Fluridone-Resistant Hydrilla (*Hydrilla verticillata*) Is Still Dominant in the Kissimmee Chain of Lakes, FL

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The invasive aquatic plant hydrilla rapidly spread through the 28,500 ha Kissimmee Chain of Lakes (KCOL) system in Florida in the late 1980s and early 1990s. Large-scale herbicide treatments with fluridone were initiated in 1993 and resulted in widespread reduction in hydrilla; however, by 2000, sustained use of fluridone resulted in dominance of fluridone-resistant strains of hydrilla throughout these lakes. The last large-scale fluridone applications on the KCOL were conducted in 2004, and in 2012, a sampling effort was initiated to determine the status of fluridone-resistant strains of hydrilla given an 8-yr period with no further selection pressure from fluridone. A total of 260 sites were sampled on the lakes during March, May, September, and December 2012. Plants were returned to the lab and exposed to fluridone at concentrations of 5, 10, and 20 μg L^{-1} and a pulse-amplitude-modulated (PAM) fluorometer was utilized to measure fluorescence yield of new shoot tissue growth following a 14-d exposure period. Results indicate that 80 to 90% of the sites sampled on the four lakes of the Kissimmee Chain remain resistant to fluridone. Three distinct patterns of response to fluridone were noted, suggesting that susceptible, moderately tolerant, and highly tolerant strains of hydrilla currently coexist on these lakes. Although fluridone-susceptible plants were present on the KCOL, this study clearly demonstrates that most of the hydrilla remained resistant despite an 8-yr period with no fluridone selection pressure.

Nomenclature: Fluridone; hydrilla, *Hydrilla verticillata* (L.f). Royle HYLLI.

Key words: Aquatic herbicide, invasive aquatic plant, submersed aquatic vegetation.

Hydrilla [*Hydrilla verticillata* (L.f.) Royle] is a submersed angiosperm that was first introduced to Florida in the late 1950s. This invasive aquatic plant has been described as the ''the perfect aquatic weed'' due to numerous physiological adaptations that result in rapid rates of growth and several asexual means of reproduction that insure both rapid spread and longevity (Langeland 1996). The semitropical climate of Florida and abundance of natural shallow lakes can support extensive hydrilla coverage that persists throughout the year. Hydrilla was present in more than 50,000 ha (125,000 ac) in Florida public waters based on surveys conducted in 2007 (FDEP 2007). In Florida, hydrilla is a dioecious biotype that produces only female flowers. Given the strict reliance on asexual reproduction, the potential for hydrilla to develop herbicide resistance received limited attention when discussing best management practices through the 1990s.

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The herbicide fluridone {1-methyl-3-phenyl-5-[3-(trifluoromethyl) phenyl]-4(1H)-pyridinone} was registered for aquatic use in Florida in 1986, and by the 1990s, it was routinely used at the whole-lake scale to target hydrilla in large public water bodies. Fluridone is a bleaching herbicide that inhibits the carotenoid synthesis pathway by targeting phytoene desaturase (PDS). Insufficient carotenoid levels result in photodestruction of chlorophyll molecules, and new plant growth typically shows strong bleaching symptoms (Bartels and Watson 1978). Laboratory and field studies suggested that long-term exposures ($>$ 60 d) to low concentrations of fluridone (5 to 15 μg ai L^{-1} [0.00000067 to 000002 oz ai gal^{-1}]) were key to providing cost-effective, selective, and extended hydrilla control (Fox et al. 1994, 1996; Netherland and Getsinger 1995; Netherland et al. 1993). For perspective, use of fluridone at the maximum label rate of 150 μg ai L^{-1} would have been cost-prohibitive and the lake-wide impacts to numerous important native plant species would not be acceptable to managers and stakeholders. As resource managers increased their reliance on low-rate fluridone strategies, anecdotal reports of reduced activity emerged in 1999. The subsequent documentation of widespread fluridone resistance by hydrilla in numerous public lakes

Management Implications

In this case study we evaluated the frequency of fluridone-resistant hydrilla on the 28,500 ha Kissimmee Chain of Lakes, FL, 8 yr following the last large-scale fluridone application. Resource managers had become highly reliant on whole-lake use patterns of fluridone, and they particularly favored the cost effectiveness, selectivity, low-use volumes, and the limited water-use restrictions on the label. We hypothesized that a series of hurricanes and large-scale hydrilla management with alternate herbicide modes of action would result in a higher frequency of fluridone-susceptible hydrilla given the 8-yr absence of fluridone pressure. The source of the susceptible hydrilla was an extensive tuber bank that can persist in the sediments for years. Our results suggest that fluridone-resistant hydrilla remains dominant throughout the KCOL. Although we did find areas of the lake with susceptible plants, resistant hydrilla continued to make up 80 to 90% of the overall population. Numerous managers have inquired about the potential for Florida lakes to revert to fluridone-susceptible populations. Our results suggest this is an unlikely scenario in the KCOL because the resistant plants remain both widespread and dominant.

in Florida (from 2000 to 2003) resulted in a dramatic reduction in the most widely used tool for hydrilla control by 2004 (Netherland et al. 2005). Fluridone resistance was shown to be due to somatic point mutations at site 304 in the gene that codes for phytoene desaturase (Arias et al. 2005; Dayan and Netherland 2005; Michel et al. 2004; Puri et al. 2007). Whereas the wild type sequence for arginine (*CGT*) results in a PDS enzyme that is highly susceptible to fluridone, three somatic point mutations (*AGT* [serine], *TGT* [cysteine], or *CAT* [histidine]) found in field populations of hydrilla conferred varying levels of tolerance to fluridone. This differential tolerance between genotypes initially confounded aquatic plant managers because some "resistant" populations continued to respond to higher-use rates of fluridone, but other populations showed a minimal response to these higher concentrations. From a practitioner's perspective, hydrilla with the serine and cysteine mutations still showed symptoms following higher-use rates of fluridone whereas plants with the histidine mutation showed very limited response to higher rates of fluridone.

The presence of fluridone-resistant hydrilla in the Kissimmee Chain of Lakes (KCOL) in central Florida was of particular concern. The KCOL consists of four large, interconnected lakes (Tohopekaliga, Cypress, Hatchineha, and Kissimmee; Table 1) covering approximately 28,500 ha that form the northernmost part of the Central and Southern Flood Control Project managed by the U.S. Army Corps of Engineers (USACE) Jacksonville District and the South Florida Water Management District (SFWMD). These lakes serve multiple purposes, including flood control, navigation, recreation, and fishing and hunting opportunities. Although flood control and water delivery are largely managed via the USACE and SFWMD, the management of hydrilla on the KCOL is coordinated and funded by the Florida Fish and Wildlife Commission Invasive Plant Management Section (FWC IPMS). Given the multiple uses of these lakes, large-scale hydrilla treatments require significant interagency coordination and communication with various stakeholder groups.

Once introduced to the KCOL, hydrilla growth and expansion was explosive throughout these shallow systems. For example, survey data from the Florida Department of Environmental Protection annual surveys indicated that 28 ha of hydrilla were reported on Lake Hatchineha in 1986, and 2,400 ha were reported by 1991, covering 90% of the lake surface area. All of the KCOL lakes had hydrilla infestations that exceeded 50% of the lake area in the early 1990s, and large-scale fluridone applications were initiated on the KCOL in 1993 (Table 2). These treatments continued through 2000 when resistance was first documented. Paradoxically, the documentation of resistance resulted in greater use of fluridone from 2001 to 2004 as managers were under increasing pressure to bring hydrilla under control (Netherland et al. 2005). Given the limited tools available at the time, fluridone was still viewed as the only viable and economic herbicide for large-scale management. The last large-scale fluridone applications on the KCOL were applied in the early spring of 2004.

Hydrilla coverage on the KCOL experienced significant fluctuations following a series of three successive hurricanes that passed directly over the lakes in the late summer of 2004. In addition to the physical uprooting of the hydrilla as the storms moved over the lakes, sustained dark water conditions (significant elevation in water color) through

Table 1. Lake size, year that hydrilla was first reported, and the peak hydrilla coverage estimated in the 1990s for the Kissimmee Chain of Lakes in Florida.

Lake	Surface area (ha)	Year hydrilla was identified, and surface area coverage (ha)	Year of peak coverage, and surface area coverage (ha)
Tohopekaliga	9,800	1983, 310	1994, 5,976
Cypress	1,800	NA[a]	NA
Hatchineha	2,600	1983, 10	1991, 2,400
Kissimmee	14,300	1983, 0.1	1995, 7,300

[a] Abbreviation: NA, not available.

Table 2. History of fluridone applications (kg) on the Kissimmee Chain of Lakes in Florida.

	Fluridone			
Year	Lake Tohopekaliga	Lake Cypress	Lake Hatchineha	Lake Kissimmee
	kg			
1993	544 (1197 lb)	—	—	—
1994	606	—	—	—
1995	1,208	—	—	787
1996	2,505	113	—	—
1997	2,466	113	889	3,123
1998	2,495	544	1,089	2,359
1999	2,486	923	1,746	2,223
2000	3,882	549	1,686	2,486
2001	3,735	1,270	1,016	1,884
2002	5,655	1,207	1,651	1,929
2003	6,160	907	1,094	1,732
2004	4,445	1,219	1,814	1,407
2005–2012	0[a]	0	0	0

[a] One application of ~ 317 kg of fluridone was applied to Lake Tohopekaliga in the fall of 2007 as part of a hydrilla tuber-suppression evaluation.

2004 and 2005 created an environment that was not conducive to survival of sprouting hydrilla tubers (Netherland 1997; Hoyer et al. 2008). As the water color transitioned to normal levels in 2006, hydrilla recovered from tubers. New herbicide-use patterns with the contact herbicide endothall were developed, and large-scale applications in 2008 through 2011 resulted in significant reductions in hydrilla coverage (Netherland and Jones 2012). Despite these hydrilla management efforts on the KCOL from 2006 to 2012, there were still thousands of hectares of the plant delineated during the fall surveys.

The significant fluctuations in hydrilla coverage from 2004 to 2012 and the lack of fluridone selection pressure since 2004 presented an opportunity to determine the frequency of fluridone-resistant hydrilla on the KCOL. We received several anecdotal reports from resource managers suggesting that sites formerly dominated by fluridone-resistant strains of hydrilla had reverted back to susceptible strains. These claims were not substantiated, but suggest that fluridone-resistant strains of hydrilla might be subject to some type of fitness penalty that would favor expansion of the susceptible plants. The claim that resistant biotypes are less fit than susceptible biotypes in the absence of herbicide pressure is generally based on observations of triazine resistance. In contrast, herbicide modes of action such as acetolactate synthesis (ALS) inhibitors have generally not been associated with a fitness penalty for herbicide-resistant plants (Sibouny and Rubin 2003). Hydrilla remains the only example of a plant with naturally occurring resistance to a PDS inhibitor (Dayan et al. 2014), and therefore the likelihood of a fitness penalty is largely unknown with this class of herbicides. Moreover, the relationship between strict asexual reproduction and a fitness penalty also is largely unknown. In order to address the claims and questions above, we sampled throughout the KCOL to determine the distribution of resistant and susceptible strains of hydrilla.

Materials and Methods

Study Area and Plant Collection. A map showing the location of KCOL (Figure 1) and a table (Table 1) provide general information on the lake size and fluridone management history in this study. Using hydrilla survey data collected by the FWC IPMS, sample areas on the four lakes were selected, and 25 hydrilla apical shoots were collected from each sample site on March 7, May 17, September 15, and December 13, 2012 (Figure 1). Once in the sample area, hydrilla beds were located and a global positioning system (GPS) coordinate was recorded for the individual collection site on each date. For each sample event, 25 sites on Lake Tohopekaliga, 10 on Lake Cypress, 10 on Lake Hatchineha, and 20 sites on Lake Kissimmee were sampled. Hydrilla shoots were placed in labeled zip-lock bags and then placed in a cooler prior to transport to facilities at the University of Florida Center for Aquatic and Invasive Plants in Gainesville, FL.

Greenhouse Culture. Hydrilla collected from the KCOL was sorted by site, and plants were cut to 4 cm (1.57 inch) apical shoots. In addition to plants collected from the KCOL, a population of hydrilla from the Rainbow River in Citrus County, FL with no history of prior exposure to

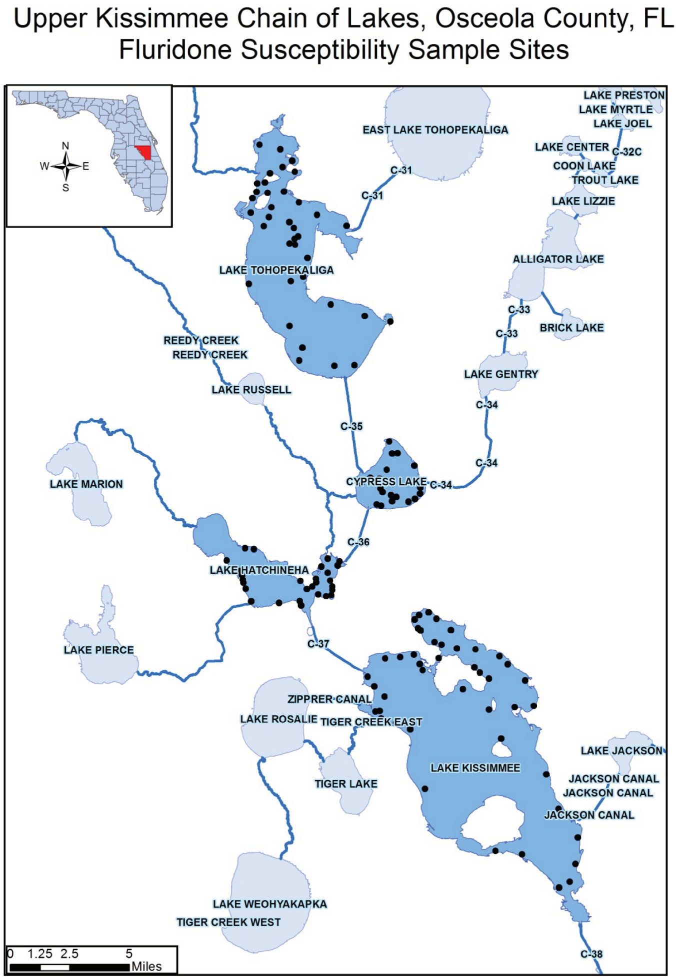

Figure 1. Location of hydrilla sample sites on the Kissimmee Chain of Lakes, FL. A global positioning system (GPS) coordinate was recorded for each sample site during the course of four sampling events. (Color for this figure is available in the online version of this paper.)

fluridone was included in each assay as a known fluridone-sensitive strain for comparative purposes. Healthy 4-cm (1.57 in) apical shoot sections were rinsed and placed in 1,000 ml clear food-grade containers that had been filled with well water containing 5% Hoagland's solution. Preliminary testing suggested that hydrilla apices grew readily in this system under ambient greenhouse conditions. Greenhouse conditions were generally consistent with outdoor ambient temperatures; however, during the March and December assays, the greenhouse heater was utilized to ensure that temperatures did not go below 15 C and greenhouse lighting was turned on in the evening to ensure day lengths were greater than 13 h. Fluridone treatments of 0, 5, 10, and 20 μg L^{-1} were applied via a stock solution of fluridone prepared from technical-grade material (99% purity) (Technical grade fluridone; SePRO Corporation, Carmel, IN). Exposure assays were run for a period of 14 d, and untreated control plants had to at least double in total length for the assay to be accepted.

After herbicide exposure, plants were measured for total length, thoroughly rinsed to remove any epiphytic algae, and the fluorescence yield of new apical or lateral shoot growth was measured for all plants using a pulse-amplitude modulated (PAM) fluorometer (Mini-PAM; Walz, Effetrich, Germany). PAM fluorometry allows for non-destructive measurement of chlorophyll fluorescence and works by focusing a saturating beam of light on the desired region of the plant. This method was recently used to distinguish fluridone tolerance in a hybrid genotype of watermilfoil compared to other accessions of Eurasian and hybrid watermilfoil (Berger et al. 2012; Berger et al. 2015). A higher fluorescence yield ratio indicates highly functioning chlorophyll, whereas lower-yield ratios indicate damaged or nonfunctioning chlorophyll. The PAM has previously been used to measure herbicide stress for seagrasses exposed to diuron and algal species exposed to metolachlor (Haynes et al. 2000; Juneau et al. 2001). The direct impact of fluridone on both β-carotene and chlorophyll (Netherland and Getsinger 1995; Netherland et al. 1993) and the use of these pigments to differentiate between fluridone-tolerant and -susceptible strains of hydrilla (Puri et al. 2006) indicated the PAM fluorometer was well-suited to rapidly quantifying differences between fluridone-susceptible and -resistant strains of hydrilla. In this study, only new apical or lateral shoot growth (beyond the initial 4 cm shoot section) was subjected to PAM readings. The PAM light source was placed in a clip so that each reading resulted in the plant tip being equidistant (10 mm [0.39 in]) and at the same angle from the saturating light beam. Light intensity was measured in the greenhouse at the time of PAM readings and values ranged between 1,045 and 1,156 μmol m^{-2} s^{-1}. Hydrilla was removed from culture for the readings and fluorescence yield was recorded in the greenhouse under daytime ambient conditions.

Data Analysis. Each treatment was replicated four times using a completely randomized design. Data from all sampling dates were combined and subjected to descriptive statistics that included mean fluorescence yield values with 95% confidence intervals. This analysis allowed us to distinguish sites containing susceptible, moderately tolerant, and highly tolerant strains of hydrilla. There were 100 samples for Lake Tohopekaliga, 40 samples for Lake Cypress, 40 samples for Lake Hatchineha, and 80 samples for Lake Kissimmee. These data were then analyzed by nonlinear regression (exponential decay) with SigmaPlot (SigmaPlot, version 11; Systat Software, Inc., San Jose, CA). Regression models were used to calculate an EC_{50} value (concentration of fluridone required to reduce fluorescence yield by 50%) based on PAM readings.

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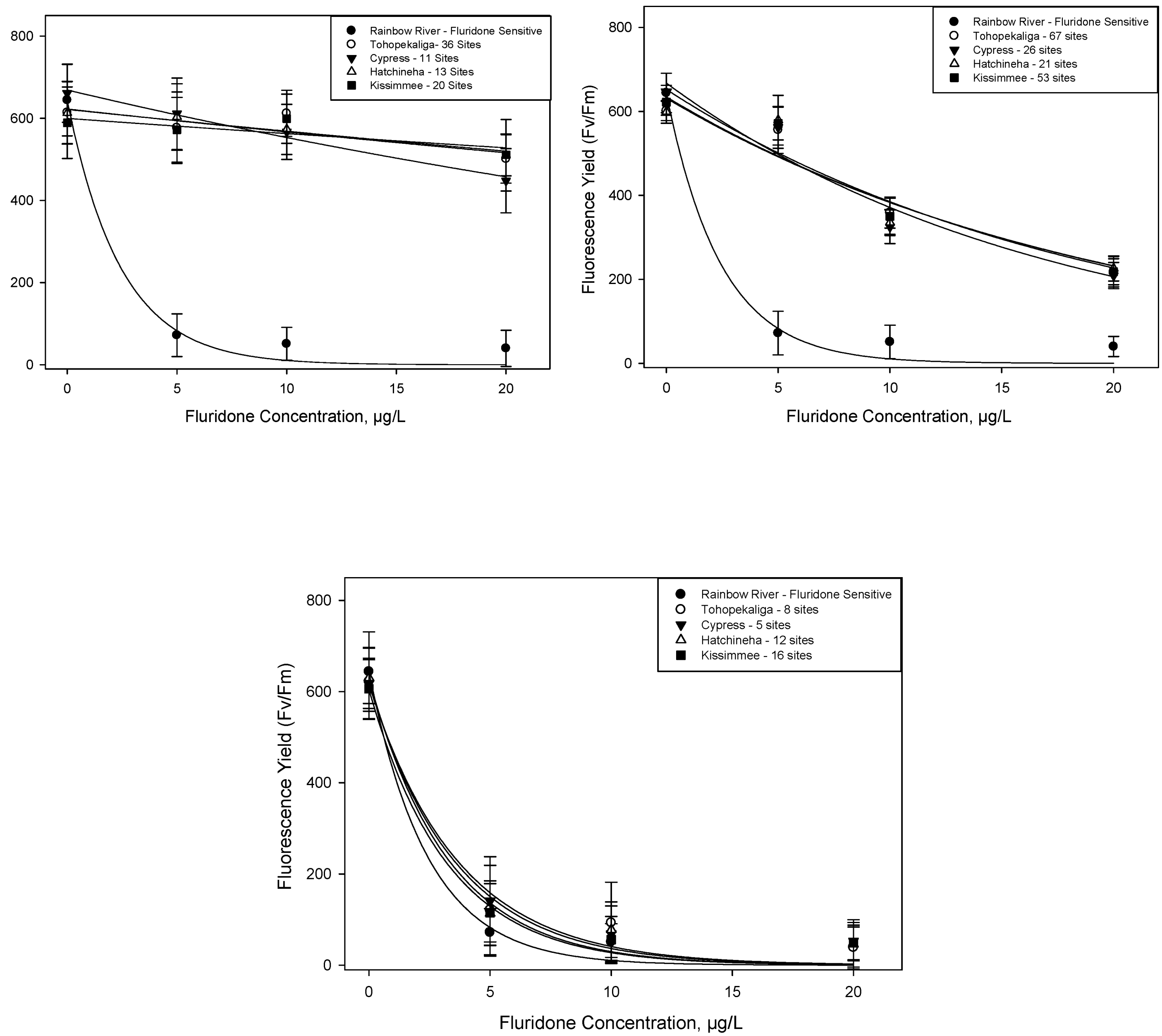

Figure 2. Fluorescence yield for hydrilla exposed to fluridone (μg L^{-1}) during a 14-d assay. Hydrilla was collected throughout the Kissimmee Chain of Lakes, FL and compared with a fluridone-susceptible strain of hydrilla from the Rainbow River, FL. Three distinct patterns of hydrilla fluorescence yield response to fluridone include highly resistant (upper left panel), moderately resistant (upper right panel), and susceptible (lower panel) strains of hydrilla.

Results and Discussion

Mean hydrilla shoot lengths (± SE) had increased by 7.6 (1.1), 19.3 (1.3), 29.6 (2.3), and 8.3 (1.0) cm , for the untreated reference plants collected during the March, May, September, and December sample events, respectively. PAM fluorescence yield results generated for the combined data on each individual lake compared to the reference population show a clear delineation between resistant and susceptible populations (Figure 2). The EC_{50} values of 2, 12, and 30 μg L^{-1} further demonstrate three distinct patterns in fluorescence yield that were detected for hydrilla in the KCOL (Table 3). Plants representing moderate and higher levels of resistance were found in all four lakes. This phenomenon of variable tolerance to fluridone was described by Michel et al. (2004), whereby different point mutations confer differing levels of tolerance to fluridone. It is important to remember that fluridone requires extended exposure periods of > 2 mo to

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Table 3. The EC_{50} values of fluridone for hydrilla phenotypes based on fluorescence yield in 14-d assays.

Phenotype[a]	EC_{50}[b] ($\mu g\ L^{-1}$)	Regression equation	r^2
Susceptible Strain $n = 45$	2	$y = 653.2 * e^{(-0.411*x)}$	0.92
Moderately Resistant $n = 163$	12	$y = 655.3 * e^{(-0.0587*x)}$	0.98
Highly Resistant $n = 62$	30	$y = 658.2 * e^{(-0.023*x)}$	0.98

[a] The terms susceptible, moderately resistant, and highly resistant are used in this table to identify the three distinct phenotypes of hydrilla based on response of fluorescence yield to fluridone concentrations.

[b] Abbreviation: EC_{50}, Concentration of fluridone required to reduce fluorescence yield by 50%.

control hydrilla. Therefore, a change in the EC_{50} value from 2 to 12 $\mu g\ L^{-1}$ fluridone is substantial because fluridone concentrations must be maintained at these higher concentrations over these extended exposure periods.

Fluridone-susceptible hydrilla was identified in all four lakes at frequencies ranging from 10 to 20% (Table 4). In lakes with confirmed fluridone resistance, it has been common for survey personnel to observe areas with hydrilla that showed strong bleaching symptoms in the midst of much larger hydrilla beds that remained green and actively growing in the presence of fluridone. Given the long-term quiescence (multiple years) of the subterranean turions formed by hydrilla, the ability of fluridone-sensitive tubers to remain in the propagule bank for years and then sprout into a population dominated by fluridone-resistant genotypes would potentially explain the patterns of response to fluridone observed above (Netherland and Haller 2006). During the course of sampling, we detected only six events in which results differed between collection dates within a sample area. In these cases, a site recognized as tolerant yielded data consistent with a susceptible population. Although the vast majority of samples produced consistent results, this finding suggests that tolerant and susceptible genotypes of hydrilla can grow in close proximity. Nonetheless, we did not generate data that would support proliferation of susceptible strains of hydrilla in the absence of selection pressure from fluridone.

Results generated from this study demonstrate the utility of the PAM fluorometer in comparing sensitivity to fluridone via a nondestructive measure. Prior analyses of fluridone activity have relied on destructive pigment analyses (β-carotene, chlorophyll, and phytoene) and photosynthetic measures to evaluate fluridone activity on hydrilla (Berger 2011; Netherland and Getsinger 1995; Puri et al. 2006). This fast and nondestructive approach could also be adapted for monitoring the influence of several other herbicide modes of action (bleaching herbicides, PSI and PSII inhibitors) on aquatic plants.

A challenge in predicting the wider effects of mutations that endow herbicide resistance is determining whether these mutations have pleiotropic effects on plant fitness (Vila-Aiub et al. 2005). In the case of hydrilla, a point mutation conferring resistance in a population that spreads via clonal means is unlikely to result in a fitness penalty. Although this study was not designed to evaluate comparative fitness of fluridone-susceptible and -resistant strains of hydrilla, the in situ results on the KCOL after 8 yr of significant fluctuations in hydrilla coverage, and no additional selection pressure from fluridone would suggest the resistant plants retain highly invasive traits.

These results present a straightforward case that a high frequency of hydrilla in the KCOL remains resistant to fluridone. Although discussion of additional fluridone use in this system is not currently warranted, there are scenarios whereby a lake can contain 90% fluridone-susceptible

Table 4. The number of fluridone-susceptible and fluridone-resistant sites detected on the Kissimmee Chain of Lakes in Florida based on fluorescence yield data.

Lake	Susceptible sites	Moderately resistant sites	Highly resistant sites	Fluridone-resistant sites
	No.	No.	No.	%
Tohopekaliga	11	65	24	89
Cypress	4	26	10	90
Hatchineha	8	21	11	80
Kissimmee	12	51	17	85

plants and 10% -resistant plants. In this case, resource managers face a difficult decision in whether to forego fluridone use due to low-frequency presence of resistant individuals, or accept that additional fluridone treatment is likely to further skew the population toward resistant individuals.

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