HYBRIDIZATION AND INVASIVENESS IN EURASIAN WATERMILFOIL (*MYRIOPHYLLUM SPICATUM*): IS PRIORITIZING HYBRIDS IN MANAGEMENT JUSTIFIED?

by

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DEDICATION

To Mom and Dad Hoff.

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ABSTRACT

Hybridization can play an important role in the evolution of invasiveness. Eurasian watermilfoil (*Myriophyllum spicatum* L.) is a widespread aquatic invasive plant species that hybridizes with native northern watermilfoil (*Myriophyllum sibiricum* Kom.). Previous studies have found mixed evidence for whether hybrid watermilfoil (*Myriophyllum spicatum* × *sibiricum*) and pure *M. spicatum* differ in vegetative growth rate and herbicide response. While several studies have emphasized variation in these traits among *M. spicatum* × *sibiricum* genotypes, variation within *M. spicatum* has not been considered. Therefore, it is unclear how important genetic variation within *M. spicatum,* versus between *M. spicatum* and *M. spicatum* × *sibiricum,* is in influencing invasive traits and management outcomes. If *M. spicatum* × *sibiricum* genotypes are always more invasive than *M. spicatum* genotypes, simply distinguishing taxa may be sufficient for identifying lake management priorities; however, if significant phenotypic overlap is observed between taxa, distinguishing individual genotypes may be important for tailoring management strategies. We performed replicated trials of a vegetative growth and 2,4-D assay to measure clonal variation in growth rate and herbicide response of *M. spicatum* and *M. spicatum* × *sibiricum*. Our results indicate that *M. spicatum* × *sibiricum* exhibits higher average vegetative growth than *M. spicatum*, whether or not it is treated with 2,4-D. We did not observe interactions between taxon and treatment or between genotype and treatment. Despite differences between *M. spicatum* and *M. spicatum* × *sibiricum* in average vegetative growth, there was substantial overlap between taxa. For example, we found that the fastest-growing genotype of pure *M. spicatum* did not differ significantly in average growth from the fastest-growing *M. spicatum* × *sibiricum* genotype. The potential for overlap between these invasive *Myriophyllum* taxa suggests that distinguishing and characterizing genotypes may be more informative for management than simply distinguishing between *M. spicatum* and *M. spicatum* × *sibiricum*.

CHAPTER ONE

INTRODUCTION

Hybridization and Invasiveness

Invasive species are increasing globally and have become an area of special concern in the field of ecology due to their ability to negatively alter the environments that they invade. Invasive species commonly cause harm to humans, the environment, and the economy, and cost the United States an estimated $120 billion annually in prevention and management (Pimentel et al. 2005). As a result, research has increasingly turned to understanding what allows a species to successfully invade and spread in new environments. Although it is context-dependent, invasion success is generally attributed to both the invasibility of recipient ecosystems and the invasiveness of the invading species (Richardson & Pyšek 2006).

The invasiveness of a species refers to its ability to reproduce, spread from its place of introduction, and establish in new locations. Invasiveness reflects how much an invasive species affects its recipient ecosystem, and thus dictates the level of prioritization in prevention and management that is needed in response (Blackburn et al. 2014). As a relatively small percentage of invading species become successful in their new environments (Williamson and Fitter 1996), it stands to reason that species differ in their levels of invasiveness. However, what determines the invasiveness of alien organisms remains among the most interesting and urgent questions in ecology (Van Kleunen et al. 2010).

Genetic variation may facilitate adaptation to the environmental pressures of the novel habitat, and thus impact invasion success (Baker 1965; Lee 2002; Mooney and Cleland 2001).

High levels of genetic variation, which is often linked to phenotypic variation, have been documented invasive species populations (see Ward et al. 2008). Intraspecific phenotypic variation can be as extreme as interspecific variation (Albert et al. 2010); therefore, intraspecific variation may influence invasiveness as much as interspecific variation (Des Roches et al. 2018; Wellband et al. 2017). However, the role of genetic variation is rarely considered in invasive species management.

Hybridization is a special case of genetic variation that is often associated with the evolution of invasiveness in cases where species become successful after a lag time or multiple introductions (Barrett and Husband 1990; Ewel et al. 1999; Kowarik 1995; Mack 1985). A hybrid taxon may exhibit increased vigor compared to its parental taxa due to high levels of heterozygosity, leading to concerns about increased invasiveness in hybrid invaders (Stebbins 1985). Further, the generation of novel hybrid genotypes increases the genetic variation of an invasive population, and one of these hybrid genotypes may harbor new combinations of genes that could make it more fit than any parental genotypes (Ellstrand and Schierenbeck 2000). Concerns about increased invasiveness and genetic diversity in hybrid invaders have led to their increased prioritization in invasive species research and management, in contrast to their parental taxa (Ellstrand and Schierenbeck 2000, Rieseberg et al. 2007, Blair and Hufbauer 2010).

While hybrid invaders are consistently prioritized, hybridization is not necessary to invasion success, and some studies suggest that the relationship between hybridization and invasiveness is relatively weak. One analysis of 256 vascular plant families found that vascular plants with higher propensity for hybridization were not more likely to produce naturalized, weedy, or invasive species than families less prone to hybridization (Whitney et al. 2009).

Darwin's observation that non-native genera are more likely to be invasive than their native counterparts supports the idea that invaders are pre-adapted, rather than evolving invasiveness following introduction (Darwin 1859). More recent studies have also suggested that invasiveness appears once an organism is released from the biological enemies of its native environment and can be reversed when a natural enemy is reintroduced (Ellstrand and Schierenbeck 2000). For example, studies have shown that the invasiveness of prickly pear (*Opuntia*) species can be reversed by a biological control agent (Dodd 1959). Some studies have also documented comparable levels of invasiveness between hybrids and their invasive parents (e.g. Tavalire 2012). Thus, while hybridization often leads to increased concern in invasive species management, its broad influence on invasiveness remains unclear.

If hybrids are consistently more invasive than their invasive parents, continued prioritization of hybrid invaders in research and management is warranted. However, if hybrid invaders and their parental invasive taxa have similar levels of invasiveness, they may warrant equal prioritization in invasive species management. The extent to which hybridization influences invasiveness, and thus whether hybrid invaders should continue to be prioritized, remains in question.

Eurasian Watermilfoil

Eurasian watermilfoil (*Myriophyllum spicatum*, L.) is an aquatic invasive plant species, and one of the most widespread and heavily managed aquatic weeds in the United States (Bartodziej and Ludlow 1998; Cofrancesco 1993). *Myriophyllum spicatum* (*sensu lato*) spreads locally through the movement of stem fragments within water bodies, and regionally through

human transport of stem fragments (Kimbel 1982; Madsen et al. 1988). *M. spicatum* is considered particularly invasive due to its fast vegetative growth rates (LaRue et al. 2013), which provide an advantage in competition with surrounding native plant species and allow *M. spicatum* to quickly establish new populations upon introduction. If left unchecked, *M. spicatum* forms dense canopies on the surface of waterbodies that it invades, effectively impeding human recreation and leading to the eutrophication of lakes (Smith and Barko 1990). As a result, *M. spicatum* is heavily managed with aquatic herbicides and its ability to withstand herbicide treatment is tied to the invasiveness of *M. spicatuam* as well.

Pure *M. spicatum* hybridizes with native northern watermilfoil (*Myriophyllum sibiricum* Kom.). Hybrid watermilfoil (*Myriophyllum spicatum* × *sibiricum*) was identified as a cryptic invader long after *M. spicatum* invasion was documented in North America due to morphological similarity with the parental taxa (Moody and Les 2002; Moody and Les 2007). As *M. spicatum* and *M. spicatum* × *sibiricum* are visually similar, genetic identification is commonly used in distinguishing between taxa (Thum et al. 2020; Wu et al. 2013). Due to concerns about hybridization in invasive species, increased attention has been paid to *M. spicatum* × *sibiricum* in invasive species research and management (e.g. LaRue et al. 2013; Taylor et al. 2017). Therefore, taxon-level genetic identification is commonly used to identify populations of *M. spicatum* × *sibiricum* for prioritization.

Myriophyllum taxa often reproduce clonally, and molecular genetic studies have identified distinct genotypes within both *M. spicatum* and *M. spicatum* × *sibiricum* (Pashnick and Thum 2020; Thum et al. 2020; Zuellig and Thum 2012) that may exhibit distinct sets of traits (Chorak and Thum 2020; Taylor et al. 2017). Due to historic prioritization of *M. spicatum*

× *sibiricum*, numerous studies have emphasized variation between *M. spicatum* × *sibiricum* genotypes in vegetative growth and herbicide response. In contrast, although numerous genotypes of pure *M. spicatum* have been identified (Thum et al. 2020; Zuellig and Thum 2012), comparatively little attention has been paid to whether there is significant and meaningful variation in invasive traits (e.g., vegetative growth) and herbicide response in *M. spicatum* (but see Chorak and Thum 2020; Thum and McNair 2018).

The goal of this thesis is to determine whether *M. spicatum* × *sibiricum* is more invasive than pure *M. spicatum* by comparing clonal variation in *M. spicatum* and *M. spicatum* × *sibiricum.* If *M. spicatum* is comparatively invasive to *M. spicatum* × *sibiricum*, prioritizing *M. spicatum* × *sibiricum* hybrids in lake management may be unwarranted. Further, while extensive study has focused on the genetic diversity of hybrid watermilfoil (e.g. (Thum et al. 2020; Zuellig and Thum 2012), comparatively little attention has been paid to whether there is significant and meaningful variation in invasive traits (e.g., vegetative growth) and herbicide response in *M. spicatum* (but see Chorak and Thum 2020; Thum and McNair 2018). Characterizing the range of invasive traits at the genotype level, rather than the taxon level, provides insight into the most appropriate management unit for controlling invasive *Myriophyllum.*

CHAPTER TWO

HYBRIDIZATION AND INVASIVENESS IN EURASIAN WATERMILFOIL (*MYRIOPHYLLUM SPICATUM*): IS PRIORITIZING HYBRIDS IN MANAGEMENT JUSTIFIED?

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Conceived the study, performed the analyses, interpreted results, and wrote the manuscript

Co-Author: Ryan A. Thum

Contributions: Conceived the study, discussed results and implications, and edited drafts

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Abstract

Hybridization can play an important role in the evolution of invasiveness. Eurasian watermilfoil (*Myriophyllum spicatum* L.) is a widespread aquatic invasive plant species that hybridizes with native northern watermilfoil (*Myriophyllum sibiricum* Kom.). Previous studies have found mixed evidence for whether hybrid watermilfoil (*Myriophyllum spicatum* × *sibiricum*) and pure *M. spicatum* differ in vegetative growth rate and herbicide response. While several studies have emphasized variation in these traits among *M. spicatum* × *sibiricum* genotypes, variation within *M. spicatum* has not been considered. Therefore, it is unclear how important genetic variation within *M. spicatum,* versus between *M. spicatum* and *M. spicatum* × *sibiricum,* is in influencing invasive traits and management outcomes. If *M. spicatum* × *sibiricum* genotypes are always more invasive than *M. spicatum* genotypes, simply distinguishing taxa may be sufficient for identifying lake management priorities; however, if significant phenotypic overlap is observed between taxa, distinguishing individual genotypes may be important for tailoring management strategies. We performed replicated trials of a vegetative growth and 2,4-D assay to measure clonal variation in growth rate and herbicide response of *M. spicatum* and *M. spicatum* × *sibiricum*. Our results indicate that *M. spicatum* × *sibiricum* exhibits higher average vegetative growth than *M. spicatum*, whether or not it is treated with 2,4-D. We did not observe interactions between taxon and treatment or between genotype and treatment. Despite differences between *M. spicatum* and *M. spicatum* × *sibiricum* in average vegetative growth, there was substantial overlap between taxa. For example, we found that the fastest-growing genotype of pure *M. spicatum* did not differ significantly in average growth from the fastest-growing *M. spicatum* × *sibiricum* genotype. The potential for overlap between these invasive *Myriophyllum* taxa

suggests that distinguishing and characterizing genotypes may be more informative for management than simply distinguishing between *M. spicatum* and *M. spicatum* × *sibiricum*.

Key words: genetic variation, vegetative growth, herbicide response, 2,4-D, *Myriophyllum sibiricum*

Management Implications

Myriophyllum spicatum × *sibiricum* (hybrid watermilfoil) has been associated with faster growth rates and increased tolerance to herbicides compared to pure invasive *M. spicatum* (Eurasian watermilfoil) and has thus become prioritized for management. Because *M. spicatum* and *M. spicatum* × *sibiricum* are morphologically similar, taxon-level genetic identification has commonly been used to identify priority populations of *M. spicatum* × *sibiricum*.

Our results suggest that *M. spicatum* × *sibiricum* exhibits higher average vegetative growth than pure *M. spicatum* both when treated with 2,4-D and when left untreated. Thus, continued prioritization of *M. spicatum* × *sibiricum* in management may be warranted. However, 2,4-D response did not differ between taxa or between genotypes. Rather, relatively fast-growing genotypes in control tanks tended to be relatively fast growers in treatment tanks, with the same pattern occurring for slow growers. These results indicate that *M. spicatum* × *sibiricum* was not more tolerant to 2,4-D in this study, and that vegetative growth in the absence of treatment may be a strong predictor of growth in the presence of 2,4-D.

We observed substantial overlap between taxa, despite differences in average growth. For example, the fastest-growing genotype of pure *M. spicatum* did not differ in average growth from

the fastest-growing *M. spicatum* × *sibiricum* genotype in control or in treatment, with the same pattern occurring for the slowest-growing genotypes of each taxon. Thus, not all *M. spicatum* × *sibiricum* genotypes are fast growers, and some pure *M. spicatum* genotypes can be as invasive as some *M. spicatum* × *sibiricum* genotypes. The potential for overlap between taxa in management relevant traits suggests that genotype, rather than taxon, is the most appropriate management unit for *M. spicatum* and *M. spicatum* × *sibiricum.*

Introduction

Evolutionary biologists have long hypothesized that genetic variation plays an important role in invasion success by facilitating adaptation to the environmental pressures of the novel habitat (Baker 1965; Lee 2002; Mooney and Cleland 2001). High levels of genetic variation have been documented in invasive species (see Ward et al. 2008), and previous studies have demonstrated that phenotypic variation within species can be as extreme as phenotypic variation across species (Albert et al. 2010). Intraspecific trait variation may therefore influence factors associated with invasion success as much as interspecific variation (Des Roches et al. 2018; Wellband et al. 2017). However, because invasions are often studied at the species level, the role of genetic variation in invasive species management is rarely considered.

In clonal species, genetic variation is described between clonal lineages (genotypes), and trait variation between these genotypes may impact invasion success. For example, with sufficient trait variation, different genotypes may successfully persist in the presence of different environmental conditions, thus extending the ecological range of the broader taxon (terHorst and Lau 2015). In clonal invasive plants, trait variation between genotypes may also be relevant to

the ability of the broader taxon to adapt to management tools such as herbicides (Michel et al. 2004).

The range of genetic diversity present in an invasive plant species may also be impacted by hybridization events (Ellstrand and Schierenbeck 2000; Pierce et al. 2017; Zalapa et al. 2010). Plant invaders capable of sexual reproduction may hybridize with related taxa in the novel habitat, and a hybrid taxon may exhibit increased vigor compared to its parental taxa due to high levels of heterozygosity (Stebbins 1985). Further, the generation of novel hybrid genotypes increases the genetic variation of an invasive population, and one of these hybrid genotypes may represent better adaptations to certain environmental conditions than any parental genotypes (Ellstrand and Schierenbeck 2000). In species with clonal ability, better-adapted genotypes may be propagated indefinitely (Balloux et al. 2003). Due to the addition of genetic variation and their potential for increased invasiveness, invasive hybrids have become a focus of invasive species research and management (Blair and Hufbauer 2010; Ellstrand and Schierenbeck 2000; Rieseberg et al. 2007).

Eurasian watermilfoil (*Myriophyllum spicatum*, L.) is an aquatic invasive plant species, and one of the most widespread and heavily managed aquatic weeds in the United States (Bartodziej and Ludlow 1998; Cofrancesco 1993). Due to concerns about impacts on native plant communities and human recreation, *M. spicatum* is heavily managed using several systemic and contact herbicides (e.g. 2,4-D, fluridone, triclopyr, florpyrauxifen benzyl, endothall).

Myriophyllum spicatum hybridizes with northern watermilfoil (*Myriophyllum sibiricum* Kom.), which is native to North America. Hybrid watermilfoil (*Myriophyllum spicatum* × *sibiricum*) was identified as a cryptic invader long after *M. spicatum* invasion was documented in

North America due to morphological similarity with the parental taxa (Moody and Les 2002; Moody and Les 2007). Since its recognition, concerns about increased vigor in *M. spicatum* × *sibiricum* compared to pure *M. spicatum* have made *M. spicatum* × *sibiricum* a focus of genetic study (LaRue et al. 2013; Moody and Les 2007; Taylor et al. 2017). As *M. spicatum* and *M. spicatum* × *sibiricum* are visually similar, taxon-level genetic identification (Grafe et al. 2014) is commonly used to identify *M. spicatum* × *sibiricum* populations for prioritization. In addition, *Myriophyllum* taxa reproduce clonally, and molecular genetic studies have identified distinct genotypes within both *M. spicatum* and *M. spicatum* × *sibiricum* that may exhibit distinct sets of traits (Pashnick and Thum 2020; Zuellig and Thum 2012).

General concerns about invasiveness of hybrid taxa have led to concerns about increased invasiveness in *M. spicatum* × *sibiricum* compared to pure *M. spicatum.* Laboratory studies comparing average growth and herbicide response in *M. spicatum* and *M. spicatum* × *sibiricum* have documented faster growth and increased tolerance to herbicide in *M. spicatum* × *sibiricum* (LaRue et al. 2013; Taylor et al. 2017). Similarly, a field study of 2,4-D treatment impacts in 23 Wisconsin lakes documented relatively better control in lakes with pure *M. spicatum* compared to most lakes with *M. spicatum* × *sibiricum* populations (Nault et al. 2017). However, two studies comparing a single genotype of *M. spicatum* to a single genotype of *M. spicatum* × *sibiricum* concluded that the two taxa did not differ in vegetative growth rate or in response to systemic herbicides 2,4-D, triclopyr, or fluridone (Poovey et al. 2007; Slade et al. 2007). A disparity thus exists between studies examining differences in invasive traits between *M. spicatum* and *M. spicatum* × *sibiricum*. This disparity suggests that variation within *M. spicatum* and *M. spicatum* × *sibiricum* may impact comparisons of invasive traits at the taxon level.

Numerous studies have emphasized variation between *M. spicatum* × *sibiricum* genotypes in vegetative growth and herbicide response (e.g. LaRue et al. 2013; Taylor et al. 2017). In contrast, although numerous genotypes of pure *M. spicatum* have been identified (Thum et al. 2020; Zuellig and Thum 2012), comparatively little attention has been paid to whether there is statistically significant and biologically meaningful variation in invasive traits (e.g., vegetative growth) and herbicide response in *M. spicatum* (but see Chorak and Thum 2020; Thum and McNair 2018). Therefore, it is unclear how important genetic variation within *M. spicatum,* versus between pure *M. spicatum* and *M. spicatum* × *sibiricum,* is in influencing invasive traits and management outcomes. In this study, we compared clonal variation within and between *M. spicatum* and *M. spicatum* × *sibiricum* for two traits associated with their invasiveness – vegetative growth rate and response to the systemic herbicide 2,4-D.

Materials and Methods

To make comparisons between and within taxa, our assay included four different genotypes each of pure *M. spicatum* and *M. spicatum* × *sibiricum* hybrids (Table 1). The microsatellite multilocus genotypes for each taxon were determined in a previous study (Thum et al. 2020), and a culture of each genotype was started from a single meristem collected in the field and vegetatively propagated in the Montana State University Plant Growth Center (Bozeman, Montana).

The pure *M. spicatum* genotypes used in our assay (E-1–E-4) are found in multiple lakes and classifying their relative vegetative growth rates and herbicide responses may thus be broadly informative for management. Additionally, due to differences in invasive traits between

genotypes, characterizing relative trait values for genotypes that co-occur in the same lake may be informative for lake-specific management decisions. Two of the *M. spicatum* genotypes used in our assay (E-3, E-4) have been documented to co-occur with a single *M. spicatum* × *sibiricum* genotype (H-1, H-3; Table 1). We thus included these pairs (E-3, H-1; E-4, H-3) in our assay. Similarly, we included a pair of co-occurring *M. spicatum*× *sibiricum* genotypes (H-2, H-4) to make applicable comparisons between genotypes of the same taxon.

To measure both vegetative growth and 2,4-D response in *M. spicatum* and *M. spicatum* × *sibiricum*, we conducted a treatment versus control assay. We replicated the whole assay twice on all eight genotypes (hereafter referred to as Trials 1 and 2). For each trial, we vegetatively propagated cultures of each genotype to generate enough meristems for the assay. We planted 10-cm stems of each genotype into 0.53-L plastic cups filled with soil (1:1:1 topsoil/sand/peat) and capped with quartz sand. To account for tank variance, we placed three cups of each genotype in each tank. For each of the two trials, we used six 378.5-L steel stock tanks filled with approximately 300 L of Smart and Barko (1985) buffered water. After planting, we allowed plants to grow for six weeks. Both trials were conducted under greenhouse conditions with ambient and supplemental incandescent lighting to maintain a 16-hour light:8-hour dark period. Air bubblers were placed in each tank and remained throughout each trial to maintain aeration of the water. Water temperatures in tanks ranged from 18 to 22 C in both trials, and pure water was replaced weekly as it evaporated.

After the six-week establishment period, we treated three random tanks in each trial with 2,4-Dichlorophenoxyacetic acid (2,4-D; Alfa Aesar, Thermo Fisher Scientific, Lancashire, LA, UK), for a target concentration exposure time (CET) of 500 µg L^{-1} for 72 hours. This CET was

chosen to mimic field concentrations for treating invasive watermilfoil populations (Green and Westerdahl 1990). Tanks in Trial 2 were underdosed, and tank concentrations in Trial 2 thus ranged from 364-371 μg L^{-1} at one-hour post-treatment and did not reach the target CET. Tanks in Trial 1 ranged from 493 to 571 μg L^{-1}. Following the 72-hour treatment exposure period, all tanks were drained, flushed with approximately 125 L of Smart and Barko (1985) buffered water, and refilled with approximately 300 L of Smart and Barko (1985) buffered water. We harvested all above-ground plant material after six additional weeks, dried them to a constant weight, and measured their dry weight.

We tested for differences in growth between taxa and among genotypes using a linear mixed-effects model in R package lme4 (Bates et al. 2015). Taxon, genotype, and 2,4-D treatment (control versus treated), as well as the interaction terms between taxon and 2,4-D treatment and between genotype and 2,4-D treatment, were treated as fixed effects. To ensure that variation in treatment concentration between Trial 1 and Trial 2 did not impact overall results, we tested main effects and interactions using a linear mixed effect model on each trial individually. As the main effect of treatment was significant in all cases and there were no significant interactions detected, we combined trials into one dataset and included trial in the linear model as a random block effect. Treatment, taxon, and genotype (nested within taxon), as well as interactions between taxon and treatment and between genotype and treatment, were included as fixed effects in the linear model. The growth assay included three replicates of each genotype in each tank, but neither tank nor replicate were included as random effects in the model because the model fit (using the Akaike information criterion) was improved without them. Genotype was nested within taxon to test for differences in growth (dry weight) between

and within taxa. Overall data were $\log_{10}$ transformed to meet the assumptions of analysis of variance (ANOVA).

We measured treatment response for each taxon and genotype as the difference in mean dry weight between control and treatment. As differences in growth and 2,4-D response between taxa were *a priori* contrasts, we used the emmeans package in R to calculate estimated marginal means and performed contrasts between taxa for mean dry weight in both control and treatment (Lenth 2020). Finally, to assess variation among genotypes, we used a Tukey's Honestly Significant Difference test to conduct post-hoc pairwise comparisons for each genotype and treatment combination.

Results and Discussion

To test whether 2,4-D has a negative impact on invasive *Myriophyllum* biomass, we included treatment as a main effect. Despite underdosing in Trial 2, plants left untreated grew significantly better than plants that were treated with 2,4-D ($F = 59.64$, $p < 0.001$; Table 2). We thus expect that 2,4-D treatment will lead to reduced overall biomass of invasive *Myriophyllum* when used in lake management.

We tested for differences in vegetative growth between pure *M. spicatum* and *M. spicatum* × *sibiricum* hybrids by including taxon as a main effect. The main effect of taxon was marginally detectable using the full model ($F = 3.217$, $p = 0.0740$; Table 2). Contrasts supported that *M. spicatum* × *sibiricum* had significantly higher average vegetative growth than *M. spicatum* in both control ($t = -2.456$, $p = 0.0147$) and 2,4-D treatment ($t = -3.105$, $p = 0.0021$;

Figure 1). This suggests that hybridization may play a role in increased growth rate, and that *M. spicatum* × *sibiricum* may be more invasive on average than pure *M. spicatum.*

Genotypes E-4 and H-3, as well as E-3 and H-1, are two *M. spicatum*-*M. spicatum* × *sibiricum* pairs of genotypes that co-occur in the same lake (Table 1). In the E-4/H-3 pair, the *M. spicatum* × *sibiricum* genotype had higher average vegetative growth compared to the *M. spicatum* genotype in treatment (Figure 1). In the E-3/H-1 pair, the *M. spicatum* × *sibiricum* genotype had higher average vegetative growth compared to the *M. spicatum* genotype in both treatment and control (Figure 1). This is consistent with the broader pattern of higher average vegetative growth in *M. spicatum* × *sibiricum* compared to *M. spicatum*. Thus, in the absence of genotype-specific data, prioritization of *M. spicatum* × *sibiricum* genotypes in lake management may be justified.

If *M. spicatum* × *sibiricum* was more tolerant to 2,4-D treatment than *M. spicatum*, we would expect to see an interaction between taxon and treatment. However, there was no difference between taxa in the degree to which 2,4-D decreased dry weight ($F = 0.090$, $p = 0.764$; Table 2). Therefore, 2,4-D applications in lake management are not likely to differentially impact these invasive *Myriophyllum* taxa. However, as we observed higher average growth in *M. spicatum* × *sibiricum* compared to *M. spicatum* when treated with 2,4-D (Figure 1), the perception that *M. spicatum* × *sibiricum* is more difficult to control may be related to its increased vegetative growth rate in comparison to pure *M. spicatum*.

We observed variation in vegetative growth rate between genotypes ($F = 14.75$, $p < 0.001$; Table 2). For example, a pair of *M. spicatum* × *sibiricum* genotypes (H-2 and H-4) that co-occur in the same lake (Table 1) differed in average vegetative growth when treated with 2,4-

D (Figure 1). Thus, genotypes of the same taxon may differ in management-relevant traits. Additionally, while genetic study has focused on variation in *M. spicatum* × *sibiricum* (LaRue et al. 2013; Moody and Les 2007; Taylor et al. 2017), we documented substantial variation in pure *M. spicatum* (Figure 1).

If some genotypes were more resistant to 2,4-D treatment than others, we would expect to see an interaction between genotype and treatment. However, we observed no difference between genotypes in the degree to which 2,4-D treatment decreased their dry weight ($F = 1.145$, $p = 0.337$; Table 2). Rather, relatively fast growers in control tanks tended to be relatively fast growers in treatment tanks, with the same pattern occurring for slow growers (Figure 1). These results are consistent with a previous study testing the effects of 500 and 1000 µg L^{-1} 2,4-D on different genotypes of *M. spicatum* × *sibiricum* (Taylor et al. 2017), and indicate that vegetative growth in the absence of treatment may be a strong predictor of growth in the presence of 2,4-D. Therefore, while we did not detect 2,4-D resistance in *M. spicatum* or *M. spicatum* × *sibiricum* genotypes, we expect that fast-growing genotypes will have relatively higher remaining biomass following 2,4-D treatment, which may lead to greater difficulty in control.

The fastest-growing genotype of *M. spicatum* (E-1) present in this experiment is geographically widespread across its novel range and has been documented in multiple states across the United States (Thum et al. 2020). E-1 also occurs in high abundance in more localized areas; for example, a survey of 62 lakes in Minnesota from 2017-2018 found that the E-1 genotype accounted for 93 percent of *M. spicatum* infestations in Minnesota (Thum et al. 2020). As growth rate has been tied to colonization success in clonal species (Herben et al. 2014), fast growth rates may have played a role in its ability to colonize and establish in new water bodies

upon introduction. Further, as fast-growing genotypes in control tanks tended to also exhibit fast growth in treatment (Figure 1), the E-1 genotype may be able to maintain relatively high biomass in lakes treated with 2,4-D and may pose similar management concerns to genotypes of *M. spicatum* × *sibiricum.*

While we observed significant differences in vegetative growth between *M. spicatum* and *M. spicatum* × *sibiricum* in control and treatment, there was significant overlap between taxa. For example, the fastest-growing *M. spicatum* genotype (E-1) did not differ significantly in average growth from the fastest-growing *M. spicatum* × *sibiricum* genotype (H-1) in control or in treatment (Figure 1). This suggests that *M. spicatum* genotypes may be comparatively invasive to some *M. spicatum* × *sibiricum* genotypes. Further, the slowest-growing *M. spicatum* genotype (E-4) did not differ significantly from the slowest-growing *M. spicatum x sibiricum* genotype (H-4) in control or in treatment (Figure 1). Thus, not all *M. spicatum* × *sibiricum* genotypes are fast growers. This overlap suggests that management decisions purely based on the *Myriophyllum* taxa that are present in a lake may fail to account for overlap between *M. spicatum* and *M. spicatum* × *sibiricum,* and that monitoring at the genotype level may be important for tailoring lake management strategies. As *M. spicatum* and *M. spicatum* × *sibiricum* are visually similar, genetic identification is commonly used in distinguishing between taxa (Grafe et al. 2014). However, fingerprinting methods that distinguish genotypes (Thum et al. 2020) may be more informative for tailoring management strategies, and genotype is likely the most appropriate management unit for *M. spicatum* and *M. spicatum* × *sibiricum.*

Overall, we observed significant differences between *M. spicatum* and *M. spicatum* × *sibiricum* in vegetative growth in control and treatment, and no difference in how the two taxa

responded to 2,4-D. Thus, prioritization of *M. spicatum* × *sibiricum* in lake management may often be warranted if monitoring at the genotype level is not possible. However, transitioning from taxon-level identification to genotype-level identification is feasible due to the development of fingerprinting methods that distinguish genotypes (Thum et al. 2020; Wu et al. 2013). The potential for overlap between taxa in management-relevant traits suggests that continued genetic monitoring at the genotype level is the most reliable way to predict how a certain genotype, or set of co-occurring genotypes, will grow and respond to management.

Acknowledgements

No conflicts of interest have been declared. This project was supported by the Michigan Invasive Species Grant Program (www.michigan.gov/invasives). We thank Greg Chorak and Ashley Wolfe for assisting in experimental setup and take down. We also thank Dr. Matt Lavin, Dr. Jane Mangold, Dr. Jennifer Lachowiec, Dr. Jeff Pashnick, Greg Chorak, Katie Gannon, Zach Kuzniar, and Ashley Wolfe for providing friendly review of this paper.

Tables

Table 1. Names and locations of lakes where genotypes used in the vegetative growth assay were collected. E=Eurasian watermilfoil (*Myriophyllum spicatum*), H=hybrid watermilfoil (*Myriophyllum spicatum* × *sibiricum).*

Genotype	Taxon	Lake	County	State
E-1	Eurasian	Langford	Gogebic	Michigan
E-2	Eurasian	Bair	Cass	Michigan
E-3	Eurasian	Coeur d'Alene	Kootenai	Idaho
E-4	Eurasian	Spring	Ottowa/Muskegon	Michigan
H-1	Hybrid	Coeur d'Alene	Kootenai	Idaho
H-2	Hybrid	White	Oakland	Michigan
H-3	Hybrid	Spring	Ottowa/Muskegon	Michigan
H-4	Hybrid	White	Oakland	Michigan

Table 2. Analysis of Variance (Type II Satterthwaite's Method) table for the linear mixed regression model (dry weight ~ taxon*treatment*genotype(taxon) + (1|trial)) determining the effects of *Myriophyllum* taxon and genotype as well as 500 µg l^{-1} 2,4-D treatment on dry weight. Headings: SumSq = Sum of Squares, MeanSq = Mean Squared Error, DF = degrees of freedom, F = F value, p = p value.

Effect	SumSq	MeanSq	DF	F	p
Taxon	0.066	0.066	1	3.217	0.074
Treatment	1.229	1.229	1	59.640	<0.001
Genotype(Taxon)	1.824	0.304	6	14.750	<0.001
Taxon x Treatment	0.002	0.002	1	0.090	0.764
Genotype(Taxon) x Treatment	0.142	0.024	6	1.145	0.337

Figures

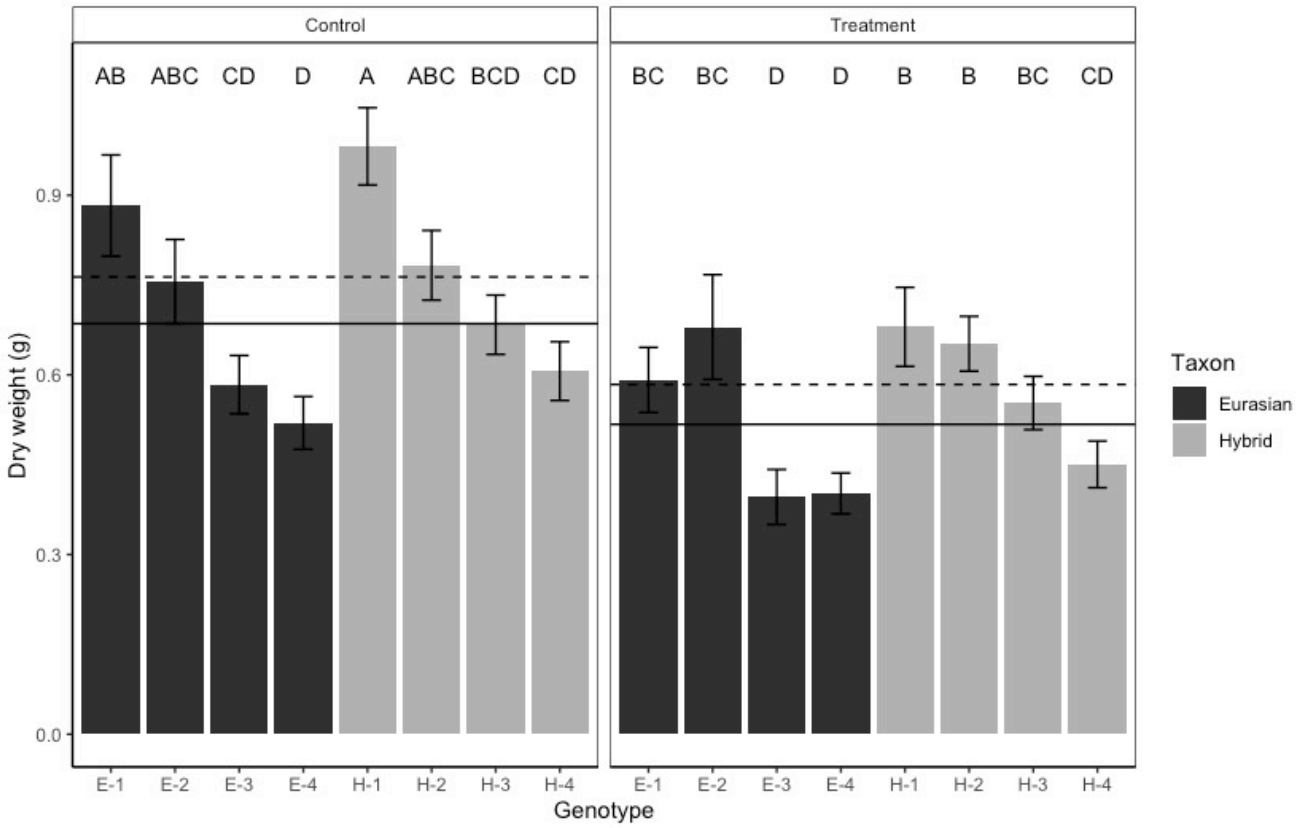

Figure 1. Average dry weight values for genotypes E-1-E-4 and H-1-H-4. The dashed line denotes grand mean for *Myriophyllum spicatum* × *sibiricum* hybrids in each treatment type, and solid line denotes grand mean for pure *Myriophyllum spicatum* in each treatment type. Error bars represent standard error of the mean. Letters represent significant differences between genotypes across both control and treatment.

CHAPTER THREE

CONCLUSION

Genetic Monitoring

Genetic monitoring refers to the use of molecular markers to i) identify individuals, genotypes, or taxa, and ii) to quantify changes in population genetic metrics (e.g. population size, genetic diversity, or population size) over time (Schwartz et al. 2007). In invasive *Myriophyllum*, genetic monitoring may be used to identify and track particularly invasive genotypes, such as those that exhibit fast growth rates or resistance to commonly-used herbicides (Schwartz et al. 2007; Stetz et al. 2011). While genetic monitoring is not commonly used in invasive species management (Kekkonen 2016), it may allow managers to tailor management strategies to the appropriate management unit of an invasive species.

In clonal species, genetic identification at the genotype level allows researchers and managers to track genotypes (clonal lineages) across the novel range. In contrast to obligate sexual species, an entire population of a primarily clonal species may be made up of a single genotype (Thum et al. 2020). Further, a single genotype can occur in high abundance locally or be geographically widespread (Thum et al. 2020). Distinguishing and characterizing genotypes, especially those that occur in multiple locations across the landscape, can thus be broadly applicable for tailoring management strategies in each population containing that genotype. Identifying particularly invasive genotypes and implementing multi-locus genotyping into

invasive species management may help to determine where these genotypes are, and how best to manage them across their invaded range.

Future Work

Genotype Characterization

The vegetative growth and 2,4-D assay outlined in Chapter 2 was done at a large mesocosm scale. Large 100-gallon stock tanks allow vertical space for plants to grow to nearly 2 feet in length before reaching the water surface, as well as horizontal space for within-tank replication, providing the necessary statistical power to detect differences between taxa and between genotypes in average vegetative growth. However, a disadvantage of the large-scale assay is the amount of time (12 total weeks) needed for plants to grow to the scale of the 100-gallon tanks. Future directions thus include testing whether vegetative growth and herbicide assays can be completed faster on smaller scales.

Performing growth and herbicide assays at smaller scales may allow us to expedite the process of characterizing genotypes of interest, if the necessary statistical power can still be reached. We are currently replicating our assay (Chapter 2) in smaller 10-gallon tanks with a total growth time of four weeks in order to compare results between the two scales. If similar results are observed in the smaller-scale assay, this design would provide a framework for increased efficiency in growth and herbicide characterization for genotypes of invasive *Myriophyllum.*

Co-occurrence

In our assay (Chapter 2), we observed variation in vegetative growth between invasive *Myriophyllum* genotypes, including pairs that co-occur in the same lake. Other studies have also documented differences between genotypes in invasive traits (see Chorak and Thum 2020; Netherland and Willey 2017; Taylor et al. 2017). In lakes containing multiple genotypes, it stands to reason different growth and herbicide responses could lead to changes in the genetic composition of invasive *Myriophyllum* populations over time (Parks et al. 2017). For example, a highly invasive *Myriophyllum* genotype (e.g. a genotype exhibiting fast growth) may increase in relative abundance over time due to its ability to quickly accumulate biomass. Further, in herbicide-managed lakes, herbicide resistant genotypes may increase in abundance following herbicide treatment. Characterizing the genetic composition, and thus the range of relative invasiveness present in a lake, may be useful in identifying priority genotypes. Finally, genetic monitoring over the course of multiple years may provide insight into whether population composition is changing over time, and whether it is changing due to treatment. An example of this is outlined in Appendix B.

Cataloging Genotypes

A shift toward genotype-level genetic monitoring may have significant implications for managing invasive *Myriophyllum*, particularly when field observation is combined with laboratory research. For example, identifying priority genotypes can occur when lake managers document difficulty in controlling a *Myriophyllum* population with herbicide. Genetic fingerprinting can be used to identify the genetic composition of that population, and previous characterization of the vegetative growth and herbicide response of genotypes present can be

used to inform management strategies. As some particularly invasive *Myriophyllum* genotypes are geographically widespread (Thum et al. 2020), genetic analysis may reveal the presence of a particularly invasive genotype that has been previously characterized for invasive traits (such as those in Chapter 2). In cases where an uncharacterized genotype is identified, performing vegetative growth and herbicide assays can provide information to managers on its relative invasiveness.

Ultimately, building a catalog of invasive *Myriophyllum* genotypes is a goal of *Myriophyllum* genetic research (Chorak and Thum 2020). As my research suggests that both pure *M. spicatum* genotypes and *M. spicatum* × *sibiricum* hybrid genotypes can be highly invasive, the inclusion of genotypes from both taxa in future growth and herbicide screens is recommended. Building this catalog may also encourage collaboration between researchers and managers, wherein problematic genotypes can be identified in both the field and laboratory, and the characterization of widespread and particularly invasive genotypes can inform management across potentially large geographic areas.

Broad Conclusions

Concerns about invasiveness in hybrid species, combined with observations that *M. spicatum* × *sibiricum* is more difficult to control, have led to the ongoing prioritization of *M. spicatum* × *sibiricum* over pure *M. spicatum* in aquatic plant management. My research suggests that *M. spicatum* × *sibiricum* exhibits faster average vegetative growth rates than *M. spicatum* and may thus be more invasive on average. However, we observed substantial variation among genotypes in vegetative growth rate, and my results suggest that some genotypes of *M. spicatum*

× *sibiricum* and *M. spicatum* are comparatively invasive. Therefore, a given genotype of *M. spicatum* × *sibiricum* is not necessarily more invasive than a given genotype of *M. spicatum*, and monitoring populations at the taxon level is not necessarily informative for tailoring management strategies.

Genetic monitoring at the taxon level has commonly been used to identify populations of *M. spicatum* × *sibiricum* hybrids for prioritization. However, genetic monitoring of *M. spicatum* and *M. spicatum* × *sibiricum* at the genotype level is still not common practice in aquatic plant management, despite the development of fingerprinting methods that distinguish genotypes (Thum et al. 2020; Wu et al. 2013). My results indicate that genotype is the most appropriate management unit for invasive *Myriophyllum*, and that integrating these fingerprinting methods into lake management plans may be an efficient way of identifying management priorities.

APPENDICES

APPENDIX A

PROTOCOL FOR LABORATORY CULTURING OF *MYRIOPHYLLUM* PLANTS

Preface

My research suggests that genotype is the most appropriate management unit for invasive *M. spicatum* and *M. spicatum* × *sibiricum*, as we observed substantial overlap in invasiveness between taxa (Chapter 2). Continued genotype-level characterization of vegetative growth rate and herbicide response in invasive *Myriophyllum* may be useful in identifying particularly invasive genotypes and in developing a more comprehensive understanding of the range of invasiveness present in both taxa.

To set up vegetative growth and herbicide assays, sufficient plant material is needed for each genotype of interest. Planting cultures of desired genotypes is a reliable way to ensure that there is enough plant material present for an experiment, and that there is sustained plant material for genotypes of continued research interest. Here, we outline the detailed methodology for starting and maintaining cultures of invasive *Myriophyllum* in a greenhouse setting.

Basic Materials

General Greenhouse Materials for Setup and Maintenance

1. 1-55-gallon plastic pickle barrel for mixing Smart and Barko (1985) buffer (see below)
2. 55-gallon plastic pickle barrels for culturing
    a. One per culture
3. 2.5-gallon plastic buckets
    a. We generally use three per culture

4. 1-L plastic paint bucket
    a. We keep a small supply (~10) around
5. Hose, with running tap water
    a. Fitted with chlorine filter attachment (We use "Rainshowr Gard'n Grow")
6. Aquarium pump (we use "YC Tech 160-1188GPH Aquarium Submersible Water Pump")
7. Bubbler units (we use VivoSun Commercial Air Pump)
    a. Flexible airline tubing cut to length for each tank
8. Lead aquarium weights (we use Awesome Aquatics plant weights)
9. Aquarium heaters (we use 300-W VivoSun Submersible Aquarium Heater)
10. Scale (grams) for measuring Smart and Barko (1985) buffer reagents (see below)
11. Plastic Ziploc bags (sandwich size)

Establishing Cultures

Materials: Establishing New Cultures

We maintain separate cultures for individual genotypes, each in a large barrel of multi-bucket plantings. The following are materials used to set up a culture from a single stem collected in the field:

A. Preparing Tanks

1. 55-gallon plastic pickle barrel for mixing SB buffer (as above)
    a. This will be reused for setting up other new cultures, and general greenhouse maintenance

2. 1-55-gallon plastic pickle barrel per genotype (culture) for planting
    a. Label with culture name (we use colored tape)
3. Reagents for Smart & Barko (1985) buffer for aquatic plant culturing
    a. $CaCl_2$
        i. Fisher Chemical: C79-3 Calcium Chloride dihydrate
    b. $MgSO_4$ (heptahydrate)
        i. Fisher Chemical Magnesium Sulfate Heptahydrate
    c. $NaHCO_3$
        i. "Arm & Hammer" pure baking soda
    d. $KHCO_3$ (crystalline)
        i. Fisher Chemical P184-500 Potassium Bicarbonate crystalline

B. <u>Preparing Planting Buckets</u>

4. 1:1:1 soil/peat/sand mix (planting substrate)
    a. We have ready access to this mix, but ~2 gallons of soil per bucket x 1 bucket per culture x number of desired cultures is needed for set-up
5. Coarse utility sand (planting substrate)
    a. We have ready access to sand, but ~3 cups of sand per bucket x 3 buckets per culture x number of desired cultures is needed for set-up
    b. For utility sand, we rinse with hose water until water runs clear
6. One-2.5-gallon plastic bucket per genotype/culture (handle removed)

Methods: Establishing New Cultures

A. Preparing Reagents:

1. Weigh correct amount of each ingredient (values are for a 50 gallon pickle barrel but ratios are listed in Smart & Barko 1985):
    a. $CaCl_2$: 17.40 g
    b. $MgSO_4$: 13.10 g
    c. $NaHCO_3$:11.10 g
    d. $KHCO_3$: 2.93 g
2. Weigh each ingredient, place in sandwich plastic bag (labeled "SB Buffer")

B. Preparing Barrels

3. Turn on hose over pickle barrel (barrel selected for mixing SB buffer), open bag of mixed SB buffer reagents, and hold bag over barrel
4. Use hose to slowly fill bag ~1/2-way with water (while holding over barrel)
5. Pour off water from the top of bag and continue this cycle of filling halfway, allowing to mix, and pouring off the top layer of water, manipulating the clumps at the bottom of the bag as needed to mix
6. Once the full bag contents have been poured out, allow hose to continue filling with water (up to ~4-5 inches below the top edge of the barrel)
7. Place aquarium pump unit in SB-mixing barrel, and hose in second barrel (barrel that will house the new culture)
8. Turn on pump, and allow to fill second barrel
    a. While barrel is filling, label second barrel with genotype name

9. Second barrel is now prepped for planting
    a. Empty first (SB mixing barrel) and rinse out before using for next culture (to prevent compounding SB reagents at bottom of barrel)

C. Preparing Substrate: Sand

10. Fill a 5 gallon bucket ~1/3 of the way with utility sand
11. Rinse sand by placing hose in bucket and stir sand while filling bucket using hose nozzle
    a. Allow to settle briefly, pour off water, and repeat until water runs nearly clear

D. Preparing Substrate: Soil

12. Fill a 2.5-gallon bucket ~2/3 of the way with soil mix (1:1:1 sand/soil/peat)
13. Fluff soil slightly (we use a large metal spoon)
14. Add a layer (~1 inch) of MSU sand (washed) over the top of soil prevent soil leaching into water column
15. Grab a paint bucket (or other small container) and fill with fresh SB water
16. Place hand ~1 inch over the top layer of sand and slowly pour the water over hand, to prevent pluming of soil layer underneath
    a. Allow water to percolate into sand and soil layer, repeating the water addition process as needed, until bubbles no longer appear and a few inches of water remains above the sand layer
17. As water is percolating, grab *Myriophyllum* stem(s) of a single genotype for planting

Methods: Planting

A. Stem Selection

1. Ideal stems have a full, healthy meristem.

   a. When starting cultures from a single stem, this may not always be attainable

2. Planting long stems (~6+ inches) is preferable, but any stem length over ~6 cm is workable when starting a culture

3. Once stems are acquired, and a bucket is fully prepared with a layer of water over soil/sand layers, grab the bottom (end opposite meristem) of one stem fragment between your pointer finger and thumb, select a location in the bucket and push ~1/2 inch-1 inch of the stem below the surface of the sand at that location

   a. Location will likely depend on how many stems are being planted

4. Stem should now be "planted" and far enough into the sediment to remain there without pushing too much of the stem below the surface of the sand

5. Repeat this process in the same bucket for any remaining stems of the same genotype (we often start a culture with only one stem to ensure that all plant material is from the same genotype)

6. Place bucket containing stem(s) of the new genotype slowly into the new barrel, tilting the bucket when placing in the water so that it slowly fills with water from the barrel

Methods: General Setup for Establishing Cultures

A. Heaters

7. Place one aquarium heater in each culture. Ensure that heater is fully submerged. We set our heaters to ~70 degrees (minimum 65, maximum 75).

B. Bubblers

8. Attach bubbler hoses to bubbler unit and anchor the end with a lead aquarium weight. We keep our bubblers at the mid-range of the bubbler speed range, somewhere between light bubbles and a rolling boil.

C. Light

9. We use ambient greenhouse lighting, and supplemental incandescent overhead lighting in the winter to maintain a 16 hour day, 8 hour night balance. We use a shade cloth across the greenhouse ceiling in the summer months to reduce direct light.

D. Ambient temperature

a. Greenhouse is kept to ~70 degrees Fahrenheit.

Maintaining Cultures

Once cultures are established, plants will need periodic transplants (taking cuttings/meristems from mature plants and propagating them) into new buckets and barrels will need periodic water (buffer) changes as part of their general maintenance. Buffer changes occur approximately once every four weeks for each culture, or as needed. Algae growth and murky water are often signs that a culture needs an immediate buffer change, but monthly routine buffer changes should often prevent these issues. Transplants often occur before meristems reach the

water surface, to prevent them from reaching the point of flowering. Transplants can also be used to address issues with plant growth; re-planting only the healthiest stems can help to "re-set" the culture. The following are methods for general greenhouse maintenance following these protocols:

Materials: Maintaining Cultures

A. Preparing Reagents
    1. Weigh each ingredient (using SB buffer quantities listed above), and place in sandwich plastic bag (labeled)
B. Buffer Changes
    2. Preparing SB water
        a. Grab 55-gallon pickle barrel, hose, and bag of SB buffer reagents
        b. Turn on hose, hold bag over barrel and use hose to fill bag ~1/2-way with water
        c. Pour off top of bag and continue this cycle of filling halfway, allowing to mix, and pouring off the top, manipulating the clumps at the bottom of the bag as needed to mix
        d. Once the full bag contents have been poured out, allow barrel to fill with water up to ~4-5 inches below the top edge of the barrel
    3. Draining and cleaning culture barrel
        a. As SB barrel is filling, grab pump and place into tank that needs a buffer change

b. Place pump unit at bottom of tank between buckets, and turn on

c. Allow to drain to slightly above bucket level, then remove buckets of plants from barrel and place on floor of greenhouse (careful to ensure that no other buckets are around that could be confused for these specific buckets), allow tank to continue draining after buckets of plants are removed

d. Grab hose, and nylon brush and scrub inside of barrel to remove algae while allowing hose to wash off the sides and create a "flushing" system in which hose water is entering the barrel as pumps are removing water

e. After tank has been scrubbed and water is running clear out of the pump, fully empty the barrel

4. Refilling culture barrel

a. Place pump unit in full barrel of fresh SB water, and place pump hose in empty plant barrel

b. Turn on pump and allow to fill to ~6-7 inches below the top edge of the barrel. Remove pump.

c. Return plant buckets carefully to culture barrel, careful to tip the bucket as it is inserted into the water to prevent a sudden influx of water into the bucket

d. Once all plant buckets have been returned to their barrel, pump can be used to top off barrel to desired water level, careful not to allow pump hose too close to buckets to prevent uprooting of plants and pluming of sediments

C. <u>Transplants</u>

1. Bucket preparation

a. Transplanting buckets use the same substrate configuration as listed above for establishing cultures
    1. 2.5-gallon buckets of 1:1:1 soil/sand/peat mix topped with utility sand should be prepared in advance of transplanting
b. Grab a small container and fill with water from the barrel that the plants are coming from (or fresh SB water)
c. Place hand ~1 inch over the top layer of sand and slowly pour the water over hand, to prevent pluming of soil layer underneath
d. Allow water to percolate into sand and soil layer, repeating the water addition process as needed, until bubbles no longer appear and a few inches of water remains above the sand layer

2. Harvesting stems
    a. As water is percolating, grab a bucket of plant material from desired culture
    b. We are targeting ~12-15 good meristems. Knowledge of what makes a “good” meristem is important (see Figure 2). Avoid meristems that are flowering, have meristem “scars,” and those that have epinasty. Often, new, healthy meristems are lower down in the culture. meristem “scars,” and those that have epinasty. Often, new, healthy meristems are lower down in the culture.

Figure 2. Examples of good meristems for planting *Myriophyllum* cultures. Meristems are feathery and full, without signs of flowering or meristem scars.

Figure 3. Examples of poor stems for planting *Myriophyllum* cultures. Left stem has epinasty and very little meristem material. Right stem has a meristem scar.

c. Begin to pick through the barrel of plant material, identifying the general status of the plants. Identify a “good” meristem, and using the thumb and pointer finger, snip off a stem fragment including that meristem as well as a section of stem below it. Stem length may depend on 1) the health of the culture (is it rotten at the bottom? Is there epinasty lower down?) 2) the desired timeline for the next harvest (is there an imminent experiment? Is this just general maintenance?) 3) how tall are the tallest stems? Ideally, we are able to select tall stem fragments (~6 inches). But, smaller fragments are workable.

d. Once a size is selected based on the above questions, and the first stem fragment is selected and snipped, that stem can be used as a length template for the rest of the stem collection. Lining up that stem with other healthy meristems, snip off ~11-14 other stems of the same length. Fewer can be used if needed (ie. there are only a few stems to begin with, or the culture is in bad shape and we are selecting only good meristems to propagate), but 12-15 total would be considered a “full” planting.

3. Planting

a. Once 12-15 stems are acquired, and the bucket is fully prepared with a layer of water, grab the bottom of one stem fragment (meristem at the top) between your pointer finger and thumb, identify the center of the sand surface, and push ~1/2 inch-1 inch of the stem below the surface of the sand at that center point. Stem should now be “planted” and far enough into the sediment to remain there without pushing too much of the stem below the surface of the sand.

b. Repeat this process for the remaining stems. A full planting includes one stem at the center of the bucket, ~4 stems forming a circle around it and spaced ~1 inch from each other and ~1 inch from the center stem, and ~10 stems around the outside, spaced ~1 inch from the edges of the bucket and ~1 inch from each other and from the middle ~4 stems (Figure 4). This layout can be adapted to smaller numbers of stems as well.

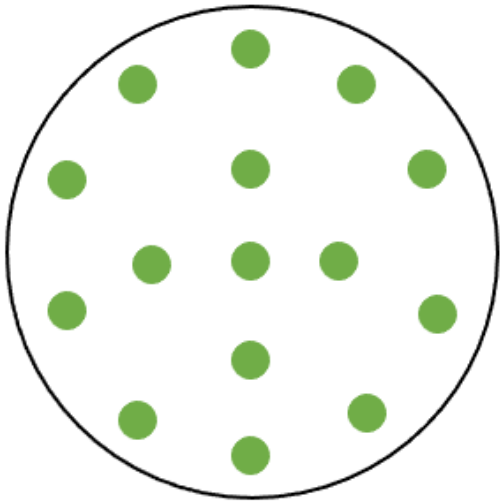

Figure 4. Planting configuration for stems of invasive watermilfoil in 2.5-gallon buckets.

Figure 5. Example of newly-transplanted watermilfoil stems in a 2.5-gallon bucket filled with 1:1:1 soil/sand/peat and topped with utility sand. Approximately 10 stems form a circle around the outside, with around four stems forming a circle around a single center stem.

4. After planting, the newly-planted bucket is ready to be placed in its barrel. Tip the bucket slightly while slowly placing it below the water surface so that water from the barrel enters the bucket slowly. Slowly lower the bucket to the bottom of the barrel.
5. The bucket of plant material that the new stems came from is now (likely) done with. To avoid confusion with other buckets, dump any remaining water out of it, and place it in a pile with other “finished” buckets.
6. Repeat this step with other pairs of buckets (one as the source of plant material and one that new stems will be planted into).

a. In some cases, we need to get more than one new bucket's worth of plant material out of one source bucket. All steps apply, other than that more than one new bucket will be needed.

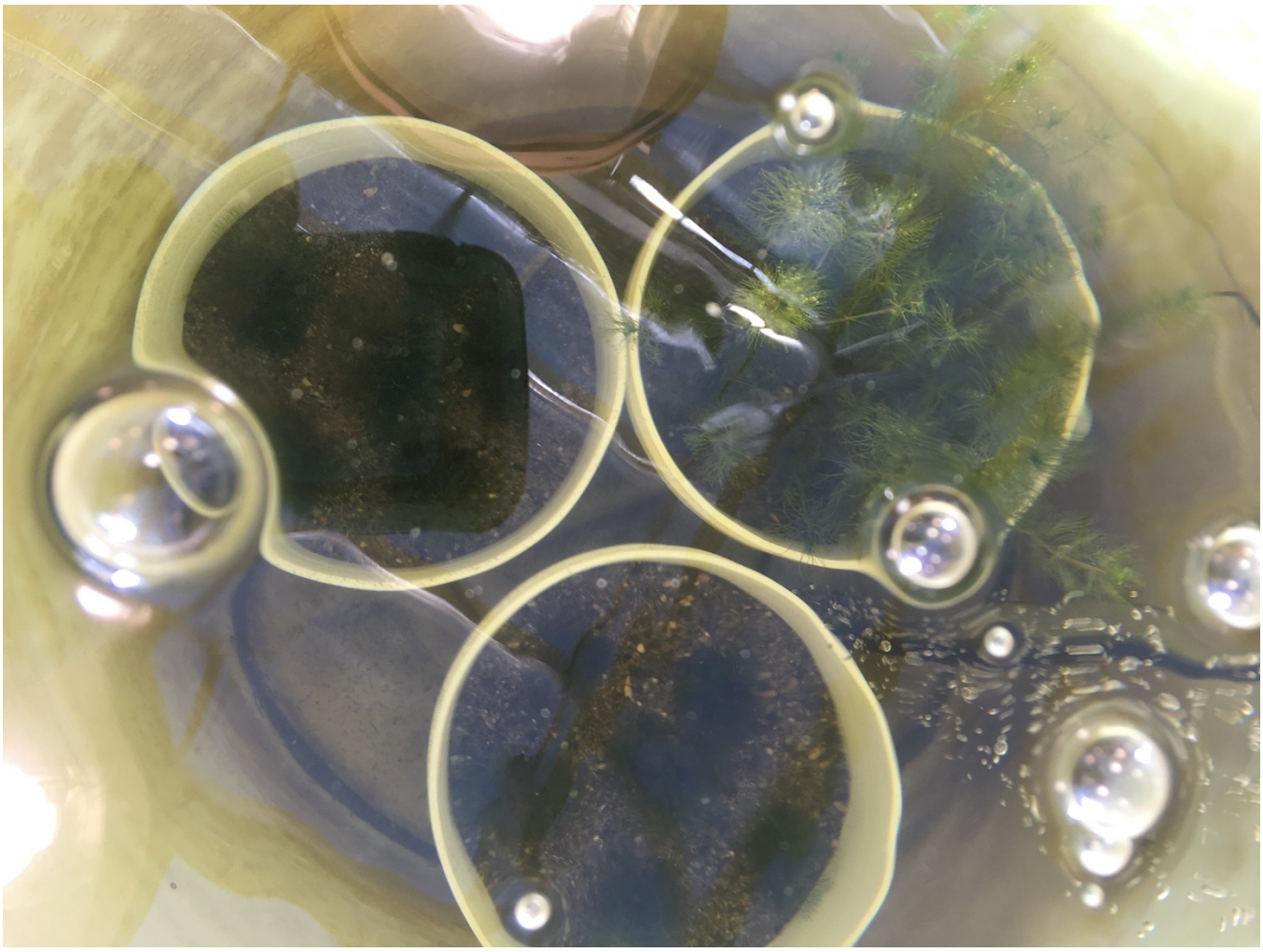

Figure 6. Culture of a single genotype (3-2.5 gallon buckets) planted in a 55-gallon barrel of SB buffer water.

APPENDIX B

OCCURRENCES OF EURASIAN AND HYBRID WATERMILFOIL STRAINS IN COEUR D'ALENE LAKE FROM 2015-2020: A REPORT TO AVISTA CORPORATION REGARDING WORK DONE FOR AVISTA CONTRACT NO. R-43238

Preface

An offshoot of the experiment described in Chapter 2 involved co-occurring genotypes from Coeur d'Alene Lake in Coeur d'Alene, Idaho (genotypes E-3 and H-1 in Chapter 2). Managers were interested in understanding how the genetic composition in Coeur d'Alene Lake changed over the period of 2015-2020, and whether management activities would lead to the selection of a more invasive genotype over time. This study may be used as an example for merging field and experimental data for lake-specific management of invasive watermilfoil.

The following is the final submitted report to Avista Corporation for the genetic analysis of invasive *Myriophyllum* genotypes present in Coeur d'Alene Lake, and outlines the potential impact of genotype-level genetic monitoring of *Myriophyllum*-invaded lakes over time (as described in Chapter 3). While this report has not yet become a manuscript, it may become one at a later date (Hoff and Thum, or Thum and Hoff, unpublished).

Introduction

Eurasian watermilfoil (*Myriophyllum spicatum*) is a well-known invasive aquatic plant that is widely spread across North American temperate lakes (Smith and Barko, 1990). Substantial amounts of money are spent annually to control this plant (Hanley and Roberts, 2019; Zipp et al., 2019), e.g. in Wisconsin an estimated $500,000 per year was spent on *M. spicatum* control between 2018 and 2020 through the state's Aquatic Invasive Species control program grants (Mikulyuk et al., 2020). *M. spicatum* is recognized as an invasive plant in Idaho

as well, as evidenced by several control programs (Laitala et al., 2012; Madsen et al., 2015; Wersal et al., 2010).

Coeur d'Alene Lake located in the panhandle of Idaho has an infestation of *M. spicatum* that has resulted in significant costs associated with control efforts in addition to reduced lakefront property values due to the presence of *M. spicatum* (Liao et al., 2016). *Myriophyllum spicatum*, as well as interspecific hybrids with northern watermilfoil (*Myriophyllum sibiricum*) were first recognized in 2004 (Moody and Les, 2007), growing in Round Lake, which is hydrologically connected to Coeur d'Alene Lake during summer lake level regulation by Post Falls Dam. The Coeur d'Alene Tribe (2007) found *M. spicatum* and hybrids (*Myriophyllum spicatum* × *sibiricum*) to be widespread across these southern waters during aquatic plant surveys conducted in 2004 and 2005. A control program began shortly thereafter in 2006 targeting *M. spicatum* and *M. spicatum* × *sibiricum*.

Between 2006 and 2013, a total of 2,003 acres of *M. spicatum* and *M. spicatum* × *sibiricum* infested waters in southern Coeur d'Alene Lake were treated with both liquid and granular formulations of 2,4-D, an auxinic herbicide. Treatment results from this period were mixed, and poor treatments were attributed to several possible factors, including inadequate herbicide contact time, sub-optimal treatment timing, and differing herbicide responses between the *M. spicatum* and *M. spicatum* × *sibiricum* (Coeur d'Alene Tribe, 2018).

Between 2014 and 2018, liquid endothall was used either exclusively or in combination with liquid 2,4-D to treat a total of 331 acres of *M. spicatum* and *M. spicatum* × *sibiricum* to address the suspected treatment issues with 2,4-D. Treatment results were better under this scheme but came at the cost of more collateral damage to native plant species, because of the less

selective nature of endothall relative to 2,4-D (Coeur d'Alene Tribe, 2018). Water potato (*Sagittaria latifolia* Willd.) is one such native plant that is highly valued by the Coeur d'Alene Tribe for its nutritious tubers harvested annually in the fall (Frey, 2000). Water potato can often be found growing near *M. spicatum* in Coeur d'Alene Lake and is sensitive to endothall (Skogerboe and Getsinger, 2001).

In 2018 the control program transitioned to controlling most *M. spicatum* and *M. spicatum* × *sibiricum* acreages with mechanical weed harvesting on a trial basis, largely because of the inconsistent herbicide results and the negative effects to valued native plants. The switch to harvesting was also driven by the ancillary benefit of removing substantial amounts of vegetation and the associated nutrients from the treatment areas (Bartodziej et al., 2017; Quilliam et al., 2015), as slowing eutrophication in Coeur d'Alene Lake is a long-term goal of the Coeur d'Alene Tribe (IDEQ and Coeur d'Alene Tribe, 2009).

Different genotypes (strains) of *M. spicatum* and *M. spicatum* × *sibiricum* can have differential vegetative growth and herbicide responses (e.g., Chorak and Thum, 2020; Netherland and Willey, 2017; Taylor et al., 2017; Thum et al., 2012). It stands to reason that different growth and herbicide response among genotypes could lead to changes in the composition of invasive *Myriophyllum* in lakes over time. For example, Parks et al. (2016) observed a large difference in the relative efficacy of auxinic herbicide treatments on *M. spicatum* versus *M. spicatum* × *sibiricum* in a large lake in Michigan (although such differences are not always observed; e.g., Thum et al., 2017). Given that both *M. spicatum* and *M. spicatum* × *sibiricum* had been identified in Coeur d'Alene Lake, and the intensity of invasive *Myriophyllum* management in Coeur d'Alene Lake, there is some concern that management activities may select for a more

invasive strain. If so, we would expect to see changes in the relative frequencies of strains over time. Therefore, the primary objective of this study was to examine the current lake-wide invasive *Myriophyllum* genetic variability relative to previous genetic surveys conducted in 2015 and 2017. In our comprehensive discussion, we include relevant data from a recent laboratory growth and 2,4-D response experiment (separate from this contract) and suggest alternative hypotheses for the observations presented in this study.

Methods

The Coeur d'Alene Tribe's Lake Management Department collected *Myriophyllum* samples for genetic analysis during routine aquatic vegetation surveys (Coeur d'Alene Tribe, 2018). For most sampling points, *Myriophyllum* was collected from a boat with a rake, but some samples were pulled from the water near boat docks. Several apical meristems were collected per sampling point from multiple rake grabs. For the 2015 and 2017 samplings, meristems from a single point were wrapped in damp paper towels, then placed in a sealable plastic bag, and kept cool until overnight shipping to the analytical laboratory in Bozeman, MT. For the 2020 sampling, meristems were shipped to the lab in a dried condition rather than fresh. The drying procedure consisted of rinsing the meristem in tap water to remove debris, gently blotting dry, then placing it in a sealable paper envelope, and placing the sealed envelope into a sealable plastic bag with approximately 25 g of silica gel beads for drying. Samples collected in 2020 were processed for drying immediately after field collection, stockpiled in an airtight container, and then shipped after all samples had been collected and dried.

Sampling points in 2015 were a repeat sampling of earlier genetics work done in 2007 and 2008. These sites were non-randomly selected and had watermilfoil with invasive growth characteristics, indeterminate leaflet pair counts (e.g., between 11 and 14) for morphological identification, or exhibited little response to herbicide treatment. The 2017 sampling was based upon a random sample of sites known to have milfoil from the Tribe's long-term point-intercept survey (Coeur d'Alene Tribe, 2018). The 2020 sampling was a repeat of 2017 sites in addition to sites sampled north of the mouth of the Coeur d'Alene River. Northern Coeur d'Alene Lake sites were chosen randomly from bays known to have *M. spicatum*. An additional set of points were sampled in 2020 to examine genetic variability at a mechanical harvesting site and a non-harvested site in Chatcolet Lake. These sites were from another Tribal point-intercept survey but at a finer grid resolution (50 m).

Molecular methods followed Thum et al. (2020). DNA extractions were performed using the Qiagen DNeasy Plant Mini Kit (Valencia, CA) following the standard plant protocol. We performed duplicate DNA extractions on ~10% of all samples to assist with scoring of microsatellites.

We genotyped eight microsatellite loci developed by Wu et al. (2013) (Myrsp 1, Myrsp 5, Myrsp 9, Myrsp 12, Myrsp 13, Myrsp 14, Myrsp 15, and Myrsp 16). Each microsatellite locus was amplified using the protocols detailed in Wu et al. (2013). Fluorescently labeled microsatellite PCR products were sent to University of Illinois – Urbana-Champaign's Core Sequencing Facility for fragment analysis on an Applied Biosystems 3730xl sequencer.

Microsatellites were scored using GeneMapper, version 5.0 (Applied Biosystems). *M. spicatum* and *M. spicatum* × *sibiricum* are hexaploid, and exact genotypes cannot be determined

because the numbers of allele copies are ambiguous. Therefore, we treated microsatellites as dominant, binary data (i.e., presence or absence of each possible allele at each locus), and we delineated distinct genotypes using the R-package POLYSAT (Clark and Jasieniuk 2011) using Lynch distances and a threshold of 0.

Results

Comparison of lake-wide composition over time

We identified a single genotype (strain) each of *M. spicatum* and *M. spicatum* × *sibiricum* in Coeur d'Alene Lake over the three genetic surveys (2015, 2017, and 2020), and one native *M. sibiricum* individual in 2017 (Figure 7). In each survey, *M. spicatum* × *sibiricum* was more widely distributed than *M. spicatum* in Coeur d'Alene Lake, with occurrences from the southern St. Joe's River inlet to the northern outlet at the Spokane River (Figures 8-10). And, the relative frequencies of the Eurasian and hybrid genotypes had not shifted significantly since 2015 (2015 vs 2020 relative frequencies $\chi^2 = 0.02$, $p = 0.89$).

Comparison of northern and southern portions of the lake

The first field identifications of invasive watermilfoil occurred in south Coeur d'Alene Lake. *Myriophyllum* is known to occur in some bays of the northern lake but has not been identified via genetic techniques. Therefore, the northern part of the lake was surveyed for the first time in 2020. The relative frequencies of *M. spicatum* versus *M. spicatum* × *sibiricum* do appear to differ between the northern (newly surveyed) and southern portions of the lake, although this pattern is not significant ($\alpha = 0.05$) (northern vs southern relative frequencies χ^2

=2.97, p = 0.09). In the southern half of the lake, *M. spicatum* and *M. spicatum* × *sibiricum* had relative frequencies of 0.26 and 0.74, respectively, whereas in the northern half of the lake, *M. spicatum* has a relative frequency of only 0.13 and *M. spicatum* × *sibiricum* a relative frequency of 0.88 (Figures 11-12).

Comparison of a harvested and unharvested area in 2020

We compared the genetic composition of one harvested and one unharvested area in the southern portion of the lake in 2020 (Chatcolet Lake). These samples were taken late in the season, after the harvesting had occurred. The proportion of *M. spicatum* × *sibiricum* was not different between the harvested area compared to the unharvested area (0.86 vs 0.78, respectively; Figure 13; $\chi^2 = 0.57$, p = 0.45). As of 2020, the harvested site had been cut annually since 2018. Both the harvested and unharvested sites have herbicide treatment history, being treated during the summers of 2015 (DMA4, Aquathol K) and 2012 (Navigate), respectively.

Discussion

While early genetic surveys in Coeur d'Alene Lake were small, observations from managers indicate that invasive watermilfoil has remained prevalent in Coeur d'Alene Lake since its first documented occurrence in 2004. Both *M. spicatum* and *M. spicatum* × *sibiricum* were recorded in Coeur d'Alene Lake in a small 2004 genetic survey, and concerns about *M. spicatum* × *sibiricum* displacement of pure *M. spicatum* have prompted further genetic study. The main concern expressed by managers is the potential that management could inadvertently

select for *M. spicatum* × *sibiricum* that display increased 'invasiveness,' manifested as being faster growing ('weedier') and/or less responsive to control efforts.

In the current study, we did not see any compelling evidence that *M. spicatum* × *sibiricum* has been displacing *M. spicatum* in the heavily managed, southern portions of Coeur d'Alene Lake over the period of 2015 to 2020. Although *M. spicatum* × *sibiricum* has increased in relative frequency nominally since 2015, this increase is not statistically significant, and it therefore appears that the relative frequencies of occurrence of *M. spicatum* and *M. spicatum* × *sibiricum* appear to have been stable over this period of time.

The relatively stable frequencies of occurrence for *M. spicatum* × *sibiricum* and *M. spicatum* in the southern part of the lake are somewhat surprising, because a recent greenhouse study found the Coeur d'Alene *M. spicatum* × *sibiricum* genotype to be a faster grower than the pure Coeur d'Alene *M. spicatum* genotype (Fig. 14; Hoff and Thum, unpublished data). The *M. spicatum* × *sibiricum* genotype grew faster than the *M. spicatum* genotype with or without 2,4-D. Furthermore, 2,4-D-treated *M. spicatum* × *sibiricum* grew as well, or better, than untreated *M. spicatum* genotype. Although we did not find any evidence of displacement by *M. spicatum* × *sibiricum* from 2015-2020, these data suggest that *M. spicatum* × *sibiricum* has the capacity to displace *M. spicatum* in treated or untreated areas of Coeur d'Alene Lake, presuming all other factors shaping *Myriophyllum* distribution are equal.

We have two hypotheses for how the greenhouse data may be reconciled with our observations of relatively stable frequencies of occurrence in the southern, heavily managed portion of Coeur d'Alene Lake. The first is that the *M. spicatum* × *sibiricum* genotype does outcompete the *M. spicatum* genotype, but that the frequencies of occurrence represent a balance

between the rate of *M. spicatum* × *sibiricum* displacement and the relative rate of input of *M. spicatum* versus *M. spicatum* × *sibiricum* from external, 'upstream' sources (i.e., dispersal-selection balance). It is important to note that Coeur d'Alene Lake has several inputs from relatively large rivers. This flow-through nature of Coeur d'Alene Lake may mean that there is a continuous input of *M. spicatum* into the lake. In that case, it is possible that the frequencies of the two genotypes have reached an equilibrium despite *M. spicatum* being the poorer competitor. Studies of surrounding areas to identify and quantify the source(s) and rate(s) of inputs warrant further study.

A second hypothesis is that the two genotypes trade-off in one or more ecological dimensions that were not accounted for in the greenhouse study. For example, perhaps the *M. spicatum* genotype outcompetes the *M. spicatum* × *sibiricum* genotype under some environmental conditions. If environmental conditions are heterogeneous across space or time, then the two strains may also coexist at some equilibrium frequencies that represent the balance of these environmental conditions. Common garden experiments in various times and locations across Coeur d'Alene Lake could be used to test this hypothesis.

It is important to note that while we did not see significant changes in the relative frequencies of the *M. spicatum* × *sibiricum* and *M. spicatum* genotypes in the southern, heavily managed portion of Coeur d'Alene Lake during the period of 2015 to 2020, we do not know whether there was a large change in their relative frequencies as a result of management before 2015. Over 2,000 acres were treated with 2,4-D in attempts to control *Myriophyllum* between 2006 and 2013 in southern Coeur d'Alene Lake. Accounts of the success were mixed from these treatments. The results of our greenhouse experiment suggest that – provided the two genotypes

present in Coeur d'Alene today were present then – the *M. spicatum* × *sibiricum* genotype would have had a competitive advantage (note that in the greenhouse experiment, *M. spicatum* × *sibiricum* treated with 2,4-D grew as well or better than untreated *M. spicatum*). In that case, it is possible that *M. spicatum* × *sibiricum* increased from a lower frequency in 2006 to the higher frequency that we observe today. Without genetic samples from before 2006 through 2013, it is unfortunately impossible to know whether the mixed results on 2,4-D efficacy from 2006 to 2013 were the result of changes in the relative frequencies of *M. spicatum* × *sibiricum* and *M. spicatum* genotypes, versus other factors such as contact time, treatment timing, etc.

We also did not identify any compelling evidence for differential response by the *M. spicatum* × *sibiricum* and *M. spicatum* genotype to mechanical harvesting in one harvested and one unharvested section of Chatcolet Lake. The relative frequency of *M. spicatum* × *sibiricum* was slightly higher in the harvested plot than in the unharvested plot, but this difference was not significant. An important caveat to this interpretation is that we assume the relative frequencies of *M. spicatum* × *sibiricum* and *M. spicatum* were similar within each plot before harvesting. However, it is possible that the two plots were different prior to the harvesting, and that changes in relative frequencies in one or both plots resulted in similar composition after harvesting. Future evaluation of the relative response of the two genotypes to harvesting should include pre- and post-treatment sampling times. In addition, we recommend sample sizes in study plots be larger (50 to 100 plants). If there is further interest in pursuing this topic, we would be willing to consult in detail on study design and subsampling strategies.

Finally, although the relative frequencies of the *M. spicatum* × *sibiricum* and *M. spicatum* genotypes appear stable in the southern portion of Coeur d'Alene Lake from 2015-2020, we did

find evidence that *M. spicatum* × *sibiricum* is relatively more frequent in the northern portion of the lake. The northern portion of the lake was not formally surveyed prior to 2020, and there is an anecdotal account that *Myriophyllum* in the northern part of the lake may represent a recent expansion. If this is a recent expansion, then the relatively higher frequency of *M. spicatum* × *sibiricum* may reflect a higher colonization potential, which is consistent with its observed faster vegetative growth rate compared to the *M. spicatum* genotype in our greenhouse study.

Figures

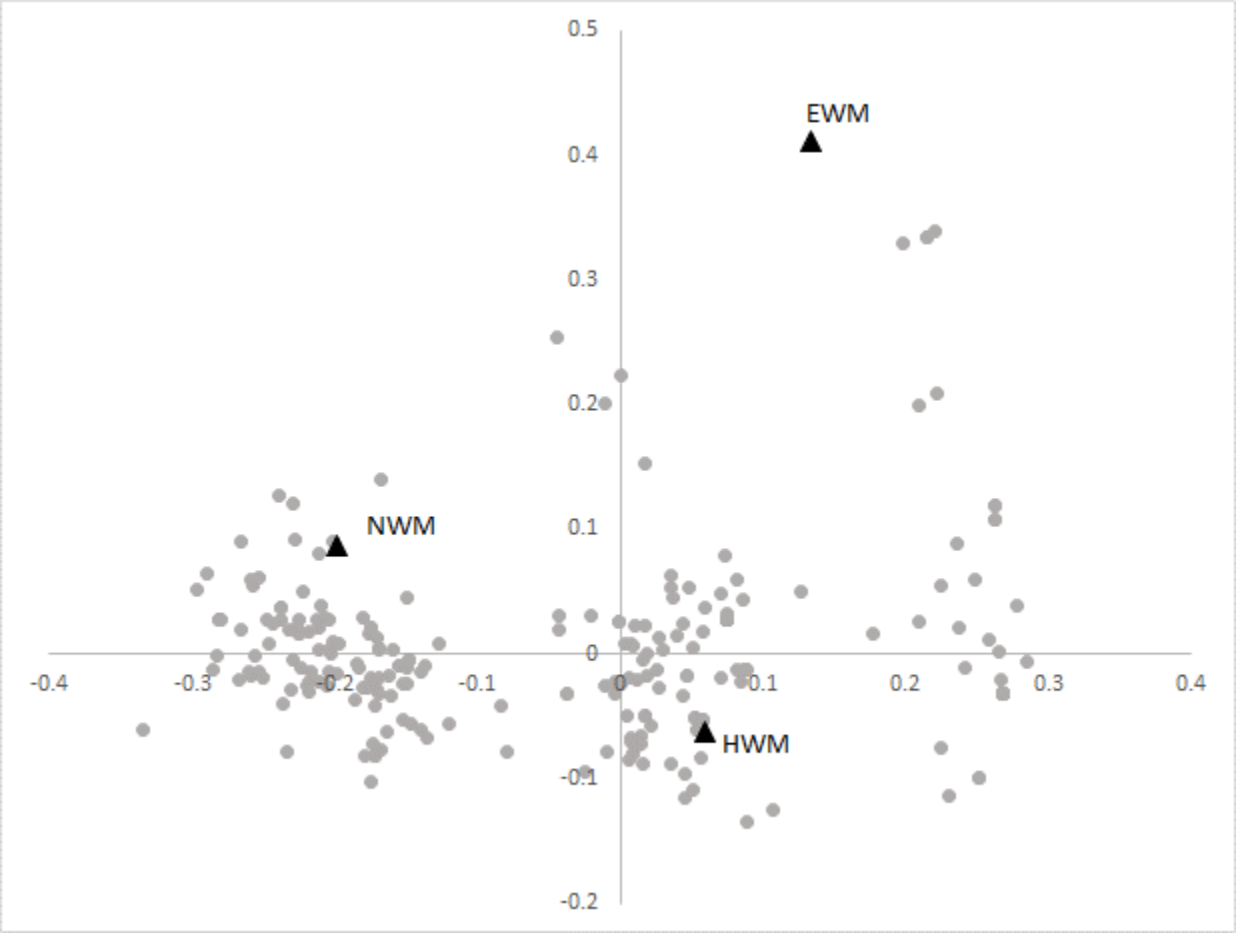

Figure 7. Principal Coordinates Analysis of microsatellite data for the three unique genotypes of *Myriophyllum* identified in Coeur d'Alene Lake during this study relative to genotypes identified in Thum et al. (2020). "EWM" = Eurasian watermilfoil (*M. spicatum*); "HWM" = hybrid watermilfoil (*M. spicatum* × *sibiricum*); "NWM" = northern watermilfoil (*M. sibiricum*).

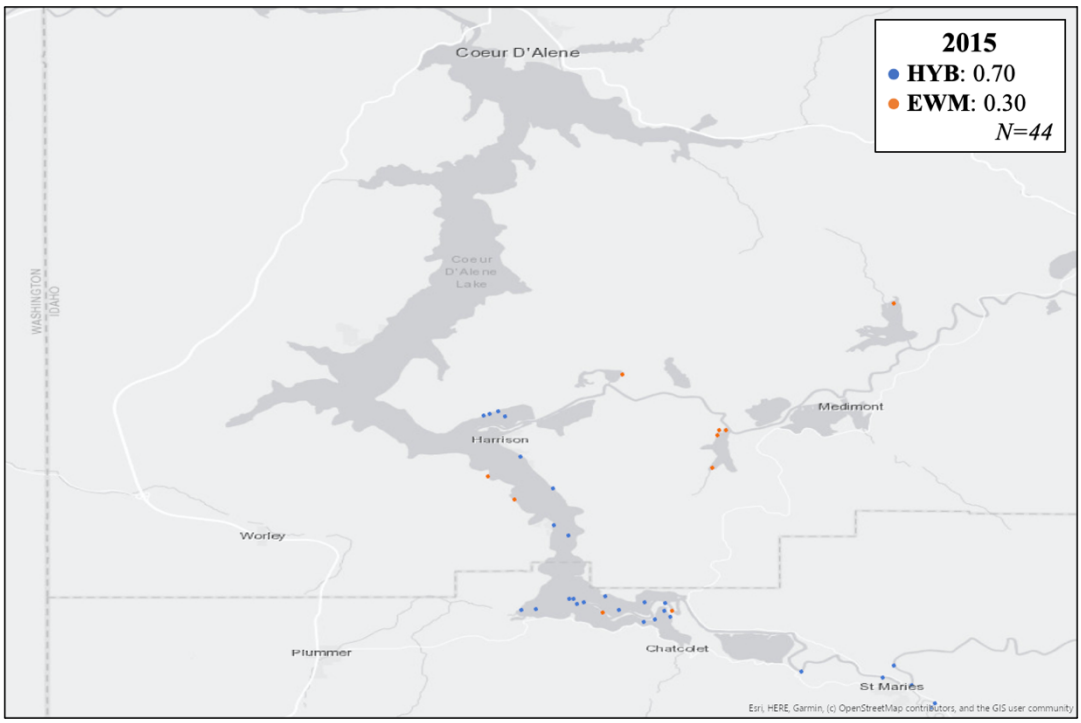

Figure 8. Distribution of *M. spicatum* and *M. spicatum* × *sibiricum* in Coeur d'Alene Lake in 2015.

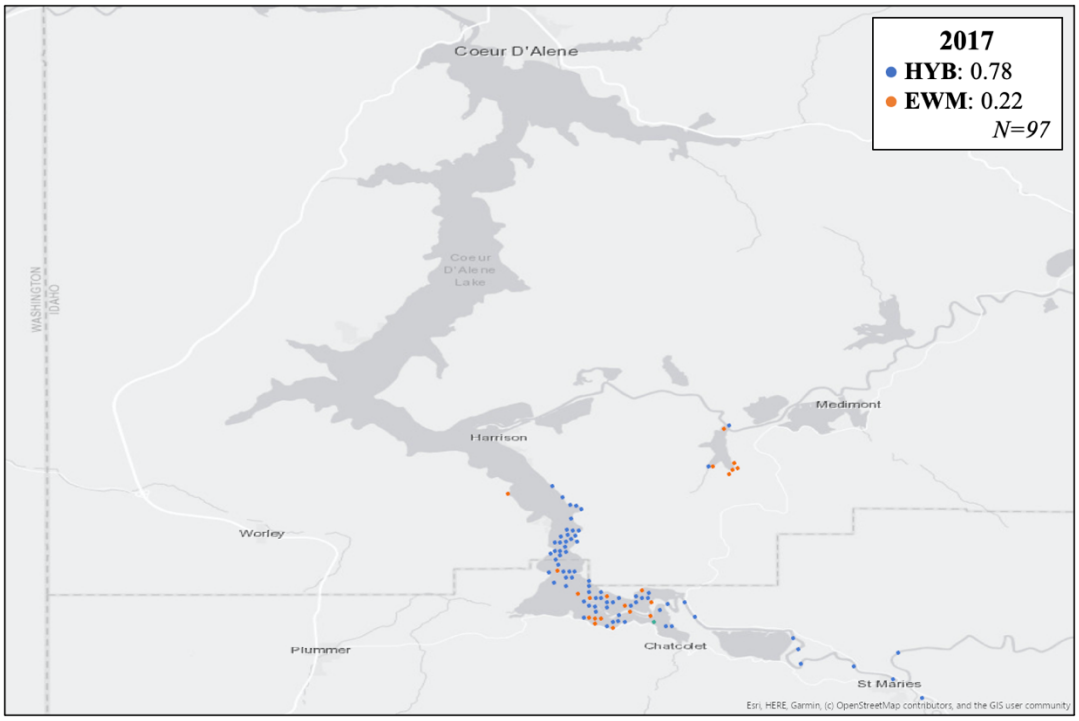

Figure 9. Distribution of *M. spicatum* and *M. spicatum* × *sibiricum* in Coeur d'Alene Lake in 2017.

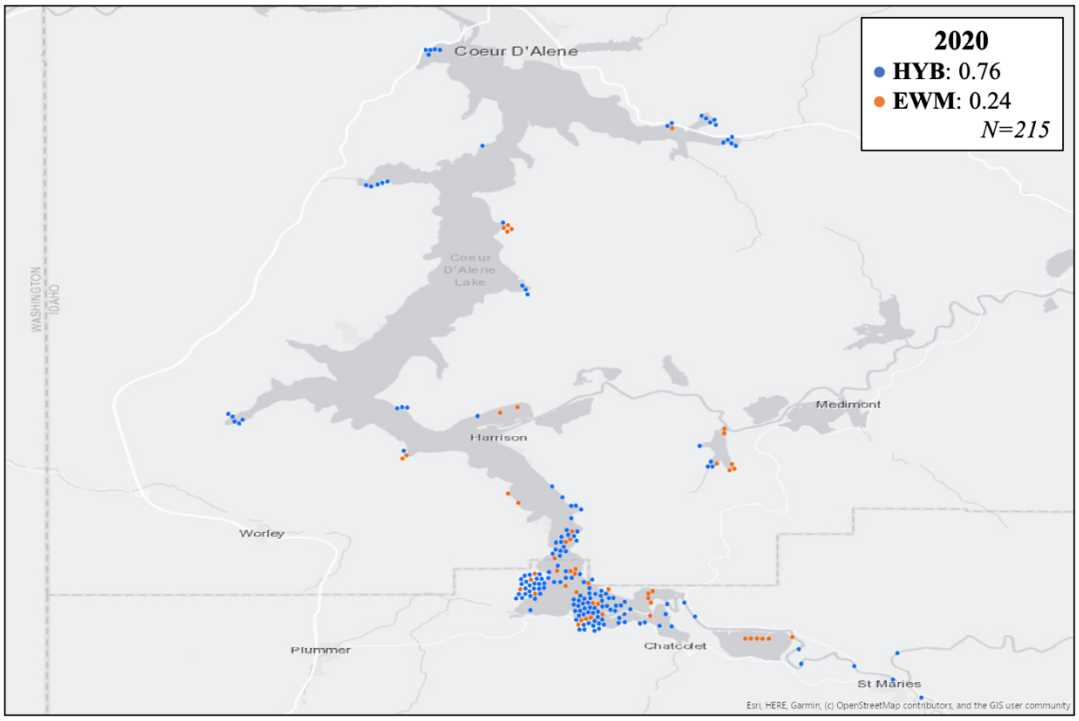

Figure 10. Distribution of *M. spicatum* and *M. spicatum* × *sibiricum* in Coeur d'Alene Lake in 2020.

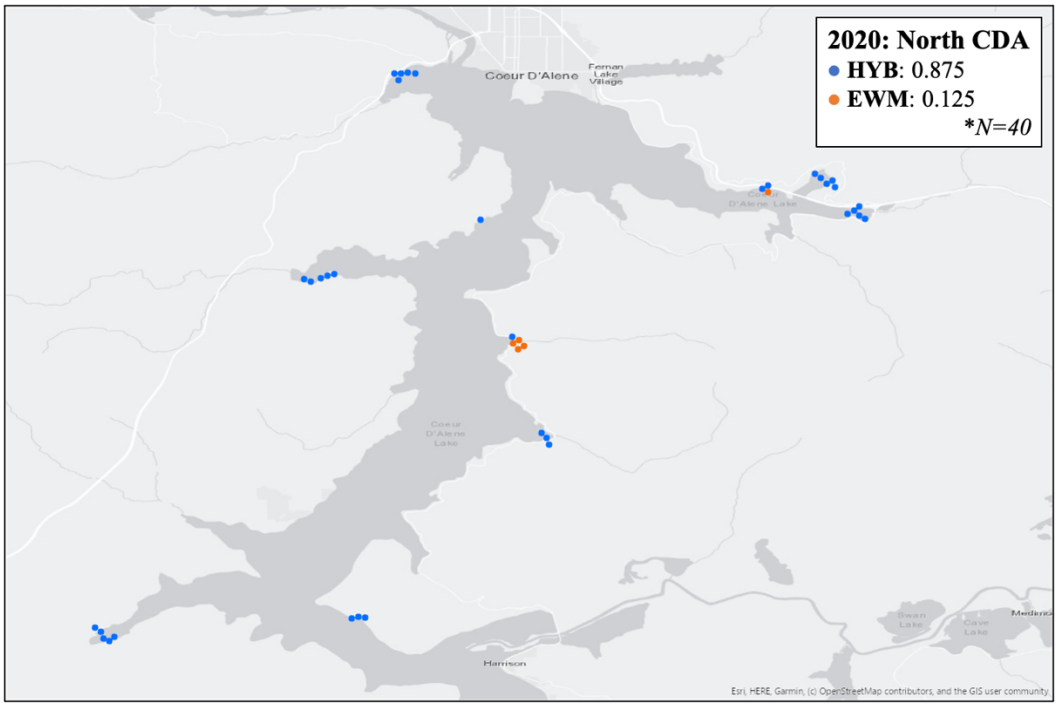

Figure 11. Distribution of *M. spicatum* and *M. spicatum* × *sibiricum* in north Coeur d'Alene Lake (north of the Coeur d'Alene River inlet) in 2020.

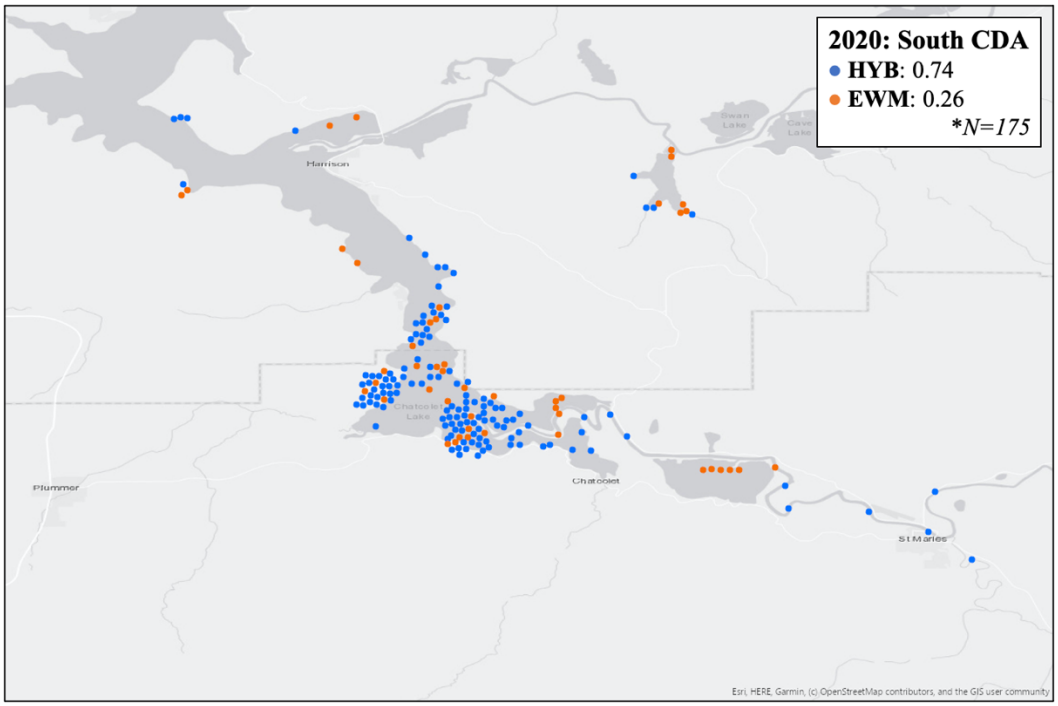

Figure 12. Distribution of *M. spicatum* and *M. spicatum* × *sibiricum* in south Coeur d'Alene Lake (south of the Coeur d'Alene River inlet) in 2020.

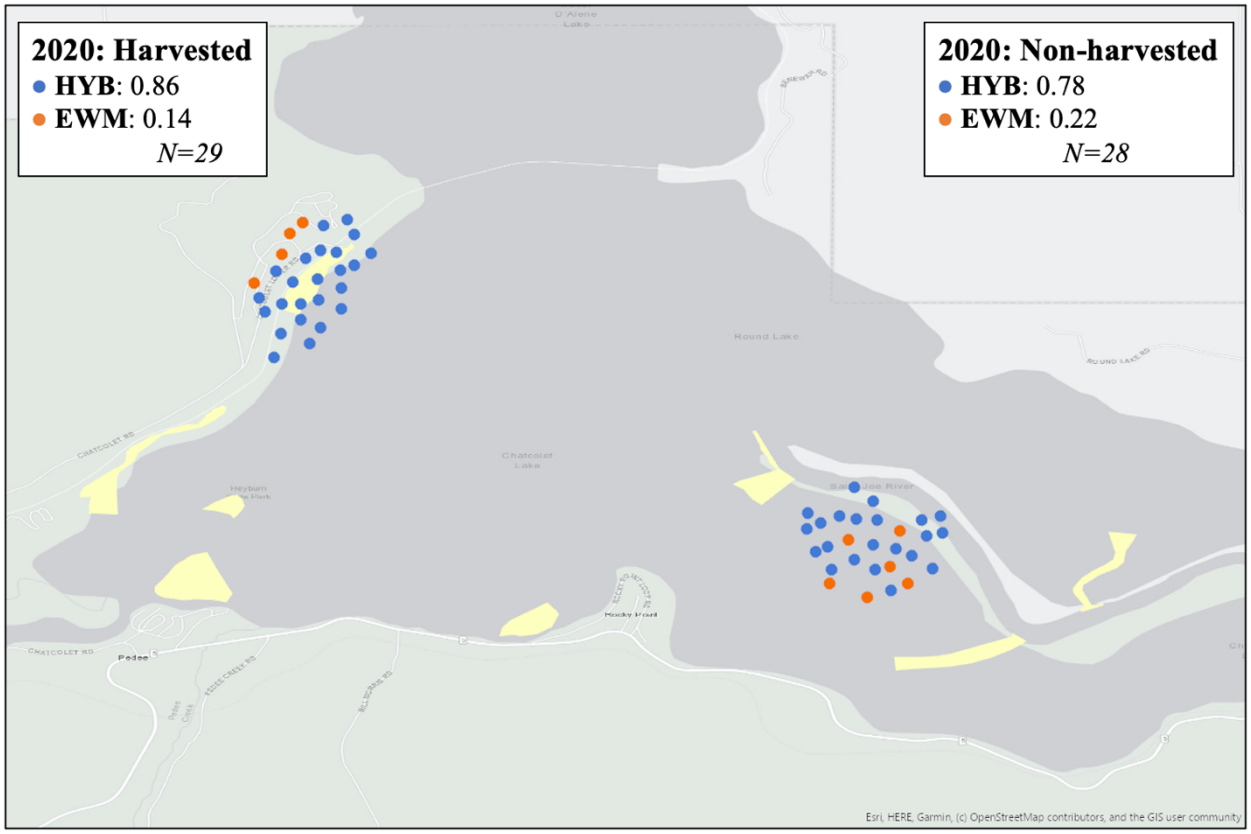

Figure 13. Distribution of *M. spicatum* and *M. spicatum* × *sibiricum* in Chatcolet Lake 2020.

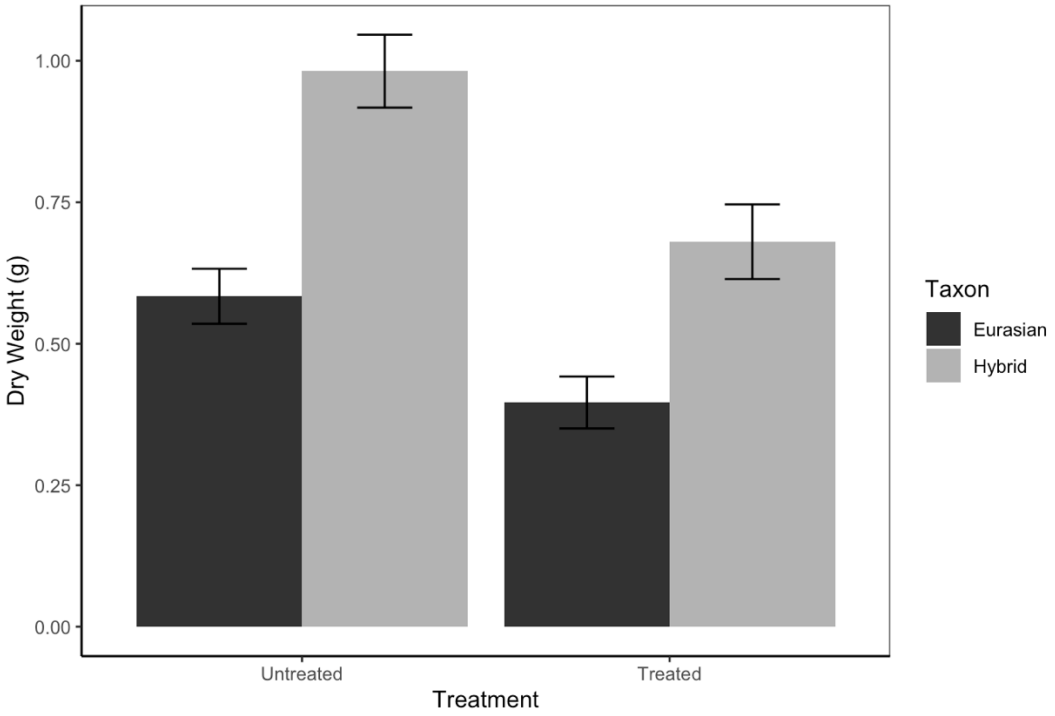

Figure 14. Greenhouse comparison of total average growth (dry weight) for the *M. spicatum* versus *M. spicatum* × *sibiricum* genotype from Coeur d'Alene Lake. At the start of the growth assay, we planted seventy-two stems of both the *M. spicatum* and *M. spicatum* × *sibiricum* genotypes from Coeur d'Alene Lake and allowed stems to establish for 6 weeks prior to treatment. Treatment tanks were treated to a target concentration exposure time (CET) of 500 µg L^{-1} for 72 hours and were allowed to grow for 6 weeks following treatment. The *M. spicatum* × *sibiricum* genotype grew faster than the *M. spicatum* genotype with or without 2,4-D. And, 2,4-D treated *M. spicatum* × *sibiricum* grew as well, or better, than untreated *M. spicatum* genotype. These data suggest that *M. spicatum* × *sibiricum* would displace *M. spicatum* in treated or untreated areas of Coeur d'Alene Lake, although we did not find any evidence of displacement by *M. spicatum* × *sibiricum* from 2015-2020.

APPENDIX C

STATUS OF THE MILFOIL WEEVIL (*EUHRYCHIOPSIS LECONTEI*) AS A BIOLOGICAL CONTROL AGENT FOR INVASIVE *MYRIOPHYLLUM*

Preface

My interest in Eurasian watermilfoil genetics stemmed from an interest in the evolution of biological control agents, and specifically the milfoil weevil (*Euhrychiopsis lecontei*). Upon starting my graduate research, I planned to study the role of genetic variation in biological control agents—specifically in *E. lecontei* on invasive *Myriophyllum*. I piloted this research with small quantities of milfoil weevils acquired from Golden Sands Research and Development Corporation and gained experience in culturing milfoil weevils of all life stages. However, due to COVID-19 research restrictions and other logistical issues, I was not able to receive an adequate quantity of milfoil weevils with which to run these larger experiments.

While milfoil weevils have demonstrated promise to be an effective biological control agent for invasive *Myriophyllum*, their efficacy has been variable in field and experimental studies. The following is an update on the status of the milfoil weevil as a biological control agent for invasive *Myriophyllum*, in terms of scientific study and use in integrated pest management.

Status of Milfoil Weevil Biocontrol

The milfoil weevil, *Euhrychiopsis lecontei* (Dietz, Coleoptera: Curculionidae) is a small, herbivorous aquatic beetle native to North America and a native herbivore of *Myriophyllum sibiricum* (northern watermilfoil). *Euhrychiopsis lecontei* is a *Myriophyllum* specialist and feeds on both native and nonnative *Myriophyllum* taxa (Creed and Sheldon 1994; 1995; Newman and Maher 1995). *Euhrychiopsis lecontei* completes all life stages submerged, feeding and developing on *Myriophyllum*, and mining into watermilfoil stems (Newman et al. 1996; Sheldon

and O'Bryan 1996). As specialist herbivores are rare among aquatic insects (Newman 1991) and native insects are preferred in biological control of invasive species due to reduced risks to non-target plants (Thorstenson 2011), *E. lecontei* has been viewed as an appealing biological control agent for invasive *Myriophyllum* taxa.

Due to its appeal as a *Myriophyllum* specialist, *Euhrychiopsis lecontei* garnered initial attention in the study of integrated pest management of invasive *Myriophyllum* (Creed and Sheldon 1993) and showed promise in its ability to negatively impact *Myriophyllum* plants. *Euhrychiopsis lecontei* showed particular promise on invasive *Myriophyllum* in some studies, documenting increased preference for (Solarz and Newman 1996), and increased fecundity on (Sheldon and Creed 2003), invasive *M. spicatum* over native *Myriophyllum* taxa. Early experiments showed that *E. lecontei* reduced milfoil buoyancy and led to plant material sinking from the water column (Creed et al. 1992). *Euhrychiopsis lecontei* was also shown to reduce carbohydrate stores due to larval stem mining, which may hinder the overwintering ability and regrowth of invasive *Myriophyllum* plants (Creed and Sheldon 1995; Newman et al. 1996). In the field, a review of declines of invasive *M. spicatum* in lakes concluded that damage by *E. lecontei* was likely responsible for a significant number of the declines (Creed 1998).

As a result of its early promise to negatively impact invasive *Myriophyllum*, *E. lecontei* has undergone extensive study, and has, in some cases, led to effective control of invasive *Myriophyllum.* For example, numerous studies have documented that *E. lecontei* treatment led to decreased biomass accumulation and plant height in *M. spicatum* (Creed and Sheldon 1995; Sheldon and Creed 1995). *E. lecontei* also caused significant declines of *M. spicatum* in tank experiments, resulting in reductions in root biomass as well as stem biomass (Newman et al.

1996). *Euhrychiopsis lecontei* treatment led to biomass reductions up to 71% in another laboratory experiment, which was comparable to the biomass reduction caused by fluridone treatment (Marko and White 2018).

Despite examples of successful control using *E. lecontei*, other studies have shown no significant impact of *E. lecontei* treatment in laboratory and field experiments. Field surveys generally have shown substantial variability of weevil efficacy to control *M. spicatum* populations (Reeves et al. 2008), with some cases showing no significant declines of *M. spicatum* (Newman et al. 1996). In another field experiment, *E. lecontei* stocking had no significant effect on *M. spicatum* or native plant biomass (Havel et al. 2017a). As a result of varying efficacy, particularly in field trials, the use of *E. lecontei* in management of invasive *Myriophyllum* has declined in recent years, with previous rearing operations (e.g. EnviroScience milfoil weevil program) permanently discontinued, and state agencies such as the Wisconsin Department of Natural Resources no longer providing grant funding for *E. lecontei* research (M. Nault, personal communication).

Numerous reasons have been proposed for the varying efficacy of *E. lecontei.* Multiple studies have attributed issues with control (particularly in natural populations) to low weevil densities (Newman et al. 1998, Havel et al. 2017a, Jester et al. 1997), and factors that limit populations of the milfoil weevil may thus need to be identified and mitigated to provide predictable control (Newman et al. 1998, Newman and Biesboer 2000). Possible factors for low *E. lecontei* densities include fish predation (Ward and Newman 2006), and other *Myriophyllum* control methods such as herbicide use (Havel et al. 2017b) and mechanical harvest (Newman and Inglis 2009). However, declines in *E. lecontei* associated with the use of other control methods

may be the result of management-related decreases in *Myriophyllum* populations rather than the control methods themselves, as long-term persistence of biocontrol agents also depends on the persistence of the target pest in the ecological community.

While numerous studies have attributed inconsistencies in *E. lecontei* biocontrol efficacy to population densities, the potential for a native herbivore to serve as a biocontrol agent for an exotic weed will depend also on its adaptability in response to its host (Sheldon and Jones 2001). In contrast to other control methods, biocontrol efficacy is dependent on the genetic variation of both the control agent and the pest, and their respective ability to adapt to reciprocal selective pressures. However, many biocontrol agents used for management purposes are bred at augmentative rearing facilities that may have high levels of inbreeding, and thus low genetic diversity (Mackauer 1976). Therefore, tests of biocontrol agent efficacy using these inbred stocks may have put control agents at an evolutionary disadvantage against their target pest. Understanding how much variation exists in both *Myriophyllum* and *E. lecontei* populations, and how this variation impacts their potential for evolution, may thus be the key to using biocontrol more effectively and reliably as a management tool for invasive *Myriophyllum*.

While varying efficacy has led to declines in its use in the integrated pest management of invasive *Myriophyllum*, *E. lecontei* has shown promise and potential as a biological control agent. We recommend future research into factors that may limit population sizes, as well as the adaptability of *E. lecontei* populations. If density or genetic variation are strongly linked to *E. lecontei* efficacy on invasive *Myriophyllum*, stocking populations of *E. lecontei* may be tailored in order to stock adequate densities or augment with genetically variable populations.

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