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MRID NO. 443648-02

DATA EVALUATION RECORD
FISH LIFE-CYCLE TOXICITY TEST
GUIDELINE 72-5

1. **CHEMICAL:** Chlorfenapyr **PC Code No.:** 129093

2. **TEST MATERIAL:** AC 303,630 technical **Purity:** 94.9%

3. **CITATION:**

Authors: J.H. Liu, F.J. Cunningham, M.R. Dunham, and J.D. Wisk
Title: Evaluation of the Toxicity of AC 303,630 During the Complete Life-Cycle of the Sheepshead Minnow (*Cyprinodon variegatus*) Under Flow-Through Test Conditions
Study Completion Date: March 31, 1997
Laboratory: Toxikon Environmental Sciences, Jupiter, FL
Laboratory Report ID: J9502004b
Sponsor: American Cyanamid Company, Princeton, NJ
MRID No.: 443648-02
DP Barcode: D239194

4. **REVIEWED BY:** William Evans, Biolgist
Ecological Hazard Branch
Environmental Fate and Effects Division
Signature: William Evans **Date:** 7/23/98

5. **APPROVED BY:** Thomas A. Bailey, Branch Chief
Ecological Hazard Branch
Environmental Fate and Effects Division
Signature: Tom A. Bailey **Date:** 8/31/98

6. **Study Parameters:**

Test Species: *Cyprinodon variegatus*
Age of Test Organism: <24 hours old embryos
Test Duration: 251 days
Study Method: Flow-Through
Type of Concentrations: Mean measured

7. **CONCLUSIONS:** Control solutions appeared to be contaminated

with AC 303,630 detected at concentrations ranging from 0.332 to 1.34 µg ai/L on Days 127, 175, and 189. In addition, measured concentrations were highly variable throughout the test. The measured concentrations in all treatment levels were more than 30% higher than the corresponding time-weighted average concentrations for more than 5% of the duration of the test (ranging from 6 to 11% of the test duration).

Although the solvent control did not appear to be contaminated, it is unclear how both controls could have mean measured concentrations reported to three significant figures and how the sensitivity of the analytical procedures could range from 0.05 to 0.3 µg/L. Since mean-measured concentrations were roughly 50% of nominal it is possible that the solvent control solution contained as much as 2.7 µg/L of AC 303,630.

It is also noteworthy that the study protocol was amended to reduce the number of turnover rates from 5 to between 3 to 4 in a 24 hour period. Aeration was required from day 14 onward to maintain a dissolved oxygen concentration above 60% saturation. This suggests that the reduction in flow rate may have played a factor in mean measured concentrations at 50% of the nominal concentrations.

Therefore, this study is not scientifically sound and does not fulfill the guideline requirements for a fish full life-cycle toxicity test using sheepshead minnows. This study is classified as **Invalid.** However, this study could be up-graded to supplemental if information on the limit of detection and quantification using the analytical technique employed could be submitted.

Results Synopsis:

Most Sensitive Endpoint: F_0 growth (8 week total length and wet weight)

NOEC: N/A
LOEC: N/A
MATC: N/A

8. **ADEQUACY OF THE STUDY:**

A. Classification: Invalid

B. Rationale: Control contamination and measured

concentrations of all treatment levels were highly variable during the test.

C. **Repairability:** No.

9. **GUIDELINE DEVIATIONS:**

1. Control solutions appeared to be contaminated with AC 303,630 at concentrations ranging from 0.332 to 1.34 µg ai/L on Days 127, 175, and 189.

2. Measured concentrations were highly variable throughout the test. Both controls had mean measured concentrations reported to three significant figures and the sensitivity of the analytical procedures ranged from 0.05 to 0.3 µg.L. In addition, the measured concentrations in all treatment levels were more than 30% higher than the corresponding time-weighted average concentrations for more than 5% of the duration of the test.

3. DO levels (50 - >100% of saturation) were periodically less than recommended (>75% of saturation).

4. The test consisted of only two replicates; four replicates are recommended.

5. Photoperiod was maintained at 12 hours light per day; 16 hours per day is recommended.

6. On exposure day 28, it was noted that 26 fish were present in four of the incubation cups rather than the recommended 25. The authors attributed the mistake to an error during the culling phase, and calculated survival based on the total number of fish.

7. Lengths and weights of F_0 fish were measured at 3 and 7 weeks post-hatch rather than 4 and 8 weeks post-hatch.

8. Spawning groups consisted of 2 male and 5 female adults; 4 males and 4 females are recommended.

9. In the second generation embryo exposure period, different numbers of embryos from each concentration level were incubated rather than the recommended 50.

10. At the initiation of the second-generation larval-juvenile exposure period (4-8 weeks), the number of larvae was not reduced to 25 per replicate growth chamber as recommended.

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11. A malfunction with the laboratory's saltwater system resulted in daily salinity values of 25-28 ‰ (Days 31-34), 18 ‰ (Day 210), and 27 ‰ (Day 213). During these malfunctions, the weekly range of salinity exceeded 6%.

10. **SUBMISSION PURPOSE:**

11. **MATERIALS AND METHODS:**

A. Biological System:

Guideline Criteria	Reported Information
Species: Prefer sheepshead minnow (*Cyprinodon variegatus*) or fathead minnow (*Pimephales promelas*).	*Cyprinodon variegatus*
Source and acclimation	Embryos were obtained from in-house brood stock and were maintained at conditions similar to the test.
Age at beginning of test: Embryos 2 to 24 hours old	<24 hours old
Feeding: Fish should be fed at least twice daily and should not be fed for at least 24 hours prior to test termination.	Newly hatched fry received live platinum brine shrimp or fatty-acid supplemented brine shrimp nauplii and commercial salmon starter at least twice daily. After day 28, fish were fed commercial salmon starter alone. Starting from day 47, fish were fed commercial flake food twice daily, with feedings increasing as fish matured.

Guideline Criteria	Reported Information
Embryo Exposure (Four-Five Days): Embryos (≤24 hours old) from at least 3 separate spawns should be randomly distributed to embryo cups. A minimum of 50 embryos (≤24 hrs old) per replicate cup, 4 cups per treatment should be used. <u>Parameters measured</u>: · Survival of embryos · Time required to hatch · Hatching success · Survival of fry for 4 weeks Dead and fungused embryos should be counted and removed daily.	Eggs were stripped from 50 adult females and fertilized with a sperm suspension from 4 adult males. Embryos (<24 hours old) were randomly distributed to embryo cups. 50 embryos per cup (randomly assigned two at a time); 2 cups per replicate aquarium; 2 replicate aquaria per treatment and control. All parameters listed at left were measured. Dead embryos were recorded and removed daily until hatching was complete.
Larval-Juvenile Exposure (From Hatch to 8 Weeks): After hatching, each group of larvae is randomly reduced to a minimum of 25 fish and released in replicate larval growth chambers. The random selection must include any fish that are lethargic or deformed. <u>Parameters measured</u>: Fish survival (determined by counting the number of live fish in each replicate growth chamber weekly). · Total lengths (mm) of all fish at 4 and 8 weeks after hatching.	After hatching was complete (day 6), larvae in the two incubation cups were impartially reduced to 25 and released into the larval growth chamber. After 56 days (post-hatch), fish in the two larval tanks were impartially reduced to 25 and released into the replicate test chamber. Both parameters at left were measured.

Guideline Criteria	Reported Information
Juvenile-Adult Exposure (From 8 wks posthatch to the end of the spawning phase [32-40 wks]): At 20-24 weeks after hatching, mature fish are placed in a spawning tank of the same concentration (4 males and 4 females randomly chosen and assigned). The spawning tank is divided into 4 individual spawning chambers with appropriate spawning substrates. The substrates are examined daily and embryos removed, counted, and recorded separately for each pair. For fathead minnow, adult exposure should be terminated when no spawning occurs for one week. For sheepshead minnow, testing should be terminated after spawning is observed for 2 weeks.	At Day 119, 2 male and 5 female adults from each control and treatment were transferred to a spawning chamber. Each spawning chamber, suspended from the sides of the test tank, consisted of two 3-gallon polyethylene pails with their bottoms removed. The pail that contained the adult fish had a 7 mm plastic screen mesh attached to the bottom, while the other pail had a 353 μm screen mesh on the bottom. The pail with fish was placed inside the pail without fish, and as fish spawned, the eggs passed through the mesh in the first pail and were contained in the second pail. Spawning chambers were removed and examined daily for newly spawned eggs, which were removed and counted. During spawning, any dead fish were removed, but not replaced. Adult exposure was terminated after four spawning sessions of at least 14 days each.
Second Generation Embryo Exposure (4-5 days): 50 embryos from each conc. level are randomly selected and transferred to incubation cups for hatch. Use the same test procedures as those for parental generation. Embryos not selected are discarded.	When possible, 100 embryos per treatment (50/replicate) were incubated and the percent hatch was determined. Not reported.

Guideline Criteria	Reported Information
Second Generation Larval-Juvenile Exposure (From Hatch to 4-8 wks): After hatching, 25 larvae are released in each growth chambers (2 chambers per treatment). Each group of 2nd generation fish is terminated 8 wks after hatching. Fish are blotted, weighed, and measured before being discarded.	After hatching, larvae were transferred into screen retention chambers within each replicate test tank (2 tanks per treatment). After 8 wks of post-hatch exposure the 2nd generation fish were terminated. Fish were measured for individual length and wet weight.

Comments:
F_1: Due to low survival of juvenile fish during two previous attempts at initiating the F_1 phase of the study, each replicate embryo chamber was placed in a solution of 10 percent formalin in seawater for 10 minutes to prevent mycobacterium infection. The number of embryos in each incubation cup was not equal, nor were the number of fry in each retention chamber equal during the F_1 juvenile exposure period.

B. **Physical System:**

Guideline Criteria	Reported Information
Test Water: Sheepshead Minnow 1. Natural seawater (sterilized and filtered) or a commercial mixture. 2. Natural seawater with a salinity of ≥15 parts per thousand (weekly range of salinity <6% and monthly pH range <0.8 pH units). Fathead Minnow 1. Reconstituted water or water from unpolluted well or spring (sterilized and tested for pollutants). 2. Hardness of 40 to 48 mg/L as $CaCO_3$ and pH of 7.2 to 7.6.	1. Natural saltwater pumped from a shallow well, then filtered and sterilized. 2. Salinity range of 18-28 ‰ and pH range of 7.7 to 8.2 during the test. N/A
Test Temperature: Fathead: 25°C and should not remain outside the range of 24 to 26°C for more than 48 hours. Sheepshead: 30°C.	Daily mean range: 26.4 - 30.9°C
Photoperiod: 16-hour light/8-hour dark. Light intensity of 10-100 lumens at water surface.	12-hour light/12-hour dark. 5.3 - 7.1 microEinsteins/m^2.

Guideline Criteria	Reported Information
Dosing Apparatus: 1. Intermittent flow proportional diluters or continuous flow serial diluters. 2. A minimum of 5 toxicant concentrations with a dilution factor ≤0.5. 3. One control should be used.	1. Proportional vacuum-siphon diluter. 2. Five with a dilution factor of 0.5. 3. A dilution water control and a solvent control.
Toxicant Mixing: 1. Mixing chamber recommended but not required. 2. Test solution completely mixed before introduction into the test system (aeration should not be used for mixing). 3. Flow splitting accuracy must be within 10% and periodically checked.	1. Mixing chambers were used 2. Not reported. 3. Flow splitting accuracy was calibrated to within 5%.
Exposure System/Test Vessels: Exposure tanks should be all glass or glass with a plastic or stainless steel frame (30.5 x 30.5 x 91.4 cm or 30.5 x 30.5 x 61 cm for fathead, and 45 x 90 x 26 cm for sheephead). Larval chambers should have glass bottoms and drains that allow water to be drawn down to 3 cm. Test water depth in adult tanks and larval chambers should be a minimum of 15 cm.	Adult glass tanks were 90 X 29 X 30.5 cm (80-L) with a test solution volume of 68 L. Glass larval chambers (28 X 11.5 X 15 cm) with approximately 4.0 L of solution were suspended within each adult tank. 26 cm maximum depth within adult tanks; 12.5 cm maximum depth within larval chambers.

Guideline Criteria	Reported Information
Embryo and Fry Chambers: 120 ml glass jars with bottoms replaced with 40 mesh stainless steel or nylon screen. Chambers can be oscillated vertically using rocker arm apparatus (2 rpm motor) or placed in separate chambers with self-starting siphons.	Incubation chambers were 60 mm diameter glass tubes with nylon mesh (355-μm) screen bottoms. Initial fry chambers consisted of 13 X 90 mm petri dish bottoms with an 18 cm high collar of 355 μm Nitex® screen; after complete hatching, fry transferred to 150 X 10 mm petri dish bottoms with a 20 cm high Nitex® screen collar. Self-starting siphons provided raising and lowering of test solution.
Flow Rate: Flow rates to larval cups should provide 90% replacement in 8-12 hours, and maintain DO at above 75% of saturation. The toxicant level cannot drop below 20% with fish in the tank.	Flow rate to larval cups provided 40.8 volume additions every 24 hours. Toxicant levels measured in the tank ranged from 44-60% of nominal. Mean DO levels ranged from 3.4 - 7.8 mg/L (50-115% saturation).
Aeration: Dilution water should be aerated to insure DO concentration at or near 100% saturation. Test tanks and embryo chambers should not be aerated.	All control and test solutions were aerated from day 14 through test termination to keep DO levels above 60% saturation.

C. <u>Chemical System</u>:

Guideline Criteria	Reported Information
Concentrations: Minimum of 5 concentrations and a control, all replicated; plus solvent control if appropriate. Toxicant conc. must be measured in one tank at each toxicant level every week.	A dilution water control, solvent control and 5 treatment concentrations: 1.90, 3.80, 7.50, 15.0, and 30.0 µg ai/L. Test solutions were sampled and measured once a week from each replicate aquarium of the controls and treatments (replicates were composited). During the reproductive and F_1 phases, samples were taken from the incubation chambers or diluter taps prior to entering test aquaria.
Other Variables: 1. DO must be measured at each conc. at least once a week. 2. Test water temp. must be recorded continuously. 3. Freshwater: A control and one conc. must be analyzed weekly for pH, alkalinity, hardness, and conductance. Natural seawater: must maintain a constant salinity and not fluctuate more than 6% weekly; monthly pH range <0.8 pH units.	1. DO and pH measured at test initiation and termination, and weekly in all test solutions. 2. Temperature measured hourly in both replicates of the negative control and also measured continuously in the water bath. 3. Salinity fluctuated >6% during Week 4 and Week 30 when the saltwater system malfunction occurred. The monthly pH range was <0.8 pH units.
Solvents: Should not exceed 0.1 ml/L in a flow-through system. Acceptable solvents are: dimethylformamide, triethylene glycol, methanol, acetone, ethanol.	Solvent conc.: 0.0067 mL/L Solvent: dimethylformamide (DMF)

12. REPORTED RESULTS:

Guideline Criteria	Reported Information
Data Endpoints must include: · survival of F_0 and F_1 embryos, time required to hatch, and hatching success; · survival and total length of F_0 fish at 4 and 8 weeks after hatching; · weights and lengths of F_1 fish at 8 weeks; · incidence of pathological or histological effects; and · observations of other effects or clinical signs.	All biological parameters listed at left with the exception of time required to hatch. Other endpoints include: · Total number eggs · Number eggs/female reproductive day

F_0 Results:

Nominal Conc. (μg ai/L)	Mean Measured Conc. (μg ai/L)	% Hatch	Day 28 (3 wks post-hatch) % Survival	Day 56 (7 wks post-hatch) % Survival	Day 187 (F_0 termination) % Survival*
Control	<0.50**	90	100	98	72
Solvent Control	<0.50	86	100	99	84
1.90	1.01	89	100	99	80
3.80	1.91	87	100	97	74
7.50	3.27	81	100	100	69
15.0	6.97	88	100	99	80
30.0	18.1	87	53	52	73

*Fish were impartially reduced to approximately 25 per replicate chamber where possible on Day 56.

**Control solutions appeared to be contaminated on Days 127, 175, and 189 (AC 303,630 was detected at concentrations ranging from 0.332 to 1.34 μg/L).

Mean MeasuredConc. (µg ai/L)	Day 28 Length (mm)	Day 56 Length (mm)	Day 56 Weight (g)	Test Termination Length (mm)	Test Termination Weight (g)
Control	17.8	26.0	0.29	53.3	2.57
Solvent Control	17.6	26.5	0.29	55.1	2.95
1.01	17.6	26.4	0.27	52.9	2.59
1.91	17.7	25.4	0.24	52.5	2.33
3.27	17.2	25.3	0.24	54.2	2.75
6.97	16.8	25.2	0.24	52.8	2.39
18.1	16.8	-	-	57.0	3.30

*None of the surviving fish at the 18.1 µg ai/L treatment were sacrificed for length and weight measurements.

Mean Measured Conc. (µg ai/L)	Total Number Eggs	Total Number of Reproductive Days	Number Eggs/ Female Reproductive Day
Control	6154	296	20.8
Solvent Control	10490	333	31.5
1.01	6579	351	18.7
1.91	6470	292	22.2
3.27	8044	292	27.5
6.97	10287	371	27.7
18.1	4713	214	22.0

F_1 Results:

Mean Measured Conc. (µg ai/L)	% Hatch	4-week Post-Hatch % Survival	4-week Post-Hatch Length (mm)	8-week Post-Hatch % Survival	8-week Post-Hatch Length (mm)	8-week Post-Hatch Wet Weight (g)

Control	93	63	21.3	60	27.2	0.34
Solvent Control	92	46	20.0	43	29.8	0.47
1.01	100	78	20.7	76	28.3	0.38
1.91	72	99	21.1	98	28.3	0.37
3.27	86	62	20.1	62	27.5	0.35
6.97	100	39	19.2	38	29.2	0.42
18.1	-*	-	-	-	-	-

*No embryos from the 18.1 μg ai/L treatment were incubated.

Toxicity Observations: There were no physical abnormalities produced or apparent behavioral changes in the F_0 or F_1 fish as a result of exposure to AC 303,630.

Statistical Results:

Statistical Method: Fisher's Exact Test or Student's t-test were used for continuous data (length and weight) and Dunnett's Test was used for survival and hatching success.

Biological Endpoint	NOEC (μg ai/L)	LOEC (μg ai/L)
F_0 hatching success	18.1	>18.1
F_0 28-day survival	6.97	18.1
F_0 28-day length	1.91	3.27
F_0 56-day survival	6.97	18.1
F_0 56-day length	1.01	1.91
F_0 56-day wet weight	1.01	1.91
F_0 termination (187 days) survival	18.1	>18.1
F_0 termination length	18.1	>18.1
F_0 termination wet weight	18.1	>18.1
F_0 eggs/female repro. day	18.1	>18.1
F_1 hatching success	6.97	>6.97
F_1 4-week survival	6.97	>6.97
F_1 4-week length	6.97	>6.97
F_1 8-week survival	6.97	>6.97
F_1 8-week length	6.97	>6.97
F_1 8-week wet weight	6.97	>6.97

NOEC: 1.01 μg ai/L **LOEC:** 1.91 μg ai/L **MATC:** 1.39 μg ai/L

Comments: At test day 56, total length of F_0 fish were determined both photographically and by measuring sacrificed fish. Statistical differences were found in total length between the solvent control and all treatments as determined by photographs.

Using the measured lengths of sacrificed fish, no statistical difference was observed between the 1.01 μg ai/L treatment and the solvent control. Therefore, the NOEC and LOEC for effects on day-56 total lengths were 1.01 and 1.91 μg ai/L, respectively.

The authors reported no treatment-related effects on total lengths or wet weights of F_0 fish at termination. A significant reduction in wet weight was observed between the pooled control and the 1.91 µg ai/L treatment, but not at the 3.27 or 6.97 µg ai/L treatments. Therefore, the reduction in wet weight at the 1.91 µg ai/L concentration was not considered treatment-related.

The authors reported significant differences compared to the solvent control in 8-week total lengths and wet weights of F_1 fish in the 1.01, 1.91, and 3.27 µg ai/L treatments, but not in the 6.97 µg ai/L treatment. They attributed the difference to differing densities of fish in the chambers, allowing some fish more food and increased growth. The number of F_1 fish per replicate after hatching ranged from 42 to 142. Guideline recommendations state that the number of fish in each replicate chamber is to be culled to 25 individuals after hatching. The fewest number of fish were established in the solvent control and the 6.97 µg ai/L treatments, which resulted in higher wet weights and total lengths in fish at these treatments.

13. **REVIEWER'S STATISTICAL RESULTS:**

Statistical Method: Since it has been concluded that this study is not scientifically sound and does not fulfill the guideline requirements for a fish full lifecycle study, statistical analysis was not performed.

Biological Endpoint	NOEC (µg-ai/L)	LOEC (µg ai/L)
F_0 hatching success	-	-
F_0 28-day survival	-	-
F_0 28-day length	-	-
F_0 56-day survival	-	-
F_0 56-day length	-	-
F_0 56-day wet weight	-	-
F_0 termination (187 days) survival	-	-
F_0 termination length	-	-
F_0 termination wet weight	-	-
F_0 eggs/female repro. day	-	-
F_1 hatching success	-	-

Biological Endpoint	NOEC (µg-ai/L)	LOEC (µg ai/L)
F_1 4 week survival	-	-
F_1 4 week length	-	-
F_1 8 week survival	-	-
F_1 8 week length	-	-
F_1 8 week wet weight	-	

Most sensitive endpoint(s):

NOEC: **LOEC:** **MATC:**

Comments:

14. **REVIEWER'S COMMENTS:** Control solutions appeared to be contaminated with AC 303,630 detected at concentrations ranging from 0.332 to 1.34 µg ai/L on Days 127, 175, and 189. In addition, measured concentrations were highly variable throughout the test. The measured concentrations in all treatment levels were more than 30% higher than the corresponding time-weighted average concentrations for more than 5% of the duration of the test (ranging from 6 to 11% of the test duration).

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    It is also noteworthy that the study protocol was amended to reduce the number of turnover rates from 5 to between 3 to 4 in a 24 hour period. Aeration was required from day 14 onward to maintain a dissolved oxygen concentration above 60% saturation. This suggests that the reduction in flow rate may have played a factor in mean measured concentrations at 50% of the nominal concentrations.

    Therefore, this study is not scientifically sound and does not fulfill the guideline requirements for a fish full life-cycle toxicity test using sheepshead minnows. This study is classified as **Invalid**. However, this study could be up-

graded to supplemental if information on the limit of detection and quantification using the analytical technique employed could be submitted.