

US EPA ARCHIVE DOCUMENT

10-4-96

DATA EVALUATION RECORD

MRID: 42694605
DP BARCODE D2424505
CASE: 007132
SUBMISSION: S490058
PC CODE: 121701
EPA ID NO.: 62552-RR, 62552-3, 62552-8, 62552-9

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Date: 10/4/96

Study Type: 72-3 [154-13] Acute Toxicity for Nontarget Organisms - Marine Fish
Biochemical No. 121701 azadirachtin

Test Material: NPI-720

Synonym: ATI-720, Azatin Technical 10%

Project No. Wildlife International Ltd. Project No. 279A-102

Sponsor: AgriDyne Technologies, Inc., 2401 S. Foothill Drive, Salt Lake City, UT 84109

Testing Facility: Wildlife International Ltd., 8598 Commerce Drive, Easton, MD 21601

Title of Report: NPI-720: A 96-HOUR FLOW-THROUGH ACUTE TOXICITY TEST WITH THE SHEEPSHEAD MINNOW, *Cyprinodon variegatus*

Authors: William Graves and James P. Swigert

Report Issued: December 23, 1992

EXECUTIVE SUMMARY:

NPI-720: 96-HOUR FLOW-THROUGH TOXICITY TEST WITH THE SHEEPSHEAD MINNOW, *Cyprinodon variegatus* NPI-720 10%; Lot #21380; Sub Lot #1088-44C

Juvenile sheepshead minnows, *Cyprinodon variegatus*, were dosed for 96 hours in a continuous-flow dilution system with azadirachtin technical (NPI-720) at concentrations of 14.1, 25.8, 44.1, 84.6, and 197 mg NPI-720/L of mesohaline water at 22±1°C. Groups of 10 organisms per chamber (in duplicates) at each concentration and in the solvent and negative controls were maintained under controlled light conditions (16/8 hours light/dark). Water flow was maintained at ~6 test volume changes / 24 hours, dissolved oxygen was greater than 60% saturation, pH ~8, and salinity was 25±2 ‰. Observations of

mortality were made at 5, 24, 48, 72, and 96 hours after initiation of the test. Cumulative percent mortality in the treatment groups were used to calculate LC₅₀ values at 24, 48, 72 and 96 hours. Sheepshead minnow mortality occurred in those chambers at highest concentrations (197 mg NPI-720/L) containing precipitated materials. No mortality or adverse effects were observed among the minnows serving as controls; one death of 20 organisms was observed in those exposed to 14.1 and 25.8 mg NPI-720/L but none were observed in those exposed to 44.1 mg NPI-720/L. Minnows were observed surfacing at 48, 72 and 96 hours at the 197 mg NPI-720/L concentration. **The 96 hour LC_{50, azadirachtin} = 129 mg NPI-720/L for *C. variegatus* [129 mg/L = 129 ppm];** the 95% confidence limits are 84.6 and 197 mg NPI-720 per liter. The 96-hour no mortality concentration and the no observed effect concentration (NOEC) and is 44.1 mg of NPI-720/L. NPI-720 is classified as **practically non-toxic** (>100 mg/L) to estuarine juvenile sheepshead minnows (*C. variegatus*). **The no observed effect concentration was 44.1 mg NPI-720/L,** the middle concentration tested. This study is **acceptable with comments** and meets the requirement of **Subdivision E: §154-13 [72-3] for an estuarine (marine) fish.**

LC ₅₀ (EC ₅₀) ppm	mg/L	Descriptor
<0.1	<0.1	very highly toxic
0.1 - 1.0	0.1 - 1.0	highly toxic
>1.0 - 10	1.0 - 10	moderately toxic
>10 - ≤100	>10 - 100	slightly toxic
→ >100	>100	practically non-toxic

[1 ppm = 1000 µg/L]

A. MATERIALS:

1. Test Material: NPI-720 10% Technical

Description: tan powder
 Lot/Batch No. Lot 21380, Sublot 1088-44C, New Wt. 546 g, Exp. 6-20-93
 Store: Dark, Cool, Dry
 Purity: azadirachtin, 10.0%
 Stability: Exp. Date 6-20-93
 Biochemical Formula: $C_{35}H_{44}O_{18}$
 Mol Wt = 720.7
 CAS No.: 11141-17-6

2. Vehicle: acetone

3. Test Animals: juvenile sheepshead minnows *Cyprinodon variegatus*
 same year class; mean wt. of 0.55 g (0.35-0.87 g, n = 10), mean length of 25 mm (21 - 28 mm, n = 10)
 Strain: Wildlife International Ltd. culture
 Age at Study Initiation: juveniles, same year class (no age stated)
 Source: Wildlife International Ltd., Easton MD 21601
 Housing: Adult mysids were maintained in culture for 14 days before selecting
 Diet: Fed flaked fish food supplied by Zeigler Brothers, Inc. P.O. Box Gardners, PA 17324; frozen brine shrimp nauplii (*Artemia* sp.) supplied by Kordon, 2242 Davis Court, Hayward CA 94545; and *Artemia* nauplii supplied by Artemia, Inc., P.O. Box 485, Newark CA 94560. The fish were not fed for 48 hours prior to the test nor during the test.

Environmental Conditions:

Temperature: 22C±1° C
Humidity: not applicable
Photoperiod: 16 h light / 8 hr dark cycle including 0.5 hour transitions
Acclimation Period: 51 hours prior to the test.
Loading: Total weight of fish per liter of solution that passed through the test chambers in 24 hours was 0.061 g of fish. Instantaneous loading was 0.37 g of fish per liter of test solution.

B. STUDY DESIGN:

1. Animal Assignment

Delivery of test solutions to the diluter was begun approximately 21 hours before the test started. Five treatment levels (14.1, 25.8, 44.1, 84.6 and 197 mg NPI-720/L) were tested along with solvent (1.5 ml acetone/L) and negative controls. Two replicates were tested with 10 fish at each dose.

The dose rationalization is based on results of range finding test and discussion with sponsor.

2. Diet: no feeding during the test

3. Statistics:

Values of LC₅₀ and 95% confidence intervals were calculated using the ERL-Duluth program (C.E. Stephan, 1978) which calculates the LC₅₀ and the 95% confidence interval by probit analysis, the moving average method, or the binomial probability. In this study, the 24, 48, 72, and 96 hour values were determined by visual inspection and the probit and binomial methods. The no mortality concentration and the no observed effect concentrations were determined by visually inspecting the mortality data.

4. Test Material:

Samples for measuring NPI-720 were collected from each replicate test chamber at test initialization, and at 24 hour intervals thereafter until the end of the test.

C. RESULTS

Mean measured concentrations were 19.4, 32.4, 54.4, 90, and 150 mg NPI-720/L and they were used for the calculations of LC₅₀.

Mortality and sub-lethal effects (surfacing) are reported in the original data (Table 3). At the highest concentration (197 mg NPI-720/L), however, sub-lethal effects (surfacing) were observed at 48, 72 and 96 hours. Mortality at 44.1 mg NPI-720 and below are considered to be insignificant and not attributable to NPI-720.

The study authors attribute biological responses at the higher concentrations (84.6 and 197 mg ATI-720/L) to turbidity and interference with respiration. They calculated LD₅₀s using the mortality data that are summarized in Table 1 and the LD₅₀s in Table 2. [The only concentration at which 50% mortality occurred was at 197 mg ATI-720/L.]

Table 1. Cumulative Per Cent Mortality of Sheepshead Minnows (*Cypinodon variegatus*) Exposed to NPI-720 for 96 Hours

(Mortality Data Summarized from Table 3 attached as an Appendix)

Mean Measured Concentration (mg NPI-720/L)	5 Hr	24 Hr	48 Hr	72 Hr	96 Hr
Negative Control	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10
Solvent Control	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10
14.1	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10	1/10 0/10
25.8	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10	1/10 0/10
44.1	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10
84.6	0/10 0/10	0/10 0/10	0/10 0/10	2/10 0/10	3/10 1/10
197	0/10 0/10	0/10 0/10	2/10 2/10	5/10 5/10	9/10 7/10

Table 2. LC₅₀ Values for *Cyprinodon variegatus* Exposed to NPI-720

Time	LC ₅₀ (µg NPI-720/L)	Lower 95% Conf Limits	Upper 95% Conf Limits	Statistical Method
24 Hours	> 197	N/A	N/A	Visual Inspection
48 Hours	> 197	N/A	N/A	Visual Inspection
72 Hours	194	149	332	Probit
96 Hours	129	84.6	197	Binomial

D. DISCUSSION

This study followed an acceptable protocol. A graded response is evident from the highest concentrations to which the minnows were exposed.

E. REVIEWER'S COMMENTS

Although this study was undertaken according to protocol, the accepted protocol did not include a positive control of known effectiveness for determining an LD₅₀.

A more definitive 96 hour LD₅₀ may possibly be determined if sheepshead minnows could be exposed to higher concentrations of NPI-720 without the "inert" materials associated with the preparation. Other materials ("inerts") may contribute to the formation of turbidity which collects over time in the experimental chambers. (Heterogeneous materials which are brought into aquatic media with the use of solubilizers, often form precipitates in, thus making quantitative measurements of the active substance difficult.) For quantitative risk assessments, the 96 hour LD₅₀ > 129 mg of NPI-720/L is a very conservative number which may be reaching the solubility

limit of the test material (active ingredient plus inerts) in saline waters. In this 96 hour test, however, greater than 50% mortality (16 of 20 exposed organisms) occurred at only the highest dose. This study does not need to be repeated.

F. CONCLUSIONS

The 96 hour $LC_{50, azadirachtin} = 129 \text{ mg/L}$ for sheepshead minnows (*Cyprinodon variegatus*) with 95% confidence limits are 84.6 and 197 mg NPI-720 per liter. The 96-hour no mortality concentration and the no observed effect concentration (NOEC) and is 44.1 mg of NPI-720/L. NPI-720 is classified as **practically non-toxic** (>100 mg/L) to sheepshead minnows. [Less than 8% mortality (only 6 of 80 organisms) occurred in animals exposed to <100 mg NPI-720/L.

This study is **acceptable** and meets the requirement of **Subdivision E: §154-13 for an estuarine [marine] vertebrate.**

LC_{50} (EC_{50})		[1 ppm = 1000 $\mu\text{g/L}$]
<u>ppm</u>	<u>mg/L</u>	<u>Descriptor</u>
<0.1	<0.1	very highly toxic
0.1 - 1.0	0.1 - 1.0	highly toxic
>1.0 - 10	1.0 - 10.0	moderately toxic
>10 - \leq 100	>10 - 100	slightly toxic
---> >100	>100	practically non-toxic

5. Quality Assurance:

A signed statement of quality assurance is included in the report.

G. APPENDED TABLES AND/OR DATA (Hard copy for reviewer)

- Table 1. Summary of Analytical Chemistry Data
- Table 3. Cumulative Percent Mortality and Treatment-Related Effects [of NPI-720 on Juvenile Sheepshead Minnows *Cyprinodon variegatus*, 96 Hours]

Table 4. LC_{50} Values [Effects of NPI-720 on Juvenile Sheepshead Minnows *Cyprinodon variegatus*]

cc: T. McClintock, P. Zubkoff, BPPD Subject File
 P. Zubkoff: (703) 308-8694: 5/21/96
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AZADIRACTIN

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Paul Zubkoff NPI-720

48-Hour Acute Toxicity Sheepshead Minnow Cyprinodon variegatus

CONC.	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB. (PERCENT)
197	20	4	20	.5908966
84.6	20	0	0	9.536742E-05
44.1	20	0	0	9.536742E-05
25.8	20	0	0	9.536742E-05
14.1	20	0	0	9.536742E-05

THE BINOMIAL TEST SHOWS THAT 197 AND +INFINITY CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS, BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 196.9999

WHEN THERE ARE LESS THAN TWO CONCENTRATIONS AT WHICH THE PERCENT DEAD IS BETWEEN 0 AND 100, NEITHER THE MOVING AVERAGE NOR THE PROBIT METHOD CAN GIVE ANY STATISTICALLY SOUND RESULTS.

Paul Zubkoff NPI-720

96-Hour Acute Toxicity Sheepshead Minnow Cyprinidon variegatus

CONC.	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB. (PERCENT)
197	20	16	80	.5908966
84.6	20	4	20	.5908966
44.1	20	0	0	9.536742E-05
25.8	20	1	5	2.002716E-03
14.1	20	1	5	2.002716E-03

THE BINOMIAL TEST SHOWS THAT 84.6 AND 197 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS, BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 129.0976

RESULTS CALCULATED USING THE MOVING AVERAGE METHOD

SPAN	G	LC50	95 PERCENT CONFIDENCE LIMITS	
2	9.753794E-02	129.0976	104.7606	172.4907

RESULTS CALCULATED USING THE PROBIT METHOD

ITERATIONS	G	H
4	1.841313	4.502499
GOODNESS OF FIT PROBABILITY		
3.658474E-03		

SINCE THE PROBABILITY IS LESS THAN 0.05, RESULTS CALCULATED USING THE PROBIT METHOD PROBABLY SHOULD NOT BE USED.

SLOPE = 2.676325
95 PERCENT CONFIDENCE LIMITS = -.9553139 AND 6.307964

LC50 = 131.0374
95 PERCENT CONFIDENCE LIMITS = 0 AND +INFINITY

LC10 = 43.94037
95 PERCENT CONFIDENCE LIMITS = 0 AND +INFINITY

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Paul Zubkoff NPI-720

72-Hour Acute Toxicity Sheepshead Minnow Cyprinidon variegatus

CONC.	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB. (PERCENT)
197	20	10	50	58.80985
84.6	20	2	10	2.012253E-02
44.1	20	0	0	9.536742E-05
25.8	20	0	0	9.536742E-05
14.1	20	0	0	9.536742E-05

THE BINOMIAL TEST SHOWS THAT 84.6 AND +INFINITY CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS, BECAUSE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS GREATER THAN 95 PERCENT.

AN APPROXIMATE LC50 FOR THIS SET OF DATA IS 196.9999

RESULTS CALCULATED USING THE MOVING AVERAGE METHOD

SPAN	G	LC50	95 PERCENT CONFIDENCE LIMITS	
1	.4957674	197.0001	144.7377	615.5791

RESULTS CALCULATED USING THE PROBIT METHOD

ITERATIONS	G	H
6	.291152	1

GOODNESS OF FIT PROBABILITY
.9723801

SLOPE = 3.857576
95 PERCENT CONFIDENCE LIMITS = 1.776086 AND 5.939066

LC50 = 194.0445
95 PERCENT CONFIDENCE LIMITS = 148.9779 AND 331.6943

LC10 = 90.9241
95 PERCENT CONFIDENCE LIMITS = 48.64956 AND 119.6429
