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Divergent responses of cryptic invasive watermilfoil to treatment with auxinic herbicides in a large Michigan Lake

Syndell R. Parks
Grand Valley State University

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Syndell Rae Parks

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Preface

Vegetation mapping surveys are an important part of managing invasive aquatic plants. They are used both to characterize the nature and extent of an infestation before designing a management plan and to assess the efficacy of management actions after implementation. This thesis research was conducted on the premise that incorporating genetics into vegetation mapping will improve the design and assessment of invasive aquatic plant control strategies.

This idea began with anecdotal reports that invasive watermilfoil had begun to show variation in its response to common herbicide treatments and the discovery, based on genetic studies, that hybrids between Eurasian and northern watermilfoil were present in many treated lakes. Subsequent laboratory studies showed that hybrid watermilfoil were generally less sensitive to the common herbicide 2,4-D. Yet hybrids exhibit so much variation in morphology that they are impossible to visually distinguish from pure Eurasian and northern watermilfoil. Based on these results, we concluded that managed watermilfoil needed to be accurately identified so that hybrids would be detected, if present, and that this could only be done using genetic methods. Currently, however, genetic methods are rarely used when evaluating watermilfoil infestations in managed lakes.

Out of the belief that genetic identifications should become an integral part of watermilfoil management, my adviser Dr. Ryan Thum, entrepreneur Linda Chamberlain, and I participated in the National Science Foundation's Innovation Corps program (iCorps). The iCorps program is intended to help researchers and students begin to transfer their research into commercial products through the validation of its value with potential customers. For our

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team, we needed to determine if managers saw value in using genetic identifications in watermilfoil assessment and treatment, and to determine why they don't use them already. During the program, we interviewed 78 people from 6 states, including state regulators who manage invasive plants and sign off on permits for management, environmental consultants who manage invasive plants on behalf of their customers, and riparian land owners who assess the initial need for management and often hire a consultant to develop and implement the strategy.

From those interactions, we determined that most environmental managers did not feel confident that the information provided by genetically distinguishing hybrid from Eurasian watermilfoil would be worth the cost. Many felt that they could accurately identify hybrid watermilfoil visually and didn't need genetic methods to accomplish the task. Additionally, available data did not convince them that hybrid and Eurasian watermilfoil differed meaningfully in their sensitivity to commonly used herbicides, because all of the current data came from laboratory experiments rather than field trials. Based on those interactions, I determined that in order for genetic monitoring of watermilfoil to be integrated into vegetation mapping, I would need to conduct a field study to demonstrate how genetic identifications could influence watermilfoil and treatment assessment. Specifically, I needed to determine whether the difference in herbicide sensitivity of hybrid and Eurasian watermilfoil found in laboratory studies also occurs in the field, and if so, how distinguishing between the two taxa could improve management.

This thesis is the summation of my research conducted on a large lake in Michigan to assess potential differences between hybrid and Eurasian watermilfoil in their response to

herbicide treatment in the field. Chapter 1 discusses broadly why cryptic invasive plants (i.e.,

invasive plants that are morphologically so similar that they cannot be reliably distinguished

visually) are a management concern in the U.S. and how incorporation of genetics into

management can improve their control. Chapter 2 provides additional background information

on watermilfoil and more detailed explanations of the methods used in managing watermilfoil

and in my thesis research. Finally, chapter 3 presents the research itself and discusses how the

results can be used to improve management of watermilfoil and cryptic invasive plants in

general.

Abstract

Invasive plants are a major concern for environmental managers. Cryptic invasive taxa present additional challenges because of their potential to respond differently to management efforts. Invasive Eurasian watermilfoil (*Myriophyllum spicatum*) and hybrid watermilfoil (*Myriophyllum spicatum* x *Myriophyllum sibiricum*) cannot be reliably distinguished based on morphological characters and are therefore cryptic taxa. Laboratory studies show that on average, hybrid watermilfoil grows faster, branches more, and is less responsive to standard control measures developed for Eurasian watermilfoil. These laboratory results predict less effective control of hybrid watermilfoil in mixed populations treated uniformly with one of these control measures. However, to date there have been no explicit comparisons of growth and response of hybrid versus Eurasian watermilfoil under operational management. In this study, I use genetic methods to distinguish morphologically cryptic Eurasian and hybrid watermilfoil, and document for the first time that the two taxa exhibit divergent responses to treatment with 2,4-dichlorophenoxyacetic acid- amine and triclopyr under operation management in Houghton Lake, Michigan. Results show that treatment was much more effective for pure Eurasian watermilfoil than for hybrids. These findings provide a concrete example of how cryptic invasive taxa can impact control efficacy and how incorporating genetic methods into monitoring can improve treatment assessment and vegetation monitoring, potentially improving management.

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Chapter 1. Introduction

Invasive plants are non-native plant species or biotypes that have negative impacts on their introduced local environment (Westbrooks 1998). They are a major concern among ecosystem managers in the U.S., where it is estimated that more than 5,000 invasive plant species have become established (Pimentel et al. 2005). Many of these were brought into the country intentionally. For example, purple loosestrife (*Lythrum salicaria*) was imported as an ornamental plant but escaped and became a major threat to marsh habitats across the U.S. (OTA 1993, Blossey et al. 2001). Kudzu (*Pueraria montana* var. *obate*) was imported as a means of preventing soil erosion but has become a major threat to woodland diversity (Westbrooks 1998). Additionally, many aquatic invasive plants were imported for aquaculture or the aquarium trade

(Les and Mehrhoff 1999). However, the majority of introductions have occurred unintentionally via incidental transport with goods or people entering the country. OTA (1993) estimated that 81% of invasive plants were introduced to the U.S. by "hitchhiking" in shipments of imported commodities or in ballast water. For example, cheat grass (*Bromus tectorum*) was introduced to the U.S. in packing material and as a contaminant in grain seed and now occurs across North America, reducing wheat production and increasing the frequency of wildfires in the Pacific Northwest (Westbrooks 1998).

While only about 10% of all introduced plant species become established as invasive populations (Barbier 2001), those that do can have severe negative impacts (Pimentel 2009, Charles and Dukes 2007) . For example, in non-agricultural terrestrial systems, invasive plants can harm or endanger native plant species, alter habitats of associated wildlife, modify nutrient

cycles, and cause soil erosion. In aquatic systems, invasive plants can diminish habitat,

endanger native plants and wildlife, and alter hydrology and nutrient cycling (Perrings 2001,

Charles and Dukes 2007). In addition to these ecological impacts, invasive plants can also cause

heavy economic losses. In terrestrial systems, infestations can reduce crop yield through

competition, reduce livestock yield through forage toxicity, reduce the areal extent of

pastureland, and lower property values (Radosevish 1987, Westbrooks 1998). They can also

hinder drainage and irrigation and reduce accessibility to many locations. In aquatic systems,

infestations can hinder hydroelectric dams and water treatment plants and lower drinking

water quality (Westbrooks 1998, Rockwell et al. 2003). They can also damage boating gear and

impede swimming and navigation, sometimes resulting in lower property values and declines in

local tourism (Rockwell et al. 2003, Zhang and Boyle 2010). Damage caused by invasive plants

annually in the United States amounts to roughly $24 billion in agricultural systems and ranges

from $1-$10 billion in non-agricultural and aquatic systems (Rockwell et al. 2003, Lovell and

Stone 2005, Pimentel et al. 2009).

Given the negative impacts of invasive plants on ecosystems and the high cost of

economic damages, control of invasive plants has become a major concern for environmental

managers. There are multiple methods of control for invasive plants: cultural, physical,

mechanical, biological, and chemical (Gettys et al. 2014). Cultural control consists of changing

actions in order to prevent establishment and reduce established populations (Gettys et al.

2014). These actions include setting restrictions on plant shipping and sale and using livestock

to eat invasive populations. Physical control involves the physical removal of invasive plant

populations (Cronk and Fuller 2001, Gettys et al. 2014). For instance, hand pulling plants or

placing barriers to remove or kill populations would be considered physical control methods.

Mechanical control consists of physical removal of plants through the use of equipment such as

mowers and harvesters (Tu et al. 2001, Gettys et al. 2014). Biological control is accomplished

through the introduction of a second species that reduces the size of the invasive plant

population (DiTomaso 2000, Ding et al. 2006, Louda et al. 2007, Gettys et al. 2014). Chemical

control reduces invasive plant populations through the application of herbicides (Swanton and

Weiss 1991, Cronk and Fuller 2001, Gettys et al. 2014).

Chemical control with herbicides is currently the most common method for invasive

plant management. Herbicides can be either systemic, meaning they are taken up and

transported throughout the plant, or contact, meaning they affect sections of the plant to

which they are directly applied (Powels and Yu 2010, Gettys et al. 2014). For example, diquat

(6,7-Dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium dibromide) is a contact herbicide, whereas

fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]pyridin-4-one) is a systemic

herbicide (Sprecher and Netherland 1995, Breitenbach et al. 2001, Gettys et al. 2014).

Herbicides also have different modes of action (what they actually *do* to the plant): some target

functions characteristic of specific plant groups (e.g., broadleaf weeds) and therefore provide

selective control, while others (broad spectrum herbicides) target processes that occur in all

plant species (Powels and Yu 2010). Diquat, for example, is a broad-spectrum herbicide that

inhibits photosynthesis and destroys cell membranes in tissues of all plant groups (Sprecher and

Netherland 1995). In contrast, the herbicide 2,4-D (2,4-Dichorohenoxyacetic acid) is a mimic of

a growth regulator hormone. Dicotyledonous (broadleaf) plants tend to be more sensitive to

this auxin mimic, allowing it to selectively control dicotyledonous plants while leaving

monocotyledonous plants unharmed at lower concentrations (EPA 1988, Sprecher and

Netherland 1995, Parsons et al. 2001). There are close to 300 herbicides registered in the

United States that are used for control of plants in terrestrial and aquatic systems (Gettys et al.

2014).

Annual costs of invasive plant control are approximately $3 billion in agricultural

systems, $1.3 billion in residential areas, and $100 million in non-agricultural and aquatic

systems combined (OTA 1993, Lovell and Stone 2005, Pimentel et al. 2005). Despite the variety

of control options available, management of invasive plants can become complicated when

different populations of the same plant species respond differently to the same control

method. For example, horseweed (*Conyza canadensis*) is considered an invasive weed in

agricultural systems and is commonly managed through the use of glyphosate (VanGessel 2001,

Feng et al. 2004, Dinelli et al. 2008). However, differences among horseweed populations in

their glyphosate sensitivity have been widely observed, and resistant biotypes are now known

(Lyon et al. 1989, Warwick 1991, VanGessel 2001, Patzoldt et al. 2002). Ineffective control in

populations of such species can result in reduced predictability of management outcomes due

to the presence of both resistant and sensitive populations, potentially wasting resources and

allowing the continued spread of less sensitive biotypes. Thus, understanding the sources of

variation among managed populations is a key component for determining which management

strategies will be most effective.

Multiple factors may contribute to variation in response to a given management

practice. Restricting attention to biological factors, there may be distinct biotypes within a

species that, as a result of physiological or ecological differences, differ in their tolerance to

control methods without prior exposure (Heinrichs et al. 2011). For example, multiple biotypes of the invasive bellyache bush (*Jatropha gossypiifolia*) are present in the United States, and while biological control is preferred for this weed, the selection of control species is complicated by the fact that no single species works well for every biotype (Heinrichs et al. 2011). Alternatively, different populations of a given species or biotype may have developed resistance to a particular control method as a direct result of repeated prior exposure (Warwick 1991). For example, as mentioned above, some (but not all) populations of horseweed exposed to repeated glyphosate treatments in agricultural fields have evolved resistance to this herbicide, resulting in resistant and sensitive populations of the same species (Lyon et al. 1989, Warwick 1991, VanGessel 2001, Patzoldt et al. 2002).

An additional contributing biological factor, and the one on which my research is focused, is the presence of cryptic taxa with different sensitivities to a given management practice. In this thesis, cryptic taxa are defined as two or more taxa that cannot be reliably distinguished morphologically but are nevertheless taxonomically distinct (Pfenninger and Schwenk 2007). As with other taxa, cryptic taxa can differ in the way they respond to a given management practice. While a population's sensitivity to a particular treatment strategy directly determines how effective control will be, the presence of cryptic taxa can lead to ambiguity regarding which taxon is being targeted and thus potentially lead to unpredictable management outcomes. Cryptic taxa can occur as a "group of species" such as Asian Yew (*Taxus spp.*), which in comprised of several closely related species that are difficult to distinguish morphologically (Lui et al. 2011). They can also occur as distinct biotypes of the same species. For example, hydrilla (*Hydrilla verticillata*) (Michel et al. 2004, Arias et al. 2005), common reed

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(*Phragmites australis*) (Saltonstall 2002, Holdredge et al. 2010), and variable leaf watermilfoil

(*Myriophyllum heterophyllum*) (Thum et al. 2011) are species that are composed of multiple

biotypes that can't be reliably distinguished morphologically but vary in their invasive

characteristics and response to management. Finally, cryptic taxa can also result from

hybridization events between closely related species. Smooth cordgrass (*Spartina alterniflora x

S. foliosa*) (Daehler and Strong 1997), hybrid salt cedar (*Tamarix ramosissima x T. canariensis or

T. gallica*) (Gaskin and Schaal 2002), bohemian knotweed (*Fallopia x bohemica*) (Walls 2010),

and hybrid cattail (*Typha x glauca*) (Woo and Zedler 2002, Holdredge et al. 2010) are examples

of cryptic hybrids that vary in invasiveness and response to management efforts compared to

their pure parental taxa.

While recognition of cryptic taxa is increasing in non-plant species and in agricultural

systems (Pfenninger and Scheenk 2007), less is known about the prevalence of cryptic plant

taxa in natural terrestrial systems and even less in aquatic systems. Genetic techniques have

therefore become a necessary tool for population assessment to determine if cryptic taxa are

present (Carlton 1996, Bastos et al. 2011) and to determine if their response to management

could influence the overall response of an invasive population. For example, the monoecious

hydrilla biotype can contain a point mutation resulting in one of three amino acid substitutions

in the phytoene desaturase (PDS) gene (Michel et al. 2004, Arias et al. 2006). Each potential

amino acid substitution can confer a different level of resistance to the common herbicide

fluridone. Determining the presence of that single mutation through genetic analysis indicates

whether the population in question is at least partially composed of a herbicide resistant

hydrilla biotype (Arias et al. 2006).

In addition to determining the presence of cryptic taxa at one point in time, genetic tools can be employed in temporal monitoring of the proportion of each taxon and its response to management to help direct management efforts. Monitoring changes in the proportions of taxa can document within-season efficacy of management for each taxon individually. For instance, if only 1% of the total hydrilla population is the resistant biotype, then the overall control may be considered high with 99% of the population responding to treatment. However, if 90% of the hydrilla population is composed of the resistant biotype, then only 10% of the population might respond to treatment and overall control may be considered low. Continued taxon-specific monitoring over multiple years of management can show how the proportion of less sensitive plants changes over time and thus indicate whether changes in management strategy are necessary. Additionally, monitoring efficacy as strategies change over time permits comparison of different treatment types to determine which strategies are most effective for each individual taxon.

The research reported in this thesis focuses on management issues created by the presence of cryptic invasive plants in the particular case of Eurasian watermilfoil (*Myriophyllum spicatum*) and its interspecific hybrid with the native northern watermilfoil (*Myriophyllum sibiricum*). Chapter 2 provides background information needed to understand the methods used in Chapter 3, including overviews of invasive Eurasian and hybrid watermilfoil, watermilfoil assessment practices, genetic assessment methods, and types of herbicides used for management. Chapter 3 presents the main research results, including the first rigorous demonstration of differential response to herbicide treatment by Eurasian and hybrid

watermilfoil in the field and a discussion of how genetic methods can provide insight into best management practices.

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Chapter 2. Background Information

1. Overview of Eurasian watermilfoil biology and invasion

Eurasian watermilfoil (*Myriophyllm spicatum* L.; EWM) is a submersed perennial aquatic plant. In lakes, it typically grows in water between 1 and 4 meters deep (up to *ca*. 12 meters), anchored to the lake bed by roots (Nichols 1975, Grace and Wetzel 1978). Leaves are finely divided and occur in groups of 3–5 (typically 4) at nodes along each stem (Aiken et al. 1979, Smith and Barko 1990). Most adult plants senesce in the fall, but intact stems sometimes overwinter (Pimentel et al. 2005). Plants typically regrow from buds on the root crowns (at the sediment-water interface) in the spring, producing branches and forming dense canopies at the surface of the water (Nichols 1975, Grace and Wetzel 1978, Nichols and Shaw 1986).

Eurasian watermilfoil has two modes of reproduction: asexual (vegetative) and sexual (seeds) (Nichols 1975). Asexual reproduction by fragmentation is thought to be the main mode (Pimentel et al. 2005). Plants naturally produce fragments (autofragmentation) that detach and establish on the bottom to become independent plants (Nichols 1975). Fragments created by disturbance (e.g., boating) can generate new plants, as well. Transport of fragments by water movements and recreational gear (especially boats) is considered to be the principal means of spreading to new locations (Nichols and Shaw 1986, Madsen 2005).

Watermilfoil can also reproduce sexually. Once the plants reach the water surface, flowers form on terminal spikes, allowing both cross- and self-pollination (Aiken et al. 1979, Madsen 2005). Once pollinated, the terminal spikes produce seeds that fall off after maturing and settle on the lake bottom (Madsen 2005). Though large numbers of viable seeds are commonly

produced, they are generally considered to contribute little to the regrowth and spread of watermilfoil observed in the field (Madsen 1998).

Due to its dispersal ability through fragmentation and its habit of forming dense mats at the water surface, Eurasian watermilfoil is one of the most heavily managed aquatic plants in the U.S. (Bartodziej and Ludlow 1997, Moody and Les 2007). Since its introduction, it has spread to 46 states and 3 Canadian Provinces. As with other invasive aquatic plants, it can out compete native species (Madsen 1991, Westbrooks 1998), increase sedimentation, reduce fish spawning and shelter habitat, and alter hydrology (Rockwell et al. 2003, Lovell and Stone 2005, Pimentel et al. 2005). Infestations can lower property values, damage personal property such as boat propellers, hinder boating, swimming, and fishing, and reduce tourism in local communities (Rockwell et al. 2003, Madsen 2005, Zhang and Boyle 2010, Westbrooks 1998).

Recent field studies have demonstrated the widespread occurrence of a hybrid between Eurasian watermilfoil and native northern watermilfoil (*Myriophyllum sibiricum* Komorov) (Moody and Les 2002, Moody and Les 2007, Sturtevant et al. 2009, Zuellig and Thum 2012). Laboratory studies have shown that hybrids generally grow faster and form more branches than the pure parental species (Danielle Grimm, unpublished data). If these characteristics are exhibited by field populations, they could result in more rapid spatial spread by hybrids.

2. Watermilfoil Control Methods

The most common method for treating watermilfoil infestations is through the use of auxinic herbicides like 2,4-dichlorophenoxyacetic acid (2,4-D) and 3,5,6-trichloro- 2-pyridinyloxyacetic acid (triclopyr) (Figure 2.1) (EPA 1988, Ghassemi 1981, Netherland and Getsinger 1992,

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Woodburn et al. 1993, Teixeira et al. 2007). 2,4-D and triclopyr are auxin mimics and thus act

systematically to affect tissue growth (Parsons et al. 2001, Gettys et al. 2014, Harrahy et al.

2014). The compounds accumulate at the actively growing sites of the plant, such as the

meristematic regions of the roots and shoots (Ghassemi 1981, Walters 1999). This

accumulation results in the stimulation of growth, rejuvenation of old cells, and overstimulation

of young cells, leading to abnormal growth patterns and sometimes death (Walters 1999). The

compounds are also converted into amino acid conjugates that obstruct nucleic acid

metabolism and protein synthesis. Those conjugates alter enzyme activity, respiration, and cell

division, resulting in inhibition of nutrient transport, malnourishment, and ultimately, death

(EPA 1988, Walters 1999). 2,4-D and triclopyr are considered to be selective herbicides because

dicotyledonous plants such as Eurasian watermilfoil tend to be more susceptible to them at low

to moderate doses than are monocotyledonous plants (Parsons et al. 2001, Netherland and

Getsinger 1992, Harrahy et al. 2014).

Figure 2.1 A: Molecular Structure of 2,4-D (2,4- Dichlorophenoxyacetic acid) (Image recreated

from: Teixeira et al. 2007). B: Molecular structure of Triclopyr (3,5,6-trichloro- 2-

pyridinyloxyacetic acid) (Image recreated from: Woodburn et al. 1993).

A.   B.
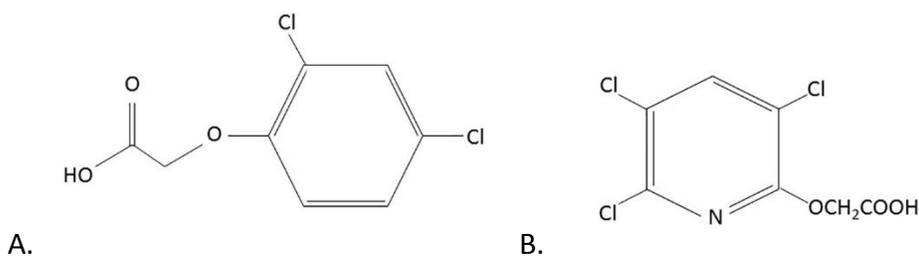
3. Variation in response to 2,4-D and Triclopyr

The heavy use of 2,4-D and triclopyr has resulted in resistance of multiple species of weeds (Whitehead and Switzer 1963, Teixeira et al. 2007, Egan et al. 2011). While resistance is well documented in agricultural systems, evidence for differences in susceptibility in aquatic populations has only more recently begun to surface. There have been anecdotal reports of variation in watermilfoil response to 2,4-D in the field, but no previous study has rigorously demonstrated such variation. The discovery of cryptic hybrid watermilfoil (see Section 4.2 below) and its widespread occurrence (Moody and Les 2007) revealed that field populations of Eurasian watermilfoil are often a mixture of pure Eurasian watermilfoil and hybrids. Recent laboratory studies have shown that Eurasian and hybrid watermilfoil differ in sensitivity to auxinic herbicides, with hybrid watermilfoil being less sensitive, on average (LaRue et al. 2013, Schulte et al. i*n review*). Though not yet confirmed in the field, these laboratory findings suggest that the presence of cryptic hybrids in populations of Eurasian watermilfoil could be a cause of variation in response and that distinguishing between hybrids and pure Eurasian watermilfoil when managing infestations in lakes could be useful in evaluating treatment efficacy.

4. Watermilfoil Assessment

4.1 Mapping watermilfoil

Typically, watermilfoil populations in lakes are mapped with a point-intercept (PI) survey (Jonasson 1988, Madsen 1999, Hauxwell et al. 2010). During a PI survey, the spatial distribution and abundance of watermilfoil is determined by recording presence/absence and semi-quantitative abundance estimates at a large number of points arranged in a regular grid that

covers the waterbody (Madsen 1999, Hauxwell et al. 2010, Mikulyuk 2010). To collect these data, watermilfoil is sampled using a rake -toss method in which a two-sided rake attached to a rope is tossed into the water, dragged a short distance along the lake bottom to collect portions of any plants that might be present, and pulled back up to the surface (Thum et al. 2012, Hauxwell et al. 2010). Presence or absence of watermilfoil at each grid point is determined by whether the rake does or does not contain watermilfoil, while abundance of watermilfoil is estimated by the proportion of the rake it covers (Thum et al. 2012). Watermilfoil typically is identified during PI surveys based on morphological traits such as leaf characteristics (Moody and Les 2007).

4.2 Distinguishing Watermilfoil

Hybrid watermilfoil cannot be reliably distinguished from Eurasian and northern watermilfoil using traditional morphological traits. While watermilfoil taxa are most commonly identified by examining the leaves, leaf morphology is so variable in hybrid populations that many individuals exhibit morphologies identical to the pure parental taxa (Figure 2.2) (Moody and Les 2007). Hybrid and pure Eurasian watermilfoil are therefore morphologically cryptic. In order to distinguish between populations of these taxa in a waterbody so that management strategies can be properly planned and evaluated, genetic methods for identification are a necessary component of vegetation mapping.

Figure 2.2 Comparison on leaf morphologies of Eurasian (bottom), northern (top), and hybrid (middle) watermilfoil. Hybrid watermilfoil morphology can look identical to Eurasian and northern making it impossible to visibly distinguish from the parental taxa.
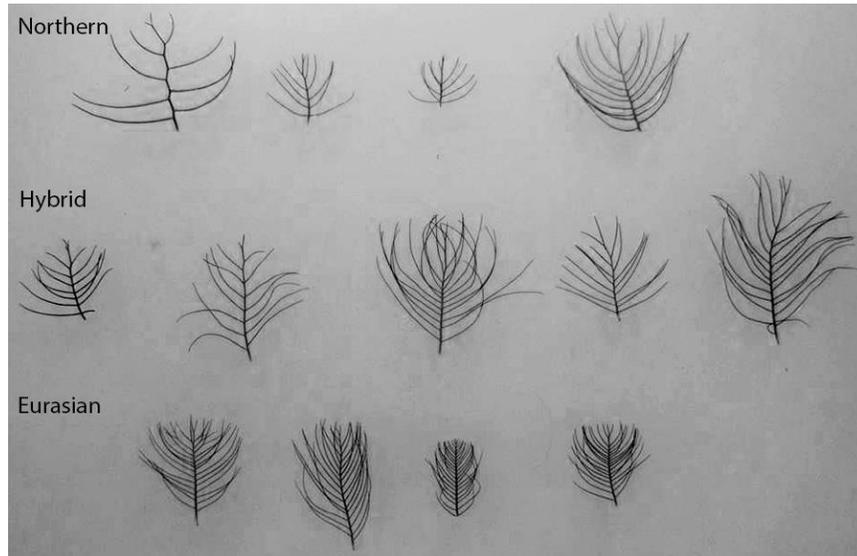
4.3 Genetic Identification

Multiple genetic techniques are available for reliably identifying cryptic taxa such as Eurasian and hybrid watermilfoil (Table 2.1; Karp et al. 1997, Willems et al. 2001, Small et al. 2004, Lowe et al. 2009, Abdel-Mawgood 2012). The method chosen for this thesis is DNA barcoding. DNA barcoding uses consistent variations in one or more target genes or sequences of an organism to assign it to a taxon (Hebert et al. 2004, Hebert and Gregory 2005). In order for DNA barcodes to be effective for detection of hybridity, they must have minimal variation to accurately and consistently distinguish taxa and be heritable from both parental taxa (Small et al. 2004). While multiple potential sequences qualify to consistently distinguish between taxa (*matK, trnK, trnLF, rbcL, ycf1*) (Moody and Les 2002, Cheng et al. 2015), nuclear ribosomal sequences are inherited

from both parental taxa and thus can determine if an individual is a hybrid (Bakkeren et al.

2000). The target gene commonly used for watermilfoil is the nuclear ribosomal internal

transcribed spacer (ITS) (Cheng et al. 2015) and as such is used for identifying hybrid and pure

Eurasian watermilfoil in this study. Analysis of the ITS sequence is typically used to distinguish

between closely related taxa of plants and fungi (Koch et al. 2003, Hebert et al. 2004). The

utility of using the ITS sequence is thought to be due to concerted evolution in which a single

variant of the ITS sequence becomes fixed within each taxon, in conjunction with a low

mutation rate (Baldwin 1992). Thus, minor differences in ITS sequences can reveal the presence

of different taxa when morphology cannot.

Table 2.1 A comparison of genetic analysis methods for use in identifying hybrid and Eurasian

watermilfoil including Internal Transcribes Spacer (ITS) digestion Amplified Fragment Length

Polymorphisms (AFLP's) and Single Nucleotide Polymorphisms (SNP's).

	ITS digestion	Sequencing	AFLP's	Microsatellites	SNP's
Time investment	medium	High	High	High	Very High
Cost	Low	High	High	High	High
Standardization	High	High	Medium	Medium	Medium
Accuracy	High	High	High	High	High
Development for Watermilfoil	Ready to use	Ready to use	Ready to use	Ready with some refinement	A lot of development necessary

For watermilfoil, we perform a restriction fragment analysis of ITS (Moody and Les. 2002, Thum et al. 2006, Moody and Les, 2007, Zuellig and Thum 2012, Grafé et al. 2015). During this analysis, restriction enzymes are used to cut the ITS region. The cut sites for these enzymes are different for each parental watermilfoil taxon, resulting in different -size fragments in each and thus a different banding pattern on a gel (Thum et al. 2006, Grafé et al. 2015). Since hybrid watermilfoil contains one copy of the ITS sequence from each parental taxon, ITS digestion of hybrid watermilfoil results in gene fragments that match the size of both parental taxa (Figure 2.3).

Figure 2.3 ITS gel Image of watermilfoil restriction gel with Northern (A), hybrid (B), Eurasian (C), and a 100 base pair reference ladder (D). Digestion of the ITS sequence results in different sized fragments for northern and Eurasian individuals. Since ITS is inherited from both parental taxa, hybrids contain fragments matching both Eurasian and northern watermilfoil.
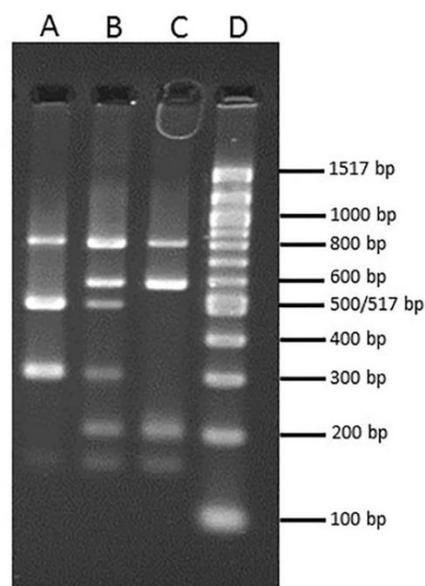
5. Management Programs

Watermilfoil is one of the most highly managed of all invasive aquatic plants, but an adaptive management approach has rarely been successfully implemented for it. Adaptive management programs use empirical data collected during the control season and rigorous statistical analysis of outcomes to evaluate the efficacy of treatment methods (Walters 1986, Williams 2010). In other words, management is viewed as an experiment and is planned, executed, and assessed accordingly (Figure 2.4). Evaluation through the collection and rigorous analysis of data can help managers determine how well each treatment method works in comparison with others and if new control methods are necessary. The fundamental goal of adaptive management is to improve control and reduce uncertainty in outcomes over time by monitoring treatment outcomes and adapting management practices to improve them (Williams and Brown 2012). Currently, Eurasian and hybrid watermilfoil are controlled using the same methods. However, if it turns out to be true that hybrid watermilfoil is generally less susceptible to treatment, as suggested by laboratory studies, then the response of these two taxa should be documented and monitored separately in order to evaluate what methods are most effective for the control of each.

Figure 2.4 Adaptive plant management cycle designed to help managers design each year's control strategy to be as effective as possible using current knowledge while collecting data necessary to improve efficacy in future years (Figure recreated from: http://www.dfg.ca.gov/erp/adaptive_management.asp)
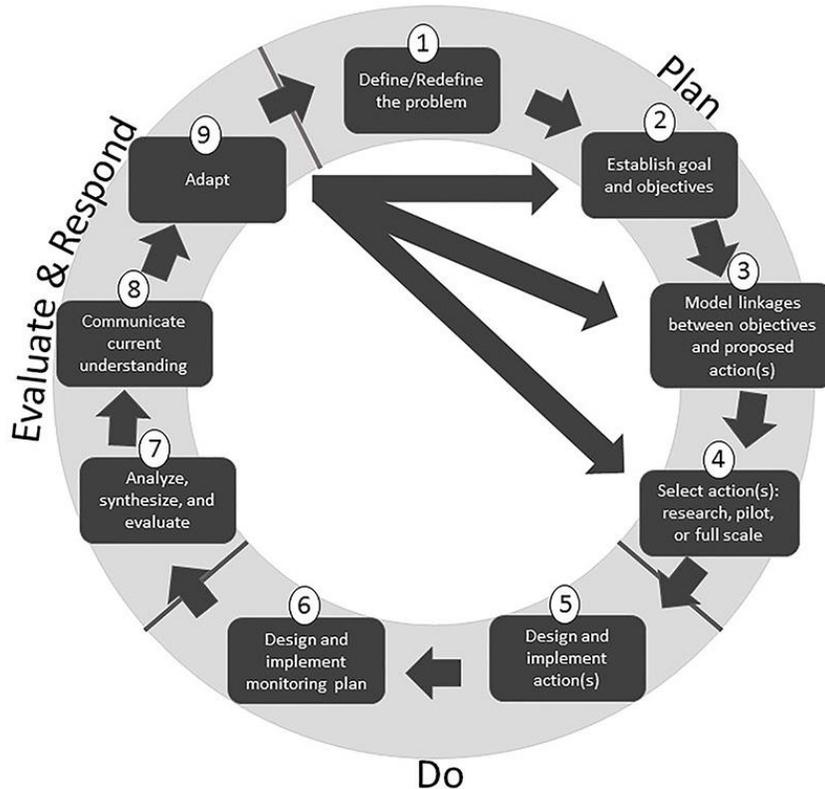
Unfortunately, while managers generally agree that adaptive management programs are beneficial for watermilfoil control, genetic identifications is currently not an integral part of watermilfoil assessment. Interactions with managers have shown that even though it is becoming more accepted that Eurasian and hybrid watermilfoil cannot be reliably distinguished morphologically, managers are not convinced that differences in treatment response occur in the field as they do in controlled laboratory studies, and they therefore feel that distinguishing

between the two taxa would provide limited useful information for management planning

(Personal Communication). The fundamental problem, then, is the current lack of field

verification of differences between Eurasian and hybrid watermilfoil in response to treatment.

In light of that feedback, the research presented in the following chapter was aimed at

determining if Eurasian and hybrid watermilfoil do in fact exhibit differential response to

auxinic herbicides during an operational field treatment and thus need to be evaluated

independently for the implementation of effective adaptive management programs.

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Chapter 3. Thesis Research

Introduction

Invasive plants are a major concern for natural resource managers due to their impacts on ecosystem services and native biodiversity and to the economic costs associated with losses and control. In aquatic systems, for example, invasive plants are responsible for billions of dollars of damages annually by impairing boating, swimming, water movement, and nutrient cycling, and reducing property values, increasing sedimentation, and eliminating habitat for native species (Lovell and Stone 2005; Zhang and Boyle 2010; Aiken et al. 1979; Bates et al. 1985; Smith and Barko 1990; Madsen et al. 1991; Gross and Sutfeld 1994). Moreover, the direct cost of managing invasive aquatic plants to mitigate these negative impacts amounts to an additional 100 million dollars annually in the United States alone (Rockwell 2003, Pimentel et al. 2005).

One complication in managing invasive plants is the fact that outcomes for a given taxon may vary markedly among managed sites. For example, variation in response of *Conyza canadensis* (horseweed) to herbicide control is seen in different populations across the United States (Szigeti et al 1996; Dinelli et al. 2008). Given the high cost of management and the limited financial resources available, it is important to understand the factors contributing to this variation in control efficacy in order to increase the predictability of management and improve outcomes.

Cryptic invasive taxa are a potentially important source of variation in treatment outcomes for widely managed invasive species. By "cryptic invasive taxa", I refer to those invasive taxa that are difficult to distinguish using traditional identification methods based on

morphological characters. For example, some invasive taxa are composed of multiple, distinct

biotypes with different introduction histories and sources (e.g., monoecious versus dioecious

Hydrilla verticillata, Madeira et al. 2004; *Phragmites australis* haplotypes, Saltonstall 2002;

Myriophyllum heterophyllum, Thum et al. 2011*; Conyza canadensis*, Szigeti et al 1996; *Tamarix*

spp, Gaskin and Schaal 2003). Similarly, invasive taxa may hybridize with closely-related and/or

morphologically similar native (or other invasive) taxa, forming distinct parental versus hybrid

invasive biotypes that are morphologically cryptic (*Fallopia x bohemica*, Walls 2010; *Tamarix*

ramosissima x T. canariensis or T. gallica, Gaskin and Schaal 2003; *Centaurea xpsammogena*,

Blair and Hufbauer 2010; Spartina angelica, Strong and Ayres 2013; *Carpobrotus edulix* x *C.*

chilensis, Weber and D'Antonio 1999).

While examples of cryptic invasive taxa have been documented, little is known about

how common it is for distinct cryptic taxa to respond differently to management efforts

implemented to control their distribution and abundance. In such cases, control plans

developed for an invasive taxon may be unknowingly applied to morphologically similar but less

sensitive cryptic taxa, resulting in variable, and often unpredictable, control efficacy, wasted

resources, and continued spread of the less sensitive taxon. A general understanding of the

magnitude of impacts by cryptic invasive taxa requires that they be identified in the first place,

and then carefully distinguished when evaluating management options to determine whether

adjustments to management approaches are warranted for different cryptic taxa.

An important example of managing cryptic invasive plant taxa in the United States is

Eurasian watermilfoil *sensu lato*, which includes both pure parental lineages (*Myriophyllum*

spicatum L.; EWM) and morphologically cryptic hybrid lineages (HWM) from crosses with native

northern watermilfoil (*Myriophyllum sibiricum* Komorov; NWM). Eurasian watermilfoil has been

one of the most highly managed invasive aquatic weeds in the United States since the late

1980s (Bartodziej and Ludlow, 1998). Initial management efforts appeared to be effective at

controlling the distribution and abundance of Eurasian watermilfoil, but anecdotal accounts of

lower treatment efficacy in some populations surfaced in the late 1990s. Subsequent genetic

analysis of some "abnormal" Eurasian watermilfoil populations identified them as HWM

(Moody and Les 2002; 2007, Sturtevant et al. 2009), which led to the hypothesis that

hybridization may play an important role in the evolution of invasiveness in Eurasian

watermilfoil (Moody and Les 2002; 2007), including the possibility that hybrids were more

difficult to control than parental EWM. Indeed, laboratory studies have demonstrated that

HWM genotypes exhibit higher vegetative growth and lower impacts of the commonly used

herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) compared to pure EWM (LaRue et al. 2013;

Schulte et al. *In review*).

   While managers increasingly view EWM and HWM as distinct taxa, the two taxa are not

routinely distinguished in management decision-making and treatment evaluations. For

example, managers commonly conduct surveys to determine the locations of invasive aquatic

plants within a waterbody and the quantities of herbicide(s) required to substantially reduce

their abundance. Similarly, they use post-treatment surveys to evaluate the efficacy of

treatments. In both types of survey, plants usually are identified using morphological

characteristics, which is unreliable for morphologically cryptic taxa such as EWM and HWM

(Moody and Les 2007). As a result, EWM and HWM are rarely distinguished from each other in

practice.

This fact is significant for two reasons. First, it highlights the importance of knowing whether the two taxa respond differently to operational management actions (e.g., herbicide treatments) to an extent that would warrant different treatment strategies. Laboratory evidence suggests they do, but no field confirmation has been performed to date. And second, it highlights the importance of finding and using a reliable method for distinguishing the two taxa. Molecular genetic studies have demonstrated that HWM can be reliably and objectively distinguished from parental EWM and NWM (Moody and Les 2002, 2007; Sturtevant et al. 2009; Zuellig and Thum 2012; Grafe et al. 2015), so it is possible that integrating genetic identification methods into aquatic plant surveys would assist treatment planning and evaluation, thereby informing adaptive management approaches for Eurasian watermilfoil and improving management outcomes in the long term.

In this study, I integrated molecular genetic identification methods into traditional pre- and post-treatment watermilfoil surveys on a large lake in central Michigan undergoing treatment with the systemic auxinic herbicides 2,4-D and triclopyr (the two most commonly used systemic herbicides to control nuisance Eurasian watermilfoil). Specifically, I tested the hypothesis that pure parental EWM would decrease in both distribution and abundance significantly more than HWM during the growing season following herbicide treatment, as predicted by previous laboratory studies (LaRue et al. 2013; Schulte et al. unpublished). My study has important implications for the debate on whether morphologically cryptic EWM and HWM warrant different treatment strategies, as well as whether incorporating genetic identifications into traditional aquatic plant survey methods could improve adaptive management of nuisance watermilfoil by providing more accurate treatment evaluations.

Methods

Study Lake

The field study was conducted on Houghton Lake in Roscommon County, Michigan. Houghton Lake has a surface area of 8,112 ha, and an average depth of 2.6 m, and has been managed for watermilfoil since 2002. The first treatment in 2002 was a whole-lake fluridone treatment followed by subsequent spot treatments using a combination of 2,4-D-amine and triclopyr (Progressive AE, personal communication with Paul Hausler). Historical genetic data show that HWM was not found prior to 2012 but comprised over 50% of the samples collected in 2013 (see Table 3.1). In June of 2014, the main portion of the lake was spot treated with 2,4-D-amine and triclopyr (Figure 3.1). Northern bay was left untreated due to presence of wild rice. Treatment areas and treatment types (concentration and 2,4-D or triclopyr) were determined by Progressive AE and implemented by a third party applicator.

Table 3.1 Number of Eurasian watermilfoil (EWM) and Hybrid watermilfoil (HWM) samples collected from Houghton Lake prior the study in 2014. All samples collected were genetically identified to ensure accuracy.

Year	Number of Sites Sampled	Number of EWM	Number of HWM
2009	2	11	0
2010	3	16	0
2011	7	13	0
2012	15	8	7
2013	28	13	27

Figure 3.1 2014 auxinic herbicide treatment map and point-intercept sampling locations for Houghton Lake, Michigan. Each point is a sample point in a 996 point intercept grid.

Data collection

Vegetation data were collected prior to treatment in June and at the end of the season in September. A total of 996 points were surveyed using a point intercept grid (Mikulyuk et al. 2010) for both sampling events (Figure 3.1). At each sampling point, a rake was tossed from each side of the boat to pull up plants (Hauxwell et al. 2010, Thum et al. 2010). The total

number of points with watermilfoil present was interpreted as the areal distribution (cover).

Based on visual assessment, the amount of watermilfoil on each rake was assigned a value of a

5-tier semi-quantitative index, as in Thum et al. (2012b). The two values of the semi-

quantitative index at each grid point were averaged and interpreted as the local abundance.

Watermilfoil from both tosses were then combined and one stem tip representing each distinct

phenotype present was collected. Tissue samples were transported to the lab and genetically

identified using an Internal Transcribed Spacer (ITS) restriction fragment analysis (Moody and

Les. 2002, Thum et al. 2006, Moody and Les, 2007, Zuellig and Thum 2012, Grafé et al. 2015).

Data analysis

Distribution and abundance measures for each watermilfoil population within Houghton Lake

were analyzed to evaluate differences between the responses of HWM and pure EWM to

treatment with auxinic herbicides. NWM was excluded from all statistical analyses because of

an insufficient sample size (n=2 pre-treatment and n=0 post-treatment (Table 3.2). Within the

main lake, data for areas treated with different herbicides and for areas treated directly versus

indirectly (via transport of herbicide by water currents and mixing (Figure. 3.1) showed no

statistical differences in watermilfoil reduction and therefore were combined for statistical

analysis. Due to differences in treatment and environmental features, the untreated northern

bay data will not be presented.

Table 3.2 The number of points containing each taxon in the main portion of Houghton Lake

before and after treatment with auxinic herbicides in Houghton Lake, MI.

	EWM	HWM	NWM
Pre-Treatment	20	211	2
Post-Treatment	0	156	0

All of the statistical tests used to assess potential changes in distribution and abundance

of watermilfoil assume that observations from different grid points are independent. Therefore,

before performing any of these tests, I assessed the degree and statistical significance of spatial

autocorrelation in the data using Moran's I statistic and a permutation test for significance (Cliff

and Ord 1981). For all watermilfoil combined and for HWM separately, I found moderate and

statistically significant positive autocorrelation ($I \approx 0.3$, $p < 0.0001$). Since positive

autocorrelation would result in artificially low p values in my statistical tests for changes in

distribution and abundance, I thinned the grid points used for these tests to increase the

minimum distance between points. This step substantially reduced Moran's I for all

components of the data (total watermilfoil, HWM, EWM; pre- and post-treatment) and

eliminated statistical significance of spatial autocorrelation. This thinned dataset was used for

all analyses.

To assess the change in distribution for each taxon individually, the corresponding

distribution data were analyzed using an exact McNemar test for dependent proportions

(Hollander et al. 2014, pp. 506-508). The null hypothesis for a given taxon (EWM, HWM, or total

watermilfoil) was that the proportion of grid cells changing from presence to absence following

treatment was the same as the proportion changing from absence to presence; the alternative

hypothesis was that the proportion of grid cells changing from presence to absence was higher.

Differences between pure EWM and HWM in the degree to which their areal

distributions changed following treatment were assessed using Zelen's exact test for a common

odds ratio (Hollander et al. 2014, p. 527-530). The odds for population i are $p_i/(1 - p_i)$, where p_i

is the success probability defined as the proportion of grid points at which population i was

present. The odds ratio for a population is the ratio of its pre-treatment and post-treatment

odds. The null hypothesis for Zelen's test was that the odds ratios are the same for pure EWM

and HWM; the alternative hypothesis was that the odds ratios differ.

Potential reduction in the abundance of each taxon following treatment was assessed

by calculating the post-treatment change in abundance at each grid point and testing the null

hypothesis that the mean change was zero against the alternative hypothesis that the mean

change was negative. I conducted these tests using a bootstrap test (with 10,000 bootstrap

samples) because of the numerous ties in these data and their evident non-normality.

Differences between EWM and HWM populations in the degree to which their

abundances changed following treatment were assessed using a large-sample test for the

difference between two success probabilities. The success probability for each taxon was

defined as the proportion of grid points at which its abundance index decreased following

treatment. The null hypothesis of no difference in success probabilities between EWM and

HWM was tested against the one-sided alternative that the success probability of EWM was

greater, using test statistic A defined by Hollander et al. (2014, p. 497).

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All statistical analyses were performed with the R language and statistical environment, version 3.1.0 (R Foundation for Statistical Computing, 2014).

Results

I was able to successfully amplify ITS and determine taxon identity for 550 pre-treatment samples (499 HWM and 51 EWM) from 383 points and 309 post treatment samples (299 HWM and 10 EWM) from 281 points. The pre-treatment survey included 368 points with HWM and 27 points with EWM while the post-treatment survey included 279 point with HWM and 2 points with EWM. Both EWM and HWM exhibited a statistically significant decrease in areal distribution following treatment (EWM: $p < 0.0001$, percent change = -100%; HWM: $p < 0.0001$, percent change = -26.2%; Figure 3.2). Zelen's test on the odds ratio confirmed that the proportions of grid points occupied by EWM and HWM in the main lake changed by different amounts following treatment ($p < 0.0001$).

The bootstrap test supported the alternative hypothesis that mean abundance decreased following treatment for both taxa in the main lake ($p < 0.0001$ for both EWM and HWM; Figure 3.3). Also, a greater proportion of points with EWM exhibited a decrease in abundance following treatment compared to HWM ($A= 3.727$; $p< 0.0001$; percent decreasing =100% for EWM and 57.8% for HWM).

Figure 3.2 The number of points (distribution) with plants present for Eurasian watermilfoil (A and B) and hybrid watermilfoil (C and D) in Houghton Lake, Michigan before (left) and after (right) treatment with auxinic herbicides. Due to differences in treatment and environmental features, the untreated northern bay data will not be presented.
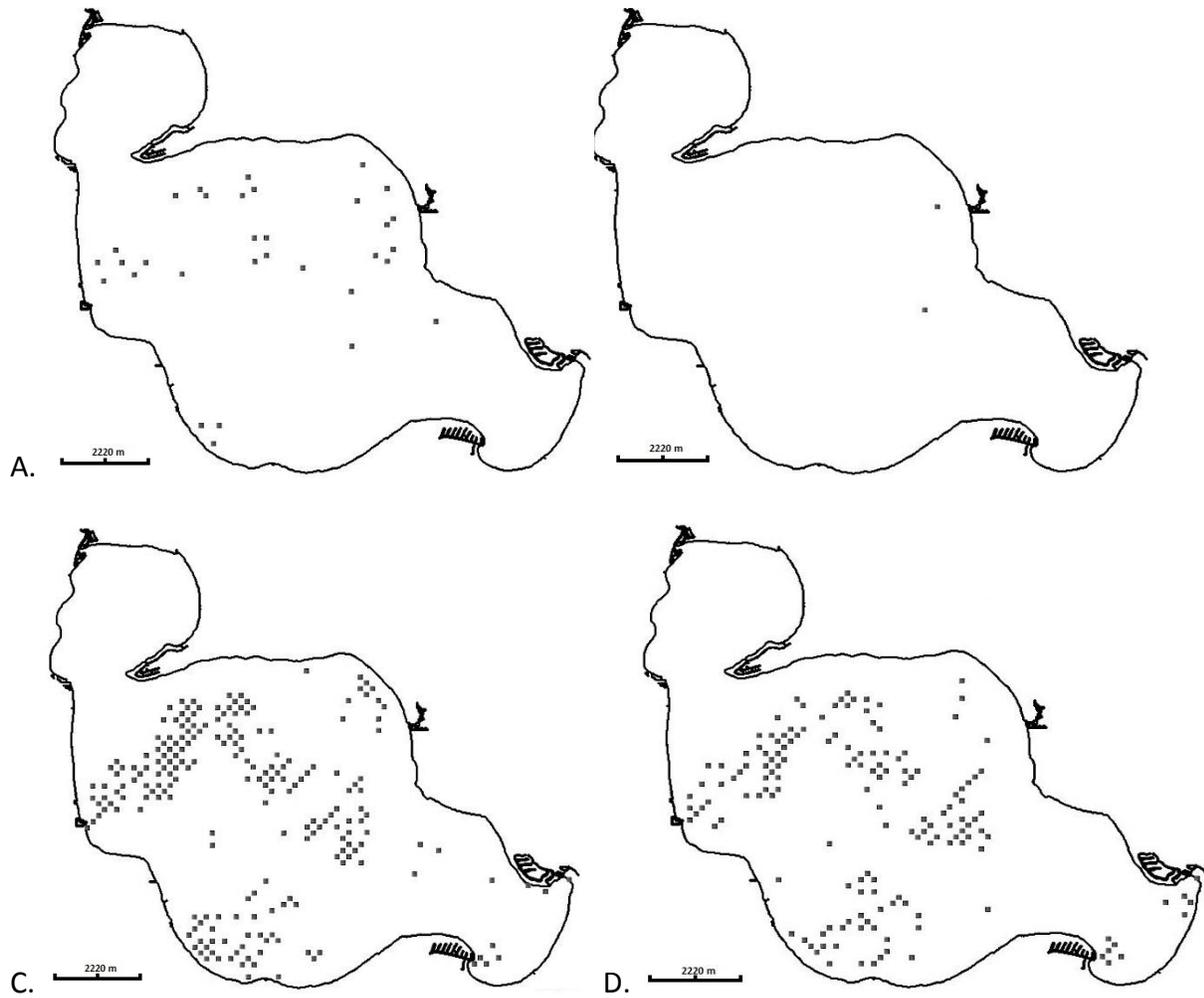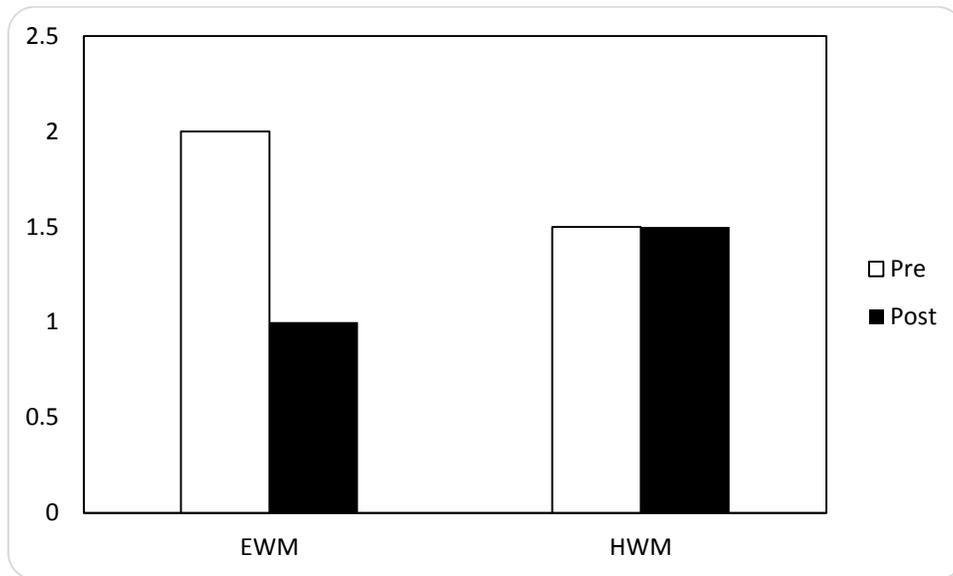
Figure 3.3 Abundance distributions for Eurasian (EWM) and hybrid (HWM) watermilfoil in the main portion of Houghton Lake before and after treatment with auxinic herbicides. Bars indicate the median abundance scores and the error bars indicate the inter-quartile range of abundance scores.

Discussion

Here, I have presented results from the first field study comparing the separate responses of EWM and HWM to treatment with auxinic herbicides. Specifically, I found that the within-season reduction in watermilfoil distribution and abundance following treatment was much greater for EWM than for HWM (Table 3.2). These field results corroborate previous laboratory studies that found comparatively higher growth of HWM than EWM in the presence of 2,4-D (LaRue et al. 2013; Schulte et al. *Unpublished data*). My results have strong implications for questions regarding whether HWM poses distinct management challenges compared to pure EWM, and I advocate integrating genetic identification methods when designing and evaluating nuisance watermilfoil management plans.

My field study explicitly demonstrates that HWM poses management challenges that are distinct from those of pure EWM in Houghton Lake. While the auxinic herbicide treatments applied in Houghton Lake provided high control of EWM (100% reduction), they did not provide comparable control of HWM (26.2% reduction). Given the general result from laboratory studies that HWM exhibits relatively higher growth than pure EWM when exposed to the auxinic herbicide 2,4-D, I predict that the Houghton Lake results will be applicable to other lakes. This study therefore provides valuable empirical support for anecdotal reports from aquatic plant managers that HWM are generally more difficult to control than pure EWM and may require new management strategies to achieve equally effective control. However, similar studies should be conducted on other lakes to determine the generality of my results.

Three common, practical questions that arise for HWM management are *if*, *when*, and *how* the watermilfoil management strategy on a given lake should change. Based on the field results reported here, results of previous laboratory studies comparing pure EWM and HWM, and the fact that these taxa are morphologically cryptic, I argue that integrating genetic identifications into routine aquatic vegetation surveys can assist the development and evaluation of aquatic plant management plans. Integrating genetic identifications into pre-treatment surveys and post-treatment evaluations would assist managers and regulators in determining whether a change in watermilfoil management strategy is warranted for a particular lake by providing objective data on the relative efficacy for pure EWM versus HWM control.

For example, my Houghton Lake results show that HWM distribution and abundance were reduced to a much lower extent compared to EWM. In the absence of genetic

identifications, however, there would have been no indication that two taxa were present and exhibited drastically different responses to management, and the factors responsible for the low reduction in watermilfoil distribution in treated portions of the lake would have been unclear. By using genetic identifications, it was clear that one critical factor affecting within season efficacy of auxinic herbicide in Houghton Lake was the preponderance of HWM compared to pure EWM (Figure 2). Based on this information, managers may decide that an alternative approach to managing HWM in Houghton Lake is warranted.

Integrating genetic identifications into aquatic plant surveys and treatment evaluations could also help determine *when* a management strategy should change, which could reduce the amount of wasted effort on ineffective treatments. The relative proportions of pure EWM and HWM may change over time, and based on the results of my study, I predict there would be a concomitant change in the overall efficacy of auxinic herbicide treatments. In fact, the present results suggest that herbicide treatments themselves may be partially responsible for changes in relative proportions of EWM and HWM in Houghton Lake. Previous genetic surveys of Houghton Lake watermilfoil were conducted annually from 2009 through 2013. While these surveys were not nearly as extensive as in 2014, the genetic analyses suggest that pure EWM was likely the dominant biotype of watermilfoil in Houghton Lake until 2012 and that HWM had become dominant by 2013 (Table 3.1), indicating a rapid shift toward increased HWM dominance since 2011. The initial discovery of hybrid presence led to increases in herbicide concentration. It is likely that incorporating more detailed genetic identifications into the aquatic plant surveys and treatment evaluations earlier would have identified the shift soon

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after hybrid establishment and thus would have provided clear and objective data identifying a year prior to 2012 as a trigger point for changing management strategies.

If/when it becomes clear that HWM requires a distinct management strategy on a given lake, the most important question then becomes what management strategy will be most effective? Increasing interest in and concern about HWM has led to proposal of numerous alternative management strategies (e.g., increased concentration, longer exposure, early versus late season timing of applications, whole lake versus spot treatment applications, herbicide mixtures, etc.). Regardless of which strategies are evaluated, accurate identification of taxa is essential and I therefore recommend integrating genetic identifications into the evaluations.

Three limitations of my study warrant further and careful investigation. First, the temporal scale of my pre- and post-treatment sampling does not allow us to distinguish the cause(s) of reduced seasonal control efficacy of HWM compared to pure EWM. It is possible that the lower reduction of HWM compared to pure EWM results from lower mortality on the standing crop of plants to which the auxinic herbicides were applied (i.e., higher survivorship of HWM). It is also possible that the lower reduction of HWM results from faster re-colonization of treated areas. For example, HWM from untreated areas may have spread more quickly to recolonize treated areas and/or plants observed in the post-treatment sampling may have colonized from the seed bank. The alternative explanations are not mutually exclusive, given that laboratory studies have demonstrated lower effects of 2,4-D on HWM, faster vegetative growth of HWM, and greater germination of HWM compared to pure EWM (LaRue et al. 2013; Grimm et al. unpublished data). Future studies should use repeated temporal sampling and methods that can distinguish among these alternatives. Second, I recognize that although HWM

on average exhibit reduced 2,4-D response compared to pure EWM (LaRue et al. 2013), there is

variation in growth and 2,4-D response among distinct hybrid genotypes (Schulte et al. 2015).

Thus, while I do not believe the results on Houghton Lake will turn out to be unusual, it is

possible that EWM and HWM would exhibit similar responses to each other on other lakes,

depending on the specific genotypes present. Third, I cannot discount impacts of environmental

factors on the reduction of each taxon. While the difference in reduction for each taxon was

consistent with what was expected based on laboratory experiments following exposure to

auxinic herbicides (LaRue et al. 2013), there may have been additional environmental variables

influencing the extent of reduction for each taxon. Future studies should collect environmental

measurements during sampling. Additionally, since it may not be feasible to find untreated

lakes with environmental factors similar enough to managed lakes to be used as controls,

future studies should also conduct sampling events over the span of multiple years of

management in order to better separate the influence of the environment and the herbicide

exposure.

Numerous plant invasions have been determined to include multiple cryptic taxa (Szigeti

et al 1996, Weber and D'Antonio 1999, Saltonstall 2002, Gaskin and Schaal 2003, Madeira et al.

2004, Blair and Hufbauer 2010, Walls 2010, Thum et al. 2011, Strong and Ayres 2013). My

results show that cryptic taxa may exhibit differential responses to common control methods.

As with watermilfoil, knowing if and where cryptic taxa occur and monitoring them separately

during management can provide valuable insights into whether different management

strategies are required and, over time, which ones are more effective. Given the morphological

similarities between cryptic taxa and the need for accurate identifications, I propose that

genetic methods should be incorporated into monitoring and management of invasive plants. Such measures will increase predictability of control outcomes and minimize the long terms costs of management.

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