Determination of the Acute Toxicity of Supaverm® to Native and Nonnative Fish Species of Southwestern Watersheds in Static Exposures

By Theresa M. Schreier, Terrance D. Hubert, Justin Smerud, and Michael A. Boogaard

Administrative Report

U.S. Department of the Interior
U.S. Geological Survey

U.S. Department of the Interior
SALLY JEWELL, Secretary

U.S. Geological Survey
Suzette M. Kimball, Director

U.S. Geological Survey, Reston, Virginia: 2016

Determination of the Acute Toxicity of Supaverm® to Native and Nonnative Fish Species of Southwestern Watersheds in Static Exposures

Study Number: AEH-14-Supaverm-01

Theresa M. Schreier, Terrance D. Hubert, Justin Smerud, and Michael A. Boogaard

U.S. Geological Survey
Upper Midwest Environmental Sciences Center
2630 Fanta Reed Road
La Crosse, Wisconsin 54603

Funding Agency: U.S. Fish and Wildlife Service

Report Date: August 2016

ABSTRACT

Many fishes native to the Gila River Basin, Arizona, are on the decline with about 70 percent of the 17 fish species Federally listed as endangered or threatened. The decline has been partly attributed to the introduction of nonnative fishes that are of recreational interest such as catfish and smallmouth bass. Effective management practices are needed to control the nuisance nonnative fishes in Southwestern United States watersheds to prevent further decline of the native species and facilitate their restoration. An effective approach is the use of chemical toxicants to control the nuisance species. One chemical mixture of interest, Supaverm®, a combination of mebendazole and closantel, has been reported to show selectivity toward nonnative fish species of concern. We conducted acute toxicity tests on native and nonnative fish species of the Gila River (Arizona). Our findings showed that Supaverm® was not selectively toxic to the nonnative fish species suggesting that the use of the chemical mixture to eradicate those fish would not be effective.

INTRODUCTION

The biodiversity of native fishes in Arizona, with approximately 30 native species recorded since the late 1800s (Minckley 1973, Rinne 1995), is low compared with the freshwater fish species of the Eastern United States. High rates of endemism characterize fishes from the Southwestern United States; specialization of form is the rule rather than the exception (Rinne 1995). In the Gila River Basin, which drains approximately 212,380 square kilometers (km2) in Arizona and New Mexico, 5 of 17 native fishes are the only species in their genus (Miller 1961, Rinne 1995). Fishes native to the Southwestern United States typically are adapted to tolerate waters of high temperature or salinity. They are also habitat specialists in areas such as thermal springs or highly erosive streams but have evolved generalizations that promote resistance to extinction (Minckley and Meffe 1987).

While habitat specialization has enabled these fishes to persist in habitats few other species can withstand, it has also left them vulnerable to habitat alterations and invasive species. As the human population has grown throughout the region and demand for water has intensified, aquatic ecosystems have been greatly altered. Numerous dams and intensive livestock grazing practices have changed water temperatures and flow regimes, usually reducing habitat quality for native fishes (Rinne and Minckley 1991). Fish introductions, mostly for sport and food, but also from aquaculture, aquarium releases, for additional forage and biological control, were also common in the Southwestern United States (Rinne 1995). The number of fish species established in Arizona has almost tripled since the beginning of the 20th century as a result of the introduction of nonnative fishes (Rinne 1991). Many of these introduced fishes are better adapted to the highly altered systems now found in the Southwestern United States than are native species (Rinne and Minckley 1991). As a result, native fishes of the Southwestern United States are becoming increasingly imperiled.

Of the approximately 17 fishes native to the Gila River Basin, the largest watershed in Arizona, about 70 percent are Federally listed as endangered or threatened (Rinne 2003), and one is extinct. The plight of native fishes in this basin is typical of most basins in the Southwestern United States. The inherent rarity of fishes native to the Southwestern United States is

exacerbated by factors such as habitat alteration and the introduction of nonnative fishes (Minckley and Meffe 1987). Along with habitat alteration and destruction, competition with and predation by nonnative fishes have been identified as the driving forces for the imperilment of many of the native fishes of concern in the Gila River Basin. Through discussion with Bureau of Reclamation personnel and literature reviews, 12 native species have been identified as most imperiled by nonnative species. Similarly, the top 12 nonnative species which pose the most substantial risk to native fauna have also been identified (Dawson and Kolar 2003). In some instances, self-sustaining populations of native fishes appear to be unable to persist in habitats where nonnative fishes have become established (Marsh and Pacey 2005). For example, in areas where the introduced red shiner is found in Arizona, two Federally threatened, native species, the spikedace and loach minnow, are absent (Minckley 1973). Spikedace and loach minnow also appear to be extirpated in areas populated by introduced fishes such as channel catfish and flathead catfish in some Arizona rivers (Rinne 1995).

To be successful, Rinne (1995) suggests that conservation of the native fishes in the Southwestern United States requires that biologists be innovative and vigilant and should include research on the interactions of native and nonnative fishes. Restrictions on the importation of nonnative fishes, incorporation of a value system for native fishes, and a focus on the conservation and restoration of habitats for native species would also be required (Rinne 1995). Where historically inhabited waters are no longer suitable for native species because of nonnative occupation, successful conservation of native fishes may rely on the removal or substantial reduction of nonnative fishes.

Effective treatments for the eradication and control of nonnative fishes include chemical renovation of stream reaches (usually in concert with installation of physical fish barriers), followed by the stocking of desired species (Rinne and Turner 1991), or application of species-specific piscicides, in rare situations. Application of a species-specific piscicide is an intuitively appealing approach for controlling nonnative fishes, but has not been practiced in the Southwestern United States because such piscicides are not available for the nonnative species of concern in the region. Chemical renovation is expensive, logistically difficult, and usually more effective in smaller headwater areas. Chemical renovation usually requires retreatment for success. Other strategies (for example, selective harvest, regulatory control) are generally not effective in controlling fishes; thus, effective management of nuisance nonnative fishes in the Southwestern United States, as well as in other ecosystems, may require an integrated approach using various methods of control in one management program. The use of piscicides combined with other innovative approaches, such as sterilants, attractants or repellants, or reproductive inhibitors, applied in an integrated manner to manage nonnative fishes may improve the probability of successful renovation of streams and rivers in the Southwestern United States.

A recent publication suggests that a certain combination of chemicals showed selectivity toward nonnative fishes that present problems in the restoration of native species in Southwestern United States watersheds (Ward 2005). The commercial formulation Supaverm® (Elanco Animal Health) contains a combination of two drugs–closantel and mebendazole (50 milligrams per milliliter [mg/mL] closantel and 75 mg/mL mebendazole). It was originally developed as a sheep dewormer and is now marketed for control of flukes in ornamental koi ponds. In laboratory tests in static treatment chambers with aeration at 20 ± 2 ° Celsius (C), Supaverm® at a concentration of 13 micrograms per liter (µg/L) killed 100 percent of nonnative

fish such as bullhead, channel catfish, and over 90 percent of fathead minnow and red shiner. The sensitivity of centrarchids varied while the native fishes of interest (longfin dace, Gila topminnow, and Gila chub) appeared unaffected for 36 hours (Ward 2005).

The objective of this study was to determine the toxicity of Supaverm® to the native and nonnative fish species of the Gila River (Arizona). The selection of native fishes tested was based on availability, threatened status or from similar families and the nonnative fishes were selected based on the introduced fish that are causing the decline of native species or availability of fish from similar families.

METHODS

Test article

Supaverm® (active ingredients, closantel and mebendazole; 1 mL of suspension = 50 milligrams [mg] closantel and 75 mg mebendazole; 12.5 percent combined active ingredient, [a.i.]). Supaverm® was obtained from Koi-Stuff, Callahan, Florida. The product was manufactured by Elanco Animal Health, United Kingdom. Reported concentrations were based on the volume of Supaverm® (a.i.) delivered to a known volume of water.

Analytical standards

Closantel (97.3 percent purity) and mebendazole (98 percent purity) were purchased from Sigma Aldrich (St. Louis, Missouri). Calibration curve standards were prepared in a 50:50 solution of water; acetonitrile with 2 percent formic acid and 10 percent dimethyl sulfoxide was added to improve solubility.

Test animals

Native and nonnative fishes were cultured from eggs at the Upper Midwest Environmental Sciences Center's (UMESC) aquaculture facility, acquired from a Federal, State, or private hatchery, or collected from the wild by seining or Aquamax®-baited metal Gee minnow traps. Culture procedures followed UMESC Standard Operating Procedures (SOPs) for fish care and maintenance. Table 1 lists the nonnative fish species and table 2 lists the native fish species that were tested including the fish source, number of fish tested, and average length and weight.

All acquired fish were transported to UMESC in insulated and aerated fish transport tanks shipped according to procedures described in Piper et al. (1982). Fish were cultured under normal rearing conditions according to UMESC SOPs in either the fish culture area or the invasive species culture complex at UMESC. All fish were held a minimum of 14 days (d) prior to testing to allow for any delayed mortality because of transport. During this holding period, fish were fed and maintained according UMESC SOPs. Fish were used without regard to gender. Prior to conducting toxicity tests, fish were fasted for 96 hours (h) (American Society

for Testing and Materials [ASTM] 2007). If necessary, fish were acclimated to test temperature of 20 °C at a rate of 3 ºC per day (ASTM 2007). Fish were designated as acceptable for admission to a test if mortalities did not exceed 0.2 percent per day during the 96-h holding period.

Test procedures

Studies were conducted at UMESC, La Crosse, Wisconsin. Acute toxicity tests were conducted in well water in static exposure baths. Water temperature was maintained near the target temperature of 20 ºC by immersing test vessels in temperature-controlled water baths. Total hardness and total alkalinity of test water were determined at least once for each test (American Public Health Association [APHA] 1985). Dissolved oxygen was monitored with a YSI Model 55 dissolved oxygen meter. The pH and temperature of the water were measured with a Beckman PHI 410 pH meter. Dissolved oxygen, pH and water temperature were monitored in the test vessels with live fish at 1, 12, 24, 48, 72, and 96 h. Aeration was added to tanks when dissolved oxygen levels dropped below 6.0 milligrams per liter (mg/L). Dead fish were removed at specific exposure intervals and cumulative mortality was recorded for each test vessel. Total length in millimeters (mm) and wet weight in grams (g) of fish in control treatments were determined at the end of each test (table 1).

Acute toxicity tests

Static toxicity tests were conducted in 20, 50, or 100-liter (L) stainless steel vessels depending on the size of the test organisms. Five to 10 fish were randomly placed in each of the test vessels at a loading rate of no more than 0.8 grams per liter (g/L) (ASTM 2007). A stock solution of Supaverm® for spiking vessels to desired nominal concentrations based on active ingredient was prepared by diluting a specific volume of Supaverm® in deionized water. One untreated water control vessel was included for each exposure replicate. Toxicity tests were initiated by exposing groups of fish to one of at least seven nominal toxicant concentrations in each set. The concentration of toxicant in each treatment vessel, except for the highest concentration and the control treatment(s), was at least 60 percent of that in the next higher one (ASTM 2007). Each concentration had three replicates. During most of the trials, exposure vessels were covered with tank lids to prevent fish from escaping. Test organisms were observed for mortality at exposure intervals of 1, 3, 6, 12, 24, 48, 72, and 96 h.

Dose verification trial

A dose confirmation trial was conducted to evaluate the accuracy of the exposure concentrations prepared during the tests. Three concentrations were evaluated, 2.5, 250, and 2,500 µg/L Supaverm® (based on a.i.). These concentrations were selected to bracket the range tested during the toxicity trials (low, mid, and high). Each concentration was prepared in triplicate with one control group. The exposure concentrations were prepared in the same manner as the acute toxicity test solutions. To incorporate biologically-mediated changes in

concentration (such as, uptake, distribution within tissues, metabolism, elimination), 10 bluegill were added to each exposure vessel. Exposure water samples (20 milliliters [mL]) were collected within 15 minutes of preparation from the center of the tank at the surface. Water samples were collected after 24, 48, 72, and 96 hours of preparation, these samples were collected from the center of the tank at middle water column depth with an Eppendorf® pipettor. Water quality (dissolved oxygen, pH, and temperature) and fish mortality readings were measured daily in all tanks. Water samples were prepared for analysis by diluting 5 mL of sample with 5 mL of acetonitrile with 4 percent formic acid and as much as 10 percent dimethyl sulfoxide, added to improve solubility of chemicals, in a centrifuge tube. The solution was mixed and centrifuged (Beckman Avanti 30) for 10 minutes at 12,000 relative centrifugal force (RCF) at 20 °C. The sample was transferred to vials for analysis on an Agilent Technologies Triple Quad LC/MS System, Model G6460A equipped with a Phenomenex Kinex EVO C18, 2.6 µm, 50 × 2.1 mm column. A 250-µg/L Supaverm® standard (based on a.i.) was prepared in well water and analyzed with all time periods except the Time 0 hour sample as a standard check.

Liquid Chromatography Mass Spectrometry Analysis

The analytical method was developed to quantitate for both closantel and mebendazole concentrations in the water samples. A mobile phase gradient (table 3) consisting of water (8 mL):acetonitrile (2 mL) with 1 percent formic acid (mobile phase A) and acetonitrile with 1 percent formic acid (mobile phase B) was generated from initial conditions of 85:15 ratio of mobile phase A:B to 20:80 (mobile phase A:B). Injection volume was 4 µL with a flow rate of 0.6 milliliters per minute (mL/min) and a column temperature of 45 °C. A five-point quadratic standard curve ($r^2 \geq 0.99$) was used to quantify the closantel and mebendazole concentrations in the samples. Supaverm® exposure concentrations for the dose verification trial were calculated by adding the closantel and mebendazole concentrations and adjusting for the 12.5 percent a.i. composition ratio. The detection limit for both closantel and mebendazole was 0.1 µg/L.

Statistical analyses

Statistical analyses involved simple descriptive statistics for means and standard deviations of nominal Supaverm® concentrations (based on a.i.) and water characteristics. Statistical calculations of toxicity data were based on nominal concentrations of the Supaverm®. The LC (lethal concentration)$_{50}$ values (lethal concentration where mortality is expected among 50 percent of the test organisms) and 95 percent confidence intervals were determined from pooled mortality data from the three replicates for all fish species as well as LC_{99} values for nonnative fish species and LC_{25} values for the native fish species (Litchfield and Wilcoxon 1949). The log-probit method (Litchfield and Wilcoxon 1949) was used to estimate LC_{25}, LC_{50}, and LC_{99} values. To evaluate the selective removal of undesired species relative to other species in the same habitat, it is convenient to compare the LC_{99} value for the target species (essentially complete removal) with the LC_{25} value for the nontarget species (maximum acceptable loss of nontarget species; Bills et al. 2003).

RESULTS

Acute Toxicity Tests

Supaverm® toxicity. The toxicity (LC_{50}'s) of Supaverm® was similar for most of the species of fish tested. Mosquitofish and gila topminnow were the least sensitive fish species at the 96-h exposure (table 4). Channel catfish and black bullhead were the most sensitive nonnative fish at the 96-h exposure and bluehead sucker was the most sensitive native fish species at the 96-h exposure (table 4). The range of LC_{50} values for the nonnative and native fish species were not sufficiently separated to permit selective removal of nonnative fish species and still provide a margin of safety that would protect native species.

The 24-h LC_{25} value for gila topminnow (1,100 µg/L) was greater than the 24-h LC_{99} values for red shiner (455 µg/L), channel catfish (500 µg/L), smallmouth bass (500 µg/L), black bullhead (660 µg/L), and flathead catfish (700 µg/L) (table 5). However, most other native fish species tested were sensitive to Supaverm® concentrations (based on a.i.) at or below LC_{99} concentrations for the nonnative fish species tested (table 5). Figure 1 and figure 2 illustrate the lack of separation between the LC_{99} and the LC_{25} concentration estimates at 24 and 96 h of exposure for the native and nonnative species.

Water quality. Mean dissolved oxygen concentration (± standard deviation [SD]) in the exposure water was 7.85 ± 1.2 mg/L and pH ranged from 7.00 to 8.70. Water temperature for the tests ranged from 18.0 to 20.0 ºC. Alkalinity ranged from 130 to 158 mg/L as calcium carbonate ($CaCO_3$) and hardness from 170 to 206 mg/L as $CaCO_3$.

Dose verification. The mean Supaverm® concentrations (based on a.i.) in the exposure vessels at the start of the trial (time 0) were 292 and 2,589 µg/L for the 250 and 2,500 µg/L treatments and the concentrations after 96 hours were 100 and 679 µg/L, respectively (table 6). The concentrations in the exposure vessels dropped significantly during the exposure period, 60 percent in the 250 µg/L and 73 percent in the 2,500 µg/L group. The 2.5-µg/L Supaverm® exposure concentration was not quantifiable due to a carryover issue of the instrument.

DISCUSSION

Supaverm® was toxic to all fish species tested. The concentrations that caused toxicity were similar among natives and nonnatives, therefore, Supaverm® would not be suitable for selective removal of nonnative fish species. Gila topminnow and mosquitofish (family, *Poeciliidae*) were the least sensitive species with toxicity only occurring in the highest Supaverm® concentrations (based on a.i.) after 96 h of exposure. Scaleless fish species (family, *Ictaluridae*) and bluehead sucker (family, *Catostomidae*) appear to be the most sensitive to Supaverm®, showing substantial mortality after 96 h of exposure to lower concentrations of Supaverm®.

The Supaverm® concentrations (based on a.i.) in the exposure vessels were up to 19 percent greater than the nominal concentration at the start of the dose verification trial but

significantly decreased (up to 73 percent) over the 96-hour exposure. The dose verification trial indicated that the procedures used to prepare the test concentrations would have resulted in actual test concentrations within 19 percent of the nominal concentrations. Supaverm® is formulated as a suspension and has limited solubility in water. Supaverm® appeared to create a suspension when initially added to the test chamber. However, settling of at least one of the active ingredients during the exposure period resulted in an overall decrease in Supaverm® concentration during the dose verification trial. For example, the amount of closantel in the 250-µg/L group was undetectable after 96 hours leaving only mebendazole as the remaining active ingredient.

Our study followed the static test procedures used by Ward 2005. Since Supaverm® forms a suspension when added to water, the static test system was not the most effective way to evaluate the toxicity. If the study was repeated, we would recommend using a flow-through test system that would minimize settling and there by maintain the Supaverm® concentration throughout the exposure duration.

The findings reported by Ward (2005) focused only on one concentration (13 µg/L) and one treatment duration (36 h). Our cursory check of Ward's (2005) calculations revealed that his actual Supaverm® concentration was 330 µg/L (Ward, personal communication, 2014) which is in the range of the concentrations tested in our toxicity trials. However, our results do not show the broad selectivity of Supaverm® reported by Ward (2005). While results indicate that red shiner, channel catfish, smallmouth bass, and black bullhead could be selectively removed in the presence of gila topminnow, other native fish species would be impacted. We recommend that alternative chemicals be evaluated to find a fish toxicant with a favorable selectivity profile.

ACKNOWLEDGMENTS

Financial support for this study was provided by the U.S Fish and Wildlife Service. We also thank the Bureau of Reclamation staff for their assistance with collection of native test organisms. Special thanks to Justin Schueller and Jeff Bernardy for developing an analytical method for determining Supaverm® concentrations in water samples.

REFERENCES

American Public Health Association (APHA), American Water Works Association, and Water Pollution Control Federation. 1985. Standard Methods for the Examination of Water and Wastewater. 17th ed., Washington, DC.

American Society for Testing and Materials (ASTM). 2007. Standard Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. Annual Book of ASTM Standards. Standard E729-96.

Bills, T. D., M. Boogaard, D. Johnson, D. Brege, R. Scholefield, R. Westman, and B. Stephens. 2003. Development of a pH/alkalinity treatment model for applications of the lampricide TFM to streams tributary to the Great Lakes. Journal of Great Lakes Research, vol. 29 (Supplement 1), pages 510–520.

Dawson, V. K. and C. S. Kolar, eds. 2003. Integrated management techniques to control nonnative fishes. U.S. Geological Survey, Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin, Dec. 2003. 146 pp. Appendixes A-F.

Litchfield, J. T. and F. A. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exper. Therap. 96:99–113.

Marsh, P. C. and C. A. Pacey. 2005. Immiscibility of native and non-native fishes. Pages 59–63 in Brouder, M. J., C. L. Springer and S. C. Leon, editors. Proceedings of two symposia: Restoring native fish to the lower Colorado River: Interactions of native and non-native fishes. July 13–14, 1999, Las Vegas, Nevada, and Restoring natural function within a modified riverine environment: The lower Colorado River. July 8–9, 1998. U.S. Fish and Wildlife Service, Southwest Region, Albuquerque, New Mexico.

Miller, R. R. 1961. Man and the changing fish fauna of the American Southwest. Papers Michigan Academy Science Arts, Letters 46:365–404.

Minckley, W. L. 1973. Fishes of Arizona. Arizona Game and Fish Department, Phoenix, Arizona.

Minckley, W. L., and G. Meffe. 1987. Differential selection by flooding in stream-fish communities of the arid American Southwest. Pages 93–104 in W. J. Matthews and D. C. Heins, editors. Community and evolutionary ecology of North American stream fishes. University of Oklahoma Press, Norman.

Piper, R. G., I. B. Mc Elwain, L. E. Orme, J. P. McCraren, L. G. Fowler, and J. R. Leonard. 1982. Fish Hatchery Management. U.S. Department of the Interior, Fish and Wildlife Service, Washington, D.C.

Rinne, J. N. 1991. An approach to management and conservation of a declining regional native fish fauna: southwestern United States. Pages 56–60 in N. Maruyama et al., editors. Wildlife Conservation: Present trends and perspectives for the 21st century. International Symposium Wildlife, 5th International Congress Zoology, Tskuba and Yokohama, Japan, August 21–25, 1990.

Rinne, J. N. 1995. The effects of introduced fishes on native fishes: Arizona, southwestern United States. Pages 149–159 in D. P. Philipp et al., editors. Protection of aquatic biodiversity. Proceedings of the World Fisheries Congress, Theme 3. Oxford & IBH Publishing Company Pvt. Ltd., New Delhi, India.

Rinne, J. N. 2003. Native and introduced fishes: their status, threats and conservation. Pages 193–213 in Malchus, Ffolliott, DeBano, and Neary, editors. Riparian areas of the Southwestern United States: hydrology, ecology, and management. Lewis Publishers, Boca Raton, Florida.

Rinne, J. N., and W. L. Minckley. 1991. Native fishes of arid lands: a dwindling resource of the desert Southwest. U.S. Department of Agriculture Forest Service General Technical Report RM-206. 45 pp.

Rinne, J. N., and P. R. Turner. 1991. Reclamation and alteration as management techniques, and a review of methodology in stream renovation. Pages 219–244 in W. L. Minckley and J. E. Deacon, editors. Battle against extinction—native fish management in the American West. The University of Arizona Press, Tucson.

Ward, D. 2005. Selective removal of nonnative fishes using Supaverm®: Toxicity screening for a candidate species-specific piscicide. J. Freshwater Ecol. 20:787–789.

Table 1. List of nonnative fish species tested for acute toxicity of Supaverm® including the fish source, number of fish tested per replicate and mean length in millimeters (mm) and weight in grams (g) (± standard deviation [STD]). UMESC = Upper Midwest Environmental Sciences Center.

Nonnative fish species (scientific name)	Fish source	Number of fish per replicate[1]	Mean length (mm)	Mean weight (g)
Bluegill sunfish (*Lepomis macrochirus*)	UMESC La Crosse, Wisconsin	10	35 (± 5.6)	0.59 (± 0.34)
Black bullhead (*Ameiurus melas*)	Osage Catfisheries, Inc. Osage Beach, Missouri	5	111 (± 11)	14.9 (± 4.6)
Channel catfish (*Ictalurus punctatus*)	UMESC La Crosse, Wisconsin	10	55 (± 3.3)	1.37 (± 0.30)
Smallmouth bass (*Micropterus dolomieu*)	UMESC La Crosse, Wisconsin	10	37 (± 2.1)	0.54 (± 0.10)
Tilapia (*Tilapia aurea*)	Aquasafra, Inc. Bradenton, Florida	10	32 (± 2.7)	0.55 (± 0.13)
Mosquitofish (*Gambusia affinis*)	Columbia Environmental Research Laboratory Columbia, Missouri	10	43 (± 5.5)	0.97 (± 0.37)
Red shiner (*Cyprinella lutrensis*)	Osage Catfisheries, Inc. Osage Beach, Missouri	10	42 (± 2.9)	0.70 (± 0.18)
Flathead catfish (*Pylodictis olivaris*)	Joe Hogan State Fish Hatchery Lonoke, Arkansas	8	95 (± 7.0)	7.43 (± 1.9)

[1] Number of fish per replicate based on availability.

Table 2. List of native fish species tested for acute toxicity of Supaverm® including the fish source, number of fish tested per replicate and mean length in millimeters (mm) and weight in grams (g) (± standard deviation [STD]).

Native fish species (scientific name)	Fish source	Number of fish per replicate[1]	Mean length (mm)	Mean weight (g)
Gila topminnow (*Poeciliopsis occidentalis*)	Arizona State University Tempe, Arizona	10	27 (± 4.3)	0.20 (± 0.11)
Longfin dace (*Agosia chrysogaster*)	Wild Aravaipa Creek, Arizona	10	55 (± 6.3)	1.67 (± 0.67)
Roundtail chub (*Gila robusta*)	Bubbling Ponds Fish Hatchery Cornville, Arizona	10	91 (± 5.8)	6.32 (± 1.28)
Speckled dace (*Rhinichthys osculus*)	Wild Fossil Creek, Arizona	7	69 (± 12)	3.31 (± 1.84)
Spikedace (*Meda fulgida*)	Bubbling Ponds Fish Hatchery Cornville, Arizona	5 or 6	71 (± 3.4)	2.52 (± 0.52)
Bluehead sucker (*Catostomus discobolus*)	Native Aquatic Species Restoration Facility Alamosa, Colorado	10	53 (± 5.0)	1.13 (± 0.36)

[1] Number of fish per replicate based on availability.

Table 3. Mobile phase gradient for Liquid Chromatography Mass Spectrometry (LC MS) analysis of closantel and mebendazole in water samples. Mobile phase A: water (8):acetonitrile (2) with 1 percent formic acid and mobile phase B: acetonitrile with 1 percent formic acid.

Time (minute)	Mobile phase A (percent)	Mobile phase B (percent)
0.75	85	15
1.5	20	80
3.25	20	80
3.4	87.5	12.5

Table 4. Acute toxicity results (lethal concentration (LC_{50} values reported in micrograms per liter [µg/L] and 95 percent confidence interval) for triplicate exposures at 24- and 96-hours of Supaverm® to nonnative and native fish species of the Gila River watershed.

Fish species	LC_{50} and 95 percent confidence levels at exposures of:	
	24 hours	96 hours
	Supaverm® concentration (µg/L, based on active ingredient)	
Mosquitofish	1,400 (1,270 – 1,543)	770 (669 – 886)
Gila topminnow[1]	1,270 (1,142 – 1,413)	790 (720 – 866)
Bluegill	550 (499 – 607)	385 (351 – 422)
Roundtail chub[1]	520 (476 – 568)	520 (476 – 568)
Black bullhead	450[2]	102 (90.0 – 116)
Longfin dace[1]	425 (367 – 492)	425 (367 – 492)
Tilapia	410 (362 – 464)	278 (252 – 306)
Bluehead sucker[1]	>375[2]	48.3 (34.4 – 67.8)
Flathead catfish (trials 1 and 2)	365 (325 – 410)	[3]
Speckled dace[1]	355 (279 – 452)	300 (231 – 390)
Spikedace[1]	335 (300 – 373)	322 (291 – 356)
Red shiner	245 (221 – 271)	232 (211 – 255)
Smallmouth bass	232 (212 – 253)	178 (162 – 195)
Channel catfish (trials 2 and 4)	233 (213 – 255)	7.00 (6.41 – 7.64)

[1] Fish species that are native to the Gila River watershed.

[2] Insufficient data to compute statistical lethal concentration or 95 percent confidence interval.

[3] LC_{50} could not be determined because of similar mortalities in a few concentrations.

Table 5. The lethal concentration (LC_{99}) values reported in micrograms per liter (µg/L) and 95 percent confidence intervals for nonnative and LC_{25} values reported in µg/L and 95 percent confidence interval for native fish species of the Gila River watershed for 24- and 96-hour exposures to Supaverm®.

Fish species	LC level	LC_{25} or LC_{99} and 95 percent confidence levels at exposures of:	
		24 hours	96 hours
		Supaverm® concentration (µg/L, based on active ingredient)	
Mosquitofish	99	2,500 (2,155 – 2,900)	1,500 (1,195 – 1,883)
Bluegill	99	1,000 (863 – 1,159)	680 (592 – 781)
Tilapia	99	910 (725 – 1,143)	500 (430 – 581)
Flathead catfish	99	700 (580 – 844)	[1]
Black bullhead	99	660[1]	180 (149 – 218)
Smallmouth bass	99	500 (437 – 572)	300 (261 – 345)
Channel catfish	99	500 (417 – 600)	<41[1]
Red shiner	99	455 (360 – 576)	410 (330 – 509)
Gila topminnow	25	1,100 (974 – 1,242)	800 (721 – 887)
Roundtail chub	25	455 (407 – 509)	455 (407 – 509)
Bluehead sucker	25	>375[1]	15.9 (8.91 – 28.3)
Longfin dace	25	350 (296 – 414)	350 (296 – 414)
Speckled dace	25	302 (232 – 393)	255 (190 – 342)
Spikedace	25	290 (249 – 338)	300 (262 – 344)

[1] Insufficient data to compute statistical lethal concentration or 95 percent confidence interval.

Table 6. Dose verification of Supaverm® concentrations in micrograms per liter (µg/L) of exposure water monitored daily during a 96-hour toxicity trial. BD = below detection limit of instrument, NA = not applicable, and NQ = not quantifiable.

Sample name	Target Supaverm® concentration (µg/L)	Supaverm® concentration (µg/L, based on active ingredient)				
		0 hour	24 hour	48 hour	72 hour	96 hour
Control	0.0	BD	BD	BD	BD	BD
2.5 A	2.5	NQ	NQ	NQ	NQ	NQ
2.5 B	2.5	NQ	NQ	NQ	NQ	NQ
2.5 C	2.5	NQ	NQ	NQ	NQ	NQ
250 A	250	287	213	123	106	103
250 B	250	290	215	116	102	93.8
250 C	250	298	223	131	109	105
2500 A	2,500	2,877	1,660	1,316	878	697
2500 B	2,500	2,531	1,581	1,215	631	673
2500 C	2,500	2,360	1,650	1,351	860	667
Spike	250	NA	272	263	263	249

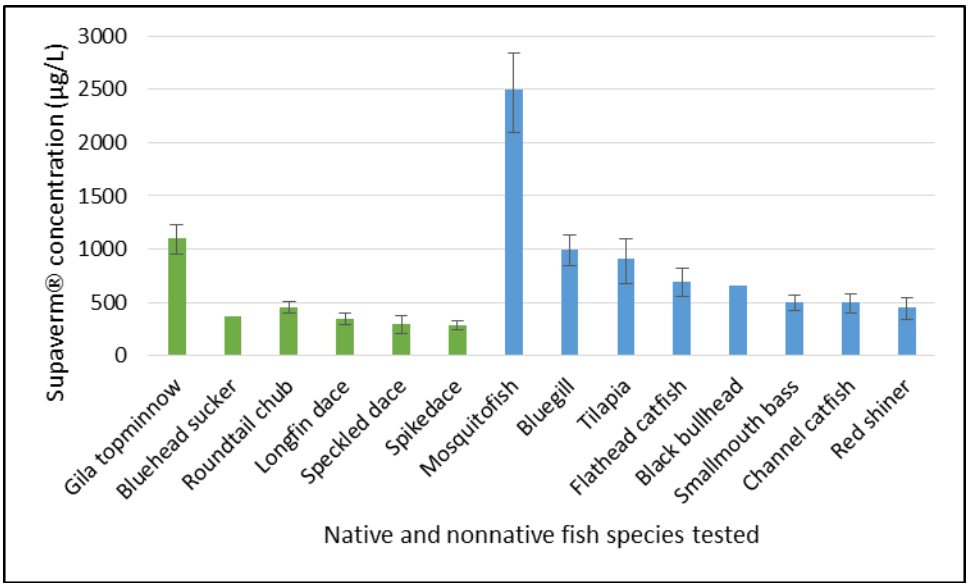

Figure 1. Lethal concentration $(LC)_{99}$ values and 95 percent confidence intervals for nonnative (blue) and LC_{25} values and 95 percent confidence interval for native (green) fish species from the Gila River watershed for 24-hour exposures to Supaverm® (concentration reported in micrograms per liter [µg/L]).

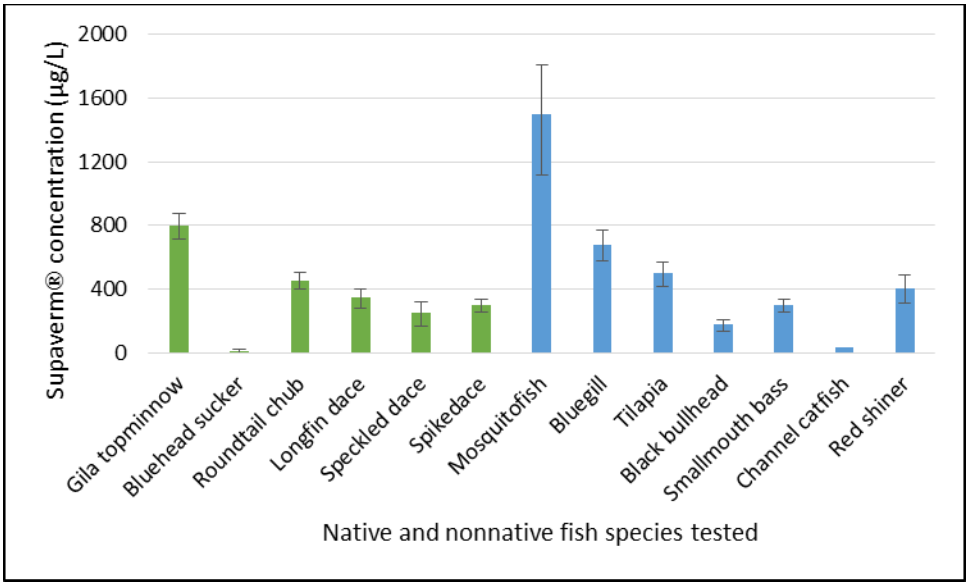

Figure 2. Lethal concentration $(LC)_{99}$ values and 95 percent confidence intervals for nonnative (blue) and LC_{25} values and 95 percent confidence interval for native (green) fish species from the Gila River watershed for 96-hour exposures to Supaverm® (concentration reported in micrograms per liter [µg/L]).