Eurasian and hybrid watermilfoil genotype distribution in Minnesota

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Raymond M. Newman, Project Manager, University of Minnesota

Ryan A Thum, Co-PI Montana State University

With assistance and input from Graduate Student Jasmine Eltawely

Department of Fisheries, Wildlife and Conservation Biology

University of Minnesota

St. Paul, MN 55108

Email Address: <u>RNewman@umn.edu</u>

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Abstract:

Eurasian watermilfoil (*Myriophyllum spicatum*) is one of the most problematic invasive aquatic plants in Minnesota. It can hybridize with the native northern watermilfoil (*M. sibiricum*) and reproduce sexually. Previous studies show that some genotypes of hybrid are resistant to specific herbicides and some may be more invasive. We determined the distribution of hybrid, Eurasian, and northern watermilfoil in Minnesota and assessed factors related to this distribution. We also assessed genetic variation (diversity) and distribution of specific genotypes and began an assessment of the response of watermilfoil and genotypes to management with herbicides. We sampled 64 lakes across the state stratified by county, size, and duration of infestation and collected milfoil from random points. The DNA from the milfoil samples was analyzed to determine taxon (Eurasian, northern or hybrid) and specific genotypes.

We found Eurasian in 43 lakes, hybrid in 28 lakes, and northern in 23 lakes. Hybrid was much more common in the metro, whereas Eurasian was broadly distributed. Northern watermilfoil was the most diverse with 84 genotypes, none shared across lakes. In contrast, we found one widespread genotype of Eurasian and six others found in individual lakes. Hybrid was intermediate in diversity with 53 genotypes; most lakes had only 1 unique genotype but 40% had multiple hybrid genotypes. Several genotypes were found in multiple lakes indicating clonal spread. The high diversity of hybrid watermilfoil indicates there is much potential for selection of problematic genotypes that are resistant to herbicides or that are competitively superior. There are numerous hybrid genotypes that could become problematic, but few have been widely distributed. We have not yet identified any clearly problematic genotypes in Minnesota but lakes with unexplained treatment failures, and populations with high diversity should be assessed. We will implement a strategy to identify and test problematic genotypes in our continuation project.

Background

Eurasian watermilfoil (*Myriophyllum spicatum*) is one of the most troublesome aquatic weeds in North America. It occurs in over 350 waterbodies in Minnesota (https://www.dnr.state.mn.us/invasives/ais/infested.html) in 35 counties. In addition to suppressing native plant communities, inhibiting recreation and use and suppressing property values, hundreds of millions are spent annually on its control, with over \$2 million per year in Minnesota. Recently concern has arisen for hybrid watermilfoil, which may respond differently to management or be more invasive than pure Eurasian (LaRue et al. 2013b, Taylor et al. 2017, Thum and McNair 2018). This study aims to determine the distribution and extent of the hybrid milfoil problem in Minnesota to define the scope of the problem and develop specific hypotheses that can be tested with future studies to improve management.

Eurasian watermilfoil hybridizes with the native northern watermilfoil (*M. sibiricum*) (Moody and Les 2002, 2007; Zuellig and Thum 2012, LaRue et al. 2013b). Hybrids are difficult to distinguish from Eurasian watermilfoil (Moody and Les 2007), and as a result, populations identified as "Eurasian watermilfoil" may be composed of "pure" Eurasian watermilfoil, hybrids, or both. Although managers and aquatic botanists increasingly recognize Eurasian and hybrid watermilfoil as distinct taxa, they are not frequently distinguished when it comes to operational management strategies, control tactics, or evaluations of management actions. Recent molecular genetic studies demonstrate that genetic diversity is much higher in watermilfoils than previously recognized (Zuellig and Thum 2012). Several studies have identified clear tolerance by some hybrid genotypes to some herbicides, including fluridone (Berger et al. 2012, 2015; Thum et al. 2012) and the auxin mimics 2,4-D (LaRue et al. 2013a; Taylor et al. 2017) and triclopyr, whereas studies on other genotypes have not found any evidence for tolerance (e.g., Poovey et al. 2007, Slade et al. 2007, Glomski and Netherland 2010, Berger et al. 2012). Netherland and Willey (2017) found that some genotypes that were relatively tolerant to one herbicide were relatively susceptible to others, and vice versa. Although hybrid watermilfoil has been documented in Minnesota since the early 2000s (Moody and Les 2002, 2007) and additional occurrences have since been reported, a comprehensive assessment of the distribution and genetic diversity of hybrid watermilfoil in Minnesota has not been conducted.

To address this gap, we assessed the distribution and occurrence of hybrid watermilfoil in Minnesota and examined relations to factors that may affect its ecology and management. Specifically, our project had the following objectives:

Objective 1: Describe the frequency of occurrence and the geographic distribution of hybrid watermilfoil in Minnesota in order to determine the extent of this AIS problem and evaluate factors that are relevant to its biology and management. Specifically, test whether it is a) geographically widespread versus restricted to the Metro Region, b) more likely to occur in lakes with native northern watermilfoil, or c) more likely to occur in lakes with a longer invasion history.

Objective 2: Delineate and quantify genetic variation in hybrids in order to determine the role different genotypes and genetic diversity might play in its distribution and management. Specifically, A) assess whether specific genotypes are associated with a) geography and distribution extent, b) invasion history, or c) management history. B) Determine whether genetic

diversity or the occurrence of specific genotypes is related to a) local environment and aquatic plant communities or b) management history or actions.

Methods

To determine the occurrence and distribution of hybrid watermilfoil in Minnesota we sampled 62 lakes with varying size and duration of infestation in 24 counties across the state. We determined the number of lakes to sample per county based on the relative numbers of lakes with documented Eurasian watermilfoil infestations (includes hybrid) as of 2017 from the Minnesota Department of Natural Resources' (MNDNR) infested waters list: https://www.dnr.state.mn.us/invasives/ais/infested.html). Lakes sampled ranged from 12.5 to 51,891 hectares in size, 2.5 to 135 m in maximum depth, and the durations of infestation ranged from 1 to 31 years (Appendix A). Because the MNDNR does not differentiate between Eurasian and hybrid when indicating invasive milfoil infestations, the year first infested may be based on sighting of either Eurasian or hybrid watermilfoil. We sampled and recorded presence of northern watermilfoil at each location, but our data does not fully reflect the distribution of northern watermilfoil in Minnesota because we sampled from only lakes listed as Eurasian/hybrid infested and northern occurs in many non-infested lakes.

Field sampling and data collection

At each lake we navigated to ~100 pre-selected random points within a predefined littoral zone (depth \leq 4.6m). At each point, at least one individual stem (top 10-15 cm of plant) was collected for each unique watermilfoil taxon found at that location and placed in a labeled sealable bag on ice in a cooler. Taxa were identified visually based on morphological features and leaflet counts. The following leaflet counts were used to identify each taxon: Eurasian 14-21 leaflet pairs, northern 5-9 pairs, and hybrid 10-13 pairs (Moody and Les 2007). At each surveyed point the depth and number of plant stems per taxa collected were recorded. Plants were returned to the laboratory, rinsed of any debris, and meristem tips (top 1-2 cm) were flash frozen and stored at -80 °C until analysis.

Genetic identifications

Total genomic DNA was extracted from cleaned plant samples using DNeasy Plant Mini Kits (Qiagen). To distinguish Eurasian, hybrid, and northern watermilfoil, plants were identified to taxon using a genetic assay based on internal transcribed spacer DNA sequence (ITS; Moody and Les 2007, Grafé et al. 2015). The same DNA samples were then used to determine genetic composition. Genetic variation was quantified and specific clones were delineated using eight microsatellite markers 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 ABI 3730xl sequencer. Microsatellites were scored using GeneMapper, version 5.0 (Applied Biosystems). Because EWM, NWM, and hybrids are hexaploid, 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) using the R-package POLYSAT (Clark and Jasieniuk 2011).

We delineated distinct genotypes using Lynch distances and a threshold of 0 in POLYSAT (Clark and Jasieniuk 2011). We genetically analyzed 20 randomly selected samples

from each lake when available; if genetic variation was present or for lakes assessed more intensively or on several occasions we analyzed additional plants. Over 1600 plants were genotyped.

Distribution Data Analysis

Based on the genetically determined taxon identifications, all surveyed lakes were mapped with ArcGIS 10.5 to indicate presence/absence of each milfoil taxon. The geographic distribution of hybrid watermilfoil was determined, as well as relative distances between infestations. Hybrid watermilfoil infestations were assessed to determine if they were more commonly found in the Twin Cities metro versus greater Minnesota. To determine the influence of lake and environmental attributes associated with the presence of hybrid watermilfoil in Minnesota and to make comparisons between lakes, we assessed the following factors for each lake (or bay of Lake Minnetonka): age of infestation, number of vehicle/trailer parking spaces at public water accesses, lake area, maximum depth and littoral area (water depth \leq 4.6m) as obtained from the MNDNR's LakeFinder database <<u>https://www.dnr.state.mn.us/lakefind/index.html></u>.

Water quality variables including mean Secchi depth and trophic state index were obtained from the Minnesota Pollution Control Agency (MPCA) lake and stream water quality assessment database <<u>https://cf.pca.state.mn.us/water/watershedweb/wdip/index.cfm</u>>. Data for both variables were based on the ten-year average from state index data collected between June and September 2008 to 2017. Lakes were given milfoil management ratings on a scale of 0-3 to describe the extent of milfoil management, which include both chemical and mechanical control, based on DNR permit approval data from 2012 to 2017. A zero indicates no management during this 6-year period, one indicates 1-2 treatments, two indicates 3-4 treatments, and three indicates 5-6 treatments. A total of four lakes were excluded from these lake attribute analyses because sampling methods were inconsistent; however they were included in the taxa distribution map and assessment to indicate presence/absence.

To assess relationships for each attribute described above, lakes were separated into groups based on milfoil taxon presence (EWM, HWM, NWM lake), making it possible for the same lake to be in more than one group if it contained multiple milfoil taxa. To determine if significant differences existed between the means of each group, a one-way analysis of variance (ANOVA) was used to compare means for the various attributes (lake area, maximum depth, littoral area, Secchi depth, distance from nearest infestation, parking spaces at water access, milfoil management rating, and age of infestation) with a p-value of 0.05 used to determine significance.

Genetic Diversity and Response to Management

We used the microsatellite genotype IDs to first look at the distribution of genetic diversity within and among taxa, and across the state and by lake attributes. We then looked at the distribution of specific genotypes among lakes and identified lakes that share genotypes.

To assess genetic variation in more detail and the potential response of hybrid watermilfoil to management with herbicides, ten lakes were selected to be intensively sampled based on recommendations by the DNR, consultants, and applicators. The five treatment lakes were Bald Eagle (62-0002), Ham (02-0053), Schmidt (27-0102), and North Arm (27-013313), and Grays' Bay (27-013301) of Lake Minnetonka. Schmidt Lake and North Arm Bay of

Minnetonka were treated with a lake-wide fluridone application, Ham Lake and Grays Bay received partial lake treatments with ProcellaCOR, and Bald Eagle had a partial lake treatment with 2-4,d. The control lakes were Christmas (27-0137), Smith's Bay of lake Minnetonka, Upper Prior (70-0072), and Otter (02-0003).

Control and treated lakes were surveyed in 2018 to characterize milfoil abundance and the plant community with Point Intercept Surveys (e.g., Madsen 1999, Nault et al. 2018; > 100 littoral points per lake) and samples of watermilfoil were collected at each site present and frozen for genetic analyses. Treated lakes were resurveyed in August to characterize the response to herbicide treatment and characterize the native plant community. Milfoil and native plant frequency of occurrence and density were compared before and after treatment lake-wide and within the areas of treatment. Changes in frequency and distribution of genotypes was also assessed.

Results

Occurrence and geographic distribution in Minnesota

A total of 62 Eurasian watermilfoil infested lakes were sampled (2 non-infested lakes containing northern watermilfoil were also sampled). We did not find any milfoil in two lakes (Gervais 62-0007 and Locke 86-0168), 43 contained Eurasian, 28 contained hybrid, and 23 contained northern (Table 1). We found various taxa combinations in surveyed lakes where milfoil was found (Table 2). Of the 28 lakes that we found containing hybrids, 13 had only hybrid watermilfoil and no other milfoil taxa, and the remaining 15 had some combination with either Eurasian, northern, or both (Table 2). In assessing all hybrid infested lakes containing one or the other parental taxon, it was found that hybrid was more likely to be present in a lake with Eurasian (13 lakes) versus northern (3 lakes). There were also significant geographic relationships. Hybrid-only infestations were mostly present in the metro (91%); only one hybrid exclusive infestation was found in greater Minnesota (Figure 1). The hybrids found in lakes outside of the metro were largely from populations that also had Eurasian and/or northern. We found four lakes that contained all three taxa, half of which were in the metro and half in greater Minnesota (Table 2).

Eurasian was evenly distributed across the state (Figure 1) and it was most commonly found in lakes that contained another taxon rather than existing alone (Table 1). In lakes where another taxon was present with Eurasian, it was more commonly found with northern (60%) over hybrid (40%). We found that 83% of lakes where both Eurasian and northern were present were outside of the metro, indicative of northern being most commonly found there as well.

Northern watermilfoil was more common outside the Twin Cities metro: 30% of lakes with northern were in the metro and 70% were outside (Figure 1). This may be due to the longer invasion history in the metro (Eurasian displacing northern) or better water clarity and more diverse plant communities outstate. Hybrid watermilfoil tended to be clustered in the metro and specifically the central and eastern metro (Figure 1). Very few lakes (5) outside the 7-county metro had hybrid (Table 2) and somewhat surprising, no lakes in Carver county (western metro) had hybrid (Table 1) despite the long occurrence of Eurasian watermilfoil in Carver county (since 1989) and large number of infestations (27).

Table 1. Summary of genetic analyses of lakes surveyed in 2017-2018. The number of each taxon identified from samples collected in each lake is presented and the number of distinct genotypes is indicated for each taxon in each lake.

			Counts per taxon		Numbe	Number of gene	
Lake	County	EWM	HWM	NWM	EWM	HWM	NWM
Coon	Anoka	11	29		1	2	
Crooked	Anoka		20			3	
Ham	Anoka		97	6		1	1
Otter	Anoka		64			2	
Ballantyne	Blue Earth	20			1		
Chub	Carlton	1		19	1		1
Auburn	Carver	24			1		
Piersons	Carver	19			1		
Riley	Carver	21			1		
Steiger	Carver	20			1		
Swede	Carver	13			1		
East Rush	Chisago		18	2		1	1
South Lindstrom	Chisago		9	19		1	4
Вау	Crow Wing	14		6	1		3
Emily	Crow Wing	2		6	1		6
Alimagnet	Dakota		20			1	
Cobblestone	Dakota		2			1	
Fish	Dakota		20			1	
Lac Lavon	Dakota		20			5	
Orchard	Dakota			5			4
Thomas	Dakota		5			2	
Oscar	Douglas	5		15	1		5
Cedar	Hennepin	5			1		
Christmas	Hennepin	48		33	1		5
Harriet	Hennepin	20			1		
Independence	Hennepin	43	44		1	1	
Minnetonka-Grays	Hennepin		54			5	
Minnetonka-North							
Arm	Hennepin		20			7	
Minnetonka-Smiths	Hennepin	14	37	6	2	10	4
Mitchell	Hennepin	24		16	1		3
Rebecca	Hennepin	21	8		1	1	
Schmidt	Hennepin		62			2	
Staring	Hennepin	8			1		
Spectacle	Isanti	3		22	1		4
Green	Kandiyohi	2			1		
German	Le Seuer	1	9	1	1	5	1
Minnie-Belle	Meeker	1		25	1		5
Mille Lacs	Mille Lacs	2		10	1		2
Pokegama	Pine	5			1		

Gilchrist	Роре	20			1		
Bald Eagle	Ramsey	35	43	50	1	1	3
Gervais	Ramsey						
Josephine	Ramsey		19			1	
McCarron	Ramsey	21	11		1	1	
Phalen	Ramsey	4			1		
Turtle	Ramsey	6	6		1	1	
Fox	Rice	20			2		
McMahon	Scott	4			1		
Upper Prior	Scott	14	10		2	2	
Mitchell	Sherburne	5		34	1		3
Gilbert Pit	St. Louis	9			1		
Little Birch	Todd	4		15	1		6
Big Carnelian	Washington			5			3
Big Marine	Washington	12		13	1		8
Bone	Washington		19			1	
Elmo	Washington	16	23		1	1	
White Bear	Washington	24	12		1	1	
Cedar	Wright			20			6
Constance	Wright	17			1		
Howard	Wright	9	10	1	1	6	1
Indian	Wright		1			1	
Locke	Wright						
Somers	Wright	2			1		
Sugar	Wright	1		19	1		5

Table 2. Occurrence of taxa in lakes in the seven county metro, greater Minnesota, and statewide for combinations present in all surveyed lakes.

EWM	HWM	NWM	EWM &	NWM &	EWM &	All three	Total
only	only	only	HWM	HWM	NWM	taxa	
8	1	1	0	2	10	2	24
10	12	0	8	1	3	2	36
18	13	1	8	3	13	4	60
	EWM only 8 10 18	EWM onlyHWM only8110121813	EWMHWMNWMonlyonlyonly8111012018131	EWM onlyHWM onlyNWM onlyEWM & HWM8110101208181318	EWM onlyHWM onlyNWM onlyEWM & HWMNWM & HWM8110210120811813183	EWM HWM NWM EWM & HWM NWM & EWM & HWM EWM & HWM EWM & NWM EWM & SU & NWM EWM & SU & S	EWM onlyHWM onlyNWM onlyEWM & HWMNWM & HWMEWM & NWMAll three taxa811021021012081321813183134



Figure 1. Statewide occurrence and proportions of Eurasian (EWM orange), hybrid (HWM red), and northern (NWM blue) watermilfoil based on genetic analyses for lakes sampled 2017-2018.

Four lakes of our total 62 lakes were left out of the environmental attribute analysis due to no milfoil being found in two lakes and limited sampling in two other lakes. Compared to lakes containing Eurasian or northern, those containing hybrid were on average smallest in size, maximum depth, and littoral area (Table 3). Average Secchi depth values for lakes with Eurasian and hybrid were similar, but lakes with northern on average had deeper Secchi depths. Across all three taxa most lakes (94%) had a trophic state index (TSI) within the range of meso- to eutrophic. Hybrid infestations were on average closer to one another in comparison to Eurasian and northern lakes across the state (Table 3).

'	Table 3. Mean values and standard errors for environmental characteristics of 58 sampled
	Minnesota lakes classified as containing either Eurasian (EWM), hybrid (HWM), or northern
1	(NWM) watermilfoil.

Lake type ^a	Lake area (ha)	Max depth (m)	Secchi Depth – water clarity of a lake (m)	Littoral Area (ha)	Trophic state index	Average distance from nearest infestation (km)
EWM	$299 \pm$	17.5 ± 3.2	2.5 ± 0.3	159 ± 28	Meso-	20.8 ± 3.5
Lakes	62				Eutrophic	
HWM	$202 \pm$	12.3 ± 1.5	2.4 ± 0.2	122 ± 29	Meso-	11.5 ± 2.2
Lakes	45				Eutrophic	
NWM	$314 \pm$	14.3 ± 1.7	2.8 ± 0.3	177 ± 31	Meso-	29.4 ± 5.3
Lakes	52				Eutrophic	

^a Lake types include all lakes with the taxon present and therefore a lake may be represented in more than one category.

We further analyzed factors associated with conditions in the metro, greater Minnesota and statewide for the same group of 58 lakes (Table 4). For all three categorized lakes (EWM, HWM, NWM), on average we found that Eurasian watermilfoil infestations were oldest in the metro in comparison to greater Minnesota, and had higher numbers of parking spaces at the water access (Table 4), however, these differences were not significant. Milfoil taxa were collected from deeper average depths from lakes in greater Minnesota versus the metro; this relationship was found across all three taxa but hybrid had the shallowest statewide average depth. Overall, sampled lakes were not heavily managed; we found that the median scores for hybrid lakes in the metro and greater Minnesota were both one. Northern lakes in the metro were less managed with a median score of 0.5 compared to greater Minnesota, which had a score of one. Eurasian lakes in the metro had a median score of zero and in greater Minnesota had a score of 0.5. The two attributes we found to be significant (p < 0.05) when comparing the three taxa were distance from nearest infestation (p = 0.01) and presence in the metro versus outstate (p = 0.0007).

	(-						
		Number	Average	Average	Median	Average	Average
		of lakes	age of	number of	milfoil	number of	depth of
			infestation	parking	management	unique	collected
			(years)	spaces at	score	genotypes per	taxa (m)
				water access		lake	
EWM	Statewide	41	16.6	22	0	1.0	1.9
	Metro	21	19.7	32	0	1.0	1.7
	Greater	20	13.2	11.5	0.5	1.0	2.0
	MN						
HWM	Statewide	26	19.2	27.7	1	2.5	1.5
	Metro	21	20.2	29.5	1	2.5	1.5
	Greater	5	15	21.6	1	2.8	1.7
	MN	-					
NWM	Statewide	21	17.8	23	1	3.6	1.8
	Metro	6	21.2	35.8	0.5	4.0	1.7
	Greater MN	15	16.4	17.8	1	3.5	1.8

Table 4. Average values of explanatory variables for Minnesota lakes classified as containing either Eurasian (EWM), hybrid (HWM), or northern (NWM).

Genetic diversity

We identified unique genotypes of each taxon based on microsatellites. Amongst the three taxa, EWM was the least diverse. Overall, we identified 7 Eurasian genotypes, 84 northern genotypes, and 53 hybrid genotypes in Minnesota (Table 5). For Eurasian watermilfoil, most lakes sampled in 2017-2018 (40 lakes) contained the same genotype that was the dominant genotype. There was very little within-lake diversity for Eurasian (2 lakes with > 1 genotype), and overall we have found six Eurasian genotypes that were different from the common widespread genotype. A unique Eurasian genotype was found in Chub, German, Smith's Bay, Upper Prior and two in Fox.

Hybrid watermilfoil showed intermediate genetic diversity in comparison to EWM and NWM (Table 1, Table 5). Twelve lakes had multiple hybrid genotypes, with there being particularly high diversity (\geq 5 genotypes) in three lakes (Lac Lavon, German and Howard) and three bays of Lake Minnetonka (Gray's, Smith's, and North Arm). The greatest number of hybrid genotypes in a single lake or bay was 10 found in Smiths' Bay of Lake Minnetonka of Hennepin County; Grays Bay had 5 genotypes and North Arm had 7 genotypes. Overall, Minnetonka had 17 different genotypes of hybrid watermilfoil. We found the same genotype in two sets of two lakes in Dakota county (Alimagnet and Lac Lavon share a genotype and Cobblestone and Lac Lavon shared a genotype). We also found a different, but common genotype in the following seven lakes: Bald Eagle, Bone, Fish, Josephine, Otter, South Lindstrom and White Bear, which spanned five counties (Ramsey, Washington, Dakota, Chisago, and Anoka). The bays in Lake Minnetonka also shared genotypes of HWM, but each also had unique genotypes. These common hybrid genotypes are indicative of clonal spread of hybrids in Minnesota. There are numerous hybrid genotypes that could become problematic, but there are relatively few hybrid genotypes that have been more widely distributed.

Northern watermilfoil was the most diverse, with most lakes having multiple different genotypes within (Table 1) and no genotypes shared between lakes (Table 5). The genetic

diversity present in hybrids is linked to this diversity in its northern parent. They further suggest that northern watermilfoil is reproducing sexually within lakes and we have no evidence of spread of northern watermilfoil between lakes.

Comparing genetic diversity by taxa and across the state, we found that northern had an average of 3.6 genotypes per lake: 3.5 different genotypes per lake in greater Minnesota and 4 in the metro (Table 4). Eurasian had an average of 1 genotype per lake statewide and in both the metro and greater Minnesota. Hybrids had an average of 2.5 genotypes per lakes in the metro and 2.5 in greater Minnesota. In comparing the ages of infestation of hybrid lakes containing a single hybrid genotype and lakes with greater than 2 hybrid genotypes, we found that the age of infestation was significantly older (p = 0.03) for hybrid lakes containing 3 or more genotypes (23.7) versus those with one genotype (15.9) (Figure 2).

			Clone: N	
Lake	County	EWM	HWM	NWM
Coon	Anoka	1:11	40:1, 55:28	
Crooked	Anoka		67:11; 68:8; 69:1	
Ham	Anoka		14:97	15:6
Otter	Anoka		3:63, 144:1	
Ballantyne	Blue Earth	1:20		
Chub	Carlton	87:1		86:19
Auburn	Carver	1:24		
Piersons	Carver	1:19		
Riley	Carver	1:21		
Steiger	Carver	1:20		
Swede	Carver	1:13		
East Rush	Chisago		88:18	89:2
South Lindstrom	Chisago		3:9	19:6; 20:10; 21:2; 22:1
Bay	Crow Wing	1:14		117:4; 118:1; 119:1
Emily	Crow Wing	1:2		75:1; 76:1; 77:1; 78:1; 79:1; 80:1
Alimagnet	Dakota		81:20	
Cobblestone	Dakota		84:2	
Fish	Dakota		3:20	
	Dakata		81:5; 82:3; 83:8;	
Lac Lavon	Dakola		84:3; 85:1	
Orchard	Dakota			50:2; 51:1; 52:1; 64:1
Thomas	Dakota		45:4; 46:1	
Oscar	Douglas	1:5		70:6; 71:3; 72:3; 73:2; 74:1
Cedar	Hennepin	1:5		
Christmas	Hennepin	1:48		105:1; 133:6; 134:4; 135:21; 136:1
Harriet	Hennepin	1:20		
Independence	Hennepin	1:43	99:44	

Table 5. Occurrence of clones (genotypes) of Eurasian (EWM), hybrid (HWM), or northern (NWM) in Minnesota. The clone number is followed by the number of plants of that clone identified in the lake (Clone:N).

Minnetonka-	Hennepin		7:10; 12:32; 137:2; 138:6: 139:4	
Minnetonka-			6·4· 7·11· 8·1· 9·1·	
North Arm	Hennepin		10:1: 11:1: 12:1	
			7:19: 9:2: 12:2:	
Minnetonka-			106:1: 107:4: 108:3:	
Smiths	Hennepin	1:13; 141:1	109:3: 114:1: 140:1:	110:1; 111:2; 112:2; 113:1
			143:1	
Mitchell	Hennepin	1:24		16:12; 17:3; 18:1
Rebecca	Hennepin	1:21	56:8	
Schmidt	Hennepin		53:61, 142:1	
Staring	Hennepin	1:8		
Spectacle	Isanti	1:3		41:19, 42:1, 43:1, 44:1
Green	Kandiyohi	1:2		
German	Le Seuer	63:1	57:1; 58:2; 59:1; 60:4; 61:1	62:1
Minnia Balla	Maakar	1.1		34:3, 35:5, 36:12, 115:1,
милле-вене	weeker	1:1		116:4
Mille Lacs	Mille Lacs	1:2		65:9; 66:1
Pokegama	Pine	1:5		
Gilchrist	Роре	1:20		
Bald Eagle	Ramsey	1:35	3:43	2:33, 4:16, 5:1
Josephine	Ramsey		3:19	
McCarron	Ramsey	1:21	13:11	
Phalen	Ramsey	1:4		
Turtle	Ramsey	1:6	54:6	
Fox	Rice	90:19; 91:1		
McMahon	Scott	1:4		
Upper Prior	Scott	1:13; 32:1	31:1; 33:9	
Mitchell	Sherburne	1:5		37:23; 38:7; 39:4
Gilbert Pit	St. Louis	1:9		
Little Birch	Todd	1.1		127:8; 128:3; 129:1; 130:1;
LITTLE DITCH	Touu	1.4		131:1; 132:1
Big Carnelian	Washington			47:1; 48:3; 49:1
Rig Marine	Washington	1.12		23:2; 24:1; 25:2; 26:1; 27:1;
Dig Marine	Washington	1.12		28:4; 29:1; 30:1
Bone	Washington		3:19	
Elmo	Washington	1:16	55:23	
White Bear	Washington	1:24	3:12	
Cedar	Wright			121:7; 122:8; 123:2; 124:1; 125:1; 126:1
Constance	Wright	1:17		
Howard	Wright	1:9	92:4; 93:1; 95:1; 96:1; 97:2; 98:1	94:1
Indian	Wright		120:1	
Somers	Wright	1:2		
Sugar	Wright	1:1		100:10; 101:3; 102:1; 103:3; 104:2



Figure 2. Hybrid watermilfoil (HWM) genotype counts per lake (blue) by average age of invasive milfoil (EWM) infestation in county (yrs).

Within lake variation and response to management

We assessed 5 reference lakes and 5 treated lakes to look at spatial and temporal changes in milfoil and hybrid genotype occurrence as well as the response of these taxa and native plants to management. All lakes had at least 1 genotype of hybrid present, except Christmas, which was previously determined to have hybrid present, but no definitive hybrids were found during our sampling in 2017-2018. Most lakes were sampled in 2017 for presence of hybrids with a random survey and then again in 2018 with point intercept surveys (higher point density) that characterized the entire plant community. The point intercept surveys will be repeated in 2019 and 2020 as part of a continuation project to assess response to management.

The lakes had a range of milfoil occurrences and densities (Table 6). In the control lakes milfoil frequency of occurrence in the littoral ranged from 4% in Upper Prior to 65% in Christmas Lake. Water clarity limited the plant community in Upper Prior (Figure 3), which also had low occurrence of native plants (31%). Both milfoil and native plant occurrence remained relatively similar between early and late summer in the two lakes that were sampled twice (Independence and Christmas) (Figure 4). These lakes have better water clarity and support a more abundant plant community than Upper Prior. Otter Lake and Smith's Bay also have good clarity and supported the most abundant native plant communities (Table 6). Milfoil was widely distributed in these lakes and northern watermilfoil was common in shallower portions of Smiths Bay (Figure 5). Milfoil was found at half the sites in Smiths Bay (Table 6).

In Otter Lake we found one hybrid genotype lake-wide in 2017 (20 samples), but with more intensive sampling in 2018 found 1 plant of a second genotype (43 plants were the same genotype found in 2017); no EWM was found. It should be noted that hybrid has been found in Otter since 1999 (Moody and Les 2001) and repeated genetic analyses since (e.g., Roley and Newman 2006, Moody and Les 2007). In Independence, one genotype of Eurasian and one of hybrid was found and no change in frequency was noted between early and late summer. In Christmas, there were no significant changes in composition of Eurasian (one genotype) and northern watermilfoil (several genotypes combined) between "early" (July) and "late" (August) samples in 2018 (χ^2 =3.40, p=0.19), or between 2016 and 2018 (χ^2 =1.27, p=0.26). The lake-wide frequency of occurrence in 2018 decreased from 65% to 45% between early and late samples (Table 6) and there was an increase in northern watermilfoil (Figure 4). Both taxa are distributed around the lake.

At Smiths Bay northern was present but, restricted to shallower sites and Eurasian/hybrid was more widespread (Figure 5). There was a significant change in the composition of Eurasian, hybrid, and northern watermilfoil between 2016 and 2018 (χ^2 =21.59, p=0.00002); specifically, there was an increase in hybrid and a decrease in Eurasian over this time. This is consistent with hybrid expansion. There was no significant change in the composition of hybrid genotypes (χ^2 =1.63, p=0.82).

Independence had a lower occurrence of milfoil (28-33%; Table 6). About half the milfoil was EWM and half was HWM (Table 5). There was no change in proportion of the two taxa between early and late summer and only one genotype of each was found. We did find some of the hybrid with 5 leaflet whirls, but there was no difference in genetic identity between the 4-and 5-leaved whirled hybrids.

For the managed lakes, Schmidt Lake and North Arm Bay of Minnetonka were treated with a lake-wide fluridone application and both had significant decreases in milfoil abundance following treatment, with almost complete elimination of milfoil (<2% frequency remaining) (Table 6, Figure 6). Only one genotype of hybrid was found in Schmidt, but future sampling can determine if other genotypes emerge. North Arm, by contrast, had much greater diversity with 7 genotypes. Previous results (Thum et al. 2017a) found a significant change in hybrid genotype composition between pre- and post-treatment with the auxin mimic triclopyr in 2015 (χ^2 =9.97, p=0.02). Specifically, the "North Arm" genotype (clone 7) increased in relative frequency post-treatment. And, concomitantly, several genotypes that were found before treatment were not found after treatment (overall diversity went down). There was not a significant change in composition between pre-treatment 2015 and 2017, although it was close (χ^2 =7.29, p=0.06). This is interesting, because although clone 7 increased after treatment in 2015, its relative abundance

decreased back to a similar level over time. The marginal significance can be attributed to an increase in relative frequency of clone 6 and some "new" clones found in 2017. This is a diverse bay, and it looks like there is some level of introduction of new genotypes (either recruitment from seed or introductions from other bays/lakes). There was also no significant change in composition between post-treatment 2015 and 2017 (χ^2 =5.23, p=0.16). The potentially tolerant clone 7, increased in 2015 after treatment, but then went back down a bit in 2017 but still stayed at a higher proportion than it was before treatment in 2015. Tolerance to herbicide and competitive or growth abilities are not necessarily correlated and further assessment of this genotype is warranted. The fluridone treatment in 2018 may have further reduced or eliminated this genotype.

The lakes treated with 2,4-D and ProcellaCOR had more focused treatments, less herbicidal coverage (8-15% of lake area treated) and less overall control (Table 6). About half of the lakewide milfoil was controlled in Bald Eagle with 2,4-D; however milfoil occurrence decreased from 53% to 5% within the treatment areas (Table 6, Figure 7). Lakewide native plant frequency increased after treatment and some northern watermilfoil expanded in the untreated areas (Figure 7). Between 2017 and pre-treatment in 2018 there was significant increase of Eurasian and hybrid relative to northern. However, there was a significant decrease in hybrid and Eurasian in 2018 from pre to post treatment and northern increased after treatment. This appears mainly due to treatments focusing on areas with abundant Eurasian and hybrid and leaving untreated areas with northern to expand (Figure 7).

There was less control observed with the use of ProcellaCOR, and lakewide milfoil abundance increased following treatment in both lakes (Table 6). On Gray's Bay the treatmentarea milfoil abundance decreased from 53% to 7% following the ProcellaCOR treatment, but on Ham Lake the treatment-area milfoil abundance increased from 47% to 82% (Table 6). The lakewide increase in occurrence at Gray's was due mainly to increases in areas outside the treatment plots, although some milfoil remained in treated areas (Figure 8). At Ham, milfoil increased within and outside the treatment plots (Figure 8) after treatment. Ham also had a significant decrease in native plant coverage (82% to 70%) following treatment including a virtual loss of northern watermilfoil (Figure 8). There were, however, no significant changes in composition of one hybrid genotype and one northern watermilfoil genotype between pre- and post-treatment in 2018 (χ^2 =2.01, p=0.16), or between 2017 and 2018 (χ^2 =0.02, p=0.86). At Gray's Bay, there was no significant change in the composition of the five hybrid genotypes that were present across sampling times in our study and in Thum et al. (2017). For these genotypes, there were no significant differences in composition between pre- and post-treatment sampling in 2018 (χ^2 =2.05, p=0.73), or between 2015 and 2018 (χ^2 =2.58, p=0.46). The hybrid clone 7 genotype that increased in North Arm in 2015 and increased in Smiths between 2016 and 2018 was present in Grays Bay in 2018; it deserves further monitoring.

Table 6. Summary of intensive lakes results including milfoil and native plant frequency of occurrence (FOC) pre- and post-treatment based on 2018 surveys within the lake wide littoral zone (shallower than 4.6m) and within treated areas.

Lake	County	Treat	Lake wide Milfoil FOC (pre- treat)	Lake wide milfoil FOC (post treat)	Native plant FOC (pre-treat	Native plant FOC (post treat)	Within treated Milfoil FOC (pre- treat)	Within treated Milfoil FOC (post treat)	
Ham	Anoka	Procella COR – 14	23%	34%	82%	70%	47%	82%	
		acres							
Gray's Minnetonka	Hennepin	Procella COR – 28 acres	22%	27%	94%	98%	53%	7%	
North Arm Minnetonka	Hennepin	Fluridone – lakewide	61%	0.6%	92%	97%	Lake wide	Lake wide	
Schmidt	Hennepin	Fluridone – lakewide	79%	2%	100%	96%	Lake wide	Lake wide	
Bald Eagle	Ramsey	2,4d – 42 acres	60%	32%	73%	92%	53%	5%	
Otter	Anoka	Control	49%		96%				
Smith's Minnetonka	Hennepin	Control	53%		97%				
Independence	Hennepin	Control	28%	33%	55%	66%			
Christmas	Hennepin	Control	65%	45%	89%	91%			
Upper Prior	Scott	Control	4%		31%				

Upper Prior 2018 Mid Summer Results



Figure 3. Occurrence and relative abundance of milfoil in Upper Prior Lake, July 2018.



Figure 4. Occurrence and relative abundance of Eurasian (includes hybrid) and northern watermilfoil in reference lakes Christmas and Independence in early and late (August) summer.



Figure 5. Occurrence and relative abundance of Eurasian (includes hybrid) and northern watermilfoil in reference lakes Otter and Smith's Bay.

ment Results



Figure 6. Pre and post-treatment occurrence and relative abundance of milfoil in North Arm Lake Minnetonka and Schmidt Lake. Both lakes were treated with fluridone in May.



Bald Eagle 2018 Pre & Post Treatment Results

Figure 7. Pre and post-treatment occurrence and relative abundance of milfoil in Bald Eagle Lake. The lake was treated with 2,4-d in localized treatment areas in July.



Grays Bay 2018 Pre & Post Treatment Results

Ham 2018 Pre & Post Treatment Results

Figure 8. Pre and post-treatment occurrence and relative abundance of milfoil in Grays Bay Lake Minnetonka and Ham Lake. Both lakes were treated with ProcellaCor in mid-summer.

Discussion

Hybrid watermilfoil is common in Minnesota occurring in almost half the lakes assessed, but it is most common in the Twin Cities metro where it occurred in more than 60% of infested lakes. Eurasian watermilfoil is more broadly distributed and northern watermilfoil is more common in greater Minnesota, beyond the metro. Northern watermilfoil is the most genetically diverse with each lake having unique genotypes and many lakes have multiple genotypes of northern. In contrast, there is one widespread and dominant Eurasian genotype and 6 other genotypes that are found only in one lake each. Hybrid watermilfoil is of intermediate diversity with 53 genotypes; it is likely that hybrid watermilfoil is reproducing sexually (LaRue et al. 2013b) and Eurasian and northern are reproducing to produce more hybrids (Zuelig and Thum 2012). Although most lakes only have one genotype of Eurasian or hybrid, there are lakes with multiple genotypes of hybrid. This genetic diversity has the potential to produce plants that a tolerant to herbicides or are more invasive.

These data indicated that the only significant differences in lakes containing hybrids, in comparison to Eurasian and northern, is that hybrid lakes on average were more common in the Twin Cities metro, and were closer to one another in distance. The analysis of all other lake attributes (lake area, maximum depth, age of infestation, littoral area, Secchi depth, parking spaces at water access, and milfoil management score) indicated that the differences in these averages between taxa were insignificant. These data inform us that the types of lakes that hybrid watermilfoil inhabits are very similar to those of Eurasian and northern in regards to these lake attributes. Wu et al. (2015) found that hybrid was more common in areas where northern and Eurasian occupied the same habitat. In Minnesota, northern was likely present in all lakes infested with Eurasian but may have subsequently disappeared from competition with Eurasian (Nichols 1994) or as non-target impacts of Eurasian herbicidal control.

We found hybrids in six of the seven counties of the Twin Cities metro. We found no hybrids in Carver County, although we did find Eurasian in numerous Carver County lakes. It is likely that hybrids will be found in Carver County, with hybrid's location dependent upon lake distance from current hybrid infestations, but the lack of hybrids in the county is puzzling. On average, the metro lakes we surveyed overall had higher parking spot counts at lake accesses in comparison to greater Minnesota, indicating that metro lakes have increased opportunities to introduce hybrids or Eurasian. In order to predict where hybrids will infest next, it is important to look at where it is currently present. Although hybrid milfoil was most common in the metro it was found in 5 lakes outside the metro, however, none were further than 80 km from Lake Minnetonka.

In lakes where hybrids were present with a parental taxon, hybrids were more often present with Eurasian rather than northern. This may be due to northern being outcompeted by the invasive milfoil species over time (Nichols 1994). It is important to note we were sampling based on documented Eurasian/hybrid infestations, so it makes sense that northern would be found in fewer lakes because our data do not truly describe its distribution. We had 13 lakes where we found hybrid watermilfoil only, which indicates that hybrids do not necessarily require their parental taxa be present in a lake. This suggests that hybrids are capable of infesting a lake through either asexual propagation, or sexual reproduction or that once present, they outcompete their parents. We had initially predicted that hybrids would most likely be present in lakes with older ages of infestation, but our analysis did not find this difference to be significant. Although Eurasian infested lakes on average had older ages of infestation, and hybrids were more commonly found in the metro, this did not directly translate to hybrid infestations being older.

LaRue et al. (2013a) found that hybrids were more common in lakes that had been treated whereas parentals were more common in lakes without treatment history. Similarly, Parks et al. (2016) found the relative frequency of Eurasian went way down following treatment whereas the relative frequency of hybrids went way up. This suggests that perhaps hybrids had a greater competitive advantage in treated lakes and can displace the pure parental genotypes. In these cases the competitive advantage may in part be due to tolerance to the herbicide.

In assessing our genetic data, we found a significant difference (p < 0.01) in average genotypes found per taxon. Hybrids were found to be intermediately diverse compared to Eurasian and northern. Hybrid had a statewide average of 2.5 genotypes present in a lake, whereas Eurasian had one and northern had 3.6. This suggests that Eurasian hybridizes more with northern than it reproduces with itself, or that hybrids undergo more sexual reproduction than Eurasian allowing it to create genetically diverse lake infestations. In terms of managing

Eurasian infestations, this is quite promising because it means that Eurasian watermilfoil is not sexually reproducing very often and therefore won't likely develop new genotypes that may later be tolerant to commonly used herbicides (although somatic mutations could confer resistance, e.g., Michel et al. 2004). The diversity in hybrid means there are more opportunities for genotypes that are tolerant of or resistant to an herbicide. This also indicates that hybrids have most likely inherited their genetic diversity from northern watermilfoil rather than Eurasian. Hybrid lakes containing a single hybrid genotype were significantly younger than hybrid lakes with more than 2 genotypes. All of the lakes with 3 or more genotypes of hybrid have been listed infested since 2003. This observation indicates that older invasive milfoil infestations are prone to developing numerous hybrid genotypes and may be locations of interest for management if herbicide tolerance becomes apparent with specific hybrid genotypes.

Although diversity of hybrid milfoil may be associated with age of infestation, many of the east metro lakes that shared hybrid genotypes were relatively new infestations, consistent with clonal spread after development in a source lake (such as White Bear, Bald Eagle or Lac Lavon). In contrast to Eurasian watermilfoil, where one genotype is dominant and widespread, we have not been able to identify any wide-spread genotype of hybrid that might be particularly problematic, but that is the aim of our ongoing work. There does not yet appear to be a few genotypes that are being widely spread. In Michigan, Thum's lab has found one hybrid genotype in six lakes across Michigan that is the same genotype as a known fluridone-resistant genotype isolated from Townline Lake, Michigan (Berger et al. 2012, 2015; Thum et al. 2012) and that also appears to exhibit diquat resistance (Netherland and Willey 2017).

There were varied responses to management and continued assessment during the next two years will provide more complete interpretation. In general, abundance and genetic structure remained fairly consistent over time in the reference lakes. As with our larger data set, hybrid diversity within lakes is not prevalent and only Smith's Bay had a number of genotypes (but the treated bays North Arm and Grays also had numerous genotypes). There was an increase in hybrid relative to Eurasian between 2016 and 2018, but no change in hybrid genotypes in this untreated bay. The fluridone treatments were quite effective at controlling milfoil and ongoing sampling will be needed to determine if there are any shifts in genetic composition. Due to the limited treatment areas, there was a more variable response to the auxin mimics 2,4-d and ProcellaCor. In Bald Eagle, Eurasian and hybrid increased across years but decreased after treatment and northern, which was largely untreated, responded conversely. Because only one genotype of Eurasian and one of hybrid has been found in Bald Eagle, shifts in genotypic composition have not been seen.

Lakewide results with ProcellaCor were more mixed. It is not known if the lesser control on Ham Lake was due to ineffective treatment or to a tolerant hybrid genotype or both. The poor control in Ham Lake was likely due to under dosing, but the Ham Lake genotype has been identified as potentially tolerant (Beets and Netherland 2018). A follow up treatment in late Fall 2018 appears to have been more effective and genetic analyses of milfoil found in early summer 2019 has not been completed. It will be important to find out whether the increase in milfoil abundance in 2018 had to do with the targeting or scale of these treatments or response of tolerant genotypes. The decrease in native plants after treatment at Ham raises questions regarding the effect ProcellaCOR has on native plant communities, or whether this has to do with specific lake dynamics on Ham. Although there was considerable genetic diversity in Grays Bay, there were no significant shifts in genetic composition despite bay-wide increases in hybrid watermilfoil. With ProcellaCOR being a new herbicide, it will be interesting to continue to monitor these two lakes to assess the milfoil population in the future.

Continued monitoring of these various herbicide treatments will be needed to determine if problematic genotypes are present in Minnesota and we will expand our statewide assessments to better identify potentially problematic genotypes in Minnesota. The response to fluridone in North Arm and Schmidt Lake suggest that fluridone tolerant genotypes were not present in these lakes but there has been limited prior use of fluridone in Minnesota and none in these lakes. It likely will be several years before we can determine what genotypes return in these lakes.

Hybrid watermilfoil is widespread in Minnesota and has much more genetic diversity than its parent Eurasian watermilfoil. The greater genetic diversity increases the likelihood that problematic genotypes will emerge. Although we have yet to identify particularly problematic genotypes this study has provided the background data and direction to better assess for problematic genotypes in Minnesota.

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