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Assessing Contaminant Sensitivity of Endangered and Threatened Aquatic Species: Part III. Effluent Toxicity Tests

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Abstract. Toxicity tests using standard effluent test procedures described by the U.S. Environmental Protection Agency were conducted with *Ceriodaphnia dubia*, fathead minnows (*Pimephales promelas*), and seven threatened and endangered (listed) fish species from four families: (1) Acipenseridae: shortnose sturgeon (*Acipenser brevirostrum*); (2) Catostomidae; razorback sucker (*Xyrauchen texanus*); (3) Cyprinidae: bonytail chub (*Gila elegans*), Cape Fear shiner (*Notropis mekistocholas*) Colorado pikeminnow (*Ptychocheilus lucius*), and spotfin chub (*Cyprinella monacha*); and (4) Poecillidae: Gila topminnow (*Poeciliopsis occidentalis*). We conducted 7-day 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 carbaryl, ammonia—or a simulated effluent complex mixture of carbaryl, copper, 4-nonylphenol, pentachlorophenol and permethrin at equitoxic proportions. In addition, Cape Fear shiners and spotfin chub were tested using diazinon, copper, and chlorine. Toxicity tests were also conducted with field-collected effluents from domestic or industrial facilities. Bonytail chub and razorback suckers were tested with effluents collected in Arizona whereas effluent samples collected from North Carolina were tested with Cape Fear shiner, spotfin chub, and shortnose sturgeon. The fathead minnow 7-day effluent test was often a reliable estimator of toxic effects to the listed fishes. However, in 21 % of the tests, a listed species was more sensitive than fathead minnows. More sensitive species results varied by test so that usually no species was always more or less sensitive than fathead minnows. Only the Gila topminnow was consistently less sensitive than the fathead minnow. Listed fish species were protected 96% of the time when results for both fathead minnows and *C. dubia* were considered, thus reinforcing the value of standard whole-effluent toxicity tests using those two species. If the responses of specific listed species are important for management decisions, our study supports the value in developing culture and testing procedures for those species.

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The U.S. Clean Water Act (CWA) specifies that ''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 the 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 protect freshwater aquatic environments from toxic discharges often include water quality standards and whole-effluent toxicity tests with commonly used test species including the cladoceran *Ceriodaphnia dubia*, the fathead minnow (*Pimephales promelas*), and algae (*Selenastrum capricornutum*). Whole-effluent toxicity tests using these test species are presumed to protect other organisms including federally listed threatened and endangered (listed) species.

Biologic 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) and thereby may not protect listed species and their habitats. The National Pollutant Discharge Elimination System permits often require toxicity tests with effluents using embryo-larval fathead minnows and *C. dubia*. However, it is unknown if the sensitivities of these species to contaminant exposure represent the sensitivities of listed species. We have conducted a number of studies comparing the sensitivity of listed species with standard test organisms (Dwyer *et al.* 1995, 1999a, 1999b,

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

Family	Species	Source	Age at start of test
Daphnidae	*C. dubia*	CERC cultures	<24 h
Cyprinidae	Fathead minnow (*P. promelas*)	CERC cultures or Aquatic Biosystems, Fort Collins, CO	<24 h
Acipenseridae	Shortnose sturgeon (*A. brevirostrum*)	Bears Bluff NFH, Wadmalaw Island, SC	2 to 3 d
Catostomidae	Razorback sucker (*X. texanus*)	Dexter NFH and Technology Center, Dexter, NM	Test 1: 7 d posthatch Test 2: 7 d posthatch
Cyprindae	Bonytail chub (*G. elegans*)	Dexter NFH and Technology Center, Dexter, NM	Test 1: 2 d posthatch Test 2: 7 d posthatch
	Cape Fear shiner (*N. mekistocholas*)	Conservation Fisheries Inc., Knoxville, TN	< 24 h
	Colorado pikeminnow (*P. lucius*)	Dexter NFH and Technology Center, Dexter, NM	Test 1: 6 d posthatch Test 2: 5 d posthatch
	Spotfin chub (*C. monacha*)	Conservation Fisheries Inc., Knoxville, TN	<24 h
Poeciliidae	Gila topminnow (*P. occidentalis occidentalis*)	Adults obtained from Dexter NFH and Technology Center, Dexter, NM	Mix: <24 h Ammonia: <24, 48, and 72 h

NFH = Natural Fish Hatchery.

1999c; 2000a, 2000b, 2004; Besser *et al.* 2004; Sappington *et al.* 2001). The objective of the present study was to determine the degree of protection afforded listed fish species through the use of species commonly used in whole-effluent toxicity tests.

We conducted 7-day static-renewal survival and growth studies with embryo-larval fathead minnows and analogous exposures using the listed species (Dwyer *et al.* 1999b, 2000a, 2000b) following U.S. Environmental Protection Agency (USEPA) standard effluent test procedures (USEPA 1994). Species tested included *C. dubia*, fathead minnows, and seven listed fish species from four families: (1) Acipenseridae: shortnose sturgeon (*Acipenser brevirostrum*); (2) Catostomidae: razorback sucker (*Xyrauchen* texanus); (3) Cyprinidae: bonytail chub (*Gila elegans*), Cape Fear shiner (*Notropis mekistocholas*), Colorado pikeminnow (*Ptychocheilus lucius*), and spotfin chub (*Cyprinella monacha*); and (4) Poecillidae: Gila topminnow (*Poeciliopsis occidentalis*). These species have been previously evaluated in static-acute 96-hour toxicity tests using juvenile fish (Dwyer *et al.* 1995, 1999a, 1999c, 2000a, 2004; Sappington *et al.* 2001). Spotfin chub have also been evaluated in chronic toxicity exposures (Besser *et al.* 2004). We now report impacts on the survival and growth of listed fish species using 7-day exposures to individual chemicals, a complex mixture, and field-collected effluents.

Materials and Methods

Test Organisms and General Test Procedures

Sources and ages of the test organisms are listed in Table 1. Fathead minnows were obtained from Columbia Environmental Research Center (CERC; U.S. Geological Survey, Columbia, MO) cultures or from a commercial source. Bonytail chub, Colorado pikeminnow, and razorback suckers were received as eggs and cultured at the CERC in heated well water (alkalinity 260 mg/L as $CaCO_3$ and hardness 290 mg/L as $CaCO_3$). Water was heated with glass aquaria heaters to a temperature of 25°C. Shortnose sturgeon, Cape Fear shiner, and spotfin chub were received as fry. Young-of-year Gila topminnows were cultured at the CERC in intermittent-flow aquaria at approximately 26°C until sexually mature and actively bearing young. *C. dubia* were cultured in ATSM water (alkalinity 110 to 120 mg/L as $CaCO_3$ hardness 160 to 180 mg/L as $CaC0_3$, and pH 7.8 to 8.0; ASTM 2003) to obtain <24-hour old neonates to start the exposures.

Fathead minnow tests were started with fish <24 hours old. Because of age requirements for fathead minnows at the start of the test, none of the fish species were acclimated to the test water before starting toxicity exposures. Instead, all fish were held in heated well water until testing began. Tests with listed fish were started approximately the time fish began to actively feed. Using this approach, the listed species and fathead minnows were at approximately the same physiologic age (i.e., just beginning to actively feed) 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 2- days posthatch and had absorbed their yolk sac. However, after starting the test, the bonytail chub did not begin to actively feed until approximately the third to fourth day of testing.

A limited number of fish were available for testing. For this reason there were only 3 or 4 replicates (10 individual / replicate) for each exposure concentration. Within an individual exposure series, the number of replicates were constant. For the ammonia test with Gila topminnows, fry produced during a 3-day period were used to start the study because we were unable to obtain a sufficient number of topminnows on a single day to start a test. Fish were kept in 24-hour age groups (0 to 24, 24 to 48, and 48 to 72), and each age group period was stocked in a separate replicate for each treatment. For the complex mixture study with Gila topminnows, enough fish were obtained in one 24-hour period to stock 2 replicates with 9 fish / replicate.

Tests were repeated (two different years) with the razorback sucker, Colorado pikeminnow, and bonytail chub. All other listed species were tested only once except Cape Fear shiner, which were tested twice with copper in ASTM hard water. Fathead minnows and *C. dubia* were tested more frequently.

All organisms were fed brine shrimp during the toxicity tests according to procedures outlined by the USEPA (1994), except for toxicity tests conducted with Cape Fear shiner. Brine shrimp in the Cape Fear shiner tests were double separated because any unhatched eggs that remained after a single separation clogged the gills of the shiners and caused death. Additionally, Cape Fear shiners are omnivorous (likely herbivorous in the wild; Snelson 1971), and initial tests in which the shiners were fed only brine shrimp were unsuccessful. Feeding was supplemented with 0.5 mL Superfood Provision

Table 2. Summary of test conditions used to conduct toxicity tests (based on USEPA 1994)

Parameter	Conditions
Species and age	See Table 1
Duration of exposure	Fish: 7 d *C. dubia*: until 3 broods in control
Temperature	25°C (except for exposures conducted with carbaryl at 22°C)
Feeding	Fish: newly hatched *Artemia* nauplii (<24 h old) fed 3 times/day *ad libitum*; except for Cape Fear shiner received in addition to brine shrimp, 0.5 mL of *Chlorella* (15 g/L stock dried powder; Now Foods, Bloomingdale, IL) *C. dubia*: Yeast-cerophyl-trout chow 0.1 mL/d (1800 mg/L stock) and 1 mL/d *S. capricornutum* (2.1×10^7 cells/mL)
Photoperiod	16 h light and 8 h darkness
Light quality	Wide-spectrum fluorescent lights (approximately 200 lux at the surface of the water)
Exposure chamber	Fish: 1-L glass beaker containing 250 mL of exposure water (except for shortnose sturgeon, which were tested in 500 mL exposure water) *C. dubia*: 30-mL plastic cup containing 15 mL exposure water
Exposures	A. Ammonia, carbaryl, chlorine, copper, diazinon and a complex mixture of carbaryl, copper, 4-nonylphenol, pentachlorophenol, permethrin at equitoxic proportions (USEPA 1995) B. Field-collected effluents
Dilution series	Fifty percent with ASTM hard water (160 to 180 mg/L as $CaCO_3$) or receiving water (for the razorback suckers and Gila topminnow exposures to the Arizona effluents)
Water renewal	Daily static renewal
Number of organism/replicate	Fish: 10 organisms in each of 3 or 4 replicates (except for Gila topminnows/complex mixture, which had 2 replicates and 9 individuals replicate) *C. dubia*: 1 organism in each of 10 replicates
Water quality monitoring	Hardness, alkalinity, conductivity, dissolved oxygen, pH, and ammonia at the start and end of exposure
Endpoints	Fish: Survival daily and dry weight on day 7 *C. dubia*: Survival and reproduction daily
Test acceptability	Fish control exposures 80% survival *C. dubia*: control exposures 80% survival and 3 broods

(an open-cell *ChIorella* microalgae; approximately 3.8 ug *Chlorella*/ 0.5 mL) added once per day to each replicate chamber.

Toxicity tests generally followed USEPA procedures described for effluents (USEPA 1994; Table 2). The carbaryl tests were conducted at 22°C (the temperature used for the previously conducted acute toxicity tests (Dwyer *et al.* 1995, 1999a, 1999c, 2000a, 2004; Sappington *et al.* 2001). Test water was reconstituted ASTM hard water (alkalinity 110 to 120 mg/L as $CaCO_3$, hardness 160 to 180 mg/L as $CaCO_3$ and pH 7.8 to 8.0; ASTM 2003). Two different hardness waters were tested in the copper exposures with Cape Fear shiner and spotfin chub (ASTM hard water and hardness 9 (mg/L as $CaCO_3$), a hardness appropriate for the species). Hardness-9 water was prepared by diluting CERC well water with deionized water.

Test solutions were prepared daily, and test chambers for fish were siphoned until approximately 20% of the original test volume remained. Fresh test solution was then added to each chamber. For *C. dubia*, organisms were transferred to fresh test solution daily. Because of their large size, shortnose sturgeon were tested in 1-L glass beakers containing 500 mL exposure water. Dissolved oxygen, temperature, and pH were measured daily on the control, low, medium, and high exposure concentrations in the fresh test solution and in a composite sample from the exposure chambers after 24 hours of exposure. During the ammonia tests, pH, temperature, and dissolved oxygen were measured in all concentrations initially and after 24 hours of exposure. Both a dilution water and, for the organic chemicals, an acetone solvent control (0.5 or 2.5 mL/L) were prepared for each test.

Individual Chemicals

Tests were conducted with individual chemicals, and not all species were tested with all chemicals. Specific chemicals included ammonia, carbaryl, chlorine, copper, or diazinon. Carbaryl was selected because it was previously tested in static acute tests with juvenile life stages of listed species (Dwyer *et al.* 1995, 1999a, 1999c; Sappington *et al.* 2001). 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 in agricultural and residential applications. Diazinon, copper, and chlorine were tested to represent additional modes of toxicologic action and for their importance as common pollutants in the listed species' habitats.

Sources and percent active ingredient for the chemicals are listed in Table 3. 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. All organic and inorganic chemical stocks were analyzed to confirm nominal concentrations. Exposure concentrations were not verified. Total ammonia concentrations as N (mg/L) were confirmed with an Orion EA940 Expandable ionAnalyzer and an Orion 95-12 ammonia electrode (Orion Thermo Electron Corp., Waltham, MA). The stock from one razorback sucker–fathead minnow test with ammonia was not confirmed. Chlorine stock concentrations were confirmed using a Fisher CL Titrimeter Model 397 (Fisher Scientific, Pittsburgh, PA) with a strip chart recorder for low concentration determinations (minimum detectable concentration was 5 ug/L; Neuderfer and Heitzman 1992).

Complex Mixture

In addition to the individual chemicals, a complex mixture was also tested. Effluents are typically complex mixtures that vary by location and time. Because a reference effluent was not available, a complex

Table 3. Sources, percent active ingredient, use, and mode of action for chemicals used in toxicity test

Chemical	Source	Active ingredient (%)	Use	Mode of action
Ammonium phosphate	EM Science, Gibbstown, NJ	12	Fertilizer, byproduct of waste-water treatment plants, and some farming practices	Respiration interference
Carbaryl	Donated by Rhone-Pôulenc Agricultural Co., Research Triangle Park, NC	99.7	Carbamate insecticide	Cholinesterase inhibitor
Chlorine (sodium hyporchlorite)	NaOCL	10-13	Disinfectant	Oxidant, disrupts cell membranes
Copper sulfate	Fisher Chemical, St. Louis, MO	25.5	Mining, industrial, fungicide	Osmoregulation interference
Diazinon	Radian International LLC	99	Organophosphate insecticide	Cholinesterase inhibitor
4-nonylphenol	Fluka Chemical, New York, NY	85.0	Nonylphenol ethoxylate detergents	Narcotic and oxidative stressor
Pentachlorophenol	Aldrich Chemical, Milwaukee, WI	99.0	Organochlorine wood preservative molluscicide	Oxidative phosphorylation uncoupler
Permethrin	Donated by ICI Americas Inc., Richmond, CA	95.2	Pyrethroid insecticide	Neurotoxin

mixture was prepared using compounds that cause toxicity by way of several different modes of action. Chemicals included in the complex mixture were carbaryl, copper, 4-nonylphenol, pentachlorophenol, and permethrin in equitoxic proportions (96-hour LC_{50}/5) as determined from previous acute toxicity tests with juvenile fathead minnows (Dwyer *et al.* 1995; Sappington *et al.* 2001). The fathead minnow 96-hour LC_{50} for each chemical 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., assuming additive toxicity, the high concentration would kill 50% of the fathead minnows after 96 hours of exposure). Results are expressed as a percent of the high concentration (identified as 100%; toxic unit = 1). The maximum volume of acetone added to any test container for the five chemical complex mixture was 2.5 mL/L.

Field Collected Effluents

Effluents (including major and minor municipal discharges and various industrial effluents) collected from Arizona or North Carolina were selected from a list of permitted discharges into waters the listed species either historically or currently inhabit. Two effluents were collected from Arizona. Each Arizona effluent was tested with fathead minnows and *C. dubia* but with only one listed species, either bonytail chub or razorback sucker. Nine North Carolina effluents were tested. Shortnose sturgeon, Cape Fear shiner, and spotfin chub were tested with three effluents, although none of the species were tested with the same effluent. All nine North Carolina effluents were also tested with fathead minnows and *C. dubia*.

For each effluent tested, three separate effluent samples were collected and used for daily renewal during the toxicity tests. Effluents were collected, placed in plastic carboys, cooled, and shipped in coolers overnight to the CERC. The first sample was collected at 2 to 3 days before starting the toxicity tests. The second sample was collected on approximately the first day of the toxicity test, and the third sample was collected on approximately the third day of the toxicity test (e.g., the first sample was used for days 0 to 3,the second sample was used for days 4 and 5; and the third sample was used for days 6 and 7). For tests with effluents from Arizona, at the same time that effluents were sampled, receiving water was also sampled to be used as the dilution water. For the tests with effluents from North Carolina, ASTM hard water was used as the dilution water.

Statistical analysis

Decrease in biomass, which combines 2 endpoints into a single endpoint (for fish, growth and survival; for *C. dubia*, reproduction and survival), were determined by calculating the inhibition concentration (ICp) for each test using a linear interpolation method (Norberg-King 1993). This approach estimates point and confidence interval estimates using a continuous model. Hypotheses testing provides no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC). NOECs and LOECs are discrete concentrations specific to the concentrations used in testing. Besser *et al.* (2004) demonstrated that effect concentrations calculated using linear interpolation were less variable than LOECs. Norberg-King (1993) reported the IC_{25} to be similar to an NOEC. Norberg-King (1993) and Besser *et al.* (2004) provided additional discussion and comparisons between hypothesis testing and linear interpolation of toxicity data. We calculated IC_{25}s for this study. An expanded confidence interval, as recommended in the ICp procedure, was calculated for our fish studies because there were <7 replicates for each test. If the expanded lower confidence limit was <0, then the lower confidence limit was reported as 0. Confidence intervals for the *C. dubia* were not expanded because 10 replicates were used.

To summarize the data, when >1 test was conducted 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 analysis. For the results with ammonia, if the total ammonia IC_{25} was >17 mg/L (the highest concentration tested), then 17 mg/L was used to calculate the geometric mean. Calculation in this manner provides a concentration lower (bias) than the actual concentration. Not including the data would bias the summarized data to a greater extent than including the data.

The number of tests conducted within this study were limited, and it would be inappropriate to conduct intensive statistical analysis of these data. Interpretation of whole-effluent tests is dependent on statistical rigor, and therefore definitive statements regarding species sensitivity could not be made (Denton and Norberg-King 1996; Baird *et al.* 1996). To summarize general trends in our data, we considered

Table 4. Average water quality characteristics of the field-collected effluents

Effluent	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Conductivity (μmho/cm)	Dissolved oxygen (mg/L)	pH	Ammonia (total/unionized; mg/L)
AZ-1	520	186	2400	7.0	8.12	ND
AZ-2	520	160	2480	9.0	8.10	ND
NC-1	75	196	1390	8.4	8.13	0.1/0.001
NC-2	80	140	691	8.3	8.21	0.4/0.003
NC-3	170	58	763	7.2	7.96	0.5/0.002
NC-4	30	100	485	8.8	8.09	0.9/0.006
NC-5	42	97	422	8.5	7.83	5.4/0.021
NC-6	122	325	2854	8.2	9.87	0.5/0.007
NC-7	1590	790	6160	9.4	8.16	4.4/0.021
NC-8	50	76	706	9.6	7.39	11.3/0.009
NC-9	20	400	1575	9.4	7.88	70.9/0.206

ND = Not determined.

results for listed fishes to be different from fathead minnow or *C. dubia* if IC_{25}s differed by more than 20%.

Results

General Test Conditions and Acceptability

For toxicity tests conducted with ASTM hard water, hardness and alkalinity were within the acceptable range (alkalinity 110 to 120 mg/L as $CaCO_3$ and hardness 160 to 180 mg/L as $CaCO_3$). The pH was typically slightly higher than the range maximum of 8.0 identified by ASTM (2003), but it was always <8.6. Dissolved oxygen concentrations were always acceptable (>40% saturation); therefore, test water was not aerated.

Control survival for all species and exposures was generally ≥80%. 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 exposure; (3) a copper–hardness-9 with fathead minnow; (4) one diazinon study (of four total) with fathead minnow; (5) an ammonia study with fathead minnow; and (6) an effluent test with fathead minnow conducted concurrently with razorback sucker and using receiving water as the diluent. In the fathead minnow–carbaryl study and the bonytail chub study, survival was 70%, and data from those tests were included in the results. The fathead minnow copper–hardness-9 study had a control survival of 79%, whereas the one fathead minnow–diazinon study had a control survival of 77%. Data from both these tests were included in the results. One fathead minnow–ammonia study had survival of only 50% in the control, and the fathead minnow toxicity data were not included. For one test with an Arizona effluent, fathead minnows had a control survival of 58% in the receiving water and 82.5% in the ASTM hard water. Control response for razorback suckers and *C. dubia* tested concurrently were ≥85% survival for both dilution waters. There are two potential reasons for the decreased survival of fathead minnows: (1) contaminants in the receiving water were toxic to fathead minnows but not to razorback suckers or *C. dubia* or (2) an inferior group of fathead minnows. The normal range of responses obtained with fathead minnows is >80% survival. Given the normal range for fathead minnow survival, and the survival for both razorback suckers and *C. dubia*, we believe that this group of fathead minnows was inferior, and the results from that group of fathead minnows was not included in the data summary.

Initial tests with other sturgeon species (Atlantic sturgeon; Dwyer *et al.* 2000a) and Cape Fear shiners were unsuccessful. Modifications to the standard test guidance, which included increasing the test volume for the shortnose sturgeon and modifying the diet for Cape Fear shiner, allowed for tests to be completed and meet test acceptability criteria (Table 2).

Analytical Results — Individual Chemical and Complex Mixture

Average per nominal concentrations for measured stock solutions were carbaryl 84% (n = 3), copper 80% (n = 4), diazinon 92% (n = 1), 4-nonylphenol 107% (n = 2), pentachlorophenol 105% (n = 2), and permethrin 99% (n = 2). Ammonia and chlorine stocks were made before each test (ammonia) or renewal (chlorine) and ranged from 90% to 110% of nominal. After 24 hours of exposure, chlorine concentrations decreased approximately 90% from initial concentrations. Toxicity values for all individual chemical tests are based on nominal concentrations and not corrected for chemical stock confirmation.

Analytical Results — Field Collected Effluents

Effluents collected from Arizona were similar in their general water quality characteristics (Table 4). Both effluents had a hardness of 520 mg/L as $CaCO_3$ and conductivity of approximately 2400 μmho/cm. AZ-1 had an alkalinity of 186 mg/L as $CaCO_3$, and AZ-2 had an alkalinity of 160 mg/L as $CaCO_3$.

The water quality characteristics of effluents collected from North Carolina varied widely (Table 4). Hardness ranged from 20 (NC-9) to 1590 (NC-7) mg/L as $CaCO_3$. Alkalinity and conductivity also varied greatly with alkalinity ranging from 58 (NC-3) to 790 mg/L (NC-7) as $CaCO_3$ and conductivity ranging from 422 (NC-5) to 6160 μmho/cm. Total ammonia concentrations ranged >2 orders of magnitude. Effluent samples identified as NC-1 had a total ammonia concentration of 0.1 mg/L, whereas samples from NC-9 had a total ammonia concentration of 70.9 mg/L.

Table 5. IC_{25} (range[a] and no. of tests) for individual chemical or complex mixture toxicity tests.

Species	IC_{25} (IC_{25} range)[a] (No. of tests)						
	Complex mixture (%)	Ammonia (mg/L)	Carbaryl (mg/L)	Chlorine (µg/L)	Copper/hard-9 (mg/L)	Copper ASTM (mg/L)	Diazinon (µg/L)
C. dubia	<6.25 (3)	1.3 (0.8–1.8) (3)	<0.33 (3)	176 (157–218) (1)		51 (22–60) (1)	<62.5
Fathead minnow	29 (21–39) (6)	7.2 (2.4– > 17) (6)	0.42 (0.22–0.81) (5)	565 (254–673) (1)	6.3 (0–12.0) (1)	103 (53–140) (1)	1176 (413–2261) (4)
Razorback sucker	33 (26–41) (2)	13.4 (10.6– >17) (2)	2.06 (1.62–2.62) (2)				
Bonytail chub	29 (14–61) (2)	11.0 (9.4–12.9) (2)	0.25 (0.23–0.28) (2)				
Cape Fear shiner	31 (26–37) (2)	8.8 (0–13.0) (1)		603 (478–649) (1)	5.3 (2.8–16.7) (1)	34 (31–37) (2)	199 (57–1269) (1)
Colorado pikeminnow	64 (64–65) (2)	8.9 (4.4–17.9) (2)	1.33 (1.17–1.52) (2)				
Spotfin chub	NC	15.8 (14.2–17.2) (1)		616 (547–635) (1)	14.3 (9.1–17.7) (1)	65 (0–68) (1)	4115 (2281–5654) (1)
Gila topminnow	54 (35–65) (1)	24.1 (19.2–25.6) (1)					

IC_{25} is the geometric mean of the IC_{25}s (number of IC_{25}s in parentheses) used in the rank analysis. For the results with ammonia, if the total ammonia IC_{25} was >17, then 17 was used in the calculation. Calculation in this manner likely provided 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.

[a] If only one test was conducted (N = 1), then the numbers presented are the 95% confidence interval (expanded for fish) from the single test. NC = Not calculated.

Individual Chemical Toxicity Results

Toxicity tests with *C. dubia* were conducted with six of the seven chemicals. Tests were not conducted using the hardness-9 water. Across the six chemical tests, *C. dubia* were almost always more sensitive (lower IC_{25}) than either the fathead minnow or any of the listed fish species (Table 5). There was one exception to this response: When copper was tested in ASTM hard water, *C. dubia* had a higher IC_{25} (51 mg/L) than Cape Fear shiner (34 mg/L).

Ammonia toxicity results were obtained for all seven fish species (Table 5). The IC_{25}s ranged from 7.2 (fathead minnow) to 24.1 mg/L (Gila topminnow). Six of the seven fish species had an IC_{25} <16 mg/L. Only Gila topminnow had an IC_{25} >16 mg/L. Four fish species were tested with carbaryl (Table 5), and carbaryl IC_{25}s ranged from 0.25 (bonytail chub) to 2.06 mg/L (razorback sucker). The IC_{25} for fathead minnows was 0.42 mg/L. Colorado pikeminnow had an IC_{25} of 1.33 mg/L.

Chlorine, copper-9, copper-ASTM, and diazinon were tested using fathead minnow, Cape Fear shiner, and spotfin chub (Table 5). The IC_{25}s for chlorine ranged from 565 (fathead minnow) to 616 ug/L (spotfin chub); copper-9 ranged from 5.3 (Cape Fear shiner) to 14.3 mg/L (spotfin chub); copper-ASTM ranged from 34 mg/L (Cape Fear shiner) to 103 mg/L (fathead minnow); and diazinon ranged from 199 (Cape Fear shiner) to 4115 ug/L (spotfin chub). The three species were most similar in their sensitivity to chlorine (range 565 to 616 ug/L), whereas the species were most dissimilar in their response to diazinon (range 199 to 4115 ug/L). As would be expected, the IC_{25} s for copper tested in water with a hardness of 9 mg/L as $CaCO_3$ were lower than the IC_{25} s for copper tested in ASTM water (hardness 160 to 180 mg/L as $CaCO_3$).

Complex Mixture Toxicity Results

Six fish species were tested using the complex mixture (Table 5). For the complex mixture, IC_{25}s ranged from 29% (fathead minnow and bonytail chub) to 64% (Colorado pikeminnow). Four of the six fish species had an IC_{25} <35% (razorback sucker 33%, Cape Fear shiner 31%, bonytail chub and fathead minnow).

Field Collected Effluent Toxicity Results

For one of the Arizona effluents (AZ-2) and four of the North Carolina effluents (NC-2, -3, -4, and -5), no toxicity to fish was observed (Table 6). As was the case with the chemical toxicity tests, *C. dubia* were generally more sensitive (IC_{25} s were lower) than the fish species. One exception to this pattern was the test conducted with the NC-8 effluent. For that test, *C. dubia* had an IC_{25} greater (>100%) than the IC_{25} for shortnose sturgeon (60%).

Six tests allowed for a comparison between fathead minnow and a listed fish species (Table 6). The IC_{25} for fathead minnow was always greater than the IC_{25} for the listed fish species. In some cases the difference was substantial. For example, the IC_{25} for bonytail chub tested with the AZ-l effluent was 11%, whereas the fathead minnow IC_{25} for the same effluent was 61%. For the test conducted with NC-6, the fathead minnow IC_{25} was >100%, whereas the spotfin chub IC_{25} was 85%. In other tests the differences were minor. For example, the IC_{25} for shortnose sturgeon tested with NC-9 was 12%, whereas the IC_{25} for fathead minnow tested with the same effluent was 14%.

Table 6. Summary of the 7-day IC_{25} estimates and 95% confidence intervals[a] for the Arizona or North Carolina effluent samples

Species	AZ-1	AZ-2	NC-1	NC-2	NC-3	NC-4	NC-5	NC-6	NC-7	NC-8	NC-9
C. dubia	6 (4–31)	44 (8–71)	24 (4–39)	>100%	>100%	>100%	>100%	40 (12–52)	33 (29–35)	>100%	10 (5–28)
Fathead minnow	61 (0–93)	NC	50 (0–77)	>100%	>100%	>100%	>100%	>100%	73 (NC)	>100%	14 (0–40)
Shortnose sturgeon									55 (48–63)	60 (53–64)	12 (4–51)
Razorback sucker		>100%									
Bonytail chub	11 (9–38)										
Cape Fear shiner			38 (NC)	>100%	>100%						
Spotfin chub						>100%	>100%	85 (70–104)			

[a] 95% confidence intervals are shown in parentheses.
NC = not calculated.

Discussion

The purpose of the study was to evaluate the relative sensitivity of listed species compared with the organisms commonly used in 7-day effluent toxicity tests, i.e., fathead minnows and *C. dubia*. The number of tests conducted within this study were limited, and interpretation of whole-effluent tests is dependent on statistical rigor (Denton and Norberg-King 1996; Baird et al. 1996). Because of these limitations, we restricted our evaluation to general observations regarding species sensitivity. In addition, because of the limited number of fish available, which decreased the number of replicates used in testing, the confidence intervals for many of the results are broad.

We were able to successfully conduct tests with a number of listed species. This direct approach may be impractical for some species and impossible for others because some species may not be amenable to culture or laboratory testing. Tests were attempted using American shad (*Alosa sapidissima*) and Atlantic sturgeon (*Acipenser oxyrhynchus*), but they were unsuccessful (Dwyer *et al.* 2000a). Initial tests with shortnose sturgeon and Cape Fear shiner were also unsuccessful, but modifications to the standard test guidance (test volume for shortnose sturgeon and diet for Cape Fear shiner) allowed for the successful completion of the tests. Before undertaking a definitive investigation, species should be evaluated in trial tests to identify modifications to culture or testing methods that may be required.

The geometric mean IC_{25} obtained for the fathead minnow complex mixture studies was 29%. The high concentration (100%) for the complex mixture was equal to a toxic unit of 1 based on 96-hour acute toxicity studies for each individual chemical conducted with a juvenile fathead minnow. Several factors likely explain these results. Mayer and Ellersieck (1986) found that 83% of the time, fish sensitivity decreased as fish size increased, with LC_{50}s increasing by up to a factor of 5. Tests in the present study were conducted with fathead minnows that were <24- hours old, whereas the acute toxicity tests were conducted with juvenile fish (approximately 0.45 mg). Also, the IC_{25} is based on biomass decrease (effects on mortality and growth) and is reported to be similar to an NOEC (Norberg-King 1993). The acute test endpoint in these previous tests was mortality after 96 hours of exposure (96-hour LC_{50}). Additionally, Mayer and Ellersieck (1986) reported that toxicity generally increased by a factor of approximately three with each 10°C increase in temperature. The acute toxicity tests were conducted at 22°C, whereas the complex mixture studies reported here were conducted at 25°C, which is the temperature recommended for conducting effluent toxicity tests. This represented only a 3°C temperature increase but could account for a portion of the greater toxicity of the complex mixture. Finally, the acute toxic unit was calculated from 96-hour exposures, whereas the IC_{25} in the present study was determined after 7 days of exposure. In summary, it is likely that the difference in life stage, endpoints used, and increases in test temperature and length of exposure are responsible for the toxicity exhibited by the complex mixture compared with the toxic unit estimated from acute tests.

Comparisons across species are difficult given the number of different types of exposures (different chemicals and effluents) conducted with the species. To compare the fathead minnow IC_{25} with the IC_{25} of listed species, we calculated a response ratio. The ratio was determined by dividing the IC_{25} for listed species by the IC_{25} for fathead minnow (Table 7). This provided a consistent and unitless approach for comparing responses. We considered results for listed fishes to be the same as fathead minnows if IC_{25} s differed by 20% or less ($0.8 \geq$ response ratio ≤ 1.2).

Because the greatest number of species were tested using the complex mixture and ammonia, these 2 exposures provide the most robust comparison of species sensitivity (Table 7). Fathead minnows and 6 listed fish (razorback suckers, bonytail chub, Cape Fear shiner, Colorado pikeminnow, spotfin chub, and Gila topminnow) were tested with the complex mixture or with ammonia (11 total comparisons). Cape Fear shiner were most similar to fathead minnows. Five of the 11 ratios (45%) were within a range of 0.8 to 1.2, thus suggesting similarity between fathead minnows and the listed species. There were no ratios <0.8 (listed species more sensitive than fathead minnow), but 6 ratios (55%) were >1.2 (listed species less sensitive than fathead minnow). These results indicate that fathead minnows may be good surrogates for the protection of listed fish species. However, this observation does not apply when considering all the data described below.

For all chemicals and effluents, 32 tests have a listed fish species response and corresponding fathead minnow response (Table 7). Four of those tests were for effluents where both the listed fish species and the fathead minnow had a response >100% (NC-2, -3, -4, and -5 effluents). Cape Fear shiner responses in 2 tests (NC-2 and -3) and spotfin chub responses in 2 tests (NC-4 and -5) were the same as fathead minnow. However, for these 4 tests, we cannot determine if the relative sensitivity of the species was the same because the highest concentration tested did not elicit a response (IC_{25} >100%

Table 7. Comparison of IC_{25} for fathead minnow with IC_{25} of listed species for all tests

	Toxicity tests																	
Species	Mix	NH_3	Carb	Cl_2	Cu-9	Cu-ASTM	Diaz	AZ-1	AZ-2	NC-1	NC-2	NC-3	NC-4	NC-5	NC-6	NC-7	NC-8	NC-9
FHM	1	1	1	1	1	1	1	1	NC	1	1[a]	1[a]	1[a]	1[a]	1	1	1	1
SNS																0.8	0.6	0.9
RBS	1.1	1.9	4.9						NC									
BTC	1.0	1.5	0.6					0.2										
CFS	1.1	1.2		1.1	0.8	0.3	0.2			0.8	1[a]	1[a]						
CPM	2.2	1.2	3.2															
SFC	NC	2.2		1.1	2.3	0.6	3.5						1[a]	1[a]	0.9			
GTM	1.9	3.3																

Comparison is the ratio of the IC_{25} for listed species divided by the IC_{25} for fathead minnow
BTC = Bonytail chub.
Carb = Carbaryl.
CFS = Cape Fear shiner.
CPM = Colorado pikeminnow.
Diaz = Diazinon.
FHM = Fathead minnow.
GTM = Gila topminnow.
NC = Not calculated.
RBS = Razorback sucker.
SFC = Spotfin chub.
SNS = Shortnose sturgeon.
[a] Test in which no effects were determined at any of the effluent concentrations (>100% effluent for both species).

effluent). For this reason, these 4 tests were not used to compare relative species sensitivity.

For the remaining 28 tests, ratios of IC_{25} s between a listed fish species and fathead minnow ranged from 0.2 (Cape Fear shiner–diazinon; bonytail chub-AZ-1) to 4.9 (razorback sucker–carbaryl). Twelve of the 28 tests (43%) were in the range of 0.8 to 1.2 (similar sensitivity between listed species and fathead minnows). Six tests (21%) had a ratio <0.8 (listed species more sensitive than fathead minnow), and 10 tests (36%) had a ratio >1.2. Four listed species (bonytail chub, Cape Fear shiner, spotfin chub and shortnose sturgeon) accounted for all the tests with a ratio <0.8. All listed fish species were within a factor of 5 (ratio 0.2 to 5.0) of the fathead minnow. Only the Gila topminnow had a ratio consistently >1.2.

To evaluate the protection provided by effluent toxicity tests that use fathead minnow and *C. dubia* concurrently, we also calculated the ratio as previously described using *C. dubia* results (Table 8). Sixty-two possible comparisons exist between fathead minnows or *C. dubia* and listed fish species. Eight of the comparisons had IC_{25} s >100%, and so we are unable to determine the relative sensitivity of the species. For the remaining 54 comparisons, only 2 (3.7%; shortnose sturgeon–NC-8 and Cape Fear shiner–copper-ASTM) of the listed fish species comparisons were not protected by the combination of fathead minnows and *C. dubia* tests. Chemical mode of action is an important issue to consider when comparing vertebrate and invertebrate results. If results from insecticides tested individually (carbaryl and diazinon) are excluded, 4.3% (2 of 46) of the listed fish species comparisons were not protected by the combination of fathead minnows and *C. dubia* tests.

Other toxicity tests have been conducted following effluent procedures with listed species of fish. The sensitivity of the listed fountain darter (*Etheostoma fonticola*) was evaluated by the Edwards Aquifer Research and Data Center (EARDC), Southwest Texas State University (San Marcos, TX). Tests were conducted with effluent collected from the San Marcos, TX, wastewater treatment plant (EARDC 1992a); a single toxicity test was conducted with glyphosate (EARDC 1992b); and five tests were conducted with cadmium chloride (EARDC 1999a, 1999b, 2000a, 2000b, 2000c). The original reports identified NOECs, LOECs, and LC_{50} s. We used the original data and calculated IC_{25} s to make comparisons consistent with the approaches used in the present study. Fathead minnows were tested concurrently with all fountain darter tests. *C. dubia* were tested only with cadmium chloride.

We were unable to calculate an IC_{25} for one of the fathead minnow–cadmium chloride exposures because none of the pooled response means were <75% of the control response. The effluent IC_{25} was 19% for fountain darter and 49% for fathead minnow. Tests conducted with glyphosate had IC_{25}s of approximately 116 mg/L for fountain darters and 100 mg/L for fathead minnows. The geometric averages for the tests conducted with cadmium chloride were fountain darter 4.7 µg/L (range 1.4 to 7.6, n = 5); fathead minnow 11.3 µg/L (range 5.7 to 20.7, n = 4); and *C. dubia* 5.2 µg/L (range 2.2 to 14.4, n = 5). We calculated IC_{25} ratios as previously described for tests conducted with the effluent and cadmium chloride. Using fathead minnow results, the ratio was approximately 0.4 whereas the ratio for the test conducted with glyphosate was 1.1. The cadmium chloride ratio using results for *C. dubia* was 0.9. These results indicate that the fountain darter is generally more sensitive than fathead minnow, but they support the idea of using a combination of *C. dubia* and fathead minnow tests.

Besser *et al.* (2004) compared the chronic toxicity of copper and pentachlorophenol using the commonly tested species fathead minnow and rainbow trout and the federally–listed endangered fountain darter. In those tests, the fountain darter was more sensitive than the commonly tested species to both chemicals. Dwyer *et al.* (2004) tested three common test spe-

Table 8. Comparison of IC_{25} for *C. dubia* with IC_{25} of fish for all tests

	Toxicity tests																
Species	Mix	NH_3	Carb	Cl_2	Cu-ASTM	Diaz	AZ-1	AZ-2	NC-1	NC-2	NC-3	NC-4	NC-5	NC-6	NC-7	NC-8	NC-9
C. dubia	1	1	1	1	1	1	1	1	1	1[a]	1[a]	1[a]	1[a]	1	1	1	1
FHM	4.6	5.5	1.3	3.2	2.0	19	10.2	NC	2.1	1[a]	1[a]	1[a]	1[a]	2.5	2.2	1	1.4
SNS															1.7	0.6	1.2
RBS	5.3	10	6.2					2.3									
BTC	4.6	8.5	NC				1.8										
CFS	5.0	6.8		3.4	0.7	3.2			1.6	1[a]	1[a]						
CPM	10	6.8	4.0														
SFC	NC[4]	12		3.5	1.3	66						1[a]	1[a]	2.1			
GTM	8.6	19															

Comparison is the ratio of the IC_{25} for listed species divided by the IC_{25} for fathead *C. dubia*.
BTC = Bonytail chub.
Carb = Carbaryl.
CFS = Cape Fear shiner.
CPM = Colorado pikeminnow.
Diaz = Diazinon.
FHM = Fathead minnow.
GTM = Gila topminnow.
NC = Not calculated.
RBS = Razorback sucker.
SFC = Spotfin chub.
SNS = Shortnose sturgeon.
[a] Test in which no effects were determined at any of the effluent concentrations (>100% effluent for all species).

cies (fathead minnow, sheepshead minnow *Cyprinodon variegatus*, and rainbow trout *Oncorhynchus mykiss)* and 17 listed or closely related species. Acute 96-hour water exposures were conducted with 5 chemicals (carbaryl, copper, 4-nonylphenol, pentachlorophenol, and permethrin). No single species was the most sensitive to all chemicals. For the 3 standard test species evaluated, rainbow trout was more sensitive than either fathead minnow or sheepshead minnow and was equal to or more sensitive than listed and related species 81 % of the time.

Conclusions

The fathead minnow 7-day effluent test was frequently (79%) a reliable estimator of toxic effects to the listed fishes. If a combination of fathead minnows and *C. dubia* were used, listed fishes were protected approximately 96% of the time. A discharge that has a history of being out of compliance with regard to the 7-day fathead minnow test and the *C. dubia* test may be contributing to the decline of listed fishes including fish from other taxonomic families.

If the responses of specific listed species are important for management decisions, our study supports the value in developing culture and testing procedures for those species (under appropriate state and federal permits). This study was conducted with listed fish that have not been routinely used in testing, 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. Therefore, results of this study should be used with some caution. Also, as previously mentioned, the number of tests conducted within this study were limited, and rigorous statistical analysis was not appropriate (Denton and Norberg-King 1996; Baird *et al.* 1996).

Comparisons using acute toxicity tests or statistical endpoints from chronic toxicity studies indicate that some listed species, including those in the current study, are much more sensitive to chemical exposure than the fathead minnow and similar to or slightly more sensitive than rainbow trout (Dwyer *et al*. 2004; Besser *et al*. 2004). This includes fish tested at temperatures greater than the temperatures used for testing rainbow trout. To best determine the effects of contaminants on listed species, various assessment approaches need to be evaluated. By conducting tests with more than one species, the sensitivity of taxonomically unrelated species may be captured by the range in responses of the species used in testing.

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