PAM fluorometry simply does not sufficiently evaluate response to norflurazon as it does with fluridone. Pigment analysis suggests that the Townline population appears to be tolerant or resistant to norflurazon at concentrations up to 40 $\mu\text{g L}^{-1}$. This would be understandable since norflurazon and fluridone work on the same enzyme, PDS, in the carotenoid biosynthesis pathway. Fluridone resistant hydrilla in Florida was also found to be cross-resistant to norflurazon (Puri et al. 2009).

Topramezone

Fluorescence yield was different between Townline and susceptible plants 10 DAT at 10, 20, and 40 $\mu\text{g L}^{-1}$ (Figure 5-3). Total chlorophyll had differences at 5, 20, and 40 $\mu\text{g L}^{-1}$ and total carotenoids had differences at 5, 10, 20, and 40 $\mu\text{g L}^{-1}$ (Figures 5-3b and 5-3c). The typically lethal rate for topramezone on watermilfoil is around 15 $\mu\text{g L}^{-1}$. It is apparent from these results that Townline is also tolerant of topramezone herbicide. This herbicide does not directly act on PDS enzyme, but blocks synthesis of a cofactor

for this enzyme. The fluridone resistant hydrilla documented in Florida did not show cross resistance to HPPD-inhibiting herbicides such as topramezone (Puri et al. 2009).

Fluridone and norflurazon inhibit the phytoene desaturase (PDS) enzyme in the carotenoid biosynthesis pathway. With reduction in carotenoid biosynthesis, chlorophyll molecules are not shielded from excess light and degrade. Topramezone is an HPPD-inhibiting herbicide that blocks synthesis of a cofactor for the PDS enzyme.

These results suggest that the Townline population of hybrid watermilfoils have some form of enhanced tolerance to, at minimum, the pigment synthesis inhibiting herbicides tested in this trial. It is unlikely that a mutation in the phytoene desaturase gene is a cause of this tolerance, as it was in hydrilla, since tolerance was also shown to a herbicide with a different mechanism of action (topramezone). The increased tolerance exhibited by Townline plants could be the result of several tolerance mechanisms such as increased metabolism of the herbicides tested or decreased uptake of the herbicides.

Herbicide resistance is defined as “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type” (WSSA 1998). Herbicide tolerance “implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant” (WSSA 1998). Some discussion has occurred debating whether the fluridone response of hybrid watermilfoil is actually resistance or tolerance. Since hybrid plants were not documented to be at one time susceptible to fluridone, it is not appropriate to refer to them as “resistant.” The Townline population of hybrid watermilfoil displays increased tolerance to the herbicides tested. The results of this study confirm the observations of resource managers that

some hybrid watermilfoil populations do not have the same response to herbicides as Eurasian watermilfoil.

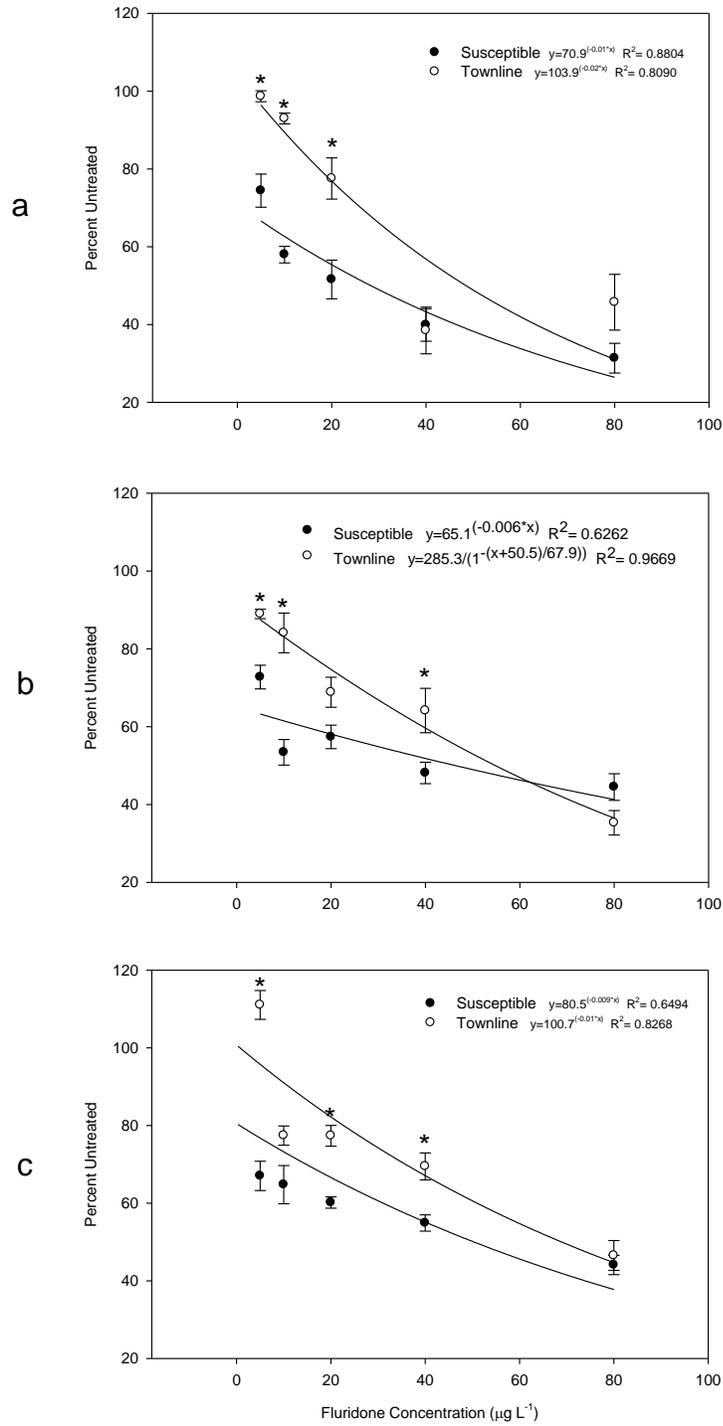


Figure 5-1. Fluorescence yield (F_v/F_m)(a), total chlorophyll (b), and total carotenoids (c), represented as percent of untreated control, in susceptible and Townline invasive watermilfoil plants as a function of fluridone concentration 10 days after treatment. Error bars indicate standard error of the mean ($n=8$). Curves indicate nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk.

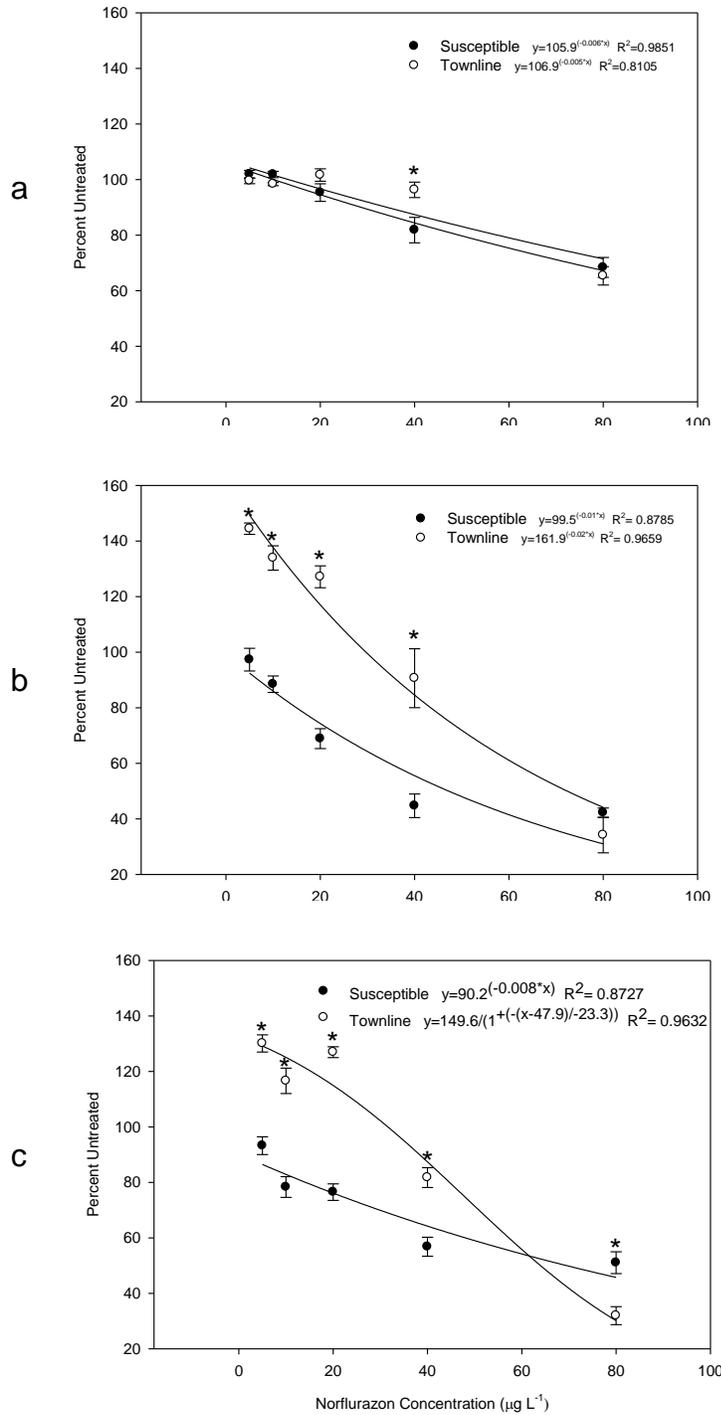


Figure 5-2. Fluorescence yield (F_v/F_m)(a), total chlorophyll (b), and total carotenoids (c), represented as percent of the untreated control, in susceptible and Townline invasive watermilfoil plants as a function of norflurazon concentration 10 days after treatment. Error bars indicate standard error of the mean ($n=8$). Curves indicate nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk.

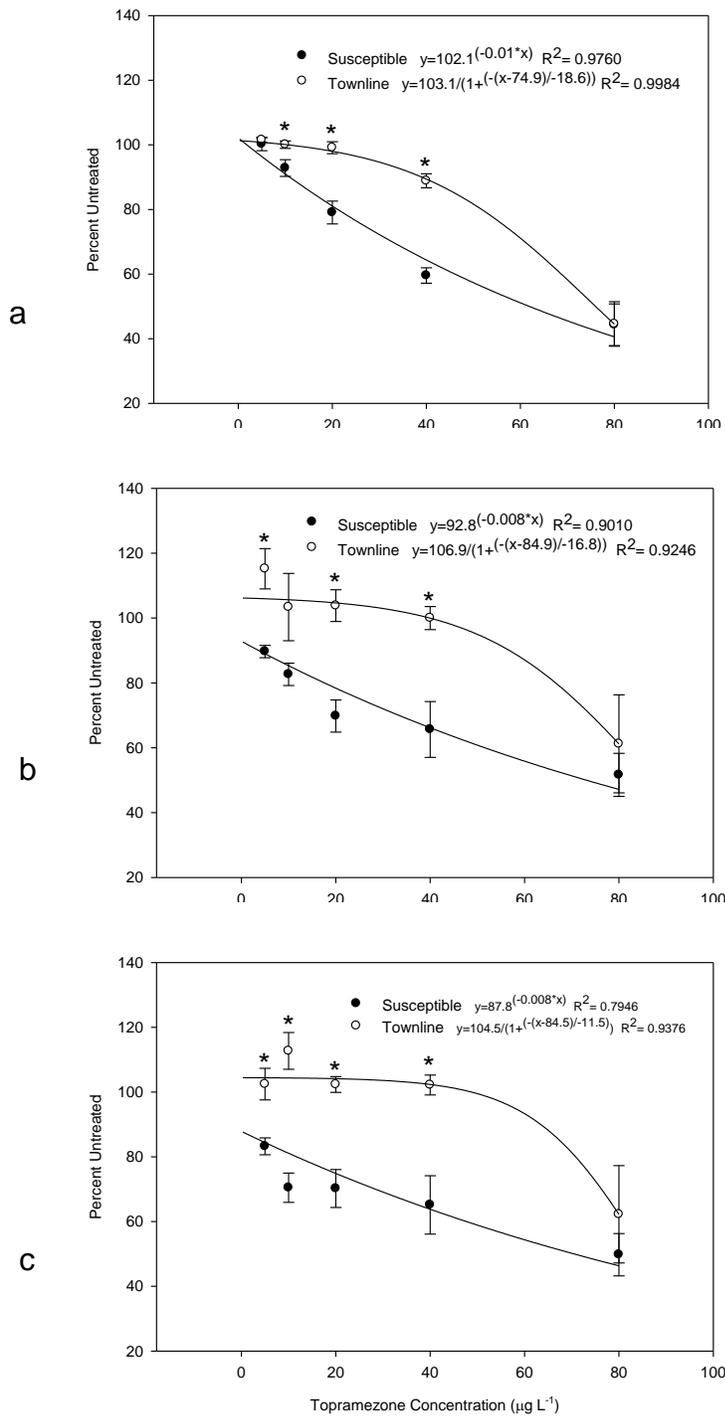


Figure 5-3. Fluorescence yield (Fv/Fm)(a), total chlorophyll (b), and total carotenoids (c), represented as percent of the untreated control, in susceptible and Townline invasive watermilfoil plants as a function of topramezone concentration 10 days after treatment. Error bars indicate standard error of the mean (n=8). Curves indicate nonlinear regression. Concentrations with significant differences as found using Fisher's Protected LSD ($\alpha=0.05$) are denoted with an asterisk.

CHAPTER 6 CONCLUSIONS

Invasive watermilfoils grow rapidly and overtake water bodies through displacement of native vegetation, inhibition of recreational use and obstruction of natural water flow. Within the past 10-15 years genetically verified hybrids of the native Northern watermilfoil (*M. sibiricum* Kom.) and the exotic invasive Eurasian watermilfoil (*M. spicatum* L.) have been documented in the Midwestern United States. The hybrid exhibits the invasive characteristics of Eurasian watermilfoil, thus requiring a similar level of control. A number of herbicides have been used to control both the hybrid and Eurasian watermilfoils, with the herbicide fluridone being a common choice by water managers due to low use rates and minimal damage to desirable native plants. Fluridone herbicide was also used extensively for hydrilla (*Hydrilla verticillata* [L.f.] Royle) management in Florida, but the development of wide-spread resistance has eliminated effectiveness.

In May 2010, a lake containing a documented hybrid watermilfoil was treated with fluridone. The population of watermilfoil on this lake, Townline, survived normally lethal rates of fluridone, raising serious concerns that fluridone resistance or tolerance has developed in another submersed aquatic species. However, confirmation and characterization of this phenomenon is needed. To characterize the level of resistance or tolerance, suspected tolerant Townline plants and susceptible populations were evaluated over a range of fluridone concentrations in both mesocosm and laboratory studies. Mesocosm growth studies confirmed increased tolerance to fluridone as compared to susceptible populations. A pulse-amplitude modulated (PAM) fluorometer was used to measure fluorescence of treated plants in an attempt to develop a less time

consuming method of fluridone resistance confirmation. Pigment analysis of chlorophyll and carotenoids confirmed the results of the PAM fluorometer and results were comparable to those found utilizing biomass experimentation.

These studies demonstrate the utility of PAM fluorometry in detecting plant tolerance to fluridone herbicide, and this method may be useful for detecting differences with other herbicides. Confirmed fluridone tolerant hybrid watermilfoil plants were found to also be tolerant to topramezone herbicide, which has a similar mode of activity but different mechanism of action than fluridone, and norflurazon herbicide, which acts on the same enzyme as fluridone. This indicates a different mechanism of resistance or tolerance in this population of hybrid watermilfoil than was found in hydrilla. This research has confirmed, characterized, and documented a herbicide tolerant hybrid watermilfoil population from Townline Lake. This finding raises serious concerns with management of this species, therefore, it is imperative that resource managers actively limit the spread of this plant.

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BIOGRAPHICAL SKETCH

Sarah Berger was born in Fremont, Ohio in 1984 to William and Patricia Berger. After graduating salutatorian from Fremont Ross High School in 2002 she attended Ohio Northern University in Ada, Ohio. Sarah received a Bachelor of Science degree majoring in biology and environmental studies in 2007. She then pursued a master's degree beginning in the summer of 2009 at the University of Florida. Upon graduation in 2011, Sarah began to pursue a doctor of philosophy degree in Agronomy at the University of Florida.