108401

acute study : core
chronic study: supplemental
raw data on "time to hatch" not submitted.
& no NOEC

79112

Data Evaluation Record

1. Chemical: Bolero

2. Formulation: Technical (SX-1127)
95.1% A.I.

3. Citation: Effects of Bolero Technical on Survival, Growth, and Development of Sheepshead Minnows (Cyprinodon variegatus), EG&G Bionomics, Marine Research Laboratory, Pensacola, Florida, September, 1979. Ref. 17 in Fish and Wildlife Safety Data to Support Registration of Bolero 10G Herbicide (acc. #241483).
MRID 79112

4. Reviewed by: Ann Rosenkranz
Aquatic Biologist
HED/EEB

5. Date Reviewed: January 17, 1980

6. Test Type:

1. a. acute aquatic toxicity study
b. early life stage (28-day posthatch) study
2. species - sheepshead minnow

7. Reported Results: (all based on measured concentrations)

a. Acute Aquatic Toxicity Study
96h LC_{50} =690 ppb
95% C.I. = 600-800ppb

b. Early Life Stage Study
1) Significant effect on hatching success
≥600ppb
2) Significant mortality of juvenile sheepshead minnows over 28 day period. ≥230 ppb
3) Significant decrease in growth of juveniles
≥230ppb (standard length)
≥150ppb (wet weight)
4) Estimated MATC of Bolero technical
≤150ppb NOEC = not determined
5) Application factor
≤0.22

8. Reviewer's Conclusion: Re-calculation of the above reported results changed the following:

1) 96-h LC_{50} value
659ppb
95% C.I. - 390-1300ppb

2) Growth of juveniles
≥150ppb (wet weight and standard length)
3) Application factor
<0.23

The study is scientifically sound and fulfills the requirements of EPA guidelines of 1978 for the acute aquatic toxicity study for estuarine species. However, it does not fulfill the requirements for the embryo-larvae study.

I. Acute Aquatic Toxicity Test

Materials/Methods

Test Procedure

The procedure was based on the one in Proposed Standard Practice for Conducting Basic Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians (ASTM, 1978). The test was conducted in an intermittent flow system using a proportional diluter (Mount and Brungs, 1967) that delivered 1 liter/cycle/test aquarium at a dilution rate of 60%. The average number of cycles was approximately 5/hour, providing approximately 2.5 volume additions in 24 hours. Groups of 10 juvenile fish, 7mm standard length, were exposed to a series of Bolero concentrations, 520, 860, 1400, 2400, 4000ppb, a saltwater control and a solvent (acetone) control. All concentrations and controls were duplicated. Photo-period was approximately 12L:12D. Dissolved oxygen, pH, salinity and temperature were measured daily. Water samples were taken from all treatment replicates on day 0 and day 4 for analysis of the concentrations of Bolero Technical.

Statistical Analysis

The 24-, 48-, 72- and 96-h LC_{50} values and their 95% confidence intervals were calculated using Stephan's program. The binomial probability method was used to analyze the 24- and 72-h data, and the moving average angle method was used for the analysis of the 48- and 96-h data.

Discussion/Results

The mean measured concentrations of Bolero Technical in seawater ranged from 41%-55% of the nominal concentrations.

Nominal conc. (ug/l) (ppb)	Mean measured conc ± S.D. (ug/l) (ppb)	% of nominal
520	260±30	50
860	390±10	45
1400	580±230	41
2400	1300±500	54
4000	2200±200	55

The water quality parameters were: temperature - 25°C, salinity - 30 °/00, pH-7.7-8.0, D.O.= ≥59% saturation. The mortality results were:

mean measured conc. (ug/l)	% mortality (N=20)			
	24h	48h	72h	96h
Control	0	0	0	0
Solvent control	0	0	0	5
260	0	0	0	0
390	0	0	0	5
580	0	10	35	40
1300	2	75	100	100
2200	100	100	100	100

The LC_{50} values and the respective 95% C.I. are:

24h	1700 ppb	(1300-2200ppb)
48h	940 ppb	(800-1100ppb)
72-h	710 ppb	(390-1300ppb)
96-h	690 ppb	(600-800ppb)

The above values are based on the measured concentrations.

Reviewer's Evaluation

Test Procedure

The procedure generally complies with the one recommended by the EPA guidelines (Stephan's). The only discrepancy between the 2 procedures is that the EPA protocol requires a flow rate through the test chambers of at least 5 volumes per 24 hours, whereas the flow rate was only 2.5 volumes per 24 hours. A high flow rate is necessary to ensure that the concentrations are relatively constant and do not fluctuate over a wide range.

Statistical Analysis

The LC_{50} values were recalculated using the same methods as the ones which were reported. The recalculated values, based on the mean measured concentrations are:

	LC_{50} Value (ug/l)	95% C.I. (ug/l)
24h	1531	1300-2200
48h	940	804-1128
72h	669	390-1300
96h	659	390-1300

The 96h LC_{50} value also includes a correction for the 5% mortality in the solvent control group. There is no indication if the author likewise corrected for this mortality.

Discussion/Results

The 5% mortality in the solvent control at 96h is within acceptable limits. However it should have been accounted for in the LC_{50} value calculation. Therefore, the recalculated LC_{50} values should be regarded as the true values.

The results also indicate that the toxicity does not increase significantly after a 72-hr exposure period.

Conclusions

1. Category: Core

2. Rationale: Although the flow rate was less than optimal, the study is acceptable and fulfills the requirements for an aquatic acute toxicity for an estuarine species.

II. Early Life Stage (28-Day Posthatch) Test
Materials/Methods

The procedure was based on the one in Proposed Standard Practice for Conducting Basic Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians (ASTM, 1978). The test was conducted in an intermittent flow system using a proportional diluter (Mount and Brungs, 1967) that delivered 1 liter/cycle/test aquarium at a dilution rate of 60%. The average number of cycles was approximately 5.4 times/hour, providing 2.7 volumes in 24 hours. Based on the results of the acute test, embryo-juveniles were exposed to nominal concentrations of 180, 300, 500, 840, and 1400 ppb Bolero Technical.

Embryos were obtained by stripping eggs from adult female fish that had been injected on 2 consecutive days with HCG. The eggs were fertilized by the addition of a sperm suspension made from macerated testes. Within 4 hours after visual confirmation of fertilization, each treatment received 4 groups of 25 embryos. The embryos were removed from each incubator cup by pipette daily and counted. This procedure was repeated until all living embryos had hatched. Embryo mortality and time to hatch were recorded.

After hatching, all surviving fish from each replicate were placed into glass growth chambers and maintained until the end of the test (28 days post hatch) by feeding on live brine shrimp (Artemia salina) nauplii daily. Survival was monitored daily and any changes in physical appearance or behavior were recorded. At the end of the test period standard lengths and wet weights were recorded.

Dissolved oxygen, pH, salinity and temperature were measured daily. Water samples were taken from alternate replicates each week for measurement of the concentrations of Bolero Technical.

Statistical Analysis

At the termination of the early life stage study, percentage hatching success and juvenile mortality were calculated. The differences between the solvent control and the test concentrations were determined by ANOVA using the arcsin transformations of the binomial percentages to angles of equal information in degrees. Differences were considered significant at the 95% confidence level ($p \leq 0.05$). Statistical comparison between the solvent control and each treatment was made by using Williams' procedure (Williams, 1971). Mean standard length and weight of the juvenile fish was also calculated and the differences determined by ANOVA. Statistical comparison between the control and each treatment was made by using Williams' procedure.

Williams' procedure tests the difference between treatment means when several dose levels are compared with a zero dose control. The following assumptions are made: The treatments comprise a control and k dose levels, denoted by integers 0 to k, with 0 representing the control and dose i greater than dose i-1 for i=1 2,...,k. The experiment in which all treatments, including the control, have the same replication r, provides unbiased estimates X_i of the mean responses. The effect of the test substance will be to increase the mean responses M_i to satisfy $M_o \leq M_1 \leq M_2 \leq \ldots \leq Mk$. If the response is a decrease in means the above formulation applies after a change in sign.

The test statistic is given by: $\bar{t}_k = (M_k - X_o)(2s^2/r)^{-1/2}$
$\hat{M}_k$ = maximum likelihood estimate of each mean. If $X_o \leq X_1 \leq X_2 \leq \ldots \leq Xk$, then $\hat{M}_i = X_i$. If $X_i > X_{i+1}$, then $M_i = M_{i+1} = 1/2\ (X_i + X_{i+1})$.
X_o = mean of control.
S^2 = error mean square
r = number of replicates/treatment

The test statistic, $\bar{t}_k$, is identical with Students' t - statistic for comparing treatment k with the control except that $\hat{M}_k$ replaces X_k. Results were considered significant at the 95% confidence level.

Discussions/Results

The mean measured concentration of Bolero Technical in seawater ranged from 68% to 83% of the nominal concentrations.

Nominal conc. (ug/l) (ppb)	Mean measured conc±SD (ug/l) (ppb)	% of Nominal
180	150±60	83
300	230±100	77
500	370±50	74
840	600±100	71
1400	960±270	68

The water quality parameters were: temperature-25°C, salinity-23°/oo (range of 18°/oo-26°/oo); pH-7.3 to 8.1, D.O.-4 to 115% of saturation. There were several low dissolved oxygen concentrations measured during the test in the solvent control and in all but the highest test concentration

chambers. The low DO concentrations are believed to be the result of growth of a slime bacteria which seemed to be enhanced by the presence of the acetone solvent. Low DO concentrations probably did not occur in the highest test concentration because all the fish died early in the test before the slime grew enough to have a significant effect. Test chambers were cleaned twice a week during the test to help maintain higher DO concentrations, but low DO levels were still recorded. However, since no excessive mortality or fish behavior normally associated with low DO concentrations were noted, it is believed that the DO concentrations had no adverse effect on the fish or the results of the test.

Exposure to mean measured Bolero Technical concentrations $\geq$ 600ppb had a significant effect on the hatching success of sheepshead minnow embryos.

Mean measured conc. (ug/l)	Percentage hatch (100 embryos/treatment)
control	75
solvent control	93
150	91
230	90
370	92
600	78
960	54

No delay in hatching time nor abnormalities in embryonic development were observed in any treatment.

Exposure to mean measured Bolero Technical $\geq$ 230 ppb significantly increased mortality of juvenile sheepshead minnows. By day 7 posthatch, all fish exposed to 960ppb of Bolero Technical had died, whereas only 56% of the fish exposed to 600ppb died. By day 14 posthatch 90% of all fish mortality observed during the 28-day posthatch exposure period had occurred. No significant physical abnormalities were observed in juvenile fish at the termination of the test at any treatment level.

Cumulative mortality of juvenile sheepshead minnow

Mean measured conc. (ug/l;ppb)	Total number fish exposed	day 7	day 14	day 21	day 28
control	75	17%	19%	19%	19%
solvent	93	17%	18%	18%	19%
150	91	25%	25%	26%	28%
230	90	32%	32%	34%	36%
370	92	22%	27%	28%	34%
600	78	56%	91%	97%	99%
960	54	100%	100%	100%	100%

Growth of fish exposed to mean measured Bolero Technical concentrations $\geq$ 150 ppb was significantly less than growth of fish in the solvent control group. Standard length was significantly decreased at concentrations greater than or equal to 230ppb Bolero Technical, and wet weight was significantly decreased at 150ppb Bolero Technical.

Mean measured conc. (ng/l; ppb)	Mean standard length (mm) ±SD	Mean wet wgt (mg)
control	13±2	56
solvent control	13±2	52
150	11.5±2	31
230	10.5±2	23
370	8.7±2	13
600	5[a]	3[a]
960	[b]	[b]

a Only 1 fish survived.
b No survivors

The estimated MATC of Bolero Technical for embryos and juveniles of sheepshead minnows was < 150 ppb, and the application factor was < 0.22, based on a calculated 96-h LC_{50} value of 690 ppb.

The purpose of this test was not to specifically assess potential reproductive effect but rather to estimate all chronic effects. However, based on the results of the early life stage test it is reasonable to expect that reproduction of sheepshead minnow could be adversely affected by exposure to Bolero concentrations as low as 150 ppb. Growth is significantly affected at this level and maturation is closely related to growth.

Reviewer's Evaluation

Material/Methods

Test Procedure

The procedure generally complies with the protocol recommended by EPA in Bioassay Procedures for the Ocean Disposal Permit Program, EPA-600/9-78-010, March, 1978.

Statistical Analysis

The results for hatching success, juvenile mortality, and growth were recalculated with the Williams procedure and Duncan's multiple range test not only to validate the results but also to compare the 2 methods. For both methods, arcsintransformations of the binomial percentages were made, and the means for the seawater control and the solvent control were compared using the t-test for paired observations. Since there were no significant differences between the 2 control groups, the solvent control data were used.

Discussion/Results

It does not appear that there was any relationship between the low dissolved oxygen levels and the results. This is based on the fact that although there are significant differences ($p \leq 0.01$) between the D.O. levels for the seawater control and the solvent control during the hatching period and 28-day juvenile study, there are no significant differences ($p \geq 0.05$) between the 2 control goups regarding any of the biological results.

Hatching success was significantly decreased ($p \leq .05$) at Bolero Technical concentrations $\geq$ 600 ppb according to the 2 methods. This agrees with the reported result.

Juvenile mortality was significantly increased at Bolero Technical concentrations $\geq$ 230 ppb according to William's procedure and at concentrations $\geq$ 600 ppb according to Duncan's test. This indicates that William's procedure may be more accurate than Duncan's test since it is based on the assumption that the responses are monotonically ordered according to test concentrations and will correct for any means that are not in order. On the other hand Duncan's test arranges the means in order without regard to the order of the test concentrations.

LC_{50} values were calculated with Stephans' program for juvenile mortalities at 7 days, 14 days, 21 days, and 28 days. Abbott's formula was used to correct for the mortality in the solvent group. The results, obtained with the moving average method are:

Time of Exposure Days	LC_{50} Value ppb	95% C.I. ppb
7	560.2	513.1-619.9
14	415.2	380.6-456.3
21	381.4	350.0-417.1
28	360.6	330.4-394.1

Growth of juvenile sheepshead minnow as measured by standard length and wet weight was significantly affected at Bolero Technical concentrations as low as 150 ppb as determined by the 2 statistical methods. The effect level for standard length differs from the reported value of 230 ppb. However, the value of 150 ppb will be regarded as the correct one. The Duncan's multiple range test also indicated that fish exposed to 370 ppb are significantly shorter than those exposed to Bolero Technical concentrations less than or equal to 230 ppb and weigh significantly less than those exposed to concentrations less than or equal to 150 ppb.

Since the 96-h LC_{50} value for the juvenile minnows was re-calculated to 659 ppb, the application factor is < 0.23. The MATC remains at $<$ 150 ppb.

Conclusions:

1. Category: ~~Supplemental~~ Upgraded to Core (See Attached) 5/30/85
2. Rationale: The study is scientifically sound. However, the results of time to hatch the embryos is missing.
3. Repairability: If the time to hatch data are submitted, as required by EPA in Bioassay Procedures for the Ocean Disposal Permit Program, EPA-600/9-78-010, March, 1978, and are found to be acceptable, then the study will be re-classified as core.

Hatching Success

No significant difference between the control and solvent control (SC). Therefore SC used as control (X_o) level.

A. Williams' Procedure

$$\bar{t}_k = (\hat{M}_k - X_0)(2S^2/r)^{-1/2}$$

$\hat{M}$= best estimate
$X_0 = \bar{x}$ control
S^2= error mean square

Mean Conc. ppb		Hatching Success (arcsin-transformations of %) A_1	A_2	B_1	B_2
X_o	SC	90	69.7	78.5	69.7
X_1	150	78.5	69.7	73.6	69.7
X_2	230	66.4	69.7	78.5	73.6
X_3	370	73.6	78.5	78.5	66.4
X_4	600	63.4	48.5	78.5	63.4
X_5	960	39.2	48.5	55.6	46.2

X_0	X_1	X_2	X_3	X_4	X_5
76.98	72.88	72.05	74.25	63.45	47.38

$$X_{2,3} = \frac{1}{2}(72.05+74.25) = 73.15$$

$$X_{1,2,3} = \frac{1}{3}[2(73.15) + 72.88] = 73.06$$

$\hat{M}_0$	$\hat{M}_1$	$\hat{M}_2$	$\hat{M}_3$	$\hat{M}_4$	$\hat{M}_5$
76.98	73.06	73.06	73.06	63.45	47.38

$$\sqrt{2S^2/r} = \sqrt{2\left(\frac{\text{error S.S.}}{\text{df}}\right)/r} = \sqrt{2(60.94)/4} = 5.52$$

$\bar{t}_4$ = - (63.45-76.98)/5.52=2.45, df = 18
significant at $p \leq .05$ (600 ppb)

$\bar{t}_3$= - (73.06-76.98)/5.52= 0.71, df=18
$p \geq 0.05$ - not significant (370 ppb)

Significant decrease in hatching success at conc. $\geq$ 600 ppb.

B. Duncan's Multiple Range Test

ppb	SC	370	150	230	600	960
x	76.98	74.25	72.88	72.05	63.45	47.38

Hatching success at 600 ppb is significantly less than that at control levels or concentrations $\leq$ 370 ppb, but it is significantly greater than that at 960 ppb.

Juvenile Mortalities

No significant difference between SW control and solvent control (SC).

A. William's Procedure

conc.	mortality (arcsin transformation of %)			
ppb	A_1	A_2	B_1	B_2
X_0 SC	23.6	17.5	24.4	36.9
X_1 150	30	39.8	36.3	22
X_2 230	35.1	25.1	42.7	41
X_3 370	46.2	32.6	32.6	29.3
X_4 600	77.1	90	90	90
X_5 960	90	90	90	90

X_0	X_1	X_2	X_3	X_4	X_5
25.60	32.03	35.98	35.18	86.78	90

$$X_{2,3} = \frac{1}{2}(35.98+35.18) = 35.58$$

$\hat{M}_0$	$\hat{M}_1$	$\hat{M}_2$	$\hat{M}_3$	$\hat{M}_4$	$\hat{M}_5$
25.60	32.03	35.58	35.58	86.78	90

$$\sqrt{2S^2/r} = \sqrt{\frac{2(865.71)}{(18)}\Big/4} = 4.90$$

$$\bar{t}_1 = \frac{32.03 - 25.60}{4.90} = \text{not significant } (p \geq 0.05, df=18)$$

$$\bar{t}_2 = \frac{35.58-25.60}{4.90} = 2.12, df = 18$$
significant ($p \leq 0.05$)

Significant increase in juvenile mortality at Bolero conc. $\geq$ 230 ppb.

B. Duncan's Multiple Range

ppb	960	600	230	370	150	SC
$\bar{x}$	90	86.78	35.98	35.18	32.03	25.60

Bolero conc. $\geq$ 600 ppb have significantly greater mortality than conc. $\leq$ 370 ppb.

Standard Length

CONC(ppb)	A_1	A_2	B_1	B_2
SC	12	14	12	14
150	11	12	11	12
230	10	10	11	11
370	9	9	9	8
600	5	5	-	-
960	-	-	-	-

ANOVA and Duncan only run on SC to 370 ppb. No significant difference between SW control and SC.

A. Duncan's Multiple Range Test df=12

ppm	SC	150	230	370
$\bar{X}$	13	11.5	10.5	8.75

Standard lengths of solvent control fish are significantly greater than those of treated fish, and standard lengths of fish exposed to 370 ppb are significantly less than those of fish exposed to lower concentrations.

B. Williams' Procedure

ppb	SC	150	230	370
$\bar{X}_k$	13	11.5	10.5	8.75
$\hat{M}_k$	13	11.5	10.5	8.75

$$\bar{t}_k = \frac{(\hat{M}k - X_0)(2S^2/r)^{-1/2}}{(2S^2/r)^{-1/2}} = \sqrt{2\left(\frac{6.75}{12}\right)}\Big/4 = 0.53$$

$$\bar{t}_k = -\frac{(11.5-13)}{0.53} = 2.83 \text{ significant } (p \leq 0.05)$$

Standard length of fish exposed to $\geq$ 150 ppb significantly less than those of solvent control fish.

Wet Weight

No significant difference between SW control solvent control (SC).

conc. (ppb)	A_1	A_2	B_1	B_2
SC	36	68	46	61
150	34	46	33	17
230	20	23	26	23
370	13	16	12	10
600	3	3	-	-
960	-	-	-	-

ANOVA and Duncan only run on SC to 370 ppb.

A. Duncan's Test

ppb	SC	150	230	370
$\bar{x}$	52.75	32.5	23	12.75

Wet weights of solvent control fish are significantly greater than those of treated fish. Weights of fish exposed to 370 ppb are significantly less than fish exposed to $\leq$ 150 ppb.

B. William's Procedure

ppb	SC	150	230	370
	X_o	X_1	X_2	X_3
X_k	52.75	32.5	23	12.75
$\hat{M}k$	52.75	32.5	23	12.75

$$\bar{t}_k = (\hat{M}_k - X_o) / (2S^2/r)^{-1/2}$$

$$(2S^2/r)^{-1/2} = \sqrt{2\left(\frac{1088.5}{12}\right)\Big/4} = 6.73$$

$$\bar{t}_1 = -(32.5-52.75)/67.3 = 3.01, \text{ df} = 12$$

Significant ($p \leq 0.05$)

Bolero conc. $\geq$ 150 ppb significantly affect wet weight.