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Field and laboratory documentation of reduced fluridone sensitivity of a hybrid watermilfoil biotype (*Myriophyllum spicatum* × *Myriophyllum sibiricum*)

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ABSTRACT

Since receiving US Environmental Protection Agency registration in 1986, the aquatic herbicide fluridone has been successfully used for selective, low dose (<10 μg L^{-1}) control of many Eurasian watermilfoil (*Myriophyllum spicatum*) populations and, in some states, continues to be a common chemical management option for larger infestations of this invasive aquatic plant. The discovery of fluridone resistance in several Florida strains of hydrilla in the late 1990s has increased awareness of potential shifts in fluridone susceptibility in managed Eurasian watermilfoil populations; however, reports of fluridone tolerance by watermilfoils remain anecdotal. We present detailed field and laboratory data that document reduced fluridone sensitivity by a strain of hybrid watermilfoil (*M. spicatum* × *M. sibiricum*) from a central Michigan lake. Overall, watermilfoil was more abundant 60 days after fluridone application at a target rate of 6 μg L^{-1} than before the application, and significantly more sites had watermilfoil post-treatment than expected under a model of at least 80% dieback. Laboratory comparisons of fluridone sensitivity of the central Michigan hybrid strain demonstrated that it grew through concentrations up to 12 μg L^{-1} whereas one Eurasian watermilfoil strain and a second hybrid watermilfoil strain were highly impacted at concentrations of 3 to 4 μg L^{-1}. This first confirmation of a fluridone-tolerant population of watermilfoil supports the value of pretreatment screening of herbicide sensitivity as part of invasive watermilfoil management. Although the tolerant watermilfoil strain was a hybrid biotype, a second tested strain of hybrid watermilfoil exhibited typical fluri-

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done sensitivity, indicating that hybridity does not necessarily confer fluridone tolerance. Thus, the factors contributing to fluridone tolerance are unknown and warrant further research.

Key words: 1-methyl-3-phenyl-5-3-(trifluoromethyl)phenyl-41H-pyridinone, aquatic herbicide, hybridity, resistance, tolerance, watermilfoil, fluridone

INTRODUCTION

Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) is one of North America's most common and problematic invasive aquatic weeds, especially in the northern tier of the United States. In addition, a large number of invasive populations of watermilfoil have been identified as hybrids between EWM and the closely related native northern watermilfoil (*Myriophyllum sibiricum* Komarov; NWM; Moody and Les 2002, 2007, Sturtevant et al. 2009; Zuellig and Thum, unpublished data). Both EWM and hybrids are frequently managed with similar methods, including chemical (e.g., Hamel et al. 2001: 2,4-D; Madsen et al. 2002: fluridone; Poovey et al. 2007: triclopyr), biological (Newman 1996: watermilfoil weevil [*Eurychiopsis lecontei*]), and mechanical controls (Unmuth et al. 1998: close-cut mechanical harvesting). In many instances, however, lake managers are unaware that they are managing hybrids because the hybrids are difficult to distinguish from EWM on the basis of morphology and require molecular identifications (Moody and Les 2007).

Since being registered by the US Environmental Protection Agency in 1986, fluridone has been effectively used to selectively control EWM and hybrid watermilfoils. In the state of Michigan, fluridone has been used since 1987 to manage EWM. From 1987 to 2003 fluridone was applied to Michigan lakes at rates estimated from 5 to 46 µg L^{-1}. After considerable investigation, the Michigan Department of Environmental Quality (the state agency responsible for approving aquatic plant management permits) concluded that fluridone concentrations between 5 and 8 µg L^{-1} were effective in controlling EWM with minimal impacts to native plant species, and that retreatment within 10 to 14 days maintained the required concentration–exposure time (MESB Sonar Investigative Panel 1999, Getsinger et al. 2001, 2002). This work culminated in a statewide standard in Michigan of whole-lake treatments at a target concentration of 6 µg L^{-1}, with retreatment 2 weeks later to raise the ambient fluridone concentration back up to 6 µg L^{-1} (known as the "6-bump-6" treatment protocol and referred to as such hereafter).

Over the past several years, anecdotal accounts of tolerance to fluridone treatment in invasive watermilfoil populations in Michigan have increased. Several Florida populations of the submersed plant hydrilla (*Hydrilla verticillata* L.f. Royle) are resistant to fluridone (Michel et al. 2004, Arias et al. 2005), reinforcing the value of sound stewardship of fluridone use for watermilfoil control. No quantitative, peer-reviewed studies have confirmed reduced fluridone response in invasive watermilfoils. The purpose of this study was to present field and laboratory data that document reduced fluridone response by a hybrid watermilfoil population in a central Michigan lake.

MATERIALS AND METHODS

Study lake. Townline Lake is a 116 ha lake located in the central portion of Michigan's Lower Peninsula. Mean depth is 3.6 m, and approximately half of the bottomland is shallow enough to support macrophyte growth. Townline Lake has been infested with invasive watermilfoil since at least 1974 (EDI Inc. 1978). Townline Lake was treated with fluridone in 1996 at an estimated concentration of 8 µg L^{-1} and in 2000 at 6-bump-6. Survey data from Michigan's Aquatic Vegetation Assessment Sites (AVAS) protocol indicated that the 2000 treatment was not completely successful; however, it is unclear whether those results reflected an insufficient dose and exposure or some difference in herbicide susceptibility of the lake's watermilfoil. Various other aquatic herbicides were used in the following years, but EWM pressure continued, and in 2009 fluridone was again considered for EWM control.

The decision to treat the Townline Lake milfoil population with fluridone led to a formal pretreatment screen of fluridone susceptibility in fall 2009 using a proprietary commercial assay offered by SePRO Corporation termed the PlanTEST™, a modified analysis of fluridone biochemical injury with methods similar to Sprecher et al. (1998). This initial screen indicated a 3- to 4-fold fluridone tolerance in Townline Lake watermilfoil and triggered additional genetic and susceptibility testing to confirm that this response of watermilfoil occurred in the lake.

Genetic identifications of plants from Townline Lake indicated that the watermilfoil population consisted of hybrids. In 2009, we sampled several scattered locations throughout the lake for genetic analysis and processed 15 plants for genetic analysis. In 2010, we obtained additional samples for genetic analysis from 10 locations in our grid surveys conducted in late April (pretreatment) and 60 days after the fluridone application from the same 10 pretreatment grid points. We identified each individual as EWM or hybrid using established protocols for ITS DNA sequences (Moody and Les 2002, Thum et al. 2006, 2011, Sturtevant et al. 2009, Zuellig and Thum unpublished data). Briefly, we compared our sequences with previously published Eurasian, northern, and hybrid watermilfoil accessions (FJ426346-FJ426357 from Sturtevant et al. 2009). EWM and NWM are separated by four fixed polymorphisms over the directly sequenced stretch of ITS DNA, and hybrids can be identified by obvious biparental sequence polymorphisms at these four sites (Moody and Les 2002, 2007, Sturtevant et al. 2009).

Laboratory herbicide screens. *Study 1.* In March 2010 a greenhouse study was conducted at the US Army Engineer Research and Development Center, Lewisville Aquatic Ecosystem Research Facility (LAERF) in Lewisville, Texas. The study was conducted with the hybrid watermilfoil from Townline Lake and EWM obtained from an LAERF pond. Two apical tips of watermilfoil (15 cm) were planted in plastic pots (750 mL) filled with LAERF pond sediment amended with 3 g L^{-1} Osmocote fertilizer (16-8-12). Pots were topped with a 1 cm layer of sand, and four pots were placed in each aquarium (66 L) on 30 March 2010. Aquariums were filled with alum-treated Lake Lewisville water and were situated in 1000 L fiberglass tanks filled with water. Water temperatures in the aquariums were maintained at 24 C by either aquarium heaters or by circulating water through a Pacific Coast Imports C-1000 chiller.

Study 2. In May 2010 a greenhouse study was conducted at the University of Florida Center for Aquatic and Invasive Plants (CAIP), in Gainesville, Florida, with the hybrid watermilfoil from Townline Lake and a separate hybrid watermilfoil collected from Otter Lake, Minnesota. Plants were established on 27 April 2010 in 95 L tanks as described above with the exception that a commercial potting soil amended with Osmocote was used as the sediment source. The greenhouse was covered in 50% shade cloth, and temperatures were allowed to fluctuate with ambient outdoor conditions. Minimum water temperature was 19 C in late April with maximum temperatures recorded at 28 C in early July.

In both studies, plants were given a 20-day pretreatment growth period and then treated with fluridone (Stock solutions were prepared using Sonar™ A.S.) at concentrations of 1.5, 3, 6, and 12 µg L^{-1} (Study 1) and at 2, 4, 6, 8, 12, and 24 µg L^{-1} (Study 2). Water samples were collected at 1, 7, and 21 days after treatment (DAT), and residues were analyzed via enzyme-linked immunosorbent assay (ELISA) technique. Following a 60-day static exposure to fluridone, plants were harvested, and viable shoot biomass was dried to a constant weight at 65 C for 72 h. Each treatment was replicated (4 replicates for Study 1 and 5 replicates for Study 2); shoot biomass data are presented as means +95% confidence intervals (C.I.). Nonlinear regression analysis was also performed to describe a fluridone treatment rate effect.

Field study of herbicide response. Fluridone was permitted in 2010 for hybrid watermilfoil control on Townline Lake under the 6-bump-6 treatment protocol. Fluridone was applied to Townline Lake on 28 April 2010 at a target concentration of 6 µg L^{-1}. Water samples were taken for estimation of fluridone concentration using FasTEST (an internal SePRO liquid chromatographic method) on 30 April 11 May, 27 May, and 24 June. Based on estimated concentrations on 11 May, a repeat application ("bump") of 3.3 µg L^{-1} fluridone was applied to increase the concentration to the target 6 µg L^{-1} (Table 1).

We monitored watermilfoil distribution and abundance within Townline Lake before and after treatment and conducted surveys on the day of treatment (28 Apr; the same day of initial fluridone application, but considered as before treatment), 3 weeks after treatment (18 May), and at the end of the summer (13 Aug). Our sampling methods are similar to those described by Hauxwell et al. (2010). Using a geographic information system, a 91 m grid was plotted on the Townline Lake bathymetric map over locations where water depth was 4.5 m or less, creating 93 sampling stations at the grid vertices. Sampling locations were programmed into a handheld Global Positioning System (GPS).

We quantified watermilfoil abundance at each sampling point within the lake using a rake-toss index. While such an index has some obvious limits to its precision, it yields a sufficient qualitative picture of watermilfoil abundance at a given location, especially for pre- and post-treatment comparisons. At each sampling location, we averaged the index from two rake tosses thrown in distinctly different directions off the bow of the boat. Watermilfoil that was clearly dead was not counted. Our index values for each throw were as follows:

(0) Rake contained no living watermilfoil.

(1) Live watermilfoil comprised <5% of the rake tine space.

(2) Live watermilfoil comprised between 5 and 25% of the tine space.

(3) Live watermilfoil was common on the rake, but occupied <50% of the rake tine space.

(4) Rake was densely covered with live watermilfoil: >50% of the rake tine space.

We also conducted a χ^2 analysis to statistically test for deviation from the expected response to fluridone. We expected at least 80% of the sites with watermilfoil in the pretreatment survey to be devoid of watermilfoil in the post-treatment survey (i.e., 80% die-back); thus, we calculated our expected number of sites with watermilfoil for the post-treatment survey to be 20% of the sites with watermilfoil in the pretreatment survey. Because fluridone may take several weeks to impact the plant population, we performed these calculations by comparing data from the initial survey (28 Apr) and last survey of the summer (13 Aug).

RESULTS

We demonstrated for the first time reduced susceptibility to fluridone in a Eurasian watermilfoil hybrid population. While reduced fluridone susceptibility by watermilfoil has been qualitatively noted in earlier reports to lake boards, no quantitative studies have confirmed its presence in both the laboratory and field. Reduced fluridone sensitivity by the Townline Lake hybrid watermilfoil population was evident in both the field and the laboratory.

Laboratory herbicide screens. As a percentage of untreated control, hybrid watermilfoil collected from Townline Lake attained greater biomass than the LAERF EWM population at 60 days after exposure to 3, 6, and 12 µg L^{-1} fluridone (Figure 1). Even at the highest test rate, Townline watermilfoil maintained >50% of the untreated control biomass through the 60-day exposure and formed an extensive surface canopy in the presence of fluridone concentrations ranging from 3 to 12 µg L^{-1}. In contrast, the LAERF plants were barely visible in the water column and in poor condition at the time of harvest. The distinct visual differences were confirmed by the biomass data. The results of Study 2 were similar to those for Study 1. The Townline watermilfoil attained >40% of the untreated control biomass despite constant exposure

Table 1. Fluridone concentrations in surface water of Townline Lake during Spring 2010 Sonar A.S. Treatment. Repeat (bump) application of 3.3 µg L^{-1} was applied 18 May. Surface samples were collected from four sites on lake (three littoral, one open water).

Date (Days after initial treatment)	30 April 2010 (2 DAT)	11 May 2010 (13 DAT)	27 May 2010 (29 DAT)	24 June 2010 (57 DAT)
µg L^{-1} Fluridone	**4.2 ± 1.1**	**2.7 ± 1.3**	**5.3 ± 0.2**	**3.7 ± 0.2**

Error is ± 1 standard deviation (n = 4).
DAT is days after initial treatment.

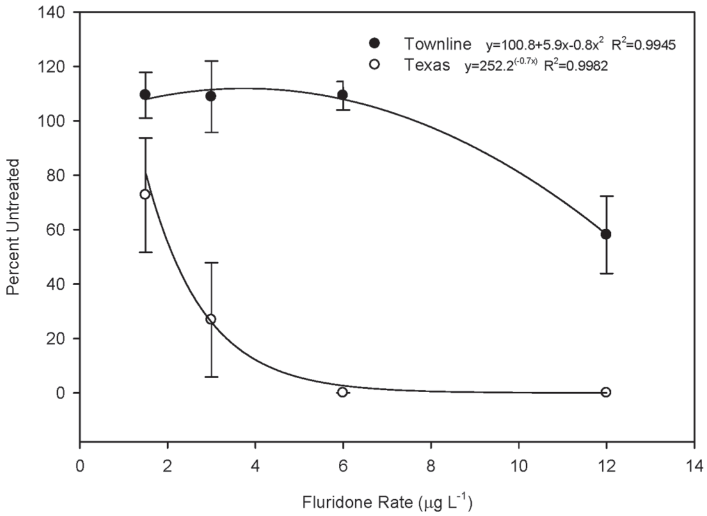

Figure 1. Hybrid watermilfoil collected from Townline Lake, MI, and a strain of Eurasian watermilfoil collected from the Lewisville Aquatic Ecosystem Research Facility, TX (Texas), were subjected to static exposures of fluridone in April 2010; shoot biomass was harvested at 60 days. Data are presented as percent biomass of the untreated control. Symbols represent means +95% confidence intervals (n = 4) and curves represent nonlinear regression.

to fluridone, while the hybrid watermilfoil from Otter Lake at concentrations of 4 μg L and greater was barely visible, and biomass was reduced to near 0% of the untreated control (Figure 2).

Field study of herbicide response. Typically, a 6-bump-6 fluridone treatment in Michigan waterbodies removes watermilfoil biomass from the water column completely or nearly so during the year of treatment. The response of Townline Lake watermilfoil to the 2010 6-bump-6 fluridone application was strongly atypical. Overall, watermilfoil was more abundant 60 days after the herbicide application than it was before the application (Figure 3). Of 82 sampling points with watermilfoil present during our study, 46 (56%) had a higher average rake-toss index post-treatment compared to pretreatment (average increase in rake index of 1.33 per site), and 13 sampling points (16%) exhibited no change pre- versus post-treatment. Twenty-three sampling points (28%) did exhibit reductions in rake toss index pre- versus post-treatment (average decrease in rake index of 1.27 per site), indicating some possible growth regulation at some points within the lake; however, only 3 of these 23 locations had post-treatment rake-toss indices of zero. Under the scenario of at least 80% die-back of watermilfoil following fluridone treatment, we expected that 65 of the 79 sites with watermilfoil in the pretreatment survey would not have watermilfoil in the post-treatment sampling; however, only three sites with watermilfoil in the pretreatment sample did not have watermilfoil in the post-treatment sample (χ^2, 1 d.f. = 59.7, $p < 0.0001$). Thus, the herbicide application clearly did not produce the expected reduction of watermilfoil in our study lake.

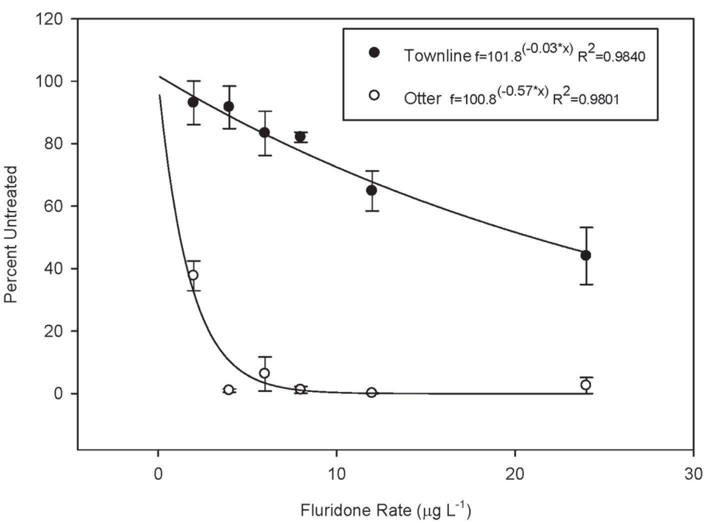

Figure 2. Hybrid watermilfoil collected from Townline Lake, MI, and a separate strain of hybrid watermilfoil collected from Otter Lake, MN (Otter) were subjected to static exposures of fluridone in May 2010 and shoot biomass was harvested at 60 days. Data are presented as percent biomass of the untreated control. Symbols represent means +95% confidence intervals (n = 5) and curves represent nonlinear regression.

Fluridone concentrations varied among the four locations where water samples were collected for analysis (Table 1), and the target concentration of 6 μg L^{-1} was never actually reached. During the first 2 weeks, fluridone concentrations varied from 2.7 to 5.4 μg L^{-1} two days after the initial application (28 Apr 2010) and varied from 1.5 to 4.1 μg L^{-1} two weeks following the application. After the bump on 18 May 2010, fluridone concentrations at the four locations where we collected water samples were much more consistent. At 9 days after the bump, fluridone residues ranged from 5.0 to 5.4 μg L^{-1}, and at 37 days after the bump fluridone residues ranged from 3.5 to 3.9 μg L^{-1}. Note that the 6-bump-6 protocol in Michigan calls for a calculation of the fluridone amount based on the volume of the top 10 feet of the water column. This practice could lead to under-dosing the 6 μg L^{-1} target if the thermocline is deeper than 10 feet. Although the measured fluridone residues indicate that the 6 μg L^{-1} fluridone concentration was not achieved, watermilfoil control is achieved in other waterbodies with similar fluridone residue measurements, and the laboratory comparisons to two other strains of watermilfoil confirm the reduced fluridone response by the Townline Lake hybrid watermilfoil.

Implications and future research. Fluridone resistance has been documented in another major invasive aquatic weed, hydrilla (*Hydrilla verticillata*), due to a single amino acid substitution in the phytoene desaturase gene (PDS; Michel et al. 2004, Arias et al. 2005). In the case of our focal watermilfoil population, it is unknown whether reduced fluridone sensitivity results from mutation(s) in the PDS gene as in hydrilla. Similarly, it is unknown whether reduced fluridone sensitivity represents natural tolerance of this particular lineage or an evolved resistance in response to its previous treatment history with fluridone.

Our study population is composed of hybrid watermilfoil (*M. spicatum* × *M. sibiricum*), but whether the reduced fluridone sensitivity in our study population is related to its hybridization history is not clear. Evolutionary biologists widely accept that hybridization can lead to rapid adaptive evolutionary change in a wide variety of traits (Anderson and Stebbins1954, Barton 2001, Rieseberg et al. 2003, Kim et al.

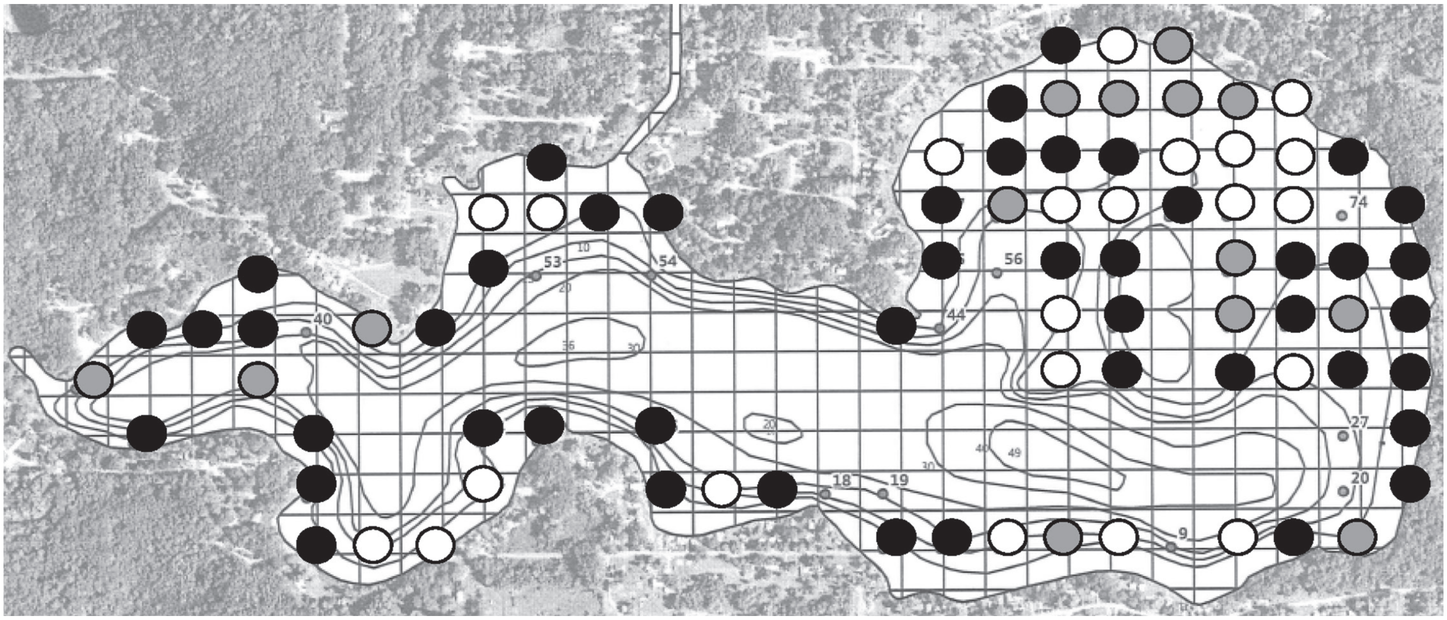

Figure 3. Change in abundance of hybrid watermilfoil in Townline Lake, MI, as determined by a rake-toss index (average of two rake tosses), before (28 Apr) vs. 107 days after (13 Aug) initial fluridone treatment at a target rate of 6 µg L^{-1}. Black circles: increase in abundance; gray circles: no change in abundance; white circles: decrease in abundance. Small gray dots with numbers are survey sites where no watermilfoil was found before or after treatment.

2008, Arnold and Martin 2010), including the evolution of invasiveness (Ellstrand and Schierenbeck 2000), but whether hybridization can confer increased tolerance to fluridone is unknown. Other waterbodies in Michigan with hybrid watermilfoil have been treated successfully with 6-bump-6 fluridone. Our laboratory study demonstrates that hybrid genotypes may not necessarily exhibit fluridone tolerance; hybrid genotypes from a second lake (Otter Lake, MN) exhibited normal sensitivity to fluridone. Genetic studies of hybrid watermilfoil populations demonstrate that hybrid watermilfoils are composed of distinct genotypes (Zuellig and Thum, unpublished data), and whether different hybrid genotypes will exhibit different levels of fluridone sensitivity warrants further research.

Due to a lack of complete information about the genetic identification of watermilfoils, susceptibility to fluridone, and field responses to fluridone treatments, it is unclear how many other tolerant strains of watermilfoil exist. The widespread development of fluridone tolerance would be a highly undesirable outcome for non-native aquatic invasive species management, especially regarding economics and selective control. Certainly, fluridone is frequently effective for watermilfoil control in the northern United States; however, we currently do not have a quantitative estimate of the number of water bodies that may have fluridone tolerant strains of watermilfoil. Our clear documentation of one fluridone tolerant population of watermilfoil indicates that further screening of fluridone sensitivities of watermilfoil strains should be conducted and that further research on factors that may contribute to increased tolerance is warranted.

SOURCES OF MATERIALS

Fluridone (SONAR): 480 g L^{-1} suspension concentrate liquid formulation Sonar A.S. ™ (SePRO Corporation, Carmel, IN)

FasTEST: Internal High Performance Liquid Chromatographic (HPLC) internal methods for fluridone and other aquatic herbicide analysis

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