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Evaluation of Chemical Control for Nonnative Crayfish at a Warm-water Fish Production Hatchery

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ABSTRACT

Invasive crayfish are known to displace native crayfish species, alter aquatic habitat and community structure and function, and are serious pests for fish hatcheries. White River Crawfish (WRC; *Procambarus acutus*) were inadvertently introduced to a warm-water fish hatchery in Missouri, USA, possibly in an incoming fish shipment. We evaluated the use of chemical control for crayfish to ensure incoming and outgoing fish shipments from hatcheries do not contain live crayfish. We conducted acute (≤24 hr) static toxicity tests to determine potency, dose-response, and selectivity of pesticides to WRC, Virile Crayfish (VC; *Orconectes virilis*), and Fathead Minnow (FHM; *Pimephales promelas*). Testing identified a formulation of cypermethrin (Cynoff®) as the most potent of five pesticides evaluated for toxicity to crayfish. A 4-hr exposure to a cypermethrin concentration of 100 $\mu g \cdot L^{-1}$ was found to kill 100% of juvenile and adult WRC; however, adult VC were not consistently killed. Concentrations of cypermethrin ≤100 $\mu g \cdot L^{-1}$ did not cause significant (>10%) mortality in juvenile FHM. Additional testing is needed to examine selectivity between crayfish and hatchery fish species. Biosecurity protocols at hatcheries that use chemical control have the potential to reliably prevent inadvertent transfers of live crayfish in fish shipments.

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INTRODUCTION

Freshwater crayfish are ecologically important and diverse, with 398 described species in the United States (US) and Canada; however, they are highly endangered, with greater than 48% of species requiring some level of conservation concern (Fetzner 2005; Taylor et al. 2007; Thoma 2016). Crayfish influence their ecosystems through their roles as consumers, predators, producers and engineers, and also because they can reach high densities (Momot 1995; Rabeni et al. 1995; Creed and Reed 2004; Lodge et al. 2012). Crayfish exert powerful influences on their environments and have high potential for invasion into new habitats and ecosystems (Chucholl 2013; DiStefano et al. 2015). Invasions by nonnative crayfish have had serious consequences including displaced or extirpated native crayfish species, contaminated native crayfish gene pools, introduced disease and parasites, and altered aquatic habitat and community dynamics (Guan and Wiles 1997; Lodge et al. 2000; Hein et al. 2007; DiStefano et al. 2009; Twardochleb et al. 2013). Controlling or eradicating invasive crayfish has been the subject of laboratory studies and field investigations globally (Bills and Marking 1988; Hyatt 2004; Peay et al. 2006; Freeman et al. 2010; Sandodden and Johnsen 2010; Twardochleb et al. 2013) and of studies investigating human-mediated pathways such as bait transfers, various trade networks or aquaculture (Lodge et al. 2000; Keller and Lodge 2007; DiStefano et al. 2009; Chucholl 2013; Dresser and Swanson 2013). The role that fish hatcheries have in transferring nonnative crayfish into aquatic ecosystems has not been as widely investigated (Bean et al. 2004; Ingersoll et al. 2013).

This project was initiated after an invasion of nonnative *Procambarus acutus* (Girard), the White River Crawfish (WRC), occurred at Blind Pond Hatchery (BPH), a Missouri Department of Conservation (MDC) warm-water fish hatchery in central Missouri, USA. The invasion is thought to have occurred through the inadvertent transfer of WRC in an incoming fish shipment

Table 1. Chemical formulations or test compounds used in testing.

Chemical	Source	Active ingredient	Chemical family	Chemical name	Chemical mode of action/effect	Chemical abstract service (CAS) number	US Environmental Protection Agency regulation number	Percent active ingredient by weight (%)
Dupont™ Altriset™ Termiticide[a]	E.I. du Pont Nemours and Company, Wilmington, Delaware, USA	Chlorantraniliprole	Anthranilic diamide insecticide	3-Bromo-N-[4-chloro-2-methyl-6-[(methylamino) carbonyl]phenyl]-1-(3-chloro-2-pyridinyl)-1H-pyrazole-5-carboxamide	Binds and activates ryanodine receptors; depletes intracellular calcium stores, eventually leading to muscle paralysis and death due to the interruption of normal muscle contraction[b]	500008-45-7	352-829	18.4
Cynoff^{EC} Insecticide	FMC Corporation, Philadelphia, Pennsylvania, USA	Cypermethrin	Pyrethroid insecticide	$C_{22}H_{19}C_{l2}NO_3$; 24.8% (±) α-cyano-(3-phenoxyphenyl) methyl(±)-cis,trans-3-(2,2-dichloroethenyl)-2, 2-dimethylcyclopropane-carboxylate[c]	Disruption of sodium ion channels in the nerve axon, resulting in hyperactivity of the nervous system[d]	52315-07-8	279-3081	24.8
Prentox® ExciteR™	Prentiss LLC, Cary, North Carolina, USA	Pyrethrin; piperonyl butoxide technical (PBO)	Pyrethrin insecticide	Technical grade pyrethrin; equivalent to min. 48% (butylcarbityl) (6-propypiperonyl) ether and 12.0% related compounds; contains petroleum distillates	Disruption of sodium ion channels in the nerve axon, resulting in hyperactivity of the nervous system[d]	121-21-1; 8003-34-7; 51-03-6	665-7984-455	6; 60
Bayer Advanced Carpenter Ant & Termite Killer Plus Concentrate	Bayer Environmental Science, Bayer Crop Science LP, Research Triangle Park, North Carolina, USA	Beta-cyfluthrin; 1,2-propanediol	Pyrethroid insecticide	ß- Cyfluthrin, Cyano(4-fluoro-3-phenoxyphenyl) methyl 3-(2, 2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate	Disruption of sodium ion channels in the nerve axon, resulting in hyperactivity of the nervous system[d]	68359-37-5; 57-55-6	72155-58	2.50; 3.22
High-Yield 38^{+}	Voluntary Purchasing Group, Bohnam, Texas, USA	Permethrin	Pyrethroid insecticide	3-phenoxyphenyl) methyl (±) cis, trans-3-(2, 2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylate[c]	Disruption of sodium ion channels in the nerve axon, resulting in hyperactivity of the nervous system[d]	52645-53-1	7401-466	38.0
Potassium chloride	Fisher Scientific, Pittsburgh, Pennsylvania, USA	Potassium chloride	Metal halide salt	KCl	Disruption of essential physiological processes; excess concentrations can result in cardiac arrest, renal failure, gastrointestinal bleeding, vomiting	7447-40-7	013904[f]	100
Formaldehyde solution AR® ACS	Mallinckrodt® Chemicals, Phillipsburg, New Jersey, USA, or Avantor Performance Materials, Inc., Center Valley, Pennsylvania, USA	Formaldehyde	Aldehyde	CH_20	Acts on proteins by denaturation and on nucleic acids by alkylation resulting in rupture of the membrane, loss of permeability and coagulation of the cytoplasm	50-00-0	50-00-0	37.0

[a] Current registrant: Syngenta Crop Protection, LLC -- Regional Headquarters, P.O. Box 18300, Greensboro, NC, USA 27409
[b] USEPA 2008a
[c] Cis/trans ratio: maximum 55% (±) cis and minimum (±) 45% trans
[d] Solomon et al. 2001; Laskowshi 2003
[e] Cis/trans ratio: maximum 55% (±) cis and minimum (±) 45% trans
[f] US Environmental Protection Agency pesticide code (Shaugnessy) number

originating from out-of-state sources (Jansen and DiStefano 2009; Pitts 2010). The WRC invasion represents a threat to successful hatchery operation because of increased labor costs and fish loss due to double handling of fish (i.e., fish are moved to intermediate tanks from ponds prior to loading fish into tanks on hatchery trucks) used to exclude WRC; damage to culture ponds (e.g., bank stability) due to burrowing; and because WRC may be inadvertently transferred in outgoing fish shipments from BPH. Our testing focused on lethal and sublethal effects of chemicals on WRC, but we also conducted tests with *Orconectes virilis* (Hagen), the Virile Crayfish (VC), a widely distributed and highly invasive North American crayfish species (Kilian et al. 2010; Larson and Olden 2011; Martinez 2012), to better understand the range in sensitivities of WRC and VC to test chemicals. The literature can identify distributions of response for invertebrates and fish (Solomon et al. 2001); however, species-specific information is needed to ensure selectivity for the particular species of interest and for the acute time interval required for this application.

Important requirements for a successful pre-shipment or shipment treatment for crayfish are 1) the chemical agent be completely effective (0% survival of crayfish); 2) highly selective (minimal impact on fish survival or condition); 3) fast acting (<4 hr) to prevent prolonged holding of fish; 4) allow for acceptable tissue residues in fish; and 5) ensure that treated water can be safely discharged. A minimum threshold dose of a chemical may be difficult to determine due to the range in sensitivities of crayfish species and the inherent variability in response within populations to chemical stressors. A particular concern for the development of a chemical control protocol is that there are no chemicals registered for crayfish control in the USA. A request to the US Environmental Protection Agency (USEPA) would be needed for special permitting. Regulatory goals will include ensuring that treated fish meet all criteria necessary for safe human consumption and that chemicals or compounds pose an acceptable level of risk to aquatic environments.

Our objectives were: 1) conduct toxicity tests to assess the potency of candidate chemicals using WRC and VC; 2) conduct dose-response tests with early life stages of WRC and VC to determine the effect of life stage and exposure duration on the behavior and survival of both species; and 3) characterize selectivity or margin of safety between crayfish and Fathead Minnow (FHM; *Pimephales promelas* Rafinesque). We also evaluated whether the current BPH biosecurity protocol for Zebra Mussel *Dreissena polymorpha* (Pallas) changed the efficacy of the chemical treatment of crayfish, as the two protocols would be used simultaneously in hatchery trucks.

MATERIALS AND METHODS

Site

Blind Pony Hatchery is located within the Blind Pony Lake Conservation Area near Sweet Springs, Missouri, USA. There are 39 earthen production ponds totaling 15.3 ha, ranging in size from 0.04 to 0.2 ha, which receive water directly from Blind Pony Lake, a man-made reservoir. Hatchery water is discharged into Pony Creek. The hatchery receives and spawns broodstock annually to produce fish for sportfish and endangered species restoration projects. Primary species produced at BPH include Pallid Sturgeon *Scaphirhynchus albus* (Forbes and Richardson), Lake Sturgeon *Acipenser fulvescens* (Rafinesque), Paddlefish *Polyodon spathula* (Walbaum), Largemouth Bass *Micropterus salmoides* (Lacepède), Bluegill *Lepomis macrochirus* (Rafinesque), and Channel Catfish *Ictalurus punctatus* (Rafinesque). Additional species, such as hybrid Striped Bass *Morone chrysops x M. saxatilis* and Walleye *Sander vitreus* (Mitchill), are produced as requested by the MDC Fisheries Division.

Source of Crayfish and Fish and Culture Methods

Juvenile and adult WRC used in testing were collected from ponds at BPH (Supplemental Table S1). Adult VC were collected from research ponds at US Geological Survey (USGS) Columbia Environmental Research Center (CERC) and MDC's Little Dixie Conservation Area (LDCA), Millersburg, Missouri, USA (Supplemental Table S1). Crayfish collected at BPH or LDCA were transported to CERC, where they were treated in a 50-ppt potassium chloride (KCl) solution twice for five minutes as part of CERC biosecurity quarantine protocols before their transfer to CERC culture facilities. All crayfish were cultured and reared in the laboratory in flow-through glass aquaria filled with CERC well water (temperature 18°C, pH 7.7, alkalinity 254 mg · L^{-1} as $CaCO_3$, hardness 286 mg · L^{-1} as $CaCO_3$). Crayfish were fed flake food (Worldwide Aquatics, Inc., Arvin, California, USA) and/or sinking salmon pellets (Rangen EXTR 450, Rangen Inc., Buhl, Idaho, USA) about 3% of body weight once per day.

Test FHM were obtained as embryo-larvae (24 hr post-hatch) from Aquatic BioSystems (Fort Collins, Colorado, USA) or seined from CERC ponds (Supplemental Table S2). Fish were held in fiberglass tanks filled with CERC well water and fed brine shrimp nauplii (Brine Shrimp Direct, Ogden, Utah, USA) ad libitum two times per day at least 6 hr apart.

Test Design

Crayfish and fish testing

Modified static test procedures were used to evaluate the potency, dose-response, and selectivity of candidate chemicals to crayfish and fish (USEPA 2002). All toxicity tests were conducted from February 2013 to November 2014. Exposures occurred in 3.8-L glass jars with test water which were maintained at constant temperature (nominally 20°C) under a 16 hr light:8 hr dark photoperiod. Crayfish and fish were acclimated to test water (CERC well in just the pilot study conducted with VC or 100 hard water, a mixture of well water and reverse osmosis or deionized water, 20°C; pH 8.2; alkalinity 100 mg · L^{-1} as $CaCO_3$; hardness 100 mg · L^{-1} as $CaCO_3$). Crayfish were tempered into 100 hard water over a minimum of 12 hr by repeatedly diluting CERC well water fifty percent with 100 hard water (dilutions done at least 4 hr apart). Feeding was discontinued at least 48 hr prior to testing. Crayfish carapace length (from the tip of rostrum to the posterior edge of the cephalothorax; mm) or total length of FHM (mm) and wet weights (g) of test organisms were measured to estimate loading rates (weight/volume of water).

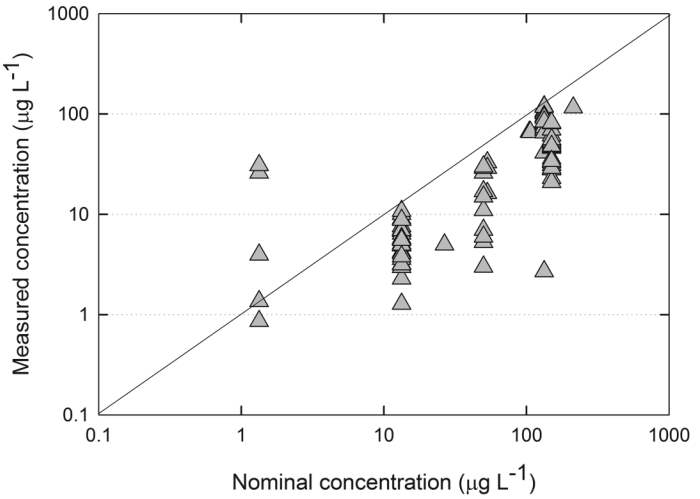

Figure 1. Recovery of nominal concentrations of cypermethrin in test solutions collected from mixing and exposure jars with and without White River Crawfish (WRC; *Procambarus acutus*) and/or Fathead Minnows (FHM; *Pimephales promelas*) at different time intervals during toxicity, fate and sequestration tests.

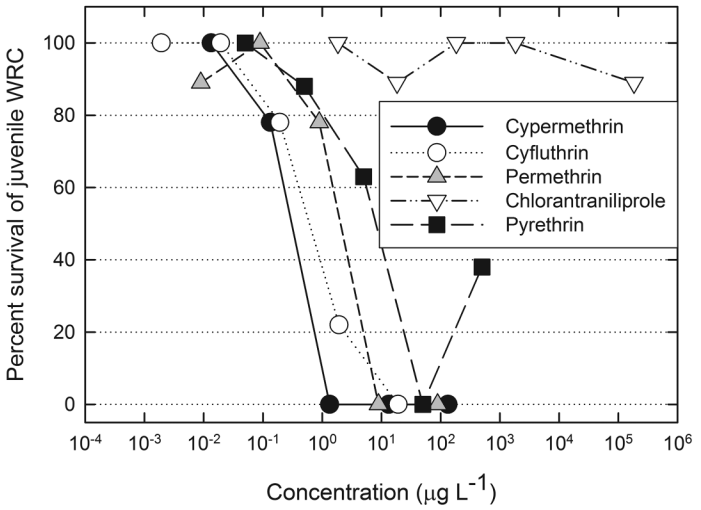

Figure 2. Potency of candidate chemicals to juvenile White River Crawfish (WRC; *Procambarus acutus*) in 24 hr exposures.

Tests examining chemical potency lasted 24 hr, which is a standard exposure time used in toxicity testing. Data from these studies would allow for comparison of the sensitives of WRC and VC relative to other crayfish and fish. Tests examining dose-response and margin of safety ran for 3 or 4 hr with 20 to 44 hr recovery periods, where crayfish and FHM were removed from test chemicals and placed in control (clean) water. The short exposure duration (3 – 4 hr) was selected to match the current protocol used for the control of Zebra Mussels, which was requested by hatchery staff. Duration of recovery periods were selected to assess recovery from 'knock down' effect of pyrethroids and pyrethrin after crayfish were provided clean water, which is generally rapid, usually with 24 – 48 hr (USEPA 2011). Survival of crayfish and FHM were measured at several time periods (0.5, 1, 2, 3, 4, 24 hr) during tests. Crayfish were transferred from test chambers to a glass tray placed on a lightbox and examined with the aid of a lighted 10x magnifying glass. Survival of FHM was determined in exposure test jars. Survival was based on movement in response to a gentle touch with a blunt probe within a 60-s period. General observations of sublethal behavioral effects in crayfish or FHM such as hyperactivity, loss of equilibrium (LOE), loss of reflex, vertical swimming, or slow movement of various body parts, including walking legs, antennae, gills or swimmerets, were also recorded (ASTM 1996; Allert et al. 2016). Crayfish were classified as juvenile (≥1 year old or <30 mm) or adults (>30 mm). Fathead minnows were classified as juveniles or adults (breeding phenotypes present).

Test chemicals

We focused our testing on pyrethrin and pyrethroid pesticides and an anthranilic diamide insecticide (Table 1). Pyrethroids are synthetic insecticides structurally similar to pyrethrins, which are botanical insecticides extracted from *Chrysanthemum cinerariifolium* (Trevir.) Vis. (Soderlund et al. 2002). We also conducted tests with adult VC and a solution of KCl with and without formalin, since these chemicals are currently added to fish shipments at BPH to prevent the inadvertent transfer of Zebra Mussels and would be used in combination with the pesticide selected for the control of crayfish.

Preparation of test solutions and analytical methods

Quality assurance blanks and spikes were prepared and analyzed for each test (Eaton et al. 2005; Supplemental Tables S3). Test solutions were prepared using standard laboratory practices (Eaton et al. 2005). Samples of test solutions were taken for analytical verification from stock solutions, mixing jars, and exposure test jars. Samples were shipped to Mississippi State Chemical Laboratory (MSCL), Mississippi State University, Mississippi, USA for analyses. Mean percent recoveries of spikes solutions for chemical analytes prepared by the MSCL ranged from 50 to 120% (Supplemental Table S4). There were no clear trends in the recovery of cypermethin over time (i.e., 2, 3, or 4 hr) in mixing or exposure jars (Figure 1). Mean percent recoveries of chemicals used in test solutions including primary and working stocks, mixing jars, and exposure jars ranged from 0 to 2322%, but were generally between 35 – 60% (Supplemental Table S5). Recoveries of chemicals may have been affected by the time of collection (i.e., inadequate mixing) or because we did not use a carrier solvent (i.e., acetone) to deliver the test chemical to test solutions.

Water-quality analyses

Measurements of temperature, pH, specific conductance, dissolved oxygen and total ammonia were measured at the initiation of tests in exposure and recovery test jars and at 3, 4, 24, and 48 hr (USEPA 2002). Temperature and dissolved oxygen were measured using a YSI Pro Dissolved Oxygen Meter (Yellow Springs, Inc., Ohio, USA). Temperature measurements were verified with a certified thermometer (ERTCO Model No. 59042).

The dissolved oxygen meter was calibrated using saturated air and known temperature and barometric pressure. In-situ water pH was measured using either an Orion Model 290A with Orion 9157BN probe or Thermo Orion 4 Star with 9107BNMD probe (Beverly, Massachusetts, USA). Mean values for in-situ water quality of test waters were similar for all tests (Supplemental Table S6). Mean temperature of test solutions was generally 20 ± 1°C. Mean dissolved oxygen was >4 mg · L^{-1} as required by acute toxicity test protocols (USEPA 2002). Alkalinity and hardness were measured by titration at 0 and 24 hr in control, low, medium, and high concentrations (USEPA 2002; Eaton et al. 2005). Mean alkalinity and hardness of 100 hard water were similar for all tests (Supplemental Table S7). Total ammonia (NH_3 as N) was analyzed using a Hach IntelliCAL Ammonia Analyzer (Loveland, Colorado, USA) or an Orion EA940 Meter with a Thermo-Orion ammonia probe (Model number 9512HPBNWP) filled with low-level ammonia filling solution. Thermo-Orion low-level ISA (ionic and pH buffer) was utilized to adjust samples for analyses. Samples for ammonia were analyzed within 24 hr of collection (Eaton et al. 2005). Total ammonia concentrations were based on a three-point standard curve. Precision and accuracy were determined based on triplicate analysis of independent, certified ammonia standards (Hach Corp., Loveland, Colorado, USA). Mean values for total ammonia concentrations were lower than the USEPA acute criteria for total ammonia nitrogen (criteria range for study = 3.2 – 10.0 mg · L^{-1} as N) and for unionized ammonia (criteria range for study = 1.15 – 4.76 mg · L^{-1} as N; Supplemental Table S8; USEPA 2002, 2013). Total ammonia and unionized ammonia concentrations were similar for all tests, regardless of species tested or test phase (exposure or recovery).

Mean percent recoveries of quality assurance data for water-quality measurements can be found at https://doi.org/10.5066/F70K26PP.

Statistical Analysis

Dose-response data are presented in plots using SigmaPlot Version 13.0 (Systat 2014) to summarize and define trends in crayfish response (lethal effects) to chemicals over time; between life stages and crayfish species; and among crayfish and fish. We attempted to evaluate toxic lethal effects by estimating median lethal concentration of the crayfish test population (LC50) and 95% confidence intervals using either Toxicity Relationship Analysis Program (TRAP; Erickson 2015) or the Probit Procedure in Statistical Analysis System (SAS Version 9.2; SAS Institute, Cary, North Carolina, USA); however, dose interval spacing, small sample sizes, and the lack of mortality after 24 hr resulted in wide confidence intervals, making the analyses ineffective.

RESULTS

Test Results

The chemical, species, source, date collected, exposure duration, life stage, number of crayfish or fish, and size of crayfish and FHM used in toxicity tests are summarized in Supplemental Tables S1 and S2. Loading rates of crayfish and FHM were higher than standard protocols (Supplemental Tables 1 and 2; >0.5 g · L^{-1}; ASTM 1996); however, survival of control organisms exceeded

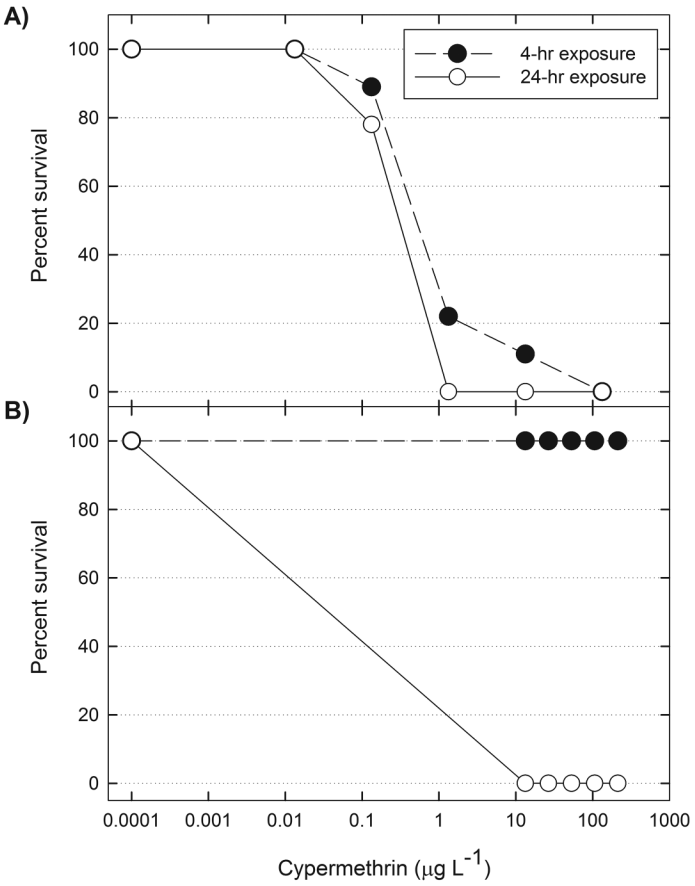

Figure 3. Mean percent survival of a) juvenile and b) adult White River Crawfish (WRC; *Procambarus acutus*) exposed to cypermethrin (µg · L^{-1}) for 4 or 24 hr.

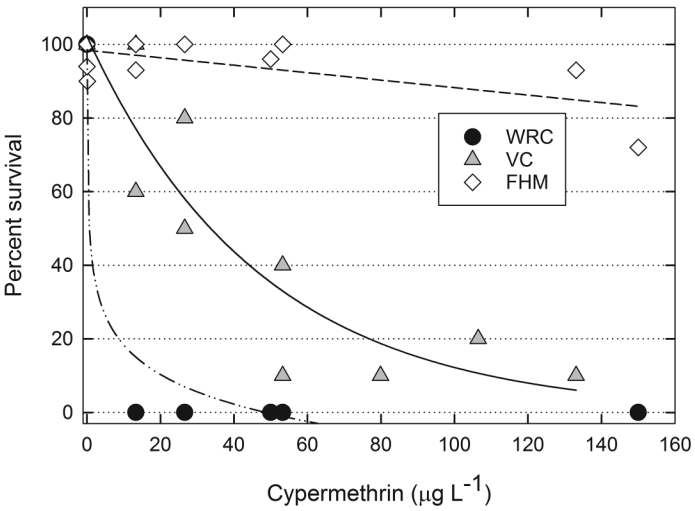

Figure 4. Mean percent survival of adult White River Crawfish (WRC; *Procambarus acutus*), adult Virile Crayfish (VC; *Orconectes virilis*), and juvenile Fathead Minnows (FHM; *Pimphales promelas*) exposed to cypermethrin (µg · L^{-1}). Organisms were exposed for 3 or 4 hr and then transferred to control (clean) water for 44 hr. Total test time = 48 hr. Trend lines are included to help visualize differences between taxa.

Table 2. Mean percent (%) sublethal behavioral effect and mortality of adult White River Crawfish (WRC; *Procambarus acutus*) exposed to cypermethrin for 0, 1, 4, and 24 hr during dose-response test. Half of test organisms exposed during the test were moved to control (recovery) water at 4 hr. Effects include hyperactivity, loss of equilibrium and small movements of walking legs, swimmerets, and gills (Allert et al. 2016). NS = no survivors.

Nominal cypermethrin concentration (µg · L^{-1})	Species	Number of crayfish	Exposure duration (hr)				
			0	1	4	4 (+20 recovery period)[a]	24[a]
Sublethal behavioral effect (% of surviving individuals)							
0[b]	WRC	8	0	0	0	0	0
13.3[b]	WRC	8	0	88	100	100	NS
26.6[b]	WRC	8	0	100	100	100	NS
53.2[b]	WRC	8	0	100	100	NS	NS
106.5[b]	WRC	8	0	100	100	NS	NS
213.0[b]	WRC	8	0	100	100	NS	NS
Mortality (%)							
0[b]	WRC	8	0	0	0	0	0
13.3[b]	WRC	8	0	0	0	50	100
26.6[b]	WRC	8	0	0	0	50	100
53.2[b]	WRC	8	0	0	0	100	100
106.5[b]	WRC	8	0	0	0	100	100
213.0[b]	WRC	8	0	0	0	100	100

[a] n =4
[b] Test =5

criterion of acute toxicity test protocols (>90%; Supplemental Table S9; USEPA 2002). Several other toxicity tests were also conducted as part of this study, the results of which were not directly related to the study conclusions. However, these toxicity tests also met USEPA acceptability criteria and provide additional information related to crayfish toxicity. Therefore, we have included these data in Supplemental Material (Supplemental Table S9).

Potency

Chlorantraniliprole was the least toxic chemical to juvenile WRC (Supplemental Table S9; Figure 2). At least one concentration of cypemethrin, cyfluthrin, permethrin, and pyrethrin caused 100% mortality of juvenile WRC during 24 hr exposures. The lowest concentration of cypermethrin that caused 100% mortality of juvenile WRC was about an order of magnitude lower than concentrations of other chemicals; therefore, cypermethrin was the chemical selected to investigate dose-responsive relationships and margin of safety between crayfish and FHM.

Dose-response for cypermethrin

Juvenile WRC (Figure 3a) were at least an order of magnitude more sensitive to cypermethrin than adult WRC (Figure 3b). Mean percent mortality was higher in juvenile and adult WRC in exposures lasting 24 hr than 4 hr. Mean percent survival in juvenile WRC was 0% for concentrations of cypermethrin >1.31 µg · L^{-1} in 24 hr exposures; however, it was only 0% in a cypermethrin concentration of 133 µg · L^{-1} for 4 hr exposures. Mean percent survival in adult WRC was 100% in all concentrations tested for 4 hr exposures; however, it was 0% in concentrations >13.3 µg · L^{-1} for exposures lasting 24 hr (Supplemental Table S9). Sublethal effects such as hyperactivity, LOE or slow movements in walking legs, swimmerets, or gills occurred within 1 hr of exposure and persisted after WRC were transferred to control (clean) water during the 20 hr recovery period (Table 2).

Margin of safety for cypermethrin in crayfish and fish

Mean percent survival of adult WRC exposed to concentrations of cypemethrin >13.31 was 0%; however, mortality in crayfish generally did not occur until after 44 hr in recovery water (Supplemental Table S9). Mean percent survival of adult VC ranged from 10 to 40% in concentrations >50 µg · L^{-1} (Supplemental Table S9). Sublethal behavioral effects in WRC (Table 3) and VC (Table 4) were consistent with previous tests, occurring within 1 hr of exposure and persisting for 44 hr after crayfish were transferred to clean water. Exposures conducted in 100 hard water plus KCl-formalin did not appear to reduce the efficacy of the cypermethrin treatment to adult crayfish (Supplemental Table S9).

In exposures lasting up to 4 hr, mean percent survival of juvenile FHM was >90% at concentrations of cypermethrin ≤133 µg · L^{-1} (Supplemental Table S9; Figure 4), but was reduced to about 70% at 150 µg · L^{-1}. Sublethal behavioral effects such as LOE or gill flaring in juvenile FHM occurred within 1 hr of exposure to cypermethrin and were near 100% in concentrations >50 µg · L^{-1} at 3 or 4 hr (Table 5). Juvenile FHM appeared to recover almost immediately and fully when placed in clean (control) water and no sublethal behavioral effects were observed at 48 hr. There appeared

Table 3. Mean percent (%) sublethal behavioral effects and mortality of adult White River Crawfish (WRC; *Procambarus acutus*) exposed to cypermethrin at 0, 1, 4, and 24 hr during margin-of-safety tests. Effects include hyperactivity, loss of equilibrium and small movements of walking legs, swimmerets, and gills (Allert et al. 2016). NS = no survivors.

Nominal cypermethrin concentration ($\mu g \cdot L^{-1}$)	Species	Number of crayfish	Exposure duration (hr)				
			0	1	4	4 (+20 recovery period)	4 (+44 recovery period)
Sublethal behavioral effects (% of surviving individuals)							
0[a]	WRC	5	0	0	0	0	0
0[b]	WRC	7	0	0	0	0	0
0[c]	WRC	10	0	0	0	0	0
0[cd]	WRC	10	0	0	0	0	0
0[e]	WRC	9	0	0[f]	0[g]	0	0
0[ed]	WRC	9	0	0[f]	0[g]	0	0
13.31[a]	WRC	5	0	100	100	NS	NS
13.31[b]	WRC	10	0	100	100	100	NS
26.62[a]	WRC	5	0	100	100	NS	NS
50[c]	WRC	10	0	100	100	100	NS
50[cd]	WRC	10	0	100	100	100	NS
50[ed]	WRC	9	0	100[f]	100[g]	100	NS
53.24[a]	WRC	5	0	100	100	NS	NS
150[c]	WRC	10	0	100	100	100	NS
150[ed]	WRC	9	0	100[f]	100[g]	100	NS
Mortality (%)							
0[a]	WRC	5	0	0	0	0	0
0[b]	WRC	7	0	0	0	0	0
0[c]	WRC	10	0	0	0	0	0
0[cd]	WRC	10	0	0	0	0	0
0[e]	WRC	9	0	0[f]	0[g]	0	0
0[ed]	WRC	9	0	0[f]	0[g]	0	0
13.31[a]	WRC	5	0	0	0	100	100
13.31[b]	WRC	10	0	0	0	80	100
26.62[a]	WRC	5	0	0	0	100	100
50[c]	WRC	10	0	0	0	80	100
50[cd]	WRC	10	0	0	0	50	100
50[ed]	WRC	9	0	0[f]	0[g]	66	100
53.24[a]	WRC	5	0	0	0	100	100
150[c]	WRC	10	0	0	0	60	100
150[ed]	WRC	9	0	0[f]	0[g]	22	100

[a] Test =9
[b] Test =11
[c] Test =12
[d] Cypermethrin in potassium chloride-formalin solution
[e] Test =13
[f] 1.5-hr exposure
[g] 3-hr exposure

Table 4. Mean percent (%) sublethal behavioral effects and mortality of adult Virile Crayfish (VC; *Orconectes virilis*) exposed to cypermethrin at 0, 1, 4, and 24 hr during margin-of-safety tests. Effects include hyperactivity, loss of equilibrium and small movements of walking legs, swimmerets, and gills (Allert et al. 2016).

Nominal cypermethrin concentration ($\mu g \cdot L^{-1}$)	Species	Number of crayfish	Exposure duration (hr)				
			0	1	4	4 (+20 recovery period)	4 (+44 recovery period)
Sublethal behavioral effects (% of surviving individuals							
0[a]	VC	5	0	0	0	0	0
0[b]	VC	10	0	0	0	0	0
0[c]	VC	10	0	0	0	0	0
13.31[a]	VC	5	0	0	80	60	40
13.31[b]	VC	10	0	100	100	100	67
26.62[a]	VC	5	0	20	100	100	100
26.62[b]	VC	10	0	60	100	100	100
53.24[a]	VC	5	0	40	100	100	100
53.24[b]	VC	10	0	100	100	100	100
79.86[b]	VC	10	0	100	100	100	100
106.5[b]	VC	10	0	100	100	100	100
133.1[c]	VC	10	0	90	100	100	100
Mortality (%)							
0[a]	VC	5	0	0	0	0	0
0[b]	VC	10	0	0	0	0	0
0[c]	VC	10	0	0	0	0	0
13.31[a]	VC	5	0	0	0	0	0
13.31[b]	VC	10	0	0	0	40	40
26.62[a]	VC	5	0	0	0	20	20
26.62[b]	VC	10	0	0	0	30	50
53.24[a]	VC	5	0	0	0	40	60
53.24[b]	VC	10	0	0	0	90	90
79.86[b]	VC	10	0	0	0	90	90
106.5[b]	VC	10	0	0	0	60	80
133.1[c]	VC	10	0	0	0	70	90

[a] Test =9
[b] Test =10
[c] Test =11

to be no reduction in the efficacy of the cypermethrin to FHM in exposures conducted in the KCl-formalin solution.

DISCUSSION

We conducted toxicity tests to identify a chemical and concentration that could be used to treat fish shipments at fish hatcheries to prevent the inadvertent transfer of live crayfish in incoming or outgoing fish shipments. Critical requirements for the chemical were that it be readily available at low cost; easily handled; have high potency to crayfish resulting in their complete mortality while providing a margin of safety for hatchery fish; and have low persistence in fish and the environment (Holdich et al. 1999; Solomon et al. 2001; Hyatt 2004). We identified Cynoff®, a commercial formulation of cypermethrin, as the pesticide that met all of the study criteria.

Our results confirmed that concentrations used to control adult crayfish would protect against early life stages of the same species (Eversole and Seller 1997) and that higher concentrations of chemical would be needed to control crayfish with shorter exposure times. A 4 hr exposure to a cypermethrin concentration in the range of 50 to 100 $\mu g \cdot L^{-1}$ resulted in 100% mortality of juvenile and adult WRC; however, adult VC were not consistently killed. There is not a large margin of safety between adult crayfish and juvenile FHM; however, survival of FHM was at least 70% for cypermethrin concentrations that were $\leq 150\ \mu g \cdot L^{-1}$ and was not significantly different from control survival for concentrations $\leq 133\ \mu g \cdot L^{-1}$. Survival of FHM was lowest in exposures where

Table 5. Mean percent (%) sublethal behavioral effects and mortality of Fathead Minnows (FHM; *Pimephales promelas*) exposed to cypermethrin at 0, 1, 4, and 24 hr during margin-of-safety tests. Effects include hyperactivity, loss of equilibrium and small movements of gills (Allert et al. 2016). ND = no data.

Nominal cypermethrin concentration ($\mu g \cdot L^{-1}$)	Species	Number of crayfish	Exposure duration (hr)				
			0	1	4	4 (+20 recovery period)	4 (+44 recovery period)
Sublethal behavioral effects (% surviving individuals)							
0[a]	FHM	15	0	0	0	0	0
0[b]	FHM	30	0	0	0	0	0
0[c]	FHM	100	0	0[d]	0[e]	1	0
0[cf]	FHM	85[g]	0	0[d]	0[e]	0	0
13.31[a]	FHM	15	0	0	40	0	0
13.31[b]	FHM	30	0	97	17	0	0
26.62[a]	FHM	15	0	20	40	0	0
50[cf]	FHM	85[g]	0	100[d]	ND[e]	0	0
53.24[a]	FHM	15	0	47	100	0	0
133.1[b]	FHM	30	0	97	100	0	5
150[cf]	FHM	85[g]	0	100[d]	100[e]	0	0
Mortality (%)							
0[a]	FHM	15	0	0	0	0	0
0[b]	FHM	30	0	0	0	0	0
0[c]	FHM	100	0	ND[d]	ND[e]	ND	10
0[cf]	FHM	85[d]	0	ND[d]	ND[e]	ND	6
13.31[a]	FHM	15	0	0	0	7	7
13.31[b]	FHM	30	0	0	0	0	0
26.62[a]	FHM	15	0	0	0	0	0
50[cf]	FHM	85[g]	0	ND[d]	ND[e]	ND	4
53.24[a]	FHM	15	0	0	0	0	0
133.1[b]	FHM	30	0	0	3	7	7
150[cf]	FHM	85[g]	0	ND[d]	ND[e]	ND	28

[a] Test =9
[b] Test =11
[c] Test =13
[d] 1.5-hr exposure
[e] 3-hr exposure
[f] Cypermethrin in potassium chloride-formalin solution
[g] One replicate n =88

adult WRC were stocked into the same jars as juvenile FHM. We observed WRC actively trying to capture FHM before they were affected by cypermethrin, possibly killing FHM.

Our observations of sublethal behavioral effects of pyrethroids were consistent with previous studies that examined crayfish, other aquatic invertebrates, and fish (Samsøe-Petersen et al. 2001; Polat et al. 2002; Sandodden and Johnsen 2010; Palmquist et al. 2012; Tiwari et al. 2012; Johnsen et al. 2013). Crayfish and FHM were affected with neurological symptoms such as hyperactivity, paralysis or LOE within 30 – 120 mins of exposures and persisted for 24 – 48 hr. However, for the development of a chemical control protocol for fish shipments, it is critical that we define lethal concentrations and not effect-based concentrations (EC) for cypermethrin. Stephenson (1982) indicated that 24 hr LC50s ranged from 1 to 70x greater than 24 hr EC50s. We examined crayfish for subtle movements (e.g., small movements in legs, antennae, gills or swimmerets) rather than assuming mortality of crayfish due only to the lack of visible activity, lack of response to tactile stimulation or the failure of the righting reflex (Stephenson 1982; Morolli et al. 2006; Sandodden and Johnsen 2010; Clark et al. 2015). This may result in overestimating the lethal concentrations of cypermethrin for WRC and VC. Lengthening (>44 hr) observation periods of crayfish in recovery (clean) water may aid in discriminating effective and lethal concentrations of cypermethrin in crayfish.

Recoveries of the active ingredient of test compounds in test chambers were generally 35 – 60%, which are within the range of other studies using static testing (Sharom and Solomon 1981).

Recoveries were most likely less than 100% due to several factors including high potency (i.e., low test concentrations), hydrophobic nature of chemicals, and formulation properties (tendency for evaporation). It is possible that we could have avoided extremely low (0%) and high (2322%) recoveries if we had used a solvent to mix test compounds into our primary stocks. Small quantities (micrograms) of the test compounds were needed to mix our primary stock solutions (1 L); however, this may not be an issue in developing protocols to be used at hatcheries, since hatchery staff will be working with much larger volumes of water (750 – 7500 L) for tanks on fish-hauling trucks.

Cypermethrin concentrations that resulted in 100% mortality of WRC (≥50 $\mu g \cdot L^{-1}$) in 4 hr exposures were at least two orders of magnitude greater than laboratory 96 hr LC50 values for *Orconectes rusticus* Girard (0.5 $\mu g \cdot L^{-1}$; Bills and Marking 1988); for *Orconectes nais* Faxon (0.069 $\mu g \cdot L^{-1}$; Munn et al. 2006), and for *Procambarus clarkii* Girard (0.14 $\mu g \cdot L^{-1}$; Morolli et al. 2006). Field-based concentrations have been reported in the range of our results. Bills and Marking (1988) reported a 96 hr LC50 of 25 $\mu g \cdot L^{-1}$ for *O. rusticus*. Sandodden and Johnsen (2010) reported complete mortality of *Pacifastacus leniusculus* (Dana) placed in a solution of BETAMAX VET® with an active ingredient (cypermethrin) concentration of 20 $\mu g \cdot L^{-1}$ within 50 min. Acute 96 hr LC50 values for cypermethrin are about 1 – 2 $\mu g \cdot L^{-1}$ for warm-water fish species such as FHM and Bluegill (Siepmann and Holm 2000; Solomon et al. 2001); however, juvenile FHM were tolerant of concentrations of cypermethrin that were 10 – 100x greater in tests lasting <4 hr during our testing. Additional studies examining the sensitivity of larger-sized fish may find a larger margin of safety than between fingerling-sized fish and crayfish.

Persistence of pyrethroid insecticides is relatively short (half-lives in order of tens to hundreds of days; Solomon et al. 2001; Spurlock and Lee 2008; Palmquist et al. 2012). There was no reduction in post-exposure growth, survival, sexual maturity, or reproduction (egg production and hatch) in *P. clarkii* exposed to permethrin concentrations in the range of 0.624 to 1.495 $\mu g \cdot L^{-1}$, suggesting that if crayfish were to survive a single potential toxic exposure (duration × dose) of permethrin, it is unlikely that they will show sublethal effects (Jarboe and Romaire 1991). Jarboe and Romaire (1995) found pond populations of *P. clarkii* recovered to pre-dosing densities and composition (sex ratio) within several months of the application of permethrin (1 – 3 $\mu g \cdot L^{-1}$). Bills and Marking (1988) found toxicity to caged FHM persisted for five weeks after the application of Baythroid, a formulation of β-cyfluthrin, in hatchery ponds.

Pyrethroids have been found to be toxic to standard toxicity test organisms at relatively low concentrations in aquatic sediments near agricultural and urban environments (Weston et al. 2005; Clark et al. 2015). There currently are no regulatory criteria for water or sediment for any pyrethroid insecticide in the USA (Spurlock and Lee 2008; TDC Environmental 2015). Fojut et al. (2012) have proposed acute (0.001 $\mu g \cdot L^{-1}$) and chronic (0.0002 $\mu g \cdot L^{-1}$) water-based criteria for cypermethrin. Single-dose events (i.e., discharge of tank water with annual or single-event fish stockings) may cross acute or chronic thresholds in small bodies of water; therefore, the potential for environmental contamination of sediment and water must be understood, if cypermethrin cannot be detoxified before hatchery truck water is released to the environment.

Additional studies are also needed to examine the rate of uptake of cypermethrin by fish during short (<3 hr) exposures, particularly for sport species that are stocked and sized for immediate consumption (McLeese et al. 1980; Fojut et al. 2011). There are no food tolerances for human consumption of fish in the USA, but there are food tolerances in the cypermethrin reregistration eligibility decision for other meat products, such as fat and meat byproducts of cattle, goat, hog, horse, and sheep (USEPA 2008b). Concentration factors (concentration in fish/concentration in water) depend on the length of exposure, dose concentrations, water temperature, and fish species. Concentration factors for whole fish for 24 to 96 hr static exposures of cypermethrin range from 2.6 to 7.1 to over 400 in flow-through exposures (McLeese et al. 1980; Polat et al. 2002; Fojut et al. 2011).

Our efforts to passively bind cypermethrin with granulated activated carbon were not more effective than what was assumed to be adsorption to glass test chambers (Sharom and Solomon 1981) or due to uptake by crayfish and FHM. There may be a simple and inexpensive method for detoxifying pyrethroids using techniques developed for toxicity identification evaluations (Weston and Amweg 2007). Esterase enzyme (carboxylesterase) rendered solutions of cypermethrin non-toxic to *Ceriodaphnia dubia* Richard (Wheelock et al. 2004) and also may decrease uptake of cypemethrin by fish due to the addition of organic matter to water in fish hauling tanks (Maund et al. 2002). There are potentially harmful side-effects, such as decreased dissolved oxygen levels and increased ammonia concentrations due to the addition of carboxylesterase into fish hauling tanks; however, effects occurred 12 hr after the addition of carboxylesterase in laboratory studies (Weston and Amweg 2007). Also, hatchery protocols require constant monitoring of dissolved oxygen concentrations in tanks during fish deliveries, which would allow for increasing the rate of supplemental oxygen, if necessary. Chemical control protocols should include additional monitoring of oxygen levels in fish hauling tanks if carboxylesterase proves effective in detoxifying cypermethrin and because pyrethroids are known to increase respiration rates in fish (Polat et al. 2002). Studies are needed to investigate short incubation times (<1 hr) to provide hatchery staff with the optimal dose of carboxylesterase to be used in chemical control protocols.

Crayfish have a high potential for invasion of new habitats (Chucholl 2013; Pearl et al. 2013). Fish shipments, which cross watershed and political boundaries, have an enormous potential for inadvertent crayfish introductions and subsequent invasions that can result in trophic disruption (Holdich et al. 1999; Lodge et al. 2000) or the extirpation of native crayfish species (Taylor et al. 2007). From 2011 to 2014, the state of Missouri averaged about 1,000 fish transfers from state hatcheries per year from their nine fish hatcheries (Miller 2014, personal communication). The number of annual fish transfers at fish hatcheries in the USA could be well over 50,000 with comparable transfer rates from other state, federal, tribal, and private hatcheries. Whereas legislation or regulation has been effective in reducing the potential for anthropogenic transfers of nonnative crayfish and is less costly

than the management of invasive crayfish (Dresser and Swanson 2013), it is currently difficult to get this type of legislation enacted in the USA (DiStefano et al. 2016). Biosecurity protocols (see links at http://www.fws.gov/fisheries/ans/ans-haccp.html; http://www.haccp-nrm.org/listplans.asp; accessed June 1, 2016) have proven to have much wider acceptance and are being implemented throughout the USA. Standardization and use of a chemical control protocol for crayfish at fish hatcheries could remove a potentially significant pathway for invasive crayfish. We are encouraged that state hatchery managers in Missouri are investigating methods to prevent the release of crayfish in fish shipments. We hope that additional testing will provide hatcheries with low-cost and environmentally safe protocols that can be approved by regulatory agencies for use, thereby reducing the risk of unintentional transfers of nonnative crayfish into receiving waters or fish hatcheries.

Conclusions

We identified Cynoff®, a commercial formulation of cypermethrin, as the pesticide that met all of the study criteria developed to assess the potential for pesticides to control crayfish in fish shipments. Determining lethal concentrations for WRC or VC was difficult due to the sublethal behavioral effects of chemicals. Our results suggest that a 4 hr exposure to a cypermethrin concentration in the range of 50 – 100 $\mu g \cdot L^{-1}$ will control WRC with minimal mortality to juvenile FHM; however, VC were not consistently killed. Additional testing with longer (>44 hr) recovery periods may help discriminate effective versus lethal concentrations in crayfish and provide greater insight into the range in sensitivities of different crayfish species. Additional testing is also needed to examine margin of safety between crayfish and hatchery fish species and possible detoxification methods for cypermethrin.

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