

EFFECT OF PH ON THE ACUTE TOXICITY OF 2,4,5-TRICHLOROPHENOL  
TO FATHEAD MINNOWS

by

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## Introduction

Acute 96-h flow-through toxicity tests with the compound 2,4,5-trichlorophenol were conducted using 28-d old fathead minnows, *Pimephales promelas* Rafinesque. Toxicity tests were conducted at six nominal pH levels of 6.2, 6.8, 7.4, 8.0, 8.6 and 9.2 to generate LC50 values at 24, 48, 72 and 96 h of exposure. Tests were conducted in two sets of three toxicity tests using nominal pH's of 6.2, 7.4 and 8.6 as one sample set and nominal pH's of 6.8, 8.0 and 9.2 for the other. Over-lapping of the pH levels within each set of toxicity tests was one method of demonstrating quality control between the two groups of fish used.

## Methods

### *Test Organisms*

The juvenile fathead minnows were 28-d old at the start of the tests and had been raised from brood cultures maintained at the UW-S culture unit. The test fish were cultured at  $24 \pm 2$  C in continual flowing laboratory water. As fry, test organisms were fed live brine shrimp nauplii (*Artemia salinus*) a minimum of 2 times daily until 14-d post-hatch, then they were fed frozen brine shrimp until 28-d old. The fish were not fed within 24 h of test start or during the tests.

### *Exposure Systems*

Tests were conducted in continual-flow proportional mini diluters (Benoit et al. 1982). A 0.5 dilution factor was used for the stock of toxicant solution with a flow rate of  $16 \text{ mL}\cdot\text{min}^{-1}$  producing 15 test chamber volume additions per day. Primary exposure chambers were constructed with glass, 24.5x10.5x10.5cm (L,W,H) and had a volume of 1.5 L. Five exposure concentrations and a clean water control were tested in duplicate in each test system. Tests were conducted at a nominal temperature of 25 with a 16L:8D photoperiod. Three proportional diluters were operated simultaneously using a common overhead gravity supply of test water.

Manipulation of the pH levels in each test system was accomplished by

introducing a concentrated acid or base solution into individual diluter water feed lines. Eighteen liters of acid or base solution were mixed and remade as needed. A sulfuric acid ( $H_2SO_4$ ) solution at pH ~1.4 was used to produce pH levels <8.0, and sodium hydroxide (NaOH) solution at pH ~12.4 was used to produce pH levels >8.0. Variable rate metering pumps (Fluid Metering, Inc., Oyster Bay, NY) were used to inject these solutions into test water feed lines between the overhead gravity water supply and the test systems. Pump rates were adjusted until desired test pH's were achieved for each diluter in the test chambers.

Stock solutions of 2,4,5-trichlorophenol were prepared by adding appropriate weights of test compound and volume of test water in 18-L glass carboys and mixing the solutions by continual mechanical stirring. After stirring for 24 h, pH of the stock solutions were adjusted to the appropriate test pH. Initial and during-test pH adjustments of 2,4,5-trichlorophenol stocks were done by addition of dilute  $H_2SO_4$  and NaOH solutions. The pump rates used to supply toxicant to the dilution cells allowed one 18 L stock to last for the duration of the test.

#### *Testing Procedures*

The laboratory water supply for organism culture, acclimation and testing was the municipal water supply for the City of Superior, WI. The water was dechlorinated by charcoal filtration and sodium sulfite addition. Water was then passed through a cation exchange resin to remove heavy metals. Residual chlorine and zinc levels are measured bimonthly to ensure quality control of culture and test water.

Measurements of total alkalinity and total hardness (EDTA) and conductivity were made during the 96 h test periods in all control test chambers and chambers containing low, medium and high toxicant concentrations. At the start of the tests, measurements of pH were taken in all exposure chambers prior to fish introductions to document pH fluctuations due to fish activity. Four hours after fish introduction and during the PM, pH was

measured again. For the remainder of the study, measurements of pH were taken in all test chambers during the AM and in one of the duplicate control, low, medium and high toxicant concentrations during the PM. Dissolved oxygen and temperature were measured daily in all exposure chambers (Table 1).

Ten fish were used in each test chamber for a total of 20 per control and each treatment level. Acclimation of test organisms was done for tests at pH's less than 7.0 and greater than 8.6. (Laboratory water pH ranges from 7.8 to 8.0.) Glass chambers containing approximately 50 L of laboratory water were used to acclimate 200 juvenile fish to the appropriate test pH. The water was pH adjusted daily during acclimation with either H<sub>2</sub>SO<sub>4</sub> or NaOH. Acclimation was done with static water conditions for a period of 5 to 7 d. Fish transfer into exposure chambers for testing was done using a beaker to reduce handling stress. Test control fish wet weights (mg) and standard length (mm) were recorded at test termination.

#### *Toxicant Analytical Procedures*

The 2,4,5-trichlorophenol [95-95-4] was obtained from Aldrich<sup>®</sup> Chemical Co., Inc., Milwaukee, WI, at a purity of 99%. An analytical stock was prepared by dissolving the chemical in HPLC grade methanol (Burdick and Jackson, Muskegan, MI).

Measurements of dissolved 2,4,5-trichlorophenol were made utilizing high pressure liquid chromatography (HPLC). The HPLC system consisted of two Waters M-45 pumps, and a Spectra-Physics SP4270 integrator. A Lichrocart 125 x 4mm cartridge column with LiChrospher 100 RP-18 (5 $\mu$ ) packing was used for the separation. The mobile phase consisted of 80% methanol:20% deionized water containing 1% acetic acid. The samples were analyzed isocratically at a flow rate of 1 mL $\cdot$ min<sup>-1</sup>.

Samples were collected with a disposable glass pipet from the center of the exposure chamber at mid-depth. The sample was transferred to a glass autoinjector vial and capped. Analysis of the samples was completed on the same day that they were sampled. All exposure chambers were sampled at 0 and

96 h. At 24, 48, and 72 h, a sample was collected from one replicate chamber of each toxicant concentration. A minimum of 20% of the samples were analyzed in duplicate, 20% analysis of replicate exposure chambers was conducted, and 10% of the samples were spiked for determination of spike recovery.

Concentrations of 2,4,5-trichlorophenol in the samples were determined by comparing peak areas for the samples to the calibration curve prepared from the analysis of a minimum of four analytical standards bracketing the range of concentrations of the samples. The working standards were prepared in deionized water from intermediate standards in HPLC grade methanol.

A limited number of samples were collected and analyzed for total 2,4,5-trichlorophenol. This analysis was conducted to provide a comparison between total and dissolved 2,4,5-trichlorophenol concentrations in the toxicity tests. Samples were collected only from the first set of toxicity tests with fathead minnows at 24 and 96 h. They were collected from the low, middle, and high exposure concentrations for each of the three tests. Before samples were collected, 50 mL of deionized water was placed into a 120 mL glass extraction bottle with a teflon lined cap. After the sample was collected, 3 drops of concentrated sulfuric acid (Fisher, Chicago, IL, Reagent Grade) was added to the solution to adjust the pH to <2. Ten milliliters of hexane (Burdick and Jackson, Non-Spectro Grade) was added to each bottle, and the sample was stirred vigorously for 30 min to extract the 2,4,5-trichlorophenol from the aqueous sample. An aliquot of the hexane layer was transferred into an autosampler vial for GC analysis. A minimum of 20% of the samples were analyzed in duplicate and 10% were spiked for determination of recovery. The GC system consisted of a Hewlett-Packard (HP) Model 5880A gas chromatograph equipped with an electron capture detector, a HP Model 7672A autosampler, and a Level 4 GC terminal. The analytical separation was conducted utilizing a J & W Scientific, Folsom, CA, 30 meter DB-Wax capillary column. The carrier gas was ultra-high purity hydrogen and the detector make-up gas was a mixture of 95% argon-5% methane. The following oven temperature program was used: initial temperature = 55 C, initial time = 1.00 min, program rate = 10 C·min<sup>-1</sup>,

final temperature = 205 C, final time = 2 min.

Concentrations of total 2,4,5-trichlorophenol were determined by use of a calibration curve prepared from the analysis of four analytical standards. The analytical standards were prepared in hexane (Burdick and Jackson, Non-Spectro) from an intermediate standard in acetone (Burdick and Jackson, High Purity).

#### *Total and Dissolved Organic Carbon*

Samples were collected for the analysis of total organic carbon (TOC) from a control and one exposure tank at low, intermediate, and high 2,4,5-trichlorophenol concentrations in each toxicity test. TOC samples were collected by pipetting 120 mL of sample from the middle of the exposure tank at mid-depth and transferring into a precleaned 120 mL glass bottle with a teflon lined cap. Dissolved organic carbon (DOC) samples were collected in a similar manner and were filtered through Gelman type A/E glass fiber filters which had been ashed at 400 C for 3 h. The filtered sample was returned to the sample bottle which had been rinsed with deionized water. Samples were preserved by the addition of 0.3 mL of a 50% phosphoric acid in deionized water. The samples were refrigerated until analyzed.

#### *Statistical Analysis of Data*

LC50 calculations with their 95% confidence limits were calculated by the trimmed Spearman-Kärber method (Hamilton et al. 1977). LC50 values were generated at each of the pH test levels for 24, 48, 72 and 96 h exposure durations. The toxicant concentrations used to calculate the LC50's were the mean for the two replicate exposures at 96 h. If 100% of the test organisms died before 96 h, then only the measured toxicant concentrations prior to the last death, plus one after, were used to calculate a mean exposure concentration.

## Results

Fathead minnows with mean standard lengths,  $16.4 \pm 2.1$  and  $15.3 \pm 1.8$ mm; mean wet weights  $54.0 \pm 26.8$  and  $40.32 \pm 17.8$  mg were measured in the test sets of nominal pH's 6.2, 7.4 8.6 and 6.8, 8.0 and 9.2, respectively.

Mean values for temperature, dissolved oxygen, conductivity, hardness and alkalinity were similar for each of the six tests (Table 1). Mean pH values for each test were within 0.1 units of the nominal pH's.

Observed cumulative mortalities at measured toxicant concentrations are reported at 4, 8, 24, 48, 72 and 96 h exposure durations. In most tests, replicate exposures gave similar mortality responses (Table 2). No control mortalities occurred. The initial responses of fathead minnows to lethal toxicant levels was rapid gilling and an increased fright response which diminished as loss of equilibrium was observed.

A reduction in toxicity of TCP was seen with increasing pH of the test water (Table 3). A ten fold decrease in toxicity was observed between tests conducted at pH 6.2 and pH 9.2.

Comparisons of total and dissolved 2,4,5-trichlorophenol concentrations (Table 4) were made for a limited number of samples from the first set of three tests (pHs 6.2, 7.4, 8.6). Samples were collected from low (2-A), medium (4-A) and high (5-A and 6-A) concentrations of 2,4,5-trichlorophenol at 24 and 96 h of exposure. The total concentrations were determined by acidification of the sample and extraction into hexane followed by gas chromatography analysis. The dissolved analysis was done on an untreated sample which was directly injected into a high pressure liquid chromatograph. The sample analyzed on the HPLC was considered to be a dissolved sample because of the filters located in the HPLC system that would not allow particulates to pass through the analytical column and into the detector. The mean (S.D.) agreement between the total and dissolved 2,4,5-trichlorophenol concentrations was  $87.2 \pm 7.3\%$  (n=18). Total 2,4,5-trichlorophenol concentrations were usually less than dissolved concentrations. This may not be real differences; rather, differences could be the result of the two

analytical techniques employed (HPLC vs. GC).

A number of duplicate and spiked samples were analyzed to provide quality assurance data for these tests (Table 5). The mean agreement for duplicates from the same exposure tank was  $98.0 \pm 2.6\%$  (n=44), the mean agreement for samples taken from replicate exposure tanks was  $97.7 \pm 3.3\%$  (n=73), and mean spike recovery was  $103.9 \pm 5.5\%$  (n=30).

TABLE 1. Mean ( $\pm$  S.D.) and Number (n) of Observations for Measured Characteristics of Test Waters Used to Exposed Fathead Minnows to 2,4,5-Trichlorophenol at Nominal pH's 6.2, 6.8, 7.4, 8.0, 8.6, at 9.2.

Measured Water Characteristics	Nominal pH of Treatment					
	6.2	6.8	7.4	8.0	8.6	9.2
Temperature (C)	24.6 ( $\pm 0.6$ ) n=60	24.1 ( $\pm 0.3$ ) n=60	25.4 ( $\pm 0.7$ ) n=60	24.6 ( $\pm 0.5$ ) n=60	26.0 ( $\pm 0.8$ ) n=60	25.1 ( $\pm 0.4$ ) n=60
Dissolved Oxygen (mg·L <sup>-1</sup> )	6.9 ( $\pm 0.4$ ) n=60	7.4 ( $\pm 0.3$ ) n=60	6.9 ( $\pm 0.3$ ) n=60	7.5 ( $\pm 0.3$ ) n=60	6.9 ( $\pm 0.4$ ) n=60	7.2 ( $\pm 0.4$ ) n=60
pH <sup>1/</sup>	6.22 ( $\pm 0.11$ ) n=90	6.83 ( $\pm 0.12$ ) n=90	7.32 ( $\pm 0.10$ ) n=90	7.99 ( $\pm 0.06$ ) n=90	8.49 ( $\pm 0.17$ ) n=90	9.10 ( $\pm 0.12$ ) n=90
Conductivity ( $\mu$ mhos·cm <sup>-1</sup> )	158.0 ( $\pm 7.0$ ) n=16	154.0 ( $\pm 3.0$ ) n=12	150.0 ( $\pm 2.0$ ) n=16	145.0 ( $\pm 4.0$ ) n=16	148.0 ( $\pm 4.0$ ) n=16	164.0 ( $\pm 7.0$ ) n=16
Hardness (mg·L <sup>-1</sup> as CaCO <sub>3</sub> )	47.7 ( $\pm 2.1$ ) n=16	49.5 ( $\pm 1.0$ ) n=16	48.5 ( $\pm 1.6$ ) n=16	49.1 ( $\pm 1.0$ ) n=16	46.2 ( $\pm 2.1$ ) n=16	49.7 ( $\pm 3.4$ ) n=16
Alkalinity (mg·L <sup>-1</sup> as CaCO <sub>3</sub> )	10.5 ( $\pm 2.6$ ) n=16	22.5 ( $\pm 2.6$ ) n=16	36.8 ( $\pm 2.3$ ) n=16	48.5 ( $\pm 1.3$ ) n=16	50.1 ( $\pm 2.1$ ) n=16	59.2 ( $\pm 4.2$ ) n=16

<sup>1/</sup> Arithmetic mean and S.D. of logarithm values.

TABLE 2. Observed Cumulative Mortalities of Fathead Minnows Exposed to Measured Concentrations of 2,4,5-Trichlorophenol at Nominal pH Levels 6.2, 6.8, 7.4, 8.0, 8.6 and 9.2. Mortalities are Reported Separately of Replicate Exposure Chambers (A and B).

Time (H)	Replicate											
	A	B	A	B	A	B	A	B	A	B	A	B
Measured Concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of 2,4,5-TCP at Nominal pH 6.2												
	<23		45 ( $\pm 7$ )		95 ( $\pm 8$ )		189 ( $\pm 6$ )		383 ( $\pm 12$ )		838 ( $\pm 19$ )	
4	0	0	0	0	0	0	0	0	0	0	10	10
8	0	0	0	0	0	0	0	0	0	0	10	10
24	0	0	0	0	0	0	0	0	0	0	10	10
48	0	0	0	0	0	0	0	0	0	0	10	10
72	0	0	0	0	0	0	0	0	1	2	10	10
96	0	0	0	0	0	0	0	0	1	2	10	10
Measured Concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of 2,4,5-TCP at Nominal pH 6.8												
	<23		186 ( $\pm 9$ )		371 ( $\pm 15$ )		716 ( $\pm 31$ )		1460 ( $\pm 57$ )		3052 ( $\pm 126$ )	
4	0	0	0	0	0	0	2	5	10	10	10	10
8	0	0	0	0	0	0	10	10	10	10	10	10
24	0	0	0	0	0	0	10	10	10	10	10	10
48	0	0	0	0	0	0	10	10	10	10	10	10
72	0	0	0	0	0	0	10	10	10	10	10	10
96	0	0	0	0	0	0	10	10	10	10	10	10
Measured Concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of 2,4,5-TCP at nominal pH 7.4												
	<23		92 ( $\pm 15$ )		230 ( $\pm 10$ )		442 ( $\pm 27$ )		785 ( $\pm 64$ )		1258 ( $\pm 94$ )	
4	0	0	0	0	0	0	0	0	4	2	10	10
8	0	0	0	0	0	0	0	0	4	2	10	10
24	0	0	0	0	0	0	1	0	4	2	10	10
48	0	0	0	0	0	0	2	0	5	2	10	10
72	0	0	0	0	0	0	2	0	6	2	10	10
96	0	0	0	0	0	0	2	0	6	2	10	10
Measured Concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of 2,4,5-TCP at pH 8.0												
	<23		324 ( $\pm 22.0$ )		805 ( $\pm 48$ )		1486 ( $\pm 87$ )		2719 ( $\pm 23.4$ )		4592 ( $\pm 22.2$ )	
4	0	0	0	0	0	0	4	5	10	10	10	10
8	0	0	0	0	0	0	10	10	10	10	10	10
24	0	0	0	0	0	0	10	10	10	10	10	10
48	0	0	0	0	0	0	10	10	10	10	10	10
72	0	0	0	0	0	0	10	10	10	10	10	10
96	0	0	0	0	0	0	10	10	10	10	10	10

TABLE 2. (Cont.)

Measured Concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of 2,4,5-TCP at Nominal pH 8.6												
	<23		149 ( $\pm 25$ )		339 ( $\pm 42$ )		816 ( $\pm 39$ )		1676 ( $\pm 43$ )		3473 ( $\pm 48$ )	
4	0	0	0	0	0	0	0	0	3	3	10	10
8	0	0	0	0	0	0	0	0	3	3	10	10
24	0	0	0	0	0	0	0	0	3	3	10	10
48	0	0	0	0	0	0	0	0	3	3	10	10
72	0	0	1	0	0	0	0	0	3	3	10	10
96	0	0	1	0	0	0	0	0	3	3	10	10

  

Measured Concentration ( $\mu\text{g}\cdot\text{L}^{-1}$ ) of 2,4,5-TCP at pH 9.2												
	<23		208 ( $\pm 47$ )		593 ( $\pm 59$ )		1641 ( $\pm 97$ )		3752 ( $\pm 133$ )		8084 ( $\pm 280$ )	
4	0	0	0	0	0	0	0	0	0	0	9	7
8	0	0	0	0	0	0	0	0	0	0	9	7
24	0	0	0	1	0	0	0	0	0	0	9	7
48	0	0	0	1	0	0	0	0	0	0	9	7
72	0	0	0	2	0	0	0	0	0	0	9	7
96	0	0	0	2	0	0	0	0	0	0	9	7

TABLE 3. LC50 Values<sup>1</sup> ( $\mu\text{g}\cdot\text{L}^{-1}$ ) and 95% Confidence Intervals (in Parenthesis) for the Exposure of Fathead Minnows to 2,4,5-Trichlorophenol Nominal pH Levels 6.2, 6.8, 7.4, 8.0, 8.6 and pH 9.2.

Exposure Duration (H)	Nominal pH of Test					
	6.2	6.8	7.4	8.0	8.6	9.2
24	567 ( <sup>2/</sup> )	515 ( <sup>2/</sup> )	824 (729-931)	1090 ( <sup>2/</sup> )	1940 (1670-2250)	6030 (5410-6730)
48	567 ( <sup>2/</sup> )	515 ( <sup>2/</sup> )	778 (677-894)	1090 ( <sup>2/</sup> )	1940 (1670-2250)	6030 (5410-6730)
72	507 (450-571)	515 ( <sup>2/</sup> )	758 (658-873)	1090 ( <sup>2/</sup> )	1940 (1660-2260)	6010 (5370-6720)
96	507 (450-571)	515 ( <sup>2/</sup> )	758 (658-873)	1090 ( <sup>2/</sup> )	1940 (1660-2260)	6010 (5370-6720)

<sup>1/</sup> The LC50 values were calculated using trimmed Spearman-Kärber analysis. LC50 values were calculated using measured mean toxicant concentrations of all samples taken from a test chamber throughout the test unless 100% mortality occurred in that chamber in <96 h. When this occurred, only concentrations measured before the last death, plus one beyond the last death, were used to calculate the mean concentration.

<sup>2/</sup> The 95% confidence intervals are not reliable because the response was all or nothing in the various treatment levels.

TABLE 4. Comparison of Dissolved and Total 2,4,5-Trichlorophenol Concentrations ( $\mu\text{g}\cdot\text{L}^{-1}$ ) in Fathead Minnow Acute Toxicity Tests Conducted at Various Nominal pH's. Measurements Were Made at 24 and 96 h of Exposure in Low, Medium and High Exposure Concentrations.

Type of Analysis (24 h of Exposure)	pH 6.2			pH 7.4			pH 8.6		
	2-A	4-A	6-A	2-A	4-A	6-A	2-A	4-A	6-A
Dissolved	35	185	855	116	435	1,340	171	802	3,510
Total	37	178	757	97	411	1,380	116	695	3,830
%Agreement	94.6	96.2	88.5	83.6	94.5	97.1	67.8	86.7	91.6

  

(96 h of Exposure)	pH 6.2			pH 7.4			pH 8.6		
	2-A	4-A	5-A	2-A	4-A	5-A	2-A	4-A	5-A
Dissolved	51	201	378	74	435	836	179	807	1,640
Total	43	164	304	90	411	692	152	731	1,450
%Agreement	84.3	81.6	80.4	82.2	94.5	82.8	84.9	90.6	88.4

TABLE 5. Summary of Quality Assurance Samples from 2,4,5-Trichlorophenol Toxicity Tests with Fathead Minnows.

Nominal Test pH	Duplicate % Agreement (from same exposure)	Duplicate % Agreement (from replicate exposure)	Spike Recovery
6.2	95.6 ± 4.2 (n=7)	97.3 ± 2.3 (n=12)	99.1 ± 8.2 (n=5)
6.8	99.0 ± 0.8 (n=7)	99.3 ± 0.9 (n=12)	101.9 ± 3.9 (n=5)
7.4	97.5 ± 2.3 (n=7)	97.2 ± 3.7 (n=12)	105.0 ± 3.8 (n=5)
8.0	98.0 ± 3.2 (n=7)	98.2 ± 2.8 (n=12)	105.7 ± 5.0 (n=5)
8.6	98.2 ± 1.6 (n=7)	97.2 ± 4.7 (n=12)	105.2 ± 4.0 (n=5)
9.2	99.2 ± 0.5 (n=9)	96.9 ± 3.8 (n=13)	106.0 ± 6.2 (n=5)