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Triclopyr Triethylamine Salt Toxicity to Life Stages of the Fathead Minnow (*Pimephales promelas* Rafinesque)

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Triclopyr [((3,5,6-trichloro-2-pyridinyl)oxy)acetic acid] is the active ingredient in GARLON® herbicides. Triclopyr exhibits herbicidal activities useful in woody plant and broodleaf weed control. Uses of GARLON herbicides include application on rights-of-way, industrial sites and forest planting sites. The application of these herbicides may result in the unintentional introduction of triclopyr into natural bodies of water. The objective of this study was to evaluate the toxicity of triclopyr triethylamine salt (triclopyr TEA salt), the active ingredient in GARLON 3A herbicide, to the embryo, larval and juvenile stages of the fathead minnow. These data will be used to help evaluate the hazard of triclopyr to fish.

MATERIALS AND METHODS

The study included a 96 hr static acute test, a 192 hr flow-through acute test and a 31 day embryo-larval test. Data obtained from the acute tests were used to select the exposure concentrations for the embryo-larval test. Testing was conducted, in part, following procedures recommended by the ASTM Subcommittee on Safety to Aquatic Organisms (ASTM, 1980) and by Benoit et al (1982).

Dilution water used in all tests was from the upper Saginaw Bay of Lake Huron and was carbon filtered and U.V. irradiated prior to use. During the course of this study the dilution water pH ranged from 7.8 to 8.0; and the ranges for total hardness and alkalinity were 101 to 132 mg/L and 81 to 104 mg/L as $CaCO_3$, respectively. Chemical measurements of the dilution water were made by following procedures described by the American Public Health Association et al (1980).

The static acute test was conducted in round glass aquaria measuring 22 cm deep and 24.5 cm in diameter. The flow-through acute and the embryo-larval exposures were conducted with an intermittent-flow proportional diluter similar to that described by Mount and Brungs (1967). The diluter had a dilution factor of 0.65 and at each cycle delivered 2 L to each of 6 exposure and 1 control flow-

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splitting chambers which delivered ∿500 mL to each of two or four replicate test aquaria at each test concentration and control. Exposure was conducted in glass aquaria that measured 16 x 8.7 x 10 cm deep. The aquaria were provided with a nylon screen-covered drain which maintained a volume of ∿750 mL. The diluter was set to cycle once every 15 minutes and resulted in a minimum volume replacement time of once every 30 minutes (Sprague, 1969). Aquaria were siphoned at least once a week to remove accumulated biological material. A 16 hr light/8 hr dark photoperiod was provided during all tests; light intensity at the water surface ranged between 860-1180 lux.

A sample of triclopyr TEA salt solution (44.9% active ingredient) was obtained from the Agricultural Products Department, The Dow Chemical Company, Midland, Michigan. In the static acute test the compound was tested and the results reported on a % active ingredient basis. In the flow-through acute and embryo-larval tests the concentrations were analyzed and reported as triclopyr TEA salt. The water solubility of triclopyr TEA salt is >400,000 mg/L. (Personal communication, J. Schultz, The Dow Chemical Company, Midland, Michigan)

In the static acute test, exposure concentrations were prepared by mixing the appropriate amount of triclopyr TEA salt stock solution with 10 L of dilution water to initiate exposure, with all concentrations reported on a nominal basis only.

In the flow-through acute and embryo-larval tests, a Micromedic® automatic pipette was used to inject the appropriate amount of triclopyr TEA salt stock solution into the toxicant mixing chamber of the diluter. This chamber was equipped with a recirculating pump which provided mixing for at least 4 minutes before the solution was diverted to the toxicant cells. The concentration of triclopyr at each test concentration was analytically determined prior to the beginning of the test; thereafter samples were analyzed at least twice per week for the duration of the study. Once during the tests, the concentration of triclopyr was measured concurrently in all replicate chambers for each test concentration, and was measured at least one additional time in each replicate. Samples were analyzed by high performance liquid chromatography (HPLC). The samples were filtered through a 0.45 µm Millipore® cellulose acetate/nitrate membrane filter, chromatographed through a reverse phase column with an eluent containing 2% acetic acid, and the eluate was monitored by UV at 280 nm. The relative standard deviations at the 95% confidence level for 5 replicate injections of 20.2 mg/L and 121 mg/L standards were 2.4 and 1.3%, respectively. The triclopyr concentrations were adjusted to reflect the concentration of triclopyr TEA salt in respective samples.

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Fish and embryos were from laboratory reared stock. Fish used in the acute tests were held in either 180 L stainless steel or 56 L glass aquaria at a water temperature of 17 ± 2°C, and were provided a 16 hr light/8 hr dark light cycle. A synthetic diet (Alexander et al, 1981) was provided ad libitum. Fish were acclimated to test temperature at least 72 hr prior to testing. Brood stock were held under similar conditions but at a temperature of 23 ± 2°C. Laying substrates were placed in the brood aquaria ∿20 hr prior to the initiation of the embryo-larval exposure so that <24 hr old eggs would be available for testing.

The static acute test was conducted at 17 ± 1°C with fish being exposed in 10 liters of test solution or water (controls). There were ten fish exposed to each of six nominal concentrations ranging from 370 to 1100 mg/L and a control. Mortality was recorded after 24, 48, 72 and 96 hr of exposure. At each observation period, temperature, pH and dissolved oxygen were recorded from at least a high, medium and low test concentration and the control. Fish were not fed during the exposure period.

The flow-through acute test was conducted at 25 ± 1°C using the proportional diluter described above. Thirty-six day old fish, acclimated to 25°C, were exposed to six triclopyr TEA salt concentrations ranging from 25.6 to 232 mg/L and a water control; 10 fish per aquarium, 2 replicate aquaria per concentration. Mortality was recorded at 24 hr intervals through 192 hr of exposure. During each observation period, temperature, pH and dissolved oxygen were measured in at least the high, medium and low test concentrations, and a control. Fish were not fed during the first 96 hr of exposure. Thereafter they were fed 5 mL of a <24 hr old brine shrimp slurry, once daily.

The embryo-larval test was conducted using the proportional diluter described previously. The diluter was set to deliver six test concentrations, ranging from 13 to 112 mg/L and a water control. There were four replicate aquaria provided at each concentration and control with 25 embryos being assigned to each replicate. The water temperature was set to maintain a test temperature of 25 ± 2°C, with excursions beyond 25 ± 1° limited to no more than 24 hours. The test was begun by indiscriminately distributing groups of 5-10 embryos (<24 hr of age) to each of the embryo incubation baskets until each contained 25 embryos. One incubation basket, which measured 70 mm in diameter and 30 mm high and had a nylon mesh bottom, was suspended in each of the 28 test aquaria. The test solutions flowed directly into the incubation baskets during each diluter cycle. Embryos were examined daily until hatching was complete; dead embryos or dead larvae were removed when observed. The day when ≥50% of the embryos at a given concentration had hatched was recorded as the mean day-to-hatch. At the completion of hatch, the total number of larvae was recorded and dead or deformed larvae were subtracted from the total to give normal

larvae at hatch. All larvae were then released into the aquaria for the larval exposure. Throughout larval exposure, approximately 0.03 grams (on a dry weight basis) of <24 hr old brine shrimp were fed to the fish three times per day on weekdays and once or twice a day on weekends. Larval exposure continued through 28 days post mean day-to-hatch. At the completion of the larval exposure period all surviving fish were sacrificed in ice water for final weight and standard-length measurements.

For each set of acute data the LC50 value and 95% confidence intervals were calculated using either Finney's (1971) method of probit analysis or Thompson's (1947) method of moving averages. For analysis of the embryo-larval test data, the percent of embryos that hatched, normal larvae at hatch and survival data were normalized by using the arcsine transformation. Transformed data and unweighted replicate means of length and weight data were evaluated by one-way analyses of variance procedure. The Dunnett's two-tailed t-test (Winer, 1971) was used to compare treatment means to control means at $\alpha = 0.05$. These data were used to estimate the maximum acceptable toxicant concentration (MATC). The MATC is the theoretical toxic threshold concentration that falls between the highest concentration showing no effect and the next highest concentration showing a toxic effect when compared to controls (McKim, 1977). The MATC is best estimated by integrating biological and statistical interpretations of the data. The MATC may be expressed as the geometric mean of the high and low chronic values.

RESULTS AND DISCUSSION

The concentration-mortality data and specific test conditions for the acute tests are presented in Tables 1 and 2. The static acute LC50 and 95% confidence interval (CI) was determined to be 245 (224-269) mg/L; the flow-through 96 and 192 hr LC50 values with 95% CI were 120 (104-140) mg/L and 101 (88.5-116) mg/L, respectively. In the flow-through test, average measured concentrations of triclopyr TEA salt were generally >96% of the predicted values. However, there was a diluter malfunction on days 6 and 8 of the flow-though test which affected the concentration at the nominal 62 mg/L level. The actual average measured concentration and the standard deviation was 44 ± 23.8 mg/L. Examination of the concentration-mortality data shows that the malfunction had little influence on the results of the test. Two fish died (10%) at the next higher concentration, 95.6 mg/L, while only 1 fish (5%) died at the 44 mg/L level.

Embryo-Larval Test: The mean-day-to-hatch for all test concentrations and the control was day three. Larval survival varied nonsystematically except at the 114 mg/L level where survival dropped precipitously and was significantly different ($\alpha = 0.05$) from the controls. Analysis of hatchability of embryos, normal

TABLE 1. Dose-Mortality Data and Test Conditions for the Static Acute Toxicity Test with Fathead Minnows Exposed to Triclopyr Triethylamine (TEA) Salt Solution

Triclopyr TEA Salt Nominal Concentration mg/L	Mortality (%)			
	24hr	48hr	72hr	96hr
494	100	100	100	100
404	100	100	100	100
323	90	100	100	100
256	10	30	40	60
207	0	0	10	10
166	0	0	0	0
0	0	0	0	0

96 hr LC50 (probit method): 245(224-269)[a] mg/L

Test Conditions

Dissolved Oxygen Concentration
(0-48 hr) >82% saturation;
(48-96 hr) >53% saturation

pH Range
(0-96 hr) 7.7-8.2

Temperature Range
(0-96 hr) 17°C-17.6°C

Test Fish

Average Weight	0.22 g
Range of Standard Length	1.6-3.1 cm
Average Loading	0.21 g/L

[a]95% confidence interval

TABLE 2. Dose-Mortality Data and Test Conditions for the Flow-through Toxicity Test with Fathead Minnows Exposed to Triclopyr Triethylamine (TEA) Salt Solution

Triclopyr TEA Salt Nominal Concentration mg/L	Triclopyr TEA Salt Measured Concentration mg/L	Mortality (%) 96 hr	Mortality (%) 192 hr
225	232 ± 4.0	100	100
146	148 ± 4.5	85	95
95	95.6 ± 1.8	5	10
62	44 ± 23.8	0	5
40	39 ± 0.9	0	0
26	25.6 ± 0.5	0	0
0	N.D.[b]	0	0
LC50 Values, mg/L (moving average method)		120(104-140)[c]	101(88.5-116)[c]

Test Conditions

Dissolved Oxygen Concentration
(0-192 hr) >70% saturation

pH Range
(0-192 hr) 7.9-8.2

Temperature Range
(0-192 hr) 24.8°C-25.8°C

Test Fish

Average Weight	0.22 g
Range of Standard Length	0.9-1.3 cm

[a]Mean ± standard deviation of combined duplicate tanks
[b]Detection limit, 6 mg/L
[c]95% Confidence interval

larvae at hatch and growth, as indexed by weight, showed no dose-related effects (Table 3). Average standard length was consistent among the treatment groups and controls with length ranging from 15.1 to 16.8 mm. Fish exposed to 72.7 mg/L were an average of 1 mm (7%) shorter than the controls. This difference is statistically significant ($\alpha = 0.05$) but has no practical biological implications and falls within the range of measurement resolution (i.e., 1 mm). The estimated MATC, based on survival, lies between 72.7 and 114 mg/L and is 91 mg/L expressed as the geometric mean of the high and low chronic values.

Test conditions during the 31 day exposure period were: temperature ranged from 24.9° to 26°C; dissolved oxygen concentration >73% saturation; and pH ranged from 7.6 to 8.2. Average measured concentrations of triclopyr TEA salt were $\geq$90% of predicted nominal concentrations.

Comparison of the 96 hr and 192 hr flow-through LC50 values (120 and 101 mg/L, respectively) shows that they differ by only a factor of 1.2. Examination of the concentration-mortality data from this test shows that 85% of all mortality occurred within the first 24 hours of exposure with additional mortality spread out over the subsequent 168 hours of exposure. These data indicate that triclopyr TEA salt solution may lack chronicity. Examination of the embryo-larval data supports this interpretation. The embryo-larval MATC is based on mortality of which >90% occurred during the first 14 days of the test. More subtle chronic end points such as embryonic development, hatchability of the embryos and growth, as indexed by weight, were not significantly different from the controls. Furthermore, the MATC, based on the geometric mean of the high and low chronic values, is 91 mg/L and falls within the 95% CI of the 192 hr flow-through LC50 (88.5-116 mg/L). The lack of a well defined sublethal response could be the result of the rapid excretion or metabolism of the triclopyr TEA salt (Tucker and Leitzke, 1979).

The hazard presented by triclopyr TEA salt to freshwater fish can be evaluated by the examination of data from a field study with triclopyr TEA salt. McKellar et al. (1982) measured residues of triclopyr in water following typical application of GARLON 3A, to an electrical transmission right-of-way. Application was by helicopter at a rate of 11.2 kg/ha. Residues of triclopyr in runoff water were followed at intervals through 510 days and ranged from nondetectable to 0.08 mg/L. The detected levels are at least 3 orders of magnitude less than those resulting in acute and/or chronic effects to the fathead minnow.

The results of this study show that triclopyr TEA salt is relatively non-toxic to fathead minnows. The concentration of triclopyr TEA salt which is toxic to the fathead minnow is well above expected environmental concentrations. Comparison of the acute and embryo-larval toxicity data indicate triclopyr TEA salt has little cummulative or chronic effect on the fathead minnow.

TABLE 3. Hatchability of Embryos, and Survival and Growth Measurements for Fathead Minnows Exposed to Triclopyr Triethylamine Salt, Means and Standard Deviation

Concentration, mg/L		Embryos[a] Hatched (%)	Normal Larvae[b] at Hatch (%)	Larval Survival[b] 28 Days (%)	Body Weight (mg)[c] 28 Days	Standard Length[c] (mm) 28 Days
Nominal	Measured					
0	N.D.[d]	95.0 ± 3.8	100.0 ± 0.0	79.0 ± 3.2	65.6 ± 2.5	16.2 ± 0.2
13	11.7 ± 0.7	95.0 ± 3.8	100.0 ± 0.0	78.8 ± 9.3	65.5 ± 5.9	16.1 ± 0.5
20	19.3 ± 1.0	95.0 ± 3.8	100.0 ± 0.0	84.3 ± 6.9	68.3 ± 6.0	16.3 ± 0.5
31	29.2 ± 0.7	99.0 ± 2	100.0 ± 0.0	67.3 ± 14.0	77.3 ± 12.9	16.8 ± 0.7
48	46.7 ± 0.6	92.0 ± 0.0	97.8 ± 2.5	77.2 ± 8.2	65.3 ± 7.4	16.0 ± 0.4
73	72.7 ± 1.0	93.0 ± 6.8	100.0 ± 0.0	65.9 ± 14.4	55.2 ± 8.6	15.1 ± 0.7*
112	114 ± 2.0	94.0 ± 4.0	97.9 ± 2.4	2.1 ± 4.2*	--	--

[a]Based on 25 embryos/replicate; 4 replicates/concentration. One replicate at 65 mg/L had only 24 embryos.

[b]Proportions analyzed after arcsine transformation.

[b]Based on number hatched

[c]Unweighted means and standard deviations of replicates; 4 replicate means. The 112 mg/L data not analyzed due to poor survival.

[d]Not detected; detection limit 6 mg/L.

*Significantly decreased from control at $\alpha = 0.05$, 2-tailed Dunnett's test.

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REFERENCES

Alexander, HC, Quick, JA, Bartlett, EA (1981) Static acute toxicity of sodium bromide to fathead minnows. Bull Environ Contam Toxicol 27:326-331

American Public Health Association, American Waterworks Association, Water Poluution Control Association (1980) Standard methods for the examination of water and wastewater. 15th edn., American Public Health Association, Washington, DC

American Society for Testing and Materials (1980) ASTM Standard 729-80, Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. ASTM, Philadelphia

Benoit, DA, Puglisi, FA, Olson, DL (1982) A fathead minnow Pimephales promelas early life stage toxicity test method evaluation and exposure to four organic chemicals. Environ Pollut 28:189-197

Finney, DJ (1971) Probit analysis, 3rd ed, Cambridge Univ Press, London

McKellar, RL, Schubert, OE, Byrd, BC, Stevens, LP, Norton, EJ (1982) Aerial application of GARLON® 3A herbicide to a West Virginia watershed. Down to Earth 38:15-19

McKim, JM (1977) Evaluation of tests with early life stages of fish for predicting long-term toxicity. J. Fish Res. Board Can 34:1148-1154

Mount, DI, Brungs, WA (1967) A simplified dosing apparatus for fish toxicology studies. Water Res 1:21-29

Sprague, JB (1969) Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Res 3:793-821

Thompson, WR (1947) Use of moving averages and interpolation to estimate median-effective dose. Bacteriol Rev 11:115-145

Tucker, RK, Leitzke, JS (1979) Comparative toxicology of insecticides for vertebrate wildlife and fish. Pharmac Ther 6:167-220

Winer, BJ (1971) Statistical principles in experimental design. 2nd ed, McGraw-Hill, New York

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