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Integrating DNA fingerprinting of invasive watermilfoil strains into aquatic vegetation monitoring and assessment

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ABSTRACT

Eurasian watermilfoil (*Myriophyllum spicatum* L.) and its hybrids with native northern watermilfoil (*Myriophyllum spicatum* L. × *Myriophyllum sibiricum* Komarov) are among the most heavily managed invasive aquatic plants in the United States. Previous genetic studies have identified numerous distinct Eurasian and hybrid strains that can spread within and among waterbodies via clonal propagation. Strains can differ in their invasiveness (e.g., growth and potential for spread) and response to herbicides. Characterizing particularly problematic or invasive strains of watermilfoil could help inform management decisions. However, identifying strains for laboratory study (e.g., herbicide response) is a significant logistical challenge. One promising tool to address this problem is strain-level monitoring. In this study, we integrated genetic fingerprinting that can distinguish different watermilfoil strains into aquatic vegetation monitoring in eight Minnesota lakes over the course of 3 yr. Specifically, we looked for changes in strain composition of watermilfoil populations over time to identify strains of specific interest for further characterization of growth and herbicide response. Using a simulation-based chi-square analysis, we documented significant changes in strain composition in six of the eight waterbodies monitored, and we identified three strains of invasive watermilfoil and two strains of native northern watermilfoil to prioritize for further investigation. Although more work is needed to determine the best sampling strategies and statistical analysis of spatiotemporal strain data, our study suggests that integrating genetic fingerprinting into aquatic vegetation management could help to more efficiently identify and manage the most troublesome watermilfoil strains.

Key words: genetic monitoring, herbicide resistance evolution, *Myriophyllum spicatum* L., northern watermilfoil, strain identification.

INTRODUCTION

Eurasian watermilfoil (*Myriophyllum spicatum* L.) is one of the most heavily managed invasive aquatic plants in the United States (Bartodziej and Ludlow 1997). Eurasian watermilfoil hybridizes with native northern watermilfoil (*Myriophyllum sibiricum* Komarov), and hybrids are also considered invasive to North America (Moody and Les 2002, Moody and Les 2007, Zuellig and Thum 2012). Invasive watermilfoils cause environmental damage by decreasing native plant and animal diversity, and they cause economic damage by inhibiting water recreation (Smith and Barko 1990, Madsen 1999, Cheruvelil and Soranno 2002). As a result, both Eurasian and hybrid watermilfoil are heavily managed, primarily through the use of herbicides.

Within Eurasian, northern, and hybrid watermilfoil, there are distinct genotypes or strains that are produced through sexual reproduction but can be maintained indefinitely by clonal propagation (Moody and Les 2002). Strains can differ in their growth, potential for spread, and response to herbicides used to control them (LaRue et al. 2013). For example, two strains have been documented as fluridone resistant, whereas many other strains are susceptible (Thum et al. 2012, Berger et al. 2015, Chorak and Thum 2020). Variation among strains in their growth rate and response to other herbicides, such as 2,4-D, diquat, and benzyl 4-amin-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate (florpyrauxifen-benzyl) has also been documented (LaRue et al. 2013, Netherland and Willey 2017, Taylor et al. 2017, Beets et al. 2019, Hoff and Thum, personal communication).

Knowledge of strains' growth, potential for spread, and response to candidate herbicides used to control them would be informative for local management planning. This information exists for a few strains. However, a significant challenge is how best to identify strains to prioritize them for laboratory characterization because strain diversity is high (Thum et al. 2020) and because logistical constraints limit the number of strains and herbicides that can be tested in a period of time in a single laboratory.

One promising approach to identify potentially problematic strains for further testing in the laboratory is to monitor the strain composition of populations over time. Some lakes can contain multiple strains (Thum et al. 2020). In addition, if the strains within a lake differ in their growth, potential for spread, or herbicide response, it stands to reason that their relative abundances could shift over time, e.g., a herbicide-resistant strain is expected to increase in

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Table 1. Lakes surveyed: the location of all waterbodies included in this study and the number of sites sampled in each waterbody. The DOW number is the lake identification number given to each waterbody by the Department of Natural Resources in Minnesota.

Lake	State	County	DOW	Latitude	Longitude	Sites sampled
Christmas	MN	Hennepin	27013700	44°53′48.4794″N	93°32′40.1994″W	113
Bald Eagle	MN	Ramsey	62000200	45°6′57.24″N	93°0′59.3994″W	151
Grays Bay	MN	Hennepin	27013301	44°57′14.0394″N	93°29′40.2″W	125
Ham	MN	Anoka	02005300	45°15′25.56″N	93°13′18.84″W	147
Independence	MN	Hennepin	27017600	45°1′45.4794″N	93°38′41.9994″W	198
North Arm	MN	Hennepin	27013313	44°57′31.32″N	93°37′12″W	229
Phelps Bay	MN	Hennepin	27013301	44°54′56.1594″N	93°38′56.4″W	148
Smith's Bay	MN	Hennepin	27013302	44°57′9.3594″N	93°34′1.9194″W	127

relative frequency after management with that herbicide. Therefore, changes in strain composition over time within a lake could be used to identify strains that might be particularly invasive or herbicide resistant, and such strains could then be prioritized for laboratory testing of herbicide response.

Although the potential for changes in genetic composition clearly exists and such changes, if documented, could help to identify more problematic/invasive strains, strain identification is not routinely integrated into management planning or evaluation. Aquatic vegetation monitoring (e.g., mapping distribution and abundance before and after control) is common for many operational watermilfoil management projects, and minimal additional effort is required to collect plants for genetic analysis during these monitoring efforts. A previous study incorporated genetic data into watermilfoil monitoring before, versus after, herbicide treatments (Parks et al. 2016), but that study only distinguished Eurasian from hybrid watermilfoil and did not track individual strains over time. However, molecular tools for genetic fingerprinting (the analysis of DNA to identify and distinguish individuals, in this case, strains) are readily available (e.g., Thum et al. 2020), and can, therefore, be used to identify and track individual strains.

Therefore, in this study, we integrated genetic fingerprinting, which can distinguish different watermilfoil strains, into aquatic vegetation monitoring in eight Minnesota lakes: five that received herbicide treatment and three that did not. We used these data to determine whether the strain composition in these invasive watermilfoil populations changed over time, and if so, to identify potentially problematic strains to prioritize for future laboratory characterization of growth and herbicide response.

MATERIALS AND METHODS

Field sampling

Between 2018 and 2020, field surveys and sample collection were carried out in eight waterbodies in Minnesota. Four of the waterbodies surveyed were separate bays in Lake Minnetonka (Table 1). However, because Lake Minnetonka is large (>5,700 ha) and because bays are the operational management units for Lake Minnetonka, we analyzed them separately (Table 1). Of the eight waterbodies surveyed, five received herbicide treatments to control watermilfoil in at least 1 of the 3 yr surveyed. Within the waterbodies that were treated with herbicides, some received whole-lake treatments, whereas others received spot treatments, meaning that the herbicide was applied only in priority areas, rather than to the waterbody as a whole. The practice of spot treatments to manage invasive watermilfoil is common in Minnesota. In four of those waterbodies, the herbicides 2,4-D, florpyrauxifen-benzyl, or diquat were applied as spot treatments. In one waterbody (North Arm Bay of Lake Minnetonka), fluridone was applied as a whole-bay treatment (see Table 2 and Supplemental Maps).

Sampling sites in each waterbody were predetermined as a point-intercept grid over the littoral zone (defined as a depth of less than 4.6 m) (Mikulyuk et al. 2010, Eltawely et al. 2020), with grid spacing set to yield approximately 150 points in the littoral zone of each waterbody. At each sampling point, a rake was thrown over each side of the boat and drawn back in to collect plant material (rake toss) (Mikulyuk et al. 2010, Thum et al. 2012, Parks et al. 2016). At each point at which watermilfoil was observed, a representative meristem was collected for genetic analysis. Although, at some survey sites, aquatic plant species other than watermilfoil were observed, we did not collect data on the presence of other species for this study. Each watermilfoil sample was given a number and a character code corresponding to the lake in which it was found and the point in that waterbody (Eltawely et al. 2020). Each meristem was placed in a sealed bag and placed on ice. Upon return to the laboratory, each sample was placed in a labeled paper envelope, which was then placed in a sealable plastic bag with silica beads to dry the samples. Samples were then sent to Montana State University.

Six waterbodies were sampled twice per year, early in the growing season (June to July) and later in the growing season (late August to September): Bald Eagle, Christmas, and Grays Bay (Lake Minnetonka) and Ham, Independence, and Phelps Bay (Lake Minnetonka). Smith's Bay in Lake Minnetonka was sampled once per year in July as a reference (untreated lake). The North Arm of Lake Minnetonka was surveyed twice in 2018 (prefluridone and postfluridone treatment), and in subsequent years, it was sampled only in August (see Table 2). Christmas Lake was not sampled in late summer (August) 2020 because of logistical constraints.

Genetic fingerprinting

Total genomic DNA was extracted from the collected meristems using DNeasy Plant Mini Kits[1] according to the

Table 2. Survey and treatment history: the sampling times and herbicide treatments for each waterbody for each year of the study. Sampling times indicate when the waterbody was visited and when samples were collected. Some waterbodies received spot treatments, meaning that only certain priority areas of the waterbody were treated with herbicide. The total treated area (sum of all spot treatments) is given in hectares/acres in parentheses after the name of the herbicide applied.

	2018		2019		2020	
Lake	Sampling	Treatment (ha/ac)	Sampling	Treatment (ha/ac)	Sampling	Treatment (ha/acres)
Christmas	June and August	None	June and August	None	June	None
Bald Eagle	June and August	2,4-D (17.08/42.2)	June and August	None	June and August	None
Grays Bay	June and August	florpyrauxifen-benzyl (17.08/42.2)	June and August	florpyrauxifen-benzyl (4.0/8.4)	June and August	Diquat (6.31/15.6) and florpyrauxifen-benzyl (7.14/17.65)
Ham	June and August	florpyrauxifen-benzyl (4.9/12)	June and August	None	June and August	None
Independence	June and August	None	June and August	None	June and August	None
North Arm	June and August	Fluridone (whole lake)	August	None	August	None
Phelps Bay	None	None	August	None	June and August	Diquat and florpyrauxifen-benzyl (8.62/21.3)
Smith's Bay	July	None	July	None	July	None

Abbreviation: florpyrauxifen-benzyl, benzyl 4-amin-3-chloro-6-(4-chloro-2-fluoro-3-methoxyphenyl)-5-fluoropyridine-2-carboxylate

manufacturer's instructions. As in Thum et al. (2020), we determined the taxon and strain for each sample by using eight microsatellite loci (Myrsp 1, Myrsp 5, Myrsp 9, Myrsp 12, Myrsp 13, Myrsp 14, Myrsp 15, and Myrsp 16) from Wu et al. (2013). Fragment analysis of the fluorescently labeled microsatellite polymerase chain reaction (PCR) products was carried out by the University of Illinois, Urbana-Champaign, Core Sequencing Facility using an ABI 3730xl sequencer.[2] Scoring of microsatellites was carried out in GeneMapper (version 5.0).[3] Microsatellites were treated as dominant, binary data, based on the presence or absence of each possible allele at each locus. Distinct strains were delineated using Lynch distances and a threshold of zero in polysat software[4] (Clark and Jasieniuk 2011).

Statistical analysis

Changes in the strain composition of watermilfoil populations were analyzed at three distinct time scales: within a single growing season (June to August), over 1 yr (e.g., June in 1 yr to June in the next year), and over a 3-yr period (June 2018 to June or August 2020). The incidence for a given strain in a waterbody was interpreted as the total number of points at which that strain was identified in each year. For each strain, we also calculated the percentage of sampled sites as strain incidence, divided by the total number of sites at which watermilfoil was collected. We refer to identified strains by a four-character code: the first two letters indicate the waterbody in which the strain was identified; the middle letter indicates whether the strain is pure Eurasian watermilfoil (E), native northern watermilfoil (N), or a hybrid (H); and the final number distinguishes strains of the same taxa within the same waterbody. The abbreviation "MC" stands for "Minnesota clones" and is used to distinguish strains that are found in multiple waterbodies included in this study. We excluded from analysis any strain that was observed only once, because, without multiple occurrences, it was unclear whether singletons represent distinct strains or if they were the result of sequencing errors.

To determine whether the strain composition of each milfoil population changed over time, we used a simulated chi-square test for homogeneity, based on 2,000 permutations, to compare each set of timepoints. Because each of our data points represents a site on the lake that was visited repeatedly, they may violate the assumption of independence necessary to have full confidence in the results of the chi-square test. However, the exact point visited on the lake likely varied over time because of the variability in survey conditions (wind and water movement, etc.) and the accuracy of the global positioning system (GPS) used. Therefore, we believe that the simulation-based chi-square test is still useful for providing an indication of strain dynamics, until more-robust sampling methods are implemented. Furthermore, chi-square analysis has been used previously in similar studies of aquatic vegetation using point-intercept surveys (Mikulyuk et al. 2010, Nault et al. 2018). All statistical analysis was carried out in R software (version 3.6.3)[5] (RStudio Team 2018).

RESULTS AND DISCUSSION

Treated lakes

Bald Eagle Lake. In Bald Eagle Lake, in June 2018, the most-abundant strain was hybrid BE-H-3 (present at 41 sites; 40% of all watermilfoil occurrences), followed closely by a Eurasian strain MC-E-1 (present at 33 sites, 32% of all watermilfoil occurrences) and northern strain BE-N-2 (present at 27 sites, 26% of all watermilfoil occurrences). After 2,4-D treatment, in July 2018, all three strains (taxa) decreased (Figure 1A; Supplemental Map 1). Then, as the population recovered, there was a shift in its genetic composition: BE-N-2 and BE-H-3 increased disproportionately relative to MC-E-1 (Figure 1A). In June 2020, BE-N-2 was present at 40 sites, making up 68% of all watermilfoil occurrences, and BE-H-3 was present at 16 sites, 27% of all watermilfoil occurrences, whereas MC-E-1was not found again after 2019. Interestingly, the relatively greater increase of the northern strain BE-N-2 is counter to the

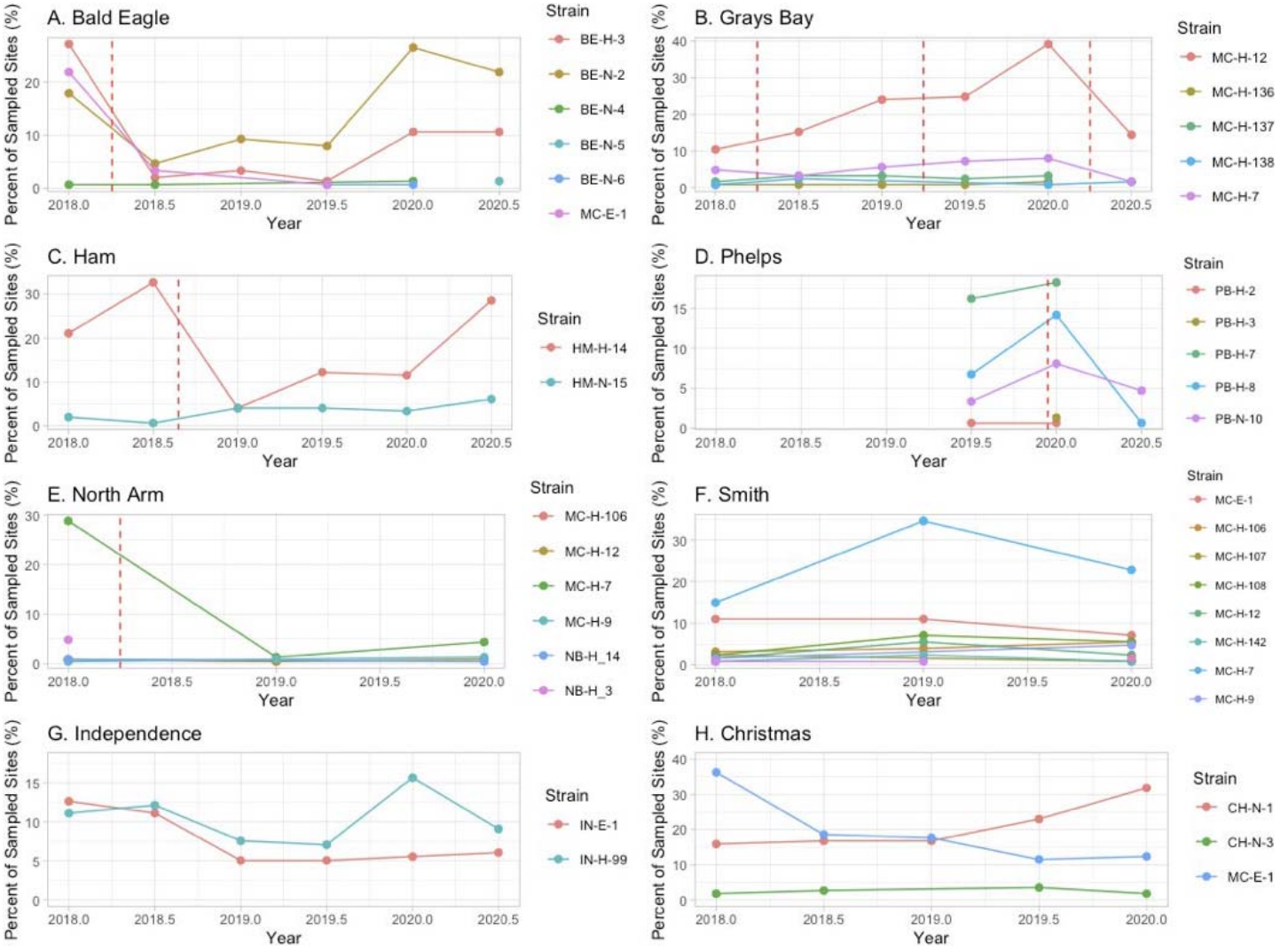

Figure 1. The strain composition of the watermilfoil population in each waterbody over time. Each panel represents a different waterbody. Point and line colors indicate different strains within the waterbody. Dashed, vertical, red lines indicate herbicide treatments. The x-axis represents the sampling timepoint, and the y-axis represents the percentage of sampled sites where each strain was found (number of sites where the strain was collected, divided by the total number of surveyed sites). Note that, although the changes in frequency are not continuous between dates, we have included lines connecting points of the same strain to make it easier to track individual strain occurrence over time. Strains are identified by a four-character code: the first two letters indicate the waterbody in which the strain was identified; the middle letter indicates whether the strain is pure Eurasian watermilfoil (E), native northern watermilfoil (N), or a hybrid (H); and the final number distinguishes strains of the same taxon within the same waterbody. The abbreviation "MC" stands for "Minnesota clones" and is used to distinguish strains that are found in multiple waterbodies included in this study.

general expectation that pure Eurasian and hybrid strains will outcompete native northern strains.

We found strong evidence for a change in the strain composition of the watermilfoil population in Bald Eagle Lake from June of 2018 to June of 2019 and from June of 2018 to June of 2020 (simulated chi-square, $P < 0.001$), but we did not find any strong evidence of a change within any of the three separate growing seasons ($P > 0.10$) (Table 3).

Both BE-H-3 and MC-E-1 are also found in other lakes around the Minneapolis metropolitan area. MC-E-1 is widespread across Minnesota, and BE-H-3 is the most commonly encountered hybrid strain in Minnesota thus far (Eltawely et al. 2020, Thum et al. 2020). The disproportionate increase of BE-N-2 and BE-H-3, compared with MC-E-1, in Bald Eagle Lake may indicate that they are relatively more-invasive strains (Figure 1A). All three strains would, therefore, be of interest for laboratory study to investigate and compare their relative growth rate and 2,4-D response.

Grays Bay. Grays Bay was spot-treated with florpyrauxifen-benzyl in all 3 yr and was also spot-treated with diquat in 2019 and 2020. Despite these repeated herbicide treatments, the bay-wide milfoil incidence continued to increase. Watermilfoil was found at 23 sites in June of 2018, 42 sites in June of 2019, and 66 sites in June of 2020. Bay-wide watermilfoil incidence did not decrease until August of 2020, when watermilfoil was found at only 22 sites. We identified five different strains of hybrid watermilfoil in Grays Bay; of these, MC-H-12 was the most common, and the other four were less common.

Table 3. Change in composition: *P* vaues indicate the amount of evidence for a change in strain composition in each waterbody at each of three timescales: within the growing season of each year (June to Sept of the same year), between years sampled (June of 1 yr to June of the subsequent year), and over the 3-yr window (June 2018 to June 2020). *P* values are based on a simulated chi-square test for homogeneity with 2,000 permutations. The treatment column indicates the years that each waterbody received a herbicide treatment (for the specific herbicide used at each timepoint see Table 2).

		P value					
		Growing season			1 yr		
Lake	Treatment	2018	2019	2020	2018 to 2019	2019 to 2020	3 yr
Bald Eagle	2018	0.136	0.347	0.276	<0.001	1	<0.001
Grays Bay (Minnetonka)	2018, 2019, and 2020	0.793	0.94	0.222	0.584	0.969	0.18
Ham	2018	0.304	0.256	0.754	0.006	0.143	0.33
North Arm (Minnetonka)	2018	NA	NA	NA	0.009	0.642	0.28
Phelps Bay (Minnetonka)	2020	NA	NA	0.008	NA	<0.001	<0.001
Smith's Bay (Minnetonka)	—	NA	NA	NA	0.061	0.054	0.36
Christmas	—	0.199	0.036	NA	0.096	0.017	<0.001
Independence	—	0.678	1	0.302	0.335	0.279	0.35

Abbreviation: NA, not available.

We did not detect a statistically significant change in genetic composition ($P > 0.10$) (Table 3). Instead, hybrid strain MC-H-12 remained the most-abundant strain across all 3 yr (Figure 1B). In June 2018, hybrid strain MC-H-12 was present at 13 sites, comprising 57% of all watermilfoil occurrences; in June 2019, it was present at 30 sites, comprising 71% of all watermilfoil occurrences, and in June of 2020, it was present at 49 sites, comprising 74% of all watermilfoil occurrences. It is notable that watermilfoil occurrence, including MC-H-12, appeared to increase primarily outside of treated areas (Supplemental Map 2). However, MC-H-12 also made up more than 60% of all watermilfoil occurrences in treated areas over all 3 yr, which indicates that its persistence is unlikely to be an artifact of simply escaping treatment. Instead, MC-H-12 may be either relatively fast to recolonize or less sensitive to the florpyrauxifen-benzyl and/or diquat, and therefore, further laboratory study of MC-H-12 is warranted.

Ham Lake. In Ham Lake, hybrid strain HM-H-14 was, by far, the most-abundant strain in June 2018 (present at 31 sites; 91% of all watermilfoil occurrences), whereas northern watermilfoil strain HM-N-15 was comparatively rare (present at three sites; 9% of all watermilfoil occurrences) (Figure 1C). Following spot-treatments with florpyrauxifen-benzyl in July 2018, hybrid strain HM-H-14 decreased from present at 48 sites (98% of all watermilfoil occurrences) in August 2018, to only six sites (50% of all watermilfoil occurrences) in June 2019. On the other hand, northern-strain HM-N-15 increased from present at a single site (2% of watermilfoil occurrences) in August 2018, to present at six sites (50% of all watermilfoil occurrences) in June 2019. This was a statistically significant change in genetic composition between 2018 and 2019 ($P = 0.006$) (Table 3). It is important to note that hybrid-strain HM-H-14 was more prevalent in treated areas compared with northern-strain HM-N-15 (areas treated in July 2018 contained eight sites with hybrid-strain HM-H-14 present in June 2018, whereas northern-strain HM-N-15 was not present in any treated areas) (see Supplemental Maps). Therefore, the disproportionate decrease of hybrid-strain HM-H-14 compared with northern-strain HM-N-15 may reflect the fact that it was disproportionately treated with herbicide. Nevertheless, even after being disproportionately treated with herbicides in 2018, in the absence of treatments in 2019 and 2020, hybrid-strain HM-H-14 rebounded to pretreatment abundance by the end of 2020 (hybrid-strain HM-H-14 was present at 42 sites in August 2020, accounting for 82% of all watermilfoil occurrences; Figure 1C). We did not find any statistical evidence for a change in composition between June 2018 and August 2020 ($P > 0.10$) (Table 3). A visual comparison of HM-H-14 incidence in treated versus untreated areas indicates that the florpyrauxifen-benzyl treatments appeared to be effective and that the persistence and increase in HM-H-14 over time reflects recolonization of treated areas from untreated areas (Supplemental Map 3). Therefore, there is no immediate concern regarding the efficacy of florpyrauxifen-benzyl on this strain, but additional monitoring and careful evaluation of the size of treated areas relative to recolonization ability of this strain is warranted.

Phelps Bay. Phelps Bay was not treated in 2019 but was treated with florpyrauxifen-benzyl in July 2020, resulting in a substantial decrease in bay-wide milfoil incidence between June 2020 (watermilfoil present at 63 sites) and August 2020 (watermilfoil present at only eight sites). We observed a concomitant, significant change in genetic composition between June and August 2020 ($P < 0.001$) (Table 3). Specifically, three hybrid strains (PB-H-2, PB-H-3, and PB-H-7), which were present before treatment, were not found at any sites after treatment, and one hybrid strain, PB-H-8 was found at only one site after treatment. Therefore, none of the hybrid strains in this bay raise any immediate concern about resistance to florpyrauxifen. In contrast, northern-watermilfoil strain PB-N-10 was present at seven sites after treatment in August 2020 and made up 88% of all watermilfoil occurrences (Figure 1D).

Of the watermilfoil identified within treated areas before treatment, 83% were hybrid strains (PB-H-7 and PB-H-8), whereas northern-strain PB-N-10 accounted for only 17% of treated sites (Supplemental Map 4). This raises the possibility that the disproportionate decrease in hybrid strains compared with northern-strain PB-N-10 reflects the formers' disproportionate treatment with florpyrauxifen-benzyl. Alternatively, the persistence of PB-N-10 (and the concomitant change in genetic composition) raises the possibility that PB-N-10 may be less susceptible to florpyr-

auxifen-benzyl compared with that of the hybrid strains present in 2018. Further genetic monitoring in Phelps Bay is warranted to determine whether the PB-N-10 will continue to outcompete and displace hybrid strains in the absence of herbicide.

North Arm. In North Arm, a bay-wide ("whole lake") fluridone treatment in 2018 virtually eliminated invasive watermilfoil (watermilfoil was observed at 84 sites in June 2018 before treatment, and no watermilfoil was observer in August 2018 after treatment). Therefore, there was no evidence for fluridone resistance by any of the strains present (Figure 1E). Further, there was no evidence for a change in genetic composition between any time points during the study period ($P > 0.10$) (Table 3). However, MC-H-7 was the dominant strain in June 2018 before treatment (of the 84 watermilfoil plants found in June 2018, 66 plants [78%] were MC-H-7), and it was also the dominant strain in 2020, when a small number of plants were found (of the 14 watermilfoil plants found in August of 2020, 8 [57%] were MC-H-7) (Figure 1E) (Supplemental Map 5). It is unclear whether the recolonization of MC-H-7 in 2020 reflects regrowth of a few plants that survived the 2018 treatment or whether MC-H-7 reflects recolonization from other Minnetonka bays in which it is present (MC-H-7 is also found in Smith's and Grays bays). Although there are no immediate concerns that MC-H-7 is fluridone resistant, further monitoring of North Arm is warranted to determine whether MC-H-7 is fast growing (more invasive), which could explain its rapid appearance in North Arm Bay after treatment.

Untreated waterbodies

Smith's Bay. In Smith's Bay, the overall milfoil abundance increased from 49 sites in 2018 to 83 sites in 2019, but then decreased to 65 sites in 2020 (Figure 1F; Supplemental Map 6). Hybrid strain MC-H-7 was the most-abundant strain in all 3 yr (hybrid-strain MC-H-7 comprised 39% of all watermilfoil occurrences in 2018, 53% in 2019, and 45% in 2020). The changes in overall milfoil abundance correspond to changes in the dominant MC-H-7 strain, which increased from present at 19 sites in 2018 to present at 44 sites in 2019 but, then, decreased to present at only 29 sites in 2020. As a result of these disproportionate changes, we observed marginally significant changes in genetic composition in Smith's Bay from 2018 to 2019 ($P = 0.054$) and from 2019 to 2020 ($P = 0.051$) (Table 2).

Lake Independence. In Lake Independence in 2018, Eurasian-strain MC-E-1 was slightly more abundant than hybrid-strain IN-H-99 (in June 2018, MC-E-1 was found at 25 sites, whereas IN-H-99 was found at 22 sites). However, IN-H-99 increased over the next 3 yr and became the more-dominant strain; by June 2020, IN-H-99 was found at 31 sites, whereas strain MC-E-1 was found at only 11 sites (Figure 1G; Supplemental Map 7). Although this change was not statistically significant ($P > 0.10$; Table 3), the switch in rank between IN-H-99 and MC-E-1 is interesting and is worth additional monitoring to determine whether the hybrid strain will outcompete MC-E-1 and displace it.

Christmas Lake. In Christmas Lake, the milfoil population was initially dominated by Eurasian-strain MC-E-1 (Figure 1H). In 2018, Eurasian-strain MC-E-1 was present at 41 sites and made up 67% of the watermilfoil occurrences, whereas the second most-abundant strain, northern-strain CH-N-1, was present at 18 sites and comprised 30% of watermilfoil occurrences. However, over the course of our study, the northern-watermilfoil strain CH-N-1 increased in incidence, whereas the Eurasian-watermilfoil strain MC-E-1 decreased (Supplemental Map 8). By 2020, northern-strain CH-N-1 was present at 36 sites and accounted for 69% of watermilfoil occurrences, whereas Eurasian-strain MC-E-1 was present at 14 sites and accounted for 27% of watermilfoil occurrences (Figure 1H). This increase in the relative frequency of CH-N-1 represents a significant change in strain composition from June to August 2019 ($P = 0.036$) and between June 2019 and June 2020 ($P = 0.017$). This pattern is surprising, because Eurasian-watermilfoil is typically assumed to outcompete native northern watermilfoil and because MC-E-1, in particular, is the widespread Eurasian strain in Minnesota (Eltawely et al. 2020).

Overall

To our knowledge, this is the first published study to document changes in strain composition of invasive watermilfoil populations over time. Two previous studies have documented changes in the relative frequency of pure Eurasian versus hybrid watermilfoil (Parks et al. 2016, Nault et al., 2018). However, none of these studies examined the invasive watermilfoil population composition beyond distinguishing pure Eurasian from hybrid watermilfoil.

In six of the eight waterbodies, we found evidence for a change in strain composition (meaning that strains increased or decreased disproportionately to one another) in a single year (June of 1 year to June of the following year): Bald Eagle Lake, Christmas Lake, Ham Lake, the North Arm of Lake Minnetonka, Smith's Bay, and Phelps Bay in Lake Minnetonka (Table 3: 1 yr). Additionally, in two waterbodies, we detected strong evidence of a change in strain composition within a single growing season: Christmas Lake in 2019 and Phelps Bay (Lake Minnetonka) in 2020 (Table 3: growing season). The fact that changes were observed indicates that the genetic composition of invasive watermilfoil populations can be dynamic over time, and the number of lakes in which changes were observed (six of eight in a 3-yr period) suggests that such changes are common when multiple strains co-occur (Table 3: 1 yr). Further, the fact that changes in composition were observed in both treated lakes and untreated lakes indicates that such changes may occur independent of management activities.

Throughout the course of this study, we identified three strains of invasive watermilfoil that merit further investigation: BE-H-3 increased disproportionately compared with other co-occurring strains in Bald Eagle Lake, MC-H-12 disproportionately increased despite repeated herbicide treatment in Grays Bay, and MC-E-1 was widespread in Bald Eagle, Christmas Lake, Independence Lake, and Smith's Bay. We, therefore, recommend that the growth rate and

herbicide response of these strains should be investigated via vegetative growth and herbicide assays.

Additionally, two strains, BE-H-3 and MC-E-1 (both identified in Bald Eagle Lake) are widespread across Minnesota (Thum et al. 2020), and BE-H-3 has been identified in seven lakes, thus far, in Minnesota (Eltawely et al. 2020). Interestingly, strain BE-H-3 has also been documented as the most-abundant strain in Otter Lake, MN. Previous studies have collected accessions from Otter Lake hybrid watermilfoil and used them in herbicide assays (Poovey et al. 2007, Berger et al. 2015). In one such study, Poovey et al. (2007) found that the Otter Lake hybrid strain response to 2,4-D was similar to that of the Eurasian strain included in this study. Although we do not have historic genetic identification to confirm whether the strain used in Poovey et al. (2007) is the same BE-H-3 strain found in Bald Eagle Lake in 2018 to 2020, it is reasonable to suspect that they may be the same. However, in Bald Eagle Lake, after a treatment with 2,4-D, strain BE-H-3 increased disproportionately compared with MC-E-1, which indicates that BE-H-3 could be more invasive (faster growing and/or more resistant) compared with at least one Eurasian strain. Because both of these strains are found in a number of other lakes in the region (Thum et al. 2020), including them in further laboratory assays could reveal insights about their relative invasiveness, which could be applied to the management of multiple lakes.

Interestingly, in three lakes, we observed native northern-watermilfoil strains that appeared to be outcompeting their invasive counterparts by disproportionately increasing or by decreasing less after treatment). In two of those lakes, the pattern may be linked to herbicide response. In Bald Eagle Lake, after 2,4-D treatment in 2018, native-strain BE-N-2 decreased less than its invasive hybrid and pure Eurasian counterparts. Additionally, over the next 2 yr, BE-N-2 continued to increase and remained the most-common strain. Similarly, in Phelps Bay, native strain PB-N-10 persisted, despite florpyrauxifen-benzyl treatment, whereas its hybrid counterparts perished. It is possible that these patterns can be explained by a disproportionate treatment of Eurasian and hybrid watermilfoil compared with the native northern strains in these lakes. However, it is also possible that the northern strains in these lakes may exhibit a decreased susceptibility to the herbicides applied, and this warrants further consideration, especially given the possibility that hybridization of resistant northern-watermilfoil strains with Eurasian watermilfoil might generate resistant hybrid strains.

In addition, in Christmas Lake, the native northern-watermilfoil strain appears to be outcompeting its invasive counter parts in the absence of herbicide treatment. Between 2018 and 2020, the native northern-watermilfoil strain CH-N-1 displaced the pure Eurasian-strain MC-E-1 as the most-common strain in the population. This pattern is surprising because long-term field observation suggests that both hybrid and pure Eurasian watermilfoil are generally more invasive and outcompete native northern watermilfoil (Aiken 1979, Nichols 1994; but see Valley and Newman 1998). In the absence of herbicide treatment, one possible explanation for the disproportionate increase of native northern watermilfoil over pure Eurasian watermilfoil may be the presence of the native milfoil weevil (*Euhrychiopsis lecontei*) because weevils prefer Eurasian watermilfoil over the native northern strain (Newman 2004). Our observations suggest that the interactions between native and invasive watermilfoils may warrant further investigation.

The herbicide response of the rest of the strains detected in this study did not raise immediate concerns. However, where feasible, we recommend continued genetic monitoring to identify potentially problematic strains before they become management concerns.

Recommendations

We advocate for more-widespread integration of genetic monitoring into invasive watermilfoil management because it can be used to detect changes in strain composition that might signify the presence of strains of watermilfoil that are particularly problematic or invasive. Additionally, genetic monitoring efforts may confirm the presence in the lake of strains that have been previously characterized from other waterbodies. We recognize that incorporating genetic monitoring into watermilfoil management programs includes additional cost and effort, and incorporating genetic monitoring for every lake is not feasible. However, aquatic vegetation mapping is common for many operational watermilfoil management projects, and for those projects, we argue that minimal additional effort is required to collect milfoil plants for genetic analysis. Because the financial cost of genetic monitoring may be prohibitive, we recommend that an initial sample of approximately 20 to 50 plants be analyzed from any given lake before undertaking any extensive genetic monitoring to determine whether monitoring might be informative. For example, genetic monitoring of lakes with only one strain is unlikely to be worth the cost investment, whereas genetic monitoring will be more informative for lakes with multiple strains. Therefore, we encourage managers to consider incorporating genetic monitoring into management projects where it is feasible for them to do so.

However, although integrating genetic fingerprinting into aquatic vegetation surveys may allow researchers to prioritize certain strains for laboratory characterization, observation of a change in composition alone is insufficient to confirm herbicide resistance, and laboratory assays for growth rate and herbicide response are still necessary. It should also be noted that, in some cases, there may be no observable change in population composition, even when problematic or invasive strains of watermilfoil are present. Here, we identify potentially confounding factors that should be accounted for when interpreting the results of genetic monitoring efforts, and we offer suggestions for further research to address these challenges.

First, control efficacy will be a function of the herbicide concentration and exposure time (CET), which may differ intentionally or unintentionally. In this study—as for most watermilfoil management—we do not have data on the herbicide concentration and exposure times achieved. Therefore, it is possible that some of the effects we observed are a function of differences in CET achieved

rather than differences between strains in their growth rate or herbicide response. Further, the same strain may exhibit better or worse control in one lake compared with another because of differences in CET achieved. Nevertheless, and because most watermilfoil management includes vegetation monitoring anyway, we feel that monitoring strain dynamics can help identify strains for further study in the laboratory.

Second, when interpreting lake-wide dynamics in spot-treated lakes, it is important to consider the spatial structure of milfoil strains and whether there is any relationship between where each strain is found and where spot treatments were applied. For example, if one strain happens to disproportionately occur inside or outside treated areas, then it may disproportionately decrease or increase, which could confound inferences about its relative sensitivity to the herbicide application. This may have been the case in Ham Lake with hybrid-strain HM-H-14 compared with northern-strain HM-N-15. We, therefore, recommend further investigation into the spatial structure of milfoil strains within lakes, and genetic monitoring efforts on whole-lake treatments may avoid some of the confounding factors associated with spot treatments. Alternatively, future studies should carry out more-concentrated sampling within treated areas to evaluate spot treatments.

Third, it is important to recognize that genetic monitoring cannot necessarily distinguish among plants that recolonized treated areas from untreated areas versus plants that survived herbicide treatment because they exhibit some level of resistance per se. For example, fast-growing strains may not exhibit resistance but may effectively recolonize treated areas over short periods, giving the appearance of resistance. This may have been the case in the North Arm Bay with hybrid-strain MC-H-7. Nevertheless, we argue that strains that occur within treated areas relatively quickly after treatment should be prioritized for laboratory study to specifically test whether they are resistant and/or fast growing (i.e., relatively more invasive).

Finally, it is important to distinguish between statistical significance and biological significance when interpreting genetic monitoring data. Logistical restrictions in sample size (time and effort) present a challenge in acquiring adequate statistical power to detect biologically significant changes. For example, rare strains will have low counts and concomitantly low statistical power to detect changes. Similarly, when herbicide treatments are efficacious overall, the number of survey points with milfoil after treatment will be low, and therefore, the power to detect changes in composition may be low, unless and until milfoil occurrence increases to pretreatment levels. With spot treatments, the number of intercept points that fall within treated areas may be low, which will limit the power to detect changes in treated areas. To address the challenge of statistical power, we recommend in silica power analyses and simulations to determine the amount of sampling necessary to detect changes of different magnitudes under different initial frequency scenarios.

Conclusion

We have shown that integrating genetic fingerprinting into aquatic vegetation management and evaluation can be used to track changes in strain composition and to identify strains that are of specific interest for further characterization of growth and herbicide response. We also provide sampling and interpretation recommendations for genetic monitoring strategies in the future. Although further research is still needed to determine the best sampling strategies and statistical analysis of spatiotemporal strain data, our study provides proof of concept that integrating genetic fingerprinting into aquatic vegetation management could facilitate efficient identification and management of the most troublesome watermilfoil strains.

SOURCES OF MATERIALS

[1]DNeasy Plant Mini Kit, Qiagen Corp., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[2]ABI 3730xl sequencer, Thermo Fisher Scientific, 168 3rd Avenue, Waltham, MA 02451.

[3]GeneMapper (version 5.0), Applied Biosystems/Thermo Fisher Scientific, 168 3rd Avenue, Waltham, MA 02451.

[4]polysat software, R Foundation for Statistical Computing, Vienna, Austria.

[5]R software (version 3.6.3), R Foundation for Statistical Computing, Vienna, Austria.

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LITERATURE CITED

Aiken SG, Newroth PR, Wile I. 1979. The biology of Canadian weeds, 34: *Myriophyllum spicatum* L. Can. J. Plant Sci. 59:201–215.

Bartodziej W, Ludlow J. 1997. Aquatic vegetation monitoring by natural resources agencies in the United States. Lake Reservoir Manage. 13(2):109–117.

Beets J, Heilman M, Netherland MD. 2019. Large-scale mesocosm evaluation of florpyrauxifen-benzyl, a novel arylpicolinate herbicide, on Eurasian and hybrid watermilfoil and seven native submersed plants. J. Aquat. Plant Mange. 57:49–55.

Berger ST, Netherland MD, MacDonald GE. 2015. Laboratory documentation of multiple-herbicide tolerance to fluridone, norflurazon, and topramazone in a hybrid watermilfoil (*Myriophyllum spicatum* × *M. sibiricum*) population. Weed Sci. 63(1):235–241.

Cheruvelil KS, Soranno PA. 2002. Plant architecture and epiphytic macroinvertebrate communities: The role of an exotic dissected macrophyte. J. N. Am. Benthol. Soc. 21(2):261–277.

Chorak GM, Thum RA. 2020. Identification of resistant clones of Eurasian (*Myriophyllum spicatum*) and hybrid (*Myriophyllum spicatum* × *Myriophyllum sibiricum*) watermilfoil to an operational rate of fluridone. Invasive Plant Sci. Manage. 13(4):247–251.

Clark LV, Jasieniuk M. 2011. POLYSAT: An R package for polyploid microsatellite analysis. Mol. Ecol. Resour. 11(3):562–566.

Eltawely JA, Newman RM, Thum RA. 2020. Factors influencing the distribution of invasive hybrid (*Myriophyllum spicatum* × *M. sibiricum*) watermilfoil and parental taxa in Minnesota. Diversity 12:120.

LaRue EA, Zuellig MP, Netherland MD, Heilman MA, Thum RA. 2013. Hybrid watermilfoil lineages are more invasive and less sensitive to a commonly used herbicide than their exotic parent (Eurasian watermilfoil). Evol. Appl. 6(3):462–471.

Madsen JD. 1999. Point Intercept and Line Intercept Methods for Aquatic Plant Management. Army Engineer Waterways Experiment Station Technical Note: ADA361270, Vicksburg MS. 16 pp.

Mikulyuk A, Hauxwell J, Rasmussen P, Knight S, Wagner KI, Nault ME, Ridgely D. 2010. Testing a methodology for assessing plant communities in temperate inland lakes. Lake Reserv. Manage. 26:54–62.

Moody ML, Les DH. 2002. Evidence of hybridity in invasive watermilfoil (*Myriophyllum*) populations. Proc. Natl. Acad. Sci. U.S.A. 99(23):14867–14871.

Moody ML, Les DH. 2007. Geographic distribution and genotypic composition of invasive hybrid watermilfoil (*Myriophyllum spicatum* × *M. sibiricum*) populations in North America. Biol. Invasions 9(5):559–570.

Nault ME, Barton M, Hauxwell J, Heath E, Hoyman T, Mikulyuk A. 2018. Evaluation of large-scale low-concentration 2,4-D treatments for Eurasian and hybrid watermilfoil control across multiple Wisconsin lakes. Lake Reserv. Manage. 34(2):115–129.

Netherland MD, Willey L. 2017. Mesocosm evaluation of three herbicides on Eurasian watermilfoil (*Myriophyllum spicatum*) and hybrid watermilfoil (*Myriophyllum spicatum* × *Myriophyllum sibiricum*): Developing a predictive assay. J. Aquat. Plant Mange. 55:39–41.

Newman RM. 2004. Invited review—Biological control of Eurasian watermilfoil by aquatic insects: basic insights from an applied problem. Arch. Hydrobiol. 159(2):145–184.

Nichols SA. 1994. Evaluation of invasions and declines of submersed macrophytes for the Upper Great Lakes Region. Lake Reserv. Manage. 10(1):29–33.

Parks SR, McNair JN, Hausler P, Tyning P, Thum RA. 2016. Divergent responses of cryptic invasive watermilfoil to treatment with auxinic herbicides in a large Michigan lake. Lake Reserv. Manage. 32(4):366–372.

Poovey AG, Slade JG, Netherland MD. 2007. Susceptibility of Eurasian watermilfoil (*Myriophyllum spicatum*) and a milfoil hybrid (*M. spicatum* × *M. sibiricum*) to triclopyr and 2,4-D amine. J. Aquat. Plant Mange. 45:111–115.

RStudio Team 2018. RStudio: Integrated development environment for R. RStudio, Inc, R Foundation for Statistical Computing, Vienna, Austria. http://www.rstudio.com/. Accessed January 10, 2020.

Smith CS, Barko JK. 1990. Ecology of Eurasian watermilfoil. J. Aquat. Plant Manage. 28:55–64.

Taylor LA, McNair JN, Guastello P, Pashnick J, Thum RA. 2017. Heritable variation for vegetative growth rate in ten distinct genotypes of hybrid watermilfoil. J. Aquat. Plant Manage. 55:51–57.

Thum R, Heilman M, Hausler PJ, Hubert LE, Tyning P, Wcisel DJ, Zuellig MP, Berger ST, Netherland MD. 2012 Field and laboratory documentation of reduced fluridone sensitivity of a hybrid watermilfoil biotype (*Myriophyllum spicatum* × *Myriophyllum sibiricum*). J. Aquat. Manage. 50:141–146.

Thum RA, Parks S, McNair JN, Tyning P, Hausler P, Chadderton L, Tucker A, Monfils A. 2017. Survival and vegetative regrowth of Eurasian and hybrid watermilfoil following operational treatment with auxinic herbicides in Gun Lake, Michigan. J. Aquat. Manage. 55:103–107.

Thum RA, Chorak GM, Newman RM, Eltawely JA, Latimore J, Elgin E, Parks S. 2020. Genetic diversity and differentiation in populations of invasive Eurasian (*Myriophyllum spicatum*) and hybrid (*Myriophyllum spicatum* × *Myriophyllum sibiricum*) watermilfoil. Invasive Plant Sci. Manage. 13(2):59–67.

Valley RD, Newman RM. 1998. Competitive interactions between Eurasian watermilfoil and northern watermilfoil in experimental tanks, J. Aquat. Plant Manage. 36:121–126.

Wu ZG, Yu D, Xu XW. 2013. Development of microsatellite markers in the hexaploid aquatic macrophyte, *Myriophyllum spicatum* (Haloragaceae). Appl. Plant Sci. 1(2):apps.1200230.

Zuellig MP, Thum RA. 2012. Multiple introductions of invasive Eurasian watermilfoil and recurrent hybridization with northern watermilfoil in North America. J. Aquat. Plant Manage. 50:1–19.