GENETIC SURVEYS IN COMBINATION WITH LABORATORY STUDIES ON

GROWTH AND RESPONSE TO HERBICIDE CAN HELP DESIGN, EVALUATE,

AND OPTIMIZE EURASIAN WATERMILFOIL MANAGEMENT PLANS

by

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DEDICATION

To Paul Ropp, Stella Gyoski Ropp, Christine Ropp Guastello, Rosario Guastello, Margret Fiorilla Guastello, and Richard Guastello, whose hard work and sacrifices laid the foundation for the opportunities I've received. Thank you, Lucia J Guastello and Lorraine Ropp Melms for your unwavering support.

"Evolution came from creation." Giovanni Bignami, former President of the *Istituto Nazionale di Astrofisica.*

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ABSTRACT

Eurasian watermilfoil (*Myriophyllum spicatum*) is a top priority for aquatic plant managers in the United States. Dense mats of Eurasian watermilfoil obstruct irrigation and recreational activities, while drastically reducing property values and potentially providing a habitat for disease-carrying mosquitos. Nuisance populations are generally managed through herbicide use. Eurasian watermilfoil is capable of both sexual and clonal reproduction, creating a unique opportunity for adaptation: sexual reproduction generates genetic variation within a population, then fit genotypes can propagate via clonal reproduction. Pure Eurasian watermilfoil is genetically diverse, and additional genotypes result from frequent hybridization with native northern watermilfoil (*Myriophyllum sibiricum*). Recent studies have shown that genetic variation in Eurasian watermilfoil populations may affect herbicide efficacy in managed populations. Given the variability in herbicide response, I propose conducting site-specific studies to evaluate the response of genotypes in a waterbody to evaluate and optimize management strategies. In my thesis, I evaluated the potential to control nuisance pure and hybrid Eurasian watermilfoil with endothall in a riverine environment (Jefferson Slough, Montana). Molecular genetic surveys indicated that only one genotype of each taxon was present throughout the slough. I first compared vegetative growth and endothall response of the hybrid and Eurasian genotypes in the greenhouse, where I did not identify a difference in endothall sensitivity. Next, I evaluated the efficacy of an operational endothall treatment in Jefferson Slough. Similar to the greenhouse study results, hybrid and Eurasian watermilfoil were reduced to the same average biomass seven weeks after operational endothall treatment. Taken together, the greenhouse and operational field treatment indicate that the genotypes in Jefferson Slough were similarly susceptible to endothall. However, vegetative growth differences may have important management implications over time. In the greenhouse, Jefferson Slough hybrids grew significantly more than Eurasian watermilfoil in the absence of endothall. Additionally, there was a qualitative shift towards higher frequency of hybrids where the taxa intermixed. These results suggest a relatively higher potential for re-growth of hybrids compared to Eurasian watermilfoil following treatment. Jefferson Slough hybrids may require additional treatment to achieve sufficient long-term control. In conclusion, I discuss integration of genetic surveys into management and research priorities.

CHAPTER ONE

INTRODUCTION

General Introduction

Invasive aquatic plants can form dense monocultures that have negative

environmental, public health, and economic consequences in waterbodies worldwide.

They can alter water nutrient and light levels, alter growth of native biota, and impede

recreation and water movement (Gordon 1998). Water quality in infested waterbodies

tends to decline due to release of excess nutrients when the plants die (Madsen 2014).

Additionally, dissolved oxygen in the water may be unable to circulate under dense plant

canopies, negatively impacting fish populations and possibly causing die-offs (Dibble

2014). Public health concerns arise when overabundant stands of aquatic weeds provide

habitat for mosquitos that may carry deadly diseases such as malaria, dengue fever,

encephalitis, yellow fever, and heartworms (Cuda 2014). These impacts are estimated to

cost billions of dollars in losses annually, making invasive aquatic plant management a

high priority for water resource managers. Additionally, management costs are estimated

at over $100 million annually (Rockwell 2003; Pimentel et al 2005; Lovell et al 2006;

Richardson 2008; Getsinger et al. 2014).

Methods to control aquatic invasive plant growth include physical, mechanical,

biological, and herbicidal, but herbicides are generally considered to be the most efficient

tactics (see Gettys et al. 2014 for a review). Several factors limit which of the only 15

active ingredients currently approved for use in aquatic habitats across the United States

(see www.aquatics.org) can be utilized for an invasive aquatic plant management project. These include: 1) state and federal laws restricting which herbicides can be used, and in which ways, 2) the intended use of the waterbody (e.g., drinking water, irrigation, recreation, etc.), 3) chemical properties of the water body (e.g., the herbicide diquat has been found to be less effective in turbid water with high concentrations of dissolved solids; Poovey and Getsinger 2002), 4) hydrological factors (e.g., riverine versus lacustrine, depth, etc.), 5) biological factors (e.g., predicted effects on non-target taxa), and 6) availability of project funds (e.g., some state programs subsidize aquatic plant management projects whereas others do not). Given the limited number of herbicide options for a suite of site-specific conditions, managers frequently employ the same or similar treatment methods multiple times in a waterbody.

A major concern associated with the use of herbicides is the potential for herbicide resistance in target populations, especially if the same active ingredients are repeatedly employed in the same system. Herbicide resistance is the inherited ability of an individual plant to survive an herbicide application that would kill a susceptible or "normal" population of the same species (Jasieniuk et al. 1996). Resistance is prevalent across invasive plants in all managed systems and increasing rapidly; as of 2018, the International Survey of Herbicide-Resistant Weeds reports that 490 unique cases of resistance to 163 different herbicides have been documented in 254 species (Heap 2018). The rapidly-growing list of herbicide-resistant weeds emphasizes that recognizing and minimizing the threat of herbicide resistance is critical to long-term control of invasive plant populations, and should be a top priority for managers.

Mutations conferring resistance can occur at target site or non-target site genes. Herbicides bind to specific active sites, known as target sites, in plants to stop physiological pathways. Mutations occurring at target site genes alter the structure of the active site so that the herbicide cannot bind, thereby inhibiting its ability to stop the associated pathway. Fluridone resistance in hydrilla (*Hydrilla verticillata* (L. f.) Royle) is a prominent example of target site resistance within aquatic plant management. Hydrilla populations in Florida have developed resistance to fluridone, a commonly-used herbicide, via mutations in the phytoene desaturase gene (Michel et al. 2004). Non-target site mutations, on the other hand, confer an ability for the plant to process the herbicide through detoxification, reduced translocation, sequestration away from the target site, or metabolic changes to the molecule (Yuan et al. 2007; Délye et al. 2013). For example, ryegrass (*Lolium rigidum*) has shown resistance to acetolactate synthase (ALS)-inhibiting herbicides. Ryegrass resistance to ALS herbicides results from mutations in cytochrome P450 enzymes which enhance the plant's ability to metabolize the herbicide (Yu et al. 2009). While researchers continually identify mutations that confer herbicide resistance, the specific mutation(s) conferring resistance in any given population are often unknown. Thus, determining the best way to incorporate genetic variation and evolutionary potential into management poses a serious practical challenge.

This thesis focuses on explicitly considering genetic variation when designing and evaluating management of invasive Eurasian watermilfoil (*Myriophyllum spicatum* L.).

Background and Study System

Eurasian watermilfoil (EWM) has been a top priority for aquatic plant managers in the United States since the late 1980's (Bartodziej and Ludlow 1998). Like many invasive aquatic plants, EWM competes with native plant taxa, lowering species diversity (Madsen et al. 1991). Dense mats of EWM form at the water surface, obstructing irrigation and recreational activities, while potentially providing a habitat for mosquitos and drastically reducing property values (Smith and Barko 1990; Zhang and Boyle 2010).

Eurasian watermilfoil is capable of both sexual and clonal reproduction. Though it reproduces prolifically through clonal fragmentation, there is a surprising amount of genetic diversity within EWM as a species. Additionally, many watermilfoil genotypes arise from frequent hybridization between EWM and native northern watermilfoil (*Myriophyllum sibiricum* Komarov; NWM) (Moody and Les 2002, 2007; Sturtevant et al. 2009; Zuellig and Thum 2012; LaRue et al. 2013a, b). Individual populations are generally each made up of their own unique combination of multiple genotypes (Thum, unpublished data). Given the amount of genetic variation seen within and between EWM and HYB, it is possible that eventually genotypes arise showing reduced herbicide sensitivity. Indeed, several studies have demonstrated that EWM and HYB genotypes can have different responses to some herbicides (Glomski and Netherland 2010; Thum et al. 2012; Berger et al. 2012, 2015; LaRue et al. 2013a; Netherland and Willey 2017).

Since EWM reproduces both sexually and clonally, it has a unique opportunity for adaptation: sexual recombination generates genetic variation within a population, then fit genotypes can rapidly propagate clonally via fragmentation. Clonal reproduction also

provides a unique opportunity for management: Once genotypes are identified for study, genetic surveys in the field can track whether individuals genetically identical to the chosen genotypes increase or decrease in proportion after treatment. For instance, a manager may find two genotypes in a given population via molecular techniques, such as microsatellite markers. Upon close examination, one appears to be less susceptible to the herbicide used in that waterbody. After treatment, the identified genotype would be expected to increase in frequency, confirming that it likely is more tolerant of the herbicide. Further evaluation of genetically identical individuals' response to other herbicides could be beneficial in designing a more successful treatment.

Herbicide treatments may fail to provide sufficient control in EWM populations where managers do not investigate the response of the specific genotypes present, wasting both time and economic resources. Repeating treatments to achieve desirable control may then compound the impact of the herbicide on non-target plants and environmental toxicity. In spite of the potential severity of these consequences, genetic variation is not commonly considered when designing operational management programs for EWM.

In this thesis, I integrate genetic survey and monitoring with a small-scale, pre-treatment laboratory growth and herbicide response experiment to help predict the response to a proposed herbicide treatment in the Jefferson Slough (Cardwell, MT). The results of this study have implications for developing aquatic plant management plans, informing future aquatic plant research, and adding to the growing body of data comparing EWM and HYB response to herbicides.

CHAPTER TWO

MESOCOSM EVALUATION OF EURASIAN AND HYBRID WATERMILFOIL

RESPONSE TO ENDOTHALL IN JEFFERSON SLOUGH, MONTANA

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Led data collection, analysis, and manuscript preparation efforts.

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Contributions: Conceived study. Assisted with data collection, analysis, and manuscript preparation.

<u>Manuscript Information Page</u>

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Introduction

Aquatic plant management decisions and outcomes are influenced by a wide range of variables, such as site- specific hydrology and water quality. In addition, genetic variation in the plant species targeted for control can play a role in management outcomes. Individuals and populations of a species may not all be genetically identical, and different genotypes may exhibit variation in management-relevant traits, such as growth and herbicide sensitivity. For example, mutations in the phytoene desaturase gene (PDS) of hydrilla (*Hydrilla verticillata* (L. f.) Royle) can confer resistance to fluridone (Michel et al. 2004). Likewise, individual biotypes of fanwort (*Cabomba caroliniana* A. Gray) exhibit different responses to some herbicides (Bultemeier et al. 2009). Explicit consideration of genetic variation may therefore facilitate predictions regarding efficacy of proposed control tactics in specific waterbodies. Yet, aquatic plant management projects rarely consider genetic variation when designing and evaluating treatment plans.

Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) is one of the most widespread and frequently-managed invasive aquatic plant species across the northern tier of the U.S. Management is primarily through herbicides, and a number of local factors are considered when making herbicide decisions. For example, herbicide formulations and use patterns will be influenced by local hydrological, chemical, and biological factors. All of these can influence the impacts on target and non-target species. However, recent research has demonstrated that EWM is more genetically diverse than originally recognized. There are numerous genotypes of at least two

genetically distinct biotypes of EWM, as well as many genotypes from frequent

hybridization between both biotypes of EWM and native northern watermilfoil

(*Myriophyllum sibiricum* Komarov) (Moody and Les 2002, 2007; Sturtevant et al. 2009;

Zuellig and Thum 2012; LaRue et al. 2013a, b). Individual populations may be

composed of a single or multiple genotypes, and different populations of EWM and

hybrid watermilfoils (HYB) are often composed of different sets of genotypes (Thum,

unpublished data). Nevertheless, genetic variation is not commonly considered during

operational management programs for EWM.

One emerging concern among water resource managers is whether, and how

often, hybrid genotypes pose unique management challenges compared to wild-type

("pure") EWM. Several studies have demonstrated that EWM and HYB can have

different responses to some herbicides (Glomski and Netherland 2010; Thum et al. 2012;

Berger et al. 2012, 2015; LaRue et al. 2013a; Netherland and Willey 2017), while other

studies have found no difference in the response of EWM and HYB from the populations

studied (Poovey et al. 2007; Slade et al. 2008). Given the evident variability in growth

and response to herbicide among genotypes, managers would benefit from studies that

can help predict whether the specific genotypes present in their waterbodies will respond

differently to proposed control tactics. This information would be especially important

for waterbodies where EWM and HYB co-occur.

Jefferson Slough, near Cardwell, Montana, U.S., is one location where EWM and

HYB co-occur. Eurasian watermilfoil was first discovered in the slough in 2011, and a

genetic survey of the slough in 2014 found that the watermilfoil population was

composed of both EWM and HYB. In the upstream reaches of the slough, only EWM

was found, while HYB dominated the downstream reaches. A small segment near the

middle of the slough contained a mixture of both (Figure 1).

Initial watermilfoil control efforts in Jefferson Slough focused on hand-pulling,

but this method was deemed ineffective after three years. Due to factors such as flowing

and turbid water, a 3mg L^{-1} endothall (7-Oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid)

treatment was proposed based on laboratory concentration and exposure time studies

(Netherland et al. 1991; Skogerboe and Getsinger 2002) and demonstrated control in the

field (Parsons et al. 2004). However, in light of other studies demonstrating the potential

for differential growth and herbicide response by EWM and HYB, Jefferson Slough

managers were interested in determining whether there was any evidence for differences

in endothall response by the two different biotypes; in particular, whether hybrids would

be tolerant to the proposed endothall treatment.

In this study, our goal was to determine whether the proposed endothall treatment

in Jefferson Slough would have similar short-term efficacy (within the growing season)

on EWM and HYB. To do so, we conducted a greenhouse assay to compare vegetative

growth and response to 3mg L^{-1} endothall by EWM and HYB collected from Jefferson

Slough in 2015. Then, we performed pre- and post-treatment sampling to evaluate the

efficacy of an operational 3mg L^{-1} endothall treatment in Jefferson Slough in 2016.

Materials and Methods

Growth and Endothall Response Study

In August 2015, we collected plants throughout Jefferson Slough to establish

cultures in the greenhouse for the growth and endothall response experiment. In 2014,

we established 100 permanent sampling points spaced at approximately 70m intervals

throughout a 9.6 km portion of the slough that was known to have watermilfoil. Based

on a 2014 genetic survey of these points, we expected to find EWM in upstream reaches

(points 1 to 39) and HYB in downstream reaches (points 56 to 100), and indeed, visual

identifications suggested this was the case for the 2015 samples. Nevertheless, we

randomly sampled 23 plants from our 2015 collections at these points to confirm their

identifications using restriction enzyme banding patterns for the internal transcribed

spacer (ITS; Thum et al. 2006; Grafé et al. 2015) (see Appendices A and B for details on

data collection). Furthermore, microsatellite genotype data on these samples (data not

shown, but see Appendix C and Taylor et al. 2017 for details on the method) indicated

that only one genotype of EWM and one genotype of HYB were present in 2015,

suggesting that the slough was dominated by a single genotype each that had extensively

spread via clonal reproduction.

After confirming identities, we established cultures in the greenhouse. We mixed

all of the plants from sampling points with EWM together. Similarly, we mixed all of the

plants from sampling points with HYB together. We then randomly selected plants from

each group and planted approximately 25 plants into each of 16, 7.6-L pots per taxon.

Pots were filled with potting soil supplemented with 2.2 mg kg^{-1} of a controlled-release

fertilizer[1]. Afterwards, we randomly assigned four pots of each taxon to each of four

568-L tanks. Tanks were filled with dechlorinated tap water from Montana State

University, supplemented with a continuous supply of CO_2 and a liquid medium based on

Smart and Barko (1985). Natural light in the greenhouse was supplemented with a full-

spectrum sodium lamp[2] to create a 14:10 h light: dark cycle. These plants were allowed

to grow in the greenhouse for approximately two months to remove or minimize any

maternal or environmental effects originating from the field collections.

Two months after planting, we harvested plants from these cultures to use for our

experiment. We randomly assigned three 12 cm apical segments to each of 48 2.4-L pots

for each taxon. Pots were filled with the same soil and Osmocote [1] formulation as

described above. Three pots of each taxon then were randomly allocated to each of 16

208-L barrels that were filled with water and nutrients as described above, and light as

described above. Within each barrel, we used a mesh netting to ensure that EWM and

HYB did not intermingle in the water column. Plants were allowed to grow for three

weeks, at which point most had grown to the water surface.

Four of the 208-L barrels were randomly assigned to the endothall treatment,

while the other four remained as untreated controls. Treated plants were exposed to 3mg

L^{-1} endothall [3] for 12 hours, after which the water was completely flushed via continuous

flow for one hour. We collected water samples from each tank at the time of treatment to

confirm that the target concentrations were reached, and immediately after flushing to

confirm that the endothall was removed. Three weeks after exposure, we harvested all

living plant material (roots and shoots) for biomass measurement. We oven-dried plant

tissue at 43°C for one week, and measured for total biomass (roots and shoots) to the nearest 0.01g. We analyzed biomass data using a split plot analysis of variance (ANOVA), with tank as the main plot, and taxon as the split-plot. We also performed two a priori contrasts: one comparing EWM and HYB in the untreated controls, and one comparing EWM and HYB in the 3 mg L^{-1} endothall treatment.

Operational Endothall Treatment and Evaluation

The operational herbicide treatment was performed by a commercial applicator on 13 July 2016. A 9.65-km stretch of the slough that covered the most upstream site known to have EWM and HYB down to the confluence with the Boulder River was treated with endothall [4] at a target concentration of 3 mg L^{-1} (3 ppm) for 12 hours. It was not a requirement of the herbicide application permit to determine endothall concentrations, so water samples were not collected. However, endothall was applied using a drip system that was calibrated to achieve the target exposure based on a rhodamine WT (RWT) fluorescent dye [5] study conducted by the applicator two days prior to the endothall application. A high correlation between endothall and RWT has been previously established.

We conducted pre- and post-treatment sampling (8-9 July 2016 and 26 August 2016, respectively) at the 100 pre-determined sampling points described above. At each point, we sampled watermilfoil biomass by tossing a rake approximately one meter from one side of the boat. Any debris or non-milfoil plant species were removed on site. We collected approximately 3 to 5 cm sections of one to three apical meristems of representative plants from each rake toss to confirm identifications using ITS as

previously described. The watermilfoil samples were then oven-dried at 43°C for one

week, and biomass was measured to the nearest 0.01g. We tested for differences in

average post-treatment biomass using an unpaired t-test and used a Zelen's test to find

changes in frequency of occurrence of each taxon. Due to a mixture of EWM and HYB

in points 39, 40, 41, 50, and 55, as well as the difficulty and unreliability in separating the

biomass of these taxa at these locations, these points were excluded from the trial.

<u>Results and Discussion</u>

An ANOVA from our greenhouse study the year before treatment (2015) detected

significant main effects of endothall treatment (0 vs 3 mg L^{-1}) and taxon (EWM vs HYB)

(Figure 2; Table 1). The interaction between taxon and endothall treatment was not

significant (p = 0.10). A priori contrasts revealed a significant difference between EWM

and HYB biomass in untreated (control) tanks (p = 0.011 Figure 2), but no significant

difference between EWM and HYB biomass in tanks treated with 3mg L^{-1} endothall for

12 hours (p = 0.41 Figure 2). Similar to our greenhouse results, EWM and HYB were

reduced to similar average biomass in Jefferson Slough after operational treatment with

endothall (unpaired t_{32} = 0.95, p = 0.35; Figure 3). Furthermore, a Zelen's test for

changes in frequency of occurrence indicated that the change in the proportion of points

occupied by EWM and HYB did not significantly differ (p = 1).

Based on the greenhouse and field results, we conclude that EWM and HYB

genotypes in Jefferson Slough at the time of treatment in 2016 were similarly susceptible

to endothall under the prescribed treatment conditions (3mg L^{-1} for approximately 12

hours). The similar response to endothall by EWM and HYB present in Jefferson Slough at the time of this study reinforces that tolerance to herbicides is not a general property of all hybrid watermilfoils, but depends on the specific genotype (Netherland and Willey 2017).

Although there was no evidence for higher tolerance to endothall by Jefferson Slough HYB, we did observe vegetative growth differences that may have important management implications in the slough over time. In the greenhouse, Jefferson Slough HYB grew significantly more than EWM under controlled conditions (contrast p = 0.01 Figure 2). This result is consistent with previous studies that have identified faster vegetative growth rates of hybrid watermilfoils compared to EWM (LaRue et al. 2013a; Taylor et al. 2017; Thum and McNair 2018). In contrast, there was significantly greater biomass of EWM compared to HYB in Jefferson Slough before endothall treatment (unpaired t_{66} = 2.72, p = 0.008). However, it is important to note that EWM and HYB were located in different areas of the slough (upstream and downstream, respectively), whereas the greenhouse comparison is more appropriate because they were grown in a common environment. Where EWM and HYB were found growing intermixed in Jefferson Slough (points 39 to 55), we did observe a qualitative shift towards a higher relative frequency of HYB (Figure 1). In this section, there were 11 HYB points and 5 EWM points pre-treatment. Post-treatment, there were 12 HYB points and 2 EWM points. The number of sample points in this portion of the slough was too small to detect whether this shift was statistically significant. However, this result, along with the greenhouse results suggest a relatively higher potential for re-growth or re-establishment

of HYB compared to EWM following treatment. This could mean that Jefferson Slough

HYB may require more frequent treatment to achieve sufficient long-term control

compared to EWM. Therefore, continued monitoring and further study of regrowth

potential is warranted to determine whether changes in frequency of occurrence differ

between EWM and HYB over the long-term (see also Parks et al. 2016).

    While the specific EWM and HYB genotypes found in Jefferson Slough at the

time of this study did not show significant differences in their response to endothall, it is

important to note that, to the best of our knowledge, there was only one genotype of each

taxon present, based on microsatellite genotyping. In our case, the comparative data from

the mesocosm trials on EWM and HYB collected from the Jefferson Slough helped

provide confidence that the proposed operational endothall treatment would be similarly

effective on the two specific genotypes present. It is important to keep in mind, however,

that watermilfoil genotypes can differ in their responses to herbicides, including endothall

(Netherland and Willey 2017), 2,4-D (Glomski and Netherland 2010; LaRue et al. 2013;

Netherland and Willey 2017), triclopyr (Glomski and Netherland 2010), diquat

(Netherland and Willey 2017), and fluridone (Berger et al. 2012, 2015; Thum et al.

2012). Thus, different responses may be found among water bodies that are composed of

different genotypes, and further studies of different populations are warranted. In

addition, it is possible for the genetic composition to change over time within the same

water body such that a watermilfoil population that is currently dominated by susceptible

genotypes could become dominated by tolerant genotypes in the future. Genetic

monitoring of populations could therefore potentially be used to determine if genetic

shifts have occurred over the course of a management program, which could be used to trigger additional growth and herbicide response comparisons of any newly-identified genotypes of concern within a given waterbody. Where feasible, we recommend that aquatic plant managers quantify and monitor genetic diversity in their system, and use that information to design small-scale evaluations to predict plant response to proposed treatment tactics prior to operational treatment.

Table 1. Analysis of variance of biomass for hybrid versus Eurasian watermilfoil (Taxon) for two levels of endothall treatment (0 and 3 mg L^{-1}) in the mesocosm study.

Factor	df	SS	MS	F- Value	P-Value
Treatment	1	713.9	713.9	20.2	0.004
Residuals	6	8.5	2.8		
Taxon	1	289.0	289.0	10.2	0.018
Treatment × Taxon	1	107.5	107.5	3.8	0.099
Residuals	6	169.7	28.2		

Figure 1. Map of the study area (Jefferson Slough, near Cardwell, MT) indicating the

occurrence of Eurasian watermilfoil (EWM) (white circles) and hybrid watermilfoil

(HYB) (black circles) during the pre- (top) and post-treatment sampling (bottom) (8-9

July 2016 and 26 August 2016, respectively). Points where the taxa co-occurred are

indicated with black-and-white checkered circles. Smaller grey circles are points where

no watermilfoil was found. Numeric labels are provided every 10 points for orientation.

Figure 2. Total biomass (root and shoot, g tank^{-1}) of hybrid watermilfoil (HYB) (black

bars) and Eurasian watermilfoil (EWM) (light grey bars) three weeks after treatment with

0 and 3 mg L^{-1} endothall treatment for 12 hours in the mesocosm study. Error bars are

one standard error of the mean; n = 4. We used a priori contrasts to test for differences

between EWM and HYB under 0 and 3 mg L^{-1}. A significant difference is indicated by

an asterisk (*). NS indicates a non-significant difference.

Figure 3. Total biomass (root and shoot, g toss^{-1}) of hybrid watermilfoil (HYB) (black

bars) and Eurasian watermilfoil (EWM) (light grey bars) before and six weeks after

operational endothall treatment in Jefferson Slough. Each bar represents the mean

biomass measurement (± standard error) of all points characterized by the taxon. We

used an un-paired t-test to test for differences between EWM and HYB average biomass

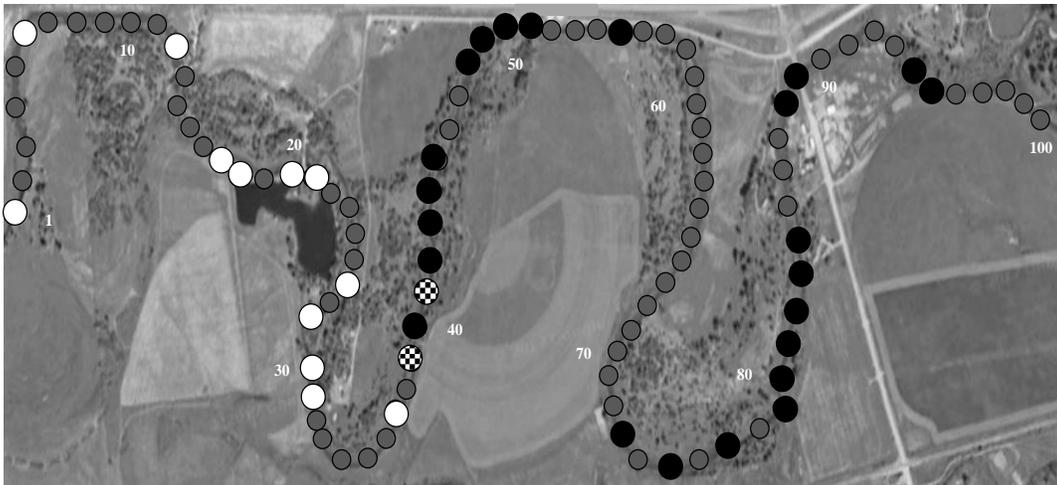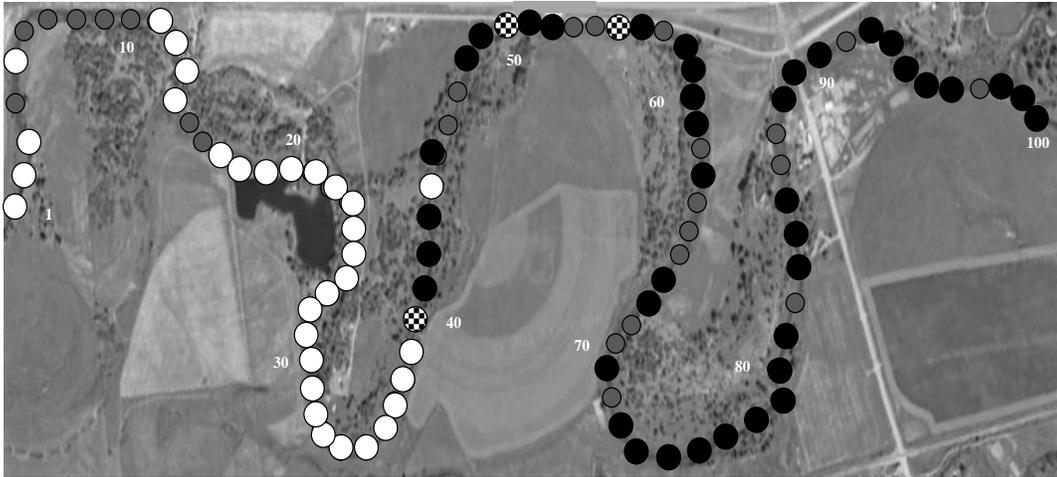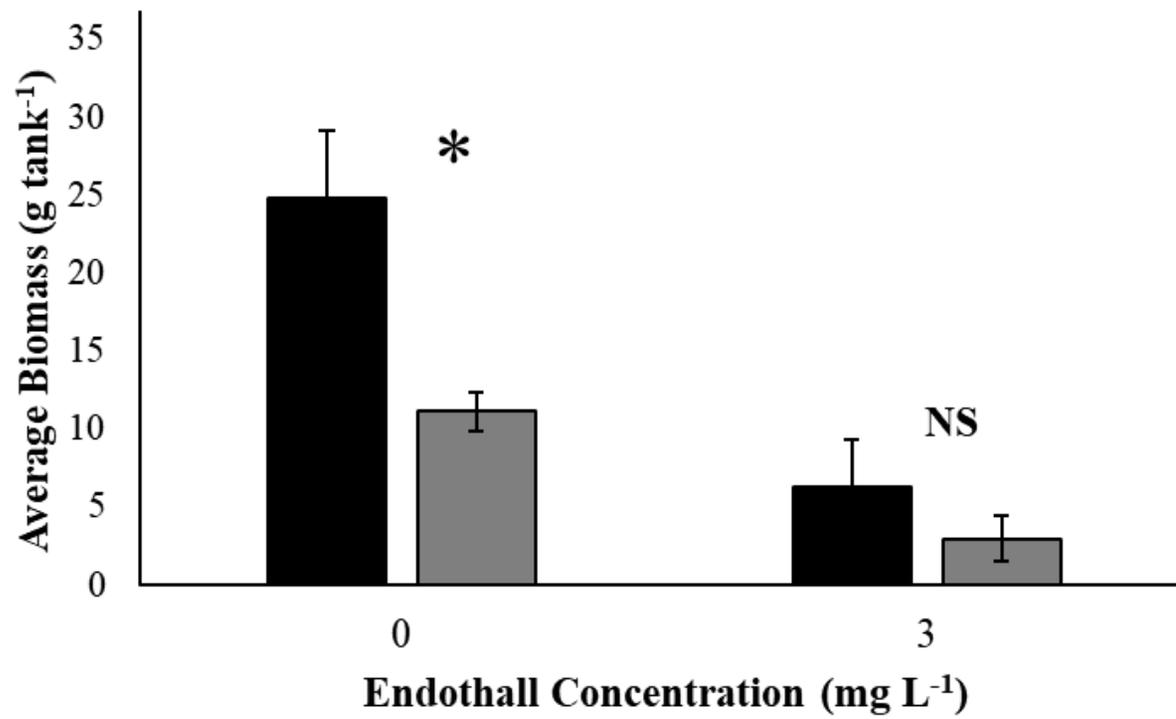post-treatment; NS indicates a non-significant difference.
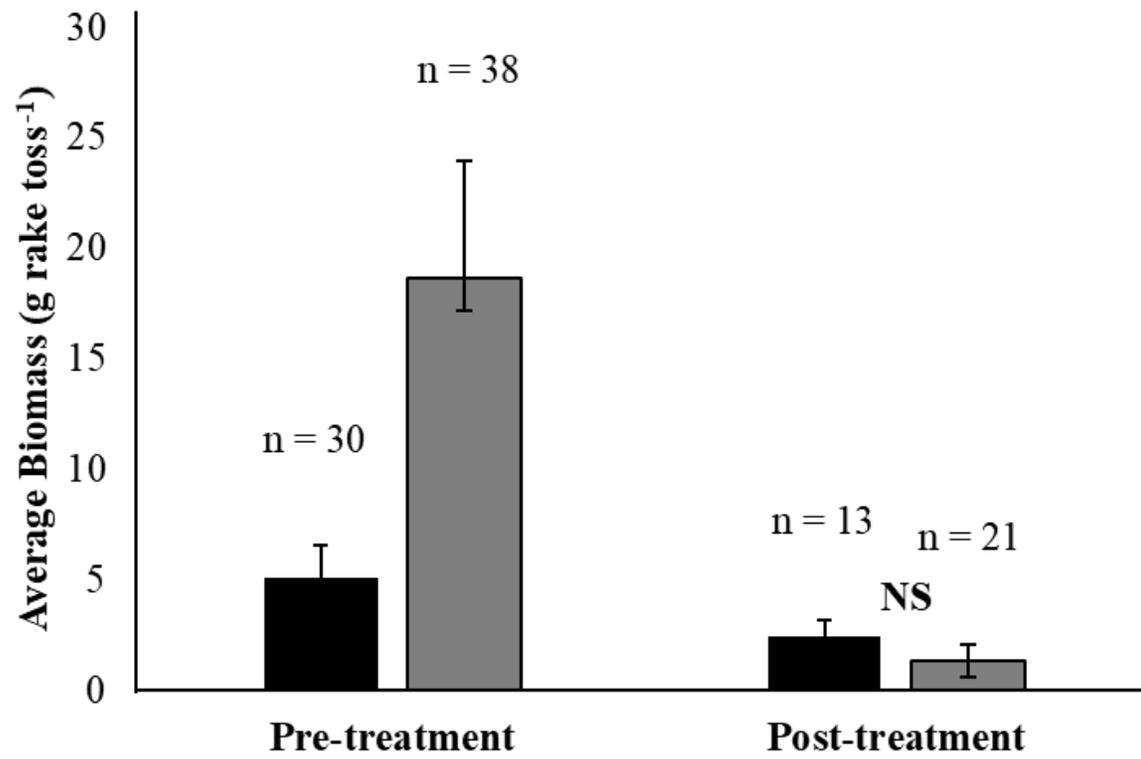
Figure 1.

Figure 2.

Figure 3.

CHAPTER THREE

CONCLUSION AND THE FUTURE OF GENETIC STUDIES IN EURASIAN

WATERMILFOIL

Integration of Genetic Variation into Eurasian Watermilfoil Management

Consideration of genetic variation in invasive Eurasian watermilfoil populations can help optimize the design and evaluation of management plans that aim to control this ecologically and economically destructive plant. Because individual EWM and HYB genotypes vary in their response to herbicide, I recommend that managers conduct small-scale surveys to quantify genetic diversity within a waterbody. Then they would use herbicide assays to infer whether the proposed treatment method will be effective in an operational treatment on those particular genotypes, and whether individuals differ in inherent growth potential. Conducting pre-treatment herbicide assays can save managers time and resources by avoiding the need for additional herbicide treatments after failed attempts at control. Additionally, adverse effects on non-target plants and other environmental concerned associated with aquatic herbicide use would be lessened by avoiding the need to repeat treatments after failure.

My findings over the course of this research project indicate that EWM managers and researchers would benefit from performing large-scale, comprehensive assays of the genotypes present in a waterbody. These assays would entail three steps: 1) Collecting samples of each EWM and HYB genotype present in the waterbody. 2) Testing the identified genotypes in a greenhouse environment, applying multiple herbicides at

various concentrations to determine which tactics provide satisfactory control. These

should also include untreated controls of those same genotypes, to determine whether

they differ in inherent growth potential. 3) Tailoring management plans based on the

results. Timing of treatment, active ingredient concentration, exposure time, and

herbicide mode of action are examples of variables managers may manipulate to optimize

control.

Waterbodies containing highly-diverse populations of EWM may pose a greater

challenge to managers. Presumably, only a limited number of plants can be evaluated at

one time due to space constraints. In these cases, individuals can be prioritized by

monitoring a treated EWM population for genetic changes. Genotypes that become more

common after treatment, or are found almost exclusively in treated areas as opposed to

untreated, may be less susceptible to treatment. These would be the most critical for

evaluation. Randomly-chosen samples from the remaining, apparently-susceptible

genotypes should be included for comparison. In cases where no obvious priorities arise

(i.e. the relative proportions of genotypes vary little after treatment), plants may be

selected randomly. Although this approach may not be ideal, managers would still be

able to gain insight into whether the genotypes present in that waterbody differ in growth

rate and susceptibility to herbicide treatment, or have even developed tolerance to a

treatment.

It is critical that managers continue to explicitly monitor genetic diversity over

time to evaluate whether populations are becoming less sensitive to herbicide, as tolerant

genotypes may arise with subsequent treatments. Given that EWM is a clonally-

reproducing species, suspicious genotypes identified through laboratory testing can be
monitored in the field over time.

<u>Knowledge Gaps and Future Research Priorities</u>

We still do not know when, where, or why specific
<u>EWM genotypes exhibit reduced response to herbicide.</u>

Ultimately, methods to rapidly assess the herbicide susceptibility of a population
would be of great benefit to managers and researchers. For instance, a genetic assay has
been developed to identify fluridone-resistant *Hydrilla verticillata*, marked by mutations
in the phytoene desaturase gene (Benoit and Les 2013).

How common is herbicide tolerance, and is it more
<u>likely to develop in certain treatment scenarios?</u>

Large-scale genetic surveys, herbicide assays, and operational treatment
evaluations of EWM populations nationwide would inform managers and researchers
how commonly tolerance develops. Those surveys should also include details about the
treatment to infer patterns associated with decreased herbicide efficacy. For instance, the
mode of action of an herbicide may play a role in whether, and how quickly, a population
develops tolerance. Contact herbicides destroy only the portions of the plant that come
into direct contact with the chemical, whereas systemic herbicides affect the entire plant
via translocation through the leaves to the meristems and roots. Multiple studies have
compared the response of genotypes to systemic herbicides, (Glomski and Netherland
2010; Thum et al. 2012; Berger et al. 2012, 2015; LaRue et al. 2013; Netherland and
Willey 2017). Aside from my study, however, only one has included contact herbicides

(diquat and endothall; Netherland and Willey 2017). In their 2017 study, Netherland and Willey found that three of the four genotypes treated for 24h with diquat were 97-100% controlled at least four weeks after treatment. The fourth genotype, from a population shown to be tolerant to three other herbicides (Townline Lake; Berger et al. 2012, 2015), far outperformed the other three. Is the fourth genotype an anomaly in its reduced sensitivity to a contact herbicide? In contrast, the same paper found far more variation in the control by endothall four weeks after treatment. Endothall-treated plants ranged from 44-121% of the untreated reference plants, meaning that some actually fared better in the presence of endothall. More studies examining EWM genotypes response to contact herbicides would establish whether one mode of action provides more widespread control over multiple genotypes, and may help inform management strategies and future herbicide production.

Herbicide use patterns may also impact the likelihood of EWM populations developing tolerance. Treatment may be applied to an entire population ("whole-lake treatment"), or only in the most critical areas, such as boat docks ("spot treatment") (Gettys et al. 2014). Is one method of application associated with more herbicide resistance? Relationships between herbicide concentration and exposure time vary as well. Herbicides may be applied at low rates for a longer period of time (for instance, a 72-hour application of endothall at 0.5mg L^{-1}), or at higher rates for a shorter period of time (a 6-hour application of endothall at 5mg L^{-1}). Efficacy differs among combinations of concentration and exposure time (Green and Westerdahl 1990; Netherland et al. 1991; Netherland and Getsinger 1992). However, there are no studies, to my knowledge,

assessing whether low concentration/long exposure or high concentration/short exposure is correlated with more cases of resistance.

What do we consider "reduced efficacy" in EWM?

Data from the herbicide assays and genetic surveys would help us determine the biological mechanisms behind populations showing "reduced efficacy". Herbicide tolerance and resistance refer to plants' inherent ability to survive and grow in the presence of herbicide, while regrowth ability is defined by their ability to grow back after the herbicide has dissipated. The results may look similar, but close observation of growth in the greenhouse can help infer which phenomenon has caused the observed reduction in treatment success.

Some parameters of herbicide treatment evaluation lack clarity and uniformity, possibly hindering large-scale comparisons among genotypes and waterbodies. For instance, over what length of time should we monitor a population to determine "efficacy"? In some waterbodies, the EWM population may appear well-controlled within the season of treatment, but grow back to pre-treatment abundance after one year (Guastello, unpublished data). Information on genotype distribution and abundance in the field can help answer the important question of whether short-term efficacy translates into longer-term control of the population. Finally, a clear, numerical definition of "reduced efficacy" should be established so populations can be more easily compared and classified in large studies. One possibility would be a threshold of percent control (based on the reduction of total biomass after treatment in the waterbody) to consider a population "susceptible" to an herbicide.

Conclusions

Continuing research on the genetics underlying variation in EWM herbicide response will be of economic and ecological benefit. Over $100 million is spent annually on controlling nuisance aquatic plant populations, so it is crucial that researchers and managers aim to ensure that the money spent on treatment is not wasted on failed treatments. Additionally, by determining the optimum herbicide concentration before treatment, managers can avoid excessive herbicide use and resulting toxicity concerns and effects on non-target plants. Simultaneously, they can more effectively mitigate the negative ecological impacts of nuisance watermilfoil. Beyond these immediate benefits, compilation of many growth and herbicide response studies will inform researchers and managers of the mechanisms behind reduced efficacy in some EWM genotypes. Resulting data may eventually be used to develop rapid assays to find tolerant individuals. Therefore, I suggest that managers and researchers alike begin genotypic evaluation and monitoring of EWM populations wherever possible to improve management strategies now and provide the foundation for future developments.

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APPENDICES

APPENDIX A

DNA EXTRACTION

For chapter 2, one vegetative meristem from each plant specimen was washed to remove debris, then stored at -80 C. Total genomic DNA was extracted from the meristem using DNEasy Plant Mini Kits (Qiagen). Extracted DNA was stored at -20 C.

APPENDIX B

INTERNAL TRANSCRIBED SPACER IDENTIFICATION

I collected species identification data from the internal transcribed spacer (ITS) in two steps (amplification and restriction), following the protocol outlined in Grafe et al. (2015). I amplified ITS regions 1 and 2, as well as the 5.8S ribosomal DNA subunit between them via polymerase chain reaction (PCR) with the forward primer ITS5 (5=-GGAAGTAAAAGTCGTAACAAGG-3=), and reverse primer ITS4 (5=-TCCTCCGCTTATTGATATGC-3=; White et al. 1990). Polymerase Chain Reaction recipes contained 1μl of template genomic DNA, 12.3μL distilled, sterile, deionized H2O, 2.5μl of 2mM dNTPs (Thermo Scientific), 1μl of each 5mM primer (Integrated DNA Technologies), 2μl of 25mM MgCl$_2$ (Promega), 5μl of 5x Colorless Flexi Reaction Buffer (Promega), and 0.2μl of GoTaq Hot Start Polymerase (Promega). Thermal cycling for DNA amplification was carried out as the following: 3 min initial denaturation at 94°C; 34 cycles of 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C; and a final extension at 72°C for 5 min.

I then digested 5μl of the amplified ITS DNA with Nhe1 and Fsp1 restriction enzymes. Restriction digest reactions contained 2.0 μL of 5x Colorless Flexi Reaction Buffer (Promega), 0.2 μL of 1 mg/ml BSA (New England BioLabs), 0.2 μL FspI (New England BioLabs), 0.1 μL of NheI (New England BioLabs), template, and 12.5 μl distilled, sterile, deionized water. The restriction digest reactions were incubated at 37 °C for one hour, 65 °C for 20 min, and then held at 12°C. I used a 2% agarose gel to visualize the restriction fragment banding patterns and assign taxonomic identifications to the samples (see figure below).

EWM **NWM** **HYB**
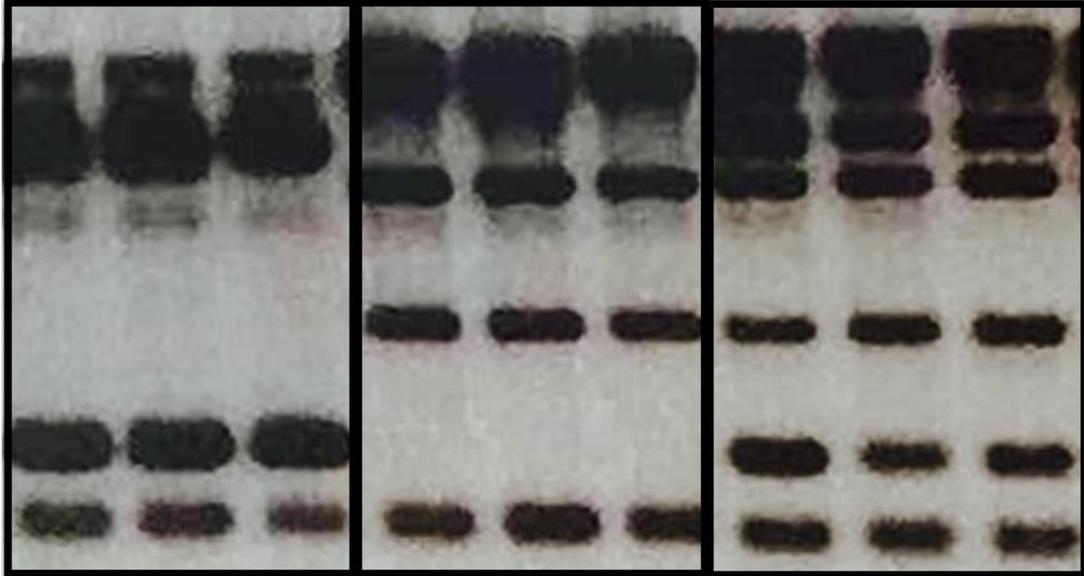
APPENDIX C

MICROSATELLITE GENOTYPING

I collected genotype data from seven microsatellite markers (Myrsp1, Myrsp5, Myrsp9, Myrsp12, Myrsp13, Myrsp15, and Myrsp16 from Wu et al. 2013). The primer pairs were labeled with fluorescent dyes 6-FAM or VIC (Applied Biosystems). Polymerase chain reaction amplifications were performed in 25μL total volume containing 80ng of genomic DNA, 11.3μl and distilled, sterile, deionized water, 2.5μl of 2mM dNTPs (Thermo Scientific), 1μl of each 5mM primer (Integrated DNA Technologies), 2□l of 25mM MgCl$_2$ (Promega), 5μl of 5x Colorless Flexi Reaction Buffer (Promega), and 0.2μl of GoTaq Hot Start Polymerase (Promega).

Thermal cycling for DNA amplification was carried out as the following: 5 min initial denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s at 52–60°C (depending on the locus; see Wu et al. 2013 for details), and 1 min at 72°C; and a final extension at 72°C for 10 min, (Wu et al. 2013). Raw microsatellite data was provided via fragment analysis on the AB 3730xl DNA Analyzer at the University of Illinois Urbana-Champaign Core Sequencing Facility using the internal size standard LIZ500. I performed genotype scoring using GeneMapper version 4.0 software (Applied Biosystems), and clone assignment was performed using the POLYSAT package for R (Clark & Jasieniuk 2011). I defined clones as individuals that have the same alleles at all markers. Presumably, those individuals sharing the same genotype at each of the seven markers were created through clonal reproduction.