DATA EVALUATION RECORD
FISH EARLY LIFE-STAGE TOXICITY TEST
§72-4(A)

1. **CHEMICAL:** Fentin hydroxide (TPTH) PC Code No.: 083601

2. **TEST MATERIAL:** TPTH Technical Purity: 97.5%

3. **CITATION:**

Author: Sousa, J.

Title: TPTH Technical - Early Life-Stage Toxicity Test with Sheepshead Minnow (*Cyprinodon variegatus*).

Study Completion Date: November 9, 2000

Laboratory: Springborn Laboratories, Inc.
790 Main Street
Wareham, Massachusetts 02571-1075

Sponsor: TPTH Task Force
c/o Landis International, Inc.
3185 Madison Highway
Valdosta, GA 31603-5126

Laboratory Report ID: 13733.6101

MRID No.: 45276001

DP Barcode: D271841

4. **REVIEWED BY:** Christie E. Padova, Staff Scientist, Dynamac Corporation

Signature: C. E. Padova **Date:** 4/17/01

APPROVED BY: Kathleen Ferguson, Ph.D., Senior Staff Scientist, Dynamac Corporation

Signature: Kathleen Ferguson **Date:** 4/17/01

5. **APPROVED BY:** Tom Steeger, Fishery Biologist, OPP/EFED/ERBIV

Signature: Thomas M. Steeger **Date:** 8/21/02

6. STUDY PARAMETERS:

Scientific Name of Test Organism: *Cyprinodon variegatus*

Age or Size of Test Organism: Embryos, ≤25 hours old

Definitive Test Duration: 33 Days

Study Method: Flow-through

Type of Concentrations: Mean-measured

7. CONCLUSIONS:

In an early life-stage toxicity test, groups of Sheepshead minnow embryos and subsequently fry were exposed for 33 days under flow-through conditions to TPTH Technical (97.5% purity) at mean-measured concentrations of 0 (negative control), 0.21, 0.42, 0.76, 1.4, and 2.7 µg a.i./L. There were 80 embryos per cup, one cup per vessel, and two replicate vessels per concentration (160 total embryos per level). On Day 5, after hatching was deemed complete, surviving fish were thinned to 40 fish per replicate (80 total fish per level).

The end of the hatching period was defined as Day 5; however, the initiation of the hatching period was not defined, and hatch-time was not compared statistically to controls. Hatch survival rates averaged 86-88%, and terminal (Day 33) survival rates averaged 96-99%. No statistical significance was observed at either endpoint. Furthermore, no sublethal effects were observed in fish from any control or treatment level during the study.

Terminal growth measurements, both length and weight, were statistically-reduced at the 1.4 and 2.7 µg a.i./L levels. Mean lengths averaged 25.3, 24.5, 24.8, 24.8, 24.1, and 23.4 mm for the control, 0.21, 0.42, 0.76, 1.4, and 2.7 µg a.i./L levels, respectively. Mean wet weights averaged 252, 221, 228, 228, 203, and 187 mg for the control, 0.21, 0.42, 0.76, 1.4, and 2.7 µg a.i./L levels, respectively.

Statistical verification by the reviewer revealed that hatching success and larval survival were not adversely affected by treatment. Length, dry weight, and wet weight were significantly reduced at all concentrations. **Thus, no NOEC could be determined. The NOEC was estimated to be lower than 0.21 µg a.i./L, the lowest concentration tested. The LOEC equaled 0.21 µg a.i./L, the lowest concentration tested. No MATC could be determined.**

This study is classified as SUPPLEMENTAL. It is scientifically valid and follows the guidelines for a fish early life-stage toxicity test using the Sheepshead minnow [Subdivision E, §72-4(a)]. However, the study failed to establish an NOEC.

8. ADEQUACY OF THE STUDY:

A. Classification: Supplemental

B. Rationale: Study failed to establish a chronic toxicity endpoint, i.e., NOEC. The NOEC could not be identified because endpoints were less than the lowest dose tested in this study.

C. Repairability: Conduct another experiment that tests concentrations lower than 0.21 µg a.i./L for purposes of defining a NOEC/MATC.

9. GUIDELINE DEVIATIONS:

1. Recovery of TPTH Technical from the exposure solutions was low, averaging 55 to 76% of the nominal levels.

2. Only two replicate vessels per concentration were used for embryo exposure; guideline requirements state 4 replicate vessels per concentration should be used.

3. Mean organism responses were used in all statistical calculations, including evaluation of growth data (length and weight).

4. The initiation of the hatching period was not defined, and hatch-time was not compared statistically to controls.

5. The flow splitting accuracy was not reported.

6. Aeration of the dilution water was not described.

10. SUBMISSION PURPOSE: To determine the effects of TPTH technical on sheepshead minnows (*Cyprinodon variegatus*) embryos and larvae during continuous aqueous exposure.

11. MATERIALS AND METHODS:

A. Test Organisms

Guideline Criteria	Reported Information
Species Any of several freshwater or estuarine fish species, including Rainbow trout, Brook trout, Bluegill sunfish, Fathead minnow, Channel catfish, Sheepshead minnow, and Silverside species.	Sheepshead minnow (*Cyprinodon variegatus*); loading rate 0.15 g/L
Supplier	Embryos were obtained from broodstock maintained at Aquatic BioSystems, Inc., Ft. Collins, CO.
Age at beginning of test Embryos, 2 to 24 hours old	≤25 Hours old

B. Test System

Guideline Criteria	Reported Information
Source of dilution water May be natural or reconstituted. Natural water should be sterilized with UV and tested for pesticides, heavy metals, and other possible contaminants.	Natural filtered seawater from Cape Cod Canal, Bourne, Massachusetts.
Does water support test animals without observable signs of stress?	Yes
Water Temperature 25-30°C; should not deviate by more than 2°C	24.6-26.0°C
pH pH should not fluctuate by more than 0.8 pH units per month	7.7-8.0
Dissolved Oxygen >75% saturation	5.2-7.6 mg/L, whereas the theoretical dissolved oxygen concentration at saturation is 6.8 mg/L at 25°C and 33‰ salinity.

Guideline Criteria	Reported Information
Salinity ≥15‰; salinity must not fluctuate by more than 6% weekly	31-33‰
Photoperiod Recommend 16 hours light, 8 hours dark	16 hours light, 8 hours dark
Test Aquaria 1. Material: Glass or glass with stainless steel frame 2. Fill depth: Generally 15-30 cm	1. Glass aquaria, 39 x 20 x 25 cm 2. 14.5 cm depth (approximately 11 L)
Embryo Cups 120 mL glass jars with bottoms replaced with 40 mesh stainless steel or nylon screen.	Glass jars, 5 cm diameter, 8 cm height (157 mL), with 40-mesh Nitex screen bottoms. The cylinders were slowly oscillated to ensure adequate flow of media around the embryos.
Type of Dilution System Intermittent flow proportional diluters or continuous flow serial diluters should be used.	A continuous-flow serial dilution system was used. A peristaltic pump delivered 1.776 mL/minute of a 0.56 µg a.i./mL stock solution into the diluter system's chemical mixing chamber, where it was mixed with 0.200 L of dilution water per minute (equivalent to 5.0 µg a.i./L) and serially diluted (50%) to produce the remaining nominal test concentrations.
Toxicant Mixing 1. Mixing chamber is recommended but not required; aeration should not be used for mixing. 2. If a mixing chamber was not employed, was it demonstrated that the test solution was completely mixed before introduction into the test system? 3. Was flow splitting accuracy within 10%?	1. A single mixing chamber was employed; test solutions were not aerated. 2. N/A 3. Not reported.

Guideline Criteria	Reported Information
Flow Rate Flow rates to larval cups should provide 90% replacement in 8-12 hours. Flow rate must maintain DO at above 75% of saturation and maintain the toxicant level.	6.5 aquaria replacements per day; 90% replacement in approximately 8 hours
Solvents Not to exceed 0.1 mL/L	N/A
Aeration Dilution water should be aerated to insure dissolved oxygen concentrations at or near 100% saturation. Test tanks and embryo cups should not be aerated.	Not specified

C. Test Design

Guideline Criteria	Reported Information
Nominal Concentrations At least one control, a solvent control (when applicable), and 5 treatment levels, one of which must adversely affect a life stage and one must not affect any life stage.	0 (negative control), 0.32, 0.63, 1.3, 2.5, and 5.0 µg a.i./L
Replicates Minimum of 20 embryos per replicate cup, 4 replicates per concentration. Minimum of 30 fish per treatment for post-hatch exposure.	80 Embryos per cup, one cup per vessel, and two replicate vessels per concentration (160 total embryos per level). On Day 5, surviving fish were thinned to 40 organisms/replicate (80 total fish per level).
Post Hatch % of embryos that produce live fry must be ≥50% in each control; % hatch in any control embryo cup must be no more than 1.6 times that in another control cup.	On Day 5, percent hatch from the two negative control replicates averaged 86.25%, with a 1.16 ratio between the replicates.
Feeding Fish should be fed at least twice daily. Fish should not be fed for at least 24 hr prior to termination on day 32.	Beginning on Day 5, the fish were offered *ad libitum* live *Artemia salina* three times daily. Feeding was discontinued approximately 24 hours to test termination.
Counts At a minimum, live fish should be counted 11, 18, 25, and 32 days after hatching.	Prior to hatching, embryos were counted daily. Following hatching, live organisms were estimated at least twice weekly. Behavior and appearance of larvae were observed daily.

Guideline Criteria	Reported Information
Controls Negative control and carrier control (when applicable) are required. Average survival at end of test must be ≥80%. Survival in any control chamber must not be < 70%.	A negative control was included. From end of hatch (Day 5) to study termination (Day 33), control survival was 97.5% in each replicate vessel.
Water Parameter Measurements 1. DO must be measured at each concentration at least once a week; 2. Salinity and pH	1. Measured daily in each replicate aquaria. 2. pH and salinity were measured daily in each replicate aquaria.
Chemical Analysis Toxicant concentration must be measured in one tank at each toxicant level every week.	Measured (HPLC/UV) in each replicate aquaria on Days 0, 6, 13, 20, 27, and 33 (day 0, 1, 8, 15, 22 and 28 post-hatch)

12. REPORTED RESULTS:

A. General Results

Guideline Criteria	Reported Information
Quality assurance and GLP compliance statement were included in the report?	Yes
Data Endpoints must include - Number of embryos hatched; - Time to hatch; - Mortality of embryos, larvae, and juveniles; - Time to swim-up (if appropriate); - Measurement of growth; - Incidence of pathological or histological effects; - Observations of other effects or clinical signs.	Data endpoints included: - Hatch survival (Day 5) - Terminal larval survival (Day 33) - Terminal length and weight (Day 33) - Sublethal effects

Guideline Criteria	Reported Information
Raw data included?	Yes, excerpted.

Survival and Time-line Data

Concentration (µg a.i./L)		Initial Number of Embryos (Day 0)	Percent Hatch Survival (Day 5)[a]	Number of Day 5 Fish (Post-thinning)	Study Termination (Day 33)		
Nominal	Mean Measured (% nominal)				Percent Survival[b]	Mean Length (mm)	Mean Wet Weight (g)
Control	—	160	86	80	98	25.3	252
0.32	0.21 (65)	160	88	80	96	24.5#	221#
0.63	0.42 (67)	160	87	80	99	24.8#	228#
1.3	0.76 (58)	160	86	80	99	24.8#	228#
2.5	1.4 (55)	160	88	80	99	24.1*#	203*#
5.0	2.7 (55)	160	88	80	98	23.4*#	187*#

*Statistically significant from control at $p<0.05$
[a]Relative to Day 0 embryos
[b]Relative to Day 5 fish (post-thinning)
#statistically significant ($p < 0.05$) using William's test
Percent recovery: 99.5 ± 6.76%

Toxicity Observations: No adverse effects were observed in any control or treatment level.

B. Statistical Results

Statistical Methods: Hatch survival (Day 5), larval survival (Day 33), and standard length and wet weight of surviving fry at test termination (Day 33) were subject to statistical analysis. Mean organism responses were used in all calculations. Comparisons for embryo survival and larval survival were performed using the Williams' Test. Statistical comparison for larval growth was performed using Dunnett's Test. Analyses were conducted at the 95% level of certainty.

Most sensitive endpoint: The study author reported that larval growth (weight and length) was the most sensitive indicator of the toxicity of TPTH Technical to Sheepshead minnow. The NOEL was 0.76 µg a.i./L, the LOEL was 1.4 µg a.i./L, and the MATC was estimated to be 1.0 µg a.i./L.

13. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method: Data were assessed for normality and homogeneity of variance prior to subsequent analysis. Williams' test was used to detect adverse effects of treatment. TOXSTAT software was used for all statistical tests.

Results Synopsis:
NOEC: <0.21 µg a.i./L
LOEC: 0.21 µg a.i./L
MATC: could not be determined

14. REVIEWER'S COMMENTS:

The study author reported that the most sensitive endpoint was growth (length and weight), and concluded that the NOEL was 0.76 µg a.i./L, the LOEL was 1.4 µg a.i./L, and the MATC was estimated to be 1.0 µg a.i./L. Statistical verification by the reviewer revealed that hatching success and larval survival were not adversely affected by treatment. Length, dry weight, and wet weight were significantly reduced at all concentrations. Thus, no NOEC could be determined. The NOEC was estimated to be lower than 0.21 µg a.i./L, the lowest concentration tested. The LOEC equaled 0.21 µg a.i./L, the lowest concentration tested. No MATC could be determined.

The reviewer's conclusions differed from those of the study author. Due to the use of a less powerful test, the study author failed to detect significant adverse effects of treatment at the lowest tested concentration for the three growth endpoints (length, dry weight, and wet weight). Thus, different estimates of the NOEC, LOEC, and MATC resulted. To be conservative, the reviewer recommends using the lower estimates.

The end of the hatching period was defined as Day 5; however, the initiation of the hatching period was not defined, and hatch-time was not compared statistically to controls. No treatment-related effect on hatch survival was observed, with averages of 86-88% of initially-exposed embryos. No sublethal effects were observed in any control or treatment level during the study. Terminal (Day 33) survival rates averaged 96-99%, with no statistical significance.

Compared to controls, terminal growth measurements were statistically-reduced at the 1.4 and 2.7 µg a.i./L levels. Mean lengths averaged 25.3, 24.5, 24.8, 24.8, 24.1, and 23.4 mm for the control, 0.21, 0.42, 0.76, 1.4, and 2.7 µg a.i./L levels, respectively. Mean wet weights averaged 252, 221, 228, 228, 203, and 187 mg for the control, 0.21, 0.42, 0.76, 1.4, and 2.7 µg a.i./L levels, respectively. Dry weights supported the same conclusions.

In this early life-stage toxicity test, Sheepshead minnow embryos and fry were exposed for 33 days to TPTH Technical (97.5% purity) at mean-measured concentrations of 0 (negative

control), 0.21, 0.42, 0.76, 1.4, and 2.7 µg a.i./L. Although recoveries of TPTH Technical from test solutions during the definitive study averaged 55-76% of nominal levels, sufficient data were provided which indicated that the integrity of the study was not compromised. This phenomenon was observed during preliminary testing and discussed with EPA via the Rapid Response Committee. The study authors reported that TPTH adsorbed "on the glass and silicon surfaces" of the test aquaria. Several factors indicate that this study represents a "best effort":

- All test aquaria exhibited a similar response.
- The variability of the measured concentrations is acceptable, with ratios ranging from 1.15 to 1.48.
- A statistically-valid endpoint was derived.
- Preliminary study data were provided.
- QC samples run concurrently with the definitive experiment resulted in recoveries of 84.3 to 110% of nominal fortified levels.
- Method validation samples using artificial seawater and analyzed prior to the start of the definitive experiment established an average recovery of 99.5 ± 6.76%.
- Recoveries of TPTH from the actual stock solutions ranged from 86-104% of nominal.

Only two replicate vessels per concentration were used for embryo exposure, instead of the required four. This deviation does not appear to have a significant effect on the results of the study and is considered minor. However, in future studies, scientifically-valid explanations should be provided justifying the use of only two replicates.

Mean organism responses were used in all statistical calculations, including evaluation of growth data (length and weight). However, raw growth data were provided in the MRID allowing for verification of statistical results.

The flow splitting accuracy was not reported. However, analysis of the negative control, and the high, middle, and low test concentrations twice prior to the start of the definitive exposure indicated that sufficient quantities of test substance were being delivered and maintained in the exposure aquaria.

Although aeration of the dilution water was not described, the dissolved oxygen levels remained sufficient throughout the definitive study.

Signed and dated GLP and Quality Assurance statements were provided.

15. REVIEWER'S STATISTICAL RESULTS:

6001 percent survival
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WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	2	86.500	86.500	86.500
2	.21	2	88.500	88.500	87.167
3	.42	2	87.000	87.000	87.167
4	.76	2	86.000	86.000	87.167
5	1.4	2	88.000	88.000	87.750
6	2.7	2	87.500	87.500	87.750

6001 percent survival
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WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
control	86.500				
.21	87.167	0.159		1.94	k= 1, v= 6
.42	87.167	0.159		2.06	k= 2, v= 6
.76	87.167	0.159		2.10	k= 3, v= 6
1.4	87.750	0.298		2.12	k= 4, v= 6
2.7	87.750	0.298		2.13	k= 5, v= 6

s = 4.193
Note: df used for table values are approximate when v > 20.

6001 larval survival
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WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	2	98.000	98.000	98.300
2	.21	2	96.500	96.500	98.300
3	.42	2	99.000	99.000	98.300
4	.76	2	99.000	99.000	98.300
5	1.4	2	99.000	99.000	98.300
6	2.7	2	97.500	97.500	97.500

6001 larval survival
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WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM

control	98.300			
.21	98.300	0.112	1.94	k= 1, v= 6
.42	98.300	0.112	2.06	k= 2, v= 6
.76	98.300	0.112	2.10	k= 3, v= 6
1.4	98.300	0.112	2.12	k= 4, v= 6
2.7	97.500	0.187	2.13	k= 5, v= 6

s = 2.677
Note: df used for table values are approximate when v > 20.

6001 length
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WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	2	25.350	25.350	25.350
2	.21	2	24.550	24.550	24.700
3	.42	2	24.750	24.750	24.700
4	.76	2	24.800	24.800	24.700
5	1.4	2	24.100	24.100	24.100
6	2.7	2	23.450	23.450	23.450

6001 length
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WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
control	**25.350**				
.21	24.700	2.517	*	1.94	k= 1, v= 6
.42	24.700	2.517	*	2.06	k= 2, v= 6
.76	24.700	2.517	*	2.10	k= 3, v= 6
1.4	24.100	4.841	*	2.12	k= 4, v= 6
2.7	23.450	7.358	*	2.13	k= 5, v= 6

s = 0.258
Note: df used for table values are approximate when v > 20.

6001 wet weight
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WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	2	252.500	252.500	252.500
2	0.21	2	222.000	222.000	225.667
3	0.42	2	227.500	227.500	225.667
4	0.76	2	227.500	227.500	225.667
5	1.4	2	203.500	203.500	203.500
6	2.7	2	187.500	187.500	187.500

6001 wet weight
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WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
control	**252.500**				
0.21	225.667	2.944	*	1.94	k= 1, v= 6
0.42	225.667	2.944	*	2.06	k= 2, v= 6
0.76	225.667	2.944	*	2.10	k= 3, v= 6
1.4	203.500	5.376	*	2.12	k= 4, v= 6
2.7	187.500	7.131	*	2.13	k= 5, v= 6

s = 9.115
Note: df used for table values are approximate when v > 20.

6001 dry weight
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WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	control	2	68.300	68.300	68.300
2	.21	2	60.050	60.050	61.783
3	.42	2	62.750	62.750	61.783
4	.76	2	62.550	62.550	61.783
5	1.4	2	57.250	57.250	57.250
6	2.7	2	53.800	53.800	53.800

6001 dry weight
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WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
control	**68.300**				
.21	61.783	2.527	*	1.94	k= 1, v= 6
.42	61.783	2.527	*	2.06	k= 2, v= 6
.76	61.783	2.527	*	2.10	k= 3, v= 6
1.4	57.250	4.285	*	2.12	k= 4, v= 6
2.7	53.800	5.623	*	2.13	k= 5, v= 6

s = 2.579
Note: df used for table values are approximate when v > 20.