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Assessing Contaminant Sensitivity of Endangered and Threatened Species: Effluent Toxicity Tests

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by

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Abstract

Toxicity tests using standard effluent test procedures were conducted (EPA 1994) with *Ceriodaphnia dubia* and fathead minnows and four endangered fish species: bonytail chub (*Gila elegans*), Colorado squawfish (*Ptychocheilus lucias*), razorback sucker (*Xyrauchen texanus*) and Gila topminnow (*Poeciliopsis occidentalis*). We conducted 7-d survival and growth studies with embryo-larval fathead minnows and analogous exposures using the listed species. Survival and reproduction were also determined with *C. dubia*. Tests were conducted with: 1) carbaryl; 2) ammonia; and 3) a mixture of carbaryl, copper, 4-nonylphenol, pentachlorophenol, and permethrin.

The fathead minnow 7-d growth and survival test appears to be a reliable estimator of effects to the listed species used in this study. Additionally, the *C. dubia* survival and reproduction test was generally more sensitive than any of the fish tested. When the listed species and fathead minnow were different, the listed species was often less sensitive than the fathead minnow. However, other studies have shown listed species to be similar to or slightly more sensitive than fathead minnows when tested using effluent procedures. This study was conducted with fish species that have not been typically tested so factors such as handling procedures, optimum feeding rates, optimum test temperature, expected test to test variation and expected survival or growth have not been previously documented, and therefore results of this study should be interpreted cautiously.

Our laboratory has evaluated only 10 aquatic vertebrate species (mostly fish) and there are over 90 fishes listed by the FWS. The database for fishes should be expanded to include additional species from different areas of the United States. Amphibian population declines have been recognized worldwide and the FWS has over 10 listed species, therefore, greater emphasis should be placed on testing additional amphibian species. Additional testing is also needed to evaluate sublethal effects of contaminants on listed species. Finally, other listed species including freshwater mussels and other invertebrates should also be examined.

Notice

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Introduction

The U.S. Clean Water Act (CWA) specifies "it is the national policy that the discharge of toxic pollutants in toxic amounts be prohibited" (Section 101(a)(3)). The CWA provides an integrated approach to protection of aquatic ecosystems through the development of water quality criteria and the control of toxic discharges (National Pollutant Discharge Elimination System - NPDES; 45 FR 33520). Programs designed to provide protection of freshwater aquatic environments from toxic discharges commonly include whole-effluent toxicity tests with the cladoceran *Ceriodaphnia dubia*, fathead minnow (*Pimephales promelas*), and algae (*Selenastrum capricornutum*). The assumption is that results of toxicity tests using these test species are protective of effects on other organisms including endangered and threatened (listed) species.

Surrogate species, such as cladocerans and fathead minnows, are the typical freshwater organisms used in standardized tests (EPA 1994). However, it is unknown if the sensitivities of these species to contaminant exposure represent the sensitivities of listed species. Biological surveys of streams and rivers in states such as Ohio indicate that effluent test protocols using standard procedures might not adequately protect aquatic ecosystems (Yoder 1989). NPDES permits often require toxicity tests with effluents using embryo-larval fathead minnows and *Ceriodaphnia dubia*. The objective of the present study was to determine the degree of protection afforded listed fish species through the use of standard species in whole-effluent toxicity tests.

Seven-d water-renewal toxicity tests were conducted using standard effluent test procedures (EPA 1994). Species tested included *C. dubia,* fathead minnows and four endangered fish species: bonytail chub (*Gila elegans*, Family Cyprinidae), Colorado squawfish (*Ptychocheilus lucias*, Family Cyprinidae), razorback sucker (*Xyrauchen texanus*, Family Catostomidae) and Gila topminnow (*Poeciliopsis occidentalis*, Family Poecillidae). These species were previously evaluated in static-acute 96-h toxicity tests (EPA 1995, Chapter 1). We conducted 7-d survival and growth studies with embryo-larval fathead minnows and analogous exposures using the listed species. Effects on survival and reproduction of *C. dubia* were also evaluated. Tests were conducted with: 1) carbaryl; 2) ammonia; and 3) a mixture of carbaryl, copper, 4-nonylphenol, pentachlorophenol, and permethrin.

Materials and Methods

Test organisms

Bonytail chub, Colorado squawfish, razorback suckers, Gila topminnows, fathead minnows, and *C. dubia* were obtained from various government sources or from Columbia Environmental Research Center (CERC) cultures (Table 1).

Table 1. Source and age of test organisms used in toxicity tests.

Species	Scientific Name	Source	Age at Start of Test
Bonytail chub	*Gila elegans*	Dexter Fish Hatchery, Dexter, NM	Test 1 - 2 days post hatch Test 2 - 7 days post hatch
Colorado squawfish	*Ptychocheilus lucius*	Dexter National Fish Hatchery, Dexter, NM	Test 1 - 6 days post hatch Test 2 - 5 days post hatch
Razorback sucker	*Xyrauchen texanus*	Dexter National Fish Hatchery, Dexter, NM	Test 1 - 6 days post hatch Test 2 - 7 days post hatch
Gila topminnow	*Poeciliopsis occidentalis*	Adults obtained from Dexter National Fish Hatchery, Dexter, NM	Mix - < 24 h old Ammonia - 3 groups <24 h, < 48 h, and <72 h
Fathead minnow	*Pimephales promelas*	CERC cultures	< 24 h old
	Ceriodaphnia dubia	CERC cultures	< 24 h old

Listed fishes were received as eggs except for the Gila topminnow which is a live bearer. All fish were held in well water (18°C, alkalinity 258 mg/L as $CaCO_3$, hardness 286 mg/L as $CaCO_3$, pH 7.8) until testing began. Young-of-year Gila topminnows were cultured at the CERC in intermittent flow aquaria at about 26°C. Topminnows were cultured until sexually mature and actively bearing young. These young were then used in toxicity tests.

Fathead minnows tests were started with fish less than 24-h old. Tests with listed fish were started with fish between five and seven days old (when they begin to actively feed). Using this procedure, listed species and fathead minnows were the same physiological age, although not the same age based on number of days post-hatch. An exception was the first test with bonytail chub. In that case, the fish were two days post-hatch (fish had absorbed their yolk-sac). However, after starting the test it was observed that they did not begin to actively feed until about the third or fourth day of testing. It was difficult to obtain a sufficient number of Gila topminnows to conduct a single test, so young produced over a three-d period were used in the toxicity assessments. *Ceriodaphnia dubia* were cultured in ASTM hard water (alkalinity 110 to 120 mg/L as $CaCO_3$, hardness 160 to 180 mg/L as $CaCO_3$; ASTM 1998) and tested when less then 24-h old.

Chemical

Tests were conducted with three different simulated effluents with all species except the Gila topminnow. These included: 1) carbaryl; 2) ammonia; and 3) a mixture of five chemicals with different modes of action. Tests with the Gila topminnow were conducted with only ammonia and the five chemical mixture. The EPA effluent toxicity tests are intended to be short-term estimates of chronic toxicity. Carbaryl was selected because it was previously tested in static acute tests with juvenile lifestages of listed species (EPA 1995, Chapter 1). Ammonia was selected for testing because it is a major constituent of many municipal and industrial effluents, and is an important non-point source pollutant from feedlots and fertilizers used for agriculture.

Effluents are typically complex mixtures that vary from one source to another. Since a reference effluent is not available to determine the comparative sensitivity of species, we prepared a chemical mixture consisting of compounds that cause toxicity through several different modes of action (Table 2). The mixture consisted of carbaryl, copper, 4-nonylphenol, pentachlorophenol, and permethrin in equitoxic proportions (96-h $LC_{50}/5$) as determined from previous acute toxicity tests conducted with these chemicals and species (EPA 1995). The fathead minnow 96-h LC_{50} for each chemical (EPA 1995) was selected as the concentration for determining equitoxic proportions. The high concentration for the mixture studies with five chemicals was equal to a toxic unit of one (i.e., if the toxicity is additive, the high concentration would kill 50% of the fathead minnows).

Sources and percent active ingredient for the chemicals are summarized in Table 2. Organic chemical stocks were prepared by dissolving the chemical in reagent grade acetone, whereas copper was dissolved in deionized water and ammonia was prepared in reconstituted hard water. Test solutions were prepared daily and tests chambers for fish were siphoned until about 15 to 20% of the original test volume remained. Fresh test solutions were then added. For *C. dubia* organisms were transferred to fresh test solution daily. The maximum volume of acetone added to any test container for the five chemical mix was 2.5 ml/L and for the carbaryl exposure was 0.5 ml/L. Both a dilution water and, when appropriate, a solvent control (0.5 and 2.5 ml/L) were prepared for each test.

Organic and inorganic chemical stocks were analyzed to confirm nominal concentrations. Organic chemical analysis was conducted at either Mississippi State Chemical Laboratory (Mississippi State, MS) or ABC Laboratories (Columbia, MO) using gas chromatography. Copper stocks were confirmed at either the CERC or Mississippi State Chemical Laboratory by atomic absorption spectrophotometry. Overall, the percent of nominal stock concentration was 91%, with a range of 63% (copper) to 107% (4-nonylphenol). Toxicity results for carbaryl are expressed as nominal concentrations. The five chemical mixture high concentration was considered 100% (toxic unit = 1) and all other mixture concentrations are expressed as a percentage of the high concentration.

Total ammonia concentrations were measured with an Orion EA940 Expandable ionAnalyzer, and Orion 95-12 ammonia electrode. The average percent of nominal ammonia stock concentration was 96%. All ammonia concentrations are expressed as total N (mg/L), corrected for the measured stock concentration, except for one test with razorback suckers and fathead minnows. For the one exception, nominal concentrations are used in all data analysis.

We conducted 7-d toxicity tests following EPA procedures described for effluents (EPA 1994). Toxicity test procedures are summarized in Table 3. Carbaryl tests were conducted at 22°C (the temperature used for the previously conducted acute toxicity tests, Chapter 1, EPA 1995). Toxicity tests with the ammonia and the chemical mixture were conducted at 25°C. Test water was reconstituted ASTM hard water (alkalinity 110 to 120 mg/L as $CaCO_3$, hardness 160 to 180 mg/L as $CaCO_3$; ASTM 1998). Alkalinity, hardness, and pH were measured on each batch of reconstituted water. Average measured water quality characteristics for the reconstituted water are summarized in Table 4. Because of age requirements for fathead minnows at the start of the test (<24 h old), none of the fish were acclimated to the test water before starting toxicity tests. However, *C. dubia* were cultured in this reconstituted hard water. Dissolved oxygen, temperature, and pH were measured on the control, low, medium, and high exposure concentrations daily in the fresh test solution and in the test solution after 24 h of exposure for carbaryl and the chemical mixture. Additionally, pH, temperature, and dissolved oxygen were measured on all concentrations of ammonia initially and after 24 h of exposure. The test was conducted in ambient light with 16 hours of light and 8 hours of dark. A test series consisted of five exposure concentrations with a 50% dilution factor. For toxicity tests with fish, each exposure concentration was tested in triplicate. Fish were counted into groups of five with two groups pooled for each exposure replicate (10 fish per 1 L beaker - 30 fish/treatment).

Table 2. Sources, percent active ingredient, use and mode of action for chemicals used in toxicity tests.

Chemical	Source	Active Ingredient (%)	Use	Mode of Action
Carbaryl	Donated by Rhone-Pôulenc Agricultural Co., Research Triangle Park, NC	99.7	Carbamate insecticide	Inhibitor of cholinesterase activity
Copper sulfate	Fisher Chemical, St. Louis, MO	25.5	Mining, industrial, fungicide	Interferes in osmoregulation
4-nonylphenol	Fluka Chemical, New York, NY	85.0	Nonylphenol ethoxylate detergents	Narcotic and oxidative stressor
Pentachlorophenol	Aldrich Chemical, Milwaukee, WI	99.0	Wood preservative, molluscicide	Uncoupler of oxidative phosphorylation
Permethrin	Donated by ICI Americas Inc., Richmond, CA	95.2	Pyrethroid insecticide	Neurotoxin
Ammonium Phosphate	EM Science, Gibbstown, NJ	12	Fertilizer, ammonia is a byproduct of waste-water treatment plants and some farming practices	Interferes in respiration

Table 3. Summary of study design for the comparative toxicity of selected chemicals to listed species.

Test type:	Renewal
Test temperature:	Carbaryl: 22°C Chemical mix and ammonia: 25°C
Water Quality:	Reconstituted ASTM hard
Chemicals:	Carbaryl Ammonia Chemical mix of carbaryl, copper, 4-nonylphenol, pentachlorophenol, permethrin in equitoxic proportions as determined from previous tests (EPA 1995).
Dilution series:	50%
Observations:	Fish: mortality every 24 h for 7 days and weight at end of study *Ceriodaphnia dubia*: mortality daily and reproduction until 3 broods in control.

Table 4. Average (+ standard deviation) measured water quality characteristics of reconstituted water used in effluent toxicity tests.

Water Quality Characteristic	Nominal Value	Measured (n = 11)
Alkalinity[1]	110 - 120	112 ± 10
Hardness[1]	160 - 180	163 ± 13
pH	7.8 - 8.0	8.3 ± 0.6

[1] mg/L as $CaCO_3$

Toxicity tests

For the ammonia test, Gila topminnows produced over a three-day period were used. Fish were kept in 24-h age groups (0-24, 24-48, 48-72) and each age group period was stocked in a separate replicate for each treatment. For the mixture study with the Gila topminnow, enough young were obtained in one 24-h period to stock two replicates with nine fish per replicate. For fish, reduction in survival or growth were the adverse effects measured. Dead fish were removed daily. Tests were repeated (two different years) with the razorback sucker, Colorado squawfish and bonytail chub. There are data for only one year for the Gila topminnow. A total of five separate tests over the two-year period, were conducted with the fathead minnows.

Ten *C. dubia* were tested in individual 30 mL beakers containing 15 mL of test solution with one animal per beaker. Survival and reproductive success were determined daily for *C. dubia* and were continued until at least 60% of the controls had a third brood (about 6 to 7 d). There were three separate tests with *C. dubia* for each chemical.

Statistical analysis

The Inhibition Concentration (ICp), integrating effects on both growth and survival of fish, and survival and reproduction of *C. dubia,* was calculated for each test using a linear interpolation method (Norberg-King 1993). The IC_{25} was used as the statistical point-estimate for this study. For the fish, an expanded confidence interval, as recommended in the ICp procedure, was calculated because there were fewer than seven replicates for each test. If the expanded lower confidence limit was less than zero, then the lower confidence limit was reported as zero. Confidence intervals for the *Ceriodaphnia dubia* were not expanded because there were 10 replicates.

The IC_{25}s could not be tested for normality due to an insufficient number of IC_{25} estimates. Thus, general linear model least square difference mean separations (SAS 1994) were determined on ranked IC_{25}s ($p \leq 0.05$, Snedecor and Cochran 1980). In order to summarize the data for a particular chemical and species, the geometric mean IC_{25} was calculated. Only those tests for which an IC_{25} could be calculated were used for statistical analysis. For the results with ammonia, if the total ammonia IC_{25} was greater than 17, then 17 was used in the calculation. Calculation in this manner will likely provide a concentration lower (bias) than the actual concentration. Not including the data (17) would bias the summarized data to a greater extent than including the data.

Results

Appendix 1 is a complete listing of IC_{25}s and confidence intervals for all individual tests. Dissolved oxygen concentrations were always at acceptable concentrations (>40% saturation); therefore no tests were aerated. The pH range for each test is listed in Appendix 2. Control survival for all species and exposures was typically 80% or greater. Exceptions included: 1) one dilution water control in a fathead minnow carbaryl exposure, 2) both the acetone and dilution water controls for Test 2 of the bonytail chub carbaryl exposures, and 3) an ammonia study with fathead minnow. In the fathead minnow-carbaryl study and the bonytail study, survival was 70% and data from those tests were included in the results for the present study. However, the fathead minnow-ammonia study had survival of only 50% in the control and therefore was not included in the results for the present study.

Carbaryl

The IC_{25}s for the Colorado squawfish (1.33 mg/L) and razorback sucker (2.06 mg/L) were significantly greater than the IC_{25} for the fathead minnow (0.42 mg/L). The IC_{25} for the bonytail chub (0.25 mg/L), while less than the IC_{25} for fathead minnows, was not significantly different than the IC_{25} for the fathead minnow. The concentration series used for tests with *C. dubia* were the same as those used for the fish. There was 100% mortality at the lowest exposure concentration indicating that *C. dubia* are much more sensitive to carbaryl than the listed fish species or fathead minnows. Additionally, because all *C. dubia* carbaryl exposure concentrations had no survival, we discontinued the controls when the mix and ammonia studies were ended (about day 6 or 7). Because the tests with carbaryl were conducted at 22°C, the controls for *C. dubia* did not produce the three broods required for test acceptability.

Norberg-King (1993) reported that the IC_{25} is typically similar to the No Observed Effect Concentration (NOEC) which is calculated using hypothesis testing. Carbaryl NOECs for fathead minnows have been reported by Carlson (1972) and Norberg-King (1989). The NOEC in those two studies were 0.21 and 0.72 mg/L, which is similar to the average IC_{25} obtained in this study of 0.42mg/L (Table 5; range 0.22 to 0.81, Appendix 1). Results from the present study further support the findings that the IC_{25} is similar to the NOEC for carbaryl.

Additionally, Norberg-King (1993) proposes that the IC_{50} is similar to the Lowest Observed Effect Concentration (LOEC). IC_{50}s calculated for the fathead minnow tests with carbaryl in the present study, ranged from 0.66 mg/L to 1.29 mg/L with a geometric mean of 1.04 mg/L. The LOEC for carbaryl ranges from 0.68 mg/L (Carlson 1972) to 1.6 mg/L (Norberg-King 1989) and are similar to the IC_{50} value calculated in the current study.

Ammonia

Table 5 summarizes the results of toxicity tests on total ammonia. Two tests, one with fathead minnows and one with razorback suckers, had an IC_{25} greater than the highest concentration tested (Appendix 1). Following those tests, exposure concentrations were increased for all subsequent testing.

Chemical Mix

The IC_{25}s for the chemical mix ranged from 28% (fathead minnows) to 64% (Colorado squawfish). Fathead minnows had the lowest IC_{25} for the fishes, however only the IC_{25} for the Colorado squawfish (64%) was significantly different than the IC_{25} for the fathead minnows (28%). Comparisons of IC_{25}s with repeated tests, indicate a fairly consistent sensitivity except for the test with the bonytail chub (Appendix 1).

Table 5. IC_{25} for three different exposures. IC_{25} is the geometric mean of the IC_{25}s (number of IC_{25}s in parentheses) used in the rank analysis. An * denotes statistically different ($p \leq 0.05$) from fathead minnow IC_{25}. For the results with ammonia, if the total ammonia IC_{25} was greater than 17, then 17 was used in the calculation. Calculation in this manner will likely provide a concentration lower (bias) than the actual concentration. Not including the data (17) would bias the summarized data to a greater extent than including the data.

Species	IC_{25}		
	Carbaryl (mg/L)	Ammonia (mg/L)[1]	Chemical mix (%)
Fathead minnow	0.42 (5)	7.21 (5)	28 (5)
Ceriodaphnia dubia	<0.33 (3)	1.29 (3)	<6.25 (3)
Bonytail chub	0.25 (2)	11.0 (2)	29 (2)
Colorado squawfish	1.33* (2)	8.9 (2)	64* (2)
Razorback sucker	2.06* (2)	13.4 (2)	33 (2)
Gila topminnow	not tested	24.1 (1)	54 (1)

1 Ammonia concentrations are total nitrogen (mg/L) and not adjusted for temperature or pH

As with carbaryl, the chemical mix concentration series used for tests with *C. dubia* were the same as those used for the fish. There was 100% mortality at the lowest exposure concentration (6.25%) indicating that *C. dubia* are more sensitive to the chemical mixture than the fish species. The IC_{25}s ranged from 1.29 mg/L for *C. dubia* to 24.1 mg/L for the Gila topminnow. None of the IC_{25}s for the listed fish species were statistically different ($p \leq 0.05$) from the IC_{25} for fathead minnows (7.21 mg/L). However, the IC_{25} for the Gila topminnow (24.1 mg/L), bonytail chub (11.0),

Un-ionized ammonia is the most toxic form of ammonia and can be determined from total nitrogen by calculation knowing pH and temperature (Thurston et al. 1977). The pH varied between tests (between species and within species), over the course of a 7-d test and over the 24-h time period between initial renewal of test solutions and just prior to siphoning (Appendix 2). For the fathead minnow the calculated un-ionized ammonia IC_{25} concentration was 0.27 mg/L (range 0.12 to 0.65). From previous studies, the NOEC for fathead minnows is 0.15 mg/L as unionized ammonia (Swigert and Spacie 1983, Thurston et al. 1986) and is slightly lower than the concentration of 0.27 mg/L calculated for the present study.

If the toxicity of chemicals is strictly additive, then only 50% mortality should occur in the highest concentration tested. In addition to the IC_{25}s reported in Table 5, we also calculated LC_{50}s on the chemical mix with fathead minnows using a nonlinear interpolative method (Stephan 1977).

These LC_{50}s had a geometric mean of 44% (chemical concentrations were 44% of their equitoxic ratio) which might indicate that the mix of chemicals was not strictly additive but synergistic. However, tests in the present study were conducted with fathead minnows that were less than 24-h old (about 0.45 mg). Mayer and Ellersieck (1986) found that 83% of the time, sensitivity of fish decreased with increased growth and LC_{50}s increased by up to a factor of 5. Therefore, the observed LC_{50} of 44% might reflect the testing of a more sensitive life-stage rather than synergism between components of the chemical mixture.

Mayer and Ellersieck (1986) also report that toxicity generally increased by a factor of about 3 with each 10°C increase in temperature. The acute toxicity tests that were used to calculate the toxic units for this study, were conducted at 22°C while these chemical mixture studies were conducted at 25°C. This represented only a 3°C temperature increase, but could account for a portion of the apparent increase in the chemical mixture toxicity. In summary, while the toxicity of the chemical mixture might be synergistic, it is more likely that the difference in life-stage or the increase in test temperature is responsible for the increased toxicity to fathead minnows.

Discussion

Of the 11 possible comparisons of fathead minnows to the listed species outlined in Table 5, only three IC_{25}s (27%) are significantly different than the IC_{25}s for fathead minnows. When there was a difference, the IC_{25} for the listed species was higher than the IC_{25} for the fathead minnow. Two of these three IC_{25}s are for the Colorado squawfish which might indicate that the Colorado squawfish is less sensitive to contaminant exposure than razorback suckers, bonytail chubs, Gila topminnows or fathead minnows. These findings are in contrast to the results of studies described in Chapter 1 and EPA (1995). These previous studies evaluated the sensitivity of listed species to fathead minnows in static 96-h acute toxicity tests. The LC_{50}s in these previous studies for listed species, including Colorado squawfish, bonytail chub, Gila topminnows, and razorback suckers were generally lower than the LC_{50}s for fathead minnows.

The sensitivity of the listed fountain darter (*Etheostoma fonticola*) has also been evaluated following EPA whole-effluent toxicity test procedures (Edwards Aquifer Research & Data Center 1992a, 1992b). Tests were conducted with effluent collected from the San Marcos, TX wastewater treatment plant and in a single compound toxicity test with glyphosate. In the test conducted with wastewater, the NOECs for both survival and growth of fountain darters was 14%. The NOEC for fathead minnow survival was 28% and the NOEC for growth was 28%. In contrast, the NOEC for growth and survival for both species was the same in the test with glyphosate. We have also conducted whole-effluent toxicity tests with razorback suckers and bonytail chub (unpublished data) with effluent samples collected from within the state of Arizona. There was no toxicity from the effluent to either fathead minnows or razorback suckers. However, in the effluent test with bonytail chub, survival of fathead minnows was somewhat higher than that of the bonytail chub. There was no effect on growth for either bonytail chub or fathead minnows.

In summary, the fathead minnow 7-d growth and survival test appear to be a reliable estimator of effects to the listed species used in this study. Additionally, the *C. dubia* survival and reproduction test was generally more sensitive than any of the fish tested. When the listed species and fathead minnow were different, the listed species was often more resistant than the fathead minnow. However, other studies have shown listed species to be similar to or slightly more sensitive than fathead minnows when tested using effluent procedures. This study was conducted with fish species that have not been typically tested so factors such as handling procedures, optimum feeding rates, optimum test temperature, expected test to test variation and expected survival or growth have not been previously documented, and therefore results of this study should be interpreted cautiously. Further testing should also be conducted with additional listed species or their U.S. Fish and Wildlife Service identified surrogate species before definitive policy decisions concerning the protection of endangered and threatened species to contaminants in aquatic environments are made.

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Appendix 1. The IC_{25}s and confidence intervals for carbaryl, ammonia and the chemical mixture for each species and test.

Carbaryl

Species	IC_{25}	Confidence Interval[1]
Ceriodaphnia dubia	Test 1 - <0.33	NC[2]
	Test 2 - <0.33	NC
	Test 3 - <0.33	NC
Fathead minnows	Test 1 - 0.22	0.05 - 1.57
	Test 2 - 0.39	0.00 - 0.90
	Test 3 - 0.30	0.14 - 1.17
	Test 4 - 0.81	0.60 - 0.91
	Test 5 - 0.60	0.30 - 1.50
Bonytail chub	Test 1 - 0.28	0.12 - 1.54
	Test 2 - 0.23	0.05 - 1.95
Colorado squawfish	Test 1 - 1.17	0.69 - 1.71
	Test 2 - 1.52	0.00 - 2.00
Razorback sucker	Test 1 - 1.62	0.77 - 2.09
	Test 2 - 2.62	1.92 - 3.10

[1] For tests with fish, confidence intervals are expanded because replicates were less than seven. confidence intervals for *Ceriodaphnia dubia* are not expanded since there were ten replicates.
[2] NC - Not calculated.

Ammonia (total measured N - mg/L)

Species	IC_{25}	Confidence Interval[1]
Ceriodaphnia dubia	Test 1 - 1.59	1.12 - 4.16
	Test 2 - 0.75	0.69 - 0.82
	Test 3 - 1.80	1.29 - 5.21
Fathead minnows	Test 1 - 14.40	NC[2]
	Test 2 - 5.82	2.29 - 8.58
	Test 3 - >17	NC
	Test 4 - 5.71[3]	4.91 - 7.60
	Test 5 - 2.40	0.89 - 22.80
Bonytail chub	Test 1 - 12.91	NC
	Test 2 - 9.4	3.45 - 33.97
Colorado squawfish	Test 1 - 4.4	3.78 - 4.83
	Test 2 - 17.9	14.71 - 19.68
Gila topminnow	Test 1 - 24.1	19.2 - 25.6
Razorback sucker	Test 1 - >17	NC
	Test 2 - 10.55[3]	0. - 12.54

[1] For tests with fish, confidence intervals are expanded because replicates were less than seven. Confidence intervals for *Ceriodaphnia dubia* are not expanded since there were ten replicates.[2]NC - Not calculated.
[3] Data have not been corrected for measured concentrations.

Appendix 1. (Continued)

Chemical mix (%)		
Species	IC_{25}	Confidence Interval[1]
Ceriodaphnia dubia	Test 1 - <6.25	NC[2]
	Test 2 - <6.25	NC
	Test 3 - <6.25	NC
Fathead minnows	Test 1 - 29.9	0 - 41.7
	Test 2 - 39.4	27.0 - 69.1
	Test 3 - 31.7	4.6 - 49.8
	Test 4 - 23.8	15.0 - 49.6
	Test 5 - 20.6	0 - 93.5
Bonytail chub	Test 1 - 60.6	37.2 - 76.3
	Test 2 - 13.9	0 - 90
Colorado squawfish	Test 1 - 63.6	35.4 - 66.35
	Test 2 - 64.6	54.1 - 67.8
Gila topminnow	Test 1 - 54.1	34.6 - 64.9
Razorback sucker	Test 1 - 26.2	0 - 37.2
	Test 2 - 41.2	20.8 - 58.9

[1] For tests with fish, confidence intervals are expanded because replicates were less than seven. Confidence intervals for *Ceriodaphnia dubia* are not expanded since there were ten replicates.
[2] NC - Not calculated.

Appendix 2. Summary of exposure water pH for each chemical. For each chemical the data includes the average low and average high (and minimum and maximum pH recorded) for all tests and species tested and includes all replicates. Initial water is defined as the water used each day for renewal. Chemical waters were mixed daily. Final water is defined as the water removed from one replicate after 24 hours of exposure and prior to renewal.

Water	Chemical	Average pH Low	Lowest pH	Average pH High	Highest pH
Initial	Ammonia	7.7	6.6	8.4	9.1
	Carbaryl	8.1	6.9	8.6	9.1
	Chemical mix	8.3	7.9	8.6	9.1
Final	Ammonia	7.7	7.1	8.3	8.6
	Carbaryl	7.9	7.3	8.4	9.0
	Chemical mix	7.7	6.4	8.4	9.0

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