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INTRASPECIFIC GENETIC VARIATION, POPULATION STRUCTURE, AND PERFORMANCE OF THE INVASIVE AQUATIC MACROPHYTE EURASIAN WATERMILFOIL (Myriophyllum spicatum) IN WATERBODIES WITH AND WITHOUT HISTORIES OF CHEMICAL HERBICIDE TREATMENT ACROSS MICHIGAN

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INTRASPECIFIC GENETIC VARIATION, POPULATION STRUCTURE, AND PERFORMANCE OF THE INVASIVE AQUATIC MACROPHYTE EURASIAN WATERMILFOIL (*Myriophyllum spicatum*) IN WATERBODIES WITH AND WITHOUT HISTORIES OF CHEMICAL HERBICIDE TREATMENT ACROSS MICHIGAN

By

Taylor A. Zallek

A THESIS

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Abstract

Populations of invasive species are often subjected to novel selective forces in the form of anthropogenic control agents in their introduced ranges. These control agents, applied unevenly among populations within a species' new range, can send invasive populations on drastically different evolutionary and ecological trajectories. In these studies, we aimed to see if different histories of chemical herbicide treatment are differentially influencing the genetic diversity, structure, and performance of populations of invasive watermilfoil (*Myriophyllum spicatum* and *M. spicatum x M. sibiricum* hybrids) from waterbodies in the state of Michigan. We sampled ten waterbodies with different histories of herbicide treatment in order to examine patterns of genetic variation amongst milfoil populations, to determine the extent of admixture among invasive watermilfoil populations, and to assess whether histories of herbicide application have an impact on the abiotic environment and/or biotic macrophyte community. We also grew invasive watermilfoil plants that were collected from waterbodies with and without histories of repeated exposure to herbicides together in mesocosms to test for tradeoffs in the expression of invasive traits. We found that genetic diversity is greater in populations with no history of herbicide treatment, populations with histories of herbicide treatment have more admixture and evidence of hybridization, and plant communities appear to be differentially shaped by histories of herbicide treatment. We also found that a history of herbicide treatment significantly affected plant survival, net growth, and mean growth rate and that these effects depended upon whether neighboring plants were from

herbicide or non-herbicide treatment waterbodies. In general, plants from waterbodies with histories of herbicide treatment were more likely to survive and expressed increased growth relative to plants collected from waterbodies with no history of herbicide treatment. These findings indicate that histories of herbicide application could be selecting for populations comprised of less genetically diverse (but more admixed) individuals with potentially higher fitness for herbicide conditions. Our results suggest that repeated exposure to chemical herbicides could be selecting for increased invasiveness among invasive watermilfoil populations. This could have drastic ecological consequences and implications for the efficacy of long-term management efforts of invasive watermilfoil.

1 Variation in genetic diversity, structure, and patterns of hybridization among and within populations of invasive Eurasian watermilfoil (*Myriophyllum spicatum*) in waterbodies with and without histories of herbicide treatment across Michigan

1.1 Abstract

Novel selective forces in the form of anthropogenic control agents (such as chemical herbicides) can dramatically influence different evolutionary trajectories among populations of invasive species. Routine exposure to herbicides can lead to the selection for herbicide resistance traits in targeted populations and impact the potential invasibility of these populations through the differential expression of invasive traits between historically targeted populations and non-targeted populations. In this study, we examined whether patterns of genetic diversity and population structure of invasive watermilfoil (*Myriophyllum spicatum* and *M. spicatum x M. sibiricum* hybrids) populations differed amongst waterbodies with and without histories of herbicide treatment. We also examined whether histories of herbicide treatment could be impacting the abiotic environment or biotic plant community. We found that genetic diversity within populations is low while genetic variation among populations is high. Invaded waterbodies with histories of herbicide treatment have more admixture than non-herbicide waterbodies. Populations are typically represented by one genetic class and evidence of hybridization is greatest in herbicide treatment waterbodies. Plant communities differ between herbicide treatment waterbodies and non-treatment waterbodies. Invasive watermilfoil populations in their introduced range demonstrate a

spectrum in genotypic diversity, admixture, and hybridization among invaded waterbodies with different histories of herbicide application. Routine use of herbicides could be sending populations of invasive watermilfoil, as well as plant communities, on different evolutionary and ecological trajectories.

1.2 Introduction

Understanding the population genetics and systematics of biological invasions can be a key aspect to unlocking the secrets of an invasive species' ability to succeed in new environments (Baker and Stebbins 1965, Barrett 1992, Sakai et al. 2001, Tsutsui et al. 2000, Lee 2002, Allendorf and Lundquist 2003, Ryan et al. 2009, Barrett 2015, Bock et al. 2015). Invasive species exist within small, genetically homogenous founding populations and many introduced populations are unsuccessful due to population bottlenecks, making it difficult for them to respond to novel selective forces found within the introduced range (Sax and Brown 2000, Frankham 2005, Estoup et al. 2016). However, recent studies have shown that some invasive populations can thrive despite their relatively low genetic diversity and the increased stress of novel anthropogenic control agents (Roman and Darling 2007, Sax et al. 2007, Schrieber and Lachmuth 2017).

As we have expanded our capabilities of transporting invasive species on a global scale, our efforts to manage them through means of control have expanded as well (Lowe et al. 2000, Donlan and Martin 2004, Pyšek and Richardson 2010). Historically, two of the most common means of controlling invasive species include biological control agents (through the introduction of predators, herbivores, or parasites) and the use of chemical herbicides (Müller-Schärer et al. 2004, Prentis et al. 2008). Biological control agents and chemical herbicides employed to manage invasive or weedy plant species can prompt rapid evolutionary responses among targeted populations (Jasieniuk et al. 1996, Richardson 2008, Powles and Yu 2010). Often these responses include the expression of specific traits that increase the tolerance of or defense against control agents (Lee et al 2002, Prentis et al. 2008). For example, some biotypes of the invasive aquatic macrophyte Hydrilla (*Hydrilla verticillata*) have evolved resistance to the herbicide fluridone as the result of somatic mutations to the gene that produces the enzyme fluridone is designed to suppress (Michel et al. 2004, Arias et al. 2005). Once established, herbicide resistant biotypes can rapidly cover large areas of water and displace native plant communities, causing significant harm to native ecosystems (Schmitz et al. 1993, Bates & Smith 1994, Schultz et al. 2012). This places managers in a difficult position for determining how to best manage invasive aquatic plants, such as Hydrilla, without sacrificing short term or long term goals for ecological stability.

Some of the traits that macrophytes have evolved to tolerate the physical stressors of their aquatic environments are often the same traits that grant them their invasive success. Physical aspects of these habitats often include strong wave action and barriers to sexual reproduction and gene flow (Santamaria 2002). In order to overcome these stressors, many invasive aquatic plants rely on asexual reproduction as a primary form of propagation (Grace 1993, Barrett et al. 1993, Santamaria 2002) and the generation of novel, successful phenotypes through genetic admixture (Lee 2002, Roman and Darling

2007). Genetic admixture in the form of hybridization between an invasive species and native congener can grant offspring with the ability to succeed in a multitude of environments (Ayres et al. 2004, Blum et al. 2007) and exhibit increased growth relative to parental species through hybrid vigor (Ellstrand and Schierenbeck 2000). Understanding the balance between alternately adaptive modes of propagation (clonal reproduction versus sexual recombination through admixture or hybridization) in invasive aquatic plants is important for understanding the mode of their success.

Eurasian watermilfoil (*Myriophyllum spicatum* L.) is a submerged aquatic macrophyte native to Europe, Asia, and North Africa (Couch and Nelson 1985) and is highly invasive in North America (Aiken et al. 1979, Smith and Barko 1990, Madsen 1994). Eurasian watermilfoil was first documented in North America in the late nineteenth century in the area surrounding Chesapeake Bay (Reed 1977), but can now be found throughout North America having currently invaded 48 out of 50 U.S. states and 3 out of 10 Canadian provinces (United States Geological Survey 2018). Eurasian watermilfoil displaces native aquatic macrophytes (Smith and Barko 1990, Madsen et al. 1991, Madsen 1994), and often forms dense stands of vegetation that alter both abiotic (oxygen levels) and biotic (invertebrate and fish communities) conditions of littoral zone communities, inhibit recreational activities, and can reduce property values (Honnell et al. 1992, Keast 1984, Lillie and Budd 1992, Madsen 1995, Eiswerth et al. 2000, Olden and Tamayo 2014).

The rapid spread and success of Eurasian watermilfoil is in part attributed to traits related to its rapid growth and modes of reproduction. Eurasian watermilfoil is particularly adept at reproducing asexually via fragmentation which produces clonal stolons that readily colonize new habitats (Aiken et al. 1979, Madsen et al. 1988). Propagule fragments can colonize separated water bodies as fragments have been shown to be transported by both human and animal vectors between lakes (Kimbel 1982, Clausen et al. 2002). Eurasian watermilfoil also exhibits high phenotypic plasticity and thrives in a multitude of aquatic environments, such as low and high nutrient environments (Aiken et al. 1979, Gerber and Les 1994, Madsen 1998, Buchan and Padilla 2000). It has recently been recognized that part of Eurasian watermilfoil's success may rely on its ability to hybridize with native Northern watermilfoil (*Myriophyllum sibiricum* Komarov). Hybirdization between the two species was first documented in North America in 2002 (Moody and Les 2002) and has since been reported several times across its introduced range (Moody and Les 2007, Strutevant et al. 2009, Zuellig and Thum 2012, LaRue et al. 2013a, Borrowman et al. 2014). Hybridization between these two species has also been documented in their co-occurring native range (central Asia) as well (Wu et al. 2015, Moody et al. 2016). Since hybrids and parental species overlap in morphology and hybrid status can only be determined through genetic analyses (Moody and Les 2010), Eurasian watermilfoil and hybrid watermilfoil will henceforth be referred to as invasive watermilfoil. Invasive watermilfoil hybrids have been shown to exhibit increased growth rates (LaRue et al. 2013b) and to be more resistant and tolerant of herbicide management efforts (Poovey et al. 2007, Slade et al. 2007, Berger et al. 2009, Glomski and Netherland 2009, Berger et al. 2012, Thum et al. 2012, LaRue et al. 2013b, Parks et al. 2016, and Thum et al. 2017). However, it is unknown whether repeated exposure to herbicides are selecting for populations comprised of more genetically diverse or hybrid, and therefore potentially more invasive, individuals in treated waterbodies.

Here, we asked whether histories of herbicide treatment influenced the genetic composition of invasive watermilfoil populations and the abiotic and biotic characteristics of the invaded waterbodies. To accomplish this, we sampled ten lakes throughout Michigan that have either been managed with herbicides to control invasive macrophytes or not in order to examine patterns of genetic variation amongst milfoil populations, to determine the extent of admixture among invasive watermilfoil populations, and to assess whether histories of herbicide application have an impact on the abiotic environment and/or biotic plant community. Specifically, we tested the following hypotheses: (1) Invasive watermilfoil populations with histories of herbicide treatment will have increased genetic diversity, (2) Invasive watermilfoil populations closer in geographic proximity will exhibit more genetic similarities than geographically distant populations, (3) Hybridization will be more prevalent in waterbodies with histories of herbicide treatment as the result of selection favoring herbicide tolerant hybrids, (4) Repeated herbicide exposure will select for unique biotic communities in invaded waterbodies with histories of herbicide treatment.

1.3 Materials and Methods

Plant material and sampling design

We collected leaf tissue from 1,362 individual invasive watermilfoil plants from 10 different water bodies (lakes and bays) spanning the Lower and Upper Peninsula of Michigan during the summers of 2015 and 2016 (Figure 1.1, names and GPS coordinates of water bodies are listed in Table A.1). We sampled from water bodies that had either been treated with herbicides (including 2,4-Dichlorophenoxyacetic acid and triclopyr) that target invasive milfoil at least once within the last five years ("herbicide treatment lakes," n = 7) or that have not previously been treated with herbicides within the last five years ("non-herbicide treatment lakes," n = 4) (MI DEQ 2018). Plant tissue was collected using a combination of rake tosses and rake twists at three spatially segregated sites per water body. Rake tosses utilized two metal leaf rakes fastened together with handles removed and replaced with a retrievable rope. The rakes were tossed over the side of the boat at a distance of approximately 3 m and the rake tines dragged the substrate of the water body and collected plants as the researcher pulled in the rope. Rake twists utilized a retractable gaff pole with its hook replaced with two metal leaf rakes fastened together. At each site, invasive watermilfoil plants were sampled at spatial intervals of 10 m in order to minimize sampling of clones and for each individual plant approximately 15 cm of tissue was stored in silica gel.

Nuclear microsatellite analysis

For each sample, total genomic DNA was extracted from approximately 2 cm^2 of dried leaf tissue using a modified (polyvinylpyrrolidone used in place of 2-mercaptoethanal) CTAB extraction method (Doyle and Doyle 1987). DNA was then spectrophotometrically (NanoDrop® ND-1000 UV-Vis spectrophotometer, Thermo Scientific, Washington, DE, USA) quantified and standardized to 20 ng/µl with deionized water.

To measure genetic diversity amongst our samples and populations, we amplified twelve polymorphic microsatellite loci that had been previously developed by Wu et al. 2013 (Table A.2). Microsatellite loci were amplified in 10 µl polymerase chain reactions (PCRs) containing 20 ng DNA, 1x Qiagen Type-it® multiplex PCR master mix (Qiagen, Hilden, Germany), and 0.2 µM each primer using the Type-it® microsatellite PCR protocol (Qiagen, Hilden, Germany). Forward or reverse primers were fluorescently labeled and markers labeled with different fluorescent dyes were simultaneously amplified. PCR products were resolved on an ABI 3730XL (Applied Biosystems, Foster City, CA, USA) using a Liz-500 internal size standard at Yale University's DNA Analysis Facility (New Haven, CT) and were visually scored using the software GeneMarker v. 2.6.3 (SoftGenetics®, LLC, State College, PA). To score marker data, we sorted alleles into bins based on the electropherogram peaks in relative fluorescence units (RFU) and repeat motif for each marker (Table A.2). To ensure repeatability in marker amplification and scoring, we replicated the whole procedure from DNA extraction through scoring twice for 72 randomly selected individuals; all data was retained because all markers were repeatable. Data from these twelve markers were subsequently combined to obtain multilocus individual genotypes for further analyses.

Plant community and environment sampling

To test if water bodies with differences in herbicide treatment history had differences in their abiotic environments and/or biotic plant communities, we collected abiotic environmental data and biotic plant community data from 6 of the 10 sampled water bodies (Figure 1.1).

Abiotic data included total dissolved nitrogen (TDN - µg/L), total dissolved phosphorus (TDP - µg/L), dissolved organic carbon (DOC - mg/L), and were determined by collecting filtered lake water followed by acidification with hydrochloric acid using a Shimadzu TOC-5000A analyzer (Shimadzu Corporation, Kyoto, Japan). Conductivity (cond. - ms/cm), temperature (temp. - degrees celsius), pH, turbidity (turb. - NTU), and dissolved oxygen (DO - mg/L) were measured using a YSI Sonde 6920 V2 (YSI Incorporated, Yellow Springs, OH, USA). All measurements were taken just below the water's surface. Samples for all variables were collected a minimum of three times per lake at the same initial 50 m plant sampling site for each transect.

Biotic data consisted of relative abundance and diversity data for other plant species in the subset of waterbodies. Plants were sampled along a minimum of three spatially separated transects per lake. The first sampling point of each transect was approximately 50 m from the shoreline and subsequent sampling locations occurred along a transect running tangent to the shoreline in intervals of 50 m until the edge of the littoral zone. Macrophytes were sampled using a standard rake toss method (Kenow et al. 2007), visually identified to species, and scored for relative abundance measured using a rake fullness ordinal scale (Figure 1.2).

STATISTICAL ANALYSES

Genetic diversity analysis

Based upon data on the number of bands observed per locus (Table 1.2), preliminary flow cytometry data (Hersch-Green unpublished results), and published chromosome karyotype reports (Löve 1961, Löve and Ritchie 1966), we suspected that all sampled plants were hexaploids (2n = 6x = 42). Because polyploidy hinders the determination of allelic dosage and genotype determination, we manually converted the microsatellite data into a dominant, presence/absence format (Lynch 1990, Falush et al. 2007) for all subsequent genetic analyses.

To assess allelic diversity per locus, we calculated the number of alleles per locus (N_A), the minimum number of alleles per locus (Min_A), the maximum number of alleles per locus (Max_A), and the mean number of alleles per locus ($Mean_A$) using the R software (R Development Core Team 2011) package POLYSAT (Clark and Jasieniuk 2011). To examine genetic diversity within and among populations, we calculated the number of genotypes (G) using the program GENOTYPE (Merimans and Van Tienderen 2004) and calculated the effective number of genotypes (G_{Eff} - Lehman & Wayne 1991), genotypic evenness (G_{Eve} - Grünwald et al. 2003), genetic diversity corrected for sample size (Nei's SS - Nei 1987), and Shannon-Weiner diversity index corrected for sample size (HSS - Chao & Shen 2003) using the program GENODIVE (Merimans and Van Tienderen 2004).

Genetic differentiation and population structure across spatially separate lakes

We performed an analysis of molecular variance (AMOVA; Excoffier et al. 1992) to assess genetic differentiation among populations and individuals and a Mantel test (Mantel 1967) to assess the relationship between genetic and geographic distance (isolation by distance, Wright 1943). Both analyses were implemented using the software GenAlEx v. 6.5 (Peakall and Smouse 2012).

We used several methods to examine patterns of genetic structure within and across water bodies. First, we used a Bayesian clustering method implemented in the program STRUCTURE version 2.3.4 (Pritchard et al. 2000). This program uses a Bayesian algorithm to determine the proportion of an individual's' allelic composition that groups into a predetermined number of clusters (*K*) whose members share similar patterns of genetic variation (Porras-Hurtado et al. 2013). We examined the probabilities of observing the data for K = 1-10, using admixture ancestry models with independent allele frequencies and 20,000 iteration burn-in period followed by 100,000 Markov Chain Monte Carlo (MCMC) iterations. We performed twenty independent runs for each *K* before incorporating posthoc analyses based on identifying the greatest rate of change in log likelihood of *K* (Evanno et al. 2005) to determine the most likely number of genetic clusters among the 10 sampled water bodies using the software STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). Individual admixture proportions (Q) for best fit *K* were then collated from the 20 STRUCTURE runs at optimal *K* using the software CLUMPP (Jakobsson and Rosenberg 2007), employing the GREEDY model with 100 repeats.

Second, because Bayesian clustering models assume Hardy-Weinberg equilibrium and are likely violated when examining structure across separate water bodies, we examined population structure using a discriminant analysis of principal components (DAPC, Jombart et al. 2010) to test the generality of our results. The DAPC was performed in R (R Development Core Team 2011) using the software package *adegenet*

(Jombart 2008). DAPC requires no group priors, does not assume Hardy-Weinberg equilibrium, and uses sequential *K*-means clustering and model selection to determine genetic clusters. Sixty principal components were retained in the initial PCA and the number of clusters (*K*) was evaluated based on the value of Bayesian Information Criteria (BIC) versus number of clusters (*K*=1 through *K*=10). Eigenvalues for the discriminant analysis were calculated using 50 principal components.

Lastly, a principal coordinate analysis (PCoA) was conducted. The PCoA was performed on pairwise genetic Lynch distances (Lynch 1990) obtained using the R package Polysat (Clark and Jasieniuk 2011) among all respective genotypes from the 10 waterbodies combined and performed using the software GenAlEx v. 6.5 (Peakall and Smouse 2012).

Patterns of hybridization and population genetic structure within lakes

To examine patterns of genetic structure and admixture within the waterbodies, we employed a Bayesian clustering method implemented in the software STRUCTURE version 2.3.4 (Pritchard et al. 2000). Since the program STRUCTURE assumes populations and their alleles are in a state of Hardy-Weinberg equilibrium, Bayesian clustering was performed on an individual water body basis so that Hardy-Weinberg assumptions were less likely to be violated. We examined the probabilities of observing the data for *K* = 1-10, using admixture ancestry models with independent allele frequencies and a 20,000 iteration burn-in period followed by 100,000 Markov Chain Monte Carlo (MCMC) iterations. Twenty independent runs for each *K* were performed before incorporating posthoc analyses based on identifying the greatest rate of change in log likelihood of *K* (Evanno et al. 2005) to determine the most likely number of clusters within a population using the software STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012). Individual admixture proportions (Q) for the greatest likelihood of *K* for each population were then collated from the 20 STRUCTURE runs using the software CLUMPP (Jakobsson and Rosenberg 2007), employing the GREEDY model with 100 repeats.

Differences in abiotic environment and biotic plant community between waterbodies

To examine whether the 6 (4 herbicide treatment, 2 non-treatment) sampled waterbodies (Figure 1.1) differed in their abiotic environments, we performed a principal component analysis (PCAs) on the means of 8 abiotic environmental variables (TDN, TDP, DOC, cond., temp., pH, turb., and D). Abiotic data was averaged using the mean values for each variable recorded per site. The PCA was performed using the software PC-ORD version 6 (McCune and Mefford 2011) and produced using correlation coefficients in the cross-products matrix.

A similar analysis was performed to see if these same 6 waterbodies differed in their biotic plant communities. We performed a PCA using the relativized abundance of 30 plant species collected at sixteen sites across the subset of 6 water bodies (4 herbicide treatment/2 non-treatment) (Figure 1.1). Biotic plant community data was relativized by taking the sum of all rake abundance scores for each plant species per site and dividing by the total number of rake tosses per site in order to account for different sampling efforts per site. The PCA was performed using the software PC-ORD version 6 (McCune and Mefford 2011) and produced using correlation coefficients in the cross-products matrix.

1.4 Results

Genetic diversity

Allelic marker polymorphism in the 10 invasive watermilfoil populations ranged from 2 to 11 alleles and although Myrsp6 had the greatest number of alleles expressed across all 10 populations ($N_A = 11$), Myrsp5 had the highest mean number of alleles per individual ($Mean_A = 4.4$) (Table 1.1). Across the 10 populations, a total of 69 unique alleles were expressed across all 12 microsatellite markers. Among the 1,362 individuals genotyped, we found 99 unique invasive watermilfoil genotypes (Table 1.2 and Figure 1.3). All genotypes were unique and exclusive to their respective waterbodies with the exception of one overlapping genotype between two waterbodies. Overall, waterbodies were dominated by one primary genotype with a few minority genotypes also present (Figure 1.3). Populations with no prior history of herbicide treatment had greater genotypic diversity (average number of genotypes = 19) than populations previously treated with herbicides (average number of genotypes = 4), (Table 1.2). These numbers could be affected by unequal sampling or the uneven distribution of genotypes (lower genotypic evenness) across waterbodies within each category. However, when controlled for sample size, the non-herbicide waterbodies had a greater Nei's genetic diversity index and Shannon-Weiner diversity index than the herbicide treatment water bodies (Table 1.2). Counter to our hypothesis that herbicide treatment waterbodies would have individuals with greater genetic diversity, populations from non-herbicide waterbodies had the greatest genetic diversity in our study.

Genetic differentiation and population structure across spatially separate lakes

The analysis of molecular variance (AMOVA) indicates that ninety-five percent of the genetic variation observed in the 1,362 individuals occurred between populations while only five percent of the total genetic variation occurred within waterbodies (Table 1.3). Genetic variation was correlated with geographic distance such that more similar genotypes were observed in waterbodies closer in geographic proximity (IBD, $r^2 = 0.216$, $P > 0.01$).

Populations with histories of herbicide treatment exhibited greater admixture (Figure 1.4-A). Optimal *K* for the entire dataset of 1,382 individuals, calculated using Evanno's ΔK method, was reported as $K = 5$. Individuals from herbicide treatment waterbodies had Q values comprised of multiple clusters while individuals from non-herbicide treatment waterbodies were represented by one primary cluster (Figure 1.4-A).

Seven discriminant functions were retained from the DAPC for an overall *K* means clustering of $K = 6$ (Figure 1.5). The DAPC showed similar results as STRUCTURE (optimal $K = 5$ versus $K = 6$) as individuals from separate waterbodies clustered in similar patterns (Figure 1.5-A). In contrast to STRUCTURE, the DAPC cluster assignment within each population was >95% towards one cluster with the exception of one waterbody, which was represented by multiple clusters (Figure 1.5-B). Compared to the results of the IBD analysis, clusters in the DAPC were not completely segregated in space as many populations shared a cluster with populations that spanned across the state of Michigan. The PCoA displayed similar groupings of individual genotypes as STRUCTURE and DAPC in graphical space (Figure 1.6).

Patterns of hybridization and population genetic structure within lakes

Performing post hoc selection of *K*, we found that *K* = 2 best described the data with the exception of 1 waterbody where optimal *K* = 3. (Figure 1.4-B). Evidence of admixture is more evident in populations from waterbodies with histories of herbicide treatment as individuals from all 6 treatment waterbodies have Q values between 0.01 and 0.99 and mean Q values approaching 0.50 (Figure 1.4-B and Table 1.4). However, the extent of admixture is difficult to determine because putative pure clusters of either Eurasian watermilfoil or Northern watermilfoil in our study could be comprised of highly advanced backcrossed hybrids. Since Q values only represent the probability of admixture and do not reflect the genetic contribution from parental species, individuals with Q values approximating 0 or 1 could still be the result of past hybridization events.

Differences in abiotic environment and biotic plant community between lakes with different histories of herbicide treatment

The PCA for abiotic environmental variables shows no discernible trends or patterns between herbicide treatment and non-herbicide waterbodies or environmental eigenvectors (Figure 1.7-A). In contrast, the PCA for biotic plant community shows stark differences between herbicide treatment and non-herbicide waterbodies across principal component axis 1 (Figure 1.7-B). Plant species that cluster towards herbicide treatment waterbodies (n = 11) across the first principal component axis are comprised entirely of monocot species with the exception of Eurasian watermilfoil (dicot) and aquatic moss (*Drepanocladus* sp.), a non-vascular plant. Plant species that cluster towards non-herbicide waterbodies across principal component axis 1 (n = 19) include a variety of monocot and dicot plants.

1.5 Discussion

Understanding how histories of management influence the genetic structure of invasive populations may help with the efficacy and long-term viability of control efforts. We found that invasive watermilfoil populations are dominated by one primary genotype often with a few, minority genotypes present. Populations with no prior history of herbicide treatment have greater genotypic diversity than populations previously treated with herbicides, yet they have less admixture. Evidence of hybridization is more apparent in waterbodies with histories of herbicide treatment, potentially as the result of repeated selection pressure. The biotic communities in waterbodies with histories of herbicide treatment are shifted towards less diverse communities represented disproportionately by monocot plant species.

Waterbodies with histories of herbicide treatment displayed reduced genetic diversity

In general, marker polymorphism is lower among sampled waterbodies in the introduced range than in the native range (Table 1.1, Wu et al. 2013). For example, Wu et al. 2013 published marker data for two waterbodies in the native range and found that the number of alleles per marker found in just one population exceeded the number of alleles per marker among all 10 of our sampled waterbodies combined. This indicates that allelic polymorphism is significantly reduced in the introduced range, potentially as the result of a post-establishment bottleneck. It also might limit the ability to properly utilize these markers in the introduced range for hybridization analyses like STRUCTURE, which often require 80-100 unique alleles to accurately assign individuals' admixture (Pritchard et al. 2000). We found that invasive watermilfoil populations are dominated by one primary genotype (Figure 1.3 and Table 1.2). This finding is significant because previous studies in the invaded and native ranges have focused on minimal sampling (approximately 10-20 individuals per water body or population) to understand genetic structure within lakes (Moody & Les 2007, Zuellig & Thum 2012, Wu et al. 2015, Wu et al. 2016). This established pattern of genotypic monomorphism across waterbodies could have implications for the efficacy of invasive watermilfoil sampling for genetic screenings in order to prescribe subsequent management efforts when resources for plant collection and processing of genetic data is limited.

On average, we found that populations with no history of targeted herbicide treatment had greater genetic diversity than populations with previous treatment histories (Figure 1.3 and Table 1.2). It's possible that the targeted removal efforts select for a single, resistant genotype that persists under the stressful conditions of herbicide application. However, lower diversity might also be due to other factors relating to the duration of invasion and number of introductions to each waterbody. The fact that almost half of the genotypes in our study (46 out of 99 genotypes) were found in just one population is worth noting (Figure 1.2 and Table 1.2). This outlier population could be gaining its genetic diversity through increased introduction of invasive watermilfoil propagules or, as the waterbody is likely fed by a stream where the invasive status of watermilfoil is unknown, could be a “sink” for exotic genotypes that occur upstream. Perhaps because this waterbody (and other non-treatment waterbodies) is relatively secluded and free from herbicides and other disturbances, the population is allowed to maintain relatively high levels of genetic diversity. Herbicides could therefore be selecting for genotypes that rely on quick, vegetative growth while populations under relaxed pressure could ultimately benefit long-term from increased genetic diversity by relying on sexual reproduction as a primary means of propagation.

Genetic structure among lakes differs based on history of herbicide treatment and geography

The AMOVA indicates that the majority of genetic variation (95%) occurs between populations (Table 1.3). These findings are consistent with other studies of submerged aquatic macrophytes (Koga et al. 2007), but is at odds with others (Martinez-Garrido et al. 2017) including a study that investigated the genetic relationships among lake populations of Eurasian watermilfoil in its native range (Cao et al. 2017). It is difficult to make interpretations on our results based on the differences observed in sources of genetic variation in other studies because they take place over different scales, but it is possible that the high genetic variation occurring between populations observed in our study could be the result of multiple introductions from geographically and genetically distant source populations. This could also be the result of significantly lower polymorphism observed within our study sites in the introduced range compared to sites in the native range (Table 1.1, Wu et al. 2013).

The statistically significant isolation by distance result (IBD, $r^2 = 0.216$, $P > 0.01$) corroborates the findings from the AMOVA and together can be interpreted to suggest that individuals within a single invasive watermilfoil population are genetically very similar to one another and are genetically dissimilar from individuals from other waterbodies. Given that macrophyte populations exist within aquatic islands surrounded by terrestrial seas, it is common for strong positive correlations to exist between genetic and geographic distances (i.e. isolation by distance) in aquatic plant communities (Barret et al. 1993, Santamaria 2002). The findings also suggest that watermilfoil colonization events into new water bodies likely occur at relatively short geographical distances. The genetic disparities between populations could be the result of multiple introductions of invasive watermilfoil across Michigan. It could also be that long histories of invasion and establishment of populations within the introduced range (Reed 1977) prevents or excludes further introduction in these same water bodies from new genotypes.

Although populations with no history of targeted herbicide treatment have greater genetic diversity than populations with previous treatment histories (Table 1.2), populations with histories of herbicide treatment exhibit greater admixture while populations with no history of herbicide treatment display less admixture between clusters (Figure 1.4-A). Admixed individuals could exhibit herbicide tolerant traits as mentioned in previous studies (Slade et al. 2007, Berger et al. 2009, Glomski and Netherland 2009, Berger et al. 2012, Thum et al. 2012) and are potentially undergoing positive selection in herbicide treated waterbodies. It could also be the result of an increased number of vectors (trailered boats, homes, visitors, etc) on herbicide treated waterbodies, which the researchers anecdotally noted appeared to have more development and recreational activity than the non-herbicide waterbodies.

In contrast to the AMOVA and IBD results, the DAPC and PCoA analyses show that populations don't always share the same cluster (or group) as the waterbody closest in geographic proximity (Figures 1.5 and 1.6). While some nearby populations shared the same cluster, some clusters stretch across large geographic areas, spanning the Upper and Lower Peninsulas of Michigan. In the southern half of the state, clusters largely overlap. This could be the result of a longer history of invasion in southern Michigan relative to the northern portion of the state. It could also be the result of an increased number of human vectors through larger human populations, connections via highways, and easy access to public boat landings that allow for increased mixing across water bodies in the south relative to the north.

Evidence of hybridization occurred more frequently in waterbodies with histories of herbicide treatment

The results of STRUCTURE run at the individual population scale indicate that most populations are dominated by a single genetic class with very little overlap or co-occurrence of multiple watermilfoil classes within the same waterbody (Figure 1.4-B). These findings are similar to the findings of Moody and Les 2010 (introduced range) and Wu et al. 2015 (native range) which showed a pattern of isolation and dominance of either Eurasian, Northern, or hybrid watermilfoil genotypes within lakes but little to no evidence of these classes co-occurring within the same lake. This pattern of limited co-occurrence of genetic classes could indicate that some form of competitive exclusion is taking place between classes in both the native and introduced ranges. As one genotype or genetic class establishes, it could quickly and significantly reduce available niche space making further colonization difficult. It could also indicate a relative rarity of pure Northern watermilfoil lineages in Michigan and the potential for a cryptic invasion as Northern watermilfoil genes and populations become supplanted by Eurasian watermilfoil genes through gradual introgression and advanced backcrossing towards Eurasian watermilfoil genomes. A similar pattern occurred during hybridization events in the species' co-occurring native range when admixed individuals showed significant backcrossing towards Eurasian watermilfoil (Wu et al. 2015).

Evidence of admixture and hybridization appears to be more frequent in waterbodies with histories of herbicide treatment (Figure 1.4-B). At least one previous study has indicated that, in its introduced range, hybrid watermilfoil occurs more frequently in 2,4-D treated waterbodies belonging to the same watershed (LaRue et al. 2013b). As studies have demonstrated that hybrid watermilfoils can display less sensitivity to herbicides, herbicides could be selecting for admixed individuals that possess these herbicide resistant traits. Given their potential dominance under these artificial selection regimes, this could also explain why hybrid lineages rarely co-occur with pure parental lineages in the same herbicide-treated waterbody (Figure 1.4-B).

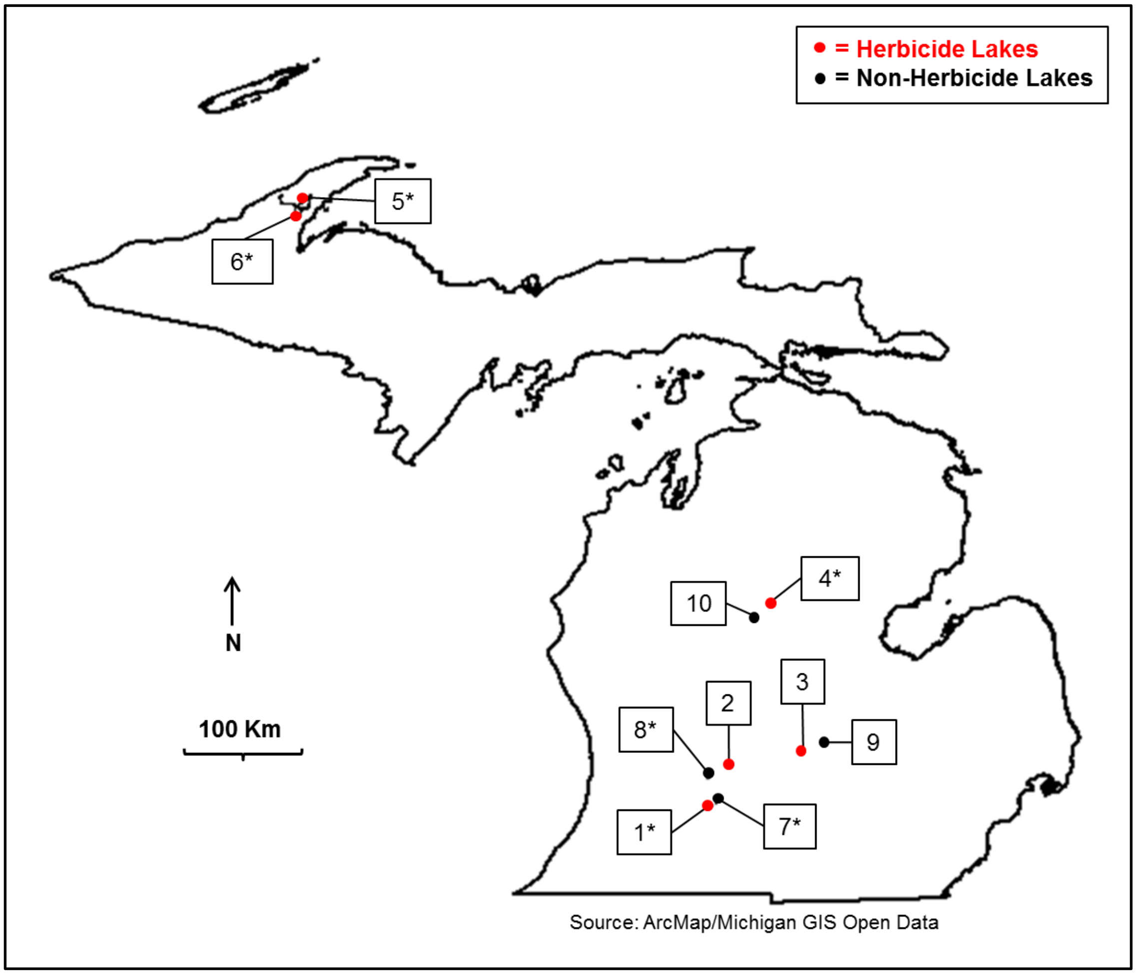
Biotic communities with histories of herbicide treatment are different than communities in non-treatment waterbodies

Waterbodies with histories of herbicide treatment group together across the first principal component axis (Figure 1.7-B). All plant species with a positive value for axis 1 and an eigenvector towards herbicide treatment waterbodies (n = 11) are monocots with the exception of Eurasian watermilfoil and a non-vascular aquatic moss species. This shift towards monocots in herbicide treatment water bodies is logical given that herbicides used in these waterbodies (2,4-D and triclopyr) are broadleaf selective herbicides meaning that they are designed to target dicots (like watermilfoils) while not harming aquatic grasses and pondweeds (monocots) (Tu et al. 2001). Similar findings of healthy monocot communities in waterbodies being treated with the herbicide fluridone in order to target invasive watermilfoil have been previously reported (Madsen et al. 2002). This shift towards monocot dominated communities could be beneficial if thin-leaved monocot species can effectively compete against invasive watermilfoils. However, the continued use of herbicides targeting dicots could suppress growth of plants such as native watermilfoils, which could potentially compete more effectively with invasive watermilfoils in a sustained management scenario.

Management implications

Our research suggests that repeated exposure to herbicides could be selecting for populations of invasive watermilfoil that have lower genetic diversity, yet possess the capacity to sustain invasions in waterbodies with histories of herbicide treatment. We found that populations with histories of herbicide treatment have more admixture than non-herbicide treatment populations. These populations could be more tolerant and better able to withstand herbicide stressors than populations comprised of non-admixed individuals. This could create some difficult scenarios for lake managers who want a fast and relatively inexpensive treatment method (such as herbicides) but might pay greater costs over time as herbicides select for increased admixture and resistance. Somewhat surprisingly, plant communities appear to be shaped by histories of herbicide treatment too. These findings indicate that herbicides are likely impacting the ecology and competitive environment for aquatic macrophytes. Further study into the competitive dynamics between plants in or from herbicide treatment environments could shed light on the potential long term ecological impacts of herbicide treatments.

1.6 Figures



Source: ArcMap/Michigan GIS Open Data

Figure 1.1 – Sampled populations of invasive watermilfoil across 10 water bodies in Michigan. (See Table A.1 for water body names and geographic positioning coordinates). *Asterisk denotes water bodies sampled for abiotic and biotic data as described in text.

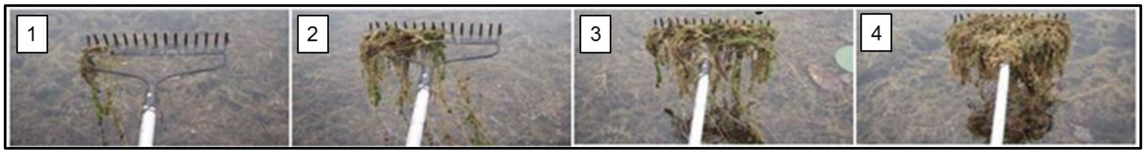
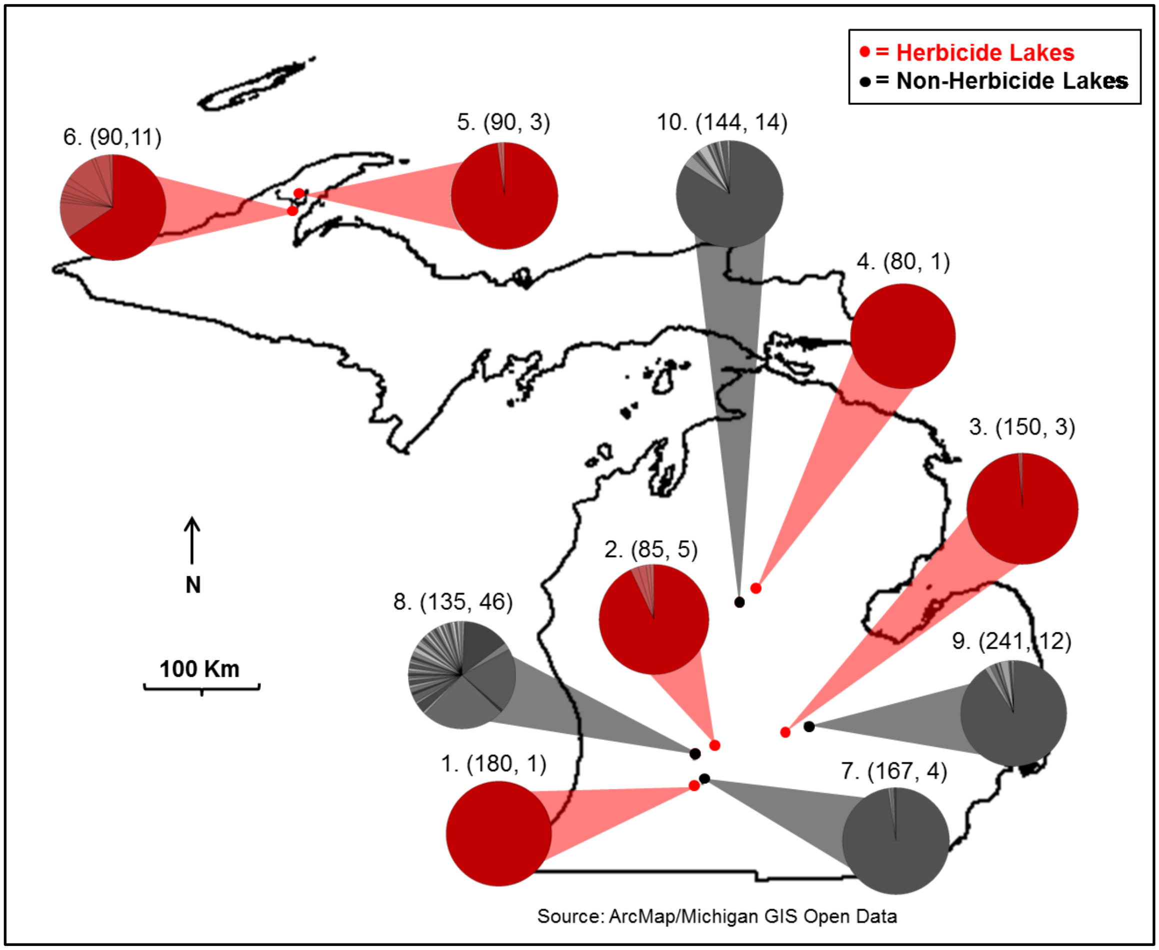


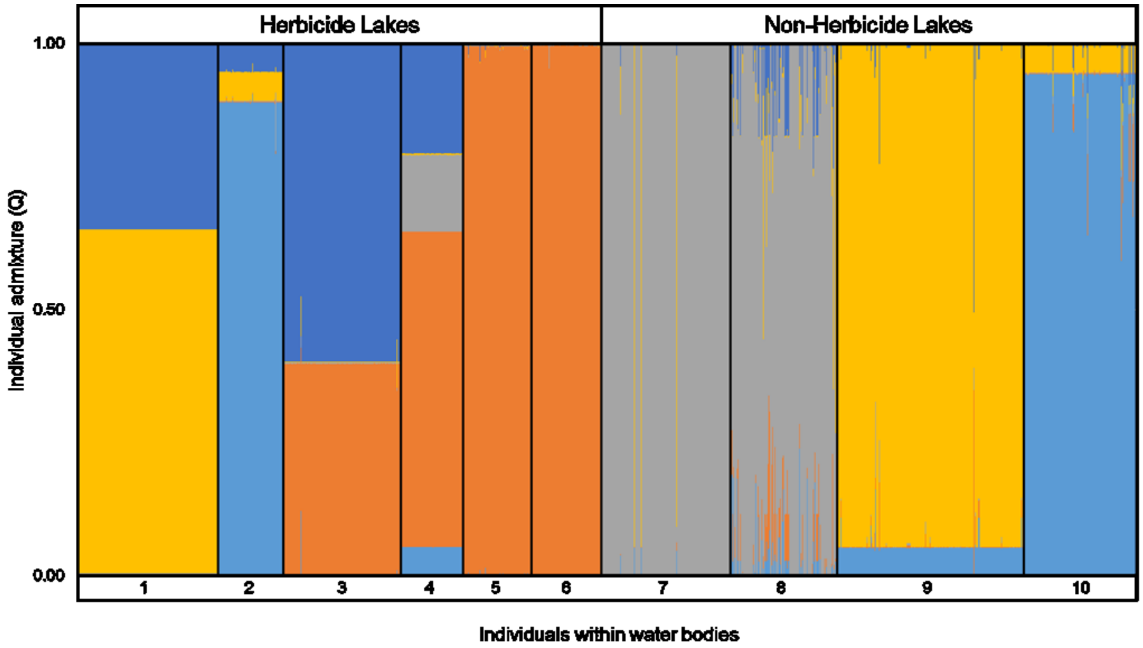
Figure 1.2 – Ordinal metric of relative aquatic macrophyte abundance retrieved during rake tosses. Images from Many Waters, LLC. and USFS Ottawa National Forest.



Source: ArcMap/Michigan GIS Open Data

Figure 1.3 – Genotypic diversity among populations of invasive watermilfoil across 10 Michigan water bodies with histories of herbicide treatment (red) and water bodies with no history of herbicide treatment (black). Pie charts represent the genotypic composition of water bodies and individual segments represent the relative proportion of an individual genotype. The first number above each pie represents the water body identification number specified in Table 1.1 followed by the (number of individuals sampled and number of genotypes identified within each water body). This map was produced using ArcMap (ESRI 2018 - v. 10.6) and geographic data taken from Michigan GIS Open Data (State of Michigan 2018).

A.


Herbicide Lakes
Non-Herbicide Lakes
Individual admixture (Q)
1.00
0.50
0.00
1
2
3
4
5
6
7
8
9
10
Individuals within water bodies

B.

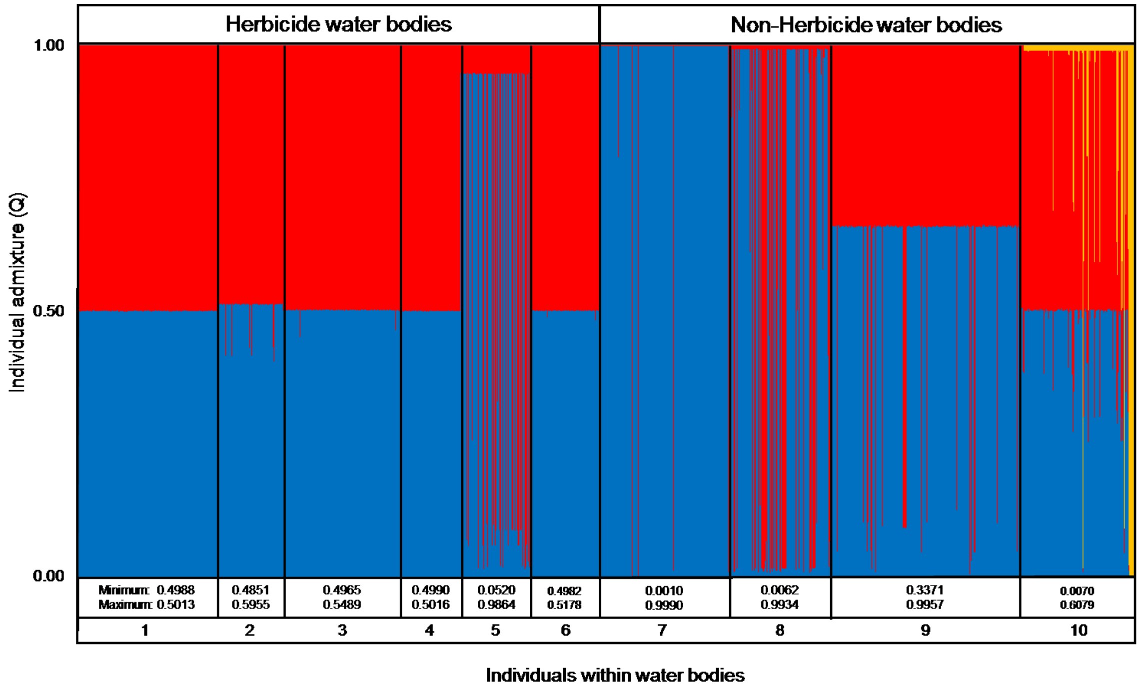

Herbicide water bodies
Non-Herbicide water bodies
Individual admixture (Q)
1.00
0.50
0.00
Minimum: 0.4988
Maximum: 0.5013
0.4851
0.5955
0.4965
0.5489
0.4990
0.5016
0.0520
0.9864
0.4982
0.5178
0.0010
0.9990
0.0062
0.9934
0.3371
0.9957
0.0070
0.6079
1
2
3
4
5
6
7
8
9
10
Individuals within water bodies

Figure 1.4 – A.) Admixture coefficient and optimal number of clusters based on rate of change in log likelihood of Δ*K* (Evanno et al. 2005) for all 1,362 sampled watermilfoil plants from 10 Michigan waterbodies run simultaneously. Optimal number of clusters was $K = 5$ for all populations. Numbers across x-axis represent individual water bodies found in (Table 1). A single vertical bar displays the membership coefficient of each individual and colors represent individual assignment to one of 5 clusters. B.) Admixture coefficient and optimal number of clusters based on rate of change in log likelihood of Δ*K* (Evanno et al. 2005) for all 1,362 sampled watermilfoil plants from 10 Michigan waterbodies run on an individual waterbody basis. Optimal number of clusters was $K = 2$ for each population with the exception of one lake which had an optimal number of clusters of $K = 3$. Numbers across x-axis represent individual water bodies (found in Table 1). A single vertical bar displays the membership coefficient of each individual. Blue represents the putative Eurasian watermilfoil (*M. spicatum*) cluster and red represents the putative Northern watermilfoil (*M. sibiricum*) cluster.

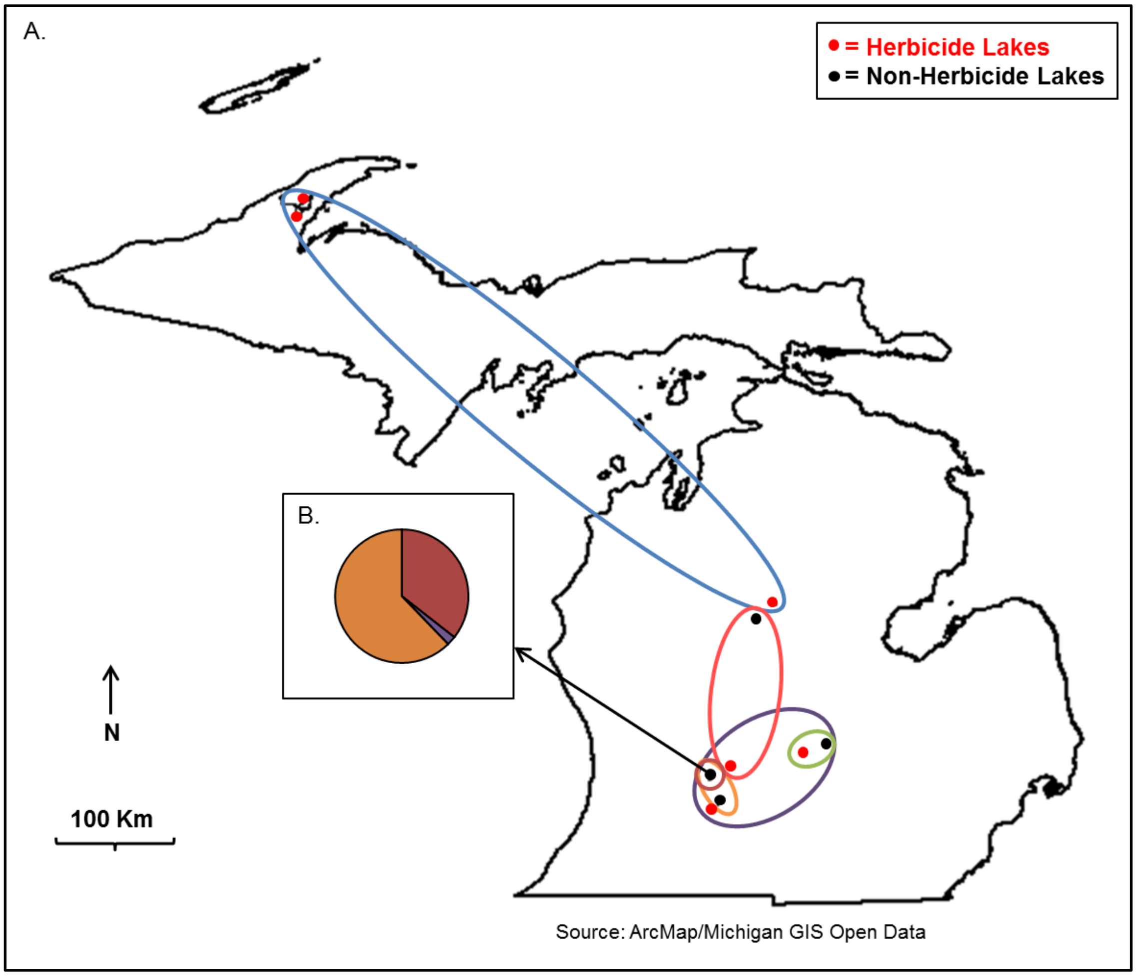


Figure 1.5 – A. Map of discriminant analysis of principle component (DAPC) clusters produced using default settings in the R package *adegenet*. Populations are represented as circles and clusters as inertia ellipses. Cluster assignment within each population was >95% towards one cluster, with the exception of Carter Lake (B.), which was represented by multiple clusters. Segments within the pie chart represent the proportion of individuals belonging to corresponding clusters with the same ellipse color. This map was produced using ArcMap (ESRI 2018 - v. 10.6) and geographic data taken from Michigan GIS Open Data (State of Michigan 2018).

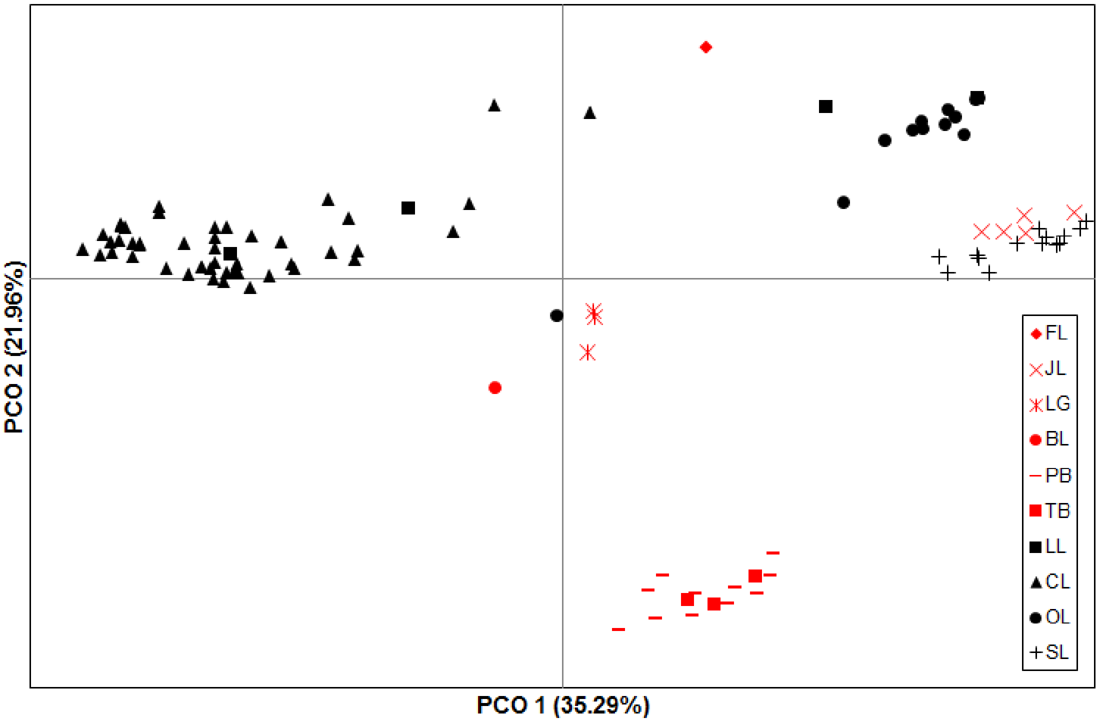
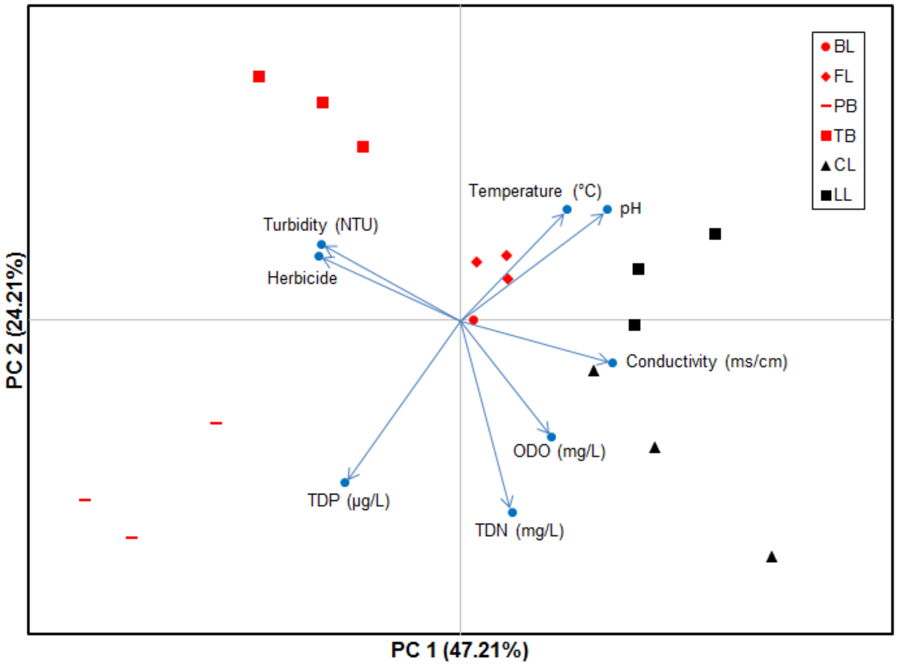


Figure 1.6 – Principal coordinate analysis (PCoA) of the pairwise genetic Lynch distances (Lynch 1990) among all sampled genotypes from 10 populations of invasive watermilfoil in Michigan water bodies. PCoA performed using the software GenAlEx v. 6.5 (Peakall and Smouse 2012) with pairwise genetic Lynch distances calculated using the R package POLYSAT (Clark and Jasieniuk 2011). Red symbols represent genotypes found in herbicide treatment water bodies and black symbols represent genotypes found in non-herbicide water bodies.

A.


BL
FL
PB
TB
CL
LL
Temperature (°C)
pH
Turbidity (NTU)
Herbicide
Conductivity (ms/cm)
ODO (mg/L)
TDP (µg/L)
TDN (mg/L)
PC 2 (24.21%)
PC 1 (47.21%)

B.

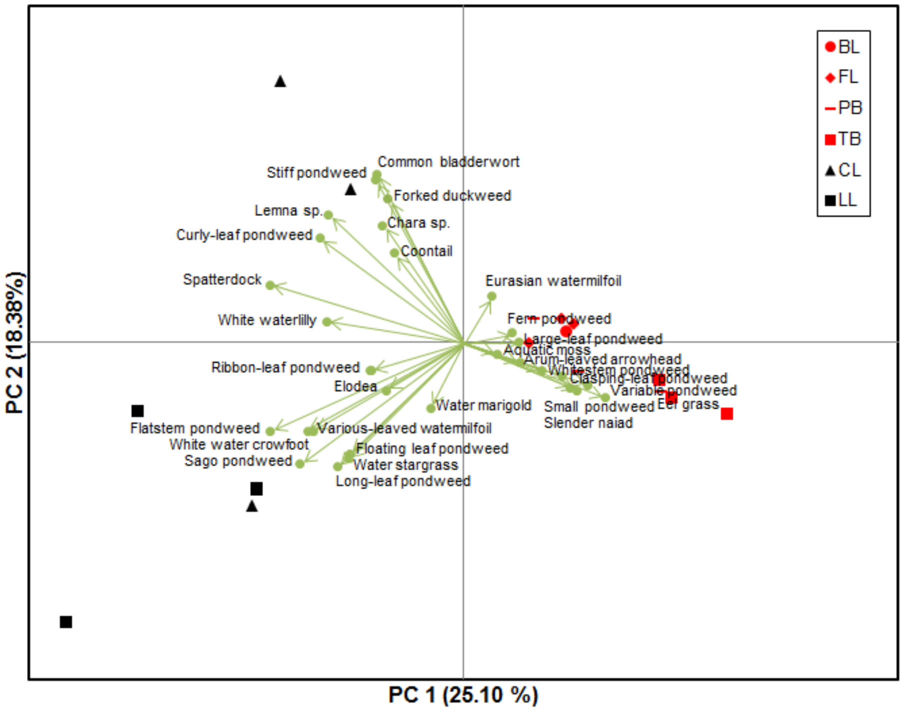

BL
FL
PB
TB
CL
LL
Common bladderwort
Stiff pondweed
Forked duckweed
Lemna sp.
Chara sp.
Curly-leaf pondweed
Coontail
Spatterdock
Eurasian watermilfoil
White waterlilly
Fern pondweed
Large-leaf pondweed
Aquatic moss
Arum-leaved arrowhead
Whitestem pondweed
Clasping-leaf pondweed
Variable pondweed
Ribbon-leaf pondweed
Elodea
Water marigold
Small pondweed
Eel grass
Slender naiad
Flatstem pondweed
Various-leaved watermilfoil
White water crowfoot
Floating leaf pondweed
Sago pondweed
Water stargrass
Long-leaf pondweed
PC 2 (18.38%)
PC 1 (25.10 %)

Figure 1.7 – Principal component analysis (PCA) of A.) abiotic environmental variables and B.) biotic plant communities per site. Red symbols represent waterbody sites with histories of herbicide treatment and black symbols represent sites with no history of herbicide treatment. Blue arrows represent the corresponding eigenvectors for PC 1 and PC 2 for abiotic variables and green arrows represent the corresponding eigenvectors for PC 1 and PC 2 for biotic plant communities.

1.7 Tables

Table 1.1 – Locus, repeat unit, and allelic diversity results per marker for 12 microsatellite loci (developed by Wu et al. 2013) used in the study of invasive watermilfoil populations in 10 Michigan water bodies. N_A = total number of alleles per loci, Min_A = minimum number of alleles per loci, Max_A maximum number of alleles per loci, $Mean_A$ = mean number of alleles per loci.

Locus	Repeat unit	N_A	Min_A	Max_A	$Mean_A$
Myrsp1	Tri	4	2	4	2.4
Myrsp4	Di	8	2	6	3.3
Myrsp5	Di	7	3	6	4.4
Myrsp6	Di	11	1	5	2.9
Myrsp8	Di	2	2	2	2.0
Myrsp9	Tri	5	2	4	2.7
Myrsp10	Tri	5	2	4	2.5
Myrsp12	Di	3	1	2	1.9
Myrsp14	Di	7	3	5	3.3
Myrsp15	Di	9	3	6	3.9
Myrsp16	Di	4	3	4	3.5
Myrsp18	Tri	4	2	3	2.4

Table 1.2 – Genetic diversity indices of 10 populations of invasive watermilfoil across Michigan in A.) water bodies with histories of herbicide treatment, and B.) water bodies with no history of herbicide treatment. N = number of individual plants sampled per water body, G = number of genotypes identified per water body using the software GENOTYPE (Merimans & Van Tienderen 2004). Genetic diversity indices were calculated using the software GENODIVE (Merimans & Van Tienderen 2004) and included, G_e = effective number of genotypes (Lehman & Wayne 1991). Eve. = genotypic evenness. Nei's SS = Nei's genetic diversity corrected for sample size (Nei 1987). HSS = Shannon-Weiner diversity index corrected for sample size (Chao & Shen 2003).

A.	Herbicide	N	G	G_{Eff}	G_{Eve}	Nei's SS	HSS
1	Fine Lake	180	1	1.00	1.00	0.00	-0.00
2	Jordan Lake	85	5	1.16	0.23	0.14	0.20
3	Lake Geneva	150	3	1.03	0.34	0.03	0.06
4	Budd Lake	80	1	1.00	1.00	0.00	-0.00
5	Pike Bay	90	11	2.20	0.20	0.20	0.63
6	Torch Bay	90	3	1.06	0.35	0.06	0.09
	Mean		**4**	**1.24**	**0.52**	**0.07**	**0.16**
B.	Non-Herbicide	N	G	G_{Eff}	G_{Eve}	Nei's SS	HSS
7	Long Lake	167	4	1.05	0.26	0.05	0.08
8	Carter Lake	135	46	7.98	0.17	0.88	1.44
9	Lake Ovid	241	12	1.21	0.10	0.17	0.27
10	Silver Lake	144	14	1.41	0.10	0.29	0.45
	Mean		**19**	**2.91**	**0.16**	**0.35**	**0.56**

Table 1.3 – Analysis of molecular variance (AMOVA) table for populations of invasive watermilfoil in 10 Michigan lakes. AMOVA calculated using the software GenAlEx v. 6.5 (Peakall and Smouse 2012).

Source	df	MS	Percent molecular variance	*P*
Among Populations	9	1287.008	95%	0.001
Within Populations	1352	0.532	5%	
Total	1361		100%	

Table 1.4 – Admixture analysis for 10 populations of invasive watermilfoil across Michigan in A.) water bodies with histories of herbicide treatment, and B.) water bodies with no history of herbicide treatment. Admixed defined as individuals with Q values 0.01<Q<0.99.

A.	Herbicide	Mean Q	Admixed individuals	Percent admixed
1	Fine Lake	0.50	180	100%
2	Jordan Lake	0.49	85	100%
3	Lake Geneva	0.50	150	100%
4	Budd Lake	0.50	80	100%
5	Pike Bay	0.36	90	100%
6	Torch Bay	0.50	90	100%
B.	Non-herbicide	Mean Q	Admixed individuals	Percent admixed
7	Long Lake	0.02	2	1%
8	Carter Lake	0.37	50	37%
9	Lake Ovid	0.39	239	99%
10	Silver Lake	0.11	138	96%

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2 Implications for Management: Herbicide treatments may influence the evolution of invasiveness in Eurasian watermilfoil (*Myriophyllum spicatum*)

2.1 Abstract

Invading populations often occupy novel environments in their introduced range, which can have dramatically different biotic and abiotic conditions relative to their natal environments. These novel environments have the potential to shape the post-establishment evolution of invading populations, further influencing their fitness and can have long-term implications for management practices. Here, we sought to understand whether repeated exposure to herbicides (a novel environment) might affect the survival and/or growth of the invasive macrophyte, Eurasian watermilfoil (*Myriophyllum spicatum*). We grew invasive watermilfoil plants that were collected from lakes with and without a history of repeated exposure to herbicides together in mesocosms. Furthermore, because nutrient levels (specifically nitrogen and phosphorus) in the water column can vary and influence growth and survival, we also experimentally manipulated nutrient levels (low or high) among mesocosms. We found that a history of herbicide treatment significantly affected plant survival, net growth, and mean growth rate and the effects depended upon whether neighboring plants were from herbicide or non-herbicide treatment lakes. Plants from lakes with histories of herbicide treatment were more likely to survive and grew faster than plants collected from lakes with no prior exposure to herbicides, but these differences were lessened when grown with other plants from herbicide treated lakes. Surprisingly, nutrients did not affect plant survival and had a marginal significant outcome on net total growth. Our results suggest that repeated

exposure to chemical herbicides could select for faster growing, and thereby more invasive, Eurasian watermilfoil genotypes. This could have implications for sustaining long-term management efforts as populations routinely treated with herbicides could exhibit increased growth and survival relative to their non-herbicide counterparts.

2.2 Introduction

Biological invasions occur when a population of an introduced species gains a competitive advantage following the removal of natural restrictions to its propagation, which allows for rapid spread and colonization of novel territory in ecosystems where it has a dominant ecological impact (Valéry et al. 2008, Lodge et al. 1993, Vitousek et al. 1996). Selective forces during biological invasions, such as abiotic and biotic environmental attributes and requirements for suitable mates, can contribute to whether some species are able to invade and/or proliferate in novel habitats (Havel et al. 2005, Lee and Gelembuik 2008, Hufbauer et al. 2012). While some invading populations might benefit from a release of constraints found in their native range, such as the removal of top-down control agents like herbivores, predators, or parasites that would regulate population sizes (Keane and Crawley 2002, Colautti et al. 2004), others may face novel forces in their introduced range in the form of control agents and uneven distribution of resources (Mooney and Cleland 2001, Davis et al. 2000). Different histories of exposure to control agents between introduced populations of the same species can influence the evolutionary trajectories of invading populations and their relative expression of invasive traits (Müller-Schärer et al. 2004, Prentis et al. 2008, Tayeh et al. 2014). Traits that are common among invasive species include relatively fast growth (Sakai et al. 2001, Van

Kleunen et al. 2010) large reproductive output (Lockwood et al. 2005), and a high degree of phenotypic plasticity (Richards et al. 2006, Davidson et al. 2011). How different histories of exposure to control agents influence the evolutionary trajectory of invading populations and their relative expression of invasive traits remains to be thoroughly tested in many invasive aquatic plant species.

Populations of invasive or weedy plants routinely exposed to chemical herbicides as control agents often evolve coping mechanisms for tolerating the stress of targeted control (Jasieniuk et al. 1996, Richardson 2008, Powles and Yu 2010). For example, some biotypes of the invasive aquatic macrophyte Hydrilla (*Hydrilla verticillata*) have evolved resistance to the herbicide fluridone as the result of somatic mutations to the gene that produces the enzyme fluridone is designed to suppress (Michel et al. 2004, Arias et al. 2005). Once established, herbicide resistant biotypes can rapidly cover large areas of water and displace native plant communities, causing significant harm to native ecosystems (Schmitz et al. 1993, Bates & Smith 1994). These herbicide resistance traits can be advantageous for invasive species, but does the expression of herbicide resistance traits come at an evolutionary or ecological expense?

In many invasive plant species, fitness tradeoffs are thought to arise because plants allocate resources to survival or stress tolerance that would otherwise be allocated to growth or reproduction if the herbicide resistance traits were not expressed (Vila-Aiub et al 2009, van Etten et al. 2016, Bingham et al. 2017). In a meta-analysis of over 200 plant species, Bergelson & Purrington (1996) found that more than 50% of the populations that they reviewed that had been exposed to herbicides showed measurable fitness tradeoffs in the form of reduced growth or reproductive output. However, on rare occasions increased growth and/or reproduction have been reported despite the development of herbicide resistance (Wang et al 2010, Vila-Aiub et al 2015, Wu et al. 2018).

Eurasian watermilfoil is an aquatic macrophyte that is native to Europe, Asia, and North Africa (Couch and Nelson 1985), but highly invasive in North America (Aiken et al. 1979, Smith and Barko 1990, Madsen 1994). Eurasian watermilfoil was first documented in North America in the late nineteenth century in the area surrounding Chesapeake Bay (Reed 1977) and is now widespread across North America, occurring in 48 out of 50 U.S. states and 3 out of 10 Canadian provinces (United States Geological Survey 2018). Eurasian watermilfoil is successful in part because it grows quickly relative to other macrophytes, forming dense canopies that outcompete and displace native vegetation through exclusion to resources such as light (Smith and Barko 1990, Madsen et al. 1991, Madsen 1994). Eurasian watermilfoil thrives in environments rich with available resources such as nitrogen and phosphorus (Omernick et al. 1991, Buchan and Padilla 2000, Feng et al. 2015) and dominates plant communities through increased competitiveness under these elevated resource conditions (Madsen 1998). It has recently been documented that part of Eurasian watermilfoil's success may rely on its ability to hybridize with native Northern watermilfoil (*Myriophyllum sibiricum* Komarov). Hybirdization between the two species was first documented in North America in 2002 (Moody and Les 2002) and has since been reported several times across its introduced range (Moody and Les 2007, Strutevant et al. 2009, Zuellig and Thum 2012, Borrowman et al. 2014) and the two species' co-occurring native range of central Asia (Wu et al.

2015). It has also been reported that hybrid watermilfoil can exhibit increased growth rates relative to its parent species (LaRue et al. 2013), possibly as the result of the novel recombination of genomes and subsequent trait expression (i.e. “heterosis”, Ellstrand and Schierenbeck 2000). Since hybrids and parental species overlap in morphology and hybrid status can only be determined through genetic analyses (Moody and Les 2010), Eurasian watermilfoil and hybrid watermilfoil will henceforth be referred to as invasive watermilfoil.

Treatments for the eradication and control of invasive watermilfoil include application of chemical herbicides (e.g., 2,4-Dichlorophenoxyacetic acid, triclopyr, and fluridone), the physical removal of plant material (e.g., mechanical and diver assisted harvesting), biological control agents (e.g., milfoil weevils, *Euhrychiopsis lecontei* and the fungus *Mycoleptodiscus terrestris*) and/or some combination of the above. While these treatments have shown short term viability, in many instances they have not proven to be successful long term and populations of invasive watermilfoil have rebounded (Nichols and Shaw 1983, Roley and Newman 2006, Nelson and Shearer 2008, Poovey et al. 2007, Berger et al. 2012). Increasing rates of herbicide resistance have been reported among populations of invasive watermilfoil, possibly as the result of hybridization (Slade et al. 2007, Berger et al. 2009, Glomski and Netherland 2009, Berger et al. 2012, Thum et al. 2012).

Here, we tested whether Eurasian watermilfoil (*Myriophyllum spicatum*) plants from waterbodies that have experienced repeated exposure to herbicides expressed reduced fitness (survival or growth rates) as compared to invasive watermilfoil plants from waterbodies that have not been treated with herbicides. We grew invasive watermilfoil plants collected from lakes with and without histories of repeated exposure to herbicides together in mesocosms where we also manipulated nutrient levels among mesocosms. We varied nutrient levels in order to examine whether any measured growth or evolutionary responses to herbicide exposure may be dependent upon nutrient availabilities as invasive watermilfoils exhibit a spectrum in their invasibility across habitats that range in available nutrients (Madsen 1998). We predicted that populations with histories of herbicide treatment would exhibit a tradeoff in their expression of invasive traits related to growth as the result of prior selection towards the allocation of resources to tolerate against repetitive herbicide exposure.

2.3 Materials and Methods

2.2 Experimental design

We collected plants from four water bodies in Michigan (Figure 2.1): two water bodies with repeated treatments of the herbicides 2,4-D and triclopyr between 2013-2016 (H) and two water bodies that have had no documented history of being previously treated with chemical herbicides (C; MI DEQ 2018). As invasive watermilfoil reproduces asexually through horizontal stolon growth, plant fragments were collected using a combination of rake tosses and rake twists (Kenow 2007) at spatial intervals of 5 m in order to reduce the number of individuals belonging to the same ramet. To acclimate plants to similar environmental conditions prior to experimentation, we planted 10-20 cm of a growing tip from a healthy plant (fragments) in 70 cm^2 plastic pots filled with potting soil and capped with sand. All pots were then placed into 2,650 liter flow-through tanks (Living Stream®, Frigid Units Inc., Toledo, OH, USA) that were continuously filled with water from the nearby Portage Lake Canal and exposed to a 12/12 hour light/dark cycle (8 Sylvania 40W Gro-Lux wide spectrum lights).

After a minimum of 3 generations of vegetative propagation, we selected 96 healthy fragments (48 from herbicide and non-herbicicde treated lakes); due to some mortality of cultured plants, the number of plants from the four different lakes were unevenly represented. Plants were planted together with another plant in a pot to represent one of three different types of neighborhood treatments: plants collected from herbicide treatment lakes grown with plants collected from herbicide treatment lakes, plants collected from herbicide treatment lakes grown with plants collected from non-herbicide control lakes, and plants collected from non-herbicide treatment lakes grown with plants collected from non-herbicide control lakes (Figure 2.2). Within each pot, plants were separated 10 cm from each other and the walls of the pot to maintain 314 cm^2/10 cm radius circle of uninhibited initial growth. All pots were filled with approximately 40% potting soil and capped with ~5 cm of sand to prevent the soil from leaching into the water column.

Pots were then divided into eight 378.5-L mesocosms (6 pots/mesocosm) and nutrients were altered such that 4 mesocosms had low nutrient additions and 4 had high nutrient additions. The low nutrient treatments were comprised of dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) in a 16 to 1 N:P molar ratio (Redfield 1934) in order to simulate concentrations of nutrients (18 µg/L DIN, 2 µg/L

SRP) found in the nearby Portage waterway (Ortiz et al. 2017). Four mesocosms were supplied with a higher dose of nutrients equivalent to concentrations of total phosphorus (20-30 µg/L) reported in lakes with littoral zones dominated by Eurasian watermilfoil (Madsen 1998) while maintaining Redfield ratio (180 µg/L DIN, 20 µg/L SRP). Nutrients were added every five days in the form of aqueous concentrated solutions to each mesocosm's water column. Mesocosms were continuously filled at an approximate rate of 1 liter per minute with water from the Portage Lake Canal. To reduce heating from sunlight radiation and to prevent release of pollen in the event of flowering, all mesocosms were covered with a 70% shade cloth.

We measured plant survival (yes or no), days from planting to mortality, plant growth metrics, and algal cover every five days for all plants for 7 weeks (August 9th to September 28th 2017). Plant growth was measured as 1.) net total growth (sum total length of all stems to the nearest 0.2 cm minus the initial length) and 2.) mean relative growth rate (net total growth rate/number of days of observation or survival). Because all plants were covered with epiphytic algae, which could influence survival or growth of plants, we also measured algae coverage on an ordinal scale with 0 = no algae coverage, 1 = 1-33% algae coverage, 2 = 34-66% algae coverage, 3 = 67-99% algae coverage, and 4 = 100% algae coverage. Plant mean algae coverage scores were calculated as the sum of the algae coverage scores divided by the number of measurement dates each plant had survived.

Statistical Analyses

We examined whether a history of herbicide treatment (H or C) for an individual focal plant, the history of herbicide treatment in their neighbor (H or C), the nutrient environment (low or high), and/or epiphytic algal load influenced mortality and growth metrics using a series of statistical tests. In all analyses, factors were treated as fixed effects and transformations were made to meet model assumptions where required (as noted below). Tukey's HSD tests were used to compare for significant differences among means when an interaction was significant ($P < 0.05$). All analyses were conducted in JMP® version 13.0 (SAS Institute Inc., Cary, NC).

To test whether a history of herbicide treatment for an individual plant, the history of herbicide treatment for their neighbor, the nutrient environment, and/or interactions among these variables influenced the probability of survival (yes or no) we used nominal logistic regression. Because many of the plants were covered with algae, we also used logistic regression to examine whether a history of herbicide treatment and/or algae coverage influenced plant survival (yes or no).

Before subsequent analyses, we excluded 8 plants that experienced broken stems during the study and we could not obtain accurate measures of growth. To test whether a history of herbicide treatment for an individual focal plant, the history of herbicide treatment for their neighbor, the nutrient environment, and/or interactions among these variables influenced net total growth rate (log10 transformed) or mean growth rate responses, we used analysis of variance (ANOVA) models.

2.4 Results

Out of the 96 plant fragments, 49 survived until the end of the experiment. Overall, the herbicide history of the focal plant had a significant effect on the likelihood of plant survival (Table 2.1) where plants from herbicide treatment lakes were more likely to survive (32 out of 48 plants survived) than plants with no history of herbicide treatment (17 out of 48 plants survived). However, the effect of the herbicide history of the focal plant depended upon the herbicide history of its neighbor. Whereas focal plants from herbicide treatment lakes were more likely to survive when the neighbor was from a non-herbicide treatment lake, focal plants from non-herbicide treatment lakes were less likely to survive when growing with plants from herbicide treatment lakes (Figure 2.1). History of herbicide treatment had a significant impact on plant survival while mean algae growth had a marginally significant impact on plant survival (Table 2.2)

Where an invasive watermilfoil plant was collected (its history of herbicide treatment) significantly affected its growth attributes (Tables 2.3 and 2.4). For net growth and mean relative growth rate, the effects depended upon whether neighbors were from herbicide treatment lakes or non-herbicide control lakes (Tables 2.3 and 2.4). Plants from lakes with histories of herbicide treatment growing with plants from the same source expressed significantly increased net growth and mean relative growth rate compared to plants from non-treatment lakes growing with other plants from non-treatment lakes (Figures 2.4 and 2.5). Nutrient treatment also had a marginal significant effect (P = 0.0543) on net total growth of plants (Figure 2.4). For net growth, plants growing in high nutrient treatments exhibited a least squares mean of 8.46 cm (± 1.204 standard error) while plants growing in low nutrients had a least squares mean of 5.1 (± 1.198 standard error) following back transformation.

2.5 Discussion

Understanding how repeated efforts to control biological invasions influence a population's expression of invasive traits is important for predicting future invasion dynamics and enlisting the best possible management practices. Our findings demonstrate that invasive watermilfoil plants from lakes with histories of repeated exposure to chemical herbicides are better survivors and exhibit increased growth relative to plants from lakes with no history of herbicide treatment.

Performance depends on treatment history and neighbors

Although plants collected from waterbodies with a history of chemical treatment performed better than plants collected from waterbodies with no history of treatment, plant performance was dependent on the neighborhood that a focal plant occupied. Plant survival was greatest among plants from herbicide treatment water bodies when growing alongside plants from non-herbicide treatment waterbodies (Figure 2.3). In contrast, plant survival was lowest among individuals from non-herbicide treatment waterbodies growing alongside other individuals from non-herbicide treatment waterbodies (Figure 2.3). These patterns of highest performance (H plant grown with C plant) and lowest performance (C plant grown with C plant) were similar for net total growth and relative growth rate (Figures 2.4 and 2.5).

Although not directly measured, more intraspecific competition could be taking place between genetically similar individuals grown together in the same neighborhood as focal plants expressed increased survivorship and growth when grown with neighbors from different populations (Figures 2.3, 2.4, 2.5). In another study that examined the population genetic structure of invasive watermilfoil in these same lakes, we found that most lake populations were composed of a few genotypes and that individuals within lakes shared more genetic similarities with each other than with individuals from other lakes (Zallek 2018, unpublished master's thesis). Populations of genetically and phenotypically similar individuals often exhibit increased intraspecific competition between individuals as they compete for shared resources in similar ways (Wilson and Turelli 1986, Abrams et al. 2008, Bolnick et al. 2011). If survivorship of individual plants is dependent upon the genetic composition of its neighbor, then we could predict that populations of invasive watermilfoil with increased genetic richness will be able to grow in higher densities than populations comprised of a single genotype.

Another explanation for the variation in expression of growth traits and survivorship among plants in this study could be the result of variation in genetic diversity and admixture among sampled source populations. At least one previous study has indicated that as Eurasian watermilfoil hybridizes with Northern watermilfoil, hybrid offspring can exhibit increased growth rate and demonstrate less sensitivity to herbicides relative to pure parental species (LaRue et al. 2013). Although the plants in this study were not genotyped or tested for admixture, our knowledge of the background levels of genetic diversity and admixture within the study populations indicates that the plants from source populations comprising the majority of our plant samples have very different patterns of genetic diversity and admixture. Herbicide treatment lakes have less genotypic diversity and more evidence of admixture relative to populations from non-herbicide control lakes (Zallek 2018, unpublished master's thesis). Plants from herbicide treatment lakes (populations with greater admixture but less genotypic richness) experienced less mortality and increased growth relative to plants from non-herbicide control lakes, which have less admixture but more genotypic diversity. Therefore, herbicides could be selecting for populations comprised of fewer genotypes exhibiting increased admixture and invasiveness. Since herbicide treatment lakes are characterized by very few genotypes, herbicides could also be selecting for invasive watermilfoil lineages that disproportionately rely on fragmentation and asexual reproduction as a primary means of propagation.

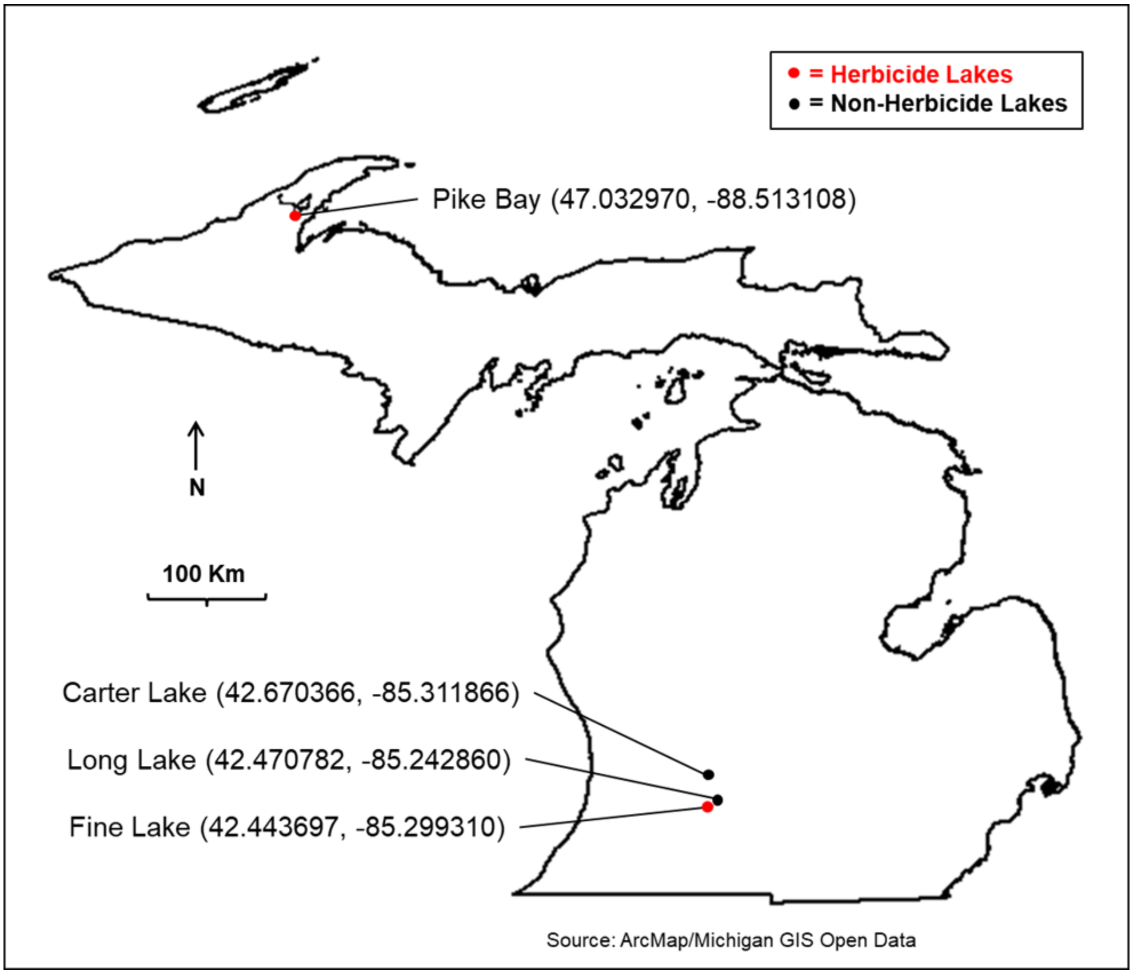
Treatment to control invasive species may influence invasiveness

Invasive watermilfoil plants from herbicide treatment lakes express significantly greater net total growth and relative growth rates when grown alongside other plants from herbicide treatment lakes versus plants from non-herbicide control lakes growing with other plants from non-herbicide control lakes. These results are counter to our initial hypothesis that invasive watermilfoil populations from non-herbicide lakes will exhibit increased invasiveness (demonstrated through increased growth) relative to populations from herbicide treatment lakes (e.g. a fitness tradeoff). Repeated exposure to herbicides could be selecting for populations of invasive watermilfoil expressing invasive traits related to growth and hardiness to novel environments as adaptive means for overcoming the stress of herbicides. Repeated exposure to herbicides could therefore be promoting invasiveness among populations of invasive watermilfoils. Counter to studies that have documented suppressed growth as an adaptive herbicide resistance trait in other plant species (Vila-Aiub et al 2009, van Etten et al. 2016, Bingham et al. 2017), increased growth rates in invasive watermilfoil could be beneficial for escaping the harmful effects of concentrated aqueous herbicides.

Management implications

Histories of exposure to herbicides could be beneficial for promoting invasive traits related to survival and growth in invasive watermilfoil. Given that invasive watermilfoil plants from herbicide treatment lakes have more invasive characteristics, this could have implications for future management of invasive populations. Lakes continually managed for invasive watermilfoil through the use of herbicides could potentially select for increasingly invasive populations that would not only make control through the use of herbicides more difficult, but could also make other forms of treatment (i.e. mechanical or biological control agents) challenging and more costly as increased growth and survivorship could impair the efficacy of those treatments as well. In addition, invasive watermilfoil populations exhibiting increased invasiveness could more effectively outcompete native vegetation, reducing native plant diversity and paving the way for future invasions of watermilfoil or other invasive aquatic plants. Our findings reveal that repeated exposure to herbicides could be generating invasive watermilfoil lineages possessing increased invasive traits. This could pose a threat to all types of waterbodies regardless of their herbicide treatment or invasion history.

2.6 Figures



Source: ArcMap/Michigan GIS Open Data

Figure 2.1 – Locations of four lakes from which invasive watermilfoil fragments were collected. Geographic data is from Michigan GIS Open Data (State of Michigan 2018), parentheses give latitude and longitude of waterbody, and the map was produced using ArcMap (ESRI 2018 - v. 10.6).

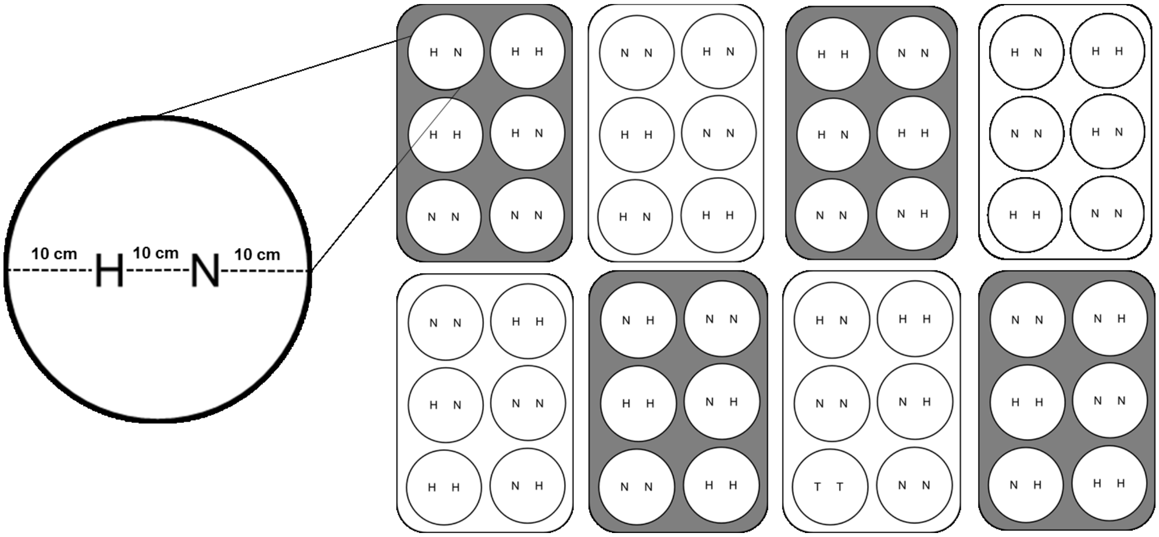


Figure 2.2 – Experimental design of eight mesocosms (large rectangles); two plants were planted per pot (circles) at 10 cm apart from each other, where C = plants collected from non-herbicide treated waterbodies and H = plants collected from herbicide treated waterbodies. Nutrients were applied to the mesocoms as low (unshaded rectangles) and high dose (shaded rectangles).

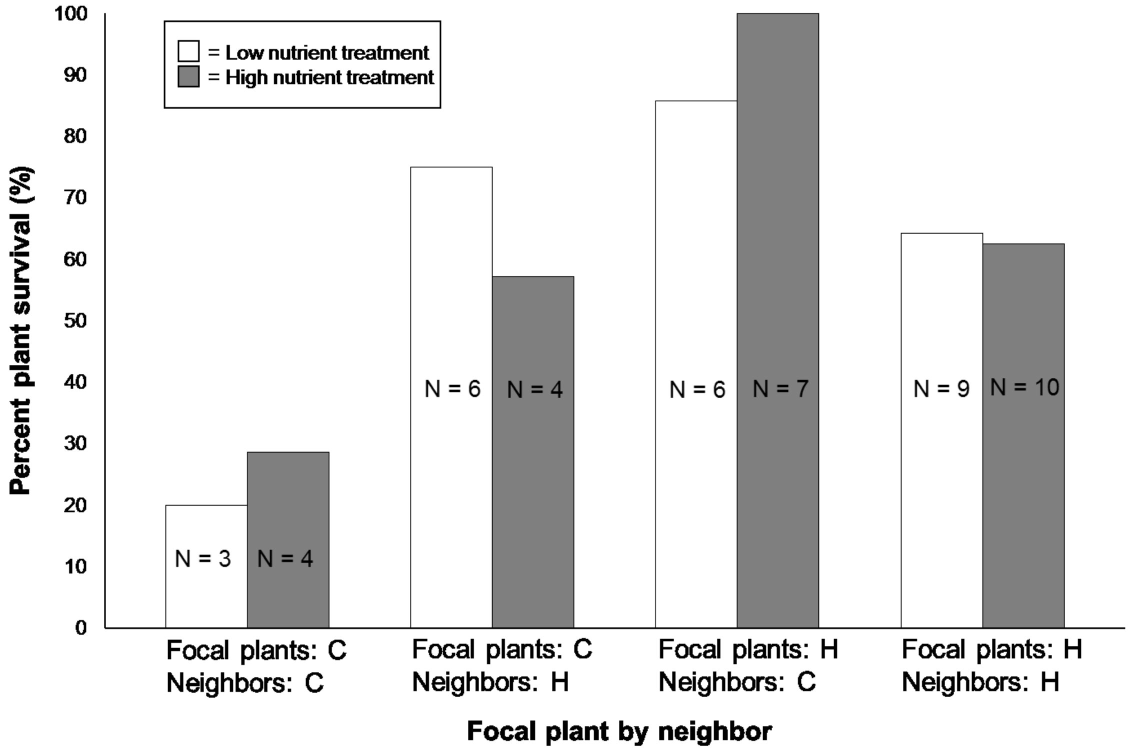


Figure 2.3 – Percent plant survival for focal plants grown with different neighbor plants where H = plants collected from herbicide treated lakes and C = plants collected from non-herbicide treated lakes. Sample sizes (N) represent the number of plants from each group that survived until the completion of the experiment.

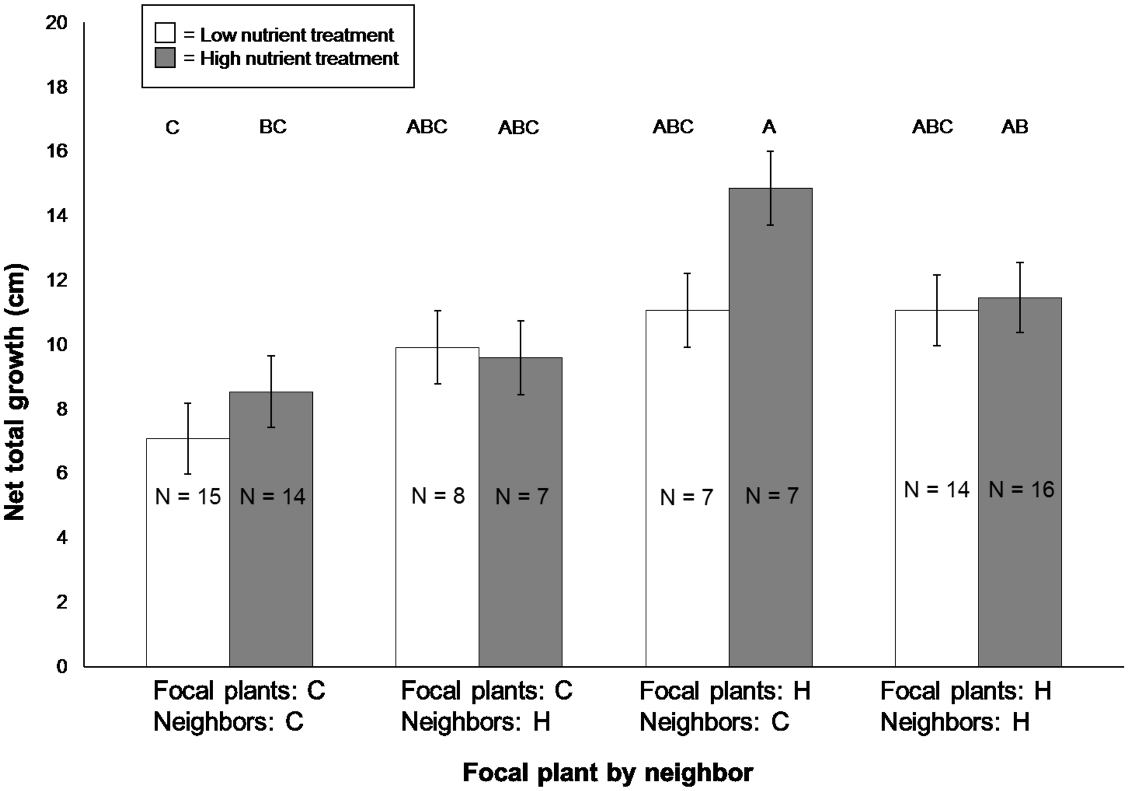


Figure 2.4 – Least square means ± 1 standard errors of net total growth (cm) measured by taking the log 10 transformed value of the final total length minus the initial total length (values were back transformed for graphical purposes, McDonald 2014.) of focal plants grown with different neighbor plants where H = plants collected from herbicide treated lakes and C = plants collected from non-herbicide treated lakes. Different letters represent significantly different least squares means according to Tukey's HSD test and sample sizes (N) are shown.

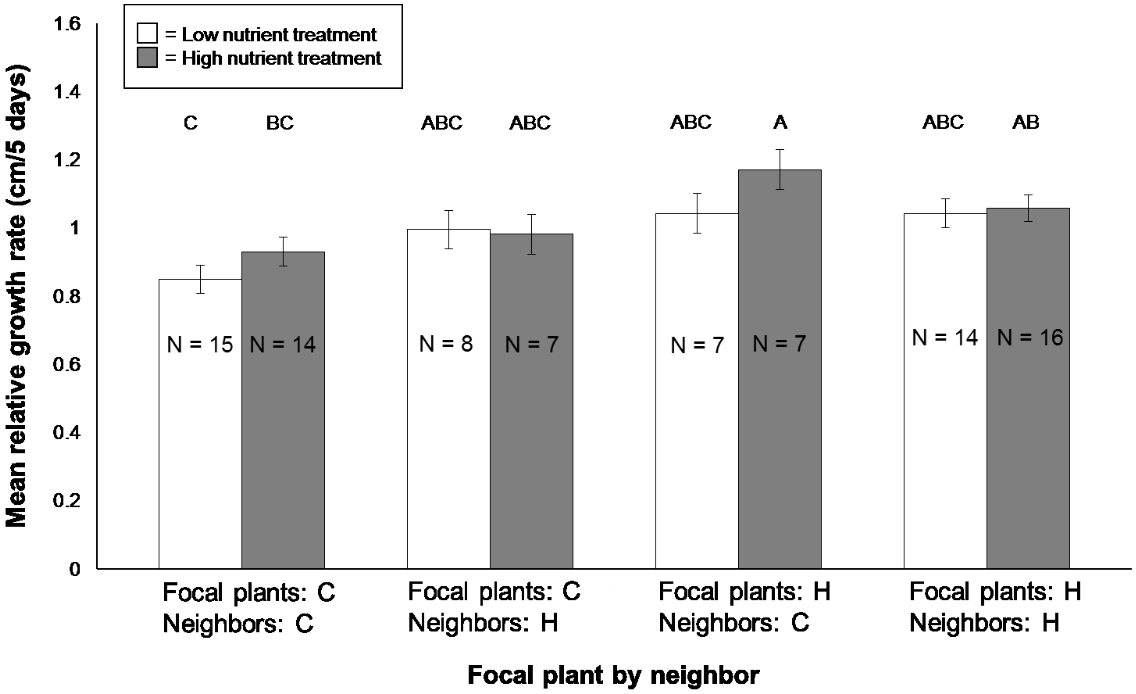


Figure 2.5 – Least square means ± 1 standard errors of mean relative growth rate (measured by taking the net total growth rate/number of days of observation or survival) of focal plants grown with different neighbor plants where H = plants collected from herbicide treated lakes and C = plants collected from non-herbicide treated lakes. Different letters represent significantly different least squares means according to Tukey's HSD test and sample sizes (N) are shown.

2.7 Tables

Table 2.1 – Logistic regression results for the effects of source of focal plant (C, H), source of neighbor (C, H), nutrient level (high, low) and/or interactions among factors on plant survival. *P*-values in bold are statistically significant at α = 0.05.

Source	**DF**	**Chi-square**	***P***
Focal source waterbody (FSW)	1	10.5310	**0.0012**
Neighbor source waterbody (NSW)	1	0.4737	0.4913
Nutrient treatment (NT)	1	0.8118	0.3676
FSWx NSW	1	13.0128	**0.0003**
FSWx NT	1	1.2330	0.2668
NSWx NT	1	2.1809	0.1397
FSWx NSW x NT	1	0.4707	0.4927
Difference	7	25.3726	0.0007

Table 2.2 – Logistic regression for the effects of source of focal plant (C, H) and individual mean algae coverage score on plant survival. *P*-values in bold are statistically significant at α = 0.05.

Source	**DF**	**Chi-Square**	***P***
Focal source water body	1	6.7379	**0.0094**
Mean algae growth	1	3.5776	0.0586
Focal source water body x Mean algae growth	1	0.1064	0.7443
Difference	3	16.2824	**0.0010**

Table 2.3 – ANOVA effect test results for the effects of source of focal plant (C, H), source of neighbor (C, H), nutrient level (high, low) and/or interactions among factors on net growth (log-transformed). *P*-values in bold are statistically significant at α = 0.05.

Source	**Parameters**	**SS**	**F Ratio**	***P***
Focal source waterbody (FSW)	1	2.7467	11.321	**0.0012**
Neighbor source waterbody (NSW)	1	0.3477	1.4331	0.2349
Nutrient treatment (NT)	1	0.9266	3.8192	0.0543
FSW x NSW	1	1.3516	5.5712	**0.0208**
FSW x NT	1	0.2951	1.2162	0.2735
NSW x NT	1	0.1679	0.6919	0.4080
FSW x NSW x NT	1	0.0012	0.0051	0.9434
Model	7	6.9721	4.1054	**0.0007**
Error	80	18.9237		

Table 2.4 – ANOVA effect test results for the effects of source of focal plant (C, H), source of neighbor (C, H), nutrient level (high, low) and/or interactions among factors on mean relative growth rate (MRGR). Bold font indicates statistically significance results at the 0.05 significance level. *P*-values in bold are statistically significant at α = 0.05.

Source	**Parameters**	**SS**	**F Ratio**	***P***
Focal source waterbody (FSW)	1	0.3785	15.384	**0.0002**
Neighbor source waterbody (NSW)	1	0.0085	0.3452	0.5585
Nutrient treatment (NT)	1	0.0540	2.1954	0.1424
FSW x NSW	1	0.1163	4.7257	**0.0327**
FSW x NT	1	0.0071	0.2885	0.5927
NSW x NT	1	0.0523	2.1269	0.1486
FSW x NSW x NT	1	0.0004	0.0162	0.8990
Model	7	0.7076	4.1088	**0.0007**
Error	80	1.9683		

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A Appendix

Table A.1 – Source water bodies for EWM samples and corresponding lake codes and approximate geographic coordinates

Water body #	Water body	Latitude	Longitude
1	Fine Lake	42.443697	-85.299310
2	Jordan Lake	42.761334	-85.146254
3	Lake Geneva	42.834216	-84.584735
4	Budd Lake	44.015803	-84.793895
5	Torch Bay	47.090681	-88.464459
6	Pike Bay	47.032970	-88.513108
7	Long Lake	42.470782	-85.242860
8	Carter Lake	42.670366	-85.311866
9	Lake Ovid	42.942351	-84.410610
10	Silver Lake	43.920779	-84.963968

Table A.2 – Characteristics of twelve microsatellite markers developed for *Myriophyllum spicatum* by Wu et al. 2013. T_a = annealing temperature (°C). Locus superscripts indicate whether forward (^{F}) or reverse (^{R}) primer was labeled with fluorescent dye. Fluorescent dye superscripts indicate the dye set group that the primers were multiplexed in.

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent dye
Myrsp1^{F}	F: GTCAAAGCAGCCACTCGG R: GGCAACAATGCAGCTAACC	$(TCA)_3(TCAGCA)_2(GCA)_3$	6-FAM^{1}
Myrsp4^{R}	F: ACTGGCTAATGATATGCTGA R: TCTTTCCACGCCTCTTC	$(TC)_{17}(AC)_9$	PET^{3}
Myrsp5^{F}	F: GGGAAGCCGACAAGAAA R: CGAAGACGGAGTTATCAAG	$(TC)_{11}$	6-FAM^{3}
Myrsp6^{R}	F: TAACAAACCGTACATTACAAGC R: TTTCTCTGGGAGCCATAAC	$(TC)_{17}$	6-FAM^{2}
Myrsp8^{F}	F: GCACCATTAGGAGGAGAAC R: CTGCCGAAGATGAAACG	$(CA)_9$	VIC^{1}
Myrsp9^{F}	F: TCCCCATCTGGTTCGTAT R: GGAAGGTAGCGGAGTGC	$(ATC)_5(TTCATC)_2(TTC)_2$	VIC^{2}
Myrsp10^{F}	F: CTAATCCCAGTCCACGG R: GCTGAAATTGAAGCCTCT	$(TCA)_4(GCA)_5$	VIC^{3}
Myrsp12^{F}	F: CGCTTCACAAGTATTCTG R: TTCATGGTAGCCGTCA	$(TC)_{18}(AC)_{10}$	NED^{1}
Myrsp14^{F}	F: TTCCCATCCTTCTCCTG R: CCAAGTAAGTGTCCCAAAC	$(TA)_2(TG)_8(TA)_8(GA)_4$	PET^{2}
Myrsp15^{F}	F: TCTTTCCACGCCTCTTC R: ACTGGCTAATGATATGCTGA	$(TG)_7(AG)_9$	NED^{2}
Myrsp16^{F}	F: GGCTGCCCTATGCTAA R: ATCCCACTGAAGTCAAACT	$(TG)_2(CA)_8(TA)_6(GA)_6$	NED^{3}
Myrsp18^{F}	F: GACGCCAAATCCAACT R: AATGATGTGCCTATACTGAA	$(TCA)_{11}$	PET^{1}